ELSEVIER

Journal of Chromatography B, 663 (1995) 353–359

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of terbinafine and its desmethyl metabolite in human plasma by high-performance liquid chromatography

J. Denouël[a,*], H.P. Keller[b], P. Schaub[b], C. Delaborde[b], H. Humbert[a]

[a]*Department of Human Pharmacology, Laboratoires Sandoz, 14 Bd Richelieu, 92500 Rueil-Malmaison, France*
[b]*Sandoz Pharma, Biopharmaceutical Department, Basle, Switzerland*

First received 5 April 1994; revised manuscript received 27 September 1994; accepted 28 September 1994

Abstract

A reliable reversed-phase high-performance liquid chromatographic method has been developed for the determination of terbinafine (Terb) and its desmethyl metabolite (DMT) in human plasma. The analytes and the internal standard (I.S.) are extracted by a liquid–liquid technique followed by an aqueous back-extraction, allowing injection of an aqueous solvent in the HPLC system. The mobile phase is acetonitrile + 0.012 *M* triethylamine −0.020 *M* orthophosphoric acid (50 : 50, v/v) and the UV detection is at 224 nm. The inter-assay precision over the concentration range 2–1000 ng/ml is between 2.9 and 9.8% for both compounds. The limit of quantification, 2 ng/ml for both compounds, is sufficient for investigating the pharmacokinetics of Lamisil in human studies. With an additional preparation step, this method can be used for assaying Terb in tissues such as nail, sebum and stratum corneum.

1. Introduction

Lamisil [terbinafine, SF 86-327; (E)-N-(6,6-dimethyl-2-hepten-4-inyl) - N-methyl-1-naphtalene methanamine hydrochloride] is a new synthetic antifungal agent of the allylamine class [1]. This compound is highly active in the treatment of dermatomycoses. The drug interferes with the cytoplasmic membrane integrity of fungi by blocking membrane ergosterol synthesis [2]. Lamisil is given to humans by the oral route, and a sensitive and specific method is mandatory to follow the kinetics of terbinafine (Terb), especially since a long terminal half-time has previously been observed after repeated administrations [3].

Terb is extensively metabolized, mainly through N-demethylation and oxidation of any of the three methyl groups (Fig. 1). About fifteen metabolites have been identified in human plasma [3]. More polar metabolites need another way of extraction, and only the desmethyl Terb (DMT), the less polar of the metabolites, can be assayed together with Terb by this method.

Previous HPLC methods involving electrochemical or UV detection combined with gradient elution were not sufficiently sensitive to follow human kinetics for more than 24 h after oral administration of 250 mg of Lamisil. An original method, with direct injection of deproteinized diluted plasma with electrochemical

* Corresponding author.

0378-4347/95/$09.50
SSDI 0378-4347(94)00449-8

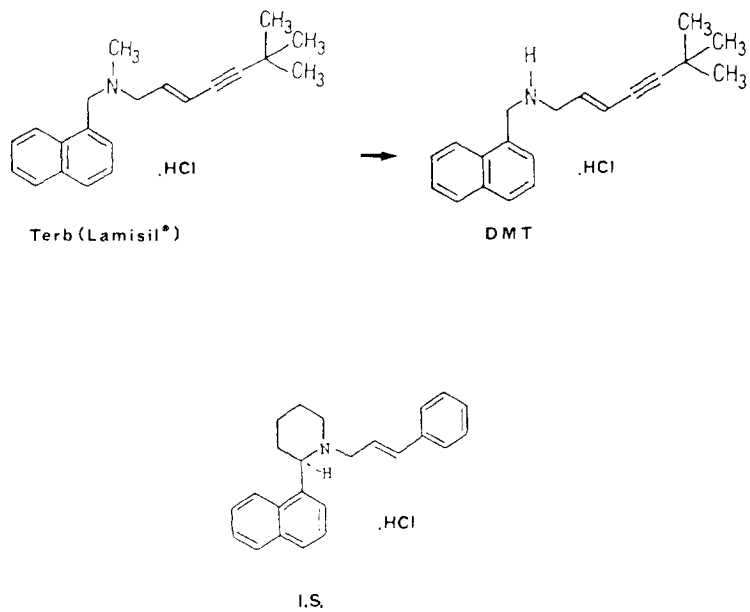

Fig. 1. Structure of terbinafine (Terb, SF 86-327), its desmethyl metabolite (DMT) and the internal standard (I.S.).

detection [4] proved to be reliable, but not sensitive enough. Another HPLC method, using electrochemical detection with addition of an internal standard, revealed good sensitivity, but did not allow determination of DMT. Furthermore, electrochemical detection did not seem to be appropriate, considering the high-voltage oxidation potential that had to be used.

A new method for the determination of Terb and DMT was needed. Therefore, a sensitive and specific method was developed which uses a double extraction and UV detection. The results obtained for the extraction from human plasma are described and an example of its application to kinetic studies is given.

2. Experimental

2.1. Chemical and reagents

Terb hydrochloride (Terb), its desmethyl metabolite hydrochloride (DMT) and internal standard IW 85-190 (I.S.) were supplied by Sandoz (Basle, Switzerland) (Fig. 1). *n*-Hexane was of UV quality (Merck, Darmstadt, Germany) and acetonitrile, 2-propanol and methanol were of HPLC quality (Carlo Erba, Milan, Italy). Sulphuric acid was of analytical quality (Merck) and other reagents: orthophosphoric acid, potassium chloride, boric acid and sodium hydroxide were also analytical reagent quality (Prolabo, Paris, France), as well as triethylamine (Fluka, Buchs, Switzerland). High quality water was obtained using a Milli Q filter system (Millipore, Milford, MA, USA). Human plasma from healthy subjects was supplied by the Blood Transfusion Center (Paris, France).

2.2. Standard solutions

Pure standard stock solutions of Terb and DMT, both as hydrochloride salt (1 mg/ml base) and I.S. were prepared in methanol and stored at 4°C. Working solutions were made by appropriate dilutions in Milli Q water or in human plasma, as required. All the results given in this report are calculated as the base equivalent.

2.3. HPLC

The chromatographic system consisted of a Model 302 pump (Gilson, Villiers le Bel, France) and a Model 231 autosampler (Gilson). The

column was a reversed-phase Pecospher C_{18} (10 cm × 4.6 mm I.D., 3 μm particle size, (Perkin–Elmer, Norwalk, CT, USA). The detector was a variable-wavelength UV–Vis detector, Model 481 (Waters Millipore, Milford, USA). The wavelength of this detector was set to 224 nm, with a response time of 1 s; the range of detection varied from 0.01 to 0.04 AUFS, depending on the quantity injected. Data acquisition was performed using the Hewlett-Packard LAS (Hewlett Packard, Palo Alto, CA, USA). The mobile phase was prepared by mixing an aqueous solution of triethylamine 0.012 *M* (3.35 ml/2 l of water) + orthophosphoric acid 0.020 *M* (2.65 ml/2 l of water) with acetonitrile (50 : 50, v/v). Elution was performed isocratically at room temperature at a flow-rate of 1 ml/min. The mobile phase was filtered through 0.6-μm membrane filters (type RC 59, Schleicher and Schuell, Dassel, Germany) before use.

2.4. Procedure for sample preparation

A 100-μl volume of I.S. solution (5 ng/μl in water) was added to 0.5 ml of plasma. After addition of 1.0 ml of 0.2 *M* borate buffer (pH 9) and 8 ml of *n*-hexane, the tubes were horizontally shaken (200 rpm) for 25 min. After centrifugation (10 min at 2000 *g*), a 7-ml aliquot of the supernatant was transferred to a tapered glass tube.

A volume of 1 ml of 0.5 *M* sulphuric acid–2-propanol (85:15, v/v) solution was added to the aliquot of hexane, and a second shaking was done for 15 min. After 5 min of centrifugation at 2000 *g*, the upper organic phase was discarded and 800 μl of the aqueous phase were carefully transferred into an ambered glass mini-injection vial. Finally, 250 μl of this sample was injected onto the analytical column.

2.5. Recovery

The recovery of Terb and DMT together, from human plasma was determined in duplicate at concentrations within the range of the calibration curve (2–2500 ng/ml). Blank plasma was spiked with known amounts of the two analytes and I.S., and results were compared with direct injections of the analytes onto the HPLC system.

2.6. Precision and accuracy

Inter-assay precision was studied in duplicate using spiked plasma samples that were independently analysed 9–21 times within a 68-day period. These spiked plasma samples were assayed together with unknown samples and were distributed among these samples on the autosampler. From the same experiments, accuracy was evaluated by comparing the calculated concentrations with the expected concentrations of the analytes. Intra-day precision and accuracy were assessed by using 10 samples spiked at two different concentrations (22 and 222 ng/ml of each compound), which were assayed within the same run.

2.7. Limit of quantification

The minimum amount of quantifiable analyte was derived from inter-assay experiments with spiked plasma samples. The LOQ was defined as being the lowest quantity of analyte determined with a precision and an accuracy equal to or better than 20%.

2.8. Stability

The stability of Terb and DMT metabolite was tested with spiked plasma samples stored at −18°C. They were assayed in duplicate on the day of preparation (day 1) and thereafter periodically during a period of 68 days.

2.9. Clinical study

The method described here for the determination of Terb and DMT in plasma was used in several pharmacokinetic studies. Typical results are presented. Plasma samples from healthy subjects were extracted like control samples. After collection and separation of the plasma, they were stored in a freezer at −18°C until their analysis.

3. Results and discussion

In previous methods, sample pretreatment consisted either of a simple protein precipitation or of a one-step solvent extraction with subsequent solvent evaporation. The method described here was developed for UV detection instead of electrochemical detection. For this reason and also because better sensitivity was needed, more extensive sample pretreatment was needed. The second step, back-extraction into an acidified aqueous phase, allowed direct injection onto the HPLC system and avoided time-consuming evaporation. However, back-extraction of the analytes into 100% aqueous solvents was poor. Therefore, investigations were undertaken with water-miscible solvents, and finally 2-propanol was chosen due to optimal extraction yields obtained. The 0.5 *M* sulphuric acid–2-propanol mixture was quite suitable for direct large-volume injection onto the reversed-phase column. The extraction recovery for Terb, DMT and I.S. were found to be 80, 95 and 92%, respectively (see Table 1). Recoveries of the analytes were reproducible throughout the calibration curve. It can be noticed that low extraction yields of Terb and DMT (100 ng/ml and 1250 ng/ml) are balanced by low extraction yields of I.S. This point shows the importance of I.S. addition.

The two-step extraction of the plasma gave very clean chromatograms (Figs. 2 and 3). Concerning chromatography, it can be seen that lipophilic compounds having an alkylated nitrogen can easily be separated on reversed-phase columns with an acetonitrile mobile phase. Triethylamine was added to the mobile phase to avoid peak tailing due to analyte interaction with free silanol groups. When setting up a new column, the combination of acetonitrile and water had to be optimised. It was observed that, depending on the batch of the column, changes in the proportion of acetonitrile (2–5%) could be necessary to maintain good separation, particularly for the separation of parent drug and internal standard. However, the Pecospher column used in this system was particularly reliable, and more than three thousand injections could

Table 1
Recovery of Terb and DMT from human plasma samples

Spiked concentration (ng/ml)	DMT recovery (%)	Terb recovery (%)	I.S.[a] recovery (%)
2	60.1	98.5	75.8
2	113.0	104.2	94.6
4	98.1	87.6	94.7
4	103.8	85.6	94.9
10	108.5	80.7	96.7
10	94.9	79.9	96.3
50	97.3	70.0	97.5
50	97.2	71.7	97.9
100	81.8	69.2	86.0
100	97.3	75.4	94.9
250	98.2	74.9	96.2
250	96.5	73.6	93.6
1000	100.0	81.0	95.1
1000	100.2	81.5	94.7
1250	93.5	72.4	92.3
1250	72.1	58.7	71.5
2500	97.0	80.7	95.2
2500	97.8	79.1	91.7
Mean	94.8	79.2	92.2
S.D.	12.4	10.6	7.3
C.V. (%)	13.0	13.3	7.9
S.E.M.	2.9	2.5	1.7
Minimum	60.1	58.7	71.5
Maximum	113.0	104.2	97.9
n	18.0	18.0	18.0

[a] For I.S., no change of concentration.

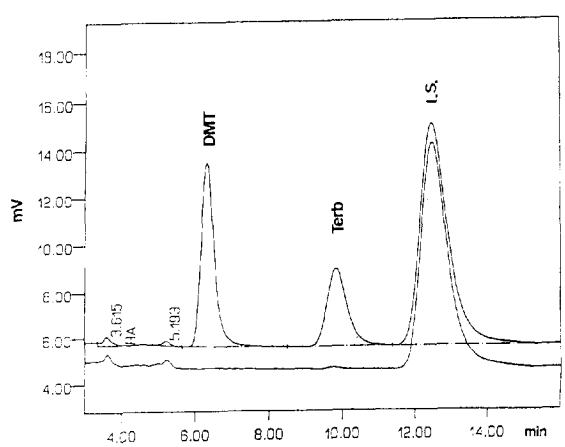

Fig. 2. Typical chromatograms of a spiked plasma sample containing 250 ng/ml of Terb and DMT, and 500 ng/ml of I.S., versus a drug-free human plasma sample.

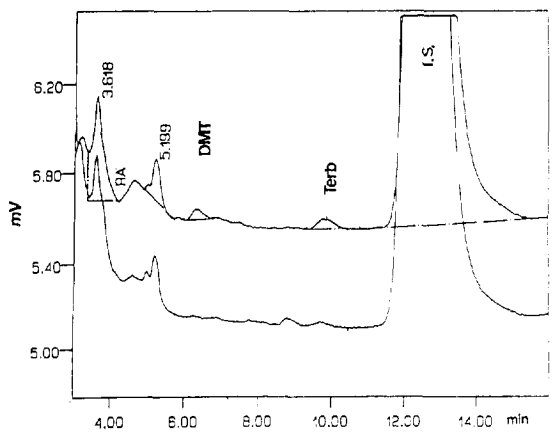

Fig. 3. Typical chromatogram of a spiked plasma sample close to the limit of quantitration, 2 ng/ml of Terb and DMT versus drug-free plasma.

be done on one column. Since its development, the method has been used for more than twenty pharmacokinetics studies and about thirty thousand samples have been assayed.

The intra-assay precision for samples containing 22 and 220 ng/ml of each compound was lower than 3.1% for Terb and better than 9.5% for DMT (Table 2). As it has been shown previously, for both compounds, addition of the I.S., improved the intra-essay reproducibility.

Inter-assay reproducibility experiments showed a mean coefficient of variation of 6.5% for the low concentration of Terb (2 ng/ml) and 9.8% for the same concentration of DMT (Table 3). A linear response for extracted plasma samples spiked with Terb and DMT was found up to

Table 2
Within-day variations of the concentrations of Terb and DMT with and without internal standard

Compound	Spiked concentration (ng.ml)	Calculation with I.S.					Calculation without I.S.
		Mean calculated concentration (ng/ml)	*n*	S.D.	Accuracy (%)	C.V. (%)	C.V. (%)
Terb	22.2	22.4	10	0.7	+0.7	3.0	6.3
	222.2	223.4	10	6.5	+0.5	2.9	10.3
DMT	22.1	22.1	10	2.1	−0.1	9.5	18.4
	220.8	220.6	9	14.5	−0.1	6.6	6.1

Table 3
Between-day variations of the concentrations of Terb and DMT

Compound	Spiked concentration (ng/ml)	Mean calculated concentration (ng/ml)	*n*	S.D.	C.V. (%)	Accuracy (%)
Terb	2	1.97	41	0.13	6.5	−1.4
	25	23.6	42	1.4	5.9	−5.6
	100	96.0	28	3.9	4.1	−4.0
	500	504.0	38	19.3	3.8	+0.8
	1000	1064.9	18	30.8	2.9	+6.5
DMT	2	2.01	38	0.2	9.8	+0.6
	25	24.4	42	1.8	7.4	−2.6
	100	97.7	28	5.9	6.0	−2.3
	500	491.4	38	27.8	5.7	−1.7
	1000	1048.8	18	83.4	8.0	+4.9

2500 ng/ml. Recent studies demonstrated a linear relationship up to 6000 ng/ml.

The lower limits of quantification in plasma were 2 ng/ml for each compound. At this concentration a good day-to-day precision was obtained (6.5% for Terb and 9.8% for DMT). From Table 4 it can be seen that Terb and DMT were stable in plasma for several weeks when stored at −18°C. Spiked and clinical samples kept frozen for one year after the first analysis, were assayed a second time without any significant change in concentrtion. Samples of extracted plasma could be left at room temperature on an autosampler for a day and a night without any decay in their concentrations. Figs. 2 to 4 show representative chromatograms of extracts of drug-free plasma spiked or unspiked with Terb, DMT and I.S. The retention times of DMT, Terb and the I.S. were 6.9, 9.9 and 12.4 min, respectively (Fig. 2). A representative extract containing concentrations close to the limit of quantification and a typical extract of plasma collected from a subject are shown in Figs. 3 and 4 respectively. The method has been successfully applied to many pharmacokinetic studies [5,6] and has proved to be reliable. Four different laboratories used it for assaying Terb and DMT in plasma samples. A representative plasma concentration–time profiles from a subject dosed orally with 250 mg of Lamisil is shown in Fig. 5. Plasma concentrations decreased from approximately 1000 to 15 ng/ml of Terb and from 800 to 15 ng/ml of DMT at 48 h postdose, but are still well within the LOQ of this assay. Due to the long terminal half-life (~20 days) of

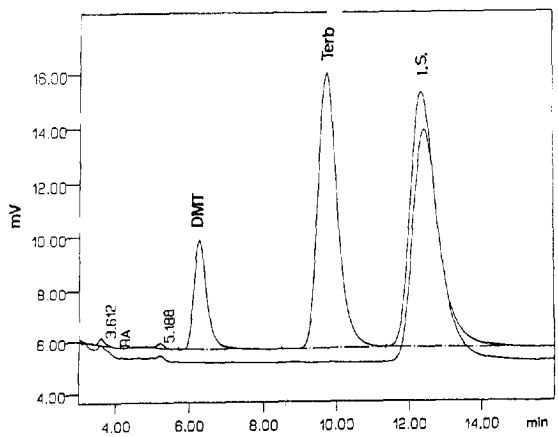

Fig. 4. Typical chromatogram of a plasma sample collected from a subject, 6 h after orally receiving 250 mg of Lamisil (130 ng/ml of DMT, 710 ng/ml of Terb) compared with plasma from the same subject before administration.

both compounds, it was necessary to obtain an LOQ low enough to follow this concentration for several days after the Lamisil administration. When very low concentrations have to be determined it is possible to optimize the HPLC system as well as the extraction (larger sample size, larger volume of injection). In this case, low concentrations (0.5 ng/ml) of both compounds can be reliably measured.

This liquid chromatographic method was also used for determination of low concentrations of Terb in nails, sebum, stratum corneum and hair. In these cases, an additional step prior to the liquid–liquid extraction was necessary [7]. Concentrations as low as 0.5 ng/mg could be de-

Table 4
Stability of Terb and DMT in human plasma stored at −18°C

Compound	Spiked concentration (ng/ml)	Concentration found (ng/ml)[a]			
		Day 0	Day 14	Day 47	Day 68
Terb	25	21.8	24.7	23.4	24.8
	500	509.5	504.0	487.1	538.2
DMT	25	27.2	23.4	24.6	24.7
	500	474.5	488.2	511.4	471.6

[a] $n = 2$ for each value.

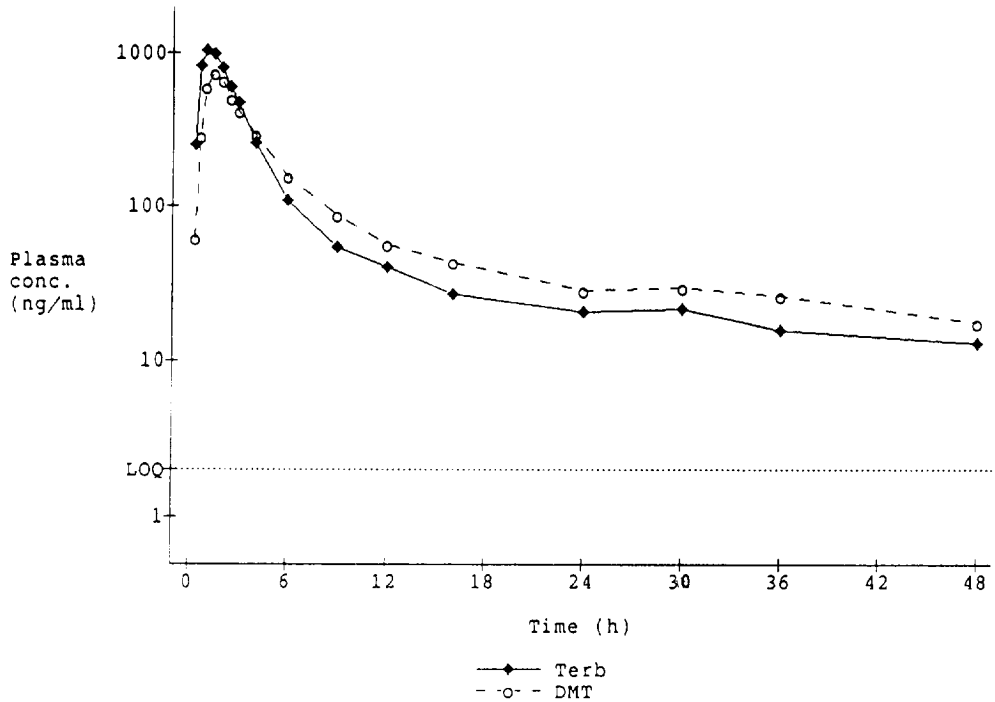

Fig. 5. Concentration–time curve for Terb + DMT in human plasma following an oral administration of 250 mg of Lamisil.

termined with good precision and accuracy in nail samples.

4. Conclusion

The HPLC method described here, was applied to the determination of Terb and DMT in plasma in many pharmacokinetic studies. It provided better sensitivity than previous HPLC assays and has been proven to be reliable and readily transferable to other laboratories. Moreover, when properly adapted, it allows precise measurement of low levels of Terb directly at the action site (nails, skin, hair).

Acknowledgements

The authors thanks Ms Cabiac and D'Amario for their technical assistance.

References

[1] G. Petrany, N.S. Ryder and A. Stütz, *Science*, 224 (1984) 1239.
[2] N.S. Ryder, *Antimicrob. Agents Chemother.*, 27 (1985) 252.
[3] F.A. Battig, M. Nefzger and G. Schulz, in R.A. Fromtling (Editor), Recent Trends in the Discovery, Development and Evaluation of Antifungal Agents, J.R. Prous, Barcelona, 1987, p. 479.
[4] F. Schatz and H. Haberl, *Arzneim. Forsch.*, 39 (1989) 527.
[5] J.C. Jensen, *J. Dermatol. Treat.*, 1 (Suppl. 2) (1990) 15.
[6] J.M. Kovarik, S. Kirkesseli, H. Humbert, P. Grass and K. Kutz, *Br. J. Dermatol.*, 126 (Suppl. 39) (1992) 8.
[7] J. Faergemann, H. Zehender, J. Denouël and L. Millerioux, *Acta Derm.-Venerol.*, 73 (1993) 305.