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Microbiological assay for terbinafine hydrochloride in tablets and creams

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Abstract

The optimization of a microbiological assay, applying the cylinder-plate method, for the determination of the antifungal terbinafine hydrochloride is described. Using a strain of *Aspergillus flavus* ATCC 15546 as the test organism, terbinafine hydrochloride at concentrations ranging from 0.125 to 0.5 μg ml^{-1} could be measured in tablets and creams. A prospective validation of the method showed that the method was linear ($r = 0.9999$), precise (intra-day: CV = 0.48%-tablets and 0.43%-creams; inter-day: CV = 0.98%-tablets and 0.64%-creams) and accurate (it measured the added quantities). The method shows results that confirm its precision, not differing significantly the others methods described in the literature. We conclude that the microbiological assay is satisfactory for quantitation of in vitro antifungal activity of terbinafine.

Keywords: *Aspergillus flavus*; Microbiological assay; Cylinder-plate method; Terbinafine

Terbinafine hydrochloride (TH) is a recent orally and topically active allylamine antifungal agent. Chemically TH is (*E*)-*N*-(6,6-dimethyl-2-hepten-4-ynil)-*N*-naphtalenemethanemine hydrochloride. The *trans* configuration is essential for in vivo and in vitro antifungal activity. This drug has broad spectrum in vitro activity against yeast, dimorphic fungi, dematiaceous fungi, moulds and dermatophytes (Petrany et al., 1984; Stütz, 1987; Balfour and Faulds, 1992; Nussbaumer et al., 1995; Abdel-Rahman and Nahata, 1997). Assays reported in the literature for the determination of TH in biological fluids include HPLC (Schatz and Haberl, 1989; Denouël et al., 1995; Zehender et al., 1995) and agar diffusion bioassay (Kan et al., 1986; Häuser et al., 1988). For the measurements in tablets formulations, the literature has reported UV-spectrophotometric method (Wang, 1996). A HPLC (Cardoso and Schapoval, 1999a) and UV-spectrophotometric (Cardoso and Schapoval, 1999b) methods are described to determine TH in tablets and creams. The microbiological assay for the determination of TH in pharmaceutical formulations has not been reported yet. Assay methods for antifungal compounds have followed the

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patterns established for the antibacterial antibiotics, measuring their effects on growth of the test organisms by turbidimetry, agar dilution and agar diffusion (Simpson, 1963). The objective of the present study was to find a sensitive and reproducible agar diffusion method to quantify TH in tablets formulations as well as in creams.

1. Materials and methods

TH substance reference (assigned purity 99.7%) was obtained from Galena (São Paulo, Brazil) while pharmaceuticals containing terbinafine were obtained commercially. Terbinafine tablets were claimed to contain 125 mg (as base) of the drug and the cream to contain 1% (as hydrochloric salt). Analytical reagents grade chemicals were used.

1.1. Organism and inoculum

The cultures of *Aspergillus flavus* ATCC 15546 (obtained of INCQS-Rio de Janeiro-Brazil) were cultivated on Sabouraud dextrose 4% agar (Oxoid) at 25°C for 7 days. The spores were harvested with a spatula, suspended in Sabouraud dextrose 4% broth using a glass homogenizer and filtered through sterile gauze. A diluted cultures suspensions of $25 \pm 2\%$ turbidity were obtained at 580 nm, using a suitable spectrophotometer (Analyser-Model 800, São Paulo, Brazil) and a 10 mm diameter test tube as an absorption cells against Sabouraud Dextrose 4% broth as blank. Portions of 1.5 ml of the inoculated Sabouraud dextrose 4% broth were added to 100 ml of Sabouraud dextrose agar at 47 ± 2°C and used as inoculated layer.

1.2. Preparation of TH reference substance

The standard solution in methanol (1 mg ml^{-1}) was diluted in 0.1 M potassium phosphate buffer, pH 4.5 and assayed at concentrations of 0.125, 0.25 and 0.5 μg ml^{-1}.

1.3. Preparation of the samples

- *Tablets:* The tablets were weighed and pulverized. An amount of powder equivalent to 50 mg of TH was transferred to 100 ml volumetric flask with 50 ml methanol and shaken for 30 min, followed by making up to volume with methanol. After filtration, the dilutions were made with 0.1 M potassium phosphate buffer, pH 4.5, to give a final concentrations of 0.125, 0.25 and 0.5 μg ml^{-1}.
- *Creams:* The samples of creams were prepared by the same method used for tablets, except that the weighed quantity of cream was equivalent to 25 mg of TH and was transferred to 50 ml volumetric flask with 30 ml of methanol.

1.3.1. Cylinder-plate assay

The agar was composed of two separate layers. The Sabouraud dextrose 4% agar (20 ml) was poured into 100×20 mm petri dish for the base layer. After solidification this layer portions of 5 ml of inoculated sabouraud 4% dextrose agar was poured to the base layer. Six stainless steel cylinders of uniform size ($8 \times 6 \times 10$ mm) were placed on the surface of inoculated medium. Three alternated cylinders were filled with 200 μl of reference concentrations solutions and the other three cylinders with the concentrations samples solutions. Ten plates were performed for each sample. After incubation (30°C for 24 h) the zone diameters (in mm) of the growth inhibition were measured using a caliper (Mitutoyo).

1.3.2. Calculation

To calculate the activity of TH in tablets and creams the Hewitt equation was used. The assay were statistically calculated by the linear parallel model and by means of regression analysis and verified using analysis of variance (Hewitt, 1977; British Pharmacopoeia, 1993; USP, 1995; European Pharmacopoeia, 1997).

1.3.3. Method validation

The method was validated by determination of linearity, precision and accuracy (USP, 1995; ICH, 1996)

- Linearity: in order to assess the validity of the assay three doses of the reference substance and three doses of the sample were used. The calculation of regression line by the method of least squares was employed.
- Precision: repeatability (intra-assay) and intermediate precision (inter-assay) were determined. Method repeatability was studied by assaying samples of tablets and creams, at same concentration, during the same day and under same experimental conditions. The intermediate precision was evaluated by comparing the assays on different days.
- Accuracy: was determined by adding known amounts of TH reference substance to the samples at the beginning of the process. An accurately weighted amounts of creams and tablets equivalent of 50 mg TH were placed in three 100 ml volumetric flask where 5.0, 10.0 and 20.0 ml of TH reference solution (240 μg ml^{-1}) were added. Methanol (50 ml) were added and the flasks were shaken for 30 min, followed by making up to volume with methanol. After filtration, the dilutions were made with 0.1 M potassium phosphate buffer, pH 4.5, to give a final concentrations of 102.4, 104.8 and 109.6%, respectively, of the sample concentrations used in the assay. The solutions were submitted the cylinder-plate assay described above. The percentage recovery of TH reference added was calculated using the formula proposed by AOAC (1990).

2. Results and discussion

The activity of antimicrobial agents may be demonstrated under suitable conditions by their inhibitory effect on microrganisms (USP, 1995). The activity of terbinafine in vitro has been tested against various microrganisms. MICs for various fungal organisms have been reported. These studies have shown that terbinafine has potent in vitro activity against dermatophytes, *Aspergillus* species and *Sporothrix schenkii* (Shadomy et al., 1985; Balfour and Faulds, 1992; Fromtling, 1992; Abdel-Rahman and Nahata, 1997). A bioassay using *Aspergillus flavus* (Häuser et al., 1988) and *Trichophyton mentagrophytes* (Shadomy et al., 1985) has been described to determine terbinafine in serum. Although the microbiological assay methods involve many steps and are time consuming, they can reveal subtle changes not demonstrable by chemical methods (USP, 1995). There are no official microbiological cylinder-plate assays described in the official codes to determine TH in pharmaceutical formulations. In this work experimental $3+3$ design using three dose levels for each standard and sample were used following the procedure described in British (1993), European (1997) and Brazilian (1988) Pharmacopoeias. The calculation procedure normally assume a direct relationship between the observed zone diameter and logarithm of applied dose. The corresponding mean zone diameters for reference solutions were: 12.5 ± 0.05 (CV% = 1.2) for low dose; 16.5 ± 0.35 (CV% = 0.81) for medium dose and 20.4 ± 0.07 (CV% = 0.93) for high dose. Corresponding zone diameters ranged between 12.2–12.7, 16.3–16.7 and 20.2–20.7 for concentrations of 0.125, 0.25 and 0.5 μg ml^{-1}, respectively (Table 1). The calibration curves for TH were constructed by plotting log of concentrations (μg ml^{-1}) versus zone diameter (mm) and showed good linearity on the 0.125 to 0.5 μg ml^{-1} range (Fig. 1). The representative linear equation for TH was $y = 5.7275 \times \ln x + 11.262$, where x is log dose and y is zone diameter. The coefficient of regression was

Table 1
Results of diameter zone of inhibition for terbinafine hydrochloride, reference solutions

Concentration (μg ml^{-1})	Mean diameter zone of inhibition (mm) ± S.E.M	Range of zone size	CV%
0.125	12.5 ± 0.05	12.2–12.7	1.20
0.25	16.5 ± 0.35	16.3–16.7	0.81
0.5	20.4 ± 0.07	20.2–20.7	0.93

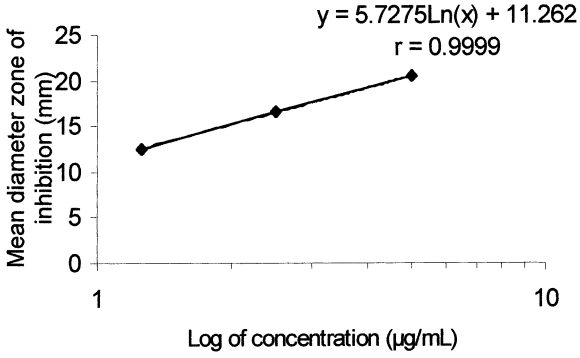

Fig. 1. Log of concentrations (µg ml^{-1}) versus mean diameter zone (mm) from the assay of TH by 3 + 3 design. The best fit line calculated by the method of least squares is shown.

Table 2
Data obtained in the analysis of terbinafine hydrochloride in tablets and creams using the microbiological assay

Sample[a]	Experimental amount (mg)[b]	% Level	CV (%)
Tablets	124.20	99.36	0.98
	123.18	98.54	
	125.90	100.72	
	126.26	101.01	
	125.14	100.11	
	124.60	99.68	
Creams	9.87	98.65	0.64
	9.95	99.54	
	9.95	99.54	
	9.98	99.84	
	10.06	100.63	
	10.11	101.06	

[a] Theoretical amount: 125 mg per tablet and 10 mg per g of cream.
[b] Mean of duplicate samples.

$r = 0.9999$. The experimental values obtained for the determination of TH in samples are present in Table 2. According British, European and Brazilian Pharmacopoeias, if a parallel-line model is chosen, the two log dose–response lines of the preparation to be examined and the reference preparation must be parallel and they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. The assays were validated by means of the analysis of variance, as described in these official codes. There are no deviation from parallelism and linearity with results obtained here ($P < 0.05$). The precision and accuracy of the assay were demonstrated. The precision is usually expressed as the variance, standard deviation or coefficient of variation (CV%) of a series of measurements (ICH, 1996). The repeatability shows mean coefficient of variation of 0.48% for tablets and 0.43% for creams indicating good intra-day precision of the method. The results obtained in different days shows coefficient of variation of 0.98% for tablets and 0.64% for creams (Table 2). The accuracy expresses the agreement between the accepted value and the value found (ICH, 1996). The mean recovery were found to be 102.0% for tablets and 100.60% for creams (Table 3). The results obtained with cylinder plate assay are comparable with declared amounts and with those obtained by HPLC and UV spectrophotometry. Analysis of variance indicated no significant difference between these methods ($P < 0.05$). To summarize, the results indicate that this method is an acceptable alternative method for the routine quality control of TH in the formulations studied.

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Table 3
Experimental values obtained in the recovery test for TH samples in the form of tablets and creams

Sample	Amount of reference (mg)		% Recovery[a]
	Added	Recovered	
Tablets	2.4	2.43	101.25
	4.8	4.90	101.88
	9.6	9.90	103.13
Creams	2.4	2.42	100.83
	4.8	4.80	100.00
	9.6	9.70	101.04

[a] Mean of three replicate analysis.

References

Abdel-Rahman, S., Nahata, M., 1997. Oral terbinafine: a new antifungal agent. Ann. Pharmacother. 31, 445–456.

AOAC, 1990. Official Methods of Analytical Chemists of AOAC, 15th edn. XVII.

Balfour, J., Faulds, D., 1992. Terbinafine — a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in superficial mycoses. Drugs 43, 259–284.

Bristish Pharmacopoeia, 1993. International edition.

Cardoso, S.G., Schapoval, E.E.S., 1999. High-performance liquid chromatographic assay of terbinafine hydrochloride in tablets and creams. J. Pharmac. Biom. Anal. 19, 809–812.

Cardoso, S.G., Schapoval, E.E.S., 1999b. UV-spectrophotometry and nonaqueous determination of terbinafine hydrochloride in dosage forms. J. AOAC International 82(4), 830–3.

Denouël, J., Keller, H.P., Schaub, P., Delaborde, C., Humbert, H., 1995. Determination of terbinafine hydrochloride and its desmethyl metabolite in human plasma by high-performance liquid chromatography. J. Chromatogr. B 663, 353–359.

European Pharmacopoeia, third ed., 1997.

Farmacopéia Brasileira, 4 ed. Atheneu, 1988.

Fromtling, R.A., 1992. Terbinafine (lamisil). Drugs Today 7, 501–508.

Häuser, M., Schmitt, H.J., Bernard, E.M., Armstrong, D., 1988. A new bioassay for terbinafine. Eur. J. Clin. Microb. Infect. Dis. 7, 531–533.

Hewitt, W., 1977. Microbiological Assay: An Introduction to Quantitative Principles and Evaluation. Academic Press, New York.

Validation of Analytical Procedures. In: Proceedings of the International Conference on Harmonisation (ICH). Commission of the European Communities, 1996.

Kan, V.L., Henderson, D.K., Bennett, J.E., 1986. Bioassay for SF 86-327, a new antifungal agent. Antimicrob. Agents Chemother. 30, 628–629.

Nussbaumer, P., Leitner, I., Mraz, K., Stütz, A., 1995. J. Med. Chem. 38, 1831–1836.

Petrany, G., Ryder, N.S., Stütz, A., 1984. Allylamine derivatives: new class of synthetic antifungal agents inhibiting fungal squalene epoxidase. Science 224, 1239–1241.

Schatz, F., Haberl, H., 1989. Analytical methods for the determination of terbinafine and its metabolites in human plasma, milk and urine. Arzneim. Forsch. 39, 527–532.

Shadomy, S., Espinel-Ingroff, A., Gebhart, J., 1985. In-vitro studies with SF-327, a new orally active allylamine derivative. Sabouraudia 23, 125–132.

Simpson, J.S., 1963. Microbiological assay using large plate methods. In: Kavanagh, F. (Ed.), Analytical Microbiology. Academic press, New York, pp. 122–123.

Stütz, A., 1987. Allylamine derivatives — a new class of active substances in antifungal chemotherapy. Angew. Chem. Int. Ed. Engl. 26, 320–328.

US Pharmacopoeia, 1995. The United States Pharmacopoeia. 23 ed. US Pharmacopoeia Convention, Rockville.

Wang, J., 1996. UV-spectrophotometry of terbinafine hydrochloride and its tablets. Zhongguo Yiyao Gongye Zazhi. 27, 363–365.

Zehender, H., Denouël, J., Roy, M., Le Saux, L., Schaub, P., 1995. Simultaneous determination of terbinafine (lamisil) and five metabolites in human plasma and urine by high-performance liquid chromatography using on-line solid-phase extraction. J. Chromatogr. B 664, 347–355.