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International Journal of Pharmaceutics 292 (2005) 195–199
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Microbiological assay of ketoconazole in shampoo

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Received 30 April 2004; received in revised form 10 September 2004; accepted 1 December 2004
Available online 20 January 2005

Abstract

Ketoconazole, an anti-fungal agent, is often incorporated in several pharmaceutical forms and in shampoo formulation it is known to be effective against fungal infection on the scalp. This paper describes a method to quantify ketoconazole in shampoo by comparing the cylinder plate assay and the HPLC method. The test organism used for the agar diffusion assay was *Candida albicans* ATCC 10231. Three different concentrations of ketoconazole were used for the diffusion assay. A mean zone diameter was obtained for each concentration. A standard curve was obtained by plotting the three values derived from the zone diameters. A prospective validation of the method showed that the method was linear ($r = 0.9982$), precise (R.S.D. = 2.57%) and accurate. The results obtained by the two methods were statistically evaluated by analysis of variance (ANOVA) and the results obtained indicate that there is no significant difference between these two methods.

Keywords: Ketoconazole shampoo; Microbiological assay; Cylinder-plate method; *Candida albicans*

1. Introduction

Ketoconazole (1-acetyl-4-[4-[[(2RS,4SR)-2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ymethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine) (Fig. 1) (European Pharmacopoeia, 2002) is an anti-fungal agent with topic and systemic action that can be incorporated into several pharmaceutical forms. As an example we could mention ketoconazole shampoo which is effective against seborrhoeic dermatitis as well as Pityriasis versicolor (Peter and Richarz, 1995).

Several different analytical procedures have been described for the determination of ketoconazole in pharmaceutical formulations: potentiometric (Abounassif and El-Shazly, 1989), spectrophotometric (Kedor-Hackmann et al., 1994; Kelani and Bebawy, 1997) and chromatographic (Kedor-Hackmann et al., 1994) methods. Heyden et al. (2002) and Nguyet et al. (2003) described an HPLC system for simultaneous determination of ketoconazole and formaldehyde, when the formulation contains imidazolidinylurea as a formaldehyde releasing preservative. However, there are no official microbiological cylinder-plate

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doi:10.1016/j.ijpharm.2004.12.001

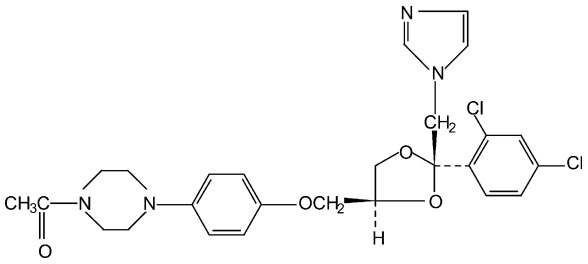

Fig. 1. Chemical structure of ketoconazole.

assays described neither in the official codes nor in the literature to determine ketoconazole in shampoo.

The activity (potency) of antibiotics may be demonstrated under suitable conditions by their inhibitory effect on microorganisms (US Pharmacopoeia, 2004). In this paper, a simple, sensitive and specific agar diffusion assay method is described for the determination of ketoconazole shampoo using an HPLC method as a comparison.

2. Materials and methods

Ketoconazole was obtained from Galena (São Paulo, Brazil). A shampoo formulation was prepared with ketoconazole 2%, pH 5.5. Ketoconazole was dissolved in hydrochloric acid, 1 N prior to the addition of shampoo. Sodium lauryl ether sulfate, coconut fatty acids diethanolamine, methyl paraben, sodium chloride and distilled water were used as excipients for shampoo preparation (shampoo base). The sample was kept in an amber glass recipient.

2.1. Preparation of the ketoconazole reference solution

Twenty-five milligrams of ketoconazole (reference substance) were transferred into a 25 ml amber volumetric flask, 1 ml HCl 1 N, 1.25 g shampoo base, 0.25 ml polysorbate 80 and 10 ml of methanol were added and shaken for 20 min, followed by sufficient quantity of methanol. After filtration, the dilutions were made with potassium phosphate buffer 1 N (1%), pH 6.0 (US Pharmacopoeia, 2004) to achieve final concentrations of 20, 100 and 500 $\mu g\,ml^{-1}$.

2.2. Preparation of the sample

The samples (ketoconazole shampoo) were prepared by weighing 1.25 g of shampoo (ketoconazole 2%) into a 25 ml amber volumetric flask, 0.25 ml polysorbate 80 and 10 ml of methanol were added and shaken for 20 min, followed by sufficient quantity of methanol. After filtration, the dilutions were made with potassium phosphate buffer 1 N (1%), pH 6.0 (US Pharmacopoeia, 2004) to achieve final concentrations of 20, 100 and 500 $\mu g\,ml^{-1}$.

2.3. Organism and inoculum

The cultures of *Candida albicans* ATCC 10231 were cultivated on Sabouraud 2% agar (Merck), maintained on slants in the refrigerator ($4 \pm 2\,^{\circ}C$) and transferred to another Sabouraud 2% agar (48 h before the assay) which was kept in incubator at 25 °C. The microorganisms were suspended in NaCl 0.9%. Diluted suspension cultures 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 cell against NaCl 0.9% as blank. Portions of 0.5 ml of the inoculated NaCl 0.9% were added to 100 ml of Sabouraud 2% agar (Merck) at $47 \pm 2\,^{\circ}C$ and used as inoculated layer.

2.4. Cylinder-plate assay

The agar was composed of two separate layers. The Sabouraud agar (20 ml) was poured into 100 mm × 20 mm Petri dishes for the base layer. After solidification of this layer, portions of 5 ml of inoculated Sabouraud 2% agar were poured to the base layer. Six stainless steel cylinders of uniform size (8 mm (o.d.) × 6 mm (i.d.) × 10 mm) were placed on the surface of inoculated medium. Three alternated cylinders were filled with 200 μl of the reference solutions (described in Section 2.1) and the other three with the samples solutions (described in Section 2.2). After incubation (37 °C for 18 h) the zone diameters (in mm) of the growth inhibition were measured using a callipers (Mitutoyo). Eight plates were used for each assay.

2.5. Calculation

To calculate the activity of ketoconazole in shampoo, the Hewitt equation was used. The assay was

statistically calculated by the linear parallel model and by means of regression analysis and verified using analysis of variance (Hewitt, 1977; US Pharmacopoeia, 2004).

2.6. Validation method

The method was validated by determination of the following operational characteristics: linearity, precision and accuracy (US Pharmacopoeia, 2004; ICH, 1996).

2.6.1. Linearity

Linearity was determined intra and inter-day. Three doses of the reference solution were used. The calculation of regression line by the method of least squares was employed.

2.6.2. Precision

Repeatability (intra-assay) and intermediate precision (inter-assay) were determined. Repeatability method was studied by assaying samples of shampoo, at the same concentration (20 mg ml^{-1}), during the same day and under same experimental conditions. The intermediate precision was evaluated by comparing the assays on two different days. The reference substance and the samples were prepared each time that the assay was performed.

2.6.3. Accuracy

The accuracy was determined by adding known amounts of ketoconazole reference substance to the samples at the beginning of the process. Amounts of 1.0, 1.5 and 1.8 ml of ketoconazole (shampoo) stock solution (2000 μg ml^{-1}) were placed in 50, 25 and 10 ml volumetric flasks, respectively, where 1.0, 1.5 and 1.8 ml of ketoconazole reference solution (500 μg ml^{-1}) were added, in this order. Dilutions were made in potassium phosphate buffer 1 N, (1%), pH 6.0 (US Pharmacopoeia, 2004), to achieve final concentrations of 50, 150 and 450 μg ml^{-1}. The recovery percentage of reference ketoconazole added was calculated using the formula proposed by AOAC (1990).

2.7. HPLC

Ketoconazole concentration analysis was performed on a Shimadzu high-performance liquid chromatograph (LC-10AD pump, UV-VisSPD-10AV detector) with the following parameters: 1 ml/min flow, UV detector at λ 225 nm, LiChrospher® column RP-8, 5 μm (150 mm × 46 mm). The system was operated at room temperature (20 ± 1°C).

The mobile phase was prepared with methanol HPLC grade, water purified (Millipore® system), monoisopropylamine and ammonium acetate with analytical grade. A solution of monoisopropylamine–methanol (2:500, v/v) (A) and a solution of ammonium acetate in water (1:200, w/v) (B) were prepared and mixed (A:B) (7:3, v/v). Final solution pH was adjusted to 5.5 with acetic acid (Staub and Bergold, 2004) The method was validated by determination of linearity, precision, specificity and accuracy (US Pharmacopoeia, 2004; ICH, 1996).

3. Results and discussion

In this experimental work a 3 × 3 design using three dose levels of each standard and sample were used, following the procedure described in the European (2002) and the Brazilian (1988) Pharmacopoeias. For the diffusion assay method, the concentration of the solutions must be chosen to ensure a linear relationship between the logarithm of the dose and the response. The corresponding mean zone diameters for the reference solutions were: 13.67 mm (R.S.D. = 4.04) for the lower dose, 17.45 mm (R.S.D. = 2.16) for the medium dose and 20.71 mm (R.S.D. = 2.34) for the higher dose (Fig. 2). The calibration curves of ketoconazole, constructed by plotting log of concentrations (μg ml^{-1}) versus zone diameter (mm) showed good linearity for concentrations of 20, 100 and 500 μg ml^{-1} (Fig. 3). The representative linear equation for ketoconazole was $y = 5.0286x + 7.2195$, where x is the log dose and y the diameter zone. The regression coefficient was $r = 0.9982$.

The experimental values obtained for the determination of ketoconazole in shampoo are presented in Table 1. According to the Brazilian (1988) and the European (2002) Pharmacopoeias, if a parallel-line model is chosen, the two log dose–response lines of the preparation must be parallel and 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

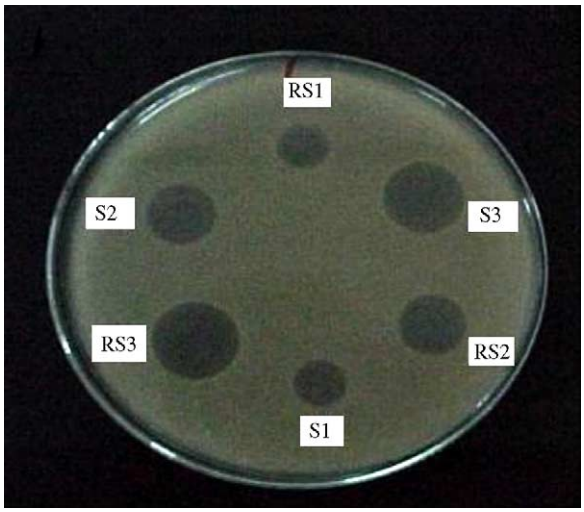

Fig. 2. Microbiological assay (cylinder plate method) using a strain of *Candida albicans* ATCC 10231 as the test organism, ketoconazole reference substance at concentrations 20 (RS1); 100 (RS2) and 500 (RS3) μg ml^{-1} and ketoconazole shampoo at concentrations 20 (S1); 100 (S2) and 500 (S3) μg ml^{-1}.

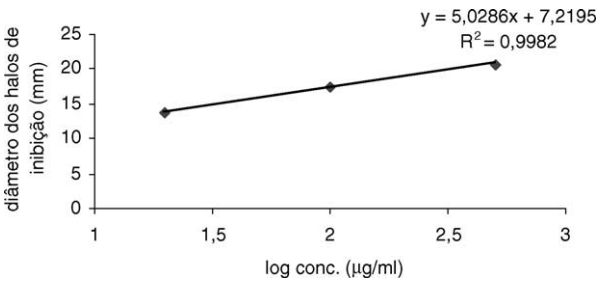

Fig. 3. Calibration curve for ketoconazole, obtained by the microbiological cylinder-plate assay.

Table 2
Recovery of a standard solution added to shampoo samples by the microbiological cylinder-plate assay

Sample	Added (μg ml^{-1})	Found (μg ml^{-1})[a]	Recovery (%)
Shampoo 1	10	9.94	99.40
Shampoo 2	30	31.71	105.70
Shampoo 3	90	90.45	100.50

[a] Each value is the mean of 10 analysis.

the analysis of variance, as described in these official codes. No deviation from parallelism and linearity were observed in our study ($p < 0.05$).

The repeatability of the method was studied; the results in the determination of ketoconazole in shampoo indicated the precision of the method with R.S.D. of 2.57%. The mean recovery was found to be 101.87% for the shampoo (Table 2).

3.1. HPLC

The calibration curve of ketoconazole (60 and 480 μg ml^{-1}) was obtained by plotting the peak area against drug concentration. The curve was linear with a linear regression coefficient of 0.9999 and a linear regression equation of $y = 56671x + 183697$. The method is precise with a coefficient of variation 0.42%. The average obtained between days was 102.60%. Recovery tests confirmed the accuracy of the HPLC proposed method (mean recovery 97.20%).

The data obtained by the microbiological assay and the HPLC method (Table 3) were statistically comparable by ANOVA test, which indicated that there is no significant difference between the two methods at $p < 0.05$.

Table 1
Data obtained in the analysis of ketoconazole in shampoo using the microbiological assay

Sample	Theoretical amount (mg ml^{-1})	Experimental amount (mg ml^{-1})	Purity (%)[a]	R.S.D. (%)
Shampoo 1	20	21.62	108.12	2.57
Shampoo 2	20	20.70	103.52	2.57
Shampoo 3	20	20.03	100.16	2.57
Shampoo 4	20	20.83	104.14	2.57
Shampoo 5	20	20.44	102.19	2.57
Shampoo 6	20	20.52	102.60	2.57

[a] Each value is the mean of eight analysis.

Table 3
Analysis of ketoconazole shampoo by two different methods

Sample	HPLC (mg ml^{-1})[a]	R.S.D. (%)	Microbiological (mg ml^{-1})[b]	R.S.D. (%)
Shampoo 1	102.23	0.29	108.12	2.64
Shampoo 2	102.94	0.39	103.52	2.71
Shampoo 3	102.90	0.41	100.16	2.77
Shampoo 4	102.42	0.39	104.14	2.06
Shampoo 5	103.13	0.25	102.19	2.48
Shampoo 6	102.01	0.52	102.60	2.46

[a] Each value is the mean of nine injections.
[b] Each value is the mean of eight plates.

4. Conclusion

The microbiological method used for ketoconazole analysis in shampoo is linear, precise and reproducible, which provides a valuable method for quality control of this drug in the studied formulation.

Acknowledgements

The authors thank CAPES, LAPPS and LEPCQ for the financial support.

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