

Comparison of microbiological assay and HPLC-UV for determination of fluconazole in capsules

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The development of a specific agar diffusion bioassay for the quantitative determination of fluconazole formulated in capsules was carried out using a strain of *Candida albicans* ATCC 18804 as the test organism. A prospective validation of the method showed adequate linearity ($r^2=0.9995$), precision (R.S.D. = 4.0% for intra-day and 4.5% for inter-day precision) and accuracy (mean recovery = 102.9%). High performance liquid chromatography was chosen as a comparison method for the fluconazole determination. The contents of fluconazole determined by both methods, for four capsule samples, showed a strong correlation, confirmed by Pearson's correlation coefficient value ($r = 0.9884$). The bioassay is a suitable method for both research and pharmaceutical industry laboratories.

Uniterms: Fluconazole/quantitative determination. Microbiological assay/agar diffusion. High performance liquid chromatography.

Este trabalho visou ao desenvolvimento e validação de um método microbiológico por difusão em ágar para quantificação de fluconazol em cápsulas utilizando o isolado *Candida albicans* ATCC 18804 como reagente biológico. O método foi validado e foi verificada linearidade ($r^2=0,9995$), precisão (D.P.R. = 4,0% para precisão intra-dia e 4,5% para precisão inter-dia) e exatidão (recuperação média = 102,9%). Concomitantemente, foi realizado o doseamento de fluconazol nas cápsulas por meio de cromatografia líquida de alta eficiência. Os teores encontrados por ambos os métodos demonstraram alta correlação, confirmada pelo Coeficiente de Correlação de Pearson ($r = 0,9884$). O ensaio microbiológico desenvolvido pode ser considerado ferramenta valiosa tanto para a pesquisa científica quanto para a rotina da indústria farmacêutica.

Unitermos: Fluconazol/quantificação. Ensaio microbiológico/por difusão em ágar. Cromatografia líquida de alta eficiência.

INTRODUCTION

Fluconazole, a triazole agent, is one of the most commonly prescribed systemic antifungals (Koks *et al.*, 1996; Marchetti *et al.*, 2001; Kim *et al.*, 2007). It is well absorbed after oral administration and shows good penetration into cerebrospinal fluid (Harris *et al.*, 1999). This agent is used in the treatment of oropharyngeal, esophageal, or vulvovaginal candidiasis, as well as other serious systemic candida infections. It is also used for

the treatment of meningitis caused by *Cryptococcus* sp. (Mathy *et al.*, 2003; Porta *et al.*, 2005). Fluconazole is available commercially for oral administration in capsules of 50, 150 and 200 mg. A sodium chloride solution of this agent for intravenous administration is also available (Porta *et al.*, 2005).

The use of this drug is widespread around the world, as the pharmaceutical industry and even compounding pharmacies produce the capsules for human usage. It is important to mention that capsules with dosage lower than that recommended are ineffective and compromise patient health. Studies focused on fluconazole determination in pharmaceutical formulation by bioassay are scarce, where the majority of papers are related to its study in clinical

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samples using a HPLC method (blood, serum, saliva or skin) (Harris *et al.*, 1989; Rex *et al.*, 1991; Garcia-Hermoso *et al.*, 1995; Koks *et al.*, 1996; Hülsewede and Dermoumi, 1996; Cohen *et al.*, 1997; Moraes *et al.*, 1999; Abdel-Moety *et al.*, 2002; Mathy *et al.*, 2003; Marchetti *et al.*, 2003; Porta *et al.*, 2005; Kim *et al.*, 2007; Lima *et al.*, 2009).

The most commonly described assay for fluconazole content in capsules are high performance liquid chromatography (HPLC), gas liquid chromatography and spectrophotometry (Harris *et al.*, 1989; Rex *et al.*, 1991; Moraes *et al.*, 1999; Porta *et al.*, 2005; Kim *et al.*, 2007). These methods require expensive equipment and considerable technician time (Rex *et al.*, 1991). Furthermore, fluconazole, as with other antibiotics, may be also quantified by bioassay for its activity assessment (Puranjoti *et al.*, 1999; Porta *et al.*, 2005), but experimental conditions such as concentrations of the inocula, incubation temperature and test microorganism are not yet well established official methods.

Previous studies focusing on fluconazole bioassay development presented methods used for determination of the antifungal levels in plasma, serum (Rex *et al.*, 1991; Porta *et al.*, 2005) and saliva (Koks *et al.*, 1996). Studies of fluconazole determination in pharmaceutical capsules have used mainly HPLC and UV (Abdel-Moety *et al.*, 2002; Porta *et al.*, 2005; Kim *et al.*, 2007), while microbiological method use is scarce. This assay can reveal subtle changes not demonstrable by chemical methods. Moreover, it gives the possibility to evaluate the potency of fluconazole, which is considered important for the analysis of antibiotics (Salgado *et al.*, 2006).

The purpose of this study was to develop and validate a microbiological method to determine the potency of fluconazole in commercial capsules. The bioassay results were compared to those obtained by HPLC, using the same samples.

MATERIAL AND METHODS

Reagents and materials

Fluconazole reference standard was kindly supplied by Pfizer. Fluconazole capsules (150 mg) were obtained from four different compounding pharmacies of Governador Valadares, Minas Gerais State, Brazil. Distilled water purified in a Millipore (Bedford, MA, USA) system was used in the analysis. Acetonitrile (HPLC grade) was purchased from Tedia (Fairfield, OH, USA) and dimethyl sulfoxide (DMSO) (analytical grade) from Synth (Diadema, São Paulo State, Brazil).

Bioassay of fluconazole

A stock standard solution of 1000 µg/mL was prepared by placing 10 mg of fluconazole reference standard into a flask and diluting in DMSO to a total volume of 10 mL. This solution was further diluted in sterile distilled water to a concentration of 625.00 µg/mL, which was used for serial two-fold dilution up to 39.06 µg/mL. The standard tested concentrations were 625.00 (S₁), 312.50 (S₂), 156.25 (R: reference concentration), 78.13 (S₄) and 39.06 (S₅) µg/ml.

A pool of each sample of fluconazole capsules was made and a stock solution of 1000 µg/mL in DMSO was prepared. This solution was diluted in sterile distilled water to a concentration of 156.25 µg/mL (identical to the standard reference concentration - R). All the fluconazole solutions (standard and test) were prepared immediately before performing the test.

Candida albicans ATCC 18804 was maintained on Sabouraud dextrose slant agar at 4 °C. Prior to use, the microorganism was transferred to Sabouraud dextrose slant agar and incubated for 24 h at 28 °C. After this period, a small portion of the yeast was transferred to a sterile saline solution (0.9%) and the transmittance was adjusted to 85% at a wavelength of 520 nm, which measured 1-5 x 10⁶ CFU/mL (Figueiredo *et al.*, 2007). Further dilution of the inocula suspension was made in 15 mL of Sabouraud dextrose agar melted at 45 °C to achieve a final concentration of 1-5 x 10⁵ CFU/mL.

Sterile Petri dishes (100 mm x 20 mm) were used in all microbiological tests. A base layer of Sabouraud dextrose agar (8 ml) was plated before the test to facilitate the visualization of inhibition zones. After base layer solidification, the next layer, to be used for inoculation, was poured into Petri dishes on top of the base layer. The agar was allowed to gel at room temperature for 10 to 15 min. After solidification, 5 mm-diameter wells were bored at six points for a 5 x 1 assay (Figure 1). Five Petri dishes were used for each assay in order to test the reference concentration (R) concomitantly with each standard or sample concentrations. Forty microliters of each standard or test solution were pipetted into individual wells. The plates were incubated at 28 °C for 24 h. The inhibition zone was measured using calipers. Assay plates were tested in triplicate (corresponding to 15 dishes in each assay), resulting in nine measures of the standards S₁, S₂, S₄ and S₅ and the test sample. The R concentration was tested forty-five times (Figure 1) in order to fit data obtained in all the dishes.

The optimization of the previously mentioned experimental conditions were achieved by testing: (i) incubation time of 24 h and 36 h; (ii) incubation temperature of

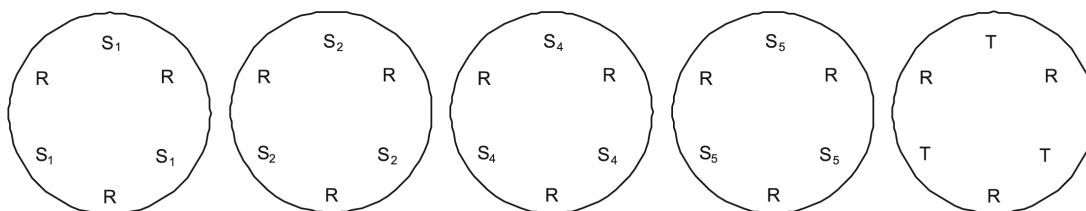


FIGURE 1 - 5 x 1 assay: five Petri dishes were used. The reference concentration (R) was tested concomitantly with all other concentrations. S₁ (625.00 µg/mL), S₂ (312.50 µg/mL), R (156.25 µg/mL), S₄ (78.16 µg/mL) and S₅ (39.06 µg/mL) represent the standard solutions. T represents the sample solution (156.25 µg/mL).

28 °C and 37 °C and (iii) inocula concentration varying from 1-5 x 10³ to 1-5 x 10⁵ CFU/mL.

Correction of the inhibition zone (IZ_c) with the reference (R) concentration

The average of all measures of R concentration (in all the dishes) was used to fit the values of inhibition zones in each individual dish.

To fit the obtained data, the following equation was applied:

$$IZ_c = IZ + (R_A - R_s)$$

where: IZ_c is the value of the corrected inhibition zone; IZ, the average value of the inhibition zone of the standard or tested sample in the studied dish; R_A, the average of the inhibition zone values for the R concentration in all dishes (a total of 45 values) and R_s, the average of inhibition zone values for the R in the studied dish (a total of nine values) (Lima *et al.*, 2009).

The data analysis was made by plotting log₁₀ of fluconazole concentration versus inhibition zones; the curve equation was obtained by regression analysis. Concentrations of test solutions were determined using the curve equation of the standard curve.

Method validation

The microbiological method was validated by evaluation of linearity, precision and accuracy, according to the procedures described in ICH guidelines Q2 (R1).

(i) Linearity: in order to assess the linearity of the assay, five concentrations of the reference standard were tested (625.00; 312.50; 156.25; 78.16 and 39.06 µg/mL). A calibration curve for log₁₀ of fluconazole concentration versus inhibition zones was plotted and the obtained data were subjected to regression analysis using the Least Squares Method.

(ii) Precision: the intra-day precision was evaluated by analyzing six replicates of fluconazole solutions (*n*=6), at 100% of the test concentration (156.25 µg/mL).

Similarly, the inter-day precision was evaluated on two consecutive days (*n*=12). The concentration of fluconazole in capsule samples was determined and the relative standard deviation (R.S.D.) was calculated.

(iii) Accuracy: this was determined by adding known amounts of fluconazole reference standard (39.06, 117.18 or 195.30 µg/mL) to a sample solution (39.06 µg/mL) at the beginning of the analyses, corresponding to 50, 100 and 150% of the test concentration. At each level, solutions were prepared in triplicate and applied to the plate assay described above. The recovery percentage of fluconazole was determined. In addition, the bioassay results were compared to a second known method (HPLC-UV).

HPLC assay for fluconazole

The HPLC analyses were carried out on a Agilent 1200 system (Santa Clara, CA, USA), composed of a quaternary pump, an auto sampler, a photodiode array detector (DAD) and HP ChemStation software. The chromatographic conditions employed were those described by Porta *et al.* (2005). The column used was a reversed-phase C₁₈ (150 × 4.6 mm I.D.; 5 µm particle size) from ACE (Aberdeen, Scotland), at 30 °C. UV-photodiode array detection was performed at 260 nm. The mobile phase was composed of acetonitrile and water (22:78), at a flow rate of 1.0 mL/min. The injection volume was 20 µL.

Approximately 25 mg of fluconazole reference standard were accurately weighed in a volumetric flask of 50 ml and dissolved in mobile phase. The flask volume was adjusted to 50 ml with the same solvent. The sample solutions were prepared by weighing a portion of the capsule's powder, equivalent to about 25 mg of fluconazole, and dissolving in mobile phase, obtaining a 500 µg/mL solution. All solutions were filtered through 0.45 µm membranes before injection.

Analysis of fluconazole capsules

Four commercial samples of fluconazole capsules were analyzed using both the developed and validated mi-

crobiological method and the HPLC method. The content results obtained by both methods were compared and the agreement between them was evaluated by the Pearson coefficient (r).

RESULTS AND DISCUSSION

Method development

In this study we developed and validated a bioassay to determine fluconazole potency in capsules. The standardization of experimental conditions is crucial to obtain reliable and reproducible results. In spite of achieving adequate results, the lack of this standardization is clearly detectable in previous studies involving potency determination of antifungals, since each paper describes a different way of performing the tests. Firstly, the presence of a base layer without the inocula provided easier-to-measure inhibition zones. The described incubation temperatures were 30 °C (Espinel-Ingroff *et al.*, 1977; Odds *et al.*, 1999), 32 °C (Purangioti *et al.*, 1999), 35 °C (Pascual *et al.*, 2007) and 37 °C (Law *et al.*, 1994; Perea *et al.*, 2000). The temperatures tested in the present paper (28 °C and 37 °C) did not yield different results, revealing that both can be used in the experiments. Published incubation times varied from 14 h (Pascual *et al.*, 2007) to 24 h (Odds *et al.*, 1999). The incubation time tested in our study (24 h) was adequate to provide sufficient yeast growth and regular inhibition zones. This was not observed when the plates were incubated for 36 h, which provided lower zones and culminated in significantly reduced concentrations (probably due to the more prominent yeast growth) which were not well correlated to the HPLC assay (data not shown).

A large number of strains have also been used as test organisms: *Candida kefyr* 706 (Garcia-Hermoso *et al.*, 1995), *Candida kefyr* ATCC46764 (Perea *et al.*, 2000), *Candida albicans* DSY1024 (Marchetti *et al.*, 2001), *Candida albicans* ATCC95020 (Pascual *et al.*, 2007) and *Sacharomyces cerevisiae* ATCC2601 (Adams, 2006). The strain *C. albicans* ATCC 18804 used in this work was considered to be adequate for fluconazole testing. In addition, it would be very helpful if researchers and industries used the same strain to perform antifungal determination so as to improve test standardization. The inocula concentration of $1-5 \times 10^5$ CFU/mL provided the best visualization of the inhibition zones compared to the inocula with 10^3 and 10^4 CFU/mL, for which zones were not clearly delineated.

In our study design, we used six wells per plate (as depicted in Figure 1), which make possible lower deviations due to the correction of the inhibition zone values by a standard concentration (R). Other researchers have

used larger plates in which 16, 36 or even 38 wells were made (Rex *et al.*, 1991; Perea *et al.*, 2000; Marchetti *et al.*, 2001; Pascual *et al.*, 2007). However, a large number of wells on a single plate makes performing the assay and the measurements of the inhibition zones more difficult. Furthermore, the excessive manipulation of each plate increases the possibility of undesirable contamination.

Bioassay

The inhibition zones were clearly delineated by using optimized testing conditions. Triplicate wells gave the same zone diameters within 1 mm. The average of the corrected inhibition zones values for standard concentrations were 16.61 mm (S_1), 13.78 mm (S_2), 10.72 mm (R), 8.07 mm (S_4) and 5.28 mm (S_5). Calibration curves were plotted and the r^2 value was > 0.9990 . DMSO, at the final concentration, did not influence the *C. albicans* ATCC 18804 growth.

Validation of bioassay

A good linear relationship ($r^2 = 0.9995$) was found between the fluconazole concentrations and growth inhibition zone diameter, in the assayed range. The regression analysis data are shown in Table I. The representative linear equation for fluconazole was $9.429x + 9.869$. The low R.S.D. value (1.04%) indicated the precision of the calibration curve.

TABLE I - Overview of the linearity data obtained for fluconazole

Parameters	Regression analysis results
Regression coefficient	0.9995
Slope \pm standard deviation	9.429 ± 0.118
Intercept \pm standard deviation	-9.869 ± 0.264
Relative standard deviation (%)	1.04
Concentration range ($\mu\text{g/ml}$)	39.06 - 625.00
Number of points	5

Precision was expressed as relative standard deviation (R.S.D.). In the intra-day precision assay ($n=6$), the mean content of fluconazole was 95.8% (R.S.D. = 4.0%). For the inter-day precision ($n=12$), the obtained mean was 96.8% (R.S.D. = 4.5%). The obtained R.S.D. values confirmed the adequate precision of the method.

Accuracy was investigated by means of a standard addition experiment, at three concentration levels, in triplicate ($n=9$). The recovery percentages varied from 101.5% to 104.4% (Table II). The mean recovery of

102.9% assured accuracy of the method. In addition, an adequate correlation with HPLC-UV method was obtained, as depicted in Table III.

TABLE II - Recovery of fluconazole in a standard addition experiment for accuracy evaluation

Sample	Added ($\mu\text{g/mL}$)	Found ($\mu\text{g/mL}$)*	%
1	39.06	40.78	104.4
2	117.18	118.95	101.5
3	195.30	200.99	102.9

* average of three determinations

TABLE III - Fluconazole contents in capsule samples obtained by bioassay and HPLC methods

Samples	Fluconazole content (%)	
	Bioassay	HPLC
1	103.9	99.1
2	95.6	90.7
3	95.5	90.7
4	103.2	100.3

HPLC assay

Samples of four commercial fluconazole 150 mg capsules were quantified in triplicate by HPLC. The obtained chromatogram (Figure 2) shows a symmetrical peak, with a tailing factor of 1.09 and retention time of

4.44 min. The fluconazole contents in each sample are presented in Table III.

Correlation of bioassay results and HPLC

The correlation between both methods was evaluated. Figure 3 demonstrates the high agreement regarding the zone inhibition of the bioassay and the peak area of the HPLC method, in five different concentrations ($r = 0.9950$). The contents of fluconazole determined by both methods, for four capsule samples, showed strong correlation, indicated by Pearson's correlation coefficient value ($r = 0.9884$).

The data in Table III indicate higher contents of fluconazole determined by bioassay in comparison to the concentrations assayed by the HPLC method. This observed deviation may be due to the considerable differences between the two distinct methods, such as experimental conditions and detection techniques. In addition, bioassays can reveal subtle changes not demonstrable by chemical methods. However, the contents of fluconazole determined by both methods showed a strong correlation ($r = 0.9884$), which is clear evidence of the correspondence in the results. Similar results were found by Warnock (1988) when comparing bioassay and HPLC for determination of itraconazole.

The bioassay, as presented, is suitable for both research and pharmaceutical industry laboratories. The major advantage of the bioassay is its low cost and relative simplicity; it is easy to perform and requires no special equipment (Rex *et al.*, 1991; Perea *et al.*, 2000). It is interesting to note that in some studies involving bioassay for fluconazole in

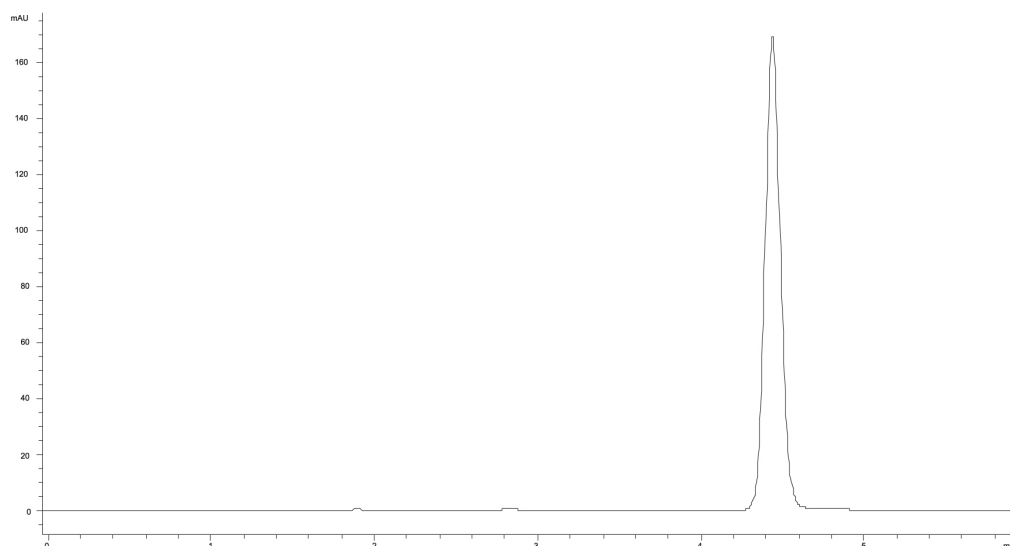


FIGURE 2 - Chromatogram obtained with acetonitrile:water (22:78), using an ACE C18 column (250 × 4.6mm I.D.; 5 μm particle size), at 30 °C, detection at 260 nm. Retention time of fluconazole peak was 4.44 min.

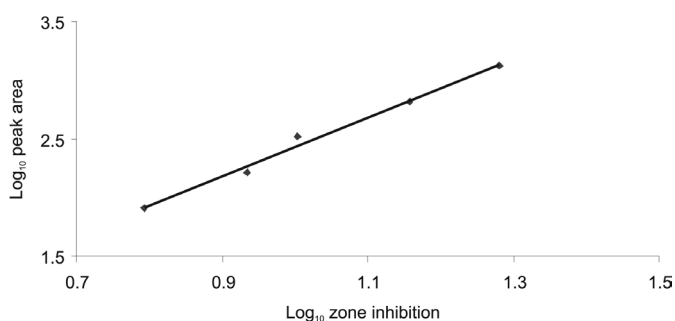


FIGURE 3 - Correlation between HPLC and bioassay: The figure demonstrates the agreement between the responses of the two tested methods ($r = 0.9950$).

plasma and sera, the r^2 values were lower than the value found in the present paper (Rex *et al.*, 1991; Hüsewede, Dermoumi, 1996). In spite of this slight difference, our results showed bioassay to be reliable, which may encourage other authors and the pharmaceutical industry to perform it for fluconazole, concomitantly with the HPLC method. This is an important step for potency verification of antifungals, even though this kind of test is not present in official pharmacopoeias (Farmacopéia Brasileira, 1988; British Pharmacopoeia, 2007; The United States Pharmacopoeia, 2007). For antibacterial drugs, pharmacopoeias recommend the performing of both HPLC and bioassays, as the tests are complementary to each other.

Bioassays play an essential role in manufacturing and quality control of antibiotic medicines (Salgado *et al.*, 2006). Therefore, the method reported here represents a simple and low cost assay to quantify fluconazole in capsules. The potential of adapting this method for use with other azole antifungals warrants further investigation.

In conclusion, the bioassay for the determination of fluconazole in capsules proved reliable when performed using an inoculum of $1-5 \times 10^5$ CFU/mL of *C. albicans* ATCC 18804 and an incubation time of 24h, at 28°C or 37°C.

REFERENCES

- ABDEL-MOETY, E. M.; KHATTAB, F. I.; KELANI, K. M.; ABOUAL-ALAMEIN, A. M. Chromatographic determination of clotrimazole, ketoconazole and fluconazole in pharmaceutical formulations. *Farmaco*, v.57, p.931-938, 2002.
- ADAMS, A. I. H.; STEPPE, M.; FRÖEHLICH, P. E.; BERGOLD, A. M. Comparison of microbiological and UV-spectrophotometric assays for determination of voriconazole in tablets. *J. AOAC Int.*, v.89, p.960-965, 1996.
- BRITISH Pharmacopoeia. London: Her Majesty's Stationary Office, 2007. p.835-836
- CLINICAL AND LABORATORY STANDARD INSTITUTE (CLSI). *M27A2*: Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard. 2.ed. Wayne: Clinical and Laboratory Standards Institute, 2002. p.1-26.
- CLINICAL AND LABORATORY STANDARDS INSTITUTE (CLSI). *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi*. Wayne: Clinical and Laboratory Standards Institute, 2002. (Approved standard M38-A).
- COHEN, L. G.; DIBIASO, A.; LISCO, S. J.; HURFORD, W. E. Fluconazole serum concentration and pharmacokinetics in an obese patient. *Pharmacotherapy*, v.17, p.1023-1026, 1997.
- ESPINEL-INGROF, A.; SHADOMY, S.; FISCHER, J. F. Bioassay for miconazole. *Antimicrob. Agents Chemother.*, v.11, p.365-368, 1977.
- FARMACOÉIA Brasileira. 4.ed. Atheneu: São Paulo, 1988. p.287.1-287.2.
- FIGUEIREDO, V. T.; SANTOS, D. A.; RESENDE, M. A.; HAMDAN, J. S. Identification and *in vitro* antifungal susceptibility testing of 200 clinical isolates of *Candida* spp. responsible for fingernail infections. *Mycopathol.*, v.164, p.27-33, 2007.
- GARCIA-HERMOSO, D.; DROMER, F.; IMPROVISIS, L.; PROVOST, F.; DUPONT, B. Fluconazole concentrations in saliva from AIDS patients with oropharyngeal candidosis refractory to treatment with fluconazole. *Antimicrob. Agents Chemother.*, v.39, p.656-660, 1995.
- HARRIS, S. C.; WALLACE, J. E.; FOULDS, G.; RINALDI, M. G. Assay of fluconazole by megabore capillary gas-liquid chromatography with nitrogen-selective detection. *Antimicrob. Agents Chemother.*, v.33, p.714-716, 1989.
- HOSTETLER, J. S.; HEYKANTS, J.; CLEMONS, K. V.; WOESTENBORGH, R.; HANSON, L. H.; STEVENS, D. A. Discrepancies in bioassay and chromatograph determinations explained by metabolism of itraconazole to hydroxyitraconazole: studies of interpatient variations in concentrations. *Antimicrob. Agents Chemother.*, v.37, p.2224-2227, 1993.

- HÜLSEWEDE, J. W.; DERMOUMI, H. Serum level determination of fluconazole by high-performance liquid chromatography and bioassay. *Zentralbl. Bakteriol.*, v.283, p.492-496, 1996.
- KEEVIL, B. G.; NEWMAN, S.; LOCKHART, S.; HOWARD, S. J.; MOORE, C. B.; DENNING, D. W. Validation of an assay for voriconazole in serum samples using liquid chromatograph-tandem mass spectrometry. *Ther. Drug Monit.*, v.26, p.650-657, 1994.
- KIM, S. S.; IM, H. T.; KANG, I. M.; LEE, H. S.; LEE, H. W.; CHO, S. H.; KIM, J. B.; LEE, K. T. An optimized analytical method of fluconazole in human plasma by high-performance liquid chromatography with ultraviolet detection and its application to a bioequivalence study. *J. Chromatogr. B.*, v.852, p.174-179, 2007.
- KOKS, C. H.; MEENHORST, P. L.; HILLEBRAND, M. J.; BULT, A.; BEIJNEN, J. H. Pharmacokinetics of fluconazole in saliva and plasma after administration of an oral suspension and capsules. *Antimicrob. Agents Chemother.*, v.40, p.1935-1937, 1996.
- LAW, D.; MOORE, C. B.; DENNING, D. W. Bioassay for serum itraconazole concentrations using hydroxyitraconazole standards. *Antimicrob. Agents Chemother.*, v.38, p.1561-1566, 1994.
- LIMA, P. M. A.; PRADO, N. D.; SILVA, M. L. M.; DINIZ, R. D. L.; QUEIROZ, K. M.; CÉSAR, I. C.; PIANETTI, G. A.; SANTOS, D. A. Determination of ketoconazole in capsules by HPLC and microbiological assay. *J. AOAC Internat.*, v.92, p.1076-1081, 2009.
- MARCHETTI, O.; MAJCHERCZYK, P. A.; GLAUSER, M. P.; BILLE, G.; MOREILLON, P.; SANGLARD, D. Sensitive bioassay for determination of fluconazole concentrations in plasma using a *Candida albicans* mutant hypersusceptible to azoles. *Antimicrob. Agents Chemother.*, v.45, p.696-700, 2001.
- MATHY, F. X.; VROMAN, B.; NTIVUNWA, D.; WINNE, A. J. D.; VERBEECK, R. K.; PRÉAT, V. On-line determination of fluconazole in blood and dermal rat microdialysates by microbore high-performance liquid chromatography. *J. Chromat. B.*, v.787, p.323-331, 2003.
- MORAES, L.A.; LERNER, F.E.; MORAES, M.E.; MORAES, M.O.; CORSO, G.; DE NUCCI G. Fluconazole bioequivalence study: quantification by tandem mass spectrometry. *Ther. Drug Monit.*, v.21, p.200-207, 1999.
- ODDS, F. C.; DUPONT, B.; RINALDI, M. G.; STEVENS, D. A.; WARNOCK, D. W.; WOESTENBORGH, R. Bioassays for itraconazole blood levels: an interlaboratory collaborative study. *J. Antimicrob. Chemother.*, v.43, p.723-727, 1999.
- PASCUAL, A.; NIETH, V.; CALANDRA, T.; BILLE, J.; BOLAY, S.; DECOSTERD, L. A.; BUCLIN, T.; MAJCHERCAYK, P. A.; SANGLARD, D.; MARCHETTI, O. Variability of voriconazole plasma levels measured by new high-performance liquid chromatography and bioassay methods. *Antimicrob. Agents Chemother.*, v.51, p.137-143, 2007.
- PEREA, S.; PENNICK, G. J.; MODAK, A.; FOTHERGILL, A. W.; SUTTON, D. A.; SHEEHAN, D. J.; RINALDI, M. G., Comparison of high-performance liquid chromatographic and microbiological methods for determination of voriconazole levels in plasma. *Antimicrob. Agents Chemother.*, v.44, p.1209-1213, 2000.
- PORTA V.; CHANG, K. H.; STORPIRTIS, S. Evaluation of the bioequivalence of capsules containing 150 mg of fluconazole. *Int. J. Pharm.*, v.288, p.81-86, 2005.
- PURANJOTI, P.; KASINA, R.; TENJARLA, S. Microbiological and HPLC analysis of miconazole in skin, serum and phase-solubility studies. *J. Clin. Pharm. Therap.*, v.24, p.445-450, 1999.
- REX, J. H.; HANSON, L. H.; AMANTEA, M. A.; STEVENS, D. A.; BENNETT, J. E. Standardization of a fluconazole bioassay and correlation of results with those obtained by high-pressure liquid chromatography. *Antimicrob. Agents Chemother.*, v.35, p.846-850, 1991.
- SALGADO, H. R. N.; LOPES, C. C. G. O.; LUCCHESI, M. B. B. Microbiological assay for gatifloxacin in pharmaceutical formulations. *J. Pharm. Biomed. Anal.*, v.40, p.443-446, 2006.
- UNITED STATES Pharmacopoeia. 30.ed. Rockville: United States Pharmacopoeial Convention, 2007. p.2137-2139

VALIDATION OF ANALYTICAL PROCEDURES: TEXT AND METHODOLOGY Q2 (R1) – ICH Harmonized Tripartite Guideline. International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, 2005. p. 1-13.

WARNOCK, D. W.; TURNER, A.; BURKE, J. Comparison of high performance liquid chromatograph and microbiological methods for determination of itraconazole. *J. Antimicrob. Chemother.*, v.21, p.93-100, 1988.

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