Fluconazol Method Validation by RP-HPLC for Determination in Biological Skin Matrices

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Abstract

The bis-triazole antifungal fluconazole (FCZ) is used in the systemic treatment of superficial mycoses. The inconvenience of drug interactions and incidence of adverse reactions occurs in approximately 16% of patients, despite several advantages against systemic fungal infections. Because its pharmacokinetics profile is favorable to cutaneous accumulation, it presents a prominent importance in the treatment of superficial mycoses. This study shows FCZ method validation by reversed-phase high-performance liquid chromatography in the linear range of 2 to 32 µg/mL, which suitable for application in biological matrices after topical permeation studies. The method is tested in simulated FCZ alcoholic solution applied to skin extracts after in vitro permeation studies using Franz cells. Recovery shows good results (in the range 75.0% ± 4.1% to 82.0% ± 6.6%) regarding the biological matrices.

Introduction

Fluconazole (FCZ) is a bis-triazole antifungal drug, which has been used in the systemic treatment of superficial mycoses, cutaneous, and cutaneo-mucous (dermatomycoses and dermatophytoses). FCZ presents several advantages in the systemic treatment of fungal infections. However, drug interactions and incidence of adverse reactions in approximately 16% of patients is an inconvenience (1–3). The FCZ pharmacokinetics profile favors cutaneous accumulation, and for this reason, a topical administration for the treatment of superficial mycoses can be an interesting alternative to overcome this problem. Because superficial mycoses have been treated topically, it traditionally occupies a prominent place in therapy to treat acute lesion with a limited extension. Thereafter, it is relevant to develop a suitable FCZ quantitation method for application in biological matrices.

Synthesized in 1982, FCZ is a polar bis-triazole and is less lipophilic (4) than the other azoles (e.g., ketoconazole, itraconazole, and miconazole) (Figure 1). The presence of a halogen–phenyl group increases its antifungal activity and aqueous solubility, leading to higher bioavailability (5). FCZ determination has mostly been reported by liquid chromatography (LC) and gas chromatography (GC). Few compendia include monographs for FCZ raw material associated with it, for instance, to chromatographic or non-aqueous assay analyses (6,7) or its pharmaceutical dosage forms associated to UV assay (7).

In the literature, complex sample preparation procedures for the determination of FCZ have been reported for biological matrices such as plasma, serum, cerebrospinal liquid, and tissue (epidermis and dermis). The use of HPLC in correlation to a microbiological determination of FCZ in plasma samples, serum, and cerebrospinal liquid have been investigated by Rex et al. (8) and Madu et al. (9). Both groups determined FCZ using a reversed-phase (RP) column (RP18, 5-µm particle size, with an isocratic mobile phase of methanol–0.025 mol/L phosphate buffer, 45:55, v/v), pH 7.0, 1.0 mL/min, 260 nm UV detector at 25°C. In these studies, separation of the samples was performed by a very laborious procedure of extractions, centrifugation, and back-extraction with organic solvent. After solvent evaporation, the final residue was dissolved in the mobile phase and injected. The detection limit was 0.1 µg/mL for both methods. On the other hand, FCZ GC–electron capture detection (ECD) determination has been reported by Faergemann and Laufen (10) in human dermis and epidermis (without stratum corneum/SC, by stripping the skin with adhesive tape several times) with greater sensitivity in results. In 1999, Faergemann (11) also determined FCZ pharmacokinetics in human fingernails and skin. Likewise

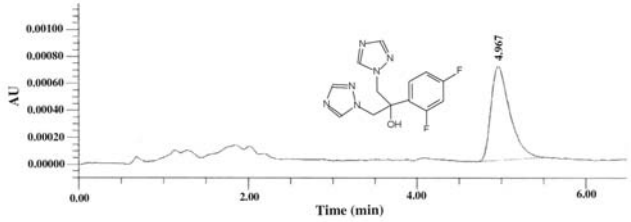

Figure 1. FCZ standard solution (2.09 µg/mL) chromatogram eluted from Lichrospher RP18 column (250 × 4 mm, 5 µm) with mobile phase methanol–0.025 mol/L PBS (45:55, pH 7.0, 1 mL/min flow) and 260 nm at circa of 5 min.

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sample pre-treatment was obtained through very laborious back extraction and clean-up processes. Nonetheless, the highly sensitive limit of quantitation (LOQ) yielded (0.025 µg/g) was fourfold lower than those obtained with high-performance liquid chromatography (HPLC) analysis (9,10). Recently, Mathy et al. (12) published a microbore RP-HPLC method using a diammonium phosphate–acetonitrile mobile phase (75:25, pH 7.0) and an UZ-view capillary flow cell (30-mm path length, LC Packings) at 210 nm detection. Good linearity, LOQ, and limits of detection (LOD) (0.15 to 20 µg/mL; 0.15 and 0.1 µg/mL, respectively) were obtained. It is noteworthy to mention that the method was applied to FCZ determination in blood and dermal rat microdialysates (probes implanted in the jugular vein and dermis) after an IV bolus in the awake, freely moving animals.

This work aimed at an RP-HPLC method validation for FCZ determination for application in biological animal skin matrices using in vitro permeation studies in Franz cells.

Experimental

Material

The following materials were used: fluconazol reference standard (Cadila Healthcare, India) and methanol (J.T. Baker, Phillipsburg, NJ); acetonitrile, concentrated hydrochloric acid, monobasic sodium phosphate monohydrate; dibasic sodium phosphate heptahydrate, sodium hydroxide (Merck, Darmstadt, Germany); 95%, v/v, ethanol (Vetec Química Fina, Brazil); 0.05 mol/L, phosphate buffer saline pH 7.4 as Franz cells receptor fluid (0.90% monobasic sodium phosphate monohydrate; dibasic sodium phosphate heptahydrate; 0.02% monobasic potassium phosphate; 0.8% sodium chloride; 0.01% mercurium chloride II) minisart SRP 15 minifilter (13 mm); and cellulose membrane (47 mm) with 0.45-µm porosity (Sartorius, Germany). Hairless male mice (HRS/J, 60 to 70 days old) were obtained at a local animal facility, and pig ears (± 4 months old pigs) were obtained at a local butcher.

FCZ ethanolic solution and an O/A FCZ emulsion prepared in-house were used as simulated drug solution and drug formulation, respectively. All chemicals and reagents were of analytical or chromatographic grade and used without ulterior purification.

An ultra-turrax agitator (T25 basic, Ika Works,Wilmington, NC) was used to dilacerate animal skin at 24000 rpm. An LC equipped with a 717 plus autosampler, a 515 HPLC isocratic pump, 486 UV detector (Waters, Milford, MA) coupled to a Lichrospher RP 18 column (5 µm, 250 × 4 mm, Merck) was used. Data were acquired and analyzed by Millennium software version 2.15.01 (Waters). Ultrasonic cleaner (1400, Unique, Brazil), vacuum pump (141, 2VC, Primar, Brazil), water-bath (E-100, Lauda, Konigshofen, Germany), centrifuge (206-R Baby II, Excelsa, Brazil), vortex agitator (MS1, Ika), UV–vis spectrophotometer (UV160A Shimadzu, Tokyo, Japan), magnetic agitator (HTR 8068, Ika, Germany), and automatic pipettes (Gilson, France) were used.

Methods

Determination of FCZ was accomplished by HPLC based on previously described methods for sample preparation (8–10) [Rex et al. (1991), Madu et al. (1994), and Faergemann (1999)] in biological samples with few modifications. Chromatographic conditions were methanol–0.025 mol/L phosphate buffer solution (PBS, 45:55), pH 7.0 mobile phase sonicated for 15 min; 1.0 mL/min flow, and 260 nm UV detection at 30°C. All solutions were diluted in mobile phase, filtered through 0.45-µm pore cellulose membrane, and a 50-µL volume was injected in the chromatograph. Measurements were performed in triplicate.

Method validation

A calibration curve was prepared by transferring 1.0, 2.0, 4.0, 8.0, and 16.0 mL aliquots of a FCZ reference standard solution (100 µg/mL) to a 50-mL volumetric flask. Final concentrations obtained, 2, 4, 8, 16, and 32 µg/mL, were injected on three different days. Linear regression was performed using the minimum square method of the average of absolute peak areas versus concentration. Statistical analysis was accomplished by analysis of variance (ANOVA) of the regression for $p < 0.05$. To verify whether the three curves could be gathered in only one standard curve, the slope of the straight line was tested, assuming no statistical difference for the null hypothesis.

The precision of the method was evaluated through the repeatability (intra-day) and the intermediate precision (inter-day) (13,14). Repeatability was performed evaluating the standard deviation (SD) and the coefficient of variation (CV) of the peak areas of FCZ standard solutions. Intermediate precision was also accomplished by evaluating the CV of the peak areas of FCZ standard solutions in three different days. The requirements are met if CV for each concentration does not exceed 15%, except for the limit of quantitation (LOQ), for which larger values (up to 20%) are admitted (13). Selectivity was accomplished by evaluating possible interference of substances present in blank samples of the receptor fluid compartment of Franz cell, as well as in the hairless mice skin (HMS) or pig ear skin (PES) sample extracts after permeation studies (14) as follows.

HMS and PES sample preparation

Fragments of the back skin of hairless mice and fragments of the ear of pig were removed with the aid of a sharp knife. After withdrawal of the subcutaneous tissue, the skins were immediately mounted on Franz diffusion cells so that the dermis was in contact with the receptor fluid (phosphate buffer saline, PBS 0.05 mol/L, pH 7.4) for 1 h hydration. Thereafter, the receptor fluid was renewed, and the skin remained in the Franz diffusion cell for 8 h. Sample aliquots were collected after intervals of 2, 4, 6, and 8 h, and volume was replaced after each collection in order to maintain constant volume. After a period of 8 h elapsed, epidermis was separated from dermis with the aid of a sharp knife and distinct extraction procedures were performed for the HMS or PES as follows.

HMS

The skin (separately, epidermis, and dermis) was placed in different flasks containing 2 mL of mobile phase each, and remained in contact for a period of 12 h. Epidermis and dermis were submitted to ultra-turrax agitation (2 and 5 min, respectively). After centrifugation (5000 rpm, 5 min), an aliquot of 1 mL of the supernatant was transferred to a 15-mL glass test tube,

added to 2 mL of 1 mol/L hydrochloric acid, and vortexed for 30 s. After addition of 4 mL of ethyl acetate, agitation by vortex (2 min), and centrifugation (4000 rpm, 3 min), the aqueous phase was transferred to another 15-mL glass test tube and added to 1 mL of 1 mol/L sodium hydroxide, vortexed for 30 s, added to 4 mL of ethyl acetate, vortexed for 2 min, and once more centrifuged (4000 rpm, 3 min). The organic phase was transferred to a glass flask and evaporated to dryness under nitrogen flow at 45°C. The residue was dissolved in 2 mL of mobile phase, under vortex agitation for 2 min and injected.

PES

Epidermis and dermis, separately placed in different flasks containing 2 and 4 mL of mobile phase, respectively, stayed in contact for a period of 12 h. Epidermis was submitted to ultraturrax agitation for 2 min, and dermis was vortexed for 2 min. After both samples were centrifuged (5000 rpm, 5 min) an aliquot of 1 mL of the supernatant was transferred to a 15-mL conical glass tube, and the extraction proceeded as previously described for the HMS. Residues of the epidermis and dermis samples were dissolved in 2 and 1 mL of mobile phase, respectively, and vortexed for 2 min and injected.

The LOQ, the lowest FCZ concentration that can be accurately determined, was theoretically calculated by the equation LOQ = 10 s_a/b (s_a, standard deviation of the curve intercept *a; b*, slope of the calibration curve). It was also experimentally determined by the evaluation of the precision and accuracy of the peak areas of FCZ concentrations in the epidermis and in the dermis of hairless mice. LOQ acceptable value should yield a CV below 20% with 80.0%–120.0% accuracy (13). The LOD was experimentally accomplished by decreasing FCZ concentrations starting from 2 µg/mL. Aliquots of 0.5, 0.25, and 0.125 mL of a 100 µg/mL FCZ standard solution were transferred to 50-mL volumetric flasks. The volume was completed with the mobile phase yielding 1.0, 0.5, and 0.25 µg/mL FCZ standard solutions, injected in triplicate.

Recovery was accomplished by comparing standard FCZ concentrations found in HMS and PES with the added theoretical value as follows. The fragments of either the back of hairless mice or the pig ear were used. This procedure was accomplished in three different days. After the removal of the subcutaneous tissue, the skins were immediately assembled in the Franz cells so that the dermis was in contact with the receptor fluid (0.05 mol/L phosphate solution, PBS, pH 7.4) for 1 h hydration. Hence, the buffer saline was replaced by a new fluid, and the skin stayed in the Franz cell for 4 h. The receptor fluid was changed every 2 h. After 4 h, epidermis was separated from the dermis with the aid of a sharp knife, and they were added to 100 µL of a 100 µg/mL FCZ ethanolic standard solution. This system remained in contact for 8 h, after which the samples were transferred to a flask and added to the mobile phase. The skin fragments extraction procedure was accomplished as described for the HMS and PES, previously described for selectivity. Receptor fluid portions were either served as blank or were added to 2 µg/mL FCZ standard solution and completed with 10 mL volume with the receptor fluid.

Results and Discussion

FCZ reference standard solution (2.09 µg/mL) was scanned in the wavelength interval of 200 to 350 nm for its best UV absorption detection at 260 nm (Figure 1). The selected mobile phase was appropriate because it provided satisfactory results in regard to peak symmetry, drug and formulation solubility, and short time of analysis. FCZ chromatogram showed a retention time of 5.1 ± 0.2 min and a capacity factor (k') of 1.78.

In the calibration curve analysis, the slope test did not show statistically different values, considering the null hypothesis set for the obtained curves. The regression analysis of the average standard curve (n = 15, equation Y = 802.24 + 5393.6X) revealed appropriate linearity (r^2 = 0.9999) in the FCZ concentration interval 2 to 32 µg/mL.

Table I presents the absolute peak areas average and the standard deviation (average ± SD) as well as the CV obtained for the calibration curve and precision, in triplicate. The lowest concentration point of the curve showed a larger CV, as expected.

Table I. FCZ Standard Solutions Results* Obtained for The Calibration Curve

Concentration (µg/mL)	Average area ± SD	Response factor	CV† (%)
2.0	10351 ± 62	5176	1.8
4.0	20587 ± 93	5147	1.4
8.0	42267 ± 63	5283	0.4
16.0	85253 ± 311	5328	1.1
32.0	171934 ± 302	5373	0.5

* Results in triplicate.
† CV = coefficient of variation.

Table II. FCZ Results* for the Selectivity Evaluated in HMS and in PES

	Average area		Concentration (µg/mL)		Percentage ± SD (%)	
Solution	HMS	PES	HMS	PES	HMS	PES
2 µg/mL FCZ standard	10471	10564	2.09	2.11		
Epidermis matrix	ND†		–		–	
Dermis matrix	ND		–		–	
Epidermis + 2.09 µg/mL FCZ	10655	10504	2.12	2.10	101.6 ± 1.8	99.5 ± 1.4
CV		1.8	1.4			
Dermis + 2.09 µg/mL FCZ	10753	10474	2.14	2.09	102.5 ± 1.9	99.1 ±1.8
CV		1.8	1.8			
Blank (receptor fluid)						
Blank + 2 µg/mL FCZ	10596	10037	2.11	2.01	105.6 ± 1.2	100.5 ± 2.2
CV		1.2	2.2			

* Results in sextuplicate.
† ND = not detected.

The average peak areas obtained for repeatability yielded a CV in the range of 0.1% to 2.0% for all concentrations, a value lower than the maximum value (15%) acceptable for biological samples. Intermediate precision showed CV inferior to 2.0%, indicating the reproducibility of the method (15).

Table II shows the selectivity results for HMS and PES ranging from 99.1 ± 1.8 to 102.5 ± 1.9 for dermis and epidermis, respectively. Figures 2A–2E show the chromatograms obtained to evaluate the selectivity of the method for HMS, as well as to identify FCZ by comparing with Figure 1. The receptor fluid chromatogram (Figure 2A) does not reveal the presence of interferences in the same retention time as FCZ. The receptor fluid chromatogram (not shown) prepared by the addition of an aliquot of FCZ standard solution showed the FCZ peak elution in the expected retention time with a correspondent concentration of 2.07 µg/mL, equivalent to 103.5% FCZ compared with 2.0 µg/mL FCZ standard solution. Figures 2B and 2D refer to the chromatograms of epidermis and dermis extracts for the HMS, respectively. They do not show the presence of UV absorbing substances in the same retention time of FCZ. This evidences no interferences in the HMS solution compared with the FCZ standard solution (Figure 1). Figures 2C and 2E refer to the chromatograms of epidermis and dermis, respectively, prepared by the addition of 2.09 µg/mL FCZ standard solution. The peaks appeared in the expected retention times with respective areas corresponding to 2.14 and 2.09 µg/mL of FCZ. This illustrated an agreement of 102.4% and 100.0%, respectively, compared with 2.09 µg/mL FCZ standard solution. A little tailing in the peaks, caused in both matrices, did not jeopardize FCZ determination.

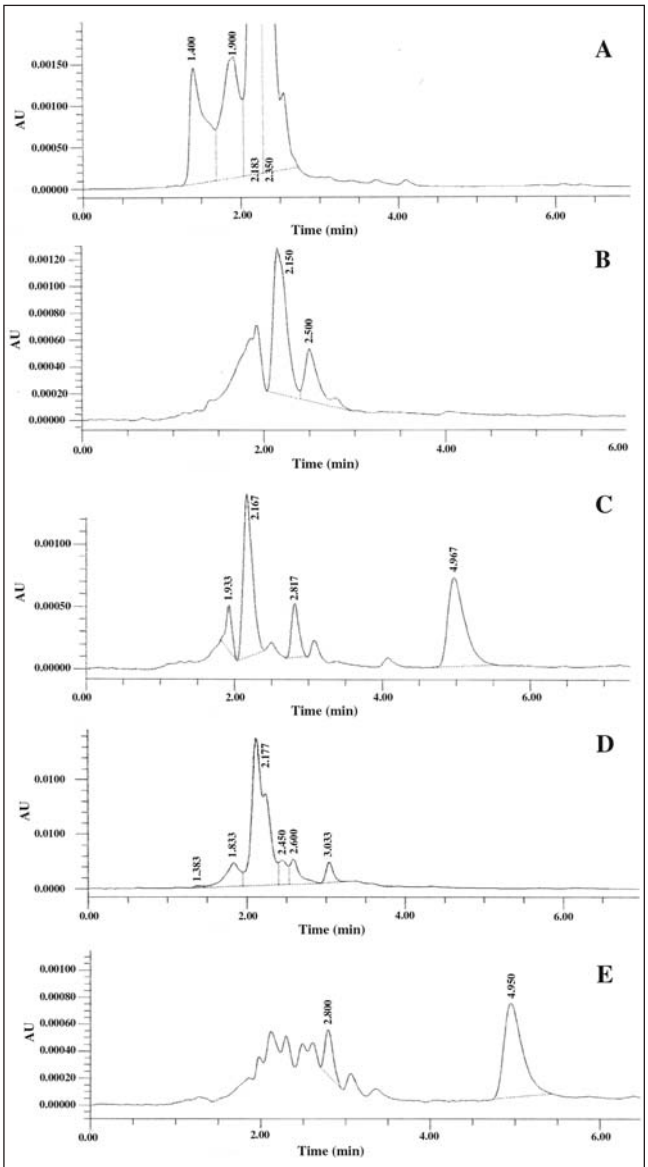

Figure 2. Representative chromatograms of skin receptor fluid (A), epidermis matrix (B), and epidermis matrix + 2.09 µg/mL FCZ standard solution (C) for HMS. Representative chromatograms of dermis matrix (D) and dermis matrix + 2.09 µg/mL FCZ standard solution (E) for HMS.

Figures 3A–3C show the evaluation of selectivity of the method obtained in the experiments with PES. Chromatograms A, B, and C (Figure 3) represent the receptor fluid, epidermis, and dermis, respectively. None of them show the presence of interferences in the UV in the same FCZ retention time.

The theoretical LOQ (2 µg/mL) calculated by the equation $LOQ = 10s_a/b$ was experimentally confirmed, showing repeatability for epidermis (CV less than 10.7% intra-day and CV equal to 6.8% inter-day) and accuracy (101.0% to 105.5%), as well as for dermis samples (CV less than 5.1% intra-day and CV equal to 4.4% inter-day) and accurate (97.6% to 103.8%) in three different days. Therefore, the results meet the requirements for CV (maximum 20%) and accuracy reproducibility (80.0% to 120.0%) (14). An LOD of 0.5 µg/mL was experimentally obtained.

Recovery was evaluated by the determination of FCZ concentration in the epidermis and dermis after 100 µL addition of a 100 µg/mL FCZ alcoholic solution and subsequent extraction. The results obtained are shown in Table III. In the hairless mice average recovery (± SD) and CV were 75.0 ± 4.1%, epidermis was

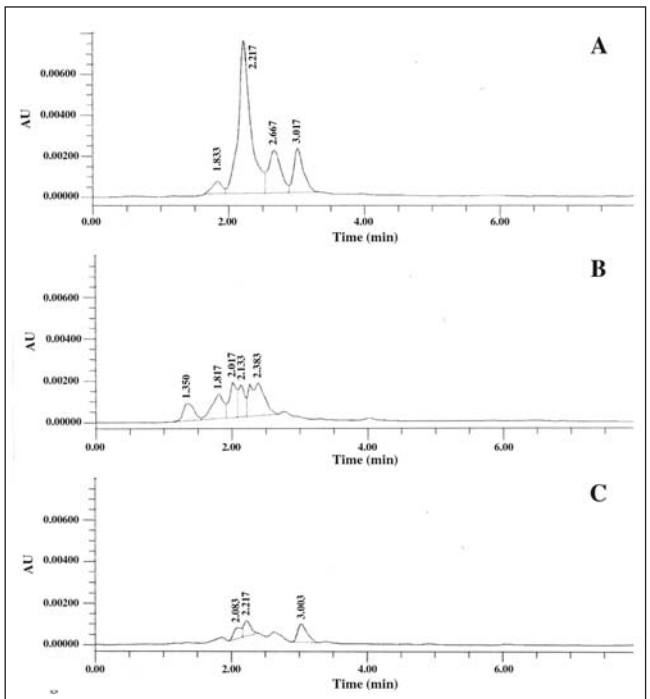

Figure 3. Representative chromatograms of epidermis matrix (A), dermis matrix (B), and receptor fluid (C) for PES.

Table III. FCZ Results* for the Recovery Test by 100 µL Addition of a 100 µg/mL FCZ Alcoholic Solution in HMS and in PES

	% Recovery	
HMS sample	**Epidermis matrix**	**Dermis matrix**
1	72.0	72.0
2	72.0	72.0
3	80.0	78.0
4	80.0	82.0
5	72.0	80.0
6	72.0	82.0
Average ± SD	75.0 ± 4.1	78.0 ± 4.6
CV (%)	5.3	5.9
PES sample		
1	82.0	76.0
2	78.0	72.0
3	84.0	88.0
4	82.0	84.0
5	82.0	84.0
6	82.0	88.0
Average ± SD	82.0 ± 2.0	82.0 ± 6.6
CV (%)	2.4	8.0

* Sextuplicate.

5.3%, and dermis was 78.0 ± 4.6% and 5.9%, respectively. For the pig ear epidermis, FCZ alcoholic solution average recovery (± SD) and CV were 82.0 ± 2.0% and 2.4% and for dermis they were 82.0 ± 6.6% and 8.0%, respectively. In spite of the larger CV value for the pig skin dermis, it still meets the limit requirement of 15.0% for the precision test (14). It is noteworthy to mention that the chromatograms of FCZ separation profile in skin after topical application has not been previously illustrated in the literature to our knowledge. The present study shows them clearly. The extraction procedures accomplished for preparation of the samples do not significantly affect the recovery of FCZ and are effective for its determination in different biological matrices.

Conclusion

Validation of the developed method for determination of FCZ was accomplished by HPLC, showing linearity, precision, and accuracy. The intra- and inter-day CV yielded low values within the established maximum limits. The method was sensitive, selective, and accurate for the HMS and PES matrices tested.

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