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Inhibition of Fluconazole *In Vitro* Antifungal Activity in Formulations Containing Propylene Glycol

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***SUMMARY*. Inhibition action of propylene glycol (PG) on the antifungal activity of fluconazole has been investigated. PG was used as cosolvent and penetration enhancer in different solutions and topical dosage forms. The interaction was analyzed by comparing with Transcutol P® (TCL), another cosolvent and penetration enhancer. Solubility of the drug was evaluated in aqueous solutions containing PG or TCL and the crystallized drug was studied by both DSC and FTIR. Solutions of the drug in the solvents were studied by FTIR and UV spectroscopy. Antifungal activity was determined for solutions with several concentrations of PG/TCL and in dosage forms with PG 10 %. *Candida albicans* was used as a model fungus and a procedure with standardized inoculum concentration was used. Results showed lower antifungal activity of fluconazole solutions and topical dosage forms when propylene glycol is included. Although crystallization is faster in PG solutions, solubility proved not to be the cause, but changes in FTIR spectra suggested that different hydrogen bond formation could explain the decrease in activity.**

INTRODUCTION

Fluconazole (FLZ) is a synthetic bistriazole antifungal agent. FLZ (Fig 1a) is a white crystalline solid, slightly soluble in water and saline solution. It is a weak base and undergoes protonation at the N-4 nitrogen [1,2]. This compound is mainly used in the treatment of topical and systemic candidiasis and cryptococcal infections [3,4]. The antifungal effects of the azoles are targeted primarily at ergosterol, the main sterol in the fungal cell membrane; the depletion of ergosterol alters membrane fluidity and structure thereby leading to increased permeability and inhibition of cell growth and replication. Azoles molecules inhibit the activity of fungal cytochrome P45014DM, also known as CYP51, by binding to the heme group in the target protein. FLZ is a highly selective inhibitor of fungal cytochrome P-450 dependent enzyme lanosterol 14-α-demethylase, which converts lanosterol to ergosterol [5-7] . FLZ has favorable pharmacokinetic; as a result of a beneficial lipophilic/hydrophilic profile, high degree of bioavailability after oral administration (90 %), and low level of binding to plasmatic proteins (12 %), this active compound readily penetrates into body tissue [8]. The drug is available as tablets for oral administration, as a powder for oral suspension, and as a sterile solution for intravenous use. Although a topical formulation with FLZ would be of interest in the management of skin fungal infections, it has not been marketed yet.

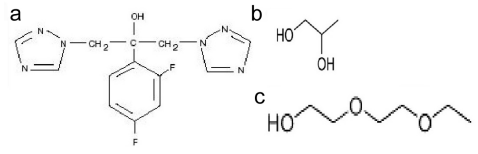

Figure 1. **a**: Fluconazole, 2,4-difluoro-α,α1-bis(1H-1,2,4-triazol-1-ylmethyl) benzyl alcohol; $C_{13}H_{12}F_2N_6O$; MW 306.3. **b**: Propylene glycol ; $C_3H_8O_2$ MW 76.09. **c**: Transcutol P® , Diethylene Glycol Monoethyl Ether; $C_6H_{14}O_3$ MW 134.17.

Propylene glycol (PG; Fig. 1b) is widely used as a constituent in dermatological and topical pharmaceutical formulations. PG has a recog-

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nized importance as cosolvent and potential enhancer; it has been shown to effectively increase the skin penetration of various topical drugs [9,10].

There is little research published about FLZ potential for topical use. Ayub *et al.* have used PG in topical dosage forms with FLZ to study dermal absorption of the drug and reported FLZ solubility in PG to be 147 ± 1 mg/mL [11]. Vidal Mussi *et al.* evaluated the efficacy of a FLZ formulation containing PG in *Leishmania* infected mice and found no *in vivo* activity [12]. In our previous experience [13], an *in vitro* assay showed that a microemulsion with PG had less antifungal activity against *Candida albicans* than one with a different cosolvent and penetration enhancer, Transcutol P® (TCL; Fig 1, c).

It is essential that the excipients used in drug formulations are compatible with the active ingredient so that effectiveness and stability are not affected. Consequently, the purpose of the present study was to further investigate the inhibition of PG on in vitro antifungal activity of FLZ. PG was used as cosolvent and penetration enhancer in solutions and semisolid topical dosage forms. We analyzed the interaction by comparing with Transcutol P®. FTIR and UV spectroscopy techniques were also performed.

MATERIALS AND METHODS

Materials

Fluconazole (FLZ) was a gift of Unifarma, Argentina; diethyleneglycol monoethyl ether (Transcutol P®, TCL) Gattefossé, France, was a gift of Ferromet S.A.; glyceryl monostearate (Cutina MD) and Polyoxyl (40) hydrogenated castor oil (CRH40) was kindly supplied by Cognis, Argentina; Castor oil, Carboxymethyl cellulose (CMC) and propylene glycol (PG) were purchased from Fabriquimica S.R.L., Argentina; Nonionic self-emulsifying wax, Cetostearyl alcohol, liquid paraffin, isopropyl miristate (IPM), jojoba oil and distilled water were all of pharmaceutical grade. All other reagents used were of analytical grade.

Methods

In order to evaluate the influence of solubility and the presence of other excipients in the antifungal activity of formulations containing FLZ and PG, several solutions and semisolids dosage forms were evaluated. Solutions with 2 and 10 mg/mL of FLZ were prepared both in phosphate-buffered saline (PBS; pH 7.4) and distilled water with 5, 10 or 15 % w/w of PG or TCL. A microemulsion, an emulsion, a lipogel, and a gel were prepared. Compositon of semisolid dosage forms is shown in Table 1. The components and methods of preparation of microemulsion, emulsion and the lipogel were selected for our previous work based on literature [13]. All the formulations had FLZ 1 %; the active compound was first dissolved in the corresponding amount of cosolvent under magnetic stirring and then added to the formulations. The gel was prepared by dissolution of fluconazole in PG and then added to a 1 % CMC gel.

pH determination

pH of solutions was determined at 25 °C with a TPA III Altronix pHmeter. For the case of semisolid dosage forms pH was determined by making a 1/10 dispersion in distilled water previously adjusted to neutral pH (7.00 ± 0.02). Results are expressed as means ± SD (n = 3).

Fluconazole solubility

FLZ 1 % aqueous solutions with PG or TCL (5, 10 or 15 % w/v) were stored at room temperature and observed for drug crystallization. Drug concentration in the solutions was determined at 25 ± 2.0 °C. An aliquot of 200 µL was taken at different time intervals for the first 8 h

Semisolid Form	Composition	pH
Microemulsion TCL	CRH40 15 %, Castor oil 5 %, TCL 10 %, CMC 3 %, Distilled water qs 100 %.	7.34 ± 0.01
Microemulsion	CRH40 15 %. Castor oil 5 %, PG 10 %, CMC 3 %, Distilled water qs 100 %.	6.77 ± 0.02
Emulsion	Cetostearyl alcohol 5 %, IPM 10 %, PG 10 %, Liquid paraffin 10 %, Non ionic self emulsifying wax 10 %, Distilled water qs 100 %	4.57 ± 0.02
Lipogel	Cutina MD 20 %, PG 10 %, Jojoba oil qs 100 %	6.34 ± 0.01
Gel	CMC 1 %, PG 10 %, Distilled water qs 100 %	7.90 ± 0.01

Table 1. Composition and pH of semisolid dosage forms.

and then on day 1, 2 and 3, 7 and 15. The final volume was adjusted to 10 ml with PBS. Concentration was measured by UV/VIS spectroscopy (UV-VIS Spectrophotometer Shimadzu UV-260) at 261 nm using a calibration curve of FLZ in PBS covering the range 20-250 µg/mL (correlation factor 0.9991-1.000) and PBS as blank. Assays were done in triplicate [14].

In vitro antifungal activity

Prior to the assay the microorganism *Candida albicans* (ATCC 10231) was subcultured from fresh stock cultures into fresh Sabouraud Dextrose Agar Medium and incubated for 44 to 52 h at 22.5 ± 2.5 °C. To harvest the cultures after incubation time, surface growth was washed with sterile saline, collected and diluted with more sterile saline to obtain a count of about 1×10^8 colony forming unit per mililiter (cfu/mL). The estimation of inoculum concentration was performed by turbidimetry according to Mc Farland test.

PBS solutions and semisolid dosage forms prepared were evaluated for antifungal activity. Appropriate dilutions of the formulations in PBS (1/10, 1/100, 1/1000 and 1/100000) were inoculated with a suspension of *Candida albicans* (ATCC 10231) in Sabouraud Dextrose Agar Medium, to a final concentration in the order of 1×10^6 cfu/mL of sample. The samples were incubated at 22.5 ± 2.5 °C and each container was observed for any change in appearance and sampled after 2, 24 and 48 h. The plate-count procedure was used to determine the number of cfu present in the test preparations at each time. Using the calculated concentration of cfu/mL present at the start of the test, the change in $\log_{10}$ values of the concentration of cfu/mL for each sample at applicable intervals was calculated. The average cfu/mL was calculated taking into account dilution factors. A standard solution of FLZ in PBS and formulations or solutions without FLZ were assayed simultaneously as standard control and blank sample, respectively. Tests were performed in triplicate [15].

Scanning electron microscopy (SEM)

FLZ raw material and crystallized drug in the aqueous solutions were affixed to carbon tape on a specimen stub and excess powder removed. The stubs were stored under vacuum overnight. The samples were sputter-coated with gold. Electron micrographs were obtained using a Field Emmission Gun Scanning Electron Microscope (FEG-SEM Zeiss Supra™ 40 apparatus Gemini column, Germany) operating at 1.0 kV accelerating voltage.

Thermal analysis

Differential scanning calorimetry (DSC, Mettler Toledo TA-400 differential scanning calorimeter, Columbus, OH, USA) was used for the solid state characterization to search out the presence of the different solid forms, solvates or hydrates from crystallization in the solutions with cosolvents. FLZ raw active compound and the solids crystallized in the solutions were analyzed. The samples (4–9 mg) were sealed in 40 µl Al-cruicible pans and heated from 25 to 160 °C under nitrogen atmosphere. DSC curves were recorded at constant heating rate of 5 °C/min [16].

FTIR spectroscopy

FTIR spectra of FLZ as received, crystallized drug from aqueous solutions, and FLZ dissolved only in PG or TCL were recorded on a Nicolet 380 FTIR Spectrometer with ATR (attenuated total reflectance) cell (ZnSe) for liquids (12 reflexions) using Nicolet Omnic software. FLZ raw drug and its crystallized form from 10 % PG or TCL aqueous solutions were weighted (3 mg) and added to 200 mg of potassium bromide (KBr). FLZ and KBr were mixed to obtain a uniform mixture, and then the mixture was converted into a pellet by applying a pressure of 9 tons for 10 min in a hydraulic pellet press. FTIR spectra of PG, TCL, ethanol and FLZ solutions (4 % w/v) in these solvents were analyzed using the ATR cell without any prior sample preparation. The patterns were recorded between 400 and 4000 cm^{-1}.

UV Spectroscopy

UV spectra of FLZ in ethanol, PBS, methanol, PG, and TCL were performed. Absorbance at 261 nm was recorded for different drug concentrations using UV-VIS Spectrophotometer Shimadzu UV-260, covering the range 20-250 µg/mL (6.53×10^{-5} – 8.16×10^{-4} M) for ethanol, PBS and methanol, and 20-500 µg/mL (6.53×10^{-5} – 1.63×10^{-3} M) for PG and TCL. Each solvent was used as blank. Assays were done in triplicate.

RESULTS AND DISCUSSION

Fluconazole solubility

FLZ previously dissolved in PG or TCL crystallized when diluted with water to 1 % final concentration of drug. Microscopic observation of the needle-like crystals that formed in both solutions showed they were similar (Fig. 2).

Figure 3 shows SEM images of FLZ as received (a) and the crystallized drug in aqueous

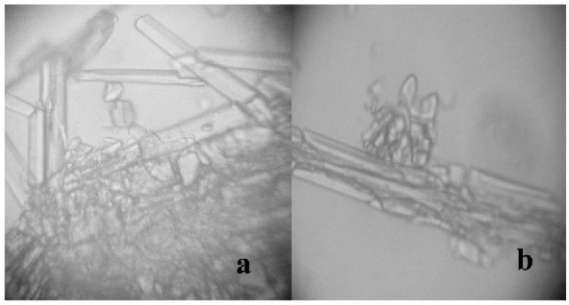

Figure 2. Optical microscope images of crystals from 5 % Propylene glycol (PG) and Transcutol P® (TCL) aqueous solutions. **a**: PG ; **b**: TCL.

solutions (b, c and d); crystals appeared quite similar but different from the raw drug. There was an important decrease in solubility by the first 8 h for PG solutions (concentration fell to 4.35, 6.43, and 8.71 mg/mL for PG 5,10 and 15 %, respectively) and after that time drug concentration remained almost constant (Fig. 4). The decrease in solubility for the case of TCL solutions by 8 h was 8.71, and 4.28 mg/mL for TCL 5 and 10 %, respectively; solubility in TCL 15 % solutions decreased only after one week. Final solubility was not < 4mg/mL for any of the tested cosolvent concentrations. On the contrary, FLZ 1 % in both solvents 100 % remained soluble for at least 15 days, without visual change in the solutions (data not shown).

In vitro Antifungal Activity

Solutions containing 2 mg/mL of FLZ with 10 and 15 % of PG were not able to reduce the initial inoculum, but the one with 5 % could reduce one logarithmic order after 48 h (Fig. 5). As in these solutions the solubility of the drug was not limiting, results indicate that there would be a concentration dependent interaction between the drug and PG and only the solution with the lower amount of PG showed scarcely any activity. None of the tested PG solutions with FLZ 10mg/mL killed the inoculum in the same extent as the standard solution which contained 2 mg/mL of the active compound (Figs. 5 and 6); PG 10 % solution could only reduce one logarithmic order after 48 h. We assumed that in the case of solutions with 5 % of PG drug solubility could have been limiting. Furthermore, TCL 5 % solution was not able to reduce the inoculum either, but 10 and 15 % ones were effective at the end of the assay. pH of all solutions was in the range 7.34 -7.56.

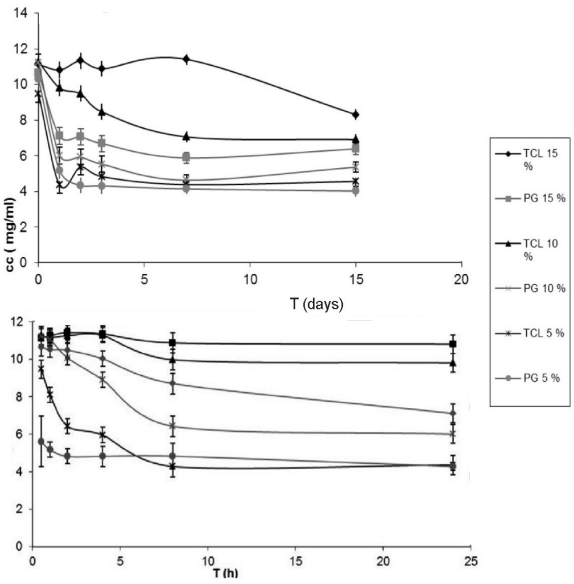

Figure 4. Fluconazole solubility in aqueous solutions at 25 °C.PG: propylene glycol; TCL: Transcutol P®

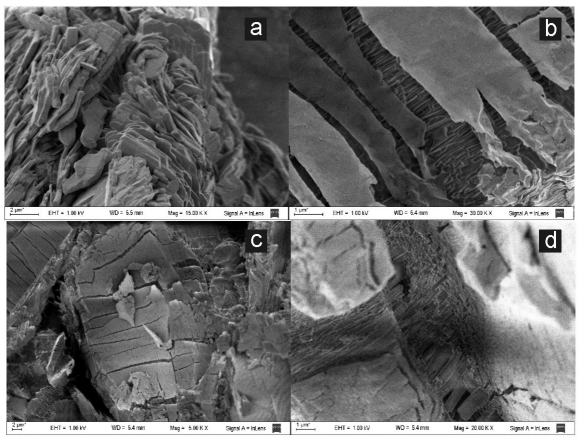

Figure 3. SEM images. **a:** fluconazole raw material; **b:** crystals from 10 % propylene aqueous solutions; **c:** crystals from 10 % Transcutol P® (TCL) aqueous solutions; **d:** magnified inner part of the hydrate crystal from 10 % TCL aqueous solutions.

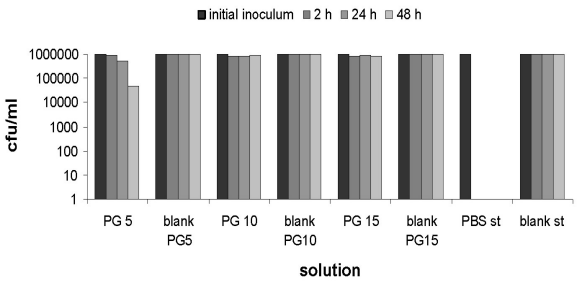

Figure 5. Antifungal activity of fluconazole 2 mg/ml phosphate-buffered saline solutions with propylene glycol (PG); cfu/ml after incubation of samples at 22.5 ± 2.5 °C; PBS st: phosphate-buffered saline standard solution.

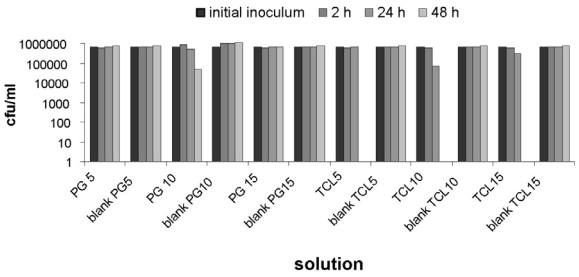

Figure 6. Antifungal activity of fluconazole 10 mg/mL phosphate-buffered saline solutions with cosolvents, expressed in cfu/ml after incubation of samples at 22.5 ± 2.5 °C. PG: propylene glycol; TCL: Transcutol P®.

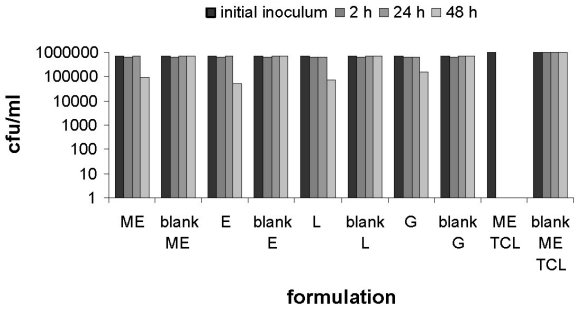

Figure 7. Antifungal activity of fluconazole 1 % dosage forms with propylene glycol 10 %; cfu/ml after incubation of samples at 22.5 ± 2.5 °C. ME: microemulsion ; E: emulsion; L: lipogel; G: gel; ME TCL: microemulsion with Transcutol P®.

As seen in our previuos work the microemulsion with 10 % PG was able to reduce one logarithmic order by 48 h, on the contrary a microemulsion containing 10 % of TCL was effective to kill the whole inoculum by 2 h (Fig. 7). The emulsion and lipogel with PG 10 % assayed in this work were not able to reduce more than one logarithmic order by the 48 h control, and the CMC gel completely failed to have any activity (Fig. 7). These results confirm that the lower antifungal activity was more related to the presence of PG rather than water content, pH (Table 1) or presence of other excipients in the formulations. Neither centrifugation nor observation with optical microscopy of the semisolids formulations showed presence of crystals.

Thermal analysis

Thermal analysis (Fig. 8) showed an endothermic peak at 138.18 °C (ΔH = – 113.30 J/g) for raw FLZ. Crystals showed two endothermic peaks at 100.91 and 136.91 °C (ΔH = – 36.47 J/g) for 10 % PG solution crystals and at 101.82

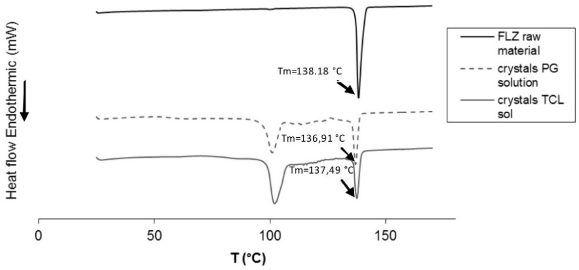

Figure 8. DSC curves; FLZ: fluconazole; PG: propylene glycol; TCL: Transcutol P®.

and 137.49 °C (ΔH= – 28.11 J/g) for 10 % TCL solution crystals, for desolvation and melting, respectively. FLZ presents polymorphism; several authors have studied its polymorphs and different solvates [16-18]. In our study results are according to previous reports for anhydrate form and monohydrate [19,20], but taking into account the fusion enthalpies we could also think that FLZ probably presents pseudo-polymorphism when crystallized in these solutions. Pseudo-polymorphism or solvatomorphism was defined as crystalline forms of a compound that differ in their elemental composition through the inclusion of solvent molecules [21-23]. However, Tong *et al.* made reference to Burger and Ramberger thermodynamic rule for enantitropic pair which states that if the higher melting form has the lower enthalpy of fusion then the two forms are related enantiotropically. Therefore, in this case acicular crystals formed in each cosolvent aqueous solution would be enantiotropically related monohydrates instead of solvatomorphs, which was confirmed by the FTIR spectra (see below) [23].

FTIR

Figure 9a shows the spectrum of FLZ as received, the main peaks reported in literature are present (3200 broad band due to hydrogen bonded O-H vibration, peak 3121 aromatic C-H stretching vibration; 1620,3 aromatic C=C stretching; 1271,9 C-F stretching;1140 C–O tertiary alcohol stretching) [1,20,24,25]. The spectrum of the crystallized drug in solutions with 10 % of PG or TCL as cosolvents showed no relevant difference (finger print range; Fig. 9b) supporting the idea that crystals in both solvents are not different pseudopolymorphs.

Figure 10a shows differential spectra of FLZ 4 % solution in PG, TCL and ethanol. In the extended graph for the "finger print" area (2000 - 600 cm^{-1}, Fig. 10b) several important differences are identified. According to Park *et al.* [25], 1419

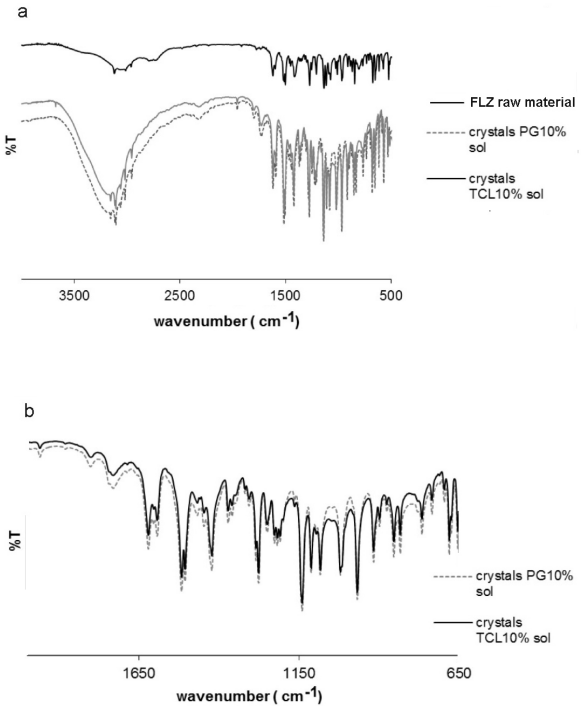

Figure 9. FTIR spectra. **a**: fluconazole (FLZ) as received and crystals from propylene glycol (PG) and Transcutol P®(TCL) 10 % water solutions; **b**: crystals from PG and TCL water solutions (finger print range).

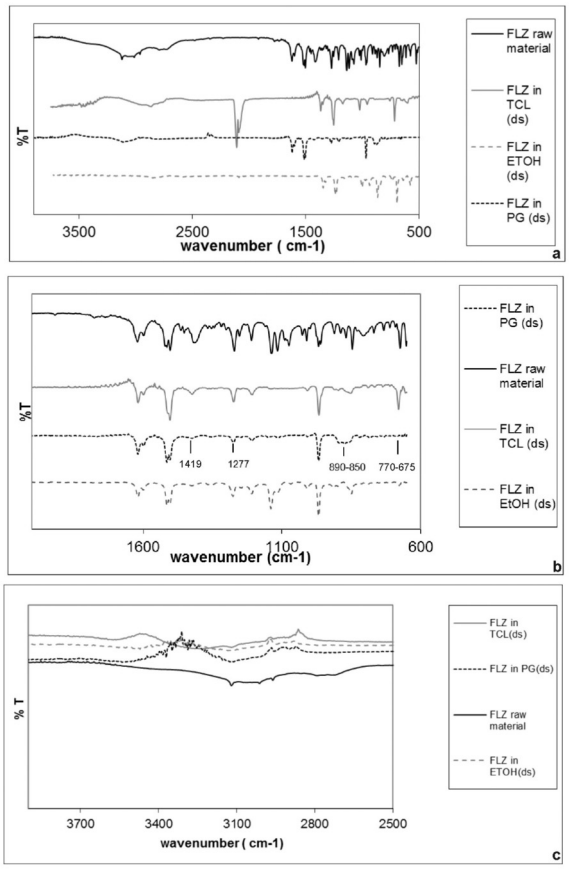

Figure 10. FTIR spectra of fluconazole (FLZ) as received and differential spectra (ds) of the drug in solutions of cosolventes. **a**: complete spectra; **b**: finger print range; **c**: H-bond region. PG: propylene glycol; TCL: Transcutol P®.

cm^{-1} peak corresponds to ring stretch of triazole group, which practically disappeared in the case of PG and ethanol. Peak at 1272-1274 cm^{-1} for C–F stretch is weaker for PG and shifted to 1277. The band for C–O stretching vibration for a tertiary alcohol disappeared for the three solvents. There is a noticeable difference in 850 cm^{-1} peak attributable to out of plane C–H bending deformation vibration of the two adjacent aromatic H (**1**) which is broadened when the drug was in PG. It is also remarkable that the band at 650-670 cm^{-1} referable to O-H bend out of plane disappeared for the case of PG [26]. Moreover, the region 3600-3000 cm^{-1} corresponding to intermolecular and intramolecular hydrogen bonding (H-bond; Fig. 10c) varies for the drug in TCL compared to PG and Ethanol. This would indicate difference in intermolecular interactions and H-bond cooperativity [27-30].

FLZ has 7 H-bond acceptor atoms and PG has two H- bond donor atoms whereas TCL has one H- bond donor atom and because of the molecular weight of the solvents the molar ratio solvent:drug is greater for PG: FLZ, so it has to be considered that there are always more molecules of PG available to interact with the drug than for the case of TCL. FTIR assay for the drug in ETOH was performed to compare with a third solvent. Ethanol has an H- bond donor atoms but its molecular structure and weight are much more similar to PG than to TCL. We could say that both in the case of PG and TCL there is evidence of H-bond formation with the drug –OH because of the change in C–O peak and also in the stretch of propane backbone at 1117 cm^{-1}, but in the case of PG there are some other changes. These differences may be attributed to different H-bond interactions with O, N, or F. The loss of triazole ring stretch would indicate that H-bond of FLZ with PG would also involve N in a position that makes the molecule more rigid. This thought is reinforced by the lack of signal for the out of plane bending of the O-H.

UV spectroscopy

UV spectra of the drug in the selected solvents exhibited no significant change either in peaks or in the considered λ_{max} for any of them

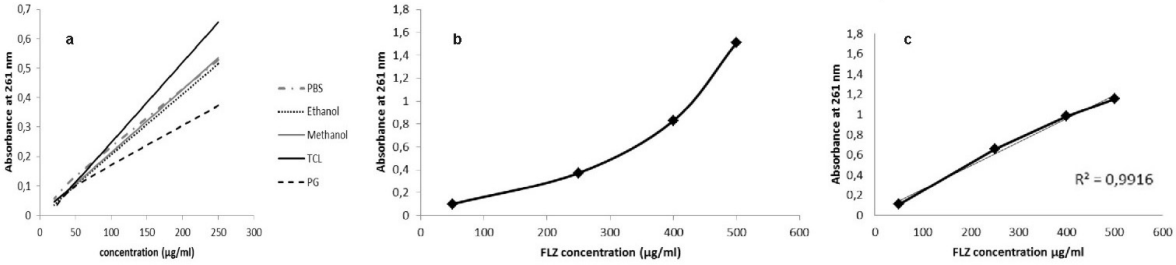

Figure 11. **a**: UV absorption of fluconazole (FLZ) in different solvents; **b**: UV absorption of FLZ in PG; **c**: UV absorption of FLZ in TCL. PBS: fosfate-buffered saline; PG: propylene glycol; TCL: Transcutol P®.

(260.5-261.5 nm). As it can be observed from the absorbance vs. concentration graph (Fig. 11a) drug absorbance at 261 nm differs in TCL and PG compared to the rest. Furthermore, in the case of PG solutions absorbance is lower for concentrations < 250 µg/mL (8.16×10^{-4} M) and it grows exponentially for more concentrated solutions (Fig. 11b). On the contrary, the absorbance of FLZ in TCL solutions follows a linear relationship in all the concentration range studied (Fig. 11c). We analyzed the graph over the expected linear range (absorbance > 0.8-1.0) because in PG solutions relationship absorbance-concentration is non-linear from 0.5 onwards. This could be explained in accordance to FTIR results. The drug would interact with the solvent by H-bond and Van der Waals forces which probably make electronic transitions to excited orbitals less favourable. In more concentrated solutions this solvent effect is not to be observed [31].

The different H-bond and Van der Waals interaction drug- solvent uncovered might be the cause of the decrease in activity. Several molecular docking designs were used to study the mode of binding of azole antifungal agents with lanosterol 14 α-demethylase (CYP51). All azole antifungals were shown to have a similar docking mode in the active site. It was reported that binding process is a process of "induced fit", and the conformations of both the target enzyme and the inhibitor would be changed during the enzyme-inhibitor interaction. The flexibilities of both the active site of the target enzyme and the inhibitor were taken into account. The docking results of azoles with *Candida albicans* CYP51 showed azole antifungals inhibited the binding of substrate to the enzyme by coordination of their azole rings (N-3 of imidazole or N-4 of 1,2,4-triazole) to the iron atom of the heme group, and according to Chen C-K et al, FLZ is bound in this active site by multiple van der Waals and aromatic stacking interactions. Another triazolyl ring of FLZ attached to C-3 had a preferable orientation toward substrate and formed indirect nonbonding interactions with the surrounding residues. The hydroxyl group attached to C-2 was important for antifungal activity, but no interaction was observed between this hydroxyl group and the active site of CYP51. A study showed that FLZ interacts with at least three water molecules, which bridge the interactions with the active site. From the docking model, it is possible that the water molecules in the active site mediate the interaction between the hydroxyl group and the P2 subsite. The difluorophenyl group of FLZ interacts deep in the same hydrophobic binding cleft as the 17-alkyl chain of substrate. Since this hydrophobic cleft showed to be narrow, the space adjacent to positions 2 and 6 of the phenyl group was limited. The pi-pi stacking interaction might exist between halogenated phenyl ring of inhibitors and the aromatic ring of residue Y132 of the enzyme. Based on these reported investigations on the structure–activity relationships of azole antifungals, it was found that 1,2,4-triazole ring and 2,4-difluorophenyl group are essential for the antifungal activity [2,32-35].

CONCLUSION

In vitro tests evidenced FLZ lack of effective antifungal activity when PG is used as cosolvent or penetration enhancer. This interaction is of interest because PG is a commonly used cosolvent, particularly in compounding pharmacy. Solubility, pH or the presence of other excipients seemed not to be the reason for the decrease in the activity. More likely the type of intermolecular interactions such as H-bond formation and Van der Waals forces between the drug and the solvent might alter the binding to the active site of the fungus enzyme. As mentioned, N4 of the triazole ring, the halogenated phenyl group, and the hydroxyl group are necessary for the activity and they seem to be modified by the

presence of PG. Van der Waals forces are involved in the binding to the enzyme as well; possibly the presence of the solvent molecules clustering around the drug molecule or the steric disposition of the drug causes these interactions to be less accessible and both the capacity of H-bond formation and the molecular size of the solvent showed to be relevant.

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