Development and Validation of Simple RP-HPLC Method for Intracellular Determination of Fluconazole Concentration and Its Application to the Study of Candida albicans Azole Resistance

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Candida albicans (strains NCTC-885-653 and ATCC-10231) long-term cultivated in the presence of antifungal agent fluconazole (FLC) and classical microbiological methods for determination of minimal inhibitory concentration (MIC) were used in this study.

A simple and sensitive method based on reverse-phase high-performance liquid chromatography (RP-HPLC) has been developed for the determination of FLC intracellular concentration in C. albicans using tinidazole as an internal standard. Following extraction with dichloromethane, the chromatographic separation was achieved on a Machery-Nagel EC250/2 Nucleodur-100-3 C18 column by gradient elution using the mobile phase consisting of (A) 0.01 M ammonium acetate buffer, pH = 5.00, and (B) acetonitrile.

Different analytical performance parameters such as linearity, precision, accuracy, limit of quantification (LOQ), and robustness were determined according to US DHHS FDA and EMEA guidelines. The method was linear for FLC (r = 0.9999) ranging from 100 to 10000 ng/mL. The intraday and interday precisions (relative standard deviation) were within 2.79 and 2.64%, respectively, and the accuracy (relative error) was less than 2.82%. The extraction recovery ranged from 79.3 to 85.5%. The reliable method was successfully applied to C. albicans azole-resistance study and it was shown that intracellular concentration of FLC correlated with a yeast drug susceptibility profile and MIC values.

1. Introduction

Acquired drug resistance by microorganisms poses a grave threat to human and animal health and has enormous economic consequences. Fungal pathogens, including the most common opportunistic fungal pathogen C. albicans, represent a particular challenge because they are eukaryotes and share many of the same mechanisms that support the growth and survival of the human host cells they infect. The number of drug classes that have unique targets in fungi is very limited, and the usefulness of current antifungal drugs is compromised by either dose-limiting host toxicity or the frequent emergence of high-grade resistance [1].

Azole compounds represent the most widely used class of antifungal drugs to treat Candida infections [2–4]. Azoles exert their action by inhibiting yeasts enzyme lanosterol 14a-demethylase and interfere with the biosynthesis of cell membrane ergosterol which causes inhibition of cell growth and finally cell death [5]. Altered interactions with the target enzyme and altered efflux pump expression are common mechanisms of azole resistance in major Candida species. Resistance can be mediated by increased efflux of azoles resulting from the overexpression of multiple drug resistance genes such as ATP-binding cassette transporters [6].

However, little is known about the mechanisms by which azoles and particularly FLC enter C. albicans. One reason for this is that the inaccessibility of the cytoplasmic face of the plasma membrane precludes direct examination of FLC intracellular transport in intact cells. To circumvent this problem, several groups have studied the ability of
C. albicans to pump fluorescent marker compounds out of the cell. These studies have provided important insights into the energetics and kinetics of these pumps, but the fluorescent compounds used in most of these studies are unrelated structurally or functionally to theazole antifungals [7, 8]. Intracellular FLC transport is biochemically characterized by studying cellular accumulation of $[^{3}H]$FLC [9]. The results suggest that $[^{3}H]$FLC enters the cell by energy-independent facilitated diffusion and import levels vary among resistant clinical isolates, suggesting that import is a conserved mechanism of resistance to azole drugs in C. albicans [10]. Thus, determination of FLC intracellular concentration in C. albicans by sensitive and selective nonradioactive method is a prerequisite not only for understanding drug intracellular transport mechanisms but also for clinical monitoring ofazole resistance of C. albicans and other medically important fungi.

A literature survey reveals some HPLC methods that are reported for the determination of FLC in pharmaceutical dosage formulations as anticipated with the variation of mobile phase, column, and detector. Different HPLC methods [11, 12] for individual assay are available for FLC in official pharmacopoeia and several LC-MS/MS methods were used for determination of FLC in human plasma [13–15]. Hence, an attempt has been made to develop a simple, efficient, and selective RP-HPLC method for intracellular determination of FLC concentration and its application to azole-resistant C. albicans.

2. Experimental

2.1. Chemicals and Reagents. FLC (Figure 1(a), USP RS, purity: 99.6%, batch: HOHO87), tinidazole (Figure 1(b), USP RS, purity: 99.9%, batch: 0312-QCS-12), ammonium acetate (Sigma-Aldrich, HPLC grade), sodium and potassium chloride and phosphate, millipore water, and methanol (HPLC grade, Alpha Chem Germany, purity: 99.9%), acetonitrile (HPLC grade, Alpha Chem Germany; purity: 99.9%), and sodium hydroxide, dichloromethane (Panreac, Spain, 99.9%) were used in this study.

2.2. Fungal Strains, Culture Media, and Antifungal Drugs. C. albicans NCTC-885-653 and C. albicans ATCC-10231 were purchased from the ATCC (LGC Standards-ATCC). Five different C. albicans clinical isolates have been obtained from Armenicum Clinical Centre (CJSC Armenicum, Yerevan, Armenia). The strains and clinical isolates were cultured in Soyabean Casein Digest (SBD) medium (HiMedia Laboratories Ltd., India) or Sabouraud Dextrose (SD) agar (Carl Roth, Germany) with aeration at 35°C. FLC (Diflucan 2 mg/mL solution for infusion, Pfizer Inc., USA), Amphotericin B (AmB, Amphocil 100 mg, 50 mg powder for injection, Penn Pharmaceuticals Ltd., UK), and Voriconazole (VRC) substance (Liqvor Pharmaceuticals, Armenia) were used in this study.

2.3. Candida albicans Cultivation and Biological Matrix Preparation. 100 mL Erlenmeyer flasks in triplicate containing $1 \times 10^5$ colony forming units (CFU) of each yeast strain cell in 100 mL SBD medium were incubated at 33°C for 48 h and yeast growth in SD agar plates at 33°C for 48 h was estimated for every 24 h by plating of 10 μL and 100 μL aliquots from the flasks. A total of 1 $\times 10^8$ CFU for each yeast strain was harvested and then cells were washed three times in ice cold PBS solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 8 mmol/L Na₂HPO₄, and 1.46 mmol/L KH₂PO₄) by centrifugation at 3000 rpm at 4°C for 15 min. Aliquots of 1000 μL samples were stored at −80°C and were brought to room temperature before use for method validation. Equal amounts of C. albicans NCTC-885-653 and ATCC-10231 strains aliquots were mixed and used for blank in biological matrix preparation.

2.4. Chromatographic Conditions. Quantity analysis was acquired by using high-performance liquid chromatography Platin Blue UPLC system (Knauer, Germany) with diode array detector. Nucleodur-100-3 C18 (250 $\times$ 2 mm, 3 μm packing, Machery-Nagel, Germany) column and guard column Nucleosil 120-5 C18 (CC 8/4) were employed. Gradient elution was employed using 0.01 mol/L ammonium acetate in water (pH = 5 ± 0.05, mobile phase A) and acetonitrile (mobile phase B). The flow rate was set at 0.3 mL/min and injection volume was 10 μL using a full loop mode for sample injection. The temperatures of column and autosampler were maintained at 30°C and 4°C, respectively.
2.5. Preparation of Standards and Quality Control Samples. Stock solutions of FLC (1 mg/mL) and tinidazole used as internal standard (IS, 1 mg/mL) were prepared independently by accurately weighing the required amounts into volumetric flasks and dissolving in methanol. The working solutions of FLC were obtained by diluting the stock solution successively with methanol. The stock solution of IS was diluted with solvent using 80:20 (v/v) (0.01 mol/L ammonium acetate buffer: acetonitrile) to make a working solution of 10 μg/mL. All solutions were stored at 4°C and were brought to room temperature before use. For preparation of standard samples for calibration curve, 50 μL of the appropriate working solutions of FLC was added to 1000 μL of blank 1 × 10⁸ CFU C. albicans to prepare concentrations of 100, 200, 500, 1000, 2000, 5000, and 10 000 ng/mL for FLC and 100 ng/mL for IS. Quality control (QC) samples at three concentration levels (low, 250 ng/mL; medium, 2500 ng/mL; high, 8000 ng/mL) were independently prepared in the same way. The standards and QC samples were freshly prepared before use.

2.6. Sample Preparation. A simple liquid-liquid extraction method was applied to extract the analyte and IS from C. albicans. Aliquots of 1000 μL 1 × 10⁸ CFU C. albicans sample were transferred to a 10 mL polypropylene tube followed by the addition of 50 μL IS working standard solution and 25 μL of 6 N NaOH solution and vortex-mixed for 15 sec. 500 μL of 0.01 mol/L sodium phosphate buffer (pH = 6.0) was added and vortex-mixed for 15 sec. Then, the mixture was extracted with 5 mL dichloromethane by vortex-mixing for 5 min. The supernatant was transferred to another tube after centrifugation at 3000 rpm at 20°C for 10 min and evaporated to dryness at 45°C under a gentle stream of nitrogen. Finally, the residue was reconstituted in 100 μL of the solvent followed by centrifugation at 3000 rpm at 20°C for 5 min. An aliquot of 10 μL of the supernatant was injected into the Platin Blue HPLC system in the full loop mode.

2.7. Method Validation. The method was validated for specificity, calibration curve, accuracy, precision, recovery, matrix effect, stability, and dilution effect in C. albicans according to the US Food and Drug Administration guidelines (US DHHS, 2001; European Medicines Agency, 2012) on bioanalytical method validation [16, 17].

2.7.1. Specificity. Comparing the chromatograms of blank 1 × 10⁸ CFU C. albicans, 1 × 10⁸ CFU C. albicans sample spiked with FLC and IS, and 1 × 10⁸ CFU C. albicans sample after long-term cultivation of yeast cells in the presence of 1/25 MIC (20 μg/mL) of FLC, no endogenous, medium components and metabolites of FLC interfered in the assay of the analyte and IS.

2.7.2. Calibration Curve. The calibration curves were constructed by plotting the peak-area ratios of each analyte to IS versus biological matrix concentrations using a 1/x² weighted least-squares linear regression model. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value, except at the LOQ, which was within ±20%.

2.7.3. Precision and Accuracy. The intraday precision and accuracy were determined by analyzing QC samples at three concentration levels (low, 250 ng/mL; medium, 2500 ng/mL; high, 8000 ng/mL) in six replicates on the same day, while the interday precision and accuracy were evaluated by analyzing QC samples at three concentration levels on three continual validation days. The precision was expressed as relative standard deviation (RSD, %) and the accuracy as the relative error (RE, %).

2.7.4. Recovery and Matrix Effect. The extraction recoveries of FLC at three QC levels with six replicates were measured by comparing the peak areas from extracted samples with those from postextracted blank C. albicans samples spiked with the analytes at the same concentration. The extraction recovery of IS was evaluated in the same way.

The matrix effect was measured at three QC levels by comparing the peak area from the postextracted blank C. albicans spiked with FLC working solutions with those of corresponding standard solutions. The matrix effect of IS was evaluated using the same procedure.

2.7.5. Stability. The stability of FLC in C. albicans was conducted at the two QC concentration levels (n = 5) in various storage conditions. Postpreparative stability was evaluated by analyzing the processed QC samples kept in an autosampler at 18°C for 48 h. Short-term and long-term stability were studied by analyzing QC samples exposed at room temperature for 4 h and stored at −80°C for 4 months, respectively. The freeze and thaw stability was tested by analyzing QC samples undergoing three freeze-thaw (−80°C to room temperature) cycles on three consecutive days.

2.7.6. Dilution Effect and Carry-Over. Dilution effect was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. Blank C. albicans samples spiked with FLC (16000 ng/mL) were diluted with pooled blank C. albicans at dilution factors of 2 in six replicates and analyzed. The six replicates should have precision of ≤15% and accuracy within ±15%. The carry-over was determined by injecting a blank C. albicans sample following the injection of an upper limit of quantification sample in three independent runs. Carry-over was considered negligible if the measured peak area was <20% of the lowest standard area.

2.8. Application to Candida albicans Azole-Resistance Study. This validated method was applied to determine the intracellular FLC concentration in C. albicans NCTC-885-653 and ATCC-10231 strains long-term serially cultivated in the presence of FLC. 5 × 10⁸ CFU/mL of cells in the SBD medium was incubated in separate tubes with total volume of 1.0 mL containing 1/25 of MIC concentration of FLC and incubated at 33°C for 48 h for obtaining one generation. The fungal strains were propagated in the presence or absence of
selecting antifungal drugs for a total of 20 generations and MIC values for FLC, VRC, and AmB of every fifth generation of each strain were estimated.

100 mL Erlenmeyer flasks in triplicate containing 1/25 of MIC concentration of FLC and 1 × 10⁶ CFU/mL of each yeast strain (NCTC-885-653 and ATCC-10231) cell at 20th generation in 100 mL SBD medium were incubated at 33°C for 48 h and yeast growth in SD agar plates at 33°C for 48 h was estimated for every 24 h by plating of 10 µL and 100 µL aliquots from the flasks. A total of 1 × 10⁸ CFU yeast cells were harvested and biological matrix was prepared as described above.

For determination of the FLC intracellular concentration in C. albicans NCTC-885-653 and ATCC-10231 strains and 5 different C. albicans clinical isolates 1 × 10⁶ CFU/mL of each yeast strain in 100 mL SBD medium were incubated at 33 °C for 30 min in triplicate containing 20 µg/mL concentration of FLC and a total of 1 × 10⁶ CFU yeast cells were harvested for biological matrix preparation.

2.8.1. Determination of Minimal Inhibitory Concentration (MIC). Fungal strains were grown in SD agar at 33°C for 48. One colony was inoculated in 5 mL of SBD medium and washed twice with 0.9% NaCl and the fungal count was determined by spotting on SD agar. The initial concentration of the fungal suspension in the SBD medium was 5 × 10⁶ CFU/mL. 0.5 mL of suspension was inoculated into separate tubes containing serial twofold dilutions of antifungal drugs. Azole antifungal drugs (FLC and VRC) dissolved in distilled water and tested in the range of 0.95 to 10000 µg/mL and AmB in the range of 0.0078 to 2 µg/mL were added to the tubes, yielding a total volume of 1 mL per tube. Drug-free medium with fungi and a fungi-free medium were used as the positive and negative controls, respectively. After incubation at 33°C for 48 h, the results were read visually, as recommended by the Clinical and Laboratory Standards Institute [18]. The MIC was considered to be the concentration that inhibited 100% of fungal growth. MIC values were confirmed by plating of 10 µL and 100 µL aliquots from the tubes with visual lack of growth on SD agar [19]. C. parapsilosis ATCC-22019 were included in each susceptibility test for quality control and assessment of reproducibility testing. Each assay was performed in triplicate on three different days.

3. Results and Discussion

3.1. Method Development. The aim of this study was to develop a simple, efficient, and selective RP-HPLC method for intracellular determination of FLC concentration in C. albicans. Various attempts were made to separate both the analyte and IS with different pH of the mobile phase buffer and composition of methanol in the mobile phase using C-18 and C-8 stationary phase columns. To ensure great resolution between known and unknown endogenous compounds, the C-18 stationary phase with an endcapping was used. Tinidazole (Figure I(b)) was selected as the IS since its structure, chromatographic behavior, and extraction efficiency were similar to those of the analyte. HPLC parameters, such as detection wavelength, ideal mobile phase, and their proportions and flow rate, were carefully studied. After trying different ratios of mixtures of acetonitrile and ammonium acetate buffer, the best results were achieved by using gradient elution. Both the analyte and IS displayed the best intensity and peak shape in the mobile phase containing 80:20 (v/v) 0.01 M ammonium acetate (solvent A) and acetonitrile (solvent B).

3.2. Method Validation

3.2.1. Specificity and Selectivity. At a flow rate of 0.3 mL/min and the detection wavelength 210 nm, the retention time was 6.8 ± 0.02 min for FLC and 5.6 ± 0.01 min (P < 0.0001) for IS. The analytes peak areas were well defined and free from tailing under the described experimental conditions (Figure 2). Typical chromatograms of FLC in C. albicans are shown in Figure 2. Blank chromatograms with UV spectra of unidentified compounds from C. albicans biomass are represented in Figure 3. Obviously, there were no significant interferences from endogenous substances and metabolites of FLC at the retention time of FLC and IS.

3.2.2. Calibration Curve. Calibration curve showed a satisfactory linearity in the range of 100–10000 ng/mL. A typical calibration curve equation was $y = 0.022x + 3.542$, with correlation coefficient of 0.9999, where $y$ is the peak-area ratio of FLC to IS and $x$ is the nominal concentration of FLC. The deviations of the back-calculated concentrations from their nominal values of LOQ ranged from −1.75 to 2.07% and standards other than LOQ were within −2.02 to 3.84%.

3.2.3. Precision and Accuracy. The intra- and interday precision and accuracy at corresponding QC levels are summarized in Table 1. The results indicated that the method showed good precision and accuracy.

3.2.4. Recovery and Matrix Effect. The extraction recoveries of FLC at the three QC levels were 79.3, 78.6, and 85.5%, respectively. The recovery of IS (100 ng/mL) was 89.4%. The extract recovery of all analytes was constant, precise, and reproducible with average percentage extraction recoveries of FLC 81 ± 4% (RSD%: 4.7). The matrix effects of FLC and IS were in the range of 85.7–90.2%, which meant that there was no significant retention time suppression or enhancement for FLC and IS.

3.2.5. Stability. The stability data for FLC are presented in Table 2. The result indicated that FLC was stable under the conditions examined.

3.2.6. Dilution Effect and Carry-Over. Diluted QC samples (16000 ng/mL) with six replicates were determined after dilution to the concentration of 8000 ng/mL, and the results of the tested samples were within the acceptable criteria. No carry-over was observed in the analysis of a blank plasma.
**Figure 2:** Typical chromatogram of IS and FLC (250 ng/mL) from extracted samples of blank *C. albicans* (a). Chromatographic separation of IS and FLC in *C. albicans* ATCC-10231 samples long-term cultivated in the presence of FLC.

**Figure 3:** Typical chromatogram of fungi biomass blank with IS tinidazole (a). UV spectra of peak with RT at approximately 13 min (b) and UV spectra of peak with RT at approximately 15 min (c).
3.3. Application to Candida albicans Azole-Resistance Study.
This validated method was applied to determine the intracellular FLC concentration in two different C. albicans strains (NCTC-885-653 and ATCC-10231) long-term cultivated in the presence of FLC. The initial susceptibility profile of the selected fungal strains was investigated by determination of MIC values for antifungal drugs FLC, VRC, and AmB (Table 3). The selected C. albicans NCTC-885-653 strain displayed high-level azole resistance as shown by 100% fungal growth inhibition in the presence of 500 μg/mL concentration of FLC and 62.5 μg/mL concentration of VRC, respectively. In contrast to this strain, C. albicans ATCC-10231 displayed approximately 2-fold low-level azole resistance as MIC values for FLC and VRC were found to be 250 μg/mL and 15.6 μg/mL, respectively. However, initial susceptibility profile of the selected fungal strains to nonazole antifungal AmB was found to be identical (0.25 μg/mL, Table 3). Resistance to azole drugs was experimentally induced in the selected fungal strains by long-term serial cultivation (for a total of 20 generations) of fungal cells in the presence of 1/25 of MIC concentration of FLC and 62.5 μg/mL concentration of VRC, respectively. In contrast to this strain, C. albicans ATCC-10231 displayed approximately 2-fold low-level azole resistance as MIC values for FLC and VRC were found to be 250 μg/mL and 15.6 μg/mL, respectively. However, initial susceptibility profile of the selected fungal strains to nonazole antifungal AmB was found to be identical (0.25 μg/mL, Table 3). Resistance to azole drugs was experimentally induced in the selected fungal strains by long-term serial cultivation (for a total of 20 generations) of fungal cells in the presence of 1/25 of MIC concentration of FLC. The development of azole resistance was achieved for initial azole susceptible C. albicans ATCC-10231 strain in approximately 20 days, and the strain at 20th generation was resistant to 750 μg/mL concentration of FLC and 500 μg/mL concentration of VRC, respectively, and also displayed high resistance to AmB (Table 3). The susceptibility profile of the C. albicans NCTC-885-653 strain at 20th generation also displayed high-level azole resistance; however, MIC values for FLC and VRC were found to be 500 μg/mL and 250 μg/mL, respectively, and approximately 4-fold low-level AmB resistance (Table 3).

The mean intracellular concentrations of FLC for these two C. albicans strains long-term cultivated in the presence or

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**Table 1: Intraday and interday precision and accuracy data for FLC assay in C. albicans matrix.**

| Concentration of analyte added (ng/mL) | Concentration of analyte found (ng/mL)* | Intraday (RSD, %)¶ | Interday (RSD, %)¶ | Relative error (RE, %)* |
|---------------------------------------|----------------------------------------|--------------------|--------------------|------------------------|
| 250                                   | 252 ± 7                                 | 2.79               | 2.64               | 0.68                   |
| 2500                                  | 2571 ± 61                               | 2.35               | 2.99               | 2.82                   |
| 8000                                  | 8038 ± 59                               | 0.73               | 1.05               | 0.47                   |

*Mean and SD representation for n = 10 standard samples for each of the mentioned analytes. ¶RSD, % = 100 × (SD/mean).

**Table 2: Stability of FLC assay in C. albicans biological matrix.**

| Nominal concentration (ng/mL) | Room temperature for 4 h | Stored at −80°C for 4 months | Three freeze and thaw cycles | Autosampler at 18°C for 48 h |
|-------------------------------|--------------------------|-------------------------------|-------------------------------|-----------------------------|
| 250                           |                          |                               |                               |                             |
| Mean ± SD                     | 249 ± 7                  | 254 ± 7                       | 234 ± 4                       | 236 ± 4                     |
| RSD, %                        | 2.7                      | 2.82                          | 1.8                           | 1.6                         |
| RE, %                         | −0.24                    | 1.6                           | −6.32                         | −5.68                       |
| QC-S/QC-R, %*                | 99.76                    | 99.82                         | 103.84                        | 103.40                      |
| 8000                          |                          |                               |                               |                             |
| Mean ± SD                     | 8041 ± 70                | 8035 ± 55                     | 8166 ± 35.38                  | 8162 ± 86                   |
| RSD, %                        | 0.68                     | 0.86                          | 0.31                          | 1.05                        |
| RE, %                         | 0.44                     | 0.51                          | 2.08                          | 2.02                        |
| QC-S/QC-R, %*                | 99.92                    | 99.91                         | 102.50                        | 100.58                      |

*Mean and SD representation for n = 5 standard samples for each of the mentioned analytes. ¶QC-S/QC-R, %: QC-S, samples exposed at room temperature for 4 h or stored at −80°C for 4 months or undergoing three freeze-thaw cycles or kept in an autosampler at 18°C for 48 h, and QC-R, reference samples, respectively.

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**Table 3: MIC* values (μg/mL) for antifungal drugs FLC, VRC, and AmB of C. albicans initial and azole-resistant strains, cultivated for 20 generations in the presence of FLC**

| Fungal strain/antifungal drug | C. albicans NCTC-885-653 | C. albicans ATCC-10231 |
|-------------------------------|---------------------------|-------------------------|
| Initial strain                |                           |                         |
| FLC                           | 500                       | 250                     |
| VRC                           | 62.5                      | 15.6                    |
| AmB                           | 0.25                      | 0.25                    |
| 20th generation of FLC-resistant strain |                 |                         |
| FLC                           | 500                       | 750                     |
| VRC                           | 250                       | 500                     |
| AmB                           | 0.125                     | 0.500                   |

*The MIC was considered to be the concentration of drug that inhibited 100% of fungal growth. ¶The fungal strains were propagated in the presence or absence of 1/25 of MIC concentration of FLC for a total of 20 generations and MIC value for FLC and VRC of each strain.
azole-resistant strains term-long cultivated in the presence or absence of FLC. All data represent mean ± SD (error bars) for each fungal culture, and are significantly different comparing initial C. albicans NCTC-885-653 and C. albicans ATCC-10231 strains at **P < 0.003 and comparing 20th generation of C. albicans NCTC-885-653 and C. albicans ATCC-10231 strains at **P < 0.005, respectively.

The results indicated that the intracellular concentrations of FLC for initially high-level azole-resistant C. albicans ATCC-10231 is statistically significantly higher comparing with the intracellular concentration of FLC for initially high-level azole-resistant C. albicans NCTC-885-653 (625 ± 51 ng/10⁸ CFU versus 445 ± 24 ng/10⁸ CFU, P < 0.003, resp.). The intracellular concentration of FLC for high-level azole-resistant C. albicans ATCC-10231 at 20th generation displayed 1.5-fold higher resistance to antifungal action of FLC, which was found to be statistically significantly lower comparing with that of another high-level azole-resistant C. albicans NCTC-885-653 with 1.5-fold lower MIC value to FLC at 20th generation (412 ± 50 ng/10⁸ CFU versus 790 ± 74 ng/10⁸ CFU, P < 0.005, resp.). FLC uptake calculation by yeast cell indicated that single CFU of high-level azole-resistant C. albicans accumulated 8.1–8.7 × 10⁶ FLC molecules while low-level azole-resistant C. albicans accumulated 12.3–15.5 × 10⁶ FLC molecules, which is highly correlated with the 1.5–2.0-fold differences between azole susceptibility profiles of the studied fungal strains.

For future confirmation that the intracellular concentration of FLC reverse-correlated with the azole-resistance profile of fungi, we compared the MIC values and fungal FLC concentration in azole-resistant NCTC-885-653 and ATCC-10231 strains at 20th generation with five different C. albicans clinical isolates. For this purpose, yeast cells were propagated in the presence of 20 µg/mL concentration of FLC for a total of 30 min incubation time (during which no significant difference has been observed in yeast cells survival between different strains, data not shown) and FLC intracellular concentration was determined for each strain (Table 4). The results indicated that the intracellular concentrations of FLC in azole-resistant C. albicans ATCC-10231 and NCTC-885-653 strains (MIC values in the 500–750 µg/mL range) are statistically significantly lower, comparing with that of azole-susceptible (MIC values in the 7.8–62.5 µg/mL range) C. albicans clinical isolates. The intracellular concentration of FLC for high-level azole-susceptible C. albicans clinical isolate number 1 (displayed 4-fold low resistance to FLC, comparing with isolates numbers 2–5) was found to be statistically significantly higher comparing with the rest of the azole-susceptible clinical isolates, and FLC uptake by 2-fold low resistance isolates number 2 and number 3 was statistically significantly higher, comparing with that of isolates number 4 and number 5. Thus, the obtained results clearly demonstrated that FLC uptake by yeast cell is highly correlated with the azole susceptibility profile of the studied fungal strains.

### Table 4: MIC values for FLC and FLC intracellular concentration (µg/mL) of C. albicans azole-resistant strains and five different C. albicans clinical isolates

| Fungal strains               | MIC for FLC | FLC intracellular concentration |
|-----------------------------|-------------|---------------------------------|
| C. albicans clinical isolates |             |                                 |
| Number 1                    | 7.81        | 13 ± 1**                        |
| Number 2                    | 31.25       | 4 ± 0.2**                       |
| Number 3                    | 31.25       | 4 ± 0.2**                       |
| Number 4                    | 62.50       | 10 ± 1                          |
| Number 5                    | 62.50       | 7 ± 0.4                         |
| ATCC-10231                  | 750         | 0.3 ± 0.02***                   |
| C. albicans azole-resistant strains |             |                                 |
| NCTC-885-653                | 500         | 0.6 ± 0.01**                     |

* C. albicans azole-resistant NCTC-885-653 and ATCC-10231 strains at 20th generation and five different C. albicans clinical isolates strains were propagated in the presence of 20µg/mL concentration of FLC for a total of 30 min and FLC intracellular concentration was determined for each strain. The MIC was considered to be the concentration of FLC that inhibited 100% of fungal growth during 48 h incubation time. All data represent mean ± SD for n = 6, for each fungal culture, and are significantly different comparing C. albicans NCTC-885-653 and ATCC-10231 strains with clinical isolate at ***P < 0.0001 and comparing C. albicans clinical isolate number 1 with numbers 2–5 at **P < 0.005 and clinical isolates number 2 and number 3 with isolates number 4 and number 5 at *P < 0.003, respectively.

### 4. Conclusion

In the present study, a simple, sensitive, and selective RP-HPLC method for intracellular determination of fluconazole concentration in C. albicans was developed for the first time. A relatively simple sample preparation procedure showed greater simplicity. Baseline separation between FLC and IS ensured the accuracy of determination. The method was successfully applied to the determination of FLC intracellular concentration in different azole-resistant C. albicans strains for the first time and will be useful for further characterization of FLC intracellular transport mechanisms and
for monitoring of drug resistance of \textit{C. albicans} and other medically important fungi.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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