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Biodistribution of free and encapsulated ^{99m}Tc-fluconazole in an infection model induced by *Candida albicans*

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ABSTRACT

Background: *Candida spp* is an etiologic agent of fungal infections in hospitals and resistance to treatment with antifungals has been extensively reported. Thus, it is very important to develop formulations that increase effectiveness with low toxicity. In this sense, nanocarriers have been investigated, once they modify drug biodistribution profile. Thus, this study aimed to evaluate the biodistribution of free and encapsulated ^{99m}Tc-fluconazole into nanocapsules (NCs) in an experimental immunosuppressed murine model of *Candida albicans* infection.

Methods: Fluconazole was radiolabeled with technetium-99 metastable (^{99m}Tc) and encapsulated into conventional (^{99m}Tc-Fluconazole-PLA-POLOX) and surface-modified (^{99m}Tc-Fluconazole-PLA-PEG) NCs by the interfacial deposition of the preformed biodegradable polymer [poly (D,L-lactic acid) (PLA) and PLA–PEG (polyethyleneglycol)] followed by solvent evaporation. The size distribution and zeta potential of the NCs preparations were determined in a Zetasizer by photon correlation spectroscopy and laser Doppler anemometry, respectively. Free and encapsulated ^{99m}Tc-fluconazole were administered intravenously in immunosuppressed mice bearing a local infection induced by *Candida Albicans* inoculation in the right thigh muscle. At pre-established time intervals, tissues and organs of interest were removed and radioactivity was measured in an automatic gamma radiation counter.

Results: The NCs diameter was between 200 and 400 nm with negative zeta potential values. Free ^{99m}Tc-fluconazole was more rapidly eliminated by the renal system compared to the encapsulated drug in NCs, which remained longer in blood circulation. The uptake of conventional NCs by mononuclear phagocyte system organs was higher than the one demonstrated by the surface-modified NCs. Both NCs remained longer in the infectious focus when compared to free ^{99m}Tc-fluconazole, but the results did not show a significant difference between NC formulations.

Conclusion: These data indicate that these NCs might represent a therapeutic alternative for the treatment of candidiasis, once they remain more time in the infectious focus, allowing high retention of ^{99m}Tc-fluconazole at this site.

Abbreviations: AIDS, acquired immunodeficiency syndrome; AUC, area under the curve; *C. albicans*, *Candida albicans*; HMPAO, hexamethylpropyleneamine oxime; MPS, mononuclear phagocyte system; NCs, nanocapsules; PEG, poly(ethylene glycol); PLA, poly(*D,L*-lactic acid); PMN, polymorphonuclear; POLOX, poloxamer; Vd, apparent volume of distribution; WBC, white blood cells

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1. Background

Immunocompromised patients due to acquired immunodeficiency syndrome (AIDS), anticancer therapy and transplantation have raised infections with fungi, such as *Candida albicans* (*C. albicans*), which compromise the mucosa and can led to systemic infections [1,2] Several classes of antifungal agents have been used in the treatment of candidiasis, such as polienic, imidazole, first and second generation triazoles, and echinocandins [3].

Triazoles' mechanism of action is due to the inhibition of fungal cytochrome P450 enzyme 14α-demethylase, which prevents the conversion of lanosterol to ergosterol, an essential component of the fungal cytoplasmic membrane. Thereafter, it results in an altered fungal membrane, impairing the functions of certain membrane-bound enzyme systems [4]. Fluconazole is an example of first-generation triazole commonly used in the treatment of candidiasis [3]. The mainly adverse effects associated to the use of fluconazole are related to the gastrointestinal tract [5]. Occasionally, alopecia is observed, especially in prolonged treatments with daily doses [6]. In AIDS and cancer patients, abnormal results of hematological tests, kidney and liver function tests are reported [7,8].

The efficacy of the antifungal therapy is directly dependent on the early diagnosis and immune status of the patient [9]. In addition, cases of resistance, especially to azole drugs, have been reported [10,11] and which are assigned to their indiscriminate use, especially in prolonged prophylactic treatment with low doses. Therefore, high doses of drugs are often required, as well as the combination of antifungal agents, increasing the risk of toxicity.

By considering the treatment difficulties, it is important to develop formulations that may increase the treatment effectiveness, minimizing toxicity risk. One strategy is to modify the biodistribution profile of the drug by means of a nanometric polymeric vector [12], such as nanocapsules (NCs), which are constituted by an oily core with an associated lipophilic drug, surrounded by a polymeric wall [13,14].

Several studies have shown *in vivo* promising results for the use of antifungal associated with colloidal vectors, such as liposomes[15] and nanospheres of amphotericin [16], liposomes of nystatin [17], nanogels [18], lipid nanoparticles of fluconazole [19] and nanoparticles of itraconazole [20], most of them for topical candidiasis treatment. However, to the best of our knowledge, *in vivo* distribution of NCs loaded with fluconazole has not been reported yet.

Thus, the aim of this work was to investigate the biodistribution of ^{99m}Tc-fluconazole free and encapsulated in conventional and surface-modified NCs after intravenous administration in experimental *C.albicans* infection model.

2. Methods

2.1. Radiolabeling of fluconazole and evaluation of radiochemical purity

The radiolabeling procedure of fluconazole (Galena, Brazil) with ^{99m}Tc (obtained from a ^{99}Mo/^{99m}Tc generator supplied by *Instituto de Pesquisas Energéticas e Nucleares – IPEN* (Brazil), was performed as it was previously described [21]. Briefly, 50 µL of a fluconazole solution (2 mg/mL), 4 µL of a $SnCl_2{\cdot}2H_2O$ (*Sigma-Aldrich*, Brazil) and sodium pyrophosphate (*Sigma-Aldrich*, Brazil) solution in 0.25 N HCl (*Sigma-Aldrich*, Brazil), 1 and 2 mg/mL, respectively, and 4 µL of a KBH_4 (*Sigma-Aldrich*, Brazil) solution in 0.1 N NaOH (*Sigma-Aldrich*, Brazil) (10 mg/mL) were added to a sealed vial. Then, $Na^{99m}TcO_4$ (74 MBq) in saline was added to the vial and the solution was gently stirred at room temperature for 120 min. The final preparation was diluted with acetic acid (*Sigma-Aldrich*, Brazil) 0.01 mol/L to a final volume of 1 mL and then it was applied to a Maxi-Clean C18 cartridge (*Alltech Associations*, EUA), previously flushed with 20 mL of acetic acid 0.01 mol/L, in order to remove the radioactive impurities, $^{99m}TcO_4^-$ and $^{99m}TcO_2$. After being rinsed with 20 mL of acetic acid 0.01 mol/L, ^{99m}Tc-fluconazole was eluted with 2 mL of methanol (*Sigma-Aldrich*, Brazil), which was evaporated. Finally, the purified 37 MBq of ^{99m}Tc-fluconazole was resuspended in 1 ml of saline solution (0.9% w/v NaCL) (*Sigma-Aldrich*, Brazil) for radiochemical purity analysis and for biodistribution study of free radiotracer. In order to obtain radiolabeled nanocapsules (NCs), 370 MBq of ^{99m}Tc-fluconazole was resuspended in a mixture of methanol:acetone (*Sigma-Aldrich*, Brazil) (1:3).

Radiochemical purity analysis of ^{99m}Tc-fluconazole was performed by instant thin-layer chromatography (ITLC) on silica gel strips (Merck®). Methyl ethyl ketone was used to determine the amount of free technetium ($^{99m}TcO_4^-$). Radioactivity was measured using an automatic gamma counter (ANSR, USA). All the solvents were analytical grade. The other chemicals were commercially available reagent grade and they were all used without further purification. Water was purified by reverse osmosis (*Simplicity 185, Millipore,* Bedford, USA).

2.2. Preparation of radiolabeled nanocapsules

The radiolabeled NCs were prepared as previously described [21,22]. The polymer poly(*D,L*-lactic acid) (PLA_{50}) with an average molecular weight (Mw) of 75.000–100.000 g/mol (*Sigma-Aldrich*, Brazil) and the surfactant poloxamer 188 (Synperonic® F68 - *Sigma-Aldrich*, Brazil) were used to prepare conventional radiolabeled NCs (^{99m}Tc-fluconazole-PLA-POLOX NCs). In this case, poloxamer 188 was only adsorbed at the surface of the NCs. The diblock polymer poly (ethylene glycol) methyl ether-block-poly(*D,L* lactide) (PLA-PEG, PLA with a Mw of 49,000 Da, containing approximately 10% w/w of PEG with a Mn of 5,000 g/mol) (*Alkermes*, USA) was used to prepare surface-modified radiolabeled NCs (^{99m}Tc-fluconazole-PLA-PEG NCs). Briefly, the polymer (0.6% w/v) was dissolved in a mixture of methanol:acetone (1:3), containing soy lecithin (Epikuron® 170 - *Lucas Meyer*, France) (0.75% w/v), Miglyol 810N (Caprylic/capric triglyceride) (*Hulls*, Germany) (2.5% v/v) and 370 MBq of ^{99m}Tc-fluconazole. For conventional and surface-modified radiolabeled NCs, the organic solution was poured with stirring into the external aqueous phase, containing or not poloxamer 188 (0.75% w/v), respectively. The solvents were evaporated to 10 mL under reduced pressure (Fisatom Rotary Evaporator, Brazil). Non-encapsulated ^{99m}Tc-fluconazole was separated from the radiolabeled NCs by ultrafiltration/centrifugation of 200 µL of the suspension of the radiolabeled NCs µL in an AMICON device (Microcon, MWCO 10.000 Da, Millipore®) at 1800 × *g* for 15 min. Non-encapsulated ^{99m}Tc-fluconazole found in the ultrafiltrate and radiolabeled NCs were retained in the upper compartment of the device. The radiolabeled NCs were resuspended in 0.9% NaCl (w/v) solution for *in vivo* biodistribution study.

2.3. Animal model

Male Swiss mice (20–25 g) were supplied by the animal facility of Faculty of Pharmacy of Federal University of Minas Gerais. Animals were kept under specific pathogen-free (SPF) conditions, with *ad libitum* access to chow and water. Mice were immunosuppressed by gamma irradiation (cobalt-60) at a dose of 6 Gray for 25 min. At 24 h post-irradiation, a blood sample was collected from the animals (n = 8) to assess the white blood cell (WBC) counts in order to evaluate immunosuppression. Non-irradiated animals were used as control. All experiments involving mice were conducted according to animal-use protocols that were approved by the local Ethics Committee in Animal Experimentation of Federal University of Minas Gerais (CETEA/UFMG). At 24 h post-irradiation, an aliquot of 100 µL containing 10^7 colony forming units (CFU) of *C. albicans*, ATTC 10231 (*American Type Culture Collection – ATCC*, USA) was inoculated into the right thigh muscle of immunosuppressed mice. Infectious focus was allowed to develop for 48 h post-inoculation of the fungus and then ^{99m}Tc-fluconazole formulations were administered followed by biodistribution studies and histopathological analysis.