



Original Article

Radiochemical pharmacokinetic profile of P10 peptide with antifungal properties

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Abstract

Paracoccidioidomycosis (PCM) is a chronic granulomatous disease that is caused by the thermally dimorphic fungus *Paracoccidioides brasiliensis*. It is endemic in some countries of Latin America and can cause a high-burden fungal infection with significant morbidity and mortality. The peptide P10, which demonstrates immune protection against experimental PCM, was radiolabeled with a radioisotope and evaluated *in vivo*. The radiolabeling was conducted to trace the pharmacokinetics of the molecule in principal organs and tissues. This was achieved with high radiochemical purity. Biodistribution and scintigraphic imaging showed fast blood clearance that was mainly renal; however, hepatobiliary excretion was also, with marked uptake in cervical lymph nodes. This profile may be useful for the development of a prophylactic drug or vaccine for patients exposed to PCM.

Key words: P10 peptide, *P. brasiliensis*, radiolabeling, biodistribution evaluation.

Introduction

Fungal infections, specifically systemic mycoses that affect internal organs, are often characteristic of certain geographical areas. They can cause major problem relating to critical illnesses, organ transplantations, hematologic malignancies, human immunodeficiency/AIDS, and even accidental contamination episodes, despite geographic location [1]. They are estimated to be the tenth most common cause of death among infectious diseases in Brazil [2].

Paracoccidioidomycosis (PCM), a systemic granulomatous disease, is caused by *Paracoccidioides brasiliensis*, a thermodynamically dimorphic fungus. PCM is widespread in Latin America, encompassing Argentina, Colombia, Mexico, Venezuela, and Brazil, affecting mainly rural workers. PCM infects primarily lung tissue [3], but hepatic and neurologic involvement are also possible [4].

Chemotherapy is usually successful at controlling granulomatous pulmonary infection. However, the efficacy of

treatment is limited by the status of the host's immune response. The inhibition of Th-2 immunity or the stimulation of Th-1 cytokines generally increases the efficacy of antifungal drugs [5]. This has been achieved by immunization with an internal peptide of the major diagnostic antigen gp43 of *P. brasiliensis*.

The 15-mer peptide, peptide 10 (P10, QTLIAHT-LAIRYAN), was identified by Taborda et al. [6]. P10, which is derived from the immunodominant antigen gp43, elicits an interferon-gamma-dependent immune protection against experimental PCM [6,7]. A P10-based vaccine might be associated with antifungal chemotherapy for enhanced response against PCM.

The evaluation of the uptake of a new drug in different tissues and organs provides essential information for drug development and dosage assays. Such investigation can be conducted using multiple physical and chemical methods including labeling with radioisotopes followed by evaluation of biodistribution *in vivo*. One advantage of such an approach is the possibility of imaging studies using a gamma camera or single-photon emission computed tomography, with the help of the same radiolabeled peptides.

The radioisotope technetium-99m (^{99m}Tc) has advantageous nuclear characteristics (6-h half-life and gamma energy of 140 keV), ready availability from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator, and well-established labeling chemistries [8]. Depending on the molecule, previous conjugation with chelating agents may be needed for the ^{99m}Tc radiolabeling procedure [9].

Our aim in this study was to characterize the uptake of the conjugated peptide10 (HYNIC-P10), radiolabeled with ^{99m}Tc , focusing on its biodistribution profile and scintigraphy imaging.

Materials and methods

The investigation was conducted at the Radiopharmacy Center, Institute of Energetic and Nuclear Research/Brazilian Commission of Nuclear Energy (IPEN/CNEN), Sao Paulo, Brazil. All animal studies were performed at the Radiopharmacy Center, IPEN/CNEN. The local animal welfare committee approved the protocol.

^{99m}Tc was eluted in 6 ml of isotonic saline solution from an alumina-based $^{99}\text{Mo}/^{99m}\text{Tc}$ generator, locally supplied by the Radiopharmacy Center. All other reagents were purchased from Sigma-Aldrich (Merck, Sao Paulo, Brazil). The conjugated HYNIC-P10, with 98% purity, was obtained from PEPTIDE 2.0 (Chantilly, VA, USA).

Radiolabeling

HYNIC-P10 was dissolved (1 mg/1 mL) in dimethyl sulfoxide. The radiolabeling method was conducted as described

by Faintuch et al. [9] with some changes. Briefly, 10 μl of HYNIC-P10 solution (0.54 mM) was added to a sealed reaction vial that contained 40 mg tricine and 2 mg of nicotinic acid, dissolved in 500 μl of nitrogenated purified water. Then, 3 μl of 14.7 mM $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ solution in 0.1N HCl (nitrogen purged), followed by addition of 500 μl of $\text{Na}^{99m}\text{TcO}_4$ (74–1850 MBq). The labeled mixture was heated to 100°C for 30 min and cooled to room temperature.

Radiochemical evaluation of the ^{99m}Tc -HYNIC-P10

Radiochemical analysis of the ^{99m}Tc -HYNIC-P10 conjugate was performed by instant thin-layer chromatography on silica gel strips (ITLC-SG; Gelman Sciences, Ann Arbor, MI, USA) with a two-solvent system (methylethylketone [MEK] and 50% acetonitrile). The ^{99m}Tc -HYNIC-P10 conjugate was also characterized by reverse-phase high-performance liquid chromatography (RP-HPLC; Shimadzu, Kyoto, Japan). RP-HPLC solvents consisted of water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.1% trifluoroacetic acid (solvent B). A Symmetry C-18 column (5.0 mm, 100Å, 4.6 \times 250 mm; Waters, Milford, MA, USA) was used with a flow rate of 1 ml/min. The RP-HPLC gradient system begins with a solvent composition of 95% solvent A and 5% solvent B and follows a linear gradient of 30% solvent A:70% solvent B from 0–25 min to 5% solvent A:95% solvent B from 25–30 min.

Determination of log *P* values

The radiotracer (100 μL) was dissolved in a mixture of equal volumes of n-octanol and water. After the mixture was vigorously stirred and centrifuged, samples (in triplicate) from both phases (n-octanol and water) were collected, radioactivity was measured using a gamma radioactivity counter, and log *P* values were then calculated.

Biodistribution study and imaging evaluation

Biodistribution studies were conducted *in vivo* with Balb/c mice, as these animals provide a good model of PCM [10]. Each animal was injected with 0.1 mL (74 MBq) of ^{99m}Tc -HYNIC-P10 via the tail vein. The animals were anesthetized with ketamine/xylazine (100 mg/10 mg/kg) and sacrificed at different times ($n = 5$ for each time) post-injection. Organs and tissues (blood, heart, lung, spleen, kidneys, liver, pancreas, stomach, large and small intestine, muscle, bone, and brain) were excised and weighed, and the radioactivity was defined by γ -counting.

The radioactivity dose was used as the standard for calculation of organ and tissue uptake, discounting the retained activity at the administration site. Results were expressed as percentage of injected dose per gram of tissue (% ID/g) and % ID per organ (% ID/organ). Total blood, muscle, and bone uptake was calculated assuming 7%, 40%, and 10% of the total body weight, respectively.

For image acquisition, the animals were anesthetized and horizontally placed under the collimator of a Mediso imaging system (Budapest, Hungary), using a low energy high resolution (LEHR) collimator. Images were acquired at 30 min, 1 h, and 2 h post-injection using a $256 \times 256 \times 16$ matrix size, with a 20% energy window set at 140 keV, for a period of 180 s.

Results

Radiolabeling

HYNIC-P10 was radiolabeled with ^{99m}Tc , with a radiochemical purity of $93.17 \pm 1.2\%$, assessed by ITLC-SG using a solvent system. In both solvents, MEK and acetonitrile 50% solution, the impurity pertechnetate ($^{99m}\text{TcO}_4$) had a retention factor (Rf) of 1; the impurity of colloid $^{99m}\text{TcO}_2$ was quantified at $Rf = 0$. The radiolabeled conjugate in the former solvent stayed together with the pertechnetate; in the latter solvent, the Rf was the same as for $^{99m}\text{TcO}_2$. Figure 1 shows the RP-HPLC radiochromatogram of the radiotracer with a retention time of 18.04 min. A smaller peak was observed at 16.35 min, which was not separated for further studies because the ratio between them was irrelevant, just 1:7. The most important radiochemical impurity was the formation of colloid $^{99m}\text{TcO}_2$ at very moderate proportions ($4.65 \pm 0.3\%$).

The lipohydrophilic character of the radioconjugate was evaluated based on the octanol/water partition coefficient. The log P value was -2.59 ± 0.07 , indicating hydrophilicity.

Blood depuration was fast; at 1-h post-injection, the result was only $0.57 \pm 0.13\%$ ID/g (Fig. 2). Figure 3 shows that uptake in blood was relatively low because of the small

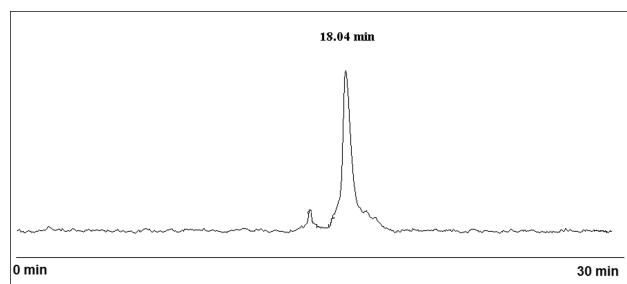


Figure 1. Radiochromatogram of technetium-99m-HYNIC-P10 obtained using reverse-phase high-performance liquid chromatography.

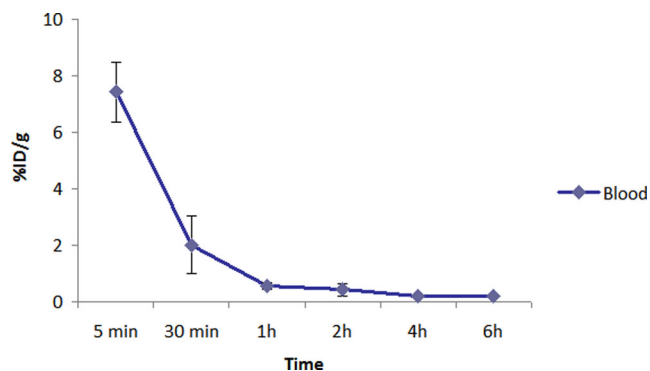


Figure 2. Blood clearance of technetium-99m peptide in healthy mice (0.1 mL/74 MBq). This Figure is reproduced in color in the online version of *Medical Mycology*.

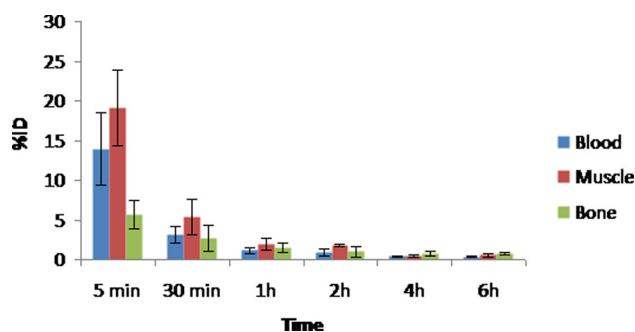


Figure 3. Total uptake of radiotracer. Results are expressed as percentage of injected dose per organ or tissue. Total blood, muscle, and bone uptake was calculated assuming 7%, 40%, and 10% of the total body weight, respectively. This Figure is reproduced in color in the online version of *Medical Mycology*.

mass of the tissue. However, when calculated per gram, uptake in blood was more remarkable than in muscle and bone, respectively, 7.42 ± 3.07 , 1.60 ± 0.75 , and $2.20 \pm 0.88\%$ ID/g (Table 1). One hour post-injection, the uptake of these tissues was $<3\%$ of the injected dose.

The excretion was mainly renal but also hepatobiliary (Fig. 4). Also at 1 h post-injection, the activity fell significantly. As depicted in Table 1, the highest uptake of the radiotracer was observed in lungs, followed by spleen and stomach. Scintigraphic images acquired at different times are shown in Figure 5. The scale of colors observed in the right side of the figure signals where radioactivity was more robust.

A small amount of radioactivity remained in the injection site (tail). High uptake was observed in the gastrointestinal tract, mainly in the liver. Nevertheless, part of the scintigraphic hot spot could correspond to lung and spleen uptake.

Most activity was seen in the bladder, as excretion was predominantly renal. After 120 min, the radiolabeled P10 was still observed in the liver as well as in cervical lymph

Table 1. Biodistribution of technetium-99m–HYNIC-P10 in healthy Balb/c mice— injected dose per gram of tissue.

Organ/Time	5 min	30 min	1 h	2 h	4 h	6 h
Blood	7.42 ± 3.07	2.00 ± 1.02	0.57 ± 0.13	0.43 ± 0.22	0.21 ± 0.02	0.22 ± 0.06
Heart	2.67 ± 0.91	0.74 ± 0.37	0.37 ± 0.18	0.18 ± 0.09	0.11 ± 0.01	0.11 ± 0.02
Lungs	12.07 ± 2.02	4.81 ± 2.90	5.50 ± 2.85	3.43 ± 2.88	1.17 ± 0.27	1.05 ± 0.19
Spleen	2.09 ± 0.29	2.80 ± 2.23	3.95 ± 2.50	2.07 ± 1.84	3.21 ± 2.51	3.58 ± 0.49
Stomach	4.14 ± 1.59	3.13 ± 0.48	1.72 ± 0.57	1.68 ± 0.55	1.10 ± 0.26	0.97 ± 0.42
Pancreas	1.72 ± 0.34	0.85 ± 0.47	0.42 ± 0.24	0.20 ± 0.08	0.12 ± 0.02	0.11 ± 0.03
Brain	0.27 ± 0.08	0.11 ± 0.05	0.05 ± 0.03	0.03 ± 0.02	0.02 ± 0.00	0.03 ± 0.01
Muscle	1.60 ± 0.75	0.49 ± 0.23	0.17 ± 0.08	0.25 ± 0.20	0.06 ± 0.04	0.06 ± 0.03
Bone	2.12 ± 0.88	0.98 ± 0.69	0.53 ± 0.23	0.56 ± 0.51	0.28 ± 0.11	0.31 ± 0.10
Liver	6.37 ± 2.42	3.73 ± 1.86	2.92 ± 1.53	2.09 ± 1.13	2.58 ± 1.31	2.72 ± 0.33
Large Intestine	2.43 ± 0.65	1.23 ± 0.64	0.77 ± 0.60	0.93 ± 0.70	0.97 ± 0.48	1.32 ± 0.56
Small Intestine	2.10 ± 0.54	2.17 ± 0.72	2.21 ± 0.89	2.66 ± 1.73	0.75 ± 0.26	0.33 ± 0.12

Data are expressed as the mean ± standard deviation ($n = 5$). The radioactivity in the stomach was evaluated after thoroughly removing the luminal contents.

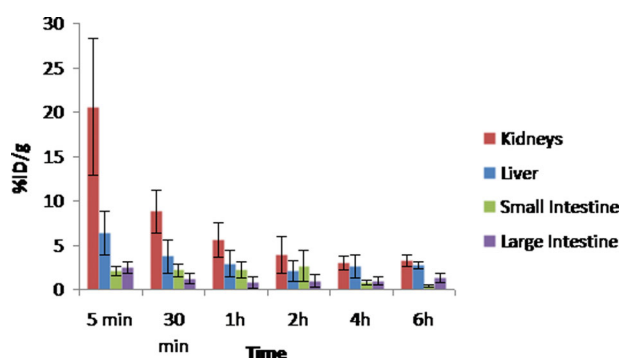


Figure 4. Excretion organ uptake of technetium-99m peptide in healthy mice (0.1 mL/74 MBq). The radioactivity in the intestines was evaluated after the luminal contents were completely removed. This Figure is reproduced in color in the online version of *Medical Mycology*.

nodes. The radioactivity in the cervical lymph nodes and thyroid was estimated in relation to the total activity in the body as the region of interest (ROI). ROI findings after 30 min, 60 min, and 120 min were 2.60%, 2.36%, and 1.54%, respectively.

Discussion

A seropositivity of as much as 27% may be observed in endemic regions, suggesting high prevalence of PCM in Latin America [11].

The P10 peptide, when used in combination with standard chemotherapy regimens, did improve treatment efficacy, with a potential to reduce the duration of treatment and avoid relapses [12]. To the best of our knowledge, this is the first radiopharmacokinetic study of P10 in an animal model. The P10 molecule was modified to present the following 15 amino acids containing (QTLIAIHTLAIRYAN), and this structure, conjugated to the chelating agent HYNIC and radiolabeled with the radioisotope ^{99m}Tc was the aim of this protocol. The HYNIC approach

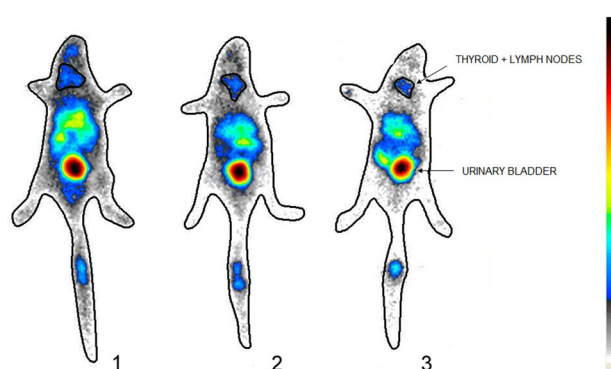


Figure 5. Scintigraphic image obtained using a gamma camera. (1) Image acquired 30 min post-injection (p.i.), (2) 60 min p.i., and (3) 120 min p.i. The hot spot, in part, corresponds to the thyroid as well as to lymph nodes. Region of interest findings after 30 min, 60 min, and 120 min were 2.60%, 2.36%, and 1.54%, respectively. The uptake by lymph nodes can be explained by the affinity of the peptide for this tissue. This Figure is reproduced in color in the online version of *Medical Mycology*.

represents a radiolabeling strategy. HYNIC–peptide conjugates can be labeled with ^{99m}Tc using different coligands. The radiotracer, with a radiochemical purity >90%, was obtained and thus adequate for *in vivo* evaluation. The principal radiochemical impurity was technetium colloid $^{99m}\text{TcO}_2$.

Several coligands have been developed to improve the ^{99m}Tc radiolabeling of HYNIC biomolecules [13]. We tried radiolabeling using tricine alone, the combination of tricine/ethylene diamino diacid, and the combination of tricine/nicotinic acid ternary ligands. The higher radiolabeling yield was obtained with the tricine/nicotinic acid labeling protocol.

The biodistribution evaluation, as close to normal as the scintigraphic images of the radiolabeled P10, revealed its pharmacokinetic pattern. One should not overlook the fact that part of the renal excretion observed could be due

to the conjugation of peptide P10 with the bifunctional chelate agent, HYNIC, which increases the lipophilicity of the molecule. In this sense, additional studies are recommended to elucidate such detail.

The uptake of the agent by cervical lymph nodes, clearly identified in the imaging step, highlights the affinity of the peptide for lymphoid tissue, which is crucial in the pathophysiology of PCM [3,4]. Such an outcome is consistent with the role of P10 in the immune response against *P. brasiliensis* and stimulates additional investigations for use in the clinical setting.

Bioactive peptides may have a role as mediators, agonists, and regulating agents. They can be cytoprotective in certain contexts and cytotoxic in others. P10 is one of several peptides derived from the major diagnostic antigen gp43 of *P. brasiliensis*. As a class, such peptides are strong modulators of local and systemic inflammatory response. Although P10 is specific for PCM, one should not rule out the possibility of effects on other infections, even in tumor models [14].

P10 peptide after conjugation with a chelating agent was successfully radiolabeled with the radioisotope ^{99m}Tc , achieving high radiochemical purity. Biodistribution showed fast blood clearance, mainly renal but also hepatobiliary excretion, along with remarkable uptake by cervical lymph nodes. Such data pave the way for the development of pharmaceutical agents, especially a vaccine for adjuvant management of patients suffering from PCM.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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