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Evaluation of Drug Interaction between [^{99m}Tc]Tc-Ketoconazole and Ketoconazole Administered for Candidiasis Treatment in Mouse Models

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ABSTRACT

The use of radiopharmaceuticals for detecting infections has gained increasing attention for their applications in nuclear medicine. The administration of [^{99m}Tc]Tc-ketoconazole may alter pharmacological aspects including drug interaction with some antifungals especially ketoconazole which is commonly used for candidiasis treatment. This study investigated the *ex vivo* biodistribution and pharmacokinetic interaction of [^{99m}Tc]Tc-ketoconazole after ketoconazole administration in BALB/c mice. In this research, the [^{99m}Tc]Tc-ketoconazole was prepared with radiochemical purity of 94.59%. The ex vivo biodistribution uptakes in infected muscle as the target organ were 0.17±0.12% ID/g for the therapy group (1h) and 0.05±0.04%ID /g (3h). However, these results were not significantly different compared with the normal muscle (p>0.05). The ex vivo biodistribution also showed the highest radioactivity uptake on the liver which was significantly different (p<0.05) for 1h (6.84±0.51 %ID/g) and 3h (5.81±0.81 %ID/g) observation in the therapy group. The Target/Non-Target (T/NT) ratio between infected muscle compared with normal muscle for the therapy group was 2.89±1.71 at 1h observation (p<0.05) as consideration for imaging applications. Pharmacokinetics analysis using *PK*Solver showed that after ketoconazole administration [^{99m}Tc]Tc-ketoconazole half-life elimination ($t_{1/2}$ β) for the therapy group was 13.78±4.77 h, which was shorter than the control group (44.77±2.74 h). Changes in pharmacokinetic parameters occurred at the area under the curve of therapy group ($AUC_{0\text{-inf}}$) of 26.10±18.97 %ID/g*h and the maximum concentration (C_{max}) of 13.05±9.48 %ID/g where C_{max} defines the peak concentration and $AUC_{0\text{-}inf}$ determines the total systemic exposure of radiopharmaceuticals, thus indicated that the radiopharmaceutical absorption rate was increased. It was demonstrated that based on biodistribution and pharmacokinetic evaluation, [^{99m}Tc]Tc-ketoconazole can be used to evaluate the progress of therapy in patients with candidiasis based on the T/NT ratio at 1h post-injection.

Keywords: Drug interaction, Radiopharmaceuticals, [^{99m}Tc]Tc-ketoconazole, Candidiasis, Ketoconazole

INTRODUCTION

Candidiasis remains a challenging problem in patients due to complications with other health issues. Candidiasis prevalence in Indonesia at Cipto Mangunkusumo hospital was discovered to be 12.3% in 2017 (Kalista, *et al*., 2017). Meanwhile, prevalence data of candidiasis was revealed in Singapore (33.3%), Taiwan (55.6%), and Japan (41%) (Arendrup, 2010). The epidemiology of candidiasis has been the subject of numerous studies as 1.2–25 cases per 100 000 population or 0.19–2.5 per 1000 admissions have been reported. Candida colonizes the mucus membranes of 30–60% of humans and is capable of causing a wide range of infections (Lim, *et al*., 2012).

Detection methods for candidiasis are classified into the conventional method with the isolation of the Candida species from clinical specimens, and molecular methods such as antibody detection and polymerase chain reaction-

restriction fragment length polymorphism (PCR–RFLP). The conventional method has limitations such as low accuracy, usually takes 2–5 days to obtain the results, and sometimes misdiagnosis of the species, meanwhile the molecular method using PCR-RFLP has some disadvantages including the high cost and relatively long analysis time, therefore the results cannot be obtained immediately (Alam *et al*., 2014). Nuclear medicine imaging is available for detecting infections and can differentiate between infectious and inflammatory processes immediately. A radiopharmaceutical used for imaging infection and inflammation accumulates in the infectious or inflammatory lesion, due to the locally changed physiological condition (Boerman, *et al*., 2001) (Becker and Meller, 2001). One of the developed radiopharmaceuticals for infections is [^{99m}Tc]Tc-ketoconazole, which was quite sensitive for the detection of Candidiasis based on previous research (Sugiharti, *et al*., 2016).

Fungal treatments are available worldwide such as amphotericin B, nystatin, clotrimazole, miconazole, fluconazole, ketoconazole, and itraconazole (Cuesta, *et al*., 2014). Ketoconazole is well known as an antifungal imidazole compound that is active against both superficial and systemic fungal infections when it is administered orally (Uno, *et al*., 1982).

Administration of radiopharmaceuticals following drug consumption may result in drug interaction. The interaction could affect biodistribution, organ visualization, and cause misdiagnosis in the patient (Olieveira, *et al*., 2008). The pharmacological interaction process affects the biodistribution of the radiopharmaceutical due to the mechanism of action of the, toxicological interaction, pharmacokinetic interaction, and physical properties of the drug (Iii, *et al*., 1982). The latest research by Zora *et al*. found that the administration of medicinal plants affected the biodistribution uptake of [^{99m}Tc]Tc-DTPA in the kidney and bladder (Zora *et al*., 2012).

This study aimed to determine drug interaction in ketoconazole administration continued with [^{99m}Tc]Tc-ketoconazole based on *in vivo* pharmacokinetics and biodistribution on BALB/c mice. The data acquired in this study can be useful for clinicians to determine the correct diagnosis and treatment for patients with candidiasis.

METHODS AND MATERIAL

Preparation of [^{99m}Tc]Tc-ketoconazole

The [^{99m}Tc]Tc-ketoconazole was prepared by adding 100µL of ketoconazole solution (20mg /mL 0.1 N HCl) into glass vials. After that, 150µL of Sn-DTPA solution (containing 75 µg $SnCl_2.2H_2O$ and 2.25mg DTPA) was added into the same vial. The solution pH was adjusted around 4-4.5 by adding HCl 0.1N or 0.1N NaOH. Thereafter, 1.75mL of freshly eluted $Na^{99m}TcO4^-$ (±74 MBq) was added to the vial. The final volume was adjusted to 2mL by adding 0.9% NaCl. The [^{99m}Tc]Tc-ketoconazole formed was tested for purity using Whatman 31 ET paper with 50% acetonitrile eluent to determine the remaining amount of TcO_2 impurities, which was found at Rf 0 and Whatman 3MM with 100% acetonitrile eluent to determine the magnitude of ^{99m}Tc, which was found at Rf of 0.9-1.

Animal model for infection

All animal experiments were carried out under a protocol approved by the Ethics Committee for The Care and Use of Laboratory Animals (KEPPHP) BATAN under protocol 004/KEPPHP-BATAN/V/2017. Six-week-old male BALB/c mice were purchase from PT. Biofarma, Tbk Bandung. The mice were maintained in a standard cage at a constant temperature (26±1°C) and the humidity was maintained at 60±5% with a 12h dark cycle. Animals were fed with standard rodent feed and water *ad libitum*.

The mouse was anesthetized by inhalation of aerosolized isoflurane mixed with oxygen. The right leg was sterilized using povidone iodine and placed on the surgical bed. Next, approximately 1x 10^7 CFU of Candida albicans in 0.1mL of saline was injected into the right thigh muscle of each mouse. The infection was assessed after 48h post-injection to obtain localized infection (Nogueira *et al*., 2018). After that, the infected organs were collected and fixed in 10% neutral buffered formalin, embedded in paraffin cut into 5µm thick axial section, and stained with hematoxylin and eosin (Efficacy *et al*., 2017).

Ex vivo biodistribution study

Ketoconazole was given peroral using oral gavage 0.1mL. Then, *ex vivo* biodistribution was assessed by evaluating the uptake of [^{99m}Tc] Tc-ketoconazole in an animal model of candidiasis at 1h and 3h after ketoconazole administration.

The mice were euthanized 1h post-injection of [^{99m}Tc]Tc-ketoconazole using accepted protocol and their organs were removed and weighed. Radioactivity was measured using 2470 Wizard 2 Automatic Gamma Counter (Perkin Elmer, Waltham, MA, USA). Results were expressed as injected dose percentage (%ID) per gram of tissue (n = 3). The use of at least three animals for each time point refers to IAEA (International Atomic Energy Agency) technical report series no.466 (IAEA, 2008).

Pharmacokinetics study

[^{99m}Tc]Tc-ketoconazole was injected into BALB/c mice via tail veins with radioactivity 3.7 MBq/ mice (n=3). Mice were divided into two groups, control, and therapy. The control group received saline continued with [^{99m}Tc]Tc-ketoconazole. The therapy group was given ketoconazole peroral and after 1h continued with [^{99m}Tc]Tc-ketoconazole. Blood samples were collected from the tail vein at 5, 10, 15, 30min and 1, 2, 3, 4, 5, 24h after [^{99m}Tc]Tc-ketoconazole administration. Radioactivity was counted using Wizard 2 Automatic Gamma Counter (Perkin Elmer, Waltham, MA, USA). The radioactivity concentration of the blood sample was expressed as the percentage of injected dose (%ID) per gram of blood. The pharmacokinetic parameters were evaluated using *PKSolver* (Zhang, *et al*., 2010).

Statistical analysis

The values are presented as means±SD. The biodistribution data and pharmacokinetic parameters between the control and therapy group were evaluated using student *t*-test, GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA). The *p*-value <0.05 is considered statistically significant.

RESULTS AND DISCUSSION

Preparation of [^{99m}Tc]Tc-ketoconazole

The use of technetium radiopharmaceutical for *in vivo* study according to the IAEA technical report series has a quality requirement of the determination of RCP (radiochemical purity) using a chromatography technique such as paper chromatography, thin-layer chromatography, HPLC (High-Performance Liquid Chromatography), or column chromatography and measured radiochemical purity >90% (IAEA, 2008).

The radio-chromatogram of [^{99m}Tc]Tc-ketoconazole, where acetone was used as a mobile phase (Figure 1 A). The [^{99m}Tc]Tc-ketoconazole and $^{99m}TcO_2$ stayed at the origin (Rf = 0) while $^{99m}TcO_4^-$ moved along with the solvent to give Rf = 1. On the other hand, in the radio-chromatogram of [^{99m}Tc]Tc-ketoconazole where the saline solution was used as a mobile phase, $^{99m}TcO_2$ stayed at the origin (Rf=0). Meanwhile, [^{99m}Tc]Tc-ketoconazole and $^{99m}TcO_4^-$ moved with the solvent to give Rf = 1 (Figure 1B). The test result showed that [^{99m}Tc]Tc-ketoconazole had 94.59% RCP (n = 3). The radiochemical impurity in this study from free $^{99m}TcO_4^-$ and free ketoconazole during the labeling process. The presence of >10% ^{99m}Tc-pertechnetate would affect biodistribution in organs and give poor imaging quality (Vallabhajosula, *et al.*, 2010). The purification was not performed because the RCP of [^{99m}Tc]Tc-ketoconazole had already met the standard requirement for *in vivo* study.

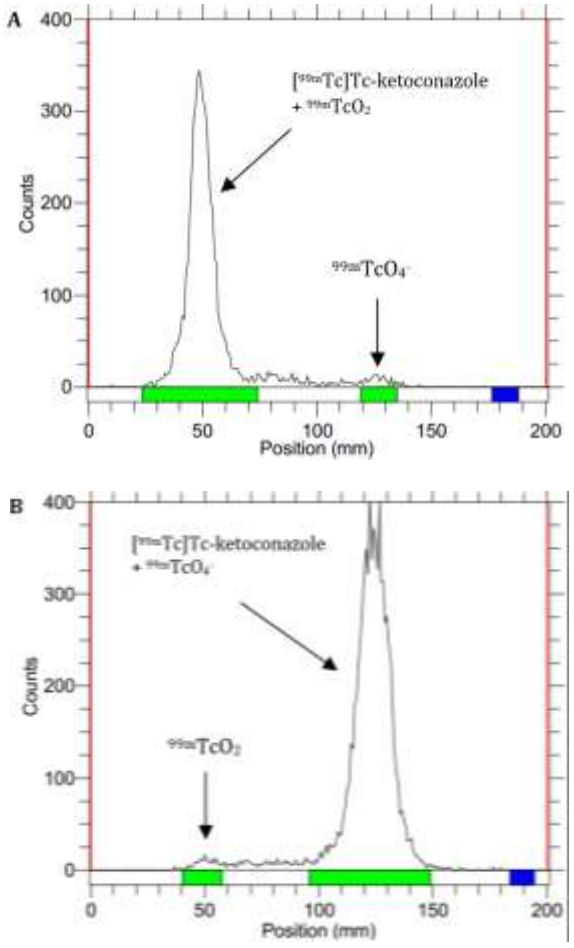

Figure 1. Radiochromatogram of [^{99m}Tc]Tc-ketoconazole using acetone as the mobile phase (A) and 0.9% NaCl as the mobile phase (B)

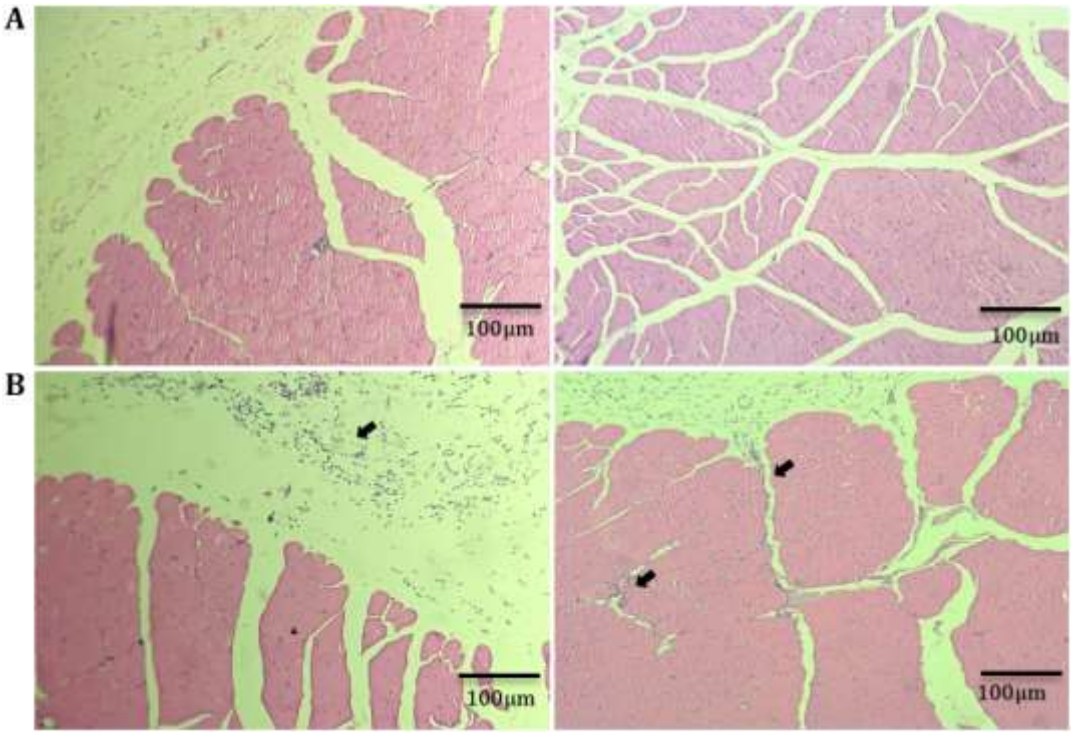

Figure 2. Histological section of normal muscle (A) and infected muscle (B) after 48 h of *Candida albicans* injections. H & E staining displayed the presence of inflammatory cells in fatty tissue around the muscles and infiltrated to muscle area (black arrows) while normal muscles showed no infiltration of inflammatory cells.

Animal model for Infection

The muscle was analyzed both in normal and infected mice (Figure 2). Histological evaluation (Figure 2A) indicated that an inflammatory cell polymorphonuclear (PMN) was found in the fatty tissue and infiltrated into tissues around the infected muscles within the 48h post injections of Candida albicans. No inflammatory cell was found in normal muscle (Figure 2B). These results confirmed that the mouse model of candidiasis was available to use for [^{99m}Tc]Tc-ketoconazole evaluations.

***Ex vivo* biodistribution and pharmacokinetics study**

The *ex vivo* biodistribution pattern of [^{99m}Tc]Tc-ketoconazole (Figure 3). The biodistribution was observed based on the comparison between the control and therapy groups at 1h and 3h post-injection. The biodistribution results showed that at 1h post-injection, high accumulation of [^{99m}Tc]Tc-ketoconazole was found in the liver, spleen, and kidney compared with other organs. The highest radioactivity uptake was revealed in the liver with $p<0.05$ which was 6.84±0.51 %ID/g for the therapy group, and 1.96± 0.29 %ID/g for the control group (Figure 3A). Meanwhile, the 3h observation results in Figure 3B showed different patterns of radioactivity accumulation in various organs. The highest accumulation was in the liver with 7.90±0.71 %ID/g in the control group and 5.81±0.81 %ID/g in the therapy group. The difference between the two groups was statistically significant with $p<0.05$. Accumulation in other organs such as the lung and stomach was also significantly different between groups with lower accumulation found in the therapy group.

The difference in radioactive accumulation pattern between the control and therapy groups indicated that interaction between ketoconazole and [^{99m}Tc]Tc-ketoconazole occurred in several organs according to the observation time interval. Significant differences at 1h and 3h were due to ketoconazole administered via oral route was subject to metabolism in the liver and thus caused high radioactivity uptake into the organ.

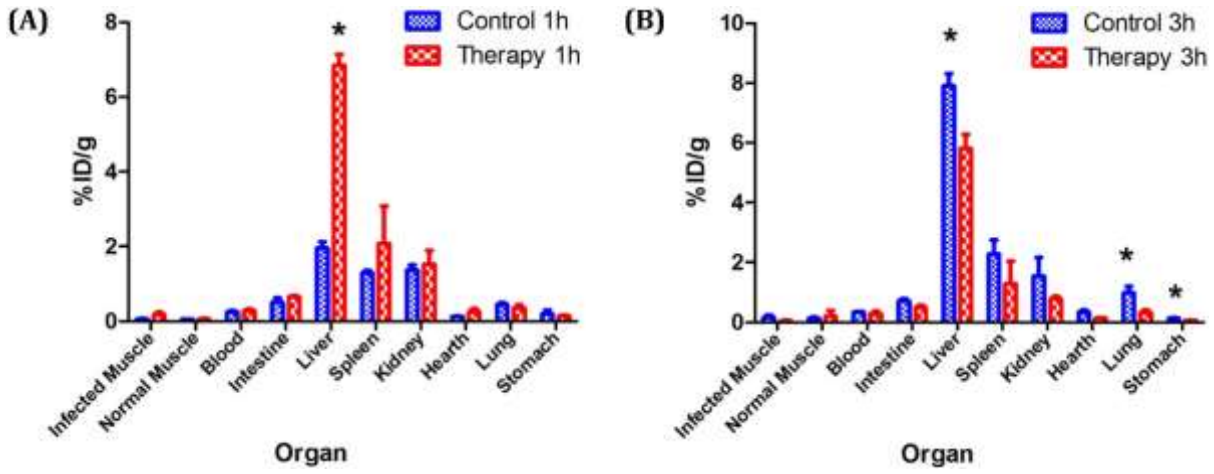

Figure 3. *Ex vivo* biodistribution of [^{99m}Tc]Tc-ketoconazole for control (saline) and therapy group (ketoconazole) 1h post-injection (A) and 3 h post-injection (B) (n = 3). Data are presented as %ID/g ± SD, **p*<0.05.

The metabolism-associated difference in radioactivity uptake into the liver is related to liver metabolic enzyme cytochrome P450-3A (CYP3A) activity and hepatic reaction due to the administration of ketoconazole continued with [^{99m}Tc]Tc-ketoconazole (Sugiharti, *et al*., 2016) (Greenblatt and Greenblatt, 2014).

High radioactivity uptake was also found in the spleen for 1h and 3h observation but the uptake amounts were not significantly different (p>0.05). Radioactivity uptake in the spleen for 1h was 1.29±0.13 %ID/g for the control group and 2.08±1.73 %ID/g for the therapy group. On the other hand, at 3h observation, the radioactivity accumulation showed 2.28±0.79 %ID/g for the control group and 1.29±1.10 %ID/g for the therapy group. The radioactivity uptake in the spleen may be due to several aspects such as the particle size (where it is known that radiopharmaceuticals with particle size between 0.1-1µm will increase the accumulation in the spleen although in this study the particle size has not been determined) and some impurities of the [^{99m}Tc]Tc-ketoconazole which could alter the biodistribution of the radiopharmaceuticals in the organ (Vallabhajosula *et al*., 2010). Based on previous research, it is also known that the high accumulation in the spleen is caused by metabolic mechanisms in the liver and spleen (Sugiharti, *et al*., 2016).

Uptakes into the kidney for 1h post-injection were 1.37±0.25 %ID/g for the control group and 1.52±0.64 %ID/g for the therapy group. Furthermore, for 3h post-injection, the radioactivity uptake in the kidney for the control group was 1.54±1.09 %ID/g compared with that in the therapy group which was 0.78±0.15 %ID/g. The uptake was revealed due to the excretion mechanism of [^{99m}Tc]Tc-ketoconazole, although the urinary excretion of ketoconazole itself was small (Tyle, 1984).

When [^{99m}Tc]Tc-ketoconazole was injected intravenously, it was distributed into organs. Infected muscle as target organ has the highest uptake at 1h post-ketoconazole administration which was 0.17±0.12 %ID/g compared with normal muscle (0.06±0.02 %ID/g). Furthermore, in the therapy group 3h post-injection, the radioactivity accumulation in infected muscle decreased to 0.05±0.04 %ID/g while in the control group the accumulation decreased to 0.16±0.13 %ID/g. [^{99m}Tc]Tc-ketoconazole accumulation in infected muscle was due to the mechanism of action of ketoconazole by inhibiting the biosynthesis of ergosterol in the Candida albicans membrane. Ketoconazole inhibits the C-14 alpha demethylase enzyme, which is important for the synthesis of ergosterol. Ergosterol deficiency leads to increased membrane permeability and affects fungal growth and replication as a key component of the fungal cell membrane (Greenblatt and Greenblatt, 2014).

The averaged organ T/NT ratio of [^{99m}Tc] Tc-ketoconazole both in the control and therapy groups (1h and 3h observation) (Figure 4).

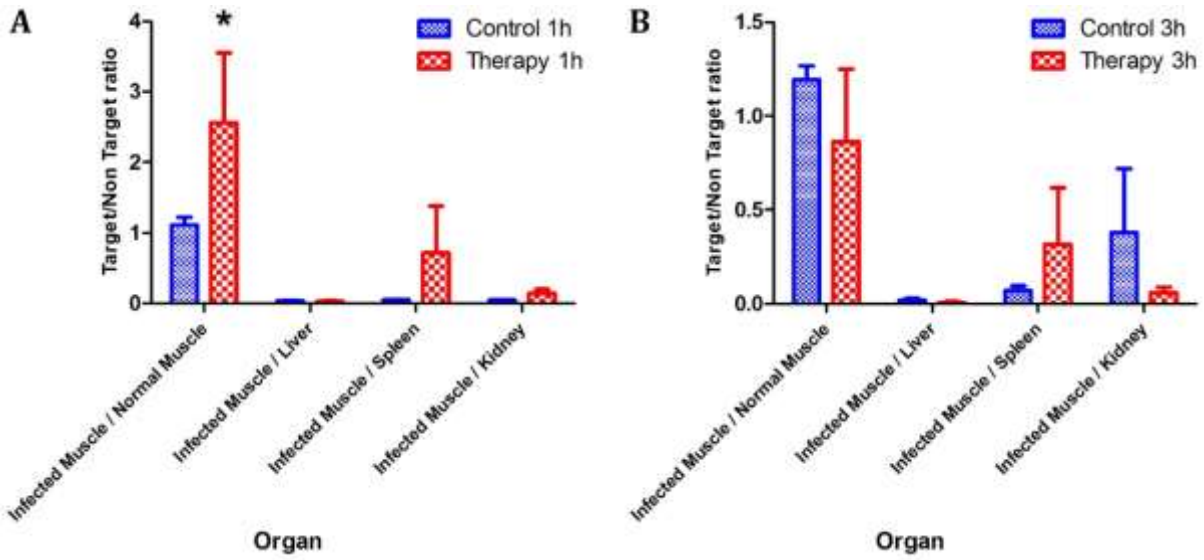

Figure 4. Ratio Target/ Non-target (T/NT) organ of [^{99m}Tc]Tc-ketoconazole with the administration of ketoconazole 1 h post-injection (A) and 3 h post-injection (B) (n=3). Data are presented as the mean ± SD from three mice with p<0.05.

Table I. Pharmacokinetics study of [^{99m}Tc]Tc-ketoconazole 1h post-injection between control (saline) and therapy group (ketoconazole)

Parameter	Unit	Control	Therapy
$K0$	µci/h	25.00±0.00	25.00±0.00
$t_{1/2}\,\alpha$	H	0.01±0.00	0.00±0.00
$t_{1/2}\,\beta$	H	44.77±2.74	13.78±4.77
C_{max}	%ID/g	7.63±4.36	13.05±9.48
$AUC_{0\text{-inf}}$	%ID/g*h	15.26±8.72	26.10±18.97
$AUMC$	%ID/g*h^2	0.14±0.11	0.16±0.18

The infected muscle as the target organ compared with normal muscle at 1h post-injection (therapy group) had the highest T/NT ratio of 2.89±1.71 as the best time-point for imaging application with p<0.05 (Figure 4A). Meanwhile, the control group showed a T/NT ratio of 1.11±0.19. Furthermore, the 3h observation groups showed a T/NT ratio of 0.86±0.67 for the therapy group and 1.19±0.13 for the control group (Figure 4B). A Low T/NT ratio was observed in comparison with other organs with high radioactive accumulation such as the liver, spleen, and kidney. The minimum T/NT ratio to obtain high-quality images in SPECT imaging is 2 (Ilem-Ozdemir, *et al.,* 2019).

The T/NT ratio obtained in this study at 1h and 3h post-injection in the control group was different when compared to previous studies by Sugiharti *et al*. Previous research showed a T/NT ratio of 3.40 for 1h and 0.33 for 3h post-injection, whereas in this study it was 1.11±0.19 at 1h and 1.19±0.13 for 3h post-injection. This difference was due to the difference in the method used to assess the *ex vivo* biodistribution. In the previous research, they used a Single Channel Analyzer to count radioactivity in the organs. While in this study, a Wizard 2 Automatic Gamma Counter 2470 was used which has better sensitivity and without any delay in sample counting. The results obtained in this research are relevant to an *in vivo* imaging study conducted by Sugiharti *et al*. according to the results of the ROI of infected muscles (1392.439) and normal muscles (1259.788). This result suggested that [^{99m}Tc]Tc-ketoconazole can be used to evaluate the progress of therapy in patients infected with candidiasis who are being treated with ketoconazole, with an optimum time for

imaging at 1h post-injection (Sugiharti, *et al*., 2016). Meanwhile, a high T/NT ratio indicated high imaging contrast and sensitivity of the radionuclide diagnostics (Vorobyeva *et al*., 2019).

Pharmacokinetic analysis was performed to measure the change in pharmacokinetic parameters between the control and therapy groups. The administration of ketoconazole continued with [^{99m}Tc]Tc-ketoconazole had similar results on the formation rate constants of metabolites (*K0*) related to the hepatic process (Table I). The radioactivity half-life distribution ($t_{1/2}$ α) in the control and therapy groups showed that [^{99m}Tc]Tc-ketoconazole had distributed rapidly to the bloodstream after injected into the tail vein. A significant result was revealed on radioactivity elimination ($t_{1/2}$ β) with p<0.05, with the therapy group showing a shorter elimination time (13.78±4.77h) compared with the control group (44.77±2.74h). Research by Tyle, JH proved that ketoconazole was eliminated from the body at 8h post-administration and reached the peak concentration at 2h (Tyle, 1984). The ketoconazole pharmacokinetics was affected the $t_{1/2}$ α and $t_{1/2}$ β of [^{99m}Tc]Tc-ketoconazole in the case of ketoconazole administration before the application of [^{99m}Tc]Tc-ketoconazole. The pharmacokinetic parameter also changed the $C_{\max}$ (13.05±9.48 %ID/g) and $AUC_{0\text{-inf}}$ (26.10±18.97 $\%ID/g*h$) compared with the control group. Previous research suggested that C_{max} was highly correlated with the $AUC_{0\text{-}inf}$ which shows that an increase in the parameter value is correlated with the absorption rates or amount of absorbed drugs (Endrenyi, *et al*., 1991). The result suggested that for the therapy group, administration of ketoconazole continued with [^{99m}Tc]Tc-ketoconazole increased the radiopharmaceutical absorption. In the animal model of candidiasis, ketoconazole was metabolized in the liver and released from the kidney. It is suggested, that the binding site in the liver was filled by ketoconazole, and [^{99m}Tc]Tc-ketoconazole was unable to attach completely and thus was released faster from the body (Greenblatt and Greenblatt, 2014). This finding is important for clinical application to determine the pattern of treatment in patients using antifungal therapy using ketoconazole and to evaluate the treatment progress using [^{99m}Tc]Tc-ketoconazole.

CONCLUSION

This study demonstrated that to evaluate the therapy progress of patients with candidiasis, the optimum time for imaging application using [^{99m}Tc]Tc-ketoconazole after ketoconazole administration was recommended at 1h. The results also suggested that interaction with ketoconazone also affected the elimination of [^{99m}Tc]Tc-ketoconazole. Further investigation using preclinical SPECT imaging is needed to evaluate the accumulation between the target organ (infected muscle) and background organs.

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