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Biological Evaluation of ^{99m}Tc-Kanamycin for Infection Imaging

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Abstract Kanamycin antibiotic was radiolabeled successfully with radioisotope technetium-99m for the potential use as radiopharmaceuticals for infection imaging. ^{99m}Tc-kanamycin complexes was prepared 93 % radiochemical purities by direct labelling using 5 mg kanamycin and 30 µg SnCl2. The reaction occurred at alkaline condition (pH=9) and under room temperature for 30 min to achieve high radiochemical purity. Radiochemical purity and stability of ^{99m}Tc-kanamycin was determined by ascending paper chromatography using Whatman 3 paper as the stationary phase, and acetone as the mobile phase to separate the radiochemical impurities in the form of ^{99m}Tc-pertechnetate. While impurities in the form of ^{99m}Tc-reduced were separated using the stationary phase ITLC-SG and 0.5 N NaOH as mobile phase. This study aimed to determine biological characteristic of ^{99m}Tc-kanamycin radiopharmaceutical. In vitro cell studies showed that the change of kanamycin structure after labeling with technetium-99m did not give a specific influence to the potency of kanamycin as an antibiotic. In addition on uptake study, a significantly higher uptake of ^{99m}Tc-kanamycin with S. aureus than E. coli. Biodistribution of ^{99m}Tc-kanamycin complexes was studied on normal and infection mice at 15, 30, 60 and 120 min post-injections. The biodistribution of ^{99m}Tc-kanamycin in infection mice showed that the complex accumulated in the infection sites. These results show that ^{99m}Tc-Kanamycin radiopharmaceutical have a potential application for infection diagnosis.

Keywords : ^{99m}Tc-kanamycin, infection, biological evaluation,

1. INTRODUCTION

According to the Global Report 2012 data, infectious diseases were responsible for the death of almost 9 million people worldwide in every year, and many of them are children under five years old (WHO, 2012). Those infection deaths occur because of late identifications of main infections which occur in very deep parts of the body (deep-seated infection). Early detection and determination of the infection location are required. Nuclear medicine come to give solution for infection disease management. The development of new radiopharmaceuticals which are able to differentiate between inflammatory and infectious with high specificity are required. Certain studies show that radiolabeled antibiotics or antimicrobial peptides are capable to distinguishing between infective and non-infective inflammations (Akhtar et al, 2012; Doroudi et al., 2014; Ebenhan et al., 2014; Fazli et al, 2012; Ilem-Özdemir, 2013; Ilem-Ozdemir et al., 2014; Mirshojaei et al., 2010; Sanad, 2014).

Kanamycin is an antibiotic belonging to the aminoglycosides that act by binding to the bacterial ribosome, thereby inhibiting protein synthesis and generating errors in the translation of the genetic code (Magnet, 2005). Its nature as a broad-spectrum antibiotic allows it to bind to Gram-negative and Gram positive bacteria. Kanamycin is used to treat infections when penicillin or other less toxic drugs cannot be used. Infections treated include: bone, respiratory tract, skin, soft tissue, and abdominal infections, complicated urinary tract infections, endocarditis, septicaemia, and enterococcal infections (Roohi et al, 2006). Kanamycin has several electron

donor fungctional groups such as –NH2, -OH and –O- that can form bonds with technetium-99m. ^{99m}Tc-kanamycin (Fig. 1) labeling studies have been carried out in the previous research by direct labeling method with the result of labeling efficiency of about 93 % (E M Widyasari et al, 2015). Besides study of physico-chemical characteristics of ^{99m}Tc-kanamycin have also been studied. In this paper biological evaluation (in vitro and in vivo study) of ^{99m}Tc-kanamycin were studied in detail experimental.

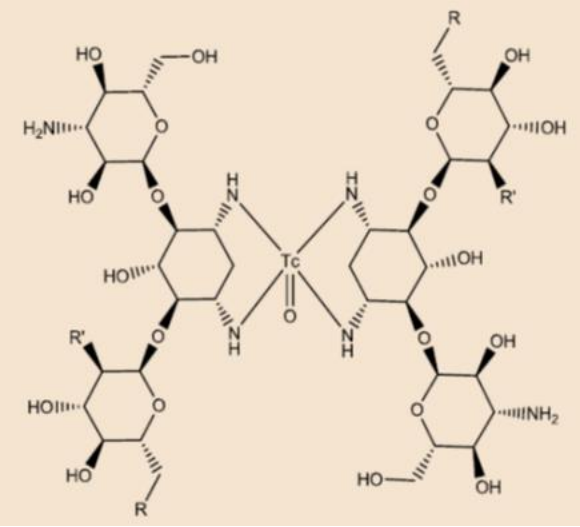


Fig. 1. Chemical structure predictions of ^{99m}Tc-kanamycin

2. MATERIALS AND METHODS

2.1 Materials and equipments

The materials used to carry out in this research were kanamycin sulphate from Meiji and stannous chloride dihydrate purchased from Sigma Aldrich Company. All other chemicals were of reagent grade and were used without further purification. ^{99}Mo/^{99m}Tc generator was obtained from BATAN Technology. Paper chromatography analysis was carried out with Whatman no. 3 paper chromatography and ITLC-SG (Agilent). The white mice (Mus musculus) were obtained by cultivation alone and biodistribution studies were performed with mus musculus mice that has infected with Staphylococcus aureus and Escherichia coli bacteria. The Animal Ethics Committee (KEPPH) of BATAN gave approval for the animal experiments.

The equipment used in this experiment consists of dose calibrator (Victoreen), vortex mixer, single channel analyzer (Ortec), and paper chromatography apparatus.

2.1 Radiolabeling and quality control techniques

The preparations procedure for ^{99m}Tc-kanamycin and the paper chromatography analysis conditions are as follows. 0.9 mL of saline containing (^{99m}TcO4- ± 2-5 mci) was added to a kanamycin wet kit containing 5 mg/mL of kanamycin and 30 µg of stannous chloride dehydrate. The mixture was kept at room temperature for 30 min. The determination of the radiochemical purity of ^{99m}Tc-kanamycin was performed using Whatman no. 3 paper as the stationary phase and acetone as the mobile phase to separate of ^{99m}Tc-pertechnetat (^{99m}TcO4-). Meanwhile, to separate the impurity of ^{99m}Tc-reduced (^{99m}TcO2), ITLC-SG was used as the stationary phase and 0.5 N NaOH as a mobile phase. The chromatograms were dried in oven at 80^{o}C for five minutes, and then paper was cut every 1 cm piece and measured using single-channel analyser (SCA) with detector NaI(Tl).

2.3 In vitro cell studies

Binding of ^{99m}Tc-kanamycin to bacteria was assessed by the method described previously (Sugiharti et al., 2015). Briefly, 2 ml of saline containing approximately 1×10^7 colony forming units (CFU) per ml viable S. aureus or E. coli was transferred to a centrifuge tube. Then, 0.1 ml ^{99m}Tc-kanamycin were added. The mixture was

incubated for 1, 3, 5 and 24 h in waterbath shaker at 37^{o}C and 120 rpm. Thereafter to each vial was added 500 µL TCA 10 % b/v and then the vials were centrifuged for 5 min. The supernatant was separated, and the radioactivity in the bacterial pellet was gently re-suspended in 1ml of PBS and re-centrifuged as above. The supernatant was sparated and the radioactivity in the bacterial pellet and supernatant were determined by Single Channel Analyzer (SCA). The radioactivity related to bacteria was expressed in percent of the added ^{99m}Tc activity bounded to viable bacteria in regard to total ^{99m}Tc (supernatant and pellet). Na^{99m}TcO^{4} was used as control.

2.4 Pharmacokinetics studies

The blood clearance study was performed in Swiss mice (normal mice) weighing about 40-50 g, after administering 400 µCi/0.4 ml of labeled product intravenously through the tail vein. At time intervals of 5 min, 15 min, 30 min, 45 min, 1 h, 2 h, 3h, 4 h, 5h, 24h and 30 h, about one drop blood was withdrawn from the tail vein of the animal. The samples were weighed accurately to the second decimal place in grams and radioactivity was measured using a SCA. The data were expressed as percent administered dose per gram of blood at each time interval.

2.5 Biodistribution studies

Biodistribution studies was prepared on three groups animal experiments, which involve group of normal mice, group of infected mice with E. coli, and group of infected mice with S. aureus. Each group consisted of 12 mice were divided in four time intervals i.e. 15, 30, 60 and 120 min after injection and each time interval consisting of three mice. To prepare infected model, the day before each study, Swiss mice with weighing 40-50 g were infected by injecting 0.1 ml of saline containing 2 × 10^{6} CFU bacteria into right thigh muscle. 24 h later, they were injected with 0.1 ml (100 µCi) of ^{99m}Tc-kanamycin via the tail vein. At 15, 30, 60 and 120 min post administration, mice were sacrificed and organs of interest were collected, weighed and radioactivity was measured with SCA. Radioactivity accumulated in each organ was expressed as percent administered dose per gram of tissue.

3. RESULTS AND DISCUSSION

In this studies labeling ^{99m}Tc-kanamycin was prepared by direct labeling method. In the previous research, ^{99m}Tc-kanamycin labeling studies have been carried out by the indirect method using pyrophosphate as a co-ligand with the result of labeling efficiency of above 95% (Sugiharti et al., 2015; E M Widyasari, 2013a; E M Widyasari, 2013b; E M Widyasari et al., 2015). However, the presence of radiochemical impurities in the form of ^{99m}Tc-pyrophosphate in this indirect labeling method may interfere imaging results. The accumulation of ^{99m}Tc-pyrophosphate in the bone made it difficult to distinguish between an infection of the bone and the uptake of ^{99m}Tc-pyrophosphate.

Radiochemical purity (RCP) was assessed by a combination of ascending paper chromatography and instant thin layer chromatography on silica gel. In paper chromatography (whatman 3) as stationary phase and aceton as the solvent, impurities $^{99m}TcO_4^-$ migrated with the front (Rf=1) while ^{99m}Tc-kanamycin and reduce/hydrolysed ^{99m}Tc remined at the origin (Rf=0). In ITLC-SG chromatography using NaOH 0.5 N as the solvent, impurities reduce/hydrolysed ^{99m}Tc remined at the origin, whereas ^{99m}Tc-kanamycin and $^{99m}TcO_4^-$ moved with the front. The mean RCP of the ^{99m}Tc-

kanamycin was 92.31 ± 1.74%. As a radiopharmaceutical to be administered intravenously it is necessary to test its stability in plasma. The result of stability test ^{99m}Tc-kanamycin in the plasma showed that up to 4 hours of incubation (Fig. 2), ^{99m}Tc-kanamycin is still providing a RCP > 90 %. This shows that plasma environment does not outlining the bond between technetium-99m and kanamycin (Eva Maria Widyasari, 2015).

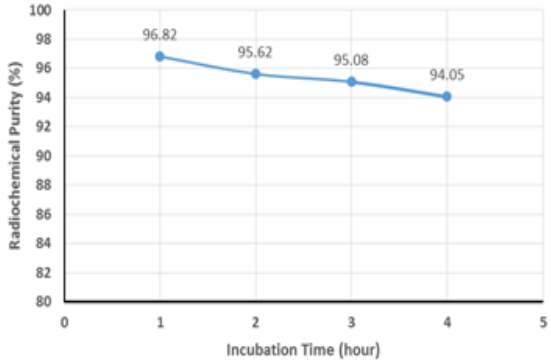


Fig. 2. ^{99m}Tc-kanamycin stability in plasma

From in vitro study, that has been reported before (Sugiharti et al., 2015), showed the change of kanamycin structure after labeling with technetium-99m did not give a specific influence to the potency of kanamycin as an antibiotic. In vitro bacteria uptake using Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) bacteria showed that decreased uptake of ^{99m}Tc-kanamycin as a function of incubation time was observed (Fig. 3 and Fig. 4). ^{99m}Tc-kanamycin had optimal uptake at 1 h post incubation and reached a high S. aureus and E. coli bacteria uptake. S. aureus bacteria uptake (53.00 ± 0.30 %) was greater than E. coli (36.99 ± 1.40 %). This was due to differences in the composition of the cell wall in bacteria S. aureus with E. coli. As the gram-positive bacteria (S. aureus) cell wall consists of peptidoglycan whereas Gram-negative bacteria (E. coli) cell wall consists of peptidoglycan and lipopolysaccharide. That difference caused S. aureus more susceptible to kanamycin so S. aureus bacteria uptake was greater than E. coli. These results are also reliable with previous research that has been done by S. Roohi, et.al which has proved that ^{99m}Tc-kanamycin has in-vivo S.aureus uptake in the range of 40-50 % (Roohi et al., 2006).

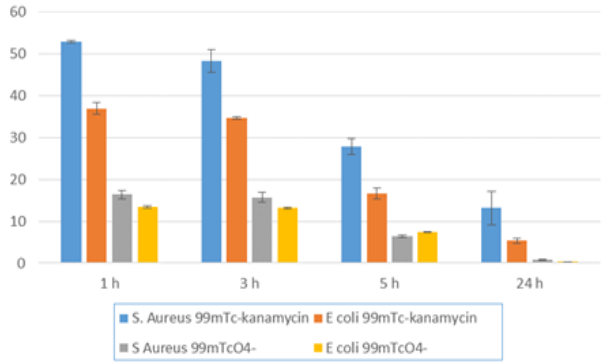


Fig. 3. Effect of incubation time on E. coli and S. aureus bacteria uptake of ^{99m}Tc-kanamycin and $^{99m}TcO_4^-$

^{99m}Tc-kanamycin as a radiopharmaceutical for detection infection is not accumulated in the normal organ or tissue and rapidly excreted from the body. The result of biodistribution of ^{99m}Tc-kanamycin are shown in Table 1 and 2. The results were expressed as the percent uptake of injected dose per gram of tissue (%ID/g) and at least in 3 mice. As described in the Table 1 and Table 2, high uptakes were found in blood, kidneys and liver at 15 min post injection. The high accumulation in the liver was probably due ^{99m}Tc-kanamycin metabolised by hepatobiliary rute i.e. intestine, liver, spleen and will be excreted through the feses. Considerable accumulation in the liver instead of caused by ^{99m}Tc-reduce (radiochemical impurities) because it based on the result of stability test, ^{99m}Tc-kanamycin fairly stable in plasma. Besides the higest accumulation in the kidney showed that

^{99m}Tc-kanamycin rapidly excreted from the body to urine. Specific accumulation in infected thigh muscles as indicated by T/NT rations, was 1.81 and 2.66 at 15 and 30 min post injection in E.coli infected mice. Whereas in S. aureus infected mice was 2.56 and 1.85 at 15 and 30 min post injection.

Table 1. Biodistribution of ^{99m}Tc-kanamycin in E. coli infected mice at 15, 30, 60 and 120 min post-injection.

Tissue	15 min	30 min	60 min	120 min
Infected muscle	2.49% ± 0.42%	1.14% ± 0.62%	0.33% ± 0.04%	0.31% ± 0.16%
Normal muscle	1.38% ± 0.59%	0.43% ± 0.10%	0.33% ± 0.06%	0.17% ± 0.06%
Blood	4.98% ± 1.39%	2.14% ± 0.97%	0.77% ± 1.10%	0.59% ± 0.43%
Liver	8.23% ± 0.72%	5.59% ± 1.44%	4.71% ± 0.50%	4.34% ± 1.10%
Kidneys	36.65% ± 3.45%	31.71% ± 6.29%	32.67% ± 2.41%	24.14% ± 9.24%
Heart	2.74% ± 1.16%	1.08% ± 0.45%	0.66% ± 0.07%	0.40% ± 0.08%
Lung	7.17% ± 1.98%	2.84% ± 1.30%	1.86% ± 0.12%	1.21% ± 0.31%
Stomach	3.33% ± 1.23%	1.29% ± 0.46%	0.89% ± 0.13%	0.57% ± 0.15%

Table 2. Biodistribution of ^{99m}Tc-kanamycin in S. aureus infected mice at 15, 30, 60 and 120 min post-injection

Tissue	15 min	30 min	60 min	120 min
Infected muscle	2.84% ± 0.89%	0.90% ± 0.02%	0.69% ± 0.01%	0.35% ± 0.11%
Normal muscle	1.11% ± 0.31%	0.49% ± 0.01%	0.35% ± 0.03%	0.20% ± 0.01%
Blood	5.65% ± 0.40%	2.60% ± 0.41%	2.49% ± 0.20%	1.59% ± 0.20%
Liver	8.94% ± 2.65%	8.93% ± 0.94%	8.91% ± 1.07%	6.31% ± 0.75%
Kidneys	44.76% ± 9.31%	41.92% ± 1.20%	59.95% ± 2.99%	48.43% ± 2.57%
Heart	2.61% ± 0.02%	1.22% ± 0.09%	0.95% ± 0.00%	0.55% ± 0.07%
Lung	5.90% ± 0.18%	2.21% ± 0.75%	2.25% ± 0.04%	1.06% ± 0.26%
Stomach	2.93% ± 0.34%	1.87% ± 0.04%	1.27% ± 0.11%	0.86% ± 0.25%

Radiopharmaceuticals for diagnosis should be quickly accumulated in the target organs and rapidly clearance from blood. Blood clearance test results of radiopharmaceutical ^{99m}Tc-kanamycin showed 5 minutes after injection of complex activity remains only 4.44% and then decreased to 1.38%, 0.73%, 0.63%, 0.60%, 0.59%, 0.21%, and 0.16% respectively at 1, 2 , 3, 4, 5, 24 to 30 hours (Fig. 5). The decline complex in the blood is due in part already excreted complex through the kidneys and partly distributed to the target organs of infected muscle and other organs.

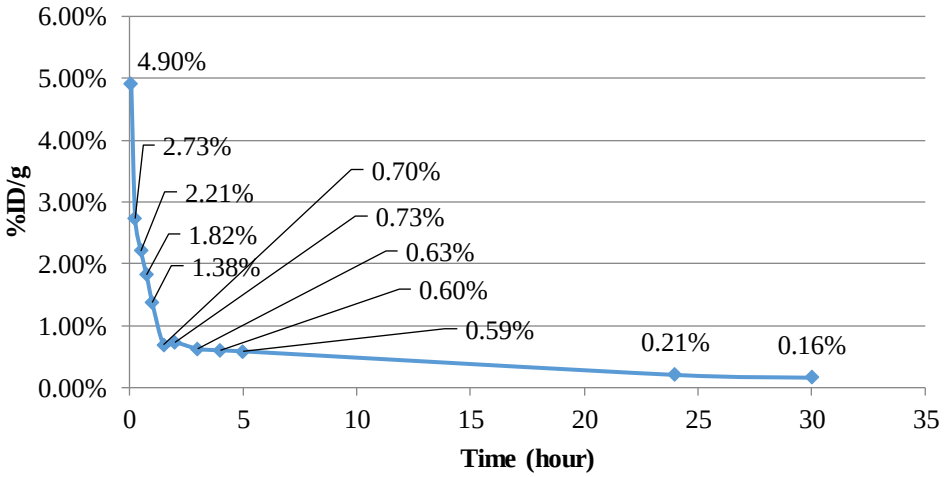
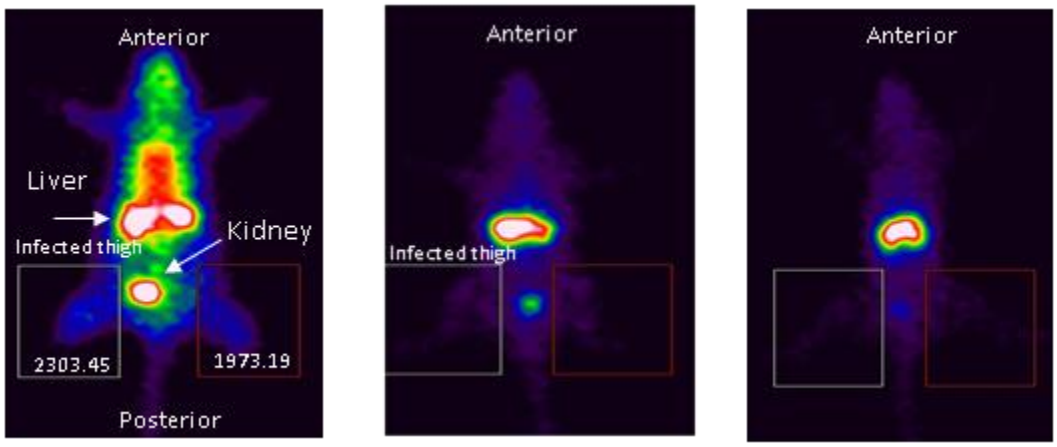


Fig 5. Blood clearance ^{99m}Tc-kanamycin in normal mice

Whole body imaging studies with a gamma camera are infected mice was presented in Fig. 6. From this result show that ^{99m}Tc-kanamycin was accumulated in the liver and kidney same as biodistribution test results. Moreover from this figure also shown that the accumulation of ^{99m}Tc-kanamycin in the thigh infected by S. aureus (left thigh) are more higer than normal thigh (right thigh). So can be concluded that ^{99m}Tc-kanamycin specific and sensitive to infections caused by bacteria.



Poling 1 hour post injection 3 hour post injection

Fig. 6. Whole body gamma camera image of infected mice (S. aureus) injected with ^{99m}Tc-kanamycin

4. CONCLUSION AND REMARKS

Kanamycin was successfully labeled with ^{99m}Tc by direct methods. In vitro study showed the change of kanamycin structure after labeling with technetium-99m did not give a specific influence to the potency of kanamycin as an antibiotic. In vitro bacteria uptake showed that decreased uptake of ^{99m}Tc-kanamycin as a function of incubation time. The result of biodistribution and imaging study show that ^{99m}Tc-kanamycin quite specific and sensitive detection infections caused by bacteria.

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