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Synthesis, quality control and biodistribution of ^{99m}Tc-Kanamycin

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Kanamycin is an antibiotic used for treatment of infections when penicillin or other less toxic drugs cannot be used. Kanamycin was labeled with technetium-99m pertechnetate using $SnCl_2 \cdot 2H_2O$ as reducing agent. The labeling efficiency depends on the ligand/reductant ratio, pH, and volume of reaction mixture. Radiochemical purity and stability of ^{99m}Tc-Kanamycin was determined by thin layer chromatography. Biodistribution studies of ^{99m}Tc-Kanamycin were performed in rats and rabbits. A significantly higher accumulation of ^{99m}Tc-Kanamycin was seen at sites of *S. aureus* infected animals (rat/rabbit).

Introduction

Nuclear medicine, with its different approaches, images pathophysiology and pathobiochemistry. New techniques, especially within immunology and molecular biology, are yielding new insights into the cascade of infection and inflammation. Nuclear medical imaging is available for whole body imaging in routine clinical practice, whereas computed tomography, magnetic resonance imaging and other techniques provide information on one part of the body. Several radiopharmaceuticals are available for imaging infection. They differ in terms of their physical characteristics, their biodistribution, their need for cell isolation and their radiation exposure. Highly sensitive relatively non-specific radiopharmaceuticals include ^{67}Ga citrate[1] and F-18 deoxyglucose.[2] The agents that are more specific for inflammation include radiolabeled white cells, whether labeled in vivo[3] or in vitro, those that make use of the permeability of the inflammatory capillaries such as the immunoglobulins,[4,5] colloids,[6] avidin,[7] dextrans[8] and liposomes,[9] or through inflammation related chemokines and cytokines.[10] Radiotracers specific to various causes of inflammation such as ^{123}I and ^{99m}Tc labeled Interleukin-2 for auto immune disease,[11–13] ^{99m}Tc-anti-E-selectin for vasculitis,[14] ^{99m}Tc-Aprotinin for amyloidosis,[15] ^{99m}Tc-Annexin V for apoptosis,[16] antimicrobial peptides,[17,18] ^{99m}Tc-Sparfloxacin,[19,20] ^{99m}Tc-Ceftizoxime[21] and ^{99m}Tc-Infecton[22] for bacterial infections have been developed. Technetium-99m Isoniazid for tuberculosis imaging is undergoing evaluation.[23]

Kanamycin sulfate is a bactericidal antibiotic, which acts by inhibiting the synthesis of protein in susceptible microorganisms. Kanamycin is used for treatment of 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, septicemia, and enterococcal infections. Treatment of enterococcal infections requires combination with penicillin.

In this paper, the labeling of Kanamycin with technetium-99m is described. Radiochemical purity, stability, bacteria binding assay and in vivo biodistribution in rats and in rabbit was also evaluated.

Experimental

Materials and methods

Kanamycin sulfate for intravenous injection was obtained from Chong Qing Medicine & Health Products, Imp & Exp. Corp. China. Rats (Sprague-Dawley) and *Staphlococcus aureus* bacteria (American Type Culture Collection, ATCC 25923) were obtained from National Institute of Health (NIH), Islamabad. The Animal Ethics Committee of the Institute gave approval for the animal experiments. Technetium-99m was obtained from a locally produced fission based PAKGEN ^{99}Mo/^{99m}Tc generator. All chemicals used were AR grade and purchased from E. Merck, Germany. Freeze-dried kits of DTPA and Ciprofloxacin were obtained from "Kit Production Group", PINSTECH, Islamabad.

Synthesis of ^{99m}Tc-Kanamycin

In the synthesis of ^{99m}Tc-Kanamycin, the amount of Kanamycin was fixed at 5 mg. To determine the optimal amount of reducing agent, 5–30 μg of $SnCl_2 \cdot H_2O$ was used, pH was adjusted by using HCl/NaOH. Reaction mixture volume used in all experiments was 2±0.1 ml. After addition of all reagents ~370 MBq $^{99m}TcO_4^-$ in saline was injected into the vial. All experiments were carried out at room temperature of 22±2 °C.

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Quality control

Radiochemical yield of ^{99m}Tc-Kanamycin was checked by thin layer chromatographic method using Whatman No. 3 paper and ITLC-SG strips (Gelman Sciences). Free $^{99m}TcO_4^-$ in the preparation was determined by using Whatman No. 3 paper as the stationary phase and acetone as the mobile phase. Reduced and hydrolyzed activity was determined by using instant thin layer chromatography (ITLC-SG strips) as the stationary phase and 0.5M NaOH as a mobile phase. Radiocolloids were also determined by passing the preparation through 0.22 μm bacteria filters (Millipore Filter Corp.). Activity remaining on the filter and in the solution was counted by a gamma-counter (Ludlum). The stability of ^{99m}Tc-Kanamycin was checked for 6 hours at room temperature. The distribution of radioactivity on chromatographic strips was measured by a 2π Scanner (Berthold, Germany). Alternatively, the strips were cut into 1 cm segments and counted by a gamma-counter.

In vitro stability

Stability of the radiotracer ^{99m}Tc-Kanamycin was studied in vitro. 1.8 ml of normal human serum was mixed with 0.2 ml of ^{99m}Tc-Kanamycin and incubated at 37 °C. 0.2 ml aliquots were withdrawn during the incubation at different time intervals up to 24 hours and subjected to chromatography for determination of ^{99m}Tc-Kanamycin, reduced/hydrolyzed ^{99m}Tc and free $^{99m}TcO_4^-$.

Bacteria Staphylococcus aureus 25923

S. aureus ATCC 25923 is the most widely used quality control organism in clinical microbiology laboratories. Being a standard reference strain, its use was similar to that of previous studies.[17] Overnight cultures of bacteria were prepared in brain heart infusion broth (BHI, Oxoid) in a shaking water bath at 37 °C. Aliquots of suspensions containing viable stationary phase bacteria were snap frozen in liquid nitrogen and stored at –70 °C. Just before use, an aliquot of this suspension was rapidly thawed in a water bath at 37 °C and diluted in sodium phosphate buffer (Na-PB).

In vitro binding of ^{99m}Tc-Kanamycin to bacteria

Binding of ^{99m}Tc-Kanamycin to *S. aureus* bacteria was assessed by the method described elsewhere.[17] Briefly, 0.1 ml of sodium phosphate buffer (Na-PB) containing ~5 MBq of ^{99m}Tc-Kanamycin was transferred to a test tube. 0.8 ml of 50% (v/v) of 0.01M acetic acid in Na-PB containing approximately $1 \cdot 10^8$ viable bacteria were added. The mixture was incubated for 1 hour at 4 °C and then centrifuged for 5 minutes at 2000 *g* at 4 °C. The supernatant was removed and the bacterial pellet was gently resuspended in 1 ml of ice cooled Na-PB and recentrifuged. The supernatant was removed and the radioactivity in the bacterial pellet was determined by a gamma-counter. The supernatants were also counted. The radioactivity related to bacteria was expressed in percent of the added ^{99m}Tc activity bound to viable bacteria in regard to total ^{99m}Tc activity. For comparison purposes binding of ^{99m}Tc-DTPA and ^{99m}Tc-Ciprofloxacin to bacteria were also performed.

Experimental thigh muscle infection

Male Sprague-Dawley rats weighing ~200 g were used in all animal experiments. A turbid suspension containing $2 \cdot 10^8$ colony-forming units (cfu) of *S. aureus* in 0.2 ml of saline was injected into the right thigh muscle of the rats. 24 hours later, when visible swelling appeared in the infected thigh, 0.2 ml of ^{99m}Tc-Kanamycin (~37 MBq) was injected via the tail vein. Four rats were used for one set of experiment. After a definite time, the rats were sacrificed after ether anesthesia and biodistribution was determined. One-milliliter samples of blood were taken by cardiac puncture and weighed. Activity in total blood was calculated by assuming blood volume = 6.5% of body weight. The whole animals were then weighed and dissected. Samples of infected muscle, normal muscle, liver, spleen, lung, kidney, stomach, and heart were weighed, and the activity was measured using a gamma counter. The results were expressed as the percent uptake of injected dose per organ.

The results of the bacterial uptake of ^{99m}Tc-Kanamycin and other compounds were analyzed by an analysis of variance. The level of significance was set at 0.05.

Induction of experimental infection in rabbits

Saline (0.3 ml) containing $2 \cdot 10^8$ cfu viable *S. aureus* ATCC 25923 was injected into the right thigh muscle of each animal followed by scintigraphy after 48 hours, when significant swelling was visible at the injection site.

^{99m}Tc-Kanamycin scintigraphy

A single headed Siemens Integrated ORBITER Gamma Camera System interfaced with high-resolution parallel hole collimator was used. It was connected to an on-line dedicated computer (Macintosh® Operating System 7.5 Software used on the ICON™ Workstation). Each animal was placed on a flat hard surface with both hind legs spread out and all legs fixed with surgical tape. Diazepam (5 mg) was injected into the left thigh muscle.

Saline (0.2 ml) containing 15 MBq of ^{99m}Tc-Kanamycin was then injected intravenously into the marginal ear vein. Immediately after injection, dynamic acquisition with both thighs in focus was done for 120 minutes. For the biodistribution study of the radiotracer, whole body acquisition was done at 15 minutes, 1 hour and 2 hours after injection.

Results and discussion

The Kanamycin molecule contains 6-deoxy-6-amino-D-glucose (6-D-glucosamine) and 3-deoxy-3-amino-D-glucose (3-D-glucosamine) moieties linked by α-glycosidic bonds to the 4 and 6 positions of deoxystreptamine. The structure of Kanamycin A[24] is shown in Fig. 1. The direct method of labeling of Kanamycin with ^{99m}Tc was exploited which is simple, rapid, efficient and does not require bifunctional chelating agents. The various chelates of ^{99m}Tc, which serve as radiopharmaceuticals are formed by interaction between specific chelating agents and a reduced form of ^{99m}Tc. In order to form bonds with technetium, the chelator must contain electron donors like nitrogen, oxygen and sulfur. Space between multiple electron donor atoms is required to allow several bonds to form with the central metal. Kanamycin has several functional groups such as $-NH_2$, –OH and –O– to form bonds with ^{99m}Tc. Although details of the chemistry of formation and molecular structure are unknown, ^{99m}Tc-Kanamycin is assumed to be a chelate complex with one or more Kanamycin ligands attached to reduced ^{99m}Tc.

Labeling efficiency and radiochemical purity and stability were assessed by a combination of ascending paper chromatography and instant thin layer chromatography on silica gel. In paper chromatography using acetone as the solvent, free $^{99m}TcO_4^-$ moved towards the solvent front ($R_f = 1$), while ^{99m}Tc-Kanamycin and reduced/hydrolyzed ^{99m}Tc remained at the point of spotting. In ITLC-SG chromatography using 0.5M NaOH as the solvent, reduced/hydrolyzed ^{99m}Tc remained at the point of spotting, whereas ^{99m}Tc-Kanamycin and free $^{99m}TcO_4^-$ moved towards the solvent front. Radiocolloids were also determined by passing the preparation through sterile filters (0.22 μm). In this technique radiocolloids were retained on the filter, while ^{99m}Tc-Kanamycin and free $^{99m}TcO_4^-$ passed through. The results obtained by both methods were in excellent agreement. The amount of radiocolloid in the final preparations was ≤2.0%.

The effects of pH are shown in Fig. 2. At low pH (2–5) the minimum labeling efficiency is 75%, while at pH 6–7 the labeling efficiency of ^{99m}Tc-Kanamycin is >97%. In basic media at pH 8 the labeling efficiency is decreased (60–72%). Hence further experiments were performed at pH 6–7.

The amount of the reducing agent, $SnCl_2 \cdot 2H_2O$, which gave the highest labeling efficiency, was 15–20 μg and a value 20 μg of $SnCl_2 \cdot 2H_2O$ was chosen (Fig. 3). To avoid colloid formation, the optimum amount of reducing agent was used. The complexation of ^{99m}Tc with Kanamycin is not rapid and maximum labeling efficiency is achieved after 30 minutes. The resulting complex of ^{99m}Tc-Kanamycin is quite stable and labeling of ≥98% is maintained for up to 6 hours (Fig. 4). When the preparation was incubated with normal human serum at 37 °C there was almost no increase in free pertechnetate or reduced/hydrolyzed ^{99m}Tc up to 24 hours. The total impurities were <5% (Table 1).

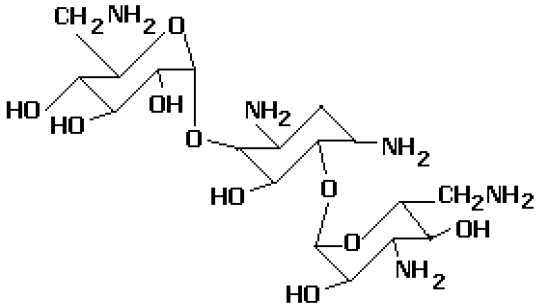

Fig. 1. Structure of Kanamycin A

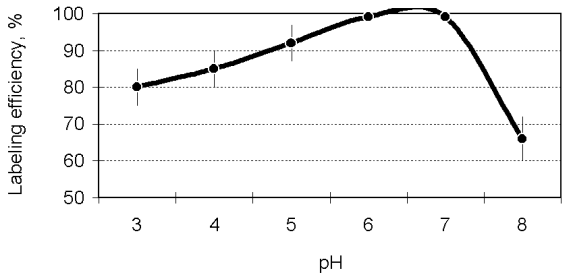

Fig. 2. Effect of pH on the labeling efficiency of ^{99m}Tc-Kanamycin ($n = 4$ per experiment)

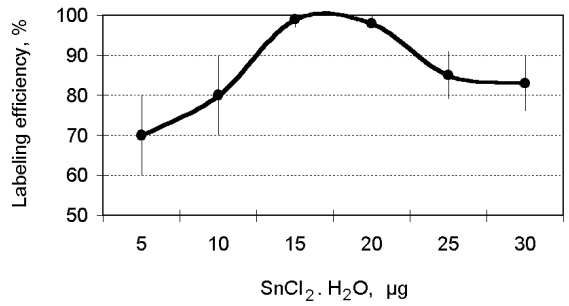

Fig. 3. Effect of $SnCl_2 \cdot 2H_2O$ amount on the labeling efficiency of ^{99m}Tc-Kanamycin ($n = 4$)

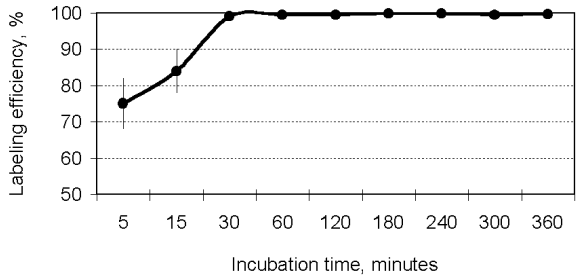

Fig. 4. Rate of complexation of ^{99m}Tc with Kanamycin and stability of ^{99m}Tc-Kanamycin ($n = 4$)

The final formulation for the radiotracer ^{99m}Tc-Kanamycin was: Kanamycin 5 mg; $SnCl_2 \cdot 2H_2O$ 20 μg; pH 6–7; ^{99m}Tc 370–500 MBq; reaction mixture volume ~2 ml; and incubation time 30 minutes at room temperature.

In vitro binding of ^{99m}Tc-Kanamycin to bacteria was comparable to ^{99m}Tc-Ciprofloxacin.[19] Binding of ^{99m}Tc-Kanamycin was in the range of 40–50% ($n = 4$), while binding of ^{99m}Tc-Ciprofloxacin, a promising agent for the diagnosis of bacterial infection[25,26] ranged from 40 to 65% ($n = 4$). In vitro binding of ^{99m}Tc-DTPA (kidney/brain imaging agent) to bacteria was <8% ($n = 3$). Varying amounts of Kanamycin (2.5–50 μg) showed similar binding efficiency with bacteria (Fig. 5).

The tissue distribution of ^{99m}Tc-Kanamycin expressed as percentage of injected dose per organ (%ID/organ) in rats, with bacterial inflammations induced, 0.5, 4 and 24 hours after intravenous administration is presented in Table 2. The ^{99m}Tc-Kanamycin was rapidly distributed after intravenous injection as shown by the renal elimination, although liver uptake is also significant. The high hydrophilic character of ^{99m}Tc-Kanamycin is in accordance with its predominant renal clearance. It is assumed that ^{99m}Tc-Kanamycin is stable in vivo, since insignificant activity was noticed in thyroid and stomach during biodistribution studies.

Table 2 also presents the infected thigh and normal thigh radioactivity obtained at 0.5, 4 and 24 hours after administration of ^{99m}Tc-Kanamycin. At 0.5 hour, 4 and 24 hours the target thigh/normal thigh radioactivity ratio indicated that higher binding affinity to the infection induced with *S. aureus* was observed. The highest target/non target ratio reached >2 at 0.5 hour and remained >2 up to 24-hour post injection of ^{99m}Tc-Kanamycin. Whole body images of infected rabbits at 15 minutes, 1 hour and 2 hours after ^{99m}Tc-Kanamycin administration are presented in Fig. 6a, b and c, respectively. *S. aureus* infection in rabbit thigh was visualized as area of increased tracer accumulation just after injection of labeled Kanamycin. The infection is clearly visible at 15-minute post administration, whereas at 1 hour and 2 hours, due to increase in urinary bladder activity, the infection sites are poorly visualized compared to the 15-minute image. Target-to-background ratios obtained from region of interest analysis of ^{99m}Tc-Kanamycin ranged 2.5 to 4:1. In vitro studies and animal experiments have shown that ^{99m}Tc-Kanamycin localizes in bacteria infected sites significantly. Due to the ease of ^{99m}Tc-Kanamycin preparation and infection uptake, it may provide an alternative to ^{99m}Tc-Ciprofloxacin in a variety of patients referred for infection evaluation.[22,27–29]

Table. 1. In vitro stability of ^{99m}Tc-Kanamycin in normal human serum ($n = 4$)

Incubation time, h	^{99m}Tc-Kanamycin	Free pertechnetate	Colloid
1	98.0 ± 1.8	1.2 ± 0.1	1.0 ± 0.2
2	97.6 ± 1.7	1.6 ± 0.3	1.4 ± 0.3
4	96.8 ± 1.7	1.9 ± 0.4	1.6 ± 0.4
24	96.4 ± 1.3	2.2 ± 0.5	1.9 ± 0.6

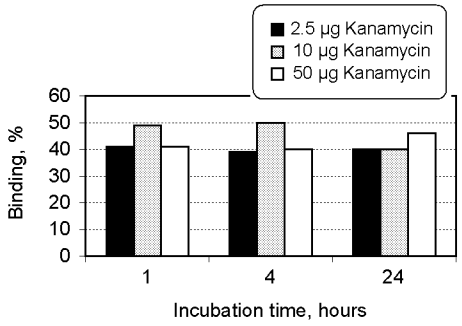

Fig. 5. In vitro binding of the ^{99m}Tc-Kanamycin to viable *S. aureus* ($n = 4$ per experiment)

Table 2. Biodistribution data in percent injected dose per total organ for ^{99m}Tc-Kanamycin at 0.5, 4 and 24 hours after intravenous administration in infected rats (means ± SD, $n = 4$)

Organ	0.5 h	4 hrs	24 hrs
Infected muscle	0.85 ± 0.13	2.71 ± 0.66	1.18 ± 0.21
Normal muscle	0.34 ± 0.08	1.15 ± 0.12	0.48 ± 0.08
Liver	22.25 ± 3.32	16.98 ± 3.45	7.98 ± 1.73
Spleen	2.57 ± 0.71	2.78 ± 0.77	1.20 ± 0.30
Lung	0.75 ± 0.23	0.88 ± 0.23	0.50 ± 0.15
Kidneys	23.45 ± 3.88	10.67 ± 2.10	2.82 ± 0.50
Urine	20.56 ± 3.56	50.55 ± 4.34	65.44 ± 6.65
Stomach	0.56 ± 0.12	0.43 ± 0.08	0.21 ± 0.02
Heart	1.12 ± 0.20	0.37 ± 0.06	0.27 ± 0.08
Blood	8.44 ± 1.55	6.54 ± 1.11	1.00 ± 0.25

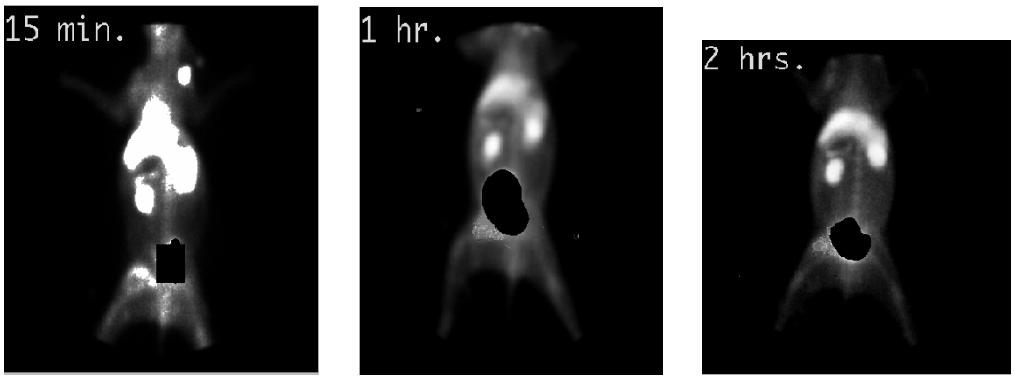

Fig. 6. Whole body gamma camera imate of rabbit injected with ^{99m}Tc-Kanamycin at 15-minute post administration (a), at 1-hour post administration (b), and 2-hour post administration (c). Urinary bladder is masked with lead foil in all figures and arrow indicates the site of infection

Conclusions

Methodology for radiolabeling of Kanamycin with ^{99m}Tc has been developed and standardized. Radiolabeling efficiency of ^{99m}Tc-Kanamycin monitored by paper and ITLC-SG was higher than 98%. The resulting complex of ^{99m}Tc-Kanamycin is quite stable and labeling of ≥98% is maintained for up to 6 hours. No post-labeling purification was required. The biological activity (in vitro) of ^{99m}Tc-Kanamycin and ^{99m}Tc-Ciprofloxacin is comparable. The radioactive preparation of ^{99m}Tc-Kanamycin is able to localize in bacterial infection induced by *S. aureus* in animal models.

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