Evaluation of ^{99m}Tc-sulfadiazine as Bacillus microorganisms infection imaging agent using animal model

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Abstract: Bacterial infection is one of the vital sources of morbidity and mortality. The development of single photon emission computed tomography (SPECT) radiotracer agents using antibiotics, for targeting *in-vivo* bacteria, helps in antibiotic dose calibration, targeted infection therapy and reduction in mortality rate. The aim of this study was to appraised ^{99m}Tc-labeling sulfadiazine as a radiopharmaceutical for bacillus infections imaging. Radiolabeling of sulfadiazine with technetium-99m was carried out by subsequent addition of 1.5 mL aqueous solution of sulfadiazine (1mg/mL), 120µg stannous tartrate, gentistic acid as stabilizing agent and 185 MBq normal saline solution of $^{99m}TcO_4^{-1}$ (pertechnetate) at pH = 5. The reaction mixture was incubated for 40 min at room temperature with light stirring. The quality control analysis (ITLC-SG and paper chromatography analysis) revealed ~ 98% labeling yield. Biodistribution and scintigraphic study was carried using bacillus bacterial infection induced New Zealand white rabbits. Due to the ease of ^{99m}Tc-sulfadiazine conjugation method, high labeling efficiency, shelf stability (>95% up to 6h), blood serum stability (~90% up to 6h) and high uptake in the infected muscle (T/NT =2.21 at 1h), ^{99m}Tc-SDZ could be used as radiopharmaceutical of choice for further pre-clinical and clinical studies.

Keywords: Bacillus infection, radiopharmaceuticals, ^{99m}Tc-sulfadiazine, imaging, nuclear medicine.

INTRODUCTION

Infectious diseases are appearing lethal and causing considerable mortality globally. Non-predictive and misuse of antibiotics are the two main factor of arising the bacterial resistance to antimicrobial agents. Out of variety of bugs, Bacillus species (Gram-positive and spore forming aerobic organisms) are more prone to go into human body and cause serious infections - as these are abundant in water, soil, dust and air. *B. cereus* are of particular interest as these microorganisms cause food spoilage and infectious diseases in human body through the production of variety of toxins (Owusu-Kwarteng *et al*., 2017) and completely resistant to penicillin antimicrobial agents. On the other hand, in most cases with respect to geographic distribution the antibiotic therapy to cure infectious ailments appears less effective and the failure level of microbial ailment therapy gradually increases. A common cause of this failure is the increased production of β-Lactamases by variety of bugs which on interaction with antimicrobial agents breaks the chemical bonds and inactivates the antibiotics - that is commonly known as microbial resistance to antibiotics (Fenselau *et al.,* 2008). Resistance to antibiotics could be overcome by avoiding misuse and the use of non-susceptible antibiotics. Particularly in case of deep-seated bacterial infections that cause the fever of unknown origin - it is necessary to locate the exact position and volume of bacterial infection which will be helpful in two ways; one to dose calibration and second in targeted therapy.

Nuclear medicine technique uses radiopharmaceuticals, offers unique procedures to locate quantitatively variety of malignancies and deep seated bacterial infections of human body using single photon emission computed tomography (SPECT) and positron emission tomography (PET) camera (Naqvi & Drlica, 2017). The technique demands excellent targeted specificity of the radiopharmaceutical. Variety of radiopharmaceuticals were developed by making complex of gamma-emitter radionuclide (commonly ^{99m}Tc) with disease target molecules (synthetics or biomolecule) to track the deep-seated microbial infections for example ^{99m}Tc-ciprofloxacin (Britton *et al.,* 2002) ^{99m}Tc-amoxicillin (Ilem *et al.,* 2016),^{99m}Tc-enorfloxacin (Siaens et al., 2004), ^{99m}Tc-sitafloxacin (Qaiser *et al.,* 2010), ^{99m}Tc-cefepime (Motaleb *et al.,* 2011), ^{99m}Tc-cefzolin (El-Tawoosy *et al.,* 2013), ^{99m}Tc-sulfadiazin (Ahmed *et al.,* 2017), ^{99m}Tcmetronidazole (Iqbal *et al.,* 2017) have been developed for infection imaging purposes. ^{99m}Tc-ciprofloxacin, one of them was used extensively to diagnose number of deep-seated bacterial infections.

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Sulfadiazine antibiotic (fig. 1) belong to sulfonamide family shows promising potential to inhibit Gram-positive and Gram-negative organisms through inactivation of bacterial folic and dihydrofolic acids required for bacterial cell membrane protein synthesis. In this study ^{99m}Tc-sulfadiazine was evaluated to imaging potential of Bacillus microbial infection induced in rabbit model after successful evaluation using *E. coli* infection imaging.

MATERIALS AND METHODS

Sulfadiazine (SDZ) were purchased from IFET, Greece; Neo Quimica, Brazil. Ammonium hydroxide (NH_4OH), Sodium hydroxides (NaOH), ascorbic acid and all other chemicals were of analytical grade and purchased from Merck(Germany). ^{99m}Tc was eluted from ^{99}Mo/$^{99m}TcO_4^{-1}$ generator in the form of saline $NaTcO_4$ solution supplied by Pakistan Institute of Nuclear Science and Technology (PINSTECH), Islamabad, Pakistan. Whatman No. 3MM Chromatographic paper (Maidstone, UK.) and instant thin layer chromatography (ITLC-SG) (Gelman sciences Institute USA), were used for radio chromatography. Bacillibacteria (ATCC 25923) were obtained from Ayub Medical Complex Hospital, Abbottabad. Rabbits were purchased from National Institute of Health (NIH), Islamabad. The animal ethics committee of the institute gave an ethical approval for the animal experiments. Rabbits were kept under standard conditions with free ingress to food and water.

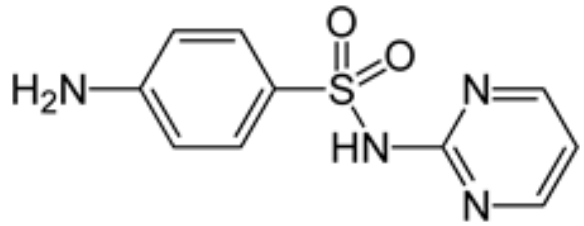

Fig. 1: Chemical structure of sulfadiazine

Labeling of SDZ with technetium-99m

The labeling sulfadiazine with ^{99m}Tc was carried out using the method reported previously (Ahmed et al., 2017). Briefly, to the 1mL solution of sulfadiazine (1.5mg/mL) added 120µg stannous tartrate (250µL), 5µL gentisic acid (8.3mg/mL) and 185MBq $^{99m}TcO_4^{-1}$. The pH of the mixture was adjusted to 5 using 0.05 N HCl/NaOH and the total volume to 2mL using saline solution. Then the reaction was allowed to react at room temperature for 40 minutes with periodic shaking.

Radiochemical Purity of ^{99m}Tc-SDZ

The purity of ^{99m}Tc-SDZ was determined by using paper and ITLC analysis. The impurities such as free $^{99m}TcO_4^{-1}$ was determined by spotting 2µL reaction mixture at base line of 3MM paper strip (dimension 2×14cm) and allowed to elute with acetone as mobile phase and the hydrolyzed fraction using saline as mobile phase was measured by spotting 2µL reaction mixture at the base line of ITLC strip (1.5×10 cm). The both strips were then air dried and scanned under 2π radio-scanner to take the counts at different R_f values.

^{99m}Tc-SDZ stability study in reaction medium

The shelf life stability of the ^{99m}Tc-SDZ complex was measured for a period of 6 h (one half-life of ^{99m}Tc) at room temperature while incubating the radio pharmaceutical in reaction solution. The study was carried out after completion of reaction time (40 min). The shelf life stability of the radiochemical was assessed with the help of chromatographic analysis as described above.

Effect of blood serum on the stability of ^{99m}Tc-SDZ

The effect of blood serum components on the stability of the ^{99m}Tc-SDZ was studied for 6 h by incubating ^{99m}Tc-SDZ and freshly harvested healthy human volunteer blood serum mixture (1:4 ratios) at 37°C in a 5% CO_2 incubator. Aliquots of 200µL were withdrawn at specified time periods such as 0.5, 1, 2, 3, 4 and 6 h and added to 800µL ice cold acetonitrile. The mixture was centrifuged, and the supernatant fluid was analyzed by paper chromatography and ITLC.

Biodistribution and scintigraphic study

Biodistribution and scintigraphic study was carried out by using single-headed Siemens' integrated ORBITER gamma camera system, interfaced with high energy general purpose collimator. For the purpose infection induced New Zealand white male rabbits (~1.0-1.5kg weight, n = 3) were used. The infection was induced into right thigh muscles of rabbit by injecting Bacillus pathogenic bacteria (36 h prior to study). When visible swelling appeared at infected thigh, the diazepam injection (5mg) was injected into left thigh muscle as an anesthetic agent. After giving anesthesia the rabbit was placed on a flat hard board with legs spread out and fixed with surgical tape under ready to scan SPECT camera. Then a solution of 300µL of ^{99m}Tc-sulfadiazine (185 MBq) was administrated through rear ear vain of rabbit. Whole body acquisition was performed both at static mode to take radioactive counts from different body organs ant different time intervals and dynamic mode to see the mode of excretion through body.

RESULTS

Radiopharmaceutical yield

The radiochemical yield was of the ^{99m}Tc-SDZ and other possible impurities such as free $^{99m}TcO_4^{-1}$ and hydrolyzed ^{99m}Tc were analyzed using chromatography (paper and ITLC) as shown in fig. 2a. The percent yield of both impurities and radiopharmaceutical were calculated using the following formula;

Table 1: Biodistribution results of ^{99m}Tc-SDZ in Bacillus bacterial induce infection rabbit model

Organs	% ID / gm organ		
	30 min	1 h	4 h
Left Kidney	3.383 ± 0.13	3.095 ± 0.23	2.542 ± 0.21
Right Kidney	2.377 ± 0.38	2.065 ± 0.28	1.263 ± 0.25
Heart	3.516 ± 0.09	3.112 ± 0.09	2.390 ± 0.12
Spleen	0.197 ± 0.04	0.144 ± 0.10	0.062 ± 0.11
Liver	2.481 ± 0.12	1.443 ± 0.21	0.600 ± 0.16
Right Lung	1.091 ± 0.07	0.909 ± 0.12	0.567 ± 0.11
Left Lung	0.973 ± 0.08	0.597 ± 0.08	0.380 ± 0.09
Urinary Bladder	6.759 ± 1.11	5.830 ± 1.82	2.688 ± 0.45
Normal thigh muscles	0.59 ± 0.03	0.51 ± 0.06	0.48 ± 0.04
Infected thigh muscles	1.23 ± 0.08	1.13 ± 0.08	1.02 ± 0.11
T/NT	2.08 ± 0.02	2.21 ± 0.02	2.13 ± 0.04

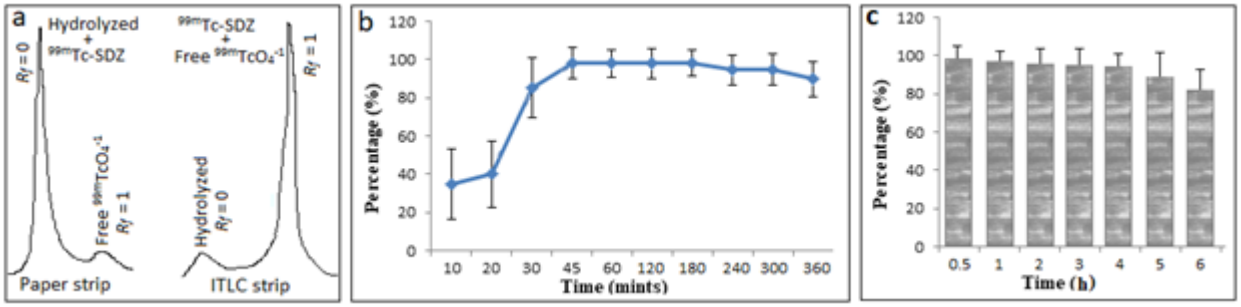

Fig. 2: a) The chromatographic analysis for radiochemical yield b) shows the ^{99m}Tc-SDZ shelf stability at room temperature and c) shows the stability of the ^{99m}Tc-SDZ against blood serum component.

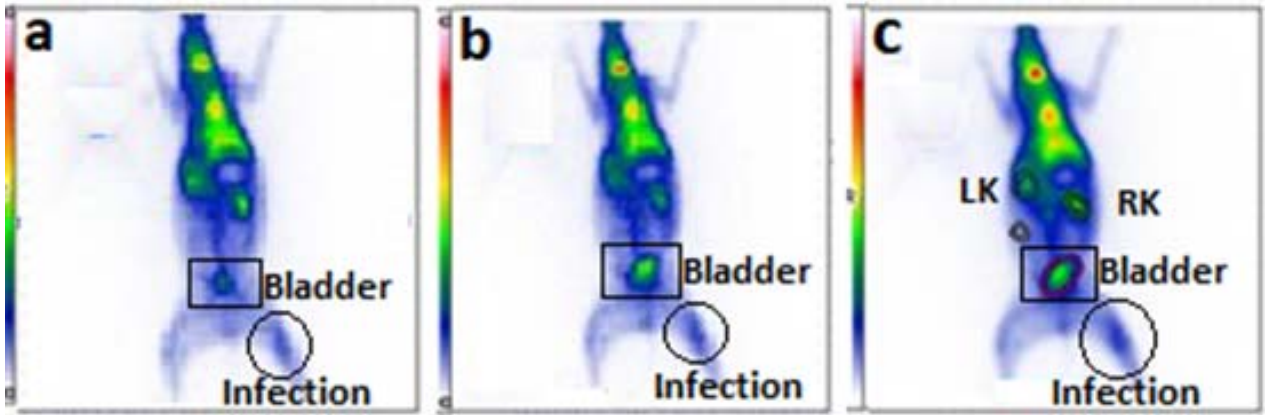

Fig. 3: Scintigraphic study of infection induced rabbit model at 30 min (a), 1 h (b) and 4h (c). In all three images the infection uptake remained same but gradual increase in activity can be noted into bladder through kidneys filtration

$$\%\ \text{Free } TcO_4^-\ (\text{PC strip}) = \frac{\text{Radioactivity counts at solvent front } (R_f=1)}{\text{Total radioactivity counts at } R_f\ 0\text{-}1}$$

$$\%\ \text{Colloids (ITLC strip)} = \frac{\text{Radioactivity counts at base line } (R_f=0)}{\text{Total radioactivity counts at } R_f\ 0\text{-}1}$$

$$\%\ \text{yield of } ^{99m}\text{Tc-SDZ} = 100 - (\%\ \text{Free } TcO_4^- + \%\ \text{Colloids})$$

^{99m}Tc-sulfadiazine stability study in reaction medium

Fig. 2b shows the shelf life stability of ^{99m}Tc-SDZ at room temperature that was start to measure at the end of reaction time when maximum yield of the ^{99m}Tc-SDZ was obtained. The fig. shows constant stability trend over study period (6h). The figure shows the maximum yield was obtained at 45min which remained stable up to three hours. However at 4, 5 and 6 hour time point slight decrease in 99mTc-SDZ complex was noted.

Effect of blood serum on the stability of ^{99m}Tc-sulfadiazine

Fig. 2c indicates the stability of the ^{99m}Tc-SDZ against blood serum component up to 6 h while incubating at the ratio of 1:4 (radiopharmaceutical to blood serum) at 37°C and 5% CO_2. The results obtained through the paper and ITLC analysis showed ^{99m}Tc-SDZ is sufficiently stable

against the action of different blood serum components. At 0.5 h time point the ^{99m}Tc-SDZ was found 97% stable while at 4 h stability value was 94%. However at subsequent time points i.e. 5 and 6 h the ^{99m}Tc-SDZ was degraded to 83%.

Biodistribution and scintigraphic study

Distribution of ^{99m}Tc-SDZ in different organs of the New Zealand white rabbit was measured by noting gamma counts at different time points using SPECT gamma camera. At the end of the count calculation the rabbits were sacrificed to weigh the body organs to calculate ^{99m}Tc-SDZ distribution in key body organs in term of % injected dose per gram organ (%ID/gm) as shown in Table 1. The results show a prominent difference between normal and infection induced thigh muscle uptake of the ^{99m}Tc-SDZ which is further elaborated by target to non-target ratio (T/NT). The scintigraphic images are shown in fig. 3 which shows uptake in heart in addition to liver and kidneys.

DISCUSSION

Our primary objective was to develop antibiotic based radiopharmaceuticals through ^{99m}Tc labeling for the detection of deep-seated bacterial infections. Deep-seated bacterial infections are hard to locate until they do not cause some entomological changes. Entomological changes then can be detected with excellent accuracy using computed tomography (CT) or magnetic resonance imaging (MRI) scan but in most cases entomological changes do not appear as a result of infections or malignancies (Naqvi & Drlica, 2017). Nuclear medicine imaging technique is a unique procedure of disease detection which does not depend on entomological changes to detect the infection or tumor lesions. In this technique SPECT or PET camera locate the origin of radiation inside the body and makes its image that provide volume and location of the disease.

In this study, ^{99m}Tc-SDZ was evaluated with regard to its in vitro and in vivo characteristics (Ahmed et al., 2017). The radiosynthesis step of ^{99m}Tc-SDZ required no any further purification step to obtain high radiochemical yield. The simple ^{99m}Tc-SDZ synthesis procedure provided >95% radiochemical yield in the presence of stannous tartrate reducing agent. The high radiochemical purity ensures the maximum target accumulation and eliminates the chance of background activity and non-target accumulation. The radioactive part of nearly 80% radiopharmaceutical is ^{99m}Tc due to its extremely favorable physical, chemical and nuclear characteristics (Lambrecht, 2011). ^{99m}Tc easily makes complex with electron donor atoms like nitrogen, oxygen and sulfur in the presence of favorable reducing agent. It can easily be obtained at low cost. The first radiolabeled antibiotic to be developed as radiopharmaceutical was ^{99m}Tc-ciprofloxacin that was extensively studied in humans. However, globally gradual increase in bacterial resistance renders the scientists to search for other target seeking molecules to develop new radiopharmaceuticals for the diagnosis of multi drug resistance bacterial infections. The main characteristics of ideal radiopharmaceuticals are high yield or purity (>90%), highly stability (~4-6 h), high target to non-target accumulation and rapid clearance from body.

Sulfadiazine targets the enzyme involve in the synthesis of bacterial cell membrane protein - that's the target of ^{99m}Tc-SDZ to locate the position of rapid growth of bacteria inside the body. In this study ^{99m}Tc-SDZ was obtained in good purity (>95%). The other impurities like hydrolyzed and free $^{99m}TcO_4^{-1}$ was found in <5% fraction. In most of the radiopharmaceutical development procedures the >90% radiochemical purity is considered sufficient because at this purity maximum imaging potential can be achieved.

The other important factor is the stability of the radiochemical that should be more that patient study period. In case of ^{99m}Tc-labeled radiopharmaceuticals it should be stable up to one half-life of ^{99m}Tc (6.02 h), as most of the nuclear medicine procedures required 2-3 h for completion. The shelf life stability of the ^{99m}Tc-SDZ was found excellent up to 6 h as shown in fig. 2b, that indicates the ^{99m}Tc-SDZ could be dispensed into patient safely to complete the imaging procedure. The blood serum stability study also indicates the chemical structure of ^{99m}Tc-SDZ is sufficiently stable against different blood serum components and enzymes. The study reveals ~90% intact radiochemical up to 4h in blood serum at physiological conditions (incubation at 37°C and 5% CO_2) that ensures safe accumulation at target. As discussed above most nuclear medicine procedures are being completed in 2-3h but in some cases 30min, 1h, 2 h, 3h and 4h points are also studied. So if these points are studied then there would be no chance of degraded product and maximum activity may accumulate at target site. The degraded products increase the background activity level as well as the non-target accumulation.

The in vivo biodistribution study of ^{99m}Tc-SDZ in bacillus bacterial infection induced rabbit model indicates that the ^{99m}Tc-SDZ has rapid accumulation at the site of infection. At 30 min SPECT acquisition reveals 1.23±0.08% ID / gm organ which slightly decreased to 1.02±0.11 at 4h study point. The infected thigh muscle to non-infected thigh muscles (T/NT) accumulation was calculated as 2.08±0.02, 2.21±0.02 and 2.13±0.04 at 0.5, 1 and 4h time points. Promising values of T/NT indicates the specificity of the radiopharmaceutical for bacillus bacterial infection which shows resistance to multiple antibiotics. The liver uptake of radioactivity is due to the metabolic path adopted by most of the foreign particles which initially at

0.5 h time point was 2.481±0.12% ID/gm organ and reduced to 0.600±0.16% ID/gm organ at 4 h time point. At 4 h there were no activity was in blood so the activity accumulated in liver for metabolism gradually decreased due to the metabolic degradation. It also reveals ^{99m}Tc-SDZ maximum metabolizes to secondary product up to 4 h. The other important factor is the continuous filtration of the ^{99m}Tc-SDZ through kidneys and gradual increase in activity in bladder. This reveals kidneys are the main excretory path of the ^{99m}Tc-SDZ. The scintigraphic images (fig. 3) show good coherence with biodistribution study. Circled areas show the accumulation of ^{99m}Tc-SDZ at infection site while the opposite thigh muscle showing absence of activity. The rapid filteration through kidneys indicates the ^{99m}Tc-SDZ is non-accumulative in kidneys which omits the chance of nephrotoxicity.

CONCLUSION

High radiochemical yield of ^{99m}Tc-SDZ at mild reaction conditions and promising stability both shelf and biological indicates the radiotracer is safe for preclinical and clinical evaluation. The key parameters of biodistribution and scintigraphic study reveals the ^{99m}Tc-SDZ can target bacillus bacterial infection foci with maximum accumulation period and rapidly wash-out from non-target organs which are the key characteristics of the radiopharmaceuticals of choice for nuclear medicine procedures. Further the rapid filtrations through the kidneys omit the chance of chronic nephrotoxicity. Overall results encourage to subject ^{99m}Tc-SDZ for pre-clinical and clinical evaluation.

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