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Synthesis, Characterization, Biodistribution and Scintigraphic Evaluation of Technetium-99m (^{99m}Tc) Labeled Pazufloxacin (PZN): A Novel Infection Radiotracer

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Abstract

In this study tagging of ^{99m}Tc with PZN was examined with regards to different concentration of PZN, stannous chloride, ^{99m}Tc and pH ranges from 5.00-6.00. The suitability of the ^{99m}Tc labeled PZN was assessed in terms of percent radiochemical purity (%RCP) yield in normal saline at concentration value of the stannous chloride as 120 mg, stability in serum (*in vitro*) at 37°C at different intervals, *in vitro* up take by live and heat killed *E. coli*, biodistribution behavior in artificially *E. coli* (live and heat killed) infected animal model rat and scintigraphic accuracy in artificially *E. coli* infected animal model rabbit. Maximum %RCP yields of 98.25 ± 0.26% was observed after 30 m of labeling using 2 mg of PZN, 125 µg stannous chloride, 2.5 m Ci sodium pertechnetate and pH 5.3.

The labeled PZN showed stable profile in serum at 37°C with a total decay of 16.40 ± 0.18%. The labeled PZN showed 72.55 ± 0.90% maximum bacterial uptake after 90 min in live and in case of heat killed *E. coli*, no significant uptake was seen. The labeled PZN showed 14.75 ± 0.40% absorption and accumulation in the infected muscle of the rat infected with live *E. coli* and infected to normal muscle ratio was 6:1. Scintigraphically, it was observed that after few minutes, the activity accumulated in the infected muscle was more than the others sites. The instant investigation substantiated that labeling PZN through this technique as compared to ^{99m}TcV:N~ and ^{99m}Tc(CO)3~ showed almost analogous %RCP yield, stability in serum at 37°C, *in vitro* binding with *E. coli*, biodistribution profile in animal modal rats and scintigraphic accuracy in animal model rabbit.

Keywords: ^{99m}Tc-PZN; *Escherichia coli* (*E. coli*); *Infection radiotracer*

Introduction

In time diagnosis of infection and its discrimination from non-infectious site is challenging for clinicians but imperative for decisive planning and suitable treatment. Routine pathological tests can identify the pathogen which caused infection but cannot offer any trace to distinguish between infection and inflammation [1,2].

Detection and monitoring of a variety of infectious diseases can be accomplished using a range of diagnostic modalities like ultra-Sound (US), Computed Tomography (CT), Magnetic Resonance Imaging (MRI), etc. [3]. These investigative modalities have proven helpful in recognizing infections because of their exceptional resolution, which facilitate the clinicians to evaluate more precisely the internal sites of indistinct infection [4].

Nevertheless, these investigative modalities depend only on morphological alterations and consequently most anomalies can only be detect at complex stages of ailment and diagnosis of in time infections and its discrimination from inflammation or other involvements can be tricky [5].

However, functional imaging can in many situations offer helpful and corresponding diagnostic details. Nuclear medicine (NM) procedures in contrast to US, CT and MRI, can find out the site and degree of infection on the basis of physiologic and metabolic alterations [6].

Gallium-67(^{67}Ga-citrate) [7], Technetium-99 (^{99m}Tc) or Indium-111 (^{111}In) labeled White Blood Cell (WBC) and peptides [8,9], have revealed some encouraging results with some disadvantages like accessibility, cumbersome labeling procedures and radiation burden.

Recently, ^{99m}Tc labeled antibiotics [10-15] have been explored for infection localization because of their high radiochemical yield, stability, binding with specific pathogen and localized imaging.

Nowadays, a need for a precise and specific infection imaging agent is still felt for in time identification of infection and its discrimination from non-infectious sites. In these lines, direct radiolabeling of a new quinolone antibiotic, Pazufloxacin (PZN) as shown in Figure 1. with ^{99m}Tc has been explored.

The rationale for PZN radiolabeling is its superior activity not only against gram-positive and gram-negative pathogen but also with other defiant strains. The higher activity of the PZN is due to the 1-aminocyclopropyl at C-10.

PZN inhibit the growth of bacteria via inhibition of bacterial DNA gyrase. These idiosyncratic characteristics posed the PZN, a drug of choice against *E. coli* when other quinolones have proven ineffective [16,17].

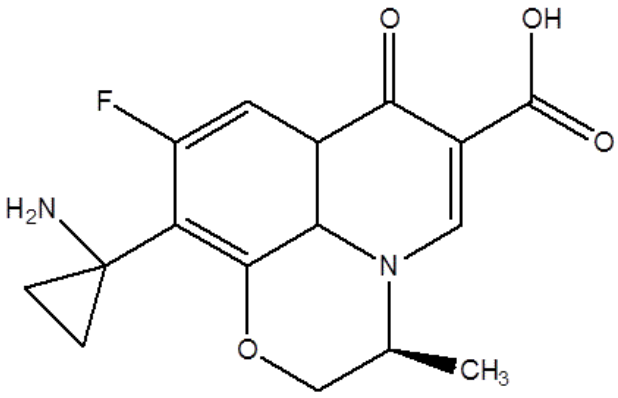

Figure 1: Structure of Pazufloxacin (PZN).

In the current investigation, the therapeutic property of PZN was utilized for diagnostic rationale by tagging PZN directly with ^{99m}Tc. The tagged moiety was characterized in terms of percent radiochemical purity, stability in normal saline at room temperature up to 24 h after reconstitution, *in vitro* stability in human serum at 37°C up to 24 h, *in vitro* binding with live and heat killed *E. coli* and biodistribution behavior in artificially *E. coli* infected animal model rats. The suitability of the ^{99m}Tc-PZN as specific *E. coli* infection radio-tracer was scintigraphically evaluated and confirmed in artificially *E. coli* infected animal model rabbits. The radiobiological characteristics of the newly prepared ^{99m}Tc-PZN were compared with the existing ^{99m}TcV:N-PZND and ^{99m}Tc(CO)$_3$-PZND, *E. coli* infection radiotracers [18,19].

Experimental

Materials

Pazufloxacin (PZN) from Hunan Jiudian Pharmaceutical Co., Ltd. China, TLC from Merk (Germany), and all the other chemicals and solvents of analytical grade from Aldrich. RP-HPLC from Shimadzu, (Japan), well counter, scalar count rate meter from Ludlum (USA), Dose calibrator from Capintech (USA) and Gamma camera (GKS-1000, GEADE) from Nuclearmedizine system (Germany).

Methods

Preparation of Technetium-99m (^{99m}Tc) labeled Pazufloxacin (PZN)

Pazufloxacin (PZN) was labeled with Technetium-99m (^{99m}Tc) using the reported method [14]. Briefly, 1.0 mCi of sodium pertechnetate ($Na^{99m}TcO_4$) was mixed with 20 µg of stannous chloride. Thereafter, 1.0 mg of pure PZN was injected to the reaction vial through sterilized syringe with the removal of same amount of nitrogen from the reaction vial. The pH was adjusted to 5.5 with 0.01 mol/L of HCl, followed by filtration through millipore filters and incubation at room temperature. The overall process was repeated by rising the amount of stannous chloride from 20 to 200 µg (i.e. 20 µg rise in each repeating procedure until the amount of stannous chloride attains a level of 200 µg), $Na^{99m}TcO_4$ from 0.5 to 5.0 mCi (i.e. 0.5 mCi increase in each repeating procedure until the amount of $Na^{99m}TcO_4$ becomes 5.0 mCi) and amount of pure PZN from 1.0 to 10.0 mg (i.e. 1.0 mg increase in each repeating procedure).

Radiochemical Purity (RCP) yield

TLC method was employed for the determination of %RCP yield of the ^{99m}Tc labeled PZN using dual mobile system. The developed strips were analyzed for %RCP yield using well counter.

In vitro stability in normal saline

High Performance Liquid Chromatography (HPLC) was used for the determination of *in vitro* stability in normal saline and characterization of the ^{99m}Tc labeled PZN. In this experiment, HPLC (Shimadzu SCL-10 AVP system) connected with well counter was used. C-18 column was employed as stationary and triethyl-aminophosphate and methyl alcohol as the mobile phase. The flow rate (ml/min) was sustained for 20 min.

In vitro stability in serum

The stability of ^{99m}Tc labeled PZN in serum at 37°C up to 24 h was evaluated using TLC method. In this investigation, 0.2 mL of the radio labeled antibiotic (^{99m}Tc-PZN) was incubated with 1.8 mL of serum. Thereafter, the strips were developed in (9:1) (v/v) CH_2Cl_2:CH_3OH as mobile phase. Subsequently, the TLC strips were analyzed for different fractions using well counter.

Further, the stability was examined in challenge assay by incubating equimolar solution of the ^{99m}Tc labeled PZN with increasing concentrations of the cystein (10^{-4}, 10^{-3}, 10^{-2}, 0.1, 10,100 and 1000 mmol/l). The stability during incubation at 37°C was determined at different intervals by TLC followed by activity estimation using well counter.

Binding with *E. coli*

Binding behavior of the ^{99m}Tc labeled PZN with *E. coli* (live and heat killed) was investigated using the reported method. Briefly, 0.5 mCi of the radiolabeled (^{99m}Tc-PZN) was mixed with 0.1 ml sodium phosphate buffer (Na-PB) followed by addition of 0.8 ml, 1×10^8 colony forming units (cfu) of the *E. coli* (live and heat killed) and 0.01 M acetic acid (50%, v/v)'. Thereafter, mixture was incubated at 4°C for 30 min, followed by centrifugation at 4000 rpm for 10 min. The percent binding was determined using well counter.

Biodistribution in animal model rat

Twenty healthy wister rats (weight, 180-210 gm) were used as experimental animals for determination of biodistribution behavior of the radiolabeled (^{99m}Tc-PZN). The selected animals were divided uniformly in two groups i.e (group A and B). Rats of group A and B rats were inflamed through intramuscular injection of pyrogen free oil into right leg of all rats. Thereafter, group A rats were infected by administering live strain of *E. coli* (0.1 mL containing 1×10^8 cfu) to the contra-lateral (left leg) of each rats and heat killed *E. coli* to the group B rats. After 12 h, 0.5 mCi of radiolabeled (^{99m}Tc-PZN) was intravenously injected to all the experimental rats. Thereafter, the experimental rats were killed as per guide lines set by US pharmacopeia. Then different organs of the rats were surgically removed and accurately measure for activity (%injected dose/g) in one gm each of the blood, infected muscle (*E. coli* live and heat killed in separate group), inflamed muscle and normal muscle, liver, spleen,

lungs stomach and intestines, kidney and urine using single well counter interface with scalar count rate meter.

Scintigraphy

Scintigraphic accuracy of the radiolabeled (^{99m}Tc-PZN) was investigated using animal model rabbits (weight 4-5 kg). Healthy rabbits were anaesthetized with 0.3 mL of ketamin i.m. followed by injecting 0.5 mL live strain of *E. coli* i.m. in to the right thigh. After 10 h, 1.0 mL pyrogen free oil was infused to the left leg of the same animal. Thereafter, 0.5 mL of the radiolabeled (^{99m}Tc-PZN) was administered to the animal model rabbit intravenously followed by whole body planner imaging at concentration value of the stannous chloride as 120 mg.

Statistical analysis

Results are expressed as %Injected dose/gram or ratios ± SEM and statistical analysis were executed using the student t -test

Results and Discussion

Percent radiochemical purity (%RCP) yield

The radio- HPLC chromatogram of ^{99m}Tc-PZN acquired at 30,6 0, 90 and 120 min demonstrated analogous prototype signals as observed in case of PZN labeled either through ^{99m}Tc≡N [18] and or ^{99m}Tc(CO)$_3$ [19] techniques . In case of ^{99m}Tc-PZN, two broader different signals were observed one at 3.8 and second one at 10.9 min. The signal observed at 3.8 entail the unwanted side product consisting of free and reduced hydrolyzed ^{99m}Tc; while the signal at 10.9 min, corresponds to main product i.e. labeled PZN, as shown in Figure 2.

The PZN labeled with ^{99m}Tc using different concentration of PZN, ^{99m}Tc, stannous chloride at different pH showed different percent radiochemical purity yield. Further, it was observed that the labeled PZN primed by mixing 2 mg PZN, 120 µg stannous chloride, and 2.5 m Ci of sodium pertechnetate at pH 5.3, showed maximum radiochemical yield in percent at 30 min subsequent to labeling and remained intact more than 90% up to 240 min in normal saline. The percent radiochemical purity yield determined between at 1 to 240 min after labeling was 97.10 ± 0.28%, 98.25 ± 0.26%, 97.30 ± 0.36%, 96.00 ± 0.28%, 94.45 ± 0.24%, and 92.15 ± 0.32%, as given in Figure 3. However, the PZN labeled through ^{99m}Tc≡N [18] and or ^{99m}Tc(CO)$_3$-[19] showed 98.40 ± 0.26% and 98.95 ± 0.28% maximum %RCP yield at 30 min after reconstitution and remain intact up to 90% up to 240 min. The percent radiochemical purity yield profile of labeled PZN, ^{99m}Tc≡N-PZND and ^{99m}Tc(CO)$_3$-PZND showed close correlation.

Figure 4 showed the effect of the concentration of the PZN on the %RCP yield profile of the labeled PZN. It was observed that the %RCP yield profile of the labeled PZN increased initially by increasing the amount of the PZN in the reaction vial. However, this increase in the %RCP yield profile of the labeled PZN was noticed up to 2.5 mg of PZN and thereafter a gradual decrease was observed.

The effect of the activity of the ^{99m}Tc on the %RCP yield profile of the labeled PZN showed a similar trailing pattern. Initially the %RCP yield profile of the labeled PZN moves up by rising the concentration of the sodium pertechnetate. However, after adding 3.00 m Ci to the reaction vial the %RCP yield decrease. It is experiential that the labeled PZN showed maximum % RCP yield by adding 2.5 mg to the reaction vial.

The effect of different concentration of stannous chloride dehydrate on the %RCP yield of the labeled PZN have shown similar pattern as observed by varying the concentration of PZN and activity. Maximum %RCP yield was observed by adding 120 µg stannous chloride to the reaction vial.

Similarly the effect of pH on the %RCP yield of the labeled PZN is not different from the ones shown in Figure 4. It was observed that the labeled PZN showed higher %RCP yield at pH 3. The percent radiochemical purity yield profile of the labeled PZN goes down either by increasing or decreasing the pH from 3.

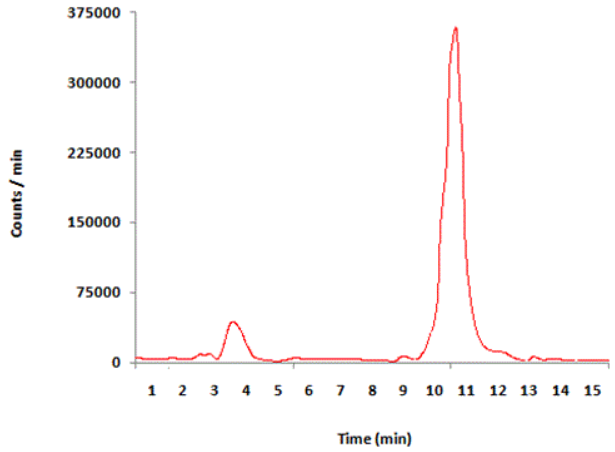

Figure 2: HPLC radiochromatogram of ^{99m}Tc-PZN.

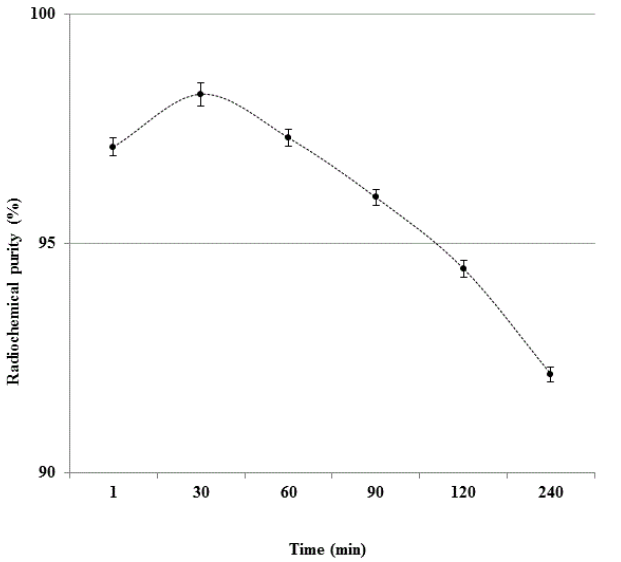

Figure 3: Radiochemical Purity (%) of Technetium-99m labeled PZN up to 240 min after reconstitution at room temperature.

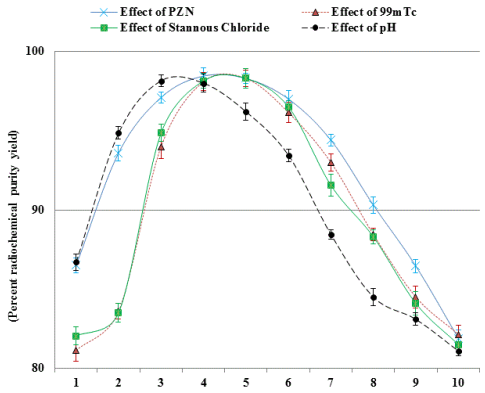

Figure 4: Effect of PZN, Technetium-99m, stannous chloride, pH on percent radiolabeling yield.

In vitro stability in serum

The *in vitro* stability profile of the PZN labeled directly with ^{99m}Tc at 37°C up to 16 h is given in Figure 5. The stability of labeled PZN in serum at 0, 2, 4, 6, 8, 10, 12, 14 and 16 h after reconstitution was 98.00 ± 0.30%, 93.30 ± 0.40%, 91.25 ± 0.35%, 88.55 ± 0.30%, 85.10 ± 0.35%, 83.45 ± 0.40%, 83.00 ± 0.36%, and 82.70 ± 0.50%. The free and colloidal ^{99m}Tc based radioactivity up to 16.40% was calculated which is in permissible acceptable limit. However, the total decay observed after 16 h of incubation for ^{99m}Tc≡N-PZND and $^{99m}Tc(CO)_3$-PZND were 15.95% and 13.35% [18,19]. The decay profile in terms of free and colloidal ^{99m}Tc based radioactivity of labeled PZN, ^{99m}Tc≡N-PZND and $^{99m}Tc(CO)_3$-PZND showed complete correlation and were in line with permissible acceptable limits.

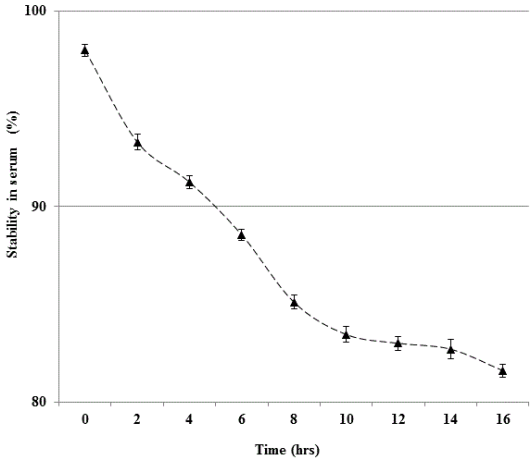

Figure 5: Stability of ^{99m}Tc-PZN at 37°C in serum.

Binding with *E. coli*

The *in vitro* live and heat killed *E. coli* uptake profile of the labeled PZN is shown in Figure 6. At 30 min, 60 min, 90 min and 120 min incubation the *in vitro* live *E. coli* bacterial up take in case of labeled PZN observed was 34.45 ± 1.20%, 66.00 ± 1.00%, 72.55 ± 0.90%, and 63.20 ± 0.88%, respectively. In case of heat killed *E. coli* no significant uptake was seen. The uptake in case of heat killed *E. coli* at 30 min, 60 min, 90 min and 120 min were 13.00 ± 1.20%, 14.70 ± 0.99%, 12.50 ± 0.98% and 12.50 ± 1.20% respectively. In comparison the ^{99m}Tc≡N-PZND [18] and $^{99m}Tc(CO)_3$-PZND [19] showed almost similar *in vitro* binding affinity at 30, 60, 90 and 120 min of incubation. No significant difference in the *in vitro* uptake was seen in the PZN labeled either directly or through $[^{99m}Tc(CO)_3(H2O)_3]^+$ or $[^{99m}Tc≡N]^{2+}$ techniques.

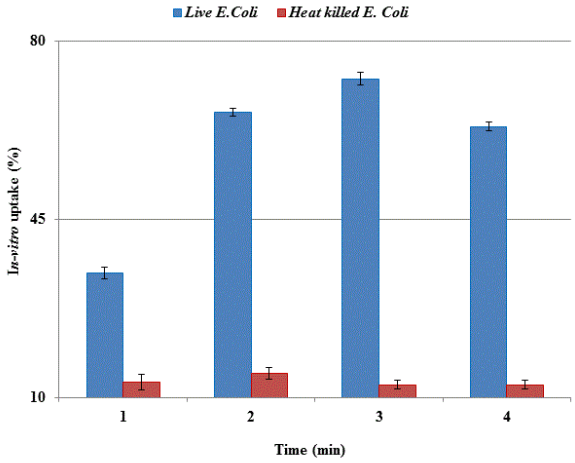

Figure 6: Bacterial binding of ^{99m}Tc-PZN.

Biodistribution in animal model rat

Table 1 Showed %*in vivo* biodistribution behavior of the labeled PZN in blood, liver, spleen, stomach, intestine, kidney, infected muscle, inflamed and normal muscle of the artificially infected model rats. Initially, the level of activity in blood was high which gradually went dawn up to 3.65 ± 0.18% and 4.25 ± 0.28%, in group A and B animal model rats respectively. No significant difference in the uptake of blood was seen in the PZN labeled either directly or through $[^{99m}Tc(CO)_3(H_2O)_3]^+$ or $[^{99m}Tc≡N]^{2+}$ technique as all showed similar trailing pattern initially the dose in blood was high which gradually went down, indicating its clearance from the circulatory system like a normal drug and the level of activity in liver, spleen, stomach and intestine of group A and B animals also disappeared gradually. Further the level of activity in liver of live and heat killed *E. coli* infected animal model rats (group A and B) showed no significant difference and also no significant difference was seen in the uptake, appearance and disappearance profile of labeled PZN, ^{99m}TcV≡N-PZND [18] and $^{99m}Tc(CO)_3$-PZND [19].

Similarly, the appearance and disappearance profile of the labeled PZN, ^{99m}TcV≡N-PZND [18] and $^{99m}Tc(CO)_3$-PZND [19] in spleen, stomach and intestine were also gone down with time and no substantial variation have been observed in the uptake live and heat killed *E. coli* infected rat. The level of activity up take in kidneys of the labeled PZN, ^{99m}TcV≡N-PZND [18] and $^{99m}Tc(CO)_3$-PZND [19] were went up with time and no considerable distinction have been observed in the uptake live and heat killed *E. coli* animal model rats (group A and B).

In the infected muscle of the animal model rats artificially infected with live *E. coli* the uptake of the labeled PZN was low in the beginning but went up gradually. The up take profile in the infected muscle of the animal model rats infected with live *E. coli* in case of labeled PZN injection were 6.35 ± 0.35%, 11.90 ± 0.21%, 14.75 ± 0.40%, and 13.80 ± 0.30% respectively. However, the level in the infected muscle of the animal model rats infected with heat killed *E. coli* in case of labeled PZN injection were 2.50 ± 0.10%, 3.00 ± 0.44%, 2.50 ± 0.15%, and 2.00 ± 0.24% respectively, which shows that the labeled PZN incorporated in the infected muscle (infected with live *E. coli*) in much higher concentration than the animal muscle infected with heat killed *E. coli*.

However no significant difference in the up take profile of the inflamed muscle of the animal infected with live and heat killed *E. coli* were seen. Similarly the difference in up take was negligible of the labeled PZN in the normal muscle of the animal model rats either infected with live or heat killed *E. coli*. The overall biodistribution of the labeled PZN, ^{99m}TcV≡N-PZND [18], and ^{99m}Tc(CO)$_3$-PZND [19] were found in close correlation with no significant difference with regards to the up take in various organs of the animal model rats. Similar to ^{99m}TcV≡N-PZND [18] and ^{99m}Tc(CO)$_3$-PZND [19], the labeled PZN also moved out from the circulatory system and appeared in the urinary system confirming the normal route of excretion.

Organs/tissues (gm)	Distribution of the 99mTc-PZN in various organs							
	Live *E. coli*				Heat killed *E. coli*			
	30	60	90	120	30	60	90	120
Infected muscle	6.35 ± 0.35	11.90 ± 0.21	14.75 ± 0.40	13.80 ± 0.30	2.50 ± 0.10	3.00 ± 0.44	2.50 ± 0.15	2.00 ± 0.24
Inflamed muscle	3.00 ± 0.28	3.50 ± 0.30	3.00 ± 0.15	3.00 ± 0.20	3.50 ± 0.22	4.00 ± 0.24	3.50 ± 0.20	3.50 ± 0.18
Normal muscle	2.00 ± 0.12	3.00 ± 0.18	2.50 ± 0.14	2.00 ± 0.16	2.00 ± 0.14	3.00 ± 0.20	2.50 ± 0.18	2.00 ± 0.22
Blood	19.55 ± 0.17	9.95 ± 0.18	8.80 ± 0.20	3.65 ± 0.18	20.10 ± 0.10	9.40 ± 0.20	7.95 ± 0.22	4.25 ± 0.28
Liver	18.15 ± 0.22	13.00 ± 0.20	11.10 ± 0.18	4.20 ± 0.22	18.55 ± 0.15	12.90 ± 0.10	11.25 ± 0.22	4.35 ± 0.18
Spleen	9.15 ± 0.18	7.10 ± 0.16	6.15 ± 0.20	4.00 ± 0.22	9.00 ± 0.22	7.55 ± 0.28	6.00 ± 0.16	4.20 ± 0.26
Kidney	10.25 ± 0.14	18.00 ± 0.18	19.50 ± 0.18	22.55± 0.20	10.55 ± 0.20	17.95 ± 0.26	20.50 ± 0.24	23.15 ± 0.18
Stomach & intestines	8.65 ± 0.18	7.20 ± 0.20	5.45 ± 0.12	3.70 ± 0.20	8.80 ± 0.26	7.35 ± 0.14	5.70 ± 0.22	4.00 ± 0.10

Table 1: Biodistribution profile of the ^{99m}Tc labeled PZN in artificially infected MWR.

Scintigraphy

Scintigraphically, it was observed that the labeled PZN showed initially normal activity uptake in the whole body of the animal model rat as shown in Figure 7.

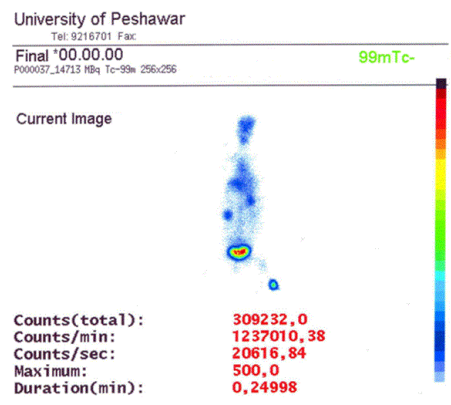

Figure 7: Scintigraphic image of artificially infected animal model rabbit.

However, after few minutes, the activity accumulated in the infected muscle more than the inflamed and normal muscle. The level of the labeled PZN was much lower in kidneys in the begging but it went up and reached to a maximum level after 120 m. The scintigraphic visualization of artificially infectious sites validated the targeted biodistribution behavior of the labeled PZN.

Conclusion

In this work PZN was labeled directly with Technetium-99m (^{99m}Tc) and compared with the existing ^{99m}TcV:N-pazufloxacin dithiocarbamate (^{99m}TcV:N-PZND) and ^{99m}Tc(CO)$_3$-pazufloxacin dithiocarbamate (^{99m}Tc(CO)$_3$- PZND) with the aim to provide an alternative radio-diagnostic agent for the deep tissue infection imaging (caused by *E. coli*) in its early stages. Based on the high radiochemical purity yield, stability in serum, *in vitro* binding with live *E. coli*, biodistribution in artificially infected animal model rats and scintigraphic assessment the labeled PZN is recommended for the diagnosis of deep tissue infection caused by *E. coli*.

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