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^{99m}Tc labeled levofloxacin as an infection imaging agent: a novel method for labeling levofloxacin using cysteine·HCl as co-ligand and in vivo study

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Levofloxacin was labeled with ^{99m}Tc using cysteine·HCl as co-ligand and $SnCl_2.2H_2O$ as reducing agent. The influence of various parameters such as amount of cysteine·HCl, reducing agent, pH value, and reaction time on labeling process was studied. After optimizing the conditions the labeling was performed at pH 5 using 1 mg of levofloxacin, 500 μg of cysteine·HCl, 50 μg of $SnCl_2.2H_2O$, and 15 min reaction time. The radiochemical purity was determined with the help of instant thin layer chromatography (ITLC) and reverse phase high performance liquid chromatography (RP-HPLC) which was more than 95% and was stable for up to 6 h. Biodistribution of ^{99m}Tc-levofloxacin (^{99m}Tc-lefx) was studied in infection induced rat models using live *Staphylococcus aureus* and heat killed *S. aureus* (sterile inflammation model). In the case of the live *S. aureus* induced abscess model, the accumulation of ^{99m}Tc-lefx at target was 3.96, which was higher than that of ^{99m}Tc-ciprofloxacin (^{99m}Tc-cifx), taken as the control.

Key Words: Infection, ^{99m}Tc-levofloxacin, co-ligand, scintigraphy, biodistribution, infection model

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Introduction

In developing countries where the environment, particularly public places, are not fully hygienic, and even in highly developed countries, bacterial action causes severe infectious diseases associated with morbidity. Early detection and mapping of the infection site allow prompt and successful treatment. After the development of radiopharmaceuticals, the risk factors of morbidity associated with infectious diseases sharply decreased.

Radiolabeled pharmaceuticals, for early detection of infections/tumors, should be associated with certain properties such as ease of labeling, high specificity, rapid accumulation at the site of infection or tumor, i.e. early diagnosis, high target to nontarget ratio, rapid blood clearance, low toxicity, low cost, and less antigenicity[1] Due to the easy availability and high compatible half-life of ^{99m}Tc with imaging period of infection foci, ^{99m}Tc is a more desirable labeling radioisotope as compared to others for diagnostic purposes.[1−3]

Certain radiolabeled agents such as ^{67}Ga-citrate,[4] white blood cells,[5] human immunoglobulin,[6] dextran,[7] and nanocolloid[8] are unable to differentiate between sterile inflammation and septic infection. Quinolones have been found to be more effective in several types of intracellular infections,[9,10] regardless of the subcellular localization of the organisms, including those caused by *Legionella pneumophila* (phagosomes),[11−14] *Listeria monocytogenes* (cytosol), or *S. aureus* (phagolysosomes).[15]

Fluoroquinolones specifically bind and inhibit bacterial DNA gyrase. Ciprofloxacin (Figure 1) is a fluoroquinolone antibiotic that was first labeled using formamidine sulfuric acid (FSA) as ^{99m}Tc reducing agent at 100 °C for 10 min for complexation.[16] Due to the instability of the FSA, stannous ion has been used to reduce ^{99m}Tc from high oxidation state to a lower one and for increasing labeling yield. Attempts have also been made to label other antibiotics of the same group with ^{99m}Tc including levofloxacin,[17,18] Norfloxacin,[19] Enorfloxacin,[20] and Sparfloxacin.[21] Levofloxacin (Figure 1), which is a very potent member of the quinolones family,[9,22,23] having the ability to fight against gram-positive and gram-negative bacteria, is used to treat infections of the sinuses, skin, lungs, ears, airways, bones, and joints caused by susceptible bacteria.[24]

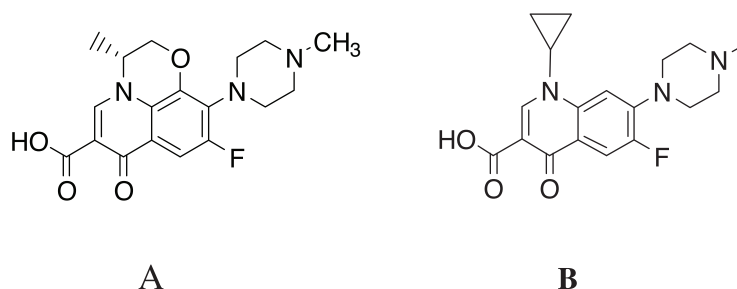

Figure 1. Structure of A: Levofloxacin, B: Ciprofloxacin.

The data obtained about the labeling conditions of ^{99m}Tc with levofloxacin and in vivo study are preliminary. Extensive research and debate are required to investigate more efficient labeling conditions to label levofloxacin with ^{99m}Tc. Further, systematic experimentations are also required to chalk out the complete profile of the efficacy and pharmacokinetics of ^{99m}Tc-lefx against a variety of gram-positive and gram-negative bacterial strain induced abscess animal models. The whole study may prove that radiolabeled levofloxacin is an excellent radiopharmaceutical candidate for early detection of chronic infections caused by gram-positive and gram-negative bacteria.

The aim of the present study was to standardize and develop a novel method to prepare a stable and easy to label kit of levofloxacin in a short time and at room temperature and in vivo study in induced abscess rat models using *S. aureus.*

Materials and methods

Reagents and equipment

Levofloxacin was purchased from Aventis Ltd (Uxbridge, UK), while Ciprofloxacin was from Bayer AG (Wuppertal, Germany). HCl, NaCl, $NaHCO_3$, NaOH, and $SnCl_2.2H_2O$ were purchased from Aldrich (Germany). Cysteine·HCl was obtained from MP, USA. All chemicals were of reagent grade and there was no need for further purification prior to use. Sprague-Dawley rats, New Zealand white rabbits, and strains of *S. aureus* were obtained from the National Institute of Health (NIH), Islamabad, Pakistan. The animal ethical committee of the institute gave ethical approval (UHS/Biochemistry/12-162) for the animal study. ^{99m}Tc was obtained from a locally situated fission based Pakistan Generator (PAKGEN) ^{99}Mo/^{99m}Tc generator.

Radiolabeling

To optimize the ^{99m}Tc-labeling conditions, experiments were carried out by dissolving 1 mg of levofloxacin in 1 mL of distilled water, followed by the addition of varying amounts of cysteine·HCl and $SnCl_2.2H_2O$. To determine the optimum amount of co-ligand and reducing agent, they were added in varying amounts. The pH was adjusted by using 0.1 N HCl/$NaHCO_3$ and NaOH solutions. The radiolabeling was performed by adding 80-1400 MBq of $Na^{99m}TcO_4$/saline, shaking for 30 s, and incubating for 10-30 min. All experiments were carried out at room temperature and under sterile conditions in a laminar flow hood.

Radiochemical analysis

The radiochemical purity and yield of ^{99m}Tc-lefx were determined by instant thin layer chromatography (ITLC) and reverse-phase high performance liquid chromatography (RP-HPLC), using Beckman HPLC systems consisting of Beckman 125 or 126 solvent-module pump systems with a Beckman 166-NM single wavelength or 168 diode array ultraviolet (UV) detector (Beckman, High Wycombe, UK) and a sodium iodide flow-through radiochemical detector attached to a Ray test gamma-radioactivity monitor. Acetone or ACN, saline (15% NaCl solution), or 0.1 M citrate buffer (pH 4.5) was used as mobile phase to separate ^{99m}Tc-lefx from free $^{99m}TcO_4^-$ and reduced/hydrolyzed ^{99m}Tc. The stability of the complex was checked over 6 h at room temperature, while the net charge on ^{99m}Tc-lefx was determined by paper electrophoresis.

Paper electrophoresis

The charge on ^{99m}Tc-lefx was determined by electrophoresis at constant voltage of 10 V/cm for 1 h using phosphate buffer of pH 7.5 and Whatman No. 1 paper as supporting medium. For this 5 μL of ^{99m}Tc-lefx

was introduced at the center of the paper impregnated in the phosphate buffer. The sample was allowed to run under the influence of the voltage for 60 min.

Blood plasma stability

For plasma stability studies, samples of ^{99m}Tc-lefx were incubated at 37 °C with freshly harvested human blood plasma and aliquots withdrawn at 0, 1, 2, 4, and 24 h time points. Ice-cold acetonitrile (ACN) was added in a 3:1 (v/v) ACN to aliquot ratio. After centrifugation, supernatant samples were analyzed by ITLC.

Biodistribution study

For biodistribution studies, 200 μL (3 × 10^8 cfu/mL) suspension of *S. aureus*, ATCC 25923 were injected into the right flank of Sprague-Dawley rats and grown for 3-5 days; at the end of the infection induced period the rats were injected with 100 μL of ^{99m}Tc-lefx via the tail vein. At 30 and 60 min and 4 h postinjection, the animals were anesthetized and dissected. Various organs were removed, washed with saline, dried over filter paper, weighed, and counted for radioactivity uptake using a well-type NaI(TI) gamma counter. For imaging studies, 300 μL of suspension of viable *S. aureus* was injected into 4 rabbits in their right flank muscles and imaged at 30 and 60 min and 4 h postinjection. The images were taken using a SPECT (single-photon emission computed tomography) animal scanner (Bioscan).

Results and discussion

Radiolabeling

Levofloxacin was labeled with ^{99m}Tc under various conditions and the labeling yield was optimized. The labeling yield was highly dependent on labeling conditions. In order to obtain the optimum concentration of each reagent for stable and efficient labeling, the pH value was chosen by hit and trial method and pH 5 was selected for labeling levofloxacin prior to checking the effect of pH on labeling. The highest labeling yield obtained was >96%, analyzed with the help of ITLC and RP-HPLC (Figure 2). The yield was also calculated and confirmed by passing the reaction mixture through a 0.22 mm filter[18] and ITLC analysis using the following expressions:

$$\%\text{colloid} = \frac{\text{Activity before filtration} - \text{Activity after filtration}}{\text{Activity before filtration}} \times 100$$

$$\%\text{free pertechnetate } (^{99m}TcO_4^-) = \frac{\text{Activity at } R_7 \text{ 0.75 to 1.0}}{\text{Total Activity}} \times 100$$

$$\%^{99m}\text{Tc-lefx} = 100 - (\%\text{colloid} + \%^{99m}\text{TcO}_4^-)$$

Effect of reducing agent on labeling

To set the optimum amount of reducing agent ($SnCl_2.2H_2O$) and to see its effect on labeling, it was used in the range of 15 μg to 300 μg. At the initial concentration of reducing agent the labeling efficiency was very poor (~56%); however, labeling efficiency was increased to about 96% at 50 μg/mL of $SnCl_2.2H_2O$ (Figure 3). This showed the lowest concentration of $SnCl_2.2H_2O$ that reduced maximum amount of $^{99m}TcO_4^-$ and offered

maximum labeling yield. Less than this concentration of $SnCl_2.2H_2O$ failed to reduce whole of the $^{99m}TcO_4^-$ for the labeling process.

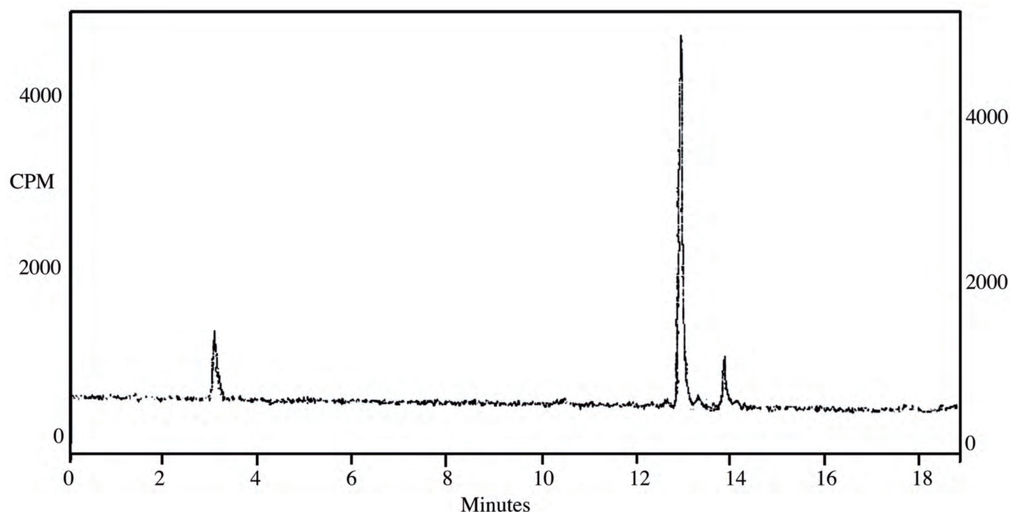

Figure 2. HPLC analysis of ^{99m}Tc-lefx; the main peaks elute at 12.89 min showing >95% labeling yield.

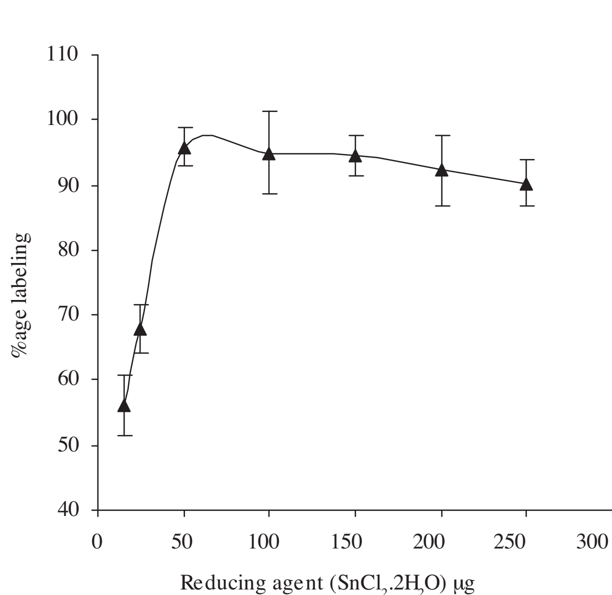

Figure 3. Effect of reducing agents ($SnCl_2.2H_2O$) on labeling efficiency.

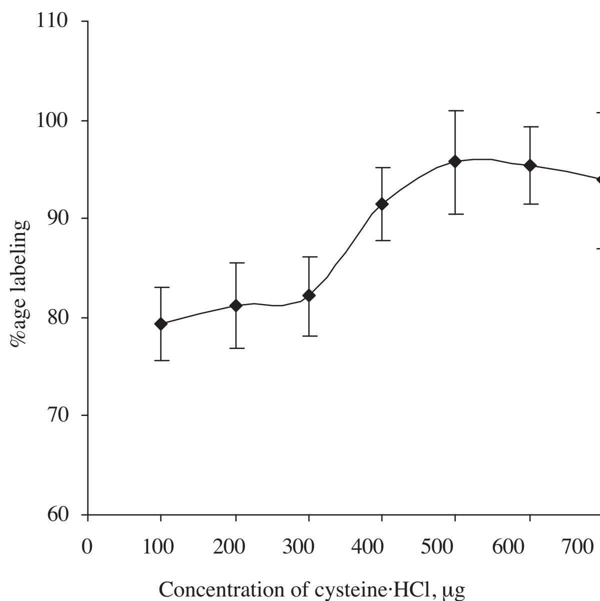

Figure 4. Effect of cysteine·HCl (Co-ligand) on labeling efficiency.

Effect of cysteine·HCl

Cysteine·HCl was used as co-ligand to reduce the reaction time and impart labeling stability to ^{99m}Tc-lefx. Concentration optimization of cysteine·HCl was carried out by studying various amounts of it (0-3.0 mg) in the

labeling process. The maximum labeling yield, 95.6%, was achieved using 500 μg of cysteine·HCl/mL (Figure 4). This fact can probably be ascribed to the ligand exchange method, whereby cysteine·HCl is labeled rapidly with a reduced form of ^{99m}Tc, resulting in an exchange of metal from cysteine·HCl to the levofloxacin molecule with long incubation times. This results in increased complex stability and radiolabeling efficiency.

Effect of pH

To establish the role of pH in radiolabeling, different pH conditions were studied. Acidic pH showed no prominent difference in labeling yield, whereas near basic pH the labeling efficiency was decreased, as shown in Figure 5. This may be attributed to a change in the structure of the levofloxacin in basic medium. The carboxylic moiety of levofloxacin may be neutralized, making it unable to make a complex with ^{99m}Tc during the metal exchange reaction process. The most favorable pH value that gave stable labeling was 5. Beyond this value the labeling efficiency and stability began to decrease.

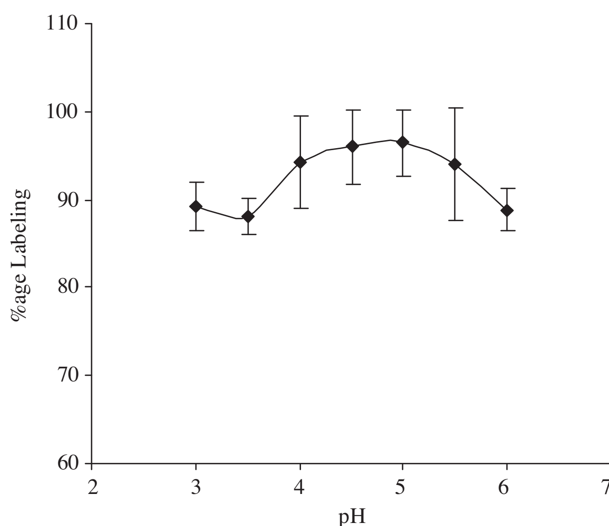

Figure 5. Effect of pH on labeling efficiency.

Incubation effect on labeling

Incubation period reveals the completion of the reaction and time of maximum yield of the radiolabeled compound. It was observed that optimal radiolabeling (>95%) was obtained at 15 min incubation period. Incubation for longer time intervals did not show any remarkable change. The stability of the labeled levofloxacin up to 6 h also showed >94% intact complex, as shown in Table 1.

Optimum parameters for levofloxacin kit formulation and radiolabeling

After setting the optimum conditions of different parameters, the following appropriate conditions were chosen for preparing the levofloxacin kit: 1 mg of levofloxacin, 500 μg of cysteine·HCl, and 2 mg of gentistic acid; all these ingredients were dissolved in 800 μL of distilled water and 50 μg of $SnCl_2.2H_2O$ was added. The

pH of the resulting solution was adjusted to 5 with 0.1 N NaOH, and monitored for 15 min with continuous stirring. This prepared kit was then filtered through a 0.22 μm filter (MILLEX® GV Filter Unit, Ireland). After lyophilization the freeze dried kits were stored at 4 °C. The whole process was carried out under sterile conditions and at room temperature. The kit was labeled with 80-1400 MBq ^{99m}Tc and analyzed using paper chromatography, (ITLC) and RP-HPLC, for further tests.

Table 1. Stability study of ^{99m}Tc-lefx kit up to 6 h.

Time (hours)	% Radioligand	% Colloid $^{99m}TcO_4^-$
15 min	95.95 ± 4.65	3.03 ± 2.08
0.5	96.02 ± 5.43	2.95 ± 1.80
1	95.12 ± 6.33	3.89 ± 2.01
2	95.88 ± 4.83	3.08 ± 1.70
3	95.66 ± 7.67	3.99 ± 1.98
4	95.47 ± 6.96	3.56 ± 2.43
5	95.12 ± 5.97	3.34 ± 2.06
6	94.71 ± 7.09	4.25 ± 3.08

Paper electrophoresis

Apparent charge on the radiolabeled molecule may change its biological interaction in the living system. This increases or decreases the binding potential of the bioactive molecule with the cell membrane. Apparent charge on the ^{99m}Tc-lefx was determined by paper electrophoresis. The experiments revealed that ^{99m}Tc-lefx carried no charge and it had a neutral moiety. Only a small amount of free pertechnetate ($^{99m}TcO_4^-$) was moved along with solvent front and all the activity (^{99m}Tc-lefx) remained at the baseline. A trace amount of colloid also appeared near the baseline due to the very small electrical charge on the colloid material. The colloid formed due to hydrolysis of tin (Sn^{+2}) and reduced ^{99m}Tc diminished the yield of ^{99m}Tc labeled levofloxacin. Total colloid %age was <1.4% in each labeling reaction, which was very poor, resulting in excellent % yield of labeled levofloxacin.

Human blood plasma stability

No significant differences in the serum stability of the radiolabeled levofloxacin were observed, with more than 90% of ^{99m}Tc-lefx remaining intact after 2 h of incubation in human serum. After 24 h only 18% ^{99m}Tc-lefx was degraded. This indicated that the blood serum enzymes were unable to degrade the ^{99m}Tc-lefx within first half-life of ^{99m}Tc and ^{99m}Tc-lefx can bind with bacterial DNA gyrase before blood protease degradation.

In vivo study

Complex stability of ^{99m}Tc-lefx and in vivo behavior were finally assessed in a *S. aureus* infection induced animal model. The biodistribution in infection-bearing rats was studied by radioactivity counting using a well-typed NaI(TI) gamma counter. Radioactivity was counted immediately after dissecting and washing at subsequent

time points up to 4 h. The results of biodistribution studies of ^{99m}Tc-lefx and ^{99m}Tc-cifx are summarized in Table 2. Following the 80 MBq of ^{99m}Tc-lefx in live *S. aureus* and heat killed *S. aureus* infected/inflamed rats, the maximal uptake was seen in the kidney and liver, which decreases with time. Uptake of activity appeared in both infected and inflamed foci at early time points (30 min and 1 h postinjection), whereas at 4 h only the infected focus showed prominent uptake. The activity uptake at the infected site in the case of both labeled antibiotics showed considerable difference, as it was 0.96%, 3.96%, and 2.18% of ^{99m}Tc-lefx and 0.76%, 2.71%, and 1.83% of ^{99m}Tc-cifx at 30 min, 1 h, and 4 h postinjection, respectively.

Table 2. (a) Biodistribution of ^{99m}Tc-lefx in live *S. aureus* and heat killed *S. aureus* infected rats. (b) Biodistribution of ^{99m}Tc-cifx in live *S. aureus* and heat killed *S. aureus* infected rats.

(a)

Organs	After 30 min		After 1 h		After 4 h	
	Live bacterial abscess	Killed bacterial abscess	Live bacterial abscess	Killed bacterial abscess	Live bacterial abscess	Killed bacterial abscess
Infected muscle	0.96 ± 0.09	0.51 ± 0.06	3.96 ± 1.79	1.35 ± 1.05	2.18 ± 1.57	0.69 ± 0.78
Normal muscle	0.20 ± 0.00	0.24 ± 0.01	0.23 ± 0.	0.22 ± 0.03	0.19 ± 0.	0.18 ± 0.00
Liver	3.01 ± 2.30	3.03 ± 1.79	2.09 ± 1.3	2.93 ± 1.62	0.41 ± 0.7	0.52 ± 0.54
Spleen	0.23 ± 0.01	0.26 ± 0.00	0.25 ± 0.00	0.30 ± 0.03	0.15 ± 0.	0.17 ± 0.04
Lungs	0.82 ± 0.01	0.87 ± 0.09	0.50 ± 0.	0.54 ± 0.02	0.29 ± 0.	0.36 ± 0.03
Stomach	0.75 ± 0.10	0.80 ± 0.01	0.37 ± 0.01	0.33 ± 0.03	0.41 ± 0.	0.52 ± 0.08
Kidney	15.10 ± 4.91	22.33 ± 4.99	17.04 ± 3.60	19.12 ± 3.05	10.64 ± 3.25	6.14 ± 2.88
Blood	0.35 ± 0.08	0.56 ± 0.14	0.51 ± 0.6	0.48 ± 0.13	0.26 ± 0.07	0.21 ± 0.10
Heart	0.43 ± 0.02	0.37 ± 0.00	0.63 ± 0.00	0.58 ± 0.03	0.16 ± 0.	0.14 ± 0.00

(b)

Organs	After 30 min		After 1 h		After 4 h	
	Live bacterial abscess	Killed bacterial abscess	Live bacterial abscess	Killed bacterial abscess	Live bacterial abscess	Killed bacterial abscess
Infected muscle	0.76 ± 0.02	0.59 ± 0.01	2.71 ± 1.87	1.41 ± 1.09	1.83 ± 1.30	0.83 ± 0.77
Normal muscle	0.20 ± 0.00	0.17 ± 0.00	0.23 ± 0.01	0.20 ± 0.00	0.21 ± 0.01	0.19 ± 0.07
Liver	7.59 ± 3.89	7.13 ± 2.31	3.31 ± 2.12	4.13 ± 2.56	0.83 ± 0.26	0.77 ± 0.09
Spleen	0.48 ± 0.00	0.42 ± 0.02	0.23 ± 0.00	0.29 ± 0.01	0.22 ± 0.00	0.31± 0.02
Lungs	0.86 ± 0.01	0.79 ± 0.07	0.46 ± 0.02	0.51 ± 0.05	0.24 ± 0.	0.29 ± 0.00
Stomach	0.89 ± 0.00	0.95 ± 0.14	0.39 ± 0.04	0.32 ± 0.01	0.32 ± 0.09	0.35 ± 0.12
Kidney	19.82 ± 4.01	19.42 ± 3.04	18.04 ± 3.44	20.12 ± 4.26	12.13 ± 3.05	05.22 ± 2.66
Blood	0.23 ± 0.01	0.38 ± 0.07	0.23 ± 0.8	0.31 ± 0.09	0.24 ± 0.10	0.18 ± 0.03
Heart	0.98 ± 0.01	0.88 ± 0.07	0.63 ± 0.06	0.58 ± 0.09	0.21 ± 0.00	0.23 ± 0.01

In previous studies,[17,18] *E. coli* infection induced animal models were used to assess the in vivo behavior of the labeled levofloxacin. EL-Ghany et al.[18] reported data about the labeling of levofloxacin and biodistribution in *E. coli* induced abscess animal models. The maximum activity that accumulated at the site of infection focus in the live *E. coli* model was 2.9%, while in the heat killed *E. coli* model it was 2.0%.

In the present study, ^{99m}Tc-lefx accumulates much more in the live *S. aureus* induced infection model (3.96%) than in the sterile inflammation dead model. The ^{99m}Tc-lefx complex appears to be more susceptible for *S. aureus* induced abscess as compared to the *E. coli* model.[18] To the best of our knowledge no study has previously been reported on the study of ^{99m}Tc labeled levofloxacin using *S. aureus* induced abscess. A study to further analyze the efficacy and stability of ^{99m}Tc-lefx using our novel labeling method is being conducted in patients with chronic infections caused by *S. aureus*/*E. coli*. Uptake at the infection focus in the initial scintigraphic study of 30 min postinjection in an infection induced rabbit model indicates ^{99m}Tc-lefx binding at the site of infection and stability of the complex (Figure 6).

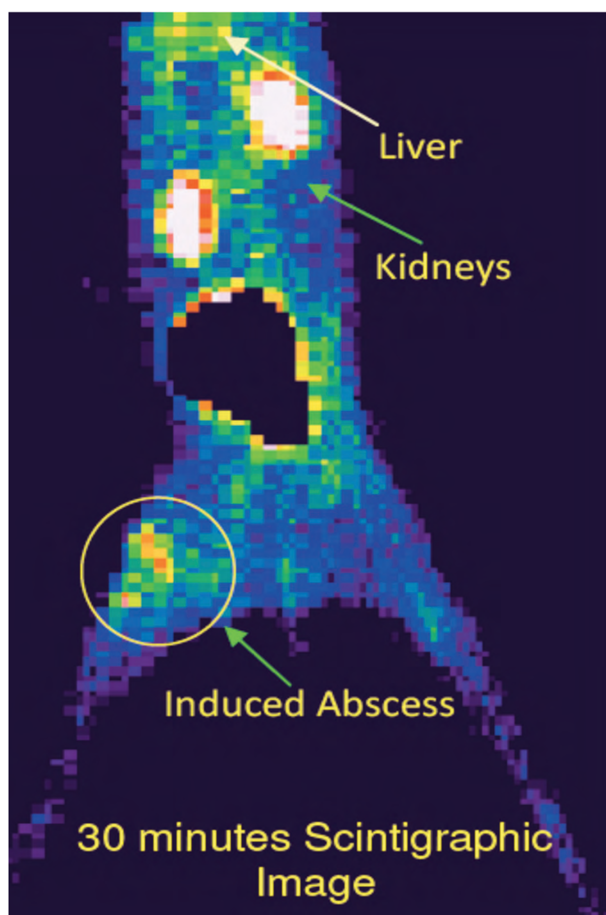

Figure 6. Scintigraphic study of rabbits using ^{99m}Tc-lefx at 30 min postinjection showing radioactivity uptake at infection site.

Conclusion

Labeled compounds have directed medical researchers toward easy and quick diagnostic strategies for infection and tumors. Of these, labeled antibiotics are particularly important in the diagnosis of infectious foci and to distinguish such foci from sterile inflammation.

Comparing the methods of labeling levofloxacin with ^{99m}Tc, as reported previously, labeling in the presence of co-ligand cysteine·HCl and reducing agent $SnCl_2.2H_2O$ is quick, stable, and efficient. All the data gathered show very good results and may further be used in clinical tests to diagnose sinus, skin, lungs, ear, airways, bone, and joint infections caused by susceptible bacteria.

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