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Labelling of ceftriaxone with ^{99m}Tc and its bio-evaluation as an infection imaging agent

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Differentiation of bacterial and sterile inflammation will have a significant impact on the current clinical practice. Ceftriaxone (CTRX) was labelled with ^{99m}Tc and assessed for its ability to depict infection on scintigraphy. Stoichiometry was performed to optimize labelling parameters. Stability and bacterial binding was verified and biodistribution pattern was seen in normal, infected/inflamed animal models. ^{99m}Tc-CTRX prepared at pH 7 with stannous chloride of 50 μg, ligand of 30 mg, and boiling for 10 min gave labelling yield of 96.2 ± 0.2% with good stability. *In vitro* binding was higher for *Escherichia coli* than *Staphylococcus aureus*. Biodistribution in normal rats showed high uptake in hepatobiliary system, gut and urinary system. In animal models induced with infection or inflammation, lesion to normal ratios at 4 h were 2.36 ± 0.21, 12.66 ± 1.44 and 1.40 ± 0.01 with *S. aureus* infection, *E. coli* infection and turpentine oil inflammation, respectively. Infection specificity especially for *E. coli* was also confirmed on scintigraphic findings.

Ceftriaxone can be labelled with ^{99m}Tc with high labelling yield at pH compatible with that of blood. Our preparation has shown stability *in vitro* and in human serum, and binds preferentially with bacteria. ^{99m}Tc-CTRX scintigraphy can be used to delineate sites of active infection and to differentiate infection and inflammation.

Keywords: radiolabelled antibiotic; ^{99m}Tc-ceftriaxone infection imaging; third-generation cephalosporin

Introduction

Imaging pathophysiology of inflammation/infection is a clear advantage of functional techniques of nuclear medicine in cases where morphological techniques such as X-ray, ultrasound, computerized tomography or magnetic resonance imaging are unable to identify infection in posttraumatic and postsurgical cases or in pyrexia of unknown origin.[1] It is also helpful in the management of osteomyelitis (acute and chronic), prosthesis infection, disc space infection, diabetic foot, intra-abdominal infection, bacterial endocarditis, pulmonary infections and infections in immune-compromised patients. Gallium–67 citrate, radiolabelled leucocytes, flourine–18 labelled fluorodeoxyglucose in positron emission tomography or radiolabelled monoclonal antibodies are currently being used to depict inflammations including the infections. They generally lack the differentiation between aseptic and septic inflammation.[2] Furthermore, the commonly used radiolabelled leucocytes technique is a cumbersome procedure and require handling of blood products. Antimicrobial agent may have more specificity for the sites of active infection and are the focus of research. Labelling with radioisotope of Technetium–99 m (^{99m}Tc) is preferred as it has favourable imaging characteristics.[3] Ciprofloxacin was the earliest antibiotic labelled with ^{99m}Tc and has been recommended for clinical use.[4–7] However, it was also found to be unable to discriminate septic and sterile lesions leading high false positives cases.[8–10] ^{99m}Tc-ciprofloxacin scan therefore did not gain significant clinical value. Several other types of antimicrobials from tetracycline, quinolone and cephalosporin groups are being evaluated for radiolabelling and clinical use.[11–13]

Cephalosporins are a group of beta-lactam antibiotics that attach with penicillin-binding-proteins (PBPs) in bacterial cell wall inhibiting its synthesis. This causes osmotic instability that kills the bacteria. Ceftriaxone (CTRX) is a third-generation cephalosporin binds primarily to PBP3 (Figure 1).[14] In this study, we present our attempt for labelling CTRX, with ^{99m}Tc and to optimize labelling parameters of ^{99m}Tc-CTRX. We also performed animal studies for its biodistribution; compared its uptake in Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria and examined its ability to differentiate infectious and sterile inflammations.

Materials and methods

Formulation, quality control and biodistribution of ^{99m}Tc-CTRX were carried out in the Isotope Production Division, Pakistan Institute of Nuclear Science and Technology, Islamabad, Pakistan. For labelling of CTRX with ^{99m}Tc, various combinations of the substrate and reducing agent at different pH levels were attempted. The prepared agent was then used to study stability in normal saline and serum, *in vitro* binding to bacteria and biodistribution in normal and infection/inflammation-induced sacrificed animals. Biodistribution was also assessed visually by scintigraphy in normal, infected and inflamed animal models in

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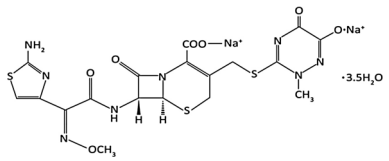

Figure 1. Chemical structure of ceftriaxone.

the Department of Medical Sciences, Pakistan Institute of Engineering and Applied Sciences, Islamabad, Pakistan.

Labelling procedure

Required amount of CTRX was dissolved in 500 μL of distilled water in a sterilized glass vial and 50 μL of aqueous solution containing 50 μg $SnCl_2.2H_2O$ was added whilst adjusting the pH to the required level by NaOH and HCl solutions. Freshly eluted sodium pertechnetate ($Na^{99m}TcO_4$) solution (≈370 MBq) in physiological saline was immediately injected into vial. The mixture was boiled for the required time followed by incubation for 10 min at the room temperature (25 ± 2 °C).

Labelling efficiency and quality control

Radiochemical purity and labelling efficiency of ^{99m}Tc-CTRX was assessed by ascending paper chromatography and instant thin-layer chromatography (TLC). Free technetium in the form of $^{99m}TcO_4^-$ in the preparation was determined using Whatmann paper No.1 strip as a stationary phase and acetone as a mobile phase (system–1). Strip measuring 14 × 2 cm^2 was spotted with 5 μL of the prepared sample and placed in a jar containing the mobile phase. When solvent reached the solvent front, the strip was scanned using a 2π scanner for radio-chromatography. The labelled CTRX and reduced/hydrolysed ^{99m}Tc remained at the origin, that is, had the retention value (R_f) of zero whilst free $^{99m}TcO_4^-$ moved towards the solvent front ($R_f = 1$). Activity of reduced/hydrolysed ^{99m}Tc was determined by TLC using instant TLC strips (14 × 2 cm^2) as stationary phase and a mixture containing five parts water, two parts ethanol and one part ammonium hydroxide as a mobile phase (system–2). The procedure was same as paper chromatography described earlier. The reduced/hydrolysed ^{99m}Tc remained at the origin whilst labelled CTRX and free $^{99m}TcO_4^-$ moved towards the solvent front. ^{99m}Tc-CTRX complex was also characterized by paper electrophoresis using 0.05 M phosphate buffer of pH 6.9 as electrolyte and Whatman No. 1 paper as support. Strip (2.5 cm × 30 cm) was soaked into phosphate buffer for few seconds. The origin was marked in the middle of strip and a spot of 5 μL of ^{99m}Tc-CTRX was placed at the point of origin. The electrophoresis was performed at constant voltage of 300 volts for 1 h. The paper strip was then scanned by 2π scanner.

Optimization

The level of pH; amount of the reducing agent; amount of the ligand; and boiling time of the solution were optimized by determining the change in labelling yield whilst varying these parameters in the solution. The pH level was set to 9, 5 or 3 whilst keeping other labelling conditions constant. To determine the optimum concentration of (stannous chloride) $SnCl_2.2H_2O$ as the reducing agent, its amount varying as 10, 20, 40, 50 and 60 μg was added to various formulations of ^{99m}Tc-CTRX keeping, pH at a constant level. Optimization of the CTRX concentration was also performed by adding varying amount of CTRX to the formulation whilst keeping the pH and stannous concentration constant. The amounts of 10, 20 or 50 mg of the drug were added labelling yields were determined. Boiling time varied from no boiling to 5 and 10 min boiling.

High-performance liquid chromatography (HPLC)

To ascertain the nature of compound after boiling HPLC of the formulation was performed in both pre and post boiling states. For this Hitachi L–6200 Intelligent Pump, L–4200 UV-VIS detector system connected to ultraviolet-visible spectrometer and a NaI gamma scanner (Ludlum®) was used. UV chromatogram and radiochromatogram of ^{99m}Tc-CTRX were obtained by performing the HPLC with C_{18} column by using an isocratic mobile system of CH_3OH. H_2O (80:20) at a flow rate of 0.5 mL/min. UV and radiometric detection was carried out to verify the nature of compound.

Assessment of the stability

^{99m}Tc-CTRX was incubated at room temperature (25 ± 2 °C) for 1, 2, 4, 6 and 24 h after preparation and percentage of remaining labelled compound was determined at each time point. The stability of the labelled compound from the same batch was also checked in human serum after same time intervals for comparison. 1.8 mL of serum of a healthy volunteer was mixed with 200 μL of ^{99m}Tc-CTRX and incubated at 37 °C. Aliquots of 0.2 mL were withdrawn after the incubation to determine the percentage of remaining labelled complex in the solution.

In vitro bacterial binding

Binding of the labelled complex with live bacteria (*S. aureus* and *E. coli*) grown in-house was performed *in vitro*, by using a previously published method.[15] 60 μg of ^{99m}Tc-CTRX was added to 0.1 mL of sodium phosphate buffer in previously tagged test tubes. Aliquots of 0.8 mL of 50% of 0.01 M acetic acid and sodium phosphate buffer were also added to the test tubes. The mixtures were prepared at varying incubation time, that is, 1, 4 or 24 h at 4 °C and centrifuged for 10 min at 2000 rpm. The supernatants were removed and bacteria at the bottom were suspended again in 1 mL of ice cooled sodium phosphate buffer. The test tubes were centrifuged again and the radioactive counts of the pellets and supernatants were then taken in a well type gamma counter (Ludlum r, model: 261). The radioactivity bound with bacteria was expressed as follows:
% (uptake) = (radioactivity of the pellet/total radioactivity) × 100

Biodistribution

A prior approval was taken from the Ethics Committee of the institute for the use of animals in these experiments which were performed in accordance with the guidelines provided by the committee. *Sprague Dawley* male rats having weight ranging from 200 to 270 g were infused in thigh muscles with nearly 10^8 colony-forming units of *S. aureus* (strain ATCC® 25923™) or *E. coli* (strain 5154552) suspended in normal saline for infection and turpentine oil for inflammation. CTRX tagged with ^{99m}Tc was injected into the tail vein 48 h after swelling, redness and hyperthermia was seen in the infused thigh. The animal was killed with chloroform overdose and dissected to obtain various organs. The radioactivity in organs was measured in the gamma counter and its per cent to injected radiation dose in

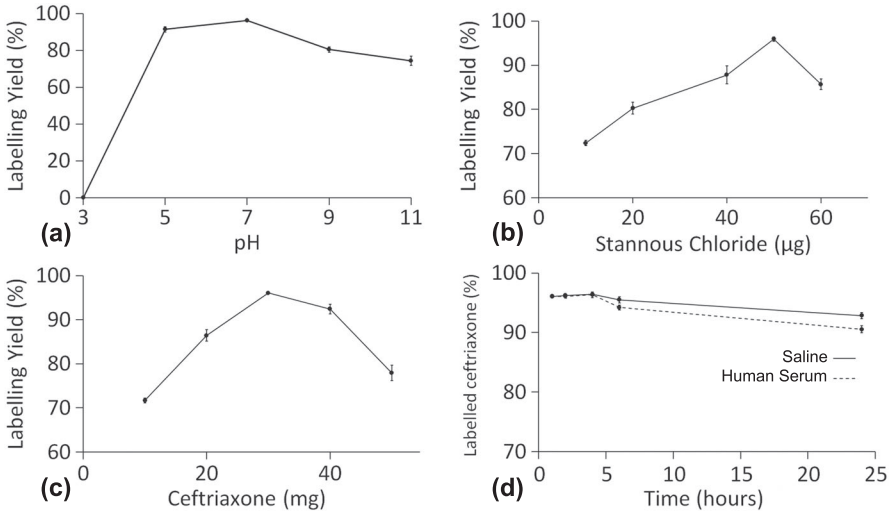

Figure 2. Yields (%) of ^{99m}Tc-ceftriaxone shown against the variations in (a) pH, (b) amount of SnCl and (c) concentration of ligand. (d) shows the stability of the complex in normal saline and human serum. ($n = 3$ at all the data points).

each gram of the organ was obtained (ID%/g). The ID%/g of was compared between the types of bacteria and inflammation. Activity per gram was also used to calculate lesions to normal ratios (L/N), which was the ration of activity in infected or inflamed thigh muscles to the contralateral normal thigh muscles. Biodistribution was assessed in three rats each for normal, *S. aureus*, *E. coli* and inflamed group.

Scintigraphic data acquisition

Biodistribution was also visually assessed scintigraphically in living Sragua Dawley male rats infected with *E. coli* and New Zealand white rabbits (average weight = 2.5 kg) inflamed with turpentine oil 2 days before scanning. Images were acquired with single headed Siemen's Integrated ORBITER Gamma Camera System interfaced with high-resolution parallel-hole collimator and fitted with dedicated Macintosh® Operation System 7.5 Software used on the ICON™ Workstation. Energy window was set at 140 ± 20 keV and 500,000 counts were obtained for each image. The animal was sedated with appropriate dose of intramuscular diazepam. Each animal was placed on the flat hard surface with both hind legs spread out and all legs fixed with adhesive tape. Saline (0.5–1.5 mL) containing 10–30 MBq of ^{99m}Tc-CTRX was injected intravenously into tail veins of the rats and ear vein of the rabbits. Five-minute whole body images were acquired 1 and 4 h after the radiotracer injection. L/N was also calculated from these digital images by sketching the regions of interest over the effected and normal thighs using the drawing tool of the computer.

Statistical analysis

Optimization, stability, bacterial binding and animal biodistribution studies were performed at least three times at each data point. Mean ± SD were calculated for all the quantities generated in these experiments. Where appropriate, means were compared by Student's *t*-test, single-factor or two-way analysis of variance with replication using Microsoft® Excel 2010. The difference in means was considered statistically significant where the value of p was ≤0.05.

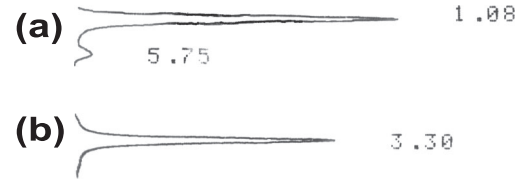

Figure 3. Radio-chromatography of ^{99m}Tc labelled ceftriaxone. (a) System–1: shows a large peak of 96.0% radioactivity of labelled ceftriaxone + reduced/hydrolysed ^{99m}Tc at 1.08 min and a small peak at 5.75 min of 4.0% free ^{99m}Tc. (b) System–2 showing a single peak of ^{99m}Tc-ceftriaxone + free ^{99m}Tc and no peak of hydrolysed/reduced ^{99m}Tc.

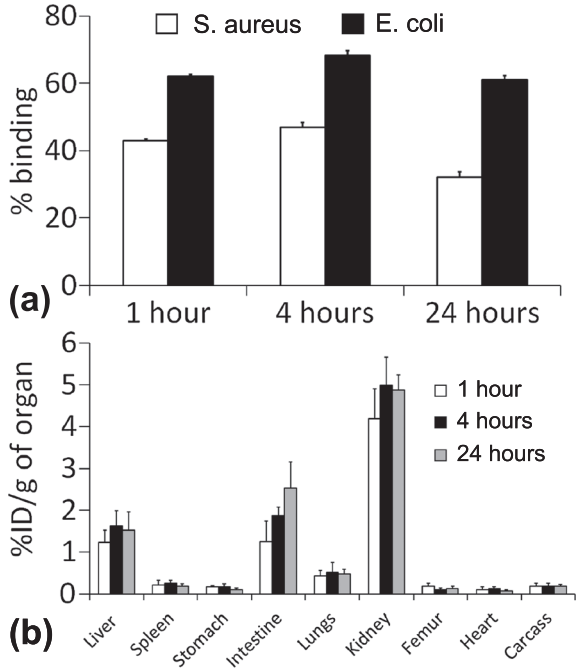

Figure 4. (a) *In vitro* binding of ^{99m}Tc-labelled ceftriaxone at various time points with *E. coli* and *S. aureus*. $n = 3$ at all time points and each type of bacteria. (b) Biodistribution of ^{99m}Tc-labelled ceftriaxone in normal Sprague Dawley male rats sacrificed 1, 4 and 24 h after the injection of radiopharmaceutical. %ID/g is the proportion of the injected activity accumulated per gram of the organ. $n = 3$ at each time point.

Table 1. Percentage of injected radiation dose accumulated in each gram (ID%/g) of muscle of thigh inoculated with pathogen and comparison with the uptake in corresponding normal thighs.

	S. aureus			E. coli			Turpentine oil		
Time (hours)	Infected thigh	Normal thigh	*p*	Infected thigh	Normal thigh	*p*	Inflamed thigh	Normal thigh	*p*
1	1.58 ± 0.24	0.84 ± 0.04	0.012	5.58 ± 0.26	0.73 ± 0.04	<0.001	0.94 ± 0.06	0.81 ± 0.05	0.035
4	3.11 ± 0.24	1.32 ± 0.03	<0.001	18.26 ± 0.38	1.46 ± 0.23	<0.001	1.76 ± 0.07	1.26 ± 0.07	0.001
24	0.68 ± 0.12	0.48 ± 0.04	0.084	2.45 ± 0.26	0.51 ± 0.12	<0.001	0.63 ± 0.02	0.61 ± 0.03	0.296

Results

The final formulation of ^{99m}Tc-CTRX was obtained with optimized levels of pH 7, stannous chloride of 50 μg and ligand of 30 mg (Figure 2a–c). Labelling yield at pH 7 was significantly more than pH 5, 9 and 11 ($p < 0.001$). It was virtually zero at pH 3 that increased to 91.3 ± 0.9% at pH 5 and 96.2 ± 0.2% at pH 7 followed by apparent decent in alkaline solutions. Labelling efficiency of 95.9 ± 0.4% was seen with 50 μg of $SnCl_2$ which was significantly higher than 10, 20, 40 and 60 μg ($p < 0.003$). Optimal amount of CTRX in the solution was 30 mg, which gave the labelling yield of 96.0 ± 0.0% showing significant difference from all the other concentration levels of the ligand ($p < 0.005$).

Another condition required for adequate labelling yield was boiling. The yield was 40.0 ± 2.4%, 82.3 ± 3.3% and 96.0 ± 1.4 when prepared at room temperature, boiling for 5 or 10 min, respectively. HPLC performed in both pre boiling and post boiling states showed same retention time of 4.25 min, which corresponded to the elution time of the compound. These optimized parameters showed labelling yield of 96.0 ± 0.3% with 0% hydrolysed/reduced ^{99m}Tc and 4.0 ± 0.3% free $^{99m}TcO_4^-$ (Figure 3).

Stability and bacterial binding

Stability of the labelled complex in normal saline and human serum is given as a function of time in Figure 2d. The labelled

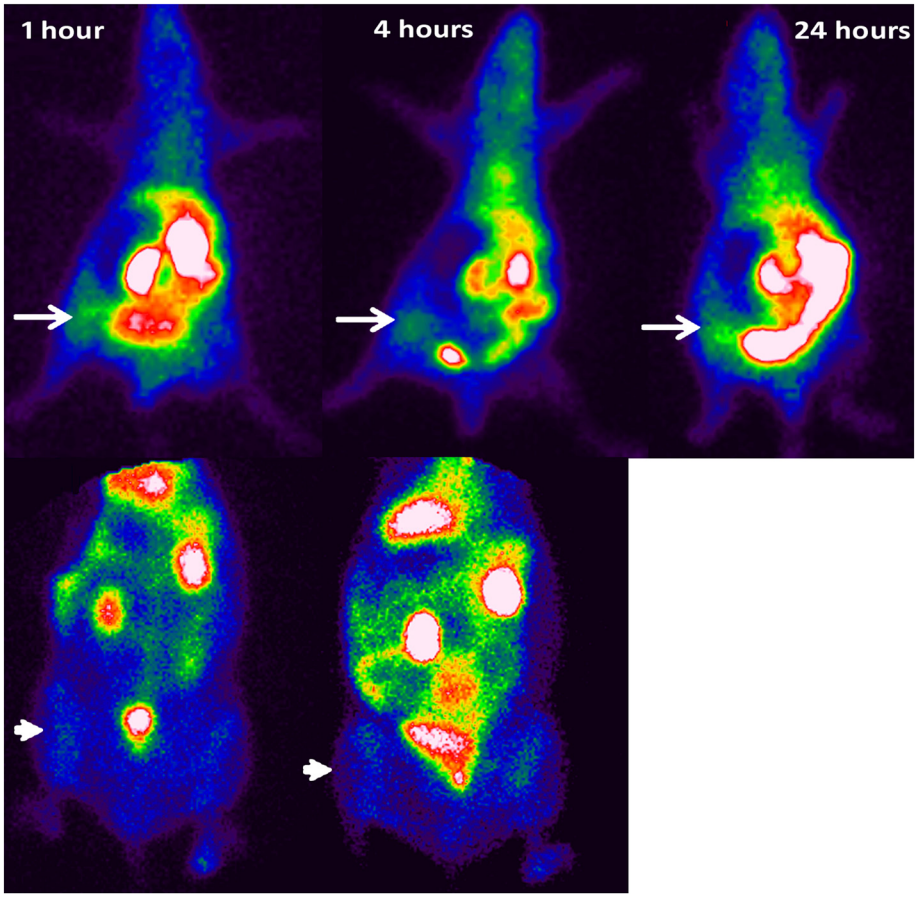

Figure 5. Scintigraphy in rats (top row) infected with *E. coli* in the left thigh showing high uptake of ^{99m}Tc-ceftriaxone at the site of infection (arrow) as compared with the normal right thigh. The bottom row are the scintigraphic images of the rabbit inflamed by the injection of turpentine oil in left thigh that shows only minimally increased uptake at the site (arrowhead) than the normal right side at 1 h. This figure is available in colour online at wileyonlinelibrary.com/journal/jlcr.

compound was stable during the first 4 h after labelling in both media with yields of more than 96% followed by a gradual reduction trend thereafter. At 24 h labelled complex was 92.8 ± 0.5% in saline and 90.5 ± 0.6% in serum ($p = 0.015$).

^{99m}Tc-CTRX bound more to *E. coli* than *S. aureus* at all the time points ($p < 0.001$) as shown in Figure 4a. The best binding was however observed at 4 h for both the bacteria (68.2% and 46.9%, respectively).

Biodistribution and scintigraphy

Liver, kidneys and intestines showed high accumulation of injected radioactivity per unit mass (ID%/g) in sacrificed animals (Figure 4b). The percentage of injected activity in the total collected urine was 0.66 ± 0.20% at 1 h, 25.4 ± 4.39% at 4 h and 40.28 ± 5.61% at 24 h. Similarly, the %ID in whole of the intestine increased from 17.77 ± 2.23 at 1 h to 23.17 ± 4.12 at 4 h and 31.09 ± 4.98 at 24 h.

Accumulation of radioactivity (ID%/g) in infected and inflamed thighs is compared with that in the contralateral normal thighs in Table 1. L/N ratios at 1, 4 and 24 h were 1.88 ± 0.24, 2.36 ± 0.21 and 1.44 ± 0.28 for *S. aureus*; 7.62 ± 0.26, 12.66 ± 1.44 and 5.05 ± 1.27 for *E. coli* and 1.17 ± 0.04, 1.40 ± 0.01 and 1.04 ± 0.01 for turpentine oil, respectively. The distribution and pharmacokinetics of radioactive CTRX in animals seen after sacrifice was also reflected visually on scintigraphic images of live animals (Figure 5). The dorsal images of Sprague Dawley rat inoculated with *E. coli* in the left thigh could differentiate normal from abnormal site of infection on all the three time points. The images of rabbit inoculated with turpentine oil in the left thigh showed no significant asymmetry. L/N ratio of radioactivity uptake in *E. coli* measured on digital images of 1, 4 and 24 h were 1.9, 5.5 and 5.3, respectively, which was 1.1 in both the images of images of turpentine oil.

Discussion

The quest for ideal infection imaging radiopharmaceutical in nuclear medicine remains open. In this study, we labelled CTRX with ^{99m}Tc, the predominantly used radioactive isotope in nuclear medicine. The primary requirement of stoichiometry was performed and in doing so several parameters, that is, pH level; amount of stannous chloride and ligand in the solution and; boiling time were optimized to have good labelling yield and stable complex. It is recommended that the injectable radiopharmaceutical should be in pH compatible with blood pH (7.4).[16] Previously, the CTRX was labelled either at pH 5 or 9.[17,18] In our analysis, we could get the best labelling yield at pH 7. The element of technetium in pertechnetate ion ($^{99m}TcO_4^-$) exists in the oxidation state of 7+, which is nonreactive specie and has to be reduced to initiate labelling reactions. The optimal amount of reducing agent ($SnCl_2$) in our preparation was 50 μg, which is consistent with previous studies. It appeared that pH of the solution affected the quantity of CTRX required to get adequate labelling yield. In our experiments, 30 mg of ligand gave the best yield, which was in agreement with alkaline formulation of Mostafa *et al*[18] but in contrast to 10 mg in acidic (pH 5) solution as reported by Fazli *et al*.[17] Our formulation at pH 7 required an additional step of boiling for 10 min to complete the reaction, a requirement that has not been reported in any of the previous studies of the solutions at pH 5 and 9. Without boiling only 40.0 ± 2.4% labelling could be achieved in our formulation.

Stability of a labelled compound has significance in terms of the shelf-life and biodistribution. *In vitro* stability is the parameter of its shelf-life after labelling the compound in the hot-lab whilst *in vivo* stability is its integrity in human body after injection. The amount of labelled compound in the shelf reduces with the dissociation of atoms of radioisotope from the ligand molecules. Such dissociation may continue or even increase in *in vivo* environment. The compounds with low labelling yields and/or significant instability inside the human body would not give required biodistribution expected for the ligand, as the dissociated radioactive atoms will accumulate in difference organs/tissues. Stability of a radiolabelled compound is affected by factors like temperature, pH and light.[16] ^{99m}Tc-CTRX complex that we prepared was formulated at pH 7 and with 10 min boiling. We performed its stability tests in normal saline at room temperature as well as in human serum at 37 °C. Our formulation of ^{99m}Tc-CTRX gave labelling yield of more than 96% with no statistically significant deterioration for first 6 h in saline ($p = 0.09$). Even after 24 h, 92.83 ± 0.5% of labelling efficiency our preparation was observed. It also remained stable in human serum with no significant loss of labelled compound at 4 h ($p = 0.26$) and 90.5 ± 0.6% labelling efficiency at 24 h. It showed significantly higher stability than the formulation prepared at pH 5 and with no boiling step.[17] The labelling yield in normal saline at these conditions reduced from 94.2 ± 5.2 % to less than 90% in 6 h and 80.6 ± 2.3% at 24 h. Similarly, in human serum, there was nearly 5% deterioration of the yield at 3 h whilst after 24 h, only 71.2 ± 1.4% of labelled compound remained in the aliquot.

No direct comparison of the affinity of ^{99m}Tc-CTRX for Gram-positive and Gram-negative bacteria could be found in literature. Cold CTRX is found to be more active against Gram-negatives.[14] This explains more *in vitro* binding of ^{99m}Tc-CTRX with *E. coli* than *S. aureus* that was also reflected as its higher accumulation in thigh muscles inoculated with *E. coli*. Binding assays performed by Fazli *et al*.[17] in *S. aureus* showed 45 ± 4.6% binding at 3 h, which is in agreement with our results at 4 h. The progressively increasing radioactivity in liver, intestine, renal tissue and urine was because biliary and urinary systems are primary routes of elimination of the antibiotic.[14] The biodistribution results of our preparation during the first 4 h were in agreement with the other preparations of the previous studies of the radiolabelled CTRX. The biodistribution did not significantly change after 24 h in our formulation.

The highest accumulation of activity in infected/inflamed muscles was seen at 4 h (Table 1). ^{99m}Tc-CTRX showed a significant ability of to differentiate the infection and inflammation in target tissue. At 4 h injected dose accumulated in tissue infected with *S. aureus* was 1.8× higher that turpentine oil inflamed muscles. But *E. coli* showed 14.5× more accumulation than inflammation suggesting more affinity and utility of ^{99m}Tc-CTRX in cases of Gram-negative bacteria. This was significantly higher than previously reported value of 5.6 for *E. coli* compared with turpentine oil.[18] Variation in the accumulation of the amount of radiopharmaceutical in infections and inflammation was also assessed by L/N ratios. The L/N ratios in case of *E. coli* were significantly higher than turpentine oil in at

all time points ($p < 0.001$, <0.001 and–0.005, respectively) whilst in *S. aureus*, they were statistically higher at 1 and 4 h (p–0.007 and =0.001, respectively) and no difference at 24 h ($p = 0.06$). Scintigraphic images of ^{99m}Tc-CTRX also suggested that it has a strong capability to delineate the site of infection and differentiate it from inflammation.

In summary, an additional boiling step is required to label CTRX with ^{99m}Tc at physiological pH. Other optimal requirements for kit preparation are 50 μg of reducing agent ($SnCl_2$) and 30 mg of the ligand. ^{99m}Tc-CTRX labelled using these parameters exhibited good stability and in clinical environment the radiopharmaceutical assembled in the morning can be used at any time during the day. It also remains stable in the human serum depicting the factual distribution of the antibiotic. ^{99m}Tc-CTRX has demonstrated significant ability to differentiate bacterial infection from inflammation. These results suggest that it can be used for further trails in human subjects.

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