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ORIGINAL ARTICLE

Preparation, optimization and pharmacological evaluation of ^{99m}Tc-4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex: as a novel potential radiopharmaceutical agent with hepatobiliary excretion

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Abstract
Schiff base ligands are biologically active compounds in having antimicrobial, antiviral and antitumor activities etc. In this study, we have synthesized a Schiff base ligand namely 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid, by reacting 3-amino-4-hydroxybenzoic acid and 2-hydroxy-3-methoxy benzaldehyde in the presence of acetic acid and refluxing it. The resulting base ligand was characterized on HPLC and used for radiolabelling with technetium-99m. The ligand 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid was labelled with ^{99m}Tc at pH 7, while reacting 230 µg of ligand with 15 mCi of $^{99m}TcO^{-}_{4}$ for 10 min at room temperature. The resulting ^{99m}Tc-ligand complex was characterized by paper chromatography, TLC, HPLC and electrophoresis technique. The stability of the complex was determined at room temperature and in human serum. The biodistribution of the complex was studied in mice and scintigraphy was performed in rabbit. The ^{99m}Tc-ligand complex showed high radiolabelling yield (up to 99 ± 1%) and high stability at room temperature and in human serum. The newly prepared complex exhibited no net charge. Our newly developed ^{99m}Tc-ligand complex demonstrated high accumulation in liver and spleen of mice as well as in rabbit. Based on these findings, we have suggested that this novel radioligand i.e., ^{99m}Tc- 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex could be used for liver and spleen imaging.

Keywords 4-Hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid · Radiolabelling · Quality control · HPLC · Electrophoresis · Biodistribution · Scintigraphy

Introduction

In the last decade, Schiff base ligands have received more attention mainly because of their wide application in the field of antimicrobial, anti-tuberculosis and anti-tumour activity etc. [1–3]. The Schiff bases are an important class of ligands in coordination chemistry because such ligands and their transition metal complexes play very crucial role and are mostly used in biological activities such as anti-fungal, antibacterial, anti-malarial, antiviral, antitumor, anti-inflammatory, anti-hypertension, antipyretic, herbicidal, enhancement of oxygen affinity with haemoglobin and myoglobin, insulin-enhancing effects, and so on [1, 4–7].

Currently, pharmaceutical chemists are trying to find more effective and safer therapeutic agents which have no side effects [8–10]. In radiopharmaceuticals, the radioactive tracers are main components to examine the function of body systems [11]. Today the main radionuclide used for preparing radio-pharmaceuticals throughout the world is ^{99m}Tc due to its physical and chemical properties, which is available from ^{99}Mo/^{99m}Tc generators [12, 13]. Many radiopharmaceuticals are available for imaging purpose. They differ in terms of their physical characteristics, biodistribution and radiation exposure [14–16].

The aim of the present study was to describe the radiolabelling method of 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid with technetium-99m. The

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ligand 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid has a suitable array of donor atoms for coordination with $^{99m}TcO^{-}_{4}$, due to this reason it was easily labelled with $^{99m}TcO^{-}_{4}$. The reaction parameters were also investigated and the biological biodistribution and scintigraphy study of the labelled compound were performed in normal mice and rabbit respectively.

Experimental

Materials and methods

All analytical chemical reagents were purchased from commercial sources and Technetium-99m was obtained from a locally produced fission based PAKGEN $^{99}Mo/^{99m}Tc$ generator. All chemicals used were AR grade and used as received.

Synthesis of 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid

For this purpose, 3-amino-4-hydroxybenzoic acid was dissolved in 20 ml methanol, in which of 2-hydroxy-3-methoxy benzaldehyde in the presence of acetic acid. Refluxed it until the colour changed and precipitates of deep orange colour appeared. The precipitated solid was filtered. The proposed mechanism is given in Fig. 1.

Radiolabelling of ^{99m}Tc-ligand

For the radiolabelling of 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex with ^{99m}Tc, the amount of ligand was 230 µg, while 25–28 µg of $SnCl_2{\cdot}2H_2O$ was used as reducing agent at pH 7 (in case of requirement, the pH 7 was achieved or maintained by using 0.05 M NaOH or 0.05 M HCl). Reaction mixture volume used in all experiments was approximately 1.5 ± 0.2 ml. After addition of all the prescribed reagents in a vial, ~ 15 mCi TcO^{-}_{4} in saline was injected and the vial was incubated for 10 min at room temperature. All experiments were carried out at room temperature (25 ± 2 °C).

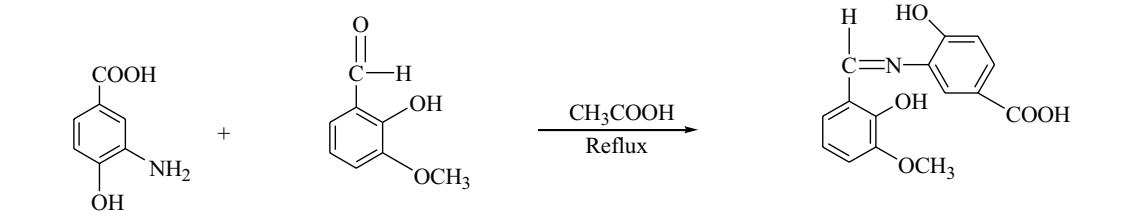
Quality control

For all radiolabelling experiments, the radiochemical yield of ^{99m}Tc-labeled 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex were checked by 'Whatman number 3' ascending paper chromatography strips (each 14×2 cm^2) and ITLC-SG strips (each 7×1 cm^2). Free $^{99m}TcO^{-}_{4}$ in the preparation was determined by using Whatman No. 3 paper as the stationary phase and acetone as a mobile phase. Reduced and hydrolysed activity was determined by using instant thin layer paper chromatography (ITLC-SG strips) as the stationary phase and 0.05 M NaOH as a mobile phase. Radiocolloids were separated by passing the preparation through 0.22 µm bacteria filter (Millipore Filter Corp). Activity remaining on the filter and in the solution was counted by a gamma-counter (Ludlum). The stability of ^{99m}Tc-labelled ligand was checked after different time intervals at room temperature. The distribution of labelled, free and hydrolyzed ligand complex 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. The results of paper chromatography showed that reduced/ hydrolysed $^{99m}TcO^{-}_{4}$ and labelled ^{99m}Tc-4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex remained at the origin whereas free $^{99m}TcO^{-}_{4}$ moved toward the solvent front. The R_f in each case was calculated by using the following formula,

$$R_f = \frac{\text{Distance travelled by the sample}}{\text{Distance travelled by the solvent}}$$

Electrophoresis of ^{99m}Tc- ligand

The charge on ^{99m}Tc-labelled 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex was studied by electrophoresis technique, using Deluxe electrophoresis chamber (Gelman) system as previously reported



3-amino-4-hydroxybenzoic acid — 2-hydroxy-3-methoxybenzaldehyde — 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino)benzoic acid

Fig. 1 Proposed chemical mechanism for the synthesis of 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid

by Akbar et al. [11]. Phosphate buffer of pH 6.0 was used in this experiment as media. Whatman No.1 paper of 30 cm long strip was moistened with phosphate buffer and then introduced in the electrophoresis chamber. A drop of ^{99m}Tc-labelled ligand mixture (sample) was placed at 12 cm far from the cathode edge of the paper sheet and electrophoresis was run for one hour at a voltage of 300 V. After completion, the strip was dried and scanned by 2π scanner to check the charge on ^{99m}Tc-labelled 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex.

HPLC of native ligand and ^{99m}Tc labelled ligand complex

The 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex and radiolabelled compound were analysed on a high-performance liquid chromatography (HPLC) instrument. HPLC of radiotracer ^{99m}Tc-ligand was studied using D-200 Elite HPLC system. The column of C-18 was used as stationary phase, 20 µl sample volume was injected and a mixture of water and acetonitrile ratio 75:25 (v/v %) was used as mobile phase. The flow rate was adjusted up to 1 ml/min at a wavelength of 254 nm.

Radiochemical stability of ^{99m}Tc-ligand complex in saline

To evaluate the stability of the ^{99m}Tc-4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex in isotonic saline after labelling, we studied its dissociation at different time intervals (0.5, 1, 2, 3, 4, 5 and 6 h) post-preparation at room temperature. Change in stability of the radiolabelled complex was analysed at each time interval by ITLC-SG and PC to determine any dissociation of the complex. The percentage (%) of free pertechnetate at a particular time, the present percentage dissociation of the complex at that time were noted.

In vitro stability (stability in human serum)

In vitro, the radiochemical stability of the ^{99m}Tc-4-hydroxy-3-(2-hydroxy-3-methoxy benzylideneamino) benzoic acid complex was also studied. For this purpose, 1.8 ml of normal human serum was mixed with 0.2 ml of ^{99m}Tc- ligand and incubated at 37 °C. Then 0.2 ml aliquots were withdrawn during the incubation at different time intervals up to 24 h and subjected to chromatography for determination of ^{99m}Tc-labelled ligand, reduced/hydrolysed ^{99m}Tc and free $^{99m}TcO^{-}_{4}$.

Factors affecting percentage of labelling yield

Several experiments were conducted to study different factors that have an effect on percentage of labelling yield such as effect of (1) pH, (2) $SnCl_2{\cdot}2H_2O$, (3) reaction time, and (4) amount of ligand. The experiments were repeated with all factors kept at optimum except the factor under study, until the optimal conditions were achieved.

Biodistribution in mice

The animal studies were approved by the Animal Ethics Committee, PINSTECH. (Pakistan Institute of Nuclear Science and Technology) according to the guidelines set out by Pakistan Atomic Energy Commission (PAEC). Male mice ($n = 5 \times 3$) were used for biodistribution studies, having weight ranges 30–70 g (4–8 weeks) and were obtained from the National Institute of Health (NIH), Islamabad, Pakistan. They were anesthetized with chloroform. Fresh ^{99m}Tc-labelled 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex was prepared and then injected into a tail vein. After injection of ^{99m}Tc-labelled ligand (2–3 mCi/mice), accumulation of radioactivity in various organs was determined. The organs were dissected, weighed and counted for radioactivity at the various time intervals (1 h, 4 h and 24 h). Data were expressed as the percentage of injected activity per gram of tissue (% IA/g).

^{99m}Tc- ligand scintigraphy in rabbit

A single-headed Siemens Integrated ORBITER Gamma Camera System interfaced with high resolution parallel hole collimator was used. It was connected to an online 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 injection (2 ml) was injected into the left thigh muscle. Saline solution (0.2 ml) containing 15 MBq of ^{99m}Tc-4-hydroxy-3-(2-hydroxy-3-methoxy benzylideneamino) benzoic acid complex was then injected intravenously into the marginal ear vein of rabbit. For the distribution study of ^{99m}Tc-4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex in living organism (rabbit), whole body gamma scan was done at 1.5 h, 4 h and 24 h after injection.

Results and discussions

The ligand 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid, used in this study has a suitable array of donor atoms for coordination with ^{99m}Tc. The obtained results have demonstrated that, the prepared Schiff base

ligand was easily labelled with ^{99m}Tc with high radiolabelling yield. Many experiments were conducted to study the different factors that affect labelling yield such as (1) amount of reducing agent, (2) amount of ligand, (3) pH and (4) reaction time. The experiments were repeated with all factors kept at optimum while changing the factor under study, until the optimal conditions were achieved. The importance of $^{99m}TcO^{-}_{4}$ can be assessed in a way that they are becoming the need of the day. Due to this reason, the production demand of the radionuclide ($^{99m}TcO^{-}_{4}$) is increasing day by day.

Effect of pH

The effect of pH on labelling of ^{99m}Tc with 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex was studied in detail. To reach the suitable pH for the maximum radiochemical yield, different experiments were carried out at different pH ranging from 4 to 11. The effects of pH on radiolabelling are shown in Fig. 2. At low pH value (4–6) the labelling efficiency was 78–91 ± 2% respectively. While at pH 7, the labelling efficiency of $^{99m}TcO^{-}_{4}$ labelled 4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid complex was 99 ± 1%. Whereas in basic media, at pH 8–11, the labelling efficiency decreased (96–73 ± 1.8% respectively). Therefore, further experiments were performed at pH 7 to obtain the highest labelling yield.

Effect of $SnCl_2{\cdot}2H_2O$

To check the effect of $SnCl_2{\cdot}2H_2O$ as a reducing agent on the labelling of ^{99m}Tc-ligand, many experiments were performed by changing the concentration of reducing agent ($SnCl_2{\cdot}2H_2O$). The amount of the reducing agent, $SnCl_2{\cdot}2H_2O$, which gave the highest labelling efficiency, was 25 µg. The change in its amount caused stark changes in radiolabelling yield and formed colloids in solution. To avoid colloid formation, the optimum amount of reducing agent was used. The effect of amount of the reducing agent on the radiolabelling yield are shown in Fig. 3.

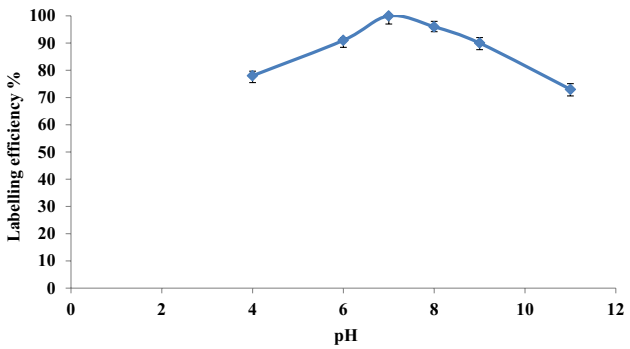


Fig. 2 Effect of pH on labelling of ^{99m}Tc with ligand

Amount of ligand

The labelling yield was low (44%) at low concentration of ligand (50 µg). Increasing the ligand concentration led to higher labelling yields. Experiments were performed with varying amount of the 4-hydroxy-3-(2-hydroxy-3-methoxy benzylideneamino) benzoic acid and obtained results have demonstrated that, 230 µg ligand gave the maximum labelling yield (up to ~ 100%). It was also observed and noted that by increasing or decreasing the amount of ligand from the optimum value, the radiolabelling yield decreased (illustrated in Fig. 4).

Effect of reaction time

The results have revealed that, the complexation of ^{99m}Tc with the prepared ligand did not occur rapidly. The resulting complex formation of ^{99m}Tc- ligand was relatively slow with a radiochemical yield of 69 ± 2% at 1-min reaction time. The Fig. 5 shows in detail the rate of formation of ^{99m}Tc-ligand

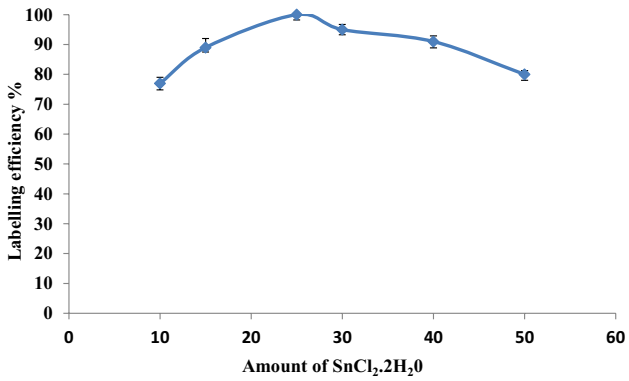


Fig. 3 Effect of reducing agent on labelling of ^{99m}Tc with ligand

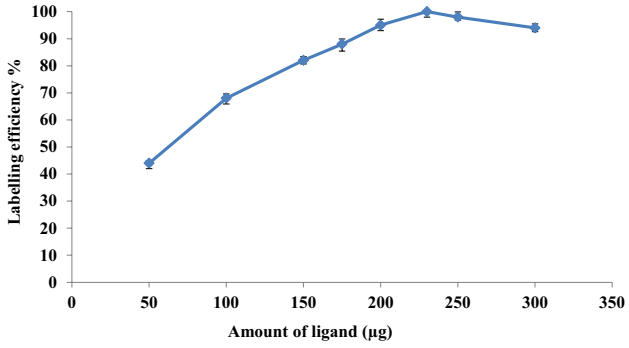


Fig. 4 Effect of the amount of ligand on labelling of ^{99m}Tc with ligand

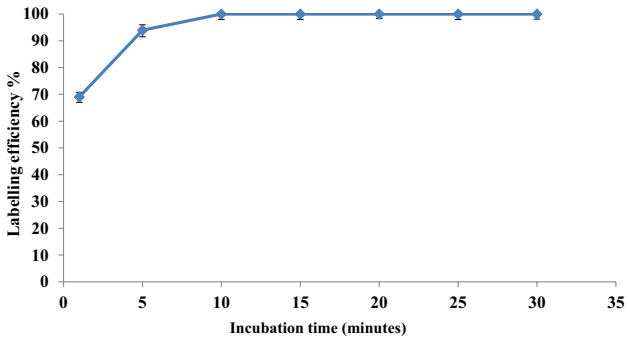


Fig. 5 Effect of the incubation time on labelling of ^{99m}Tc with ligand

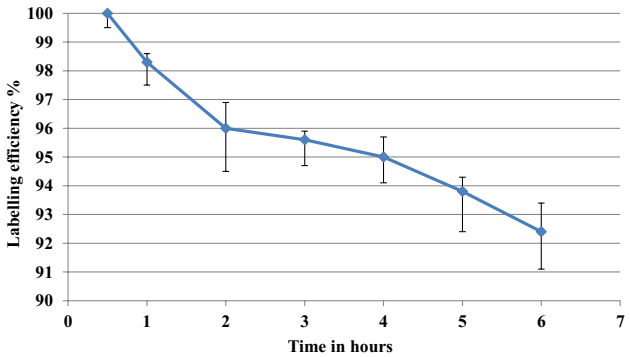


Fig. 6 Stability of ^{99m}Tc- ligand complex at room temperature

and that the maximum labelling efficiency was achieved after 10-min reaction time.

Stability and purity

The formed complex was quite stable in saline solution and a slight decrease was observed with the passage of time. The labelling of > 92% was maintained for up to 6 h (Fig. 6). Radiochemical purity of the prepared product was high with a good stability at room temperature (Fig. 6) and in normal human serum (Fig. 7). The ^{99m}Tc-labelled ligand complex displayed no charge on paper electrophoresis as shown in Fig. 8. Because the sample drop of prepared ^{99m}Tc-4-hydroxy-3-(2-hydroxy-3-methoxybenzylideneamino) benzoic acid did not move towards anode or cathode from the point of origin at the applied condition, as shown in Fig. 8. The purity of pure (native) ligand and radiochemical purity of ^{99m}Tc- labelled ligand were 100%, which were determined by using HPLC as shown in Figs. 9 and 10. In both these figures, the single solitary peaks against a nearly flat background illustrated the high purity of the aforementioned ligand moieties.

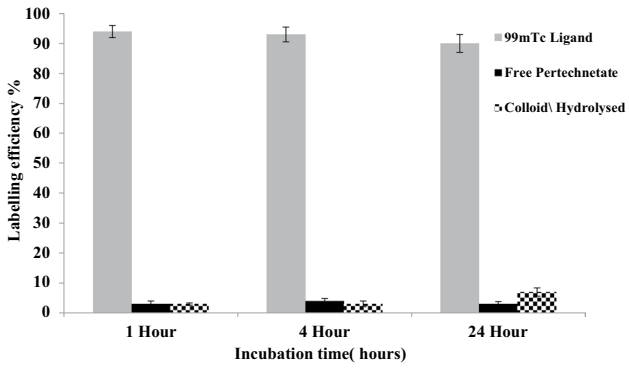


Fig. 7 Stability of ^{99m}Tc- ligand in normal human serum

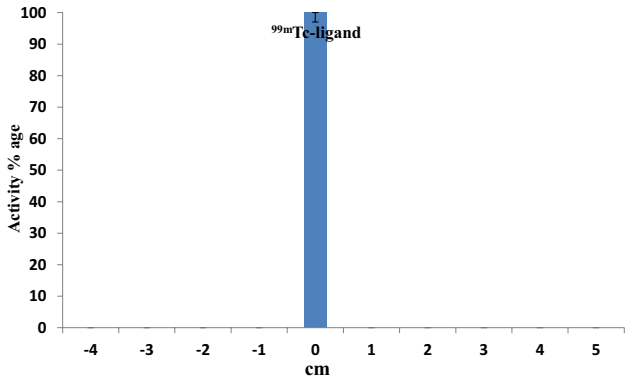


Fig. 8 Electrophoresis of the ^{99m}Tc- ligand

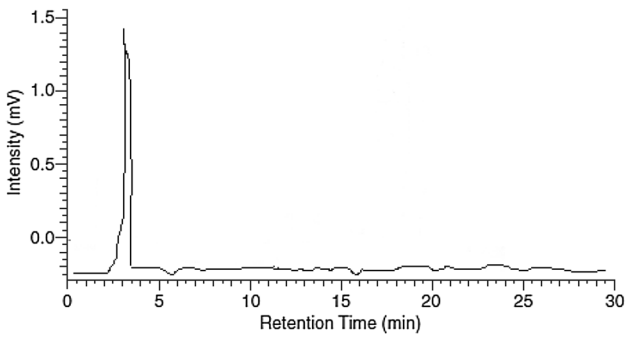


Fig. 9 HPLC analysis of ligand

Biodistribution and scintigraphy

The biodistribution of the developed radio ligand complex was studied in normal mice. The biodistribution of ^{99m}Tc-ligand was found to be the highest in liver, spleen and lungs, as shown in Fig. 11. Similarly, in rabbit scintigraphy, the highest activity was found in liver, spleen and lungs, as shown in Fig. 12. In both studies, our prepared radio-ligand complex showed significant radioactivity in spleen, which

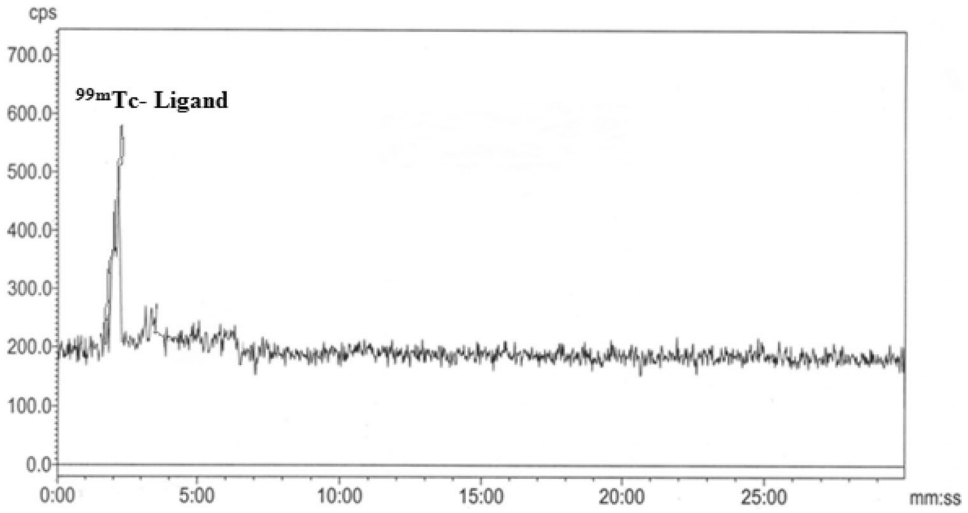


Fig. 10 HPLC analysis illustrates the percentage labelling of ligand with ^{99m}Tc

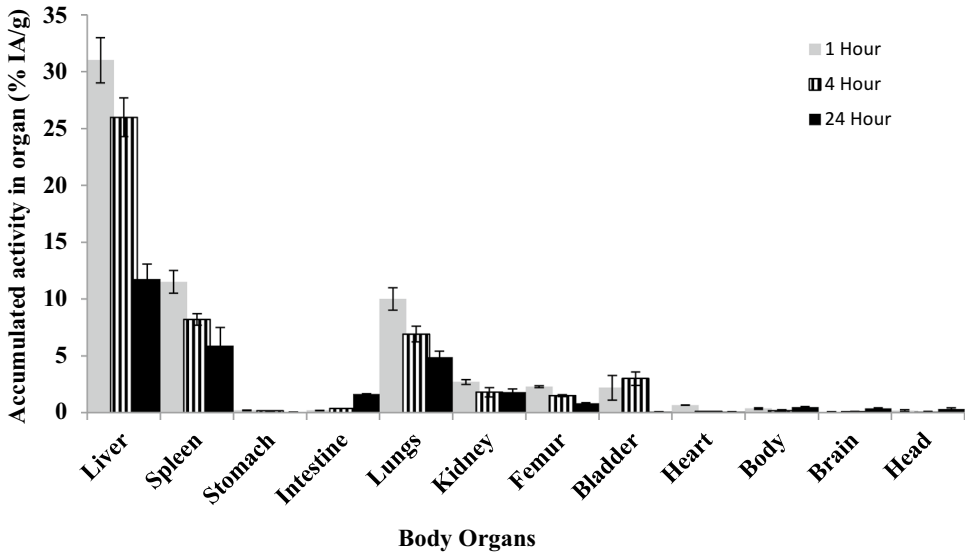


Fig. 11 Biodistribution of ^{99m}Tc- ligand in body organs of mice at 1 h, 4 h and 24 h after the injection

is uncommon for ^{99m}Tc-labelled diagnostic compounds. We suggest that further studies should be carried out in order to study the full pharmacological and diagnostic potential of this radioligand complex, in response to various diseases, disorder and syndrome of spleen, liver and lungs. However, owing to the given data, our novel complex exhibited the potential to be a spleen and liver diagnostic agent.

Conclusion

In this study, a Schiff base ligand 4-hydroxy-3-(2-hydroxy-3 methoxy-benzylideneamino) benzoic acid was synthesized and labelled with technetium-99m (radioisotope). The radiolabelling of 4-hydroxy-3-(2-hydroxy-3 methoxy-benzylideneamino) benzoic acid with ^{99m}Tc was done by a simple method with a labelling yield of $99 \pm 1\%$. The ^{99m}Tc labelled ligand was stable up to 92.4% at 6 h post preparation. The radioligand was also stable in normal human serum. A significant value of radioactivity was detected in the spleen and liver of mice and rabbit used for biodistribution study, at different intervals of post injection. This novel radioligand complex proves to have a hepatobiliary excretion.

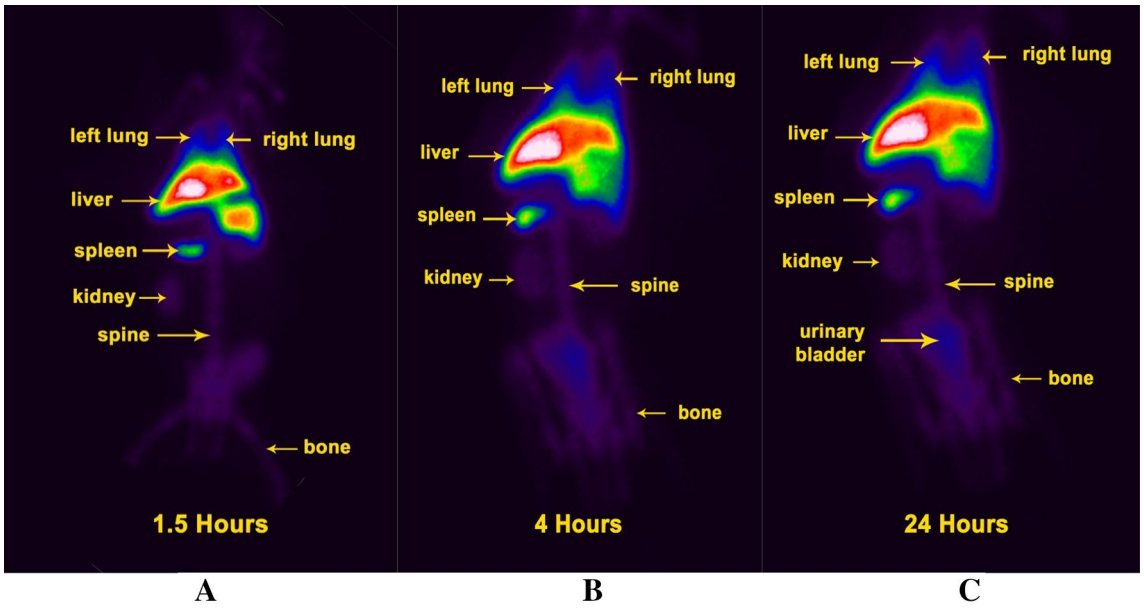


Fig. 12 Whole body gamma camera image of rabbit injected with ^{99m}Tc- ligand at 1.5 h post administration (**a**), 4 h post administration (**b**), and 24 h post administration (**c**)

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Compliance with ethical standards

Conflict of interest Author declare that do not have any conflict of interest for this article.

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