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Synthesis and bio-evaluation of a new fatty acid derivative for myocardial imaging

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ABSTRACT

Development of a ^{99m}Tc-fatty acid analogue is of interest, as ^{99m}Tc is logistically advantageous over the cyclotron-produced ^{11}C and ^{123}I. Synthesis of a 16 carbon fatty acid derivative and its radiolabeling with the novel $[^{99m}TcN(PNP)]^{2+}$ core is described here. Hexadecanedioic acid was conjugated to cysteine in an overall yield of 55%. This ligand could be labeled with ^{99m}Tc via the $[^{99m}TcN(PNP)]^{2+}$ core, in 80% yield, as a mixture of two isomers (syn and anti). The major isomer isolated by HPLC was used for bioevaluation studies in swiss mice and compared with radioiodinated iodophenyl pentadecanoic acid (IPPA), an established agent for myocardial metabolic imaging. ^{99m}Tc-labeled complex cleared faster from the non-target organs, namely, liver, lungs, and blood compared to that of $[^{125}I]$-IPPA. However, the complex exhibited lower uptake and faster washout from the myocardium as compared to $[^{125}I]$-IPPA.

1. Introduction

Fatty acids are the major source of energy in the normal myocardium, accounting for up to 90% of aerobic metabolism,[1] via the β-oxidation pathway. This pathway is adversely affected in the dysfunctional myocardium, in a more prolonged fashion than perfusion abnormalities ('Ischemic Memory'). Hence, imaging of fatty acid metabolism is potentially more advantageous than perfusion imaging in early identification of high-risk patients.[1,2] The straight chain fatty acids with 15–21 carbon atoms have shown high myocardial extraction desirable for diagnostic myocardial applications.[3] Fatty acids to be used in imaging of myocardial viability require suitable modification in order to increase the residence time of the tracer inside the myocardium.[4] Radiolabeled fatty acids currently used for imaging myocardial viability are ^{123}I-iodophenylpentadecanoic acid (IPPA), ^{123}I-beta methyl iodophenylpentadecanoic acid (BMIPP), and ^{11}C-palmitic acid.[4–6] However, the cyclotron-produced ^{123}I and ^{11}C have inherent economic and logistic disadvantages, which adversely limit the use of the radiopharmaceuticals made using these radioisotopes, particularly in nuclear medicine centers located far away from the cyclotron. The generator produced ^{99m}Tc, due to its desirable characteristics as a diagnostic radionuclide, provides an attractive alternative. In this direction, a number of $^{99\ m}$Tc-based analogues have been prepared and tested for their potential toward use in assessing myocardial viability.[7–14] However, thus far none has qualified for use in imaging myocardial viability. The reason for this could be possibly attributed to poor extraction and/or rapid washout of the radiotracer. Therefore, development of new ^{99m}Tc-labeled fatty acid analogues is an interesting relevant field of research. The recent strategies of labeling with ^{99m}Tc involves use of various metal cores such as $[^{99m}Tc{=}O]^{3+}$, $[^{99m}Tc(CO)_3(H_2O)_3]^{+}$, $[^{99m}Tc(CO)_3$cyclopentadienyl], and $[^{99m}Tc{\equiv}N]^{2+}$. Among these, $[^{99m}TcN]^{2+}$ is a relatively less explored synthon in the labeling of fatty acids for metabolic cardiac imaging. It forms symmetrical square pyramidal complexes of type $[^{99m}TcNL_2]$ with bidentate ligands (L), containing soft donor atoms such as S and P.[15] This symmetric labeling approach is well suited for small molecules. However, for big molecules like fatty acids, the symmetric labeling approach could result in bulky complexes due to the involvement of two ligands. This in turn, may affect the uptake and retention characteristics of the molecule in the target organ and may also lead to undesirable in vivo pharmacokinetics. The introduction of the PNP ligand opens up the possibility of preparing asymmetric [2 + 2] square pyramidal complexes via the formation of a new $[^{99m}TcN(PNP)]^{2+}$ core.[16] This core has two vacant positions, suitable for coordination with a bidentate ligand. Complexes prepared using this core and bidentate ligands, with donor atoms like SS or NS or SO, have been reported to be stable. Cysteine, dithiols, and dithiocarbamates have been found to be suitable as bidentate chelators for complexation with the $[^{99m}TcN(PNP)]^{2+}$ core.[17–20]

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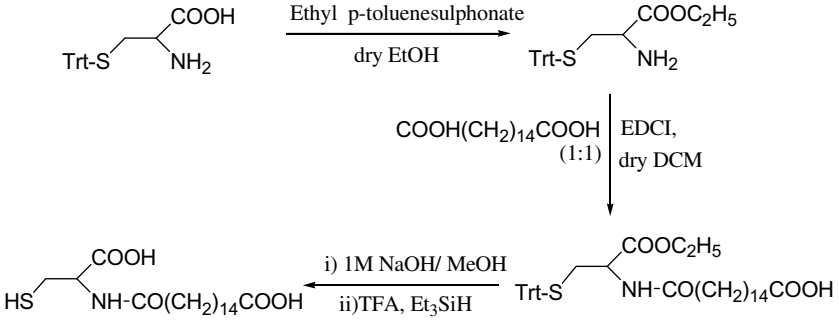
In the present study, the envisaged strategy involves derivatization of the one carboxylic acid terminal of hexadecanedioic acid with the amino group of cysteine. In the conjugate thus formed, the carboxylic acid and thiol moieties of the cysteine residue could be involved as a bidentate donor in forming a heterocomplex with the $[^{99m}TcN(PNP)]^{2+}$ core resulting in a neutral complex. The asymmetric [2 + 2] complex thus obtained has been characterized and evaluated as a marker for myocardial imaging.

2. Results and discussion

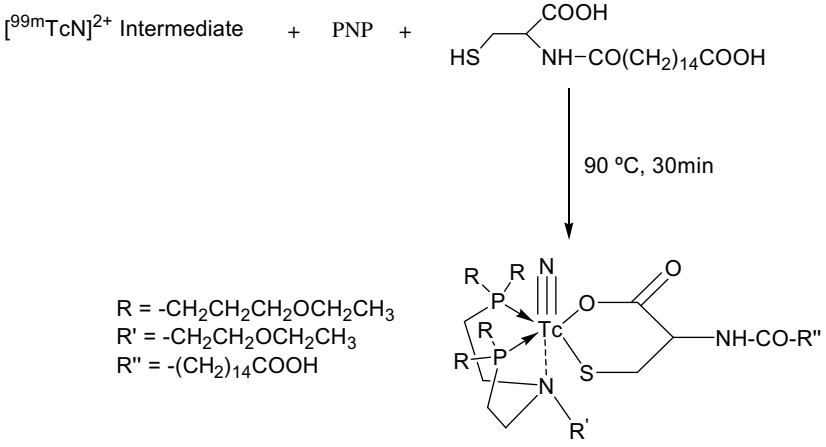
In the present study, hexadecanedioic acid was modified at one of the terminal carboxylic acid. The procedure followed is shown in Scheme 1. The carboxylic acid group was conjugated to the amino group of *S*-trityl cysteine ester using *N*-Ethyl, *N*′-(3-dimethylamino) carbodiimide hydrochloride (EDCI) as the activating agent. The coupled product, after purification, was obtained as an oil in an overall yield of 55% and characterized by ^{1}H NMR. The ester and trityl groups of the fatty acid cysteine conjugate were deprotected following a reported procedure and the resultant target ligand was used directly for radiolabeling without further characterization. The $[^{99m}TcN]^{2+}$ core was prepared using succinic dihydrazide (SDH), which acts as a nitride donor (N^{3-}), and stannous chloride. The complex was prepared by simultaneous addition and mixing of PNP ligand, fatty acid–cysteine conjugate and the $[^{99m}TcN]^{2+}$ core in a vial and heating at 90 °C for 30 min (Scheme 2). According to the literature, cysteine derivatives, in which thiol and acid groups of cysteine are free, form ideal bifunctional chelators for complexation with the $[^{99m}TcN(PNP)]^{2+}$ core.[17,18] In the present study, fatty acid derivative has been prepared with the cysteine residue with free thiol and acid group. It is therefore logical to presume that the structure of the final complex formed is similar to the ones reported earlier. Formation of the intermediate $[^{99m}TcN(PNP)]^{2+}$ core [Fig. 1(a)] and final complex [Fig. 1(b)] was ascertained by HPLC. The labeling yield of ^{99m}TcN(PNP)–fatty acid complex was 80%. The asymmetric centre in the cysteine residue leads to the formation of a mixture of syn and anti isomers of ^{99m}TcN(PNP)–fatty acid complex.[18] The biological studies were carried out with the major isomer (61%) which was isolated using HPLC. However, stereochemistry of isomers can be ascertained unambiguously only after preparing the complex in macroscopic level and subsequent characterization.

A comparative study of the ^{99m}TcN(PNP)–fatty acid complex and ^{125}I-IPPA was carried out to evaluate the potential of the newly formed asymmetric complex. For this purpose, IPPA was labeled with ^{125}I via nucleophilic isotopic exchange in presence of Cu(I). ^{125}I-IPPA could be obtained in 57% radiolabeling yield, as ascertained by paper electrophoresis (R_f = 0) and HPLC. The HPLC elution profile of ^{125}I-IPPA showed $^{125}I^-$ peak at 3 min and the labeled product appeared as a sharp peak at 19 min. The labeled product was purified by extraction in dichloromethane. The radiochemical purity of purified ^{125}I-IPPA was found to be more than 98% by paper electrophoresis.

The stability of the ^{99m}TcN(PNP)–fatty acid complex in presence of challenging ligands such as cysteine was studied in vitro. A 10–50-fold excess of cysteine showed no transchelation of the complex with free cysteine as confirmed by HPLC, wherein the retention of radioactive peak was observed. However, at 500-fold excess of cysteine, appreciable exchange (~28%) was observed. The stability of the complex in serum was also assessed. The ^{99m}TcN(PNP)–fatty acid complex incubated in serum showed no



Scheme 1. Synthesis of fatty acid–cysteine.



Scheme 2. Radiolabeling of FA–cysteine conjugate with $[^{99m}TcPNP]^{2+}$ core.

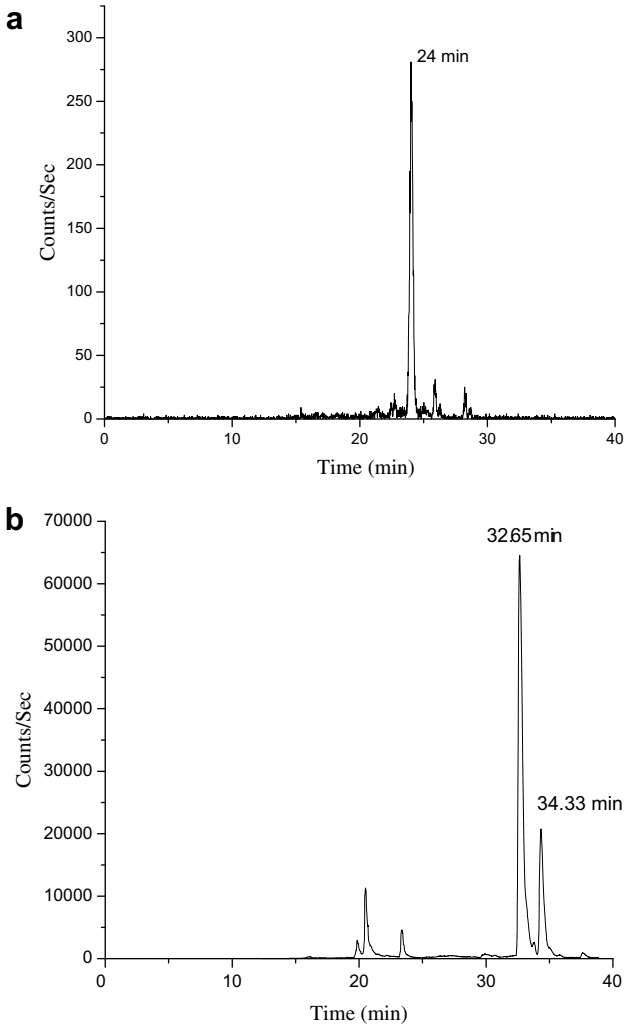


Figure 1. HPLC patterns of (a) $[^{99m}TcN(PNP)]^{2+}$ core and (b) $[^{99m}TcN(PNP)$–fatty acid] complex.

appreciable degradation throughout the period of study (1 h). An insignificant amount of activity (<4%) was found to be associated with the precipitated serum proteins.

The major isomer of ^{99m}TcN(PNP)–fatty acid complex isolated by HPLC was evaluated in swiss mice (Table 1) and the results were

Table 1
Biodistribution of ^{99m}TcNPNP–fatty acid derivative in Swiss mice

	% injected dose per gram (SD) (n = 3)			
	2 min p.i.	5 min p.i.	10 min p.i.	30 min p.i.
Organ				
Liver	38.80 (2.88)	29.88 (5.62)	24.01 (2.49)	5.98 (1.55)
Intestine	5.84 (1.53)	17.05 (3.20)	17.52 (0.24)	38.91 (4.10)
Stomach	1.26 (0.35)	1.42 (0.42)	0.61 (0.26)	1.52 (0.30)
Kidney	23.48 (5.88)	12.94 (1.18)	5.75 (1.02)	5.38 (2.53)
Heart	3.75 (0.28)	1.38 (0.29)	0.51 (0.08)	0.39 (0.05)
Lungs	5.44 (0.66)	2.70 (0.54)	1.18 (0.15)	1.14 (0.22)
Spleen	2.63 (0.18)	2.64 (0.68)	0.56 (0.04)	0.51 (0.26)
Muscle	0.95 (0.06)	0.86 (0.07)	0.32 (0.04)	0.23 (0.05)
Blood	12.18 (1.54)	3.20 (0.36)	1.52 (0.26)	0.89 (0.06)
Ratios				
Heart/blood	0.31 (0.03)	0.43 (0.05)	0.34 (0.02)	0.44 (0.05)
Heart/muscle	3.96 (0.28)	1.60 (0.20)	1.57 (0.09)	1.75 (0.56)
Heart/lungs	0.69 (0.07)	0.52 (0.12)	0.43 (0.04)	0.35 (0.04)
Heart/liver	0.10 (0.01)	0.05 (0.01)	0.02 (0)	0.07 (0.02)

Table 2
Biodistribution of ^{125}I-IPPA in Swiss mice

	% injected dose per gram (SD) (n = 3)			
	2 min p.i.	5 min p.i.	10 min p.i.	30 min p.i.
Organ				
Liver	49.39 (6.67)	34.03 (3.45)	47.94 (10.04)	30.40 (6.82)
Intestine	2.09 (0.62)	2.65 (0.46)	2.73 (0.24)	4.81 (1.52)
Stomach	1.57 (0.14)	2.94 (0.94)	4.33 (0.21)	6.11 (0.29)
Kidney	9.96 (2.58)	11.02 (0.1)	15.11 (0.04)	11.25 (1.18)
Heart	9.51 (1.61)	7.84 (0.93)	9.16 (0.17)	7.10 (1.79)
Lungs	23.93 (5.26)	14.47 (3.34)	12.23 (2.39)	11.39 (2.55)
Spleen	13.47 (2.35)	8.31 (1.54)	15.97 (1.13)	10.23 (1.91)
Muscle	1.57 (0.23)	1.91 (0.16)	2.06 (0.01)	1.94 (0.34)
Blood	5.12 (0.52)	5.53 (0.57)	6.36 (1.12)	6.42 (1.59)
Ratios				
Heart/blood	1.86 (0.24)	1.43 (0.25)	1.46 (0.29)	1.11 (0.07)
Heart/muscle	6.03 (0.16) 0.4.15 (0.88)	4.44 (0.11)	3.66 (0.78)	
Heart/lungs	0.4 (0.05)	0.56 (0.13)	0.77 (0.16)	0.62 (0.02)
Heart/liver	0.2 (0.06)	0.23 (0.05)	0.20 (0.04)	0.23 (0.01)

compared with that of ^{125}I-IPPA (Table 2). The complex showed a maximum uptake of 3.75 ± 0.28% ID/g at 2 min p.i. as compared to the 9.51 ± 1.61%ID/g uptake shown by ^{125}I-IPPA at the same time interval post injection. Further, the ^{99m}TcN(PNP)–fatty acid complex showed faster washout with only 0.39%ID/g remaining at the end of 30 min p.i. The complex showed high initial uptake in liver, lungs, and blood, but showed a faster clearance in comparison to that of [^{125}I]-IPPA. However, the critical ratios, namely, heart/liver, heart/lung, and heart/blood remained suboptimal due to fast clearance of the ^{99m}Tc complex from the myocardium. The faster clearance of the ^{99m}TcN(PNP)–fatty acid complex from the liver is possibly attributable to the ether residues in the PNP ligand, which undergo metabolism in the liver making it less lipophilic. This is perhaps the reason for the observed difference in clearance of injected activity of $[^{99m}TcN(PNP)]^{2+}$-based myocardial agents when compared to other ^{99m}Tc fatty acid derivatives reported. A very relevant and recently published paper by Emiliano Cazzola et al. describes the use of $[^{99m}TcN(PNP)]^{2+}$ labeled fatty acids for myocardial imaging studies,[20] similar to what we are reporting. It could be observed that when compared with the above-reported complexes, the clearance of the injected activity of the present complex from different organs, including heart, is similar, apart from the fact that our compound is showing higher initial uptake compared to the reported compounds. This could be presumably due to the higher lipophilicity of the present complex (longer C-15 fatty acid and use of more lipophilic PNP6 ligand) as compared to the C-11, C-12 fatty acids and relatively less lipophilic PNP3 and PNP5 ligands used by Emiliano et al. The log P value of the present complex in octanol/water was determined following a reported procedure[21] and was found to be 1.8. However, this value alone cannot be utilized to account for the observed differences in the in vivo behavior of the present complex with that of the complexes reported by Emiliano et al., as the log$P$ values of those complexes have not been mentioned. Although the in vivo behavior of the ^{99m}Tc labeled fatty acid currently studied was not good enough for use in myocardial imaging, these studies have provided an insight into preparation and behavior of ^{99m}Tc labeled fatty acids using $[^{99m}TcN(PNP)]^{2+}$ core.

3. Conclusions

A fatty acid–cysteine derivative was synthesized in moderate yield (~55%) by a four-step synthetic procedure. The prepared derivative was labeled via the $[^{99m}TcN(PNP)]^{2+}$ core leading to formation of a [2 + 2] asymmetric complex in over 80% radiolabeling yield. The complex was obtained as a mixture of two isomers and

the major fraction (61%) was isolated, by HPLC, and evaluated in animal model. Biodistribution studies in swiss mice showed uptake of the complex in the myocardium, but was found to be lower than that of ^{125}I-IPPA. Retention of activity in myocardium was also found to be lower for the complex under investigation. An interesting observation which emerges from the in vivo studies is the rapid washout from liver and lungs, an important factor that determines the critical ratios, namely, heart/lung and heart/liver, which could lead to significant improvement in the quality of the image. The results indicate the need for synthetic modification of the parent fatty acid molecule with a view to increasing its residence time in the myocardium, with retention of its other favorable features.

4. Experimental

4.1. Synthesis

4.1.1. General

Hexadecanedioic acid, ethyl *p*-toluene sulfonate, *S*-trityl cysteine, triethyl silane, succinic dihydrazide and stannous chloride were obtained from Aldrich, USA. EDCI was purchased from Fluka, Germany. Iodophenyl pentadecanoic acid (IPPA) was purchased from Emka Chemicals, Germany. All other reagents used were of analytical grade. Solvents used for syntheses were dried as per the standard procedure prior to use. Sodium pertechnetate ($Na^{99m}TcO_4$) was eluted with saline just before use from a ^{99}Mo–^{99m}Tc gel generator supplied by Board of Radiation and Isotope Technology, India. $Na^{125}I$ was obtained from Radiochemicals section, Radiopharmaceuticals Division, BARC, India. Bis[(diethoxypropylphosphanyl)ethyl]ethoxyethylamine (PNP6) was obtained as a gift as a part of a Coordinated Research Project. Silica gel plates (Silica Gel 60 F_{254}) were obtained from Merck, India. Whatman No. 1 chromatography paper was used for paper electrophoresis. Electrophoresis was carried out at 300 V using 0.05 M sodium acetate solution for 30 min. HPLC characterization of the prepared complex was carried out on a JASCO PU 1580 HPLC system, with a JASCO 1575 tunable absorption detector and radiometric detector system, using a C18 reversed phase HiQ Sil (5 mm, 4 × 250 mm) column and water (A): acetonitrile (B) mixture as the mobile phase under gradient elution (0 min 90% A, 28 min 10% A, 50 min 10% A). Characterization of ^{125}I-IPPA was carried out under isocratic condition using acetonitrile (0.1% TFA): water (0.1% TFA) (90:10) mixture as the mobile phase. IR spectra of the synthesized ligands were recorded on a JASCO FT-IT/420 spectrophotometer, Japan. ^{1}H NMR spectra were recorded on a 200 MHz Bruker spectrophotometer.

4.1.2. Synthesis of fatty acid cysteine conjugate

About 100 mg (0.35 mmol) of hexadecanedioic acid and 136 mg (0.35 mmol) of *S*-trityl cysteine ester, synthesized following a reported procedure,[18] were dissolved in 15 mL of dry dichloromethane, in a 100 mL round-bottomed flask, under nitrogen atmosphere. The reaction mixture was cooled to 0 °C after which 75 mg (0.38 mmol) of EDCI, dissolved in minimum amount of dimethyl formamide, was added and the reaction mixure kept stirring at 0 °C for one hour. The reaction mixture was then brought to room temperature and the reaction continued overnight. The progress of the reaction was monitored by TLC. Upon completion of the reaction, the solvent was removed under vacuum, water was added and extracted with dichloromethane (3 × 10 mL). The dichloromethane extracts were pooled, dried over anhydrous sodium sulfate, and filtered. The filtrate was then evaporated to yield the crude coupled product, which was purified on silica gel column using 10% ethyl acetate–chloroform mixture. The product was characterized using ^{1}H NMR. R_f (ethylacetate:chloroform, 10:90; v/v) = 0.3; IR (neat, cm^{-1}) 3056 (w); 2919 (s); 2849 (m); 1740 (s); 1651 (s). ^{1}H NMR ($CDCl_3$, δ ppm) 7.20–7.39 (m, 15H, $(C_6\underline{H}_5)_3C$); 5.96–6.00 (d, 1H, CON$\underline{H}$); 4.58–4.62 (m, 1H, NH–C$\underline{H}$–C$\underline{H}_2$–S); 4.10–4.20 (q, 2H, COOC$\underline{H}_2$CH$_3$); 2.59–2.63 (m, 2H, –CH–C$\underline{H}_2$S); 2.28–2.36 (t, 2H, –CH$_2$C$\underline{H}_2$CONH); 2.11–2.18 (t, 2H, –CH$_2$C$\underline{H}_2$COOH); 1.58 (s, 4H, –C$\underline{H}_2$–CH$_2$–COOH and –C$\underline{H}_2$CH$_2$CONH); 1.20–1.24 (s, 23H, $(C\underline{H}_2)_{10}$& COOCH$_2$C$\underline{H}_3$). The ester and trityl deprotection of the fatty acid–cysteine conjugate were carried out as reported in the literature.[18] The target ligand formed was used as such for radiolabeling without further characterization.

4.2. Radiolabeling

4.2.1. Preparation of ^{99m}TcN(PNP) fatty acid complex

About 5 mg of succinic dihydrazide, 0.1 mg of stannous chloride, and 250 μL of ethanol were taken in a vial to which 750 μL of $^{99m}TcO_4^-$ (1.85 GBq; 50 mCi) was added. The reaction mixture was kept at room temperature for 20 min for formation of $[^{99m}TcN]^{2+}$ intermediate. To this intermediate, ~3 μL of PNP6 ligand and 4 mg of fatty acid cysteine conjugate, each dissolved in 250 μL of ethanol (purged with nitrogen before use), were added simultaneously and the reaction mixture was heated at 90 °C for 30 min. The final complex was characterized by HPLC.

4.2.2. Preparation of ^{125}I-IPPA

The labeling of IPPA with ^{125}I was carried out by heating 2–3 mg of IPPA with 68 μg of $CuSO4.5H_2O$, 4 mg sodium metabisulfite, 50 μL of glacial acetic acid, and 50 μL (~185 MBq; 5 mCi) of $Na^{125}I$ to a temperature of 160 °C for 35–40 min. After cooling, 2 mL of 0.76 M sodium acetate solution was added to the reaction mixture. Labeling yield was ascertained by paper electrophoresis and HPLC. The labeled product was separated from unreacted iodide by solvent extraction, using dichloromethane. The organic extract was flushed with nitrogen to remove dichloromethane and reformulated in 5% ethanol. The pure product was again characterized by paper electrophoresis and HPLC.

4.3. Stability studies

4.3.1. Ligand exchange studies

The stability of the complex toward ligand exchange was studied using cysteine as the challenging ligand. For this, ~100 μL ^{99m}TcN(PNP) fatty acid complex was added to 900 μL of phosphate-buffered saline containing 10–500-fold molar excess of cysteine over that of the ligand. The samples were incubated at 37 °C for 60 min and then analyzed by HPLC.

4.3.2. Serum stability

Stability of the ^{99m}TcN(PNP) fatty acid complex in serum was tested in vitro. About 50 μL of the radiolabeled preparation was added to 450 μL serum, and the mixture was incubated at 37 °C for 60 min. To this mixture, equal volume of cold ethanol was added to precipitate the serum proteins and centrifuged at 10,000g (4 °C, 20 min). The supernatant was analyzed by HPLC to assess stability of the complex in serum. The precipitate was washed twice with ethanol and counted in the NaI(Tl) scintillation detector to determine the activity associated with serum protein.

4.4. Biodistribution studies

Normal Swiss mice (20–25 g body weight) were used for the in vivo distribution studies of ^{99m}TcN (PNP)–fatty acid complex and ^{125}I-IPPA. Prior to the experiment, the mice were fasted overnight, although water was given ad libitum. About 100 μL (~1.85 MBq; 50 μCi) of the prepared complex was administered intravenously in each animal. Different sets of animals (n = 3) were

kept for the studies at various time points (2, 5, 10, and 30 min). The animals were sacrificed immediately at the end of the respective time points, and the relevant organs and tissue excised for measurement of associated activity. Radioactivity measurements were carried out in a flat-bed-type NaI(Tl) scintillation counter with a suitable energy window for ^{99m}Tc. Accumulated activity was expressed in terms of percentage of total injected dose per gram associated with the specific organ/tissue. All procedures performed and reported herein were in accordance with the national laws pertaining to the conduct of animal experimentation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.07.074.

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