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RESEARCH ARTICLE

Preparation and Biological Evaluation of ^{99m}Tc-TMPP as a novel Agent for Tumor Diagnosis

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Abstract

5, 10, 15, 20 tetrakis {4-methoxyphenyl} 21H, 23H porphyrin (TMPP) was labeled with technetium-99m via direct labeling technique using stannous chloride as a reducing agent. The optimum conditions that gave high labeling yield (95.2%) of ^{99m}Tc-TMPP complex were achieved by using 3mg TMPP, 100 g $SnCl_2.2H_2O$, at pH 3 and 30min reaction time. Molecular geometry was used to illustrate the interaction between ^{99m}Tc and TMPP. The preclinical evaluation and biodistribution in solid tumor bearing mice showed high biological accumulation in solid tumor cells (22.66 % injected activity/ g tissue) and high T/NT ratio equal to 3.21 ± 0.15 at 30 minutes post-injection. Data described before could recommend ^{99m}Tc-TMPP as a potential targeting radiopharmaceutical for tumor imaging.

INTRODUCTION

Porphyrins are important in many biological processes of normal metabolism of living organisms, such as oxygen transport , photosynthesis, etc. because they form highly stable complexes with many metals and so have many applications in biomedical and chemical analysis [1]. Also, porphyrins have characteristics of selective accumulation in tumor tissues of animals and human models [2]. The ability of porphyrin derivatives to accumulate in the neoplastic cells is demonstrated in photodynamic therapy (PDT) which is a medical treatment technique using a combination of light and photosensitizing drug to develop reactive oxygen species (ROS) in tumor cells [3-5]. ROS created by photosensitizing drugs are preferentially accumulated in tumor cells, leading to cell damage in several subcellular organelles [3,4,6]. Porphyrins are well-recognized photosensitizing drugs for the treatment of cancer [3-5]. Furthermore, porphyrins have the advantages of no toxicity in the dark, stable composition, selective accumulation in neoplastic tissue and high creation of ROS [4,7]. Although PDT is clinically used, this modality has many disadvantages such as induction of hemorrhage, low efficacy in treating of large tumors, low sensitivity of detection ,etc. [8-10]. These advantages of porphyrins and disadvantages of PDT open the way to make labeling of porphyrins derivatives with suitable radionuclides that may improve the efficacy of porphyrins in diagnosis of tumors.

^{99m}Tc is a widely used radionuclide in radioactive tracer investigations as single-photon emission computed tomography (SPECT) imaging agent owing to its suitable half-life about (6 hours) that ensures that the patient is not exposed to unnecessary radiation. ^{99m}Tc also is of favorable energy (140 KeV) of -ray yielding a high counting efficacy [11-18].

This work aims at labeling TMPP with ^{99m}Tc under different experimental conditions, using of molecular modeling in confirmation of reaction between ^{99m}Tc and TMPP and investigation of the potential use of ^{99m}Tc-TMPP (prepared under the optimum conditions) as a tumor imaging agent using a mouse model.

EXPERIMENTAL

Chemicals

TMPP was purchased from Aldrich Chemical Company. $^{99}Mo/^{99m}Tc$ generator was purchased from (Elutic, Brussels, Belgium). All other chemicals were purchased from Merck and they were reactive grade. A NaI (Tl) γ - ray scintillation counter (Scaler RatemeterSR7 model, England) was used for the measurement of γ-ray radioactivity. Whatman No.1 paper chromatography (PC), Whatman International Ltd, Maidstone, Kent, UK.

Methods

Method of labeling

TMPP was dissolved in N_2-purged DMSO in an evacuated penicillin vial. Three milligrams of TMPP was transferred to a 10ml vial then the vial was evacuated. A solution containing 100 g $SnCl_2.2H_2O$ was added and the pH of the reaction mixture was adjusted to 3. One ml of freshly eluted $^{99m}TcO_4^-$ (400MBq) was added to the above mixture. The reaction mixture was shaken and let to react at room temperature for adequate time (30min) desired completing the reaction.

Analysis

Paper and HPLC chromatographic techniques are used to determine the percent labeling yield of the labeled ^{99m}Tc-TMPP complex.

Paper chromatographic technique

The ascending paper chromatographic technique is used to determine the percent labeling yield by using strips of Whatman No.3 paper chromatography, 10 cm length and 1.5 cm width, were marked gently with a pencil at a distance of 2 cm from the lower end lined into sections 0.5 cm each up to 7 cm. A spot from the reaction mixture was applied by a hypodermic syringe and then the strip was developed in an ascending method in a closed jar filled with N_2 gas to prevent oxidative decomposition of the labeled ^{99m}Tc-TMPP spot. The developing solvents; acetone and saline were purged with N_2 gas for the same purpose. After complete development, the strips were dried and cut into fragments of 0.5 cm each. Then the sections were counted in a well-type -scintillation counter. The organic solvent acetone was used to calculate the percent of free $^{99m}TcO_4^-$ and saline was used to calculate the amount of reduced hydrolyzed technetium-99m (colloid).

HPLC chromatographic technique

HPLC was applied to guarantee that the labeled molecule was present as a single species and to ascertain the complexation yield. Before the HPLC analysis of the labeled compound, a cold solution of TMPP was injected to the column (C-18 reversed phase column) and UV spectrophotometer detector (SPD-6A) adjusted to the 270 nm wavelength. The column was eluted with mobile phase (water (A) and acetonitrile (B) mixed with 0.1% trifluoroacetic acid as the mobile phase.) [19] and the flow rate was adjusted to 1 ml / min. TMPP gives a peak at R_t = 23 min. Then, 10 µl of the reaction mixture containing ^{99m}Tc-TMPP was injected to the column of HPLC under the same condition mentioned before, after 0.20 µm Millipore filtration, and the fractions of 1 ml were collected and counted using 3-inch NaI (TI) well-type crystal coupled to SR-7 scaler ratemeter so that a radiochromatogram can be obtained.

In-vitro stability study in serum:

The effect of time on the in-vitro stability of ^{99m}Tc-TMPP complex was studied in order to decide the proper time during which the complex can be used. Oxidation or hydrolysis of ^{99m}Tc-TMPP complex may occur during storage time after labeling with technetium, besides the effect of ionizing γ-radiation (radiolysis). Therefore, stability of the ^{99m}Tc-TMPP complex was studied in serum by mixing 0.2 ml of ^{99m}Tc-TMPP complex and 1.8 ml of serum and incubated at 37 °C for 8 h. Exactly 0.2 ml aliquots were withdrawn during the incubation at different time intervals up to 8 h and assayed using P.C. for determination of the in vitro stability of ^{99m}Tc-TMPP in serum.

Computational method

The optimized molecular structures of ^{99m}Tc-TMPP complex and corresponding energies were studied using GAUSSIAN 09 program package [20] using Hartee-Fock level combined with three different basis sets without any restriction on the geometries. The standard 3-21G basis set was used for carbon and hydrogen atoms and the standard 6-31G basis set was used for oxygen and nitrogen atoms. anywise, the last basis set was LANL2MB and it was used for technetium atom. The LANL2MB basis set treated electrons near the nuclei via effective core

potentials (ECPs) and it also involves some relativistic effect, which are important for heavy elements like technetium [21–24]. Gaussview program [24] has been used to have visual the optimized structures. It is significant to mention that, for ^{99m}Tc-TMPP complex, all the optimized structures were found to be true minima, i.e. no imaginary frequency modes were acquired.

Induction of tumor in mice

Exactly 0.2 ml solution of Ehrlich Ascites Carcinoma was injected intramuscularly in the right thigh of female Swiss Albino mice to induce a solid tumor. The animals were well-kept till the tumor development was visible (10-15 days). The parent tumor line (Ehrlich Ascites Carcinoma) was withdrawn from 7 days old donor female Swiss Albino mice and diluted with sterile physiological saline solution to give 12.5 x 10^6 cells/ml.

Biodistribution study in mice

In vivo biodistribution studies were done in groups of five female Albino mice where each animal was injected in the tail vein with 0.2 ml solution containing 5-10 kBq of ^{99m}Tc-TMPP. The mice were then put in metabolic cages for the required time. The mice were sacrificed by cervical dislocation in groups at various time intervals after injection and the organs or tissues of interest were removed, weighed and counted. Correction was made for background radiation and physical decay during the experiment.

RESULTS and DISCUSSION

Radiochemical purity and stability of ^{99m}Tc-TMPP complex were evaluated by paper and HPLC chromatographic methods. Is used in paper chromatography as the developing solvent, free $^{99m}TcO_4^-$ moved with the solvent front (R_f=1), while ^{99m}Tc-TMPP and reduced hydrolyzed technetium remained at the point of spotting. Saline as the mobile phase was used to determine the reduced hydrolyzed technetium where reduced hydrolyzed technetium remained at the origin (R_f = 0) while other species move with the solvent front (R_f =1). By subtracting the sum of the percent of colloid and free pertechnetate from 100% the labeling yield was calculated. The radiochemical purity (labeling yield) is the mean value of three experiments.

HPLC analysis of the ^{99m}Tc-TMPP

The radiochromatogram was presented in figure (1) and showed two peaks, one at fraction No. 6 which relates to the free pertechnetate, while the second peak was collected at fraction No. 22 that relates to ^{99m}Tc-MPP which was found to identical with the UV signal. The radiochromatogram showed 95.2% labeling yield of ^{99m}Tc-TMPP complexes, which was agreeing with the results of the analysis using ascending paper chromatography.

Factors affecting the percent labeling yield

Effect of TMPP amount:

Fig.2. shows that at 1mg TMPP, the labeling yield of ^{99m}Tc-TMPP complex was 76.2% where this low labeling yield was because the fact that the substrate concentration is low and insufficient to complex all reduced technetium. By increasing the amount of TMPP, the labeling yield increased and reached the maximum value of 95.2% at 3 mg TMPP. By increasing the TMPP amount over 3mg, the labeling yield somewhat decreased again till reaching 66.7% at 10mg TMPP.

Effect of $SnCl_2.2H_2O$ content

Fig.3. shows that below 10□□g $SnCl_2.2H_2O$, the percent labeling yield was low because $SnCl_2.2H_2O$ is not enough for entire reduction of pertechnetate to form ^{99m}Tc-TMPP complex and this was proved by two points, at 50µg $SnCl_2.2H_2O$ the quantity of free pertechnetate was 17.9% then decreased to 6.6% at 75µg of $SnCl_2 2H_2O$. The optimum labeling yield was obtained at 100□g $SnCl_2.2H_2O$ at which the highest labeling yield of 95.2% was obtained. When excess $SnCl_2.2H_2O$ content is used, >100□g, the labeling yield decreased again (77.3% at 150□g $SnCl_2.2H_2O$) and reduced hydrolyzed technetium (21.1% at 150□g $SnCl_2.2H_2O$) was the main impurity because excess $SnCl_2.2H_2O$ was turned into colloid.

Effect of pH of the reaction medium

Figure 4 shows that, the labeling yield of ^{99m}Tc-TMPP depends upon the pH of the reaction mixture in the range from 2 to 6. At pH 2 the difference between free pertechnetate and ^{99m}Tc-RH$^-$ was not so high, the free $^{99m}TcO_4^-$ and ^{99m}Tc-RH$^-$ was equal to (2.8 and 6.7%, respectively). The optimum labeling yield of 95.2% was obtained at pH 3. Above pH 3, the labeling yield decreased again due to colloid formation. At pH 6, ^{99m}Tc-RH$^-$ was the main impurity and was equal to 29.6%.

Effect of Reaction time and In-vitro stability

As shown in Fig 5. Stability of ^{99m}Tc-TMPP complex in serum was determined using P.C. the results showed that, ^{99m}Tc-TMPP complex was stable in serum showing maximum labeling yield at the optimum conditions which is (3mg TMPP , 100 g $SnCl_2.2H_2O$ and pH 3), the reaction is completed at 30 min reaching labeling yield 95.2% which slightly decreased after 60min to 86.2% and then remains stable at 85% for 8 hours

Molecular geometry

Determination of the exact structure and composition of the reaction product of ^{99m}Tc-TMPP complex is impossible because the amount of ^{99m}Tc eluted from ^{99}Mo/^{99m}Tc generator is very little ca. 10-9 mol/L [25]. Thus, we use theoretical background and methods of molecular modeling to predict and purpose ^{99m}Tc-TMPP complex. Moreover, tin chloride was added to reduce the oxidation state of technetium from IIV to V, IV or III in order to perform labeling of the chelating agent with ^{99m}Tc, However, technetium oxidation states in the chelate systems were inspected in presence of $SnCl_2$ and it was concluded and proved that Tc (V) and Tc (IV) were the most stable oxidation states [26, 27]. Moreover, pH of the reaction mixture is important in the labeling process. Over and above, stability of complexes containing none or fewer chelate rings is less than those complexes containing chelate rings due to the chelate effect [28]. According to this discussion, we can expect that: Technetium will like to interact with the four nitrogen atoms of the core of the porphyrin molecule (H_2L) due to the chelate effect; since the optimum pH for the labeling reaction medium is an acidic pH, the coordinated technetium atom will complete its coordination sphere with O_2^- or H_2O species and the oxidation state of the coordinated technetium will be +4 or +5.

Accordingly, the geometry of the possible complexes for TMPP are {$[LTcOH_2]^{+2}$, **1**; $[LTcOH_2]^+$, **2**; $[LTc(OH_2)_2]^-$, **3**; $[LTc(OH_2)_2]$, **4**; [(L)TcO], **5**; $[(L)TcO]^+$, **6**; $[(L)OTcOH_2]$, **7**; $[(L)OTcOH_2]^-$, **8**; $[(L)OTcO]^{-2}$, **9**; and $[(L)OTcO]^-$, **10**} was optimized, in a singlet and doublet state for Tc(V) and Tc(IV) complexes, respectively, by the HF method with the B3LYP function. All optimized geometries of the suggested complexes are shown in Fig. 6. The energy, for the geometrically optimized complexes are expressed in Table 1. It is clear that complex 4 has the lowest energy (-2756.27805630 a.u.) and so this is the most stable structure of ^{99m}Tc-TMPP complex. The optimized geometrical structure and the atomic numbering of $[(L)H_2OTcOH_2]$, 4, complex is shown in Fig. 7. The structure of complex 4 presents nearly linear H_2O–Tc–OH_2 unit with an angle of 125.876° and a coplanar Tc (N1N2N3N4) unit as shown in table (1). Tables (2 an 3) show some selected parameters of the optimized geometrical structure complex 4. The calculated bond lengths and angles for Tc–O and Tc–N are in quite good agreement with the values reported for other oxotechnetium and oxorhenium complexes of related structure [29–31].

Biodistribution of ^{99m}Tc-TMPP complex

In-vivo biodistribution studies of ^{99m}Tc-TMPP in solid tumor bearing mice was found to be greatest in blood, heart and stomach (18.97, 11.49 and 2.72%, respectively) at 15 min post injection and lowest in left normal muscle and bone (3.39±0.14 and 7.07±0.35% ID/g tissue at 15 min and 30 min , respectively) (Table 4). The bioretension of ^{99m}Tc-TMPP in the right thigh (inoculated) was greater than that of the left thigh. The uptake of ^{99m}Tc-TMPP in right thigh was significantly increased with time and was equal to 9.33±0.46, 22.66±1.03, 15.99±0.79 and 14.7%±0.38% ID/g tissue at 15, 30 60, and 240min, respectively, indicating that ^{99m}Tc-TMPP has high stable T/TN ratio for which approximately 3 and delivers ^{99m}Tc to the tumor sites with an adeqaute percentage for imaging. The urinary pathway is the way through it body clearance of ^{99m}Tc-TMPP is done. This preclinical study suggests that ^{99m}Tc-TMPP can be used as solid tumor imaging agent

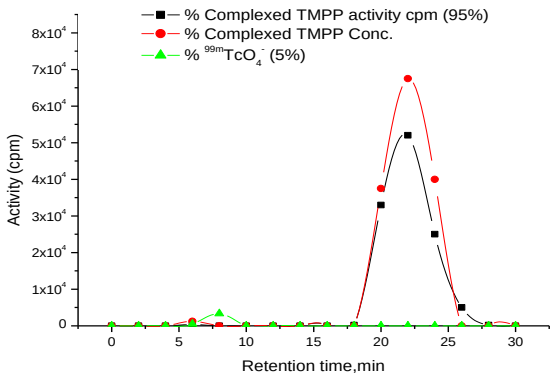

Fig 1: HPLC radiochromatogram and U.V. profile of ^{99m}Tc-TMPP complex.

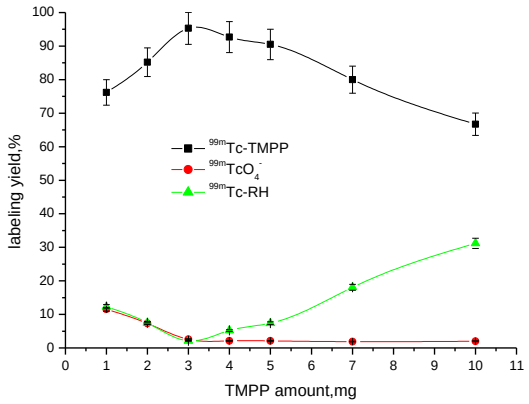

Fig 2: Effect of TMPP amount on the labeling yield of ^{99m}Tc-TMPP.

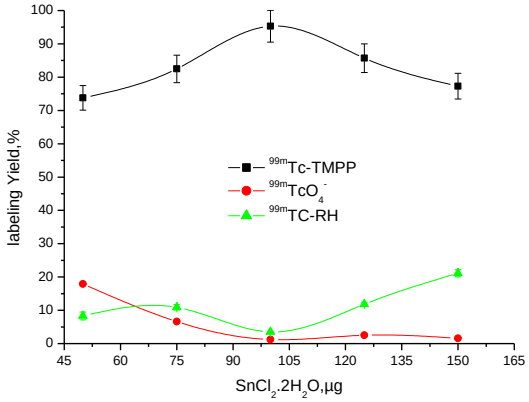

Fig 3: Effect of $SnCl_2.2H_2O$ on the percent labeling yield of ^{99m}Tc-TMPP.

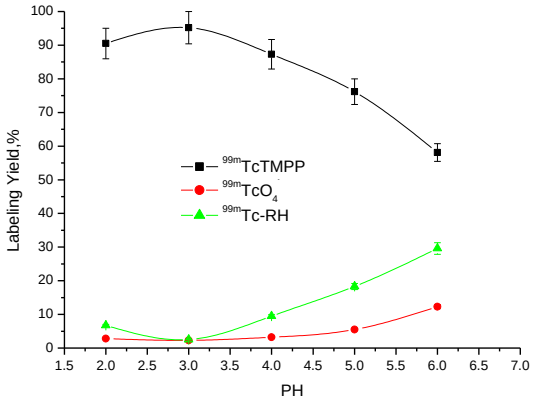

Fig 4: Effect of pH of the reaction mixture on the labeling yield of ^{99m}Tc-TMPP

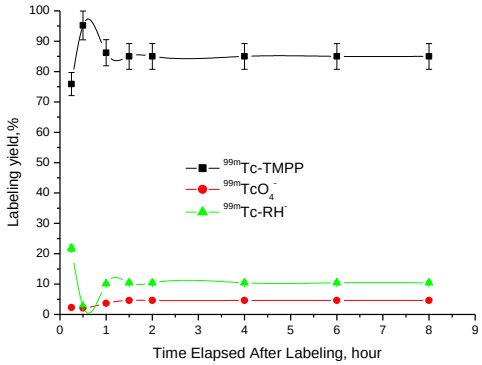

Fig 5: Effect of time on the labeling yield of ^{99m}Tc-TMPP complex

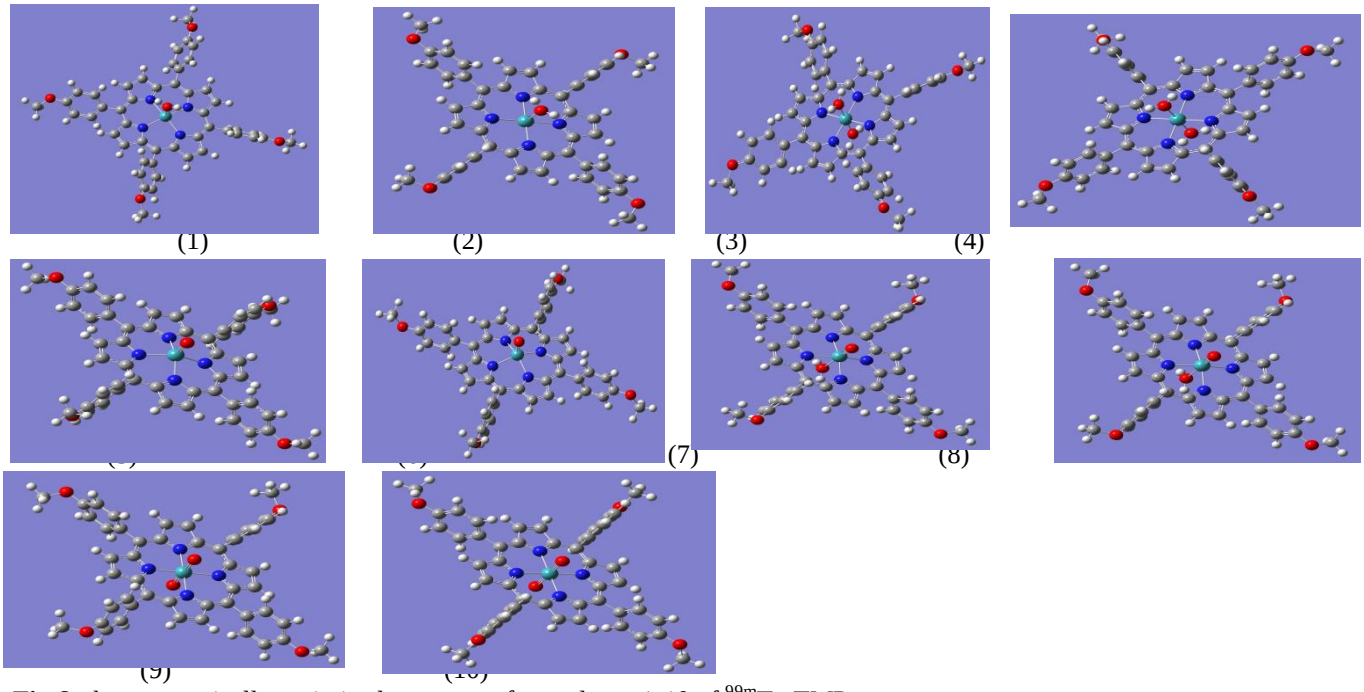

Fig 6: the geometrically optimized structure of complexes 1-10 of ^{99m}Tc-TMP

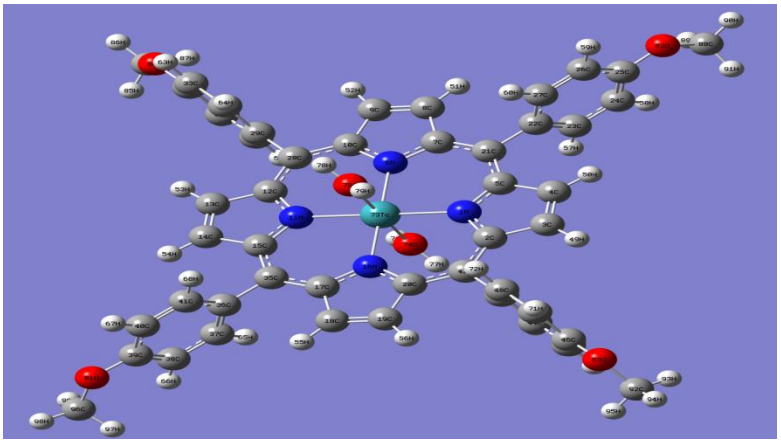

Fig 7: Optimized structure of [LTc(OH_2)$_2$], 4,with numbering of atom

Complex	Energy(a.u.)
1	-2501.831015
2	-2499.73470936
3	-2575.79348748
4	**-2576.27805630**
5	-2574.18175037
6	-2574.0936279
7	-2575.20519528
8	-2575.40955751
9	-2574.18175037
10	-2574.09362790

Tab 1 shows suggested complexes energy

Bond	Bond Length
R3 R(1,73)	2.1317
R13 R(6,73)	2.1317
R23 R(11,73)	2.1317
R89 R(73,74)	2.2263
R90 R(73,75)	2.2265
R91 R(74,76)	0.9507

Angle	Angle size
A(2,1,73)	125.8766
A(5,1,73)	124.378
A(7,6,73)	124.377
A(10,6,73)	125.8771
A(12,11,73)	125.8768
A(15,11,73)	124.3778
A(17,16,73)	124.3778

Tab 2 and 3 show optimized parameters of the most stable complex

organs and (Body fluid)	% ID/g tissue (fluid) at time intervals post injection			
	15 min	30 min	1 h	4 h
Blood	18.97±0.94	8.73±0.4365	2.4±0.12	1.99±0.11

Kidneys	6.32±0.316	17.43±0.87	17.57±0.87	23.18±1.15
Liver	2.98±0.14	5.99±0.29	7.85±0.19	8.39±0.1 6
Spleen	6.3±0.315	3.21±0.16	1.94±0.11	4.4±0.22
Intestine	2.39±0.42	4.71±0.23	8.45±0.11	8.41±0.42
Stomach	2.72±0.13	0.649±0.032	0.35±0.017	1.02±0.051
Lungs	4.2±0.21	4.72±0.23	0.51±0.025	0.31±0.015
Heart	4.51.±0.57	4.46±0.22	2.28±0.064	2.3±0.12
Bone	6.95±0.34	7.58±0.37	7.71±0.38	7.78±0.73
Tumor muscle	9.33±0.46	22.66±1.03	15.99.±0.79	14.7±0.38
Normal muscle	3.93±0.14	7.07±0.35	5.63±0.28	5.32±0.26
T/NT Ratio	2.56±0.03	3.21±0.15	2.84±0.17	2.76±0.18

Reaction conditions 3mg TMPP, 10□□g $SnCl_2.2H_2O$.pH 3 and 30 min reaction time, n=5

Tab 4 Biodistribution of ^{99m}Tc-TMPP complex in Albino mice bearing EAC.

CONCLUSION

^{99m}Tc-TMPP was prepared via direct labeling technique and a high labeling yield of 95.2% was obtained using (3mg TMPP, 10□□g $SnCl_2.2H_2O$.pH 3 and 30 min reaction time). The molecular modeling study of ^{99m}Tc-TMPP showed that ^{99m}Tc-TMPP has almost linear H_2O–Tc–OH_2 unit with an angle of 125.876° and a coplanar Tc(N1N2N3N4) unit. The biodistribution study of ^{99m}Tc-TMPP showed that ^{99m}Tc-TMPP has high T/NT ratio. All these findings are very promising to suggest that ^{99m}Tc-TMPP is a potential radiopharmaceutical for solid tumor imaging.

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