Preparation and biological evaluation of $^{99m}$Tc-TMPP as a novel agent for tumor diagnosis

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ABSTRACT

Compound 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, 100µg SnCl$_2$·2H$_2$O, at pH 3 and 30min reaction time. Molecular geometry was used to illustrate the structure of complex which is formed 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.

Key Words: Technetium-$^{99m}$/ TMPP / Labeling / Biodistribution /Imaging/Molecular geometry

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 (Konirova et al., 2003). Also, porphyrins have characteristics of selective accumulation in tumor tissues of animals and human models (Shetty et al., 1995). 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. ROS created by photosensitizing drugs are preferentially accumulated in tumor cells, leading to cell damage in several subcellular organelles (Sternberg and Dolphin, 1998; Nyman et al., 2004; Riccheli, 1995).

Porphyrins are well-recognized photosensitizing drugs for the treatment of cancer (Macdonald and Dougherty, 2001). Furthermore, porphyrins have the advantages of no toxicity in the dark, stable composition, selective accumulation in neoplastic tissue and high creation of ROS (Byrne et al., 1990). 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. (Bonnett et al., 1989; Tapas et al., 2010; Banerjee et al., 2001). 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 (Das et al., 2008; Wang et al., 2010; Kavali et al., 2005). 99mTc also is of favorable energy (140 KeV) of γ-ray yielding a high counting efficacy (Yu et al., 2012; Mohamed et al., 2014).

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

MATERIALS and METHODS

Chemicals
TMPP was purchased from Aldrich Chemical Company. 99Mo/99mTc 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

1. Method of labeling
TMPP was dissolved in N2-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 100µg SnCl2·2H2O was added and the pH of the reaction mixture was adjusted to 3. One ml of freshly eluted 99mTcO4⁻ (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.

2. Analysis
Paper and HPLC chromatographic techniques are used to determine the percent labeling yield of the labeled 99mTc-TMPP complex.

2.1. 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 N2 gas to prevent oxidative decomposition of the labeled 99mTc-TMPP spot. The developing solvents; acetone and saline were purged with N2 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 γ-scintillation counter. The organic solvent acetone was used to calculate the percent of free 99mTcO4⁻ and saline was used to calculate the amount of reduced hydrolyzed technetium-99m (colloid) (Motaleb, 2007)

2.2. 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 in ratio 30:5:65 respectively. (El Shaboury et al., 2007) 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 ⁹⁹mTc-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.

3. In-vitro stability study in serum:
The effect of time on the in-vitro stability of ⁹⁹mTc-TMPP complex was studied in order to decide the proper time during which the complex can be used. Oxidation or hydrolysis of ⁹⁹mTc-TMPP complex may occur during storage time after labeling with technetium, besides the effect of ionizing γ-radiation (radiolysis). Therefore, stability of the ⁹⁹mTc-TMPP complex was studied in serum by mixing 0.2 ml of ⁹⁹mTc-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 ⁹⁹mTc-TMPP in serum (Van Der Laken et al., 2000).

4. Computational method
The optimized molecular structures of ⁹⁹mTc-TMPP complex and corresponding energies were studied using GAUSSIAN 09 program package (Frisch et al., 2010) 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 (Hehre et al., 1969; Collins et al., 1976; Hay and Wadt, 1985). Gaussview program (computer program Gaussview Version 5.0.9 Gaussian, Wallingford) has been used to have visual the optimized structures. It is significant to mention that, for ⁹⁹mTc-TMPP complex, all the optimized structures were found to be true minima, i.e. no imaginary frequency modes were acquired.

5. In-Vivo Study
5.1. 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 10⁶ cells/ml (Van Der Laken et al., 2000).

5.2. 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 ⁹⁹mTc-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
RESULTS and DISCUSSION
Radiochemical purity and stability of $^{99m}\text{Tc}$-TMPP complex were evaluated by paper and HPLC chromatographic methods. It is used in paper chromatography as the developing solvent, free $^{99m}\text{TcO}_4^-$ moved with the solvent front ($R_f=1$), while $^{99m}\text{Tc}$-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.

1. HPLC analysis of the $^{99m}\text{Tc}$-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 $^{99m}\text{Tc}$-MPP which was found to identical with the UV signal. The radiochromatogram showed 95.2% labeling yield of $^{99m}\text{Tc}$-TMPP complexes, which was agreeing with the results of the analysis using ascending paper chromatography.

2. Factors affecting the percent labeling yield

2.1. Effect of TMPP amount:

Fig.2. shows that at 1mg TMPP, the labeling yield of $^{99m}\text{Tc}$-TMPP complex was 76.2% where this low labeling yield was due to 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.

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

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

2.2. Effect of $\text{SnCl}_2\cdot2\text{H}_2\text{O}$ content

Fig.3. shows that below 100µg $\text{SnCl}_2\cdot2\text{H}_2\text{O}$, the percent labeling yield was low because $\text{SnCl}_2\cdot2\text{H}_2\text{O}$ is insufficient for entire reduction of pertechnetate to form $^{99m}\text{Tc}$-TMPP complex and this was proved by two points, at 50µg $\text{SnCl}_2\cdot2\text{H}_2\text{O}$ the quantity of free pertechnetate was 17.9% then decreased to 6.6% at 75µg of $\text{SnCl}_2\cdot2\text{H}_2\text{O}$. The optimum labeling yield was obtained at 100µg $\text{SnCl}_2\cdot2\text{H}_2\text{O}$ at which the highest labeling yield of 95.2% was
obtained. When excess SnCl₂·2H₂O content is used, >100µg, the labeling yield decreased again (77.3% at 150µg SnCl₂·2H₂O) and reduced hydrolyzed technetium (21.1% at 150µg SnCl₂·2H₂O) was the main impurity because excess SnCl₂·2H₂O was turned into colloid.

**Fig 3: Effect of SnCl₂·2H₂O on the percent labeling yield of ⁹⁹mTc-TMPP.**

### 2.3. Effect of pH of the reaction medium

Figure 4 shows that the labeling yield of ⁹⁹mTc-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 ⁹⁹mTc-RH⁻ was not so high, the free ⁹⁹mTcO₄⁻ and ⁹⁹mTc-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, ⁹⁹mTc-RH⁻ was the main impurity and was equal to 29.6%.

**Fig 4: Effect of pH of the reaction mixture on the labeling yield of ⁹⁹mTc-TMPP**

### 2.4. Effect of Reaction time and In-vitro stability

As shown in Fig 5. Stability of ⁹⁹mTc-TMPP complex in serum was determined using P.C. the results showed that, ⁹⁹mTc-TMPP complex was stable in serum showing maximum labeling yield at the optimum conditions which is (3mg TMPP, 100µg SnCl₂·2H₂O 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 hour.

**Fig 5: Effect of time on the labeling yield of ⁹⁹mTc-TMPP complex**

### 3. Molecular geometry

Determination of the exact structure and composition of the reaction product of ⁹⁹mTc-TMPP complex is impossible because the amount of ⁹⁹mTc eluted from ⁹⁹Mo/⁹⁹mTc generator is very little ca. 10-9 mol/L (Stanik and Benkovsky, 2011). Thus, we use theoretical background and methods of molecular modeling to suggest and purpose ⁹⁹mTc-TMPP complex. Moreover, tin chloride was added to reduce the oxidation state of technetium from IIIV 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 (Fisˇer et al., 1985; Fisˇer et al., 1985). 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 (Cotton et al., 1999). 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$_2$L) 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$_2$O species and the oxidation state of the coordinated technetium will be +4 or +5.

Accordingly, the geometry of the possible complexes for TMPP are $\{[LTcOH_2]^+, [LTcOH_2]^+, 1; [LTcOH_2]^+, 2; [LTc(OH_2)_2]^+, 3; [LTc(OH_2)_2]^+, 4; [(L)TcO], 5; [(L)TcO]^+, 6; [(L)OTcOH]^+, 7; [(L)OTcOH]^+, 8; [(L)OTcO]^+, 9; and [(L)OTcO]^+\}$ 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$_2$O–Tc–OH$_2$ unit with an angle of 125.876° and a coplanar Tc (N1N2N3N4) unit as shown in table (1).

![Fig 6: the geometrically optimized structure of complexes 1-10 of $^{99m}$Tc-TMP](image_url)
Table 1 suggested complexes energy

<table>
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<tr>
<th>Complex</th>
<th>Energy (a.u.)</th>
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<tr>
<td>2</td>
<td>-2499.73470936</td>
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</tr>
<tr>
<td>10</td>
<td>-2574.09362790</td>
</tr>
</tbody>
</table>

Table 1 suggested complexes energy

oxorhenium complexes of related structure (Liu et al., 1991; Chatterjee et al., 2011; Gancheff et al., 2002).

![Fig 7: Optimized structure of [LTc(OH₂)₂], 4, with numbering of atom](image)

Biodistribution of ⁹⁹ᵐTc-TMPP complex

*In-vivo* biodistribution studies of ⁹⁹ᵐTc-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 ⁹⁹ᵐTc-TMPP in the right thigh (inoculated) was greater than that of the left thigh. The uptake of ⁹⁹ᵐTc-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 240 min, respectively, indicating that ⁹⁹ᵐTc-TMPP has high stable T/TN ratio for which approximately 3 and delivers ⁹⁹ᵐTc to the tumor sites with an adequate percentage for imaging. The urinary pathway is the way through it body clearance of ⁹⁹ᵐTc-TMPP is done. This preclinical study suggests that ⁹⁹ᵐTc-TMPP can be used as solid tumor imaging agent.

CONCLUSION

Compound ⁹⁹ᵐTc-TMPP was prepared via direct labeling technique and a high labeling yield of 95.2% was obtained using (3mg TMPP, 100µg SnCl₂·2H₂O, pH 3 and 30 min reaction time). The molecular modeling study of ⁹⁹ᵐTc-TMPP showed that ⁹⁹ᵐTc-TMPP has almost linear H₂O–Tc–OH₂ unit with an angle of 125.87° and a coplanar Tc (N1N2N3N4) unit. The biodistribution study of ⁹⁹ᵐTc-TMPP showed that ⁹⁹ᵐTc-TMPP has high T/TN ratio. All these findings are very promising to suggest that ⁹⁹ᵐTc-TMPP is a potential radiopharmaceutical for solid tumor imaging.
Tab 2 and 3 optimized parameters of the most stable complex

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<th>Bond</th>
<th>Bond Length</th>
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<td>R3</td>
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<tr>
<td>R13</td>
<td>R(6,73) 2.1317</td>
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<tr>
<td>R23</td>
<td>R(11,73) 2.1317</td>
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<tr>
<td>R89</td>
<td>R(73,74) 2.2263</td>
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<td>R90</td>
<td>R(73,75) 2.2265</td>
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<tr>
<td>R91</td>
<td>R(74,76) 0.9507</td>
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<table>
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<td>A(17,16,73)</td>
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Tab 4 Biodistribution of $^{99m}$Tc-TMPP complex in Albino mice bearing EAC.

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<td></td>
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<tr>
<td>Blood</td>
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<tr>
<td>Kidneys</td>
<td>6.32±0.316</td>
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<tr>
<td>Liver</td>
<td>2.98±0.14</td>
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<tr>
<td>Spleen</td>
<td>6.3±0.315</td>
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<tr>
<td>Intestine</td>
<td>2.39±0.42</td>
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<tr>
<td>Stomach</td>
<td>2.72±0.13</td>
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<tr>
<td>Lungs</td>
<td>4.2±0.21</td>
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<tr>
<td>Heart</td>
<td>4.51±0.57</td>
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<tr>
<td>Bone</td>
<td>6.95±0.34</td>
</tr>
<tr>
<td>Tumor muscle</td>
<td>9.3±0.46</td>
</tr>
<tr>
<td>Normal muscle</td>
<td>3.93±0.14</td>
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<tr>
<td>T/NT Ratio</td>
<td>2.56±0.03</td>
</tr>
</tbody>
</table>

Reaction conditions 3mg TMPP, 100µg SnCl2.2H2O .pH 3 and 30 min reaction time, n=5

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al-اعداد والتقييم البيولوجي لـ تي ام بي بي – تكنسيوم-99م كمركب جديد لتشخيص الورم

محمد فايد الصباغ – محمد عبداللطيب – محمد طه القللي - لبنى محمد عبدالعزيز* و السيد منصور لاشين*

قسم المركبات المرقمة – مركز المعامل الحارة – هيئة الطاقة الذرية

قسم الكيمياء الطبية - كلية الصيدلة - جامعة الزقازيق - مصر

يعتبر نظير التكنيسيوم-99م من أهم النظائر المشعة المستخدمة في الطب النووي التشخيصي وذلك لخواصه الإشعاعية المميزة مثل: عمر نصف مناسب (6.02 ساعة) وتحلل اشعاعي بأشعة جاما والتى لها طاقة مناسبة (140كليلوالكترون فولت).

تتضم هذا البحث ترقيم تي ام بي بي كأحد مشتقات للبورفيرين بنظير التكنيسيوم-99م ودراسة العوامل المؤثرة في عملية الترقيم. وقد تم عمل تقييم لكفاءة التوجيه لـ تي ام بي بي لنظير التكنيسيوم-99م في أنواع مختلفة من الأورام المستحثة في فئران التجارب.

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