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Synthesis and Biodistribution of ^{99m}Tc-PyDA as a Potential Marker for Tumor Hypoxia Imaging[1]

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Abstract—The ^{99m}Tc-pyrimidine-4,5-diamine (^{99m}Tc-PyDA) complex was prepared. The yield under the optimum conditions (5 mg of PyDA, pH 8, 25 μg of $SnCl_2 \cdot 2H_2O$) was 96 ± 3% . The complex is stable in vitro for 24 h. The complex was tested on mice as potential marker for tumor hypoxia imaging. The complex showed high tumor hypoxia uptake with the target/nontarget (T/NT) ratio as high as ~3.

Keywords: technetium-99m, pyrimidine, labeling, biodistribution, hypoxia, tumor, imaging

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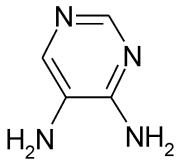
Molecular imaging is a well-defined technique which can visualize, characterize, and measure the biological processes at the molecular and cellular levels in humans and other living systems [1]. Radioisotope applications become involved in many life disciplines like industry, agriculture, biology, chemistry, and nuclear medicine [2]. Nuclear medicine field is interested in the design and synthesis of new radiolabeled agents for diagnosis and therapy [3]. The oxygen level in tissues is a key factor for the evaluation of the criticality of the disease progress and the treatment planning; however, its accurate noninvasive in vivo measurement is difficult. Nuclear medicine techniques introduce very good tools able to give informative images about deep and superficial hypoxic tumors using single photon emission computed tomography (SPECT) and positron emission tomography (PET). The corresponding equipment is already in routine use in many cancer centers [4, 5]. The development of hypoxia-sensitive radiotracer markers for noninvasive nuclear medicine imaging will allow hypoxic tumors to be revealed in early stages. Among such markers are ^{99m}Tc-citrofolate [6], ^{99m}Tc-N_2S_2-Tat(49–57)-bombesin [7] ^{18}F-fluoromisonidazole (FMISO) [8, 9], ^{123}I-iodoazomycin arabinoside (IAZA) [10], ^{99m}Tc-bombesin [11], and ^{99m}Tc-meropenem [12]. Due to the excellent physical properties of ^{99m}Tc (ideal half-life of 6.02 h and ideal γ-ray energy of 140 keV) and to its low cost and good availability, researchers' interest in ^{99m}Tc-labeled compounds as hypoxic tumor radiotracer markers increases [13, 14]. As organic compounds containing amine nitrogen atoms are able to bind ^{99m}Tc very strongly, numerous complexes with ligands bonded to ^{99m}Tc via one or more amine nitrogen atoms have been reported, such as ^{99m}Tc-HL91 [15. 16], ^{99m}Tc-2-nitroimidazole cyclam derivatives [17], ^{99m}Tc-nitroimidazole complexes [18], ^{99m}Tc-polyamine analogs [19], ^{99m}Tc$(CO)_3 \cdot$ (IDA–PEG3–CB)]$_2$ [20], 2-, 4-, 5-substituted nitroimidazole–iminodiacetic acid–^{99m}Tc$(CO)_3$ complexes [21], ^{99m}Tc-labeled bisnitroimidazole propylenamine oxime complexes [22], ^{99m}Tc-BnAO-NI [23], ^{99m}Tc(CO_3)-labeled chlorambucil analog [24], and ^{99m}TcN-MAG-AMCPP [25]. The pyrimidine group is the key fragment of many drugs such as antimicrobial, analgesic, antiviral, antiinflammatory, anti-HIV, antitubercular, antitumor, antimalarial, diuretic, and cardiovascular agents and hypnotic drugs, so many researches are focusing on the synthesis of new compounds containing pyrimidine moiety [26. 27]. As diaminopyrimidine is a precursor for different pharmacologically active drugs [28–30], we performed experiments to label this agent. In this study, pyrimidine-4,5-diamine (PyDA, see below) was successfully radiolabeled with ^{99m}Tc, and the labeled product was evaluated as novel radiotracer for tumor hypoxia imaging.

[1] The text was submitted by the authors in English.

Factors affecting the radiolabeling process were studied in detail.



Pyrimidine-4,5-diamine

EXPERIMENTAL

Pyrimidine-4,5-diamine ($C_4H_6N_4$) was purchased from Sigma–Aldrich. All chemicals were of analytical grade and were used without additional purification. Deionized water was used in all experiments for preparing all the solutions. Technetium-99*m* was eluted as $^{99m}TcO_4^-$ from a $^{99}Mo/^{99m}Tc$ generator, Gentech, Turkey. A NaI(Tl) γ-ray scintillation counter (Scaler Ratemeter SR7 model, England) was used for the measurement of γ-ray radioactivity. HPLC was performed with a Hitachi device using Alphabond C18 125A 10U column (i.d. 3.9, length 300 mm, Japan).

Synthesis of ^{99m}Tc-PyDA complex. PyDA was labeled with ^{99m}Tc via direct labeling technique using $SnCl_2 \cdot 2H_2O$ to reduce ^{99m}Tc(VII) to lower oxidation state suitable for the complexation with PyDA. PyDA was dissolved in N_2-purged distilled water in an evacuated penicillin vial. The required amounts (1–25 mg) of PyDA were transferred into clean 10-mL vials which were kept under positive N_2 pressure. Then, 1 mL of phosphate buffer with required pH (3–9) was added. After that, the required amount of $SnCl_2 \cdot 2H_2O$ (5–100 μg) and then 100 μL of freshly eluted $^{99m}TcO_4^-$ solution (195 MBq) were added to each vial. The effect of the reaction time on the yield and the in vitro stability of the complex were also studied.

Determination of the labeling yield and in vitro stability was performed by ascending paper chromatography (PC) and HPLC.

Ascending paper chromatography was performed using Whatman no. 1 paper (Whatman International Ltd., Maidstone, Kent, the United Kingdom). Strips of Whatman no. 1 paper (13 cm long and 0.5 cm wide) were marked at a distance of 2 cm from the lower end and lined into 1-cm sections up to 10 cm. 1–2 μL of the reaction mixture was seeded using hypodermic syringe, and then the strips were developed in an ascending manner in a closed jar filled with N_2. One strip was developed using acetone as a developing solvent, from which the percent of free $^{99m}TcO_4^-$ was determined [it moved with the solvent front (R_f = 1), whereas ^{99m}Tc-PyDA and hydrolyzed reduced colloid remained at the origin], while the other strip was developed using an ethanol : water : ammonium hydroxide mixture (2 : 5 : 1) to determine the percent of reduced hydrolyzed ^{99m}Tc colloid [it remained at the origin (R_f = 0), whereas the complex and free $^{99m}TcO_4^-$ moved with the solvent front]. After complete development, the strips were dried and cut into 1-cm fragments, which were analyzed with a γ-ray scintillation counter. The yield of the ^{99m}Tc-PyDA complex was determined by subtracting the relative content of the colloid and free $^{99m}TcO_4^-$ from 100%.

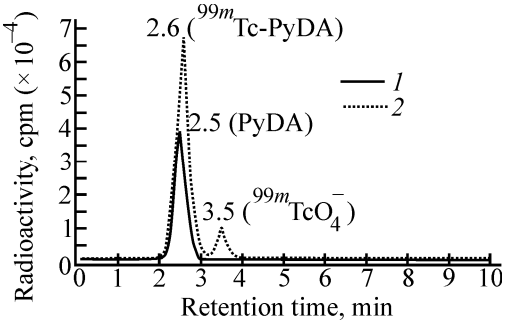


Fig. 1. Radiochromatogram of ^{99m}Tc-PyDA complex: (*1*) UV absorption and (*2*) radioactivity.

The labeling yield was further confirmed by HPLC. The sample volume was 10 μL. We used an SPD-6A UV detector operating at 254 nm. The mobile phase was a mixture of acetonitrile and 20 mM ammonium acetate buffer adjusted to pH 6.8 (45 : 55 by volume). The flow rate was 1 mL min^{-1}. Fractions of volume 0.1 mL were collected separately using a fraction collector up to a volume of 10 mL and counted with a γ-ray scintillation counter. The retention times of ^{99m}Tc-PyDA and free $^{99m}TcO_4^-$ were 2.6 and 3.5 min, respectively (Fig. 1).

Biodistribution. Experiments were performed using the procedure that was approved by the animal ethics committee and was in accordance with the guidelines set out by the Egyptian Atomic Energy Authority. Male Albino Swiss mice weighing 20–25 g were used.

Tumor hypoxia induction in mice. The parent tumor line (Ehrlich ascites carcinoma) was withdrawn from a 7-day-old donor female Swiss Albino mouse and was diluted with sterile physiological saline solution to a concentration of 12.5×10^6 cells mL^{-1}. Exactly 0.2 mL of the solution was then injected intramuscularly in the right thigh to produce a solid tumor. The animals were

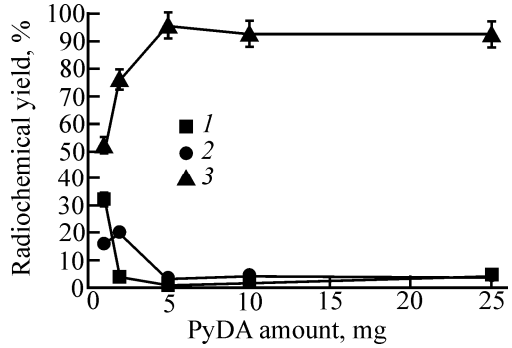


Fig. 2. Yield of ^{99m}Tc-PyDA as a function of PyDA amount. Reaction conditions: 25 μg of $SnCl_2 \cdot 2H_2O$, 100 μL (~195 MBq) of $^{99m}TcO_4^-$ solution, pH 8, room temperature, 35 min. (*1*) $^{99m}TcO_4^-$, (*2*) colloid, and (*3*) ^{99m}Tc-PyDA; the same for Figs. 3–5.

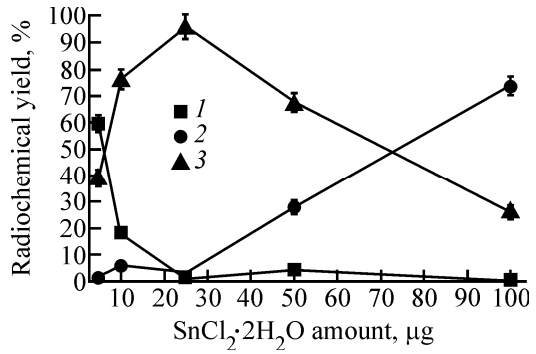


Fig. 3. Effect of $SnCl_2 \cdot 2H_2O$ amount on the yield of ^{99m}Tc-PyDA. Conditions: 5 mg of PyDA, 100 μL (~195 MBq) of $^{99m}TcO_4^-$ solution, pH 8, room temperature, 35 min.

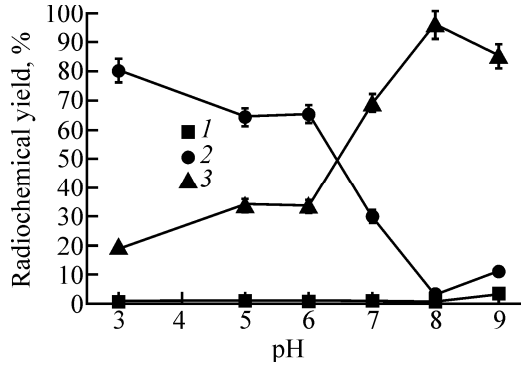


Fig. 4. Yield of ^{99m}Tc-PyDA as a function of pH. Reaction conditions: 5 mg of PyDA, 25 μg of $SnCl_2 \cdot 2H_2O$, 100 μL (~195 MBq) of $^{99m}TcO_4^-$ solution, room temperature, 35 min.

maintained until the tumor development was apparent (4–6 days).

Biodistribution assay. A 0.1-mL portion of a solution containing ^{99m}Tc-PyDA complex (185–1850 kBq) was injected intravenously into the tail vein of mice. Then, the animals were anesthetized at the predesigned time intervals (5, 15, 30, 60, and 120 min post injection), their body organs and fluids were separated and weighed, and their radioactivities were measured. The percentages of the injected dose per gram organ or fluid (% ID/g) were calculated. Differences in the data were evaluated with the Student's *t*-test (2-tailed test, level of significance $P < 0.05$). All the results are given as mean ± SEM.

RESULTS AND DISCUSSION

Factors affecting the labeling yield. *PyDA amount.* PyDA was successfully labeled with ^{99m}Tc via direct labeling technique, in which the reduced ^{99m}Tc species reacted with PyDA to form the labeled chelate, ^{99m}Tc-PyDA. The PyDA amount was varied in the range 1–25 mg. As seen from Fig. 2, at low PyDA amount (1 mg), the labeling yield was low (52.2 ± 1.3%), because this amount was insufficient to chelate all the reduced ^{99m}Tc species. The optimum PyDA amount was 5 mg, at which the maximum labeling yield was obtained (96 ± 3%). With a further increase in the PyDA amount to 25 mg, the labeling yield slightly decreased (to 92.2 ± 2.1%).

$SnCl_2 \cdot 2H_2O$ amount. The effect of the reducing agent ($SnCl_2 \cdot 2H_2O$) amount on the yield of ^{99m}Tc-PyDA is shown in Fig. 3. At the $SnCl_2 \cdot 2H_2O$ amount of 5 μg, the yield of ^{99m}Tc-PyDA was low, 39.0 ± 1.2%, because of incomplete reduction of $^{99m}TcO_4^-$ (the relative content of free $^{99m}TcO_4^-$ was 59.5 ± 1.4%). The labeling yield significantly increased as the $SnCl_2 \cdot 2H_2O$ amount was increased from 5 to 25 μg, reaching a maximum of 96 ± 3%. With a further increase in the $SnCl_2 \cdot 2H_2O$ amount, the labeling yield drastically decreased (to 26.3 ± 1.1% at 100 μg of $SnCl_2 \cdot 2H_2O$) because of the formation of colloidal species (73.6 ± 1.4%).

pH. The influence of pH of the reaction mixture on the yield of ^{99m}Tc-PyDA is shown in Fig. 4. At low pH, the labeling yield was low (19.0 ± 1.6% at pH 3). The maximum yield of ^{99m}Tc-PyDA was attained at pH 8 (96 ± 3%).

Reaction time. Figure 5 shows that the minimum reaction time needed to reach the maximum labeling yield of ^{99m}Tc-PyDA (96 ± 3%) was 35 min.

Thus, the maximum yield of ^{99m}Tc-PyAo was attained using 5 mg of PyDA and 25 μg of $SnCl_2 \cdot 2H_2O$ at pH 8 and reaction time of 35 min.

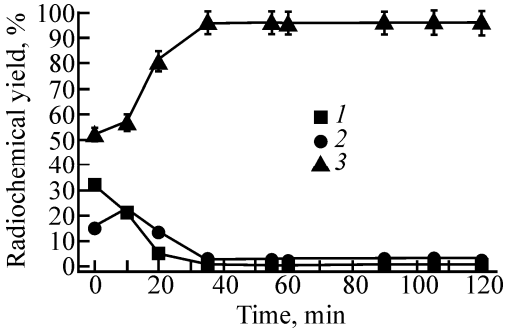


Fig. 5. Yield of ^{99m}Tc-PyDA as a function of time. Reaction conditions: 5 mg of PyDA, 25 μg of $SnCl_2 \cdot 2H_2O$, 100 μL (~195 MBq) of $^{99m}TcO_4^-$ solution, pH 8, room temperature.

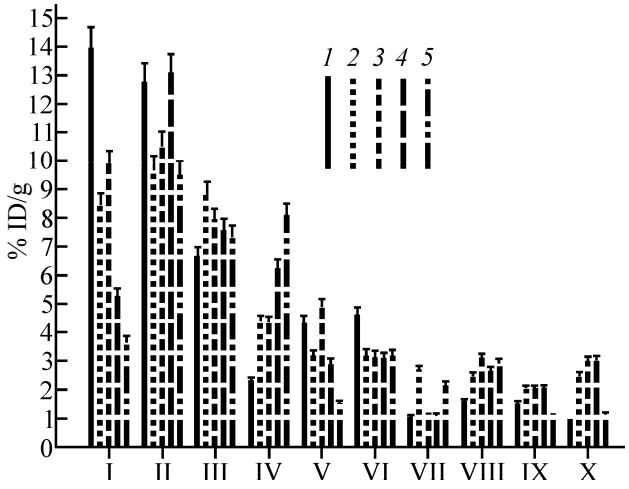


Fig. 6. In vivo biodistribution of ^{99m}Tc-PyDA in tumor hypoxia bearing Albino mice at different time intervals post injection (% ID/g, n = 3). (I) Blood, (II) kidneys, (III) liver, (IV) intestine, (V) heart, (VI) lungs, (VII) stomach, (VIII) bones, (IX) spleen, and (X) muscles. Time post injection, min: (*1*) 5, (*2*) 15, (*3*) 30, (*4*) 60, and (*5*) 120.

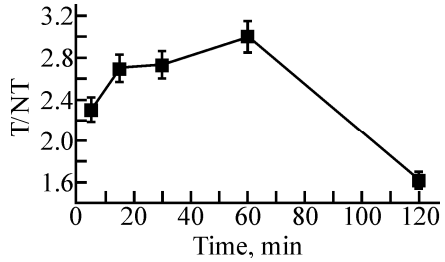


Fig. 7. T/NT ratio of ^{99m}Tc-PyDA in tumor hypoxia bearing Albino mice model as a function of time post injection.

In-vitro stability of ^{99m}Tc-PyDA was studied in order to determine the suitable time during which the preparation can be used. The complex appeared to be stable for 24 h after labeling.

Biodistribution. Figure 6 shows the biodistribution of ^{99m}Tc-PyDA in tumor hypoxia bearing mice (% ID/g) at 5, 15, 30, 60, and 120 min post injection. As can be seen, ^{99m}Tc-PyDA was mainly excreted via both urinary and hepatobiliary pathways, as kidneys showed 13.1 ± 0.7% ID/g at 60 min, liver showed 8.8 ± 0.4% ID/g at 15 min, and intestine showed 8.1 ± 0.4% ID/g at 120 min. ^{99m}Tc-PyDA was not specifically accumulated in any organs other than the tumor hypoxia tissue.

The main parameter for evaluating the selectivity and sensitivity of ^{99m}Tc-PyDA as tumor hypoxia imaging agent is the target/nontarget (T/NT) ratio between the tumor muscle (mouse right leg muscle) and normal muscle (mouse left leg muscle). Figure 7 shows the T/NT ratio of ^{99m}Tc-PyDA in tumor hypoxia bearing mice, which indicates that ^{99m}Tc-PyDA is highly selective to the tumor cells: The T/NT ratio is ~2.3 at 5 min post injection and reaches the highest value of ~3 at 60 min post injection. Thus, ^{99m}Tc-PyDA as a potential radiotracer marker for tumor hypoxia is not inferior to many other agents (the T/NT ratio and time post injection, respectively, are indicated): ^{99m}Tc-BnAO-NI (2.59, 2 h), $^{99m}Tc(CO)_3$-labeled chlorambucil analog (3.2, 3 h), ^{99m}TcN-MAG-AMCPP (1.83, 1 h), ^{99m}Tc-DETA (2.47, 4 h), ^{99m}Tc-TETA (2.45, 4 h), ^{99m}Tc-TEPA (2.91, 4 h), $[^{99m}Tc(CO)_3(IDA–PEG3–CB)]_2$ (3.45, 3 h), and ^{99m}Tc-nitride-pyrazolo[1,5-*a*]pyrimidine (2.2, 60 min) [20, 21, 24–26].

Thus, PyDA was radiolabeled with ^{99m}Tc by the direct labeling technique with a high yield, 96 ± 3%. The complex ^{99m}Tc-PyDA showed high in vitro stability and selective uptake in tumor cells (T/NT ~3 at 60 min post injection). The complex shows promise for clinical noninvasive evaluation of tumor hypoxia in vivo.

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