

Preparation of ^{99m}Tc -PQQ and preliminary biological evaluation for the NMDA receptor

Yanyan Kong · Xingqin Zhou · Guoxian Cao ·
Xijie Xu · Meifen Zou · Xiaofeng Qin ·
Rongjun Zhang

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Abstract Pyrroloquinoline quinone (PQQ), an essential nutrient, antioxidant, redox modulator and nerve growth factor found in a class of enzymes called quinoproteins, was labeled with ^{99m}Tc by using stannous fluoride (SnF_2) method. Radiolabeling qualification, quality control and characterization of ^{99m}Tc -PQQ and its biodistribution studies in mice were performed and discussed. Effects of pH values, temperature, time and reducing agents concentration on the radiolabeling yield were investigated. The quality control procedure of ^{99m}Tc -PQQ was determined by thin layer chromatography (TLC), radio high-performance liquid chromatography (RHPLC) and paper electrophoresis methods. The average radiolabeling yield was $94 \pm 1\%$ under optimum conditions of 0.99 mg of PQQ, 30 μg of SnF_2 , 0.5 mg of ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) and 18.5 MBq of $\text{Na}^{99m}\text{TcO}_4$ at pH 6 and 25 °C with a response volume of 1 ± 0.1 mL. ^{99m}Tc -PQQ was stable and anionic. Lipid–water partition coefficient of ^{99m}Tc -PQQ was -1.49 ± 0.16 . The pharmacokinetics parameters of ^{99m}Tc -PQQ were $t_{1/2\alpha} = 18.16$ min, $t_{1/2\beta} = 100.45$ min, $K_{12} = 0.013$ min $^{-1}$, $K_{21} = 0.017$ min $^{-1}$, $K_e = 0.016$ min $^{-1}$, AUC (area under the curve) = 1040.78 ID% g $^{-1}$ min and CL (plasma clearance) = 0.096 mL min $^{-1}$. The dual-exponential equation was $Y = 10.88e^{-0.038t} + 5.21e^{-0.0069t}$. The biodistribution

of ^{99m}Tc -PQQ was studied in ICR (Institute for Cancer Research 7701 Burhelme Are., Fox Chase, Philadelphia, PA 1911 USA) mice. In vitro autoradiographic studies clearly showed that the ^{99m}Tc -PQQ radioactivity accumulated predominantly in the hippocampus and cortex, which had a high density of *N*-methyl-D-aspartate Receptor (NMDAR). The enrichment can be blocked by NMDAR redox modulatory site antagonists-ebesen (EB) and ^{99m}Tc -PQQ is therefore a promising candidate for the molecular imaging of NMDAR. To date, however, there have been no studies characterizing ^{99m}Tc -PQQ.

Keywords Pyrroloquinoline quinone · ^{99m}Tc · ^{99m}Tc -PQQ · NMDAR

Introduction

PQQ (4,5-dihydro-4,5-dioxo-1H-pyrrolo [2,3-f] quinoline-2,7,9-tricarboxylic acid, Fig. 1) or methoxatin, existing in various plants and animal tissues, was identified as a coenzyme of methanol dehydrogenase from the methylo-trophic soil bacterium *Pseudomonas* sp. in 1979 [1]. PQQ is a well-characterized, free, heat-stable, anionic, water-soluble vitamin and redox cycling planar orthoquinone that possesses a variety of chemical properties [2]. PQQ is analogous to combining some of the best chemical features of ascorbic acid (reducing potential), riboflavin (redox reactions), and pyridoxal (PL) (carbonyl reactivity) cofactors into one molecule and is more efficient than those substance correspondingly [3].

It has been shown that PQQ could be involved in the treatment of neurological and psychological disorders. For example, it was demonstrated to have excellent

Y. Kong
Department of Nuclear Medicine, Huashan Hospital,
Fudan University, Shanghai 200040, China

X. Zhou · G. Cao (✉) · X. Xu · M. Zou · X. Qin · R. Zhang
Key Laboratory of Nuclear Medicine, Ministry of Health,
Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu
Institute of Nuclear Medicine, Wuxi, Jiangsu 214063, China
e-mail: jswxcx@126.com

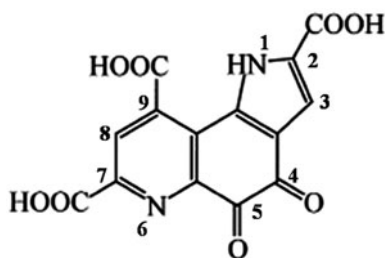


Fig. 1 The chemical structure of PQQ, shown here as 4,5-dioxo-H-pyrrolo(2,3-f) quinoline-2,7,9-tricarboxylic acid

neuroprotective efficacy in vitro and in vivo models of NMDAR-mediated or other neurotoxicant, such as methylmercury and 6-hydroxydopamine, induced neurotoxicity and electrical responses [4], to reduce the infarct size in animals with hypoxia and ischemia [5], to protect hippocampal neurons from the increase of the intracellular Ca^{2+} level and to be effective in an animal model of epilepsy [6]. PQQ significantly decreased the oxidative products of free radicals, reactive oxygen species (ROS), nitric oxide, peroxynitrite, [7] and so on. It also suppressed the lipid peroxidation, increased the antioxidant enzyme activities, attenuated the percentage of neuronal necrosis or apoptosis [8] and engaged in ameliorating the mitochondrial dysfunction [9]. On the other hand, it was also reported that PQQ had the ability to prevent fibril formation in the causative proteins of neurodegenerative proteins to prevent common neurodegenerative diseases [10]. PQQ also increased production of nerve growth factor (NGF) in some cell lines [11]. PQQ was potentially effective for preventing cognitive deficit in neurodegeneration caused by oxidative stress [12].

Aizenman et al. [13] have shown that PQQ can potentially oxidize the NMDAR redox site and thereby decrease activity of the NMDAR. Due to the unique physical characteristics, $\text{Na}^{99\text{m}}\text{TcO}_4$ was used in labeling process. Preparation of $^{99\text{m}}\text{Tc}$ -PQQ has been achieved by reacting the free ligand, PQQ (0.99 mg) and $\text{Na}^{99\text{m}}\text{TcO}_4$ in an acidic medium (HCl) in the presence of SnF_2 and EDTA-2Na. Labeling PQQ with $^{99\text{m}}\text{Tc}$ for nuclear medical imaging is yielding new insights into the diagnosis and treatment of neuropsychic diseases. Based on its water solubility and negative charge, PQQ in its free form would not be expected to easily cross the blood–brain barrier. However, it was reported that PQQ was capable of entering the brain following systemic administration, and particularly notable was the work by Smidt et al. [14]. In addition, possible metabolic products such as the oxazole derivative of PQQ cross the blood–brain barrier in order to exert effects on NGF and may be also neuroprotective [2]. With a molecular weight of 330 and specific interaction with

NMDAR redox site, PQQ may be a potential small molecule NMDAR tracer.

Experimental

Materials and methods

Materials

The free ligand, PQQ, was synthesized by our research team. SnF_2 anhydrous was obtained from J & K Chemical Technology Co. Ltd. EDTA-2Na was obtained from Sinopharm Chemical Reagent Co. Ltd. Ebselen (EB) was purchased from U.S. sigma company. All the chemicals used were analytical grade. $^{99\text{m}}\text{Tc}$ pertechnetate obtained from locally produced fission based $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator system (Chengdu Nuclear Isotope Qualcomm Inc.) was used for PQQ labeling.

Method

PQQ was synthesized as previously reported [15]. The structure characterization was measured by ^1H NMR spectra (AM2400 nuclear magnetic resonance apparatus, America BRU KER., Ltd.), ^{13}C NMR spectra (AM2400 nuclear magnetic resonance apparatus, America BRU KER., Ltd.) and MS spectra (MAT 2.2 mass spectrometer, America Varian company). The purity of PQQ was assayed by a HPLC performed on a Waters 600-type high-performance liquid chromatography (the United States Waters Corporation) equipped with a multi λ fluorescence detector (Waters 2475). An analytical Amethyst C18-H column (PN:320185-4615, $4.6 \times 150 \text{ mm } 5 \mu\text{m}$, SN:06060815870, Sepax Technologies, Inc.) was used. UV detector was set to 249 nm. The flow rate was adjusted to 0.8 mL min^{-1} after an injection volume of $5 \mu\text{L}$ PQQ with solvents 0.1% trifluoroacetic acid (TFA) in H_2O (solvent A) and 0.1% TFA in CH_3CN (solvent B). The HPLC gradient system started with a solvent composition of 95% A and 5% B and reached 25% A and 75% B in 20 min.

A series of studies were carried out to optimize labeling efficiency by varying a combination of factors: (a) varying the amount of PQQ ligand, (b) adjusting the reaction pH, (c) titrating the amount of SnF_2 and EDTA-2Na, (d) investigating the specific activity and volume of $^{99\text{m}}\text{Tc}$ pertechnetate used for the labeling reaction to match the concentration of PQQ ligand used in the formulation, (e) finding the optimized temperature and time for reaction. Each parameter was examined carefully for its effects on radiolabeling yield of the final product, $^{99\text{m}}\text{Tc}$ -PQQ. Radiolabeling of PQQ was completed in the presence of

SnF_2 and a sample was taken from the mixture for quality control by TLC, paper electrophoresis and RHPLC.

Labeling procedure

In the labeling of ^{99m}Tc -PQQ, 20, 50, 100, 200, 300, 400, 500, 600, 700 μL PQQ (9.9 mg of PQQ was dissolved in 3.0 mL of distilled water) were added to nine vials, respectively. To this red solution, quantitative SnF_2 solution and phosphate solution were added and then followed by the addition of EDTA-2Na and $\text{Na}^{99m}\text{TcO}_4$. To determine the optimal amount of reducing agent, 5 mg of SnF_2 was dissolved in 5 mL distilled water. The effect of its concentration (5, 10, 20, 40, 60, 80, 100, 120, 140 μL) on labeling efficiency was observed under the condition of quantitative PQQ, phosphate solution, EDTA-2Na and $\text{Na}^{99m}\text{TcO}_4$. Labeling was carried out at different pH values (3, 4, 5, 6, 7, 8, 9, 10) by adjusting the phosphate buffer pH with 0.1 mol L^{-1} ammonium hydroxide. 10 mg of EDTA-2Na was dissolved in 1 mL distilled water. Take 5, 10, 20, 50, 100, 200, 400, 600 μL of it to evaluate the effect of EDTA-2Na on the radiolabeling yield. After evaluation of all reagents, the total activity (1.85, 3.7, 7.4, 37, 55.5, 111, 185 and 370 MBq with the volume of 0.4 mL) and volume of $\text{Na}^{99m}\text{TcO}_4$ (0.1, 0.5, 1, 1.5 and 2 mL with the total activity of 18.5 MBq) was tested, respectively. All of these procedures mentioned above were incubated at 100 °C for 30 min. Above all, SnF_2 solution used in every procedure was always prepared freshly before use. Finally, labeling yield was studied by varying time interval (5, 10, 20, 30, 40 and 60 min at 100 °C and 0, 5, 10, 20, 40 and 60 min at room temperature, 25 °C).

Quality control of ^{99m}Tc -PQQ

The labeling yield, charge and radiochemical purity (RCP) were determined by TLC, paper electrophoresis and RHPLC.

TLC

Radiochemical yield of ^{99m}Tc -PQQ was assessed by TLC method using Xinhua No. 1 paper strips as stationary phase while acetone as the mobile phase. The reaction product was spotted on Xinhua No. 1 paper strips (10 \times 0.8 cm^2 sheets) and developed in acetone solution. After developing, the strips were dried at room temperature. Then, they were cut into 1 \times 0.8 cm^2 pieces and counted by Wizard 1470 automatic gamma counter (U.S. Perkin Elmer Company) equipped with a multi-channel analyzer. Retention factor (Rf) and labeling yield were determined from TLC chromatogram data.

Paper electrophoresis

The charge of ^{99m}Tc -PQQ was determined by paper electrophoresis using kalium phosphate buffer solution: alcohol: distilled water, 1:1:1 (V:V:V), of pH 7.4 as electrolyte and Xinhua No. 1 papers strips (19 \times 1 cm^2 sheets) as a support. The sample was run at a constant voltage of 150 V for 3 h of standing time. The strip was scanned by a Cd (Te) detector. For comparison, a sample of $\text{Na}^{99m}\text{TcO}_4$ and reduced/hydrolyzed ^{99m}Tc were also run under identical condition.

RHPLC

The RCP of ^{99m}Tc -PQQ was determined by a RHPLC performed on a Waters 600-type high-performance liquid chromatography (the United States Waters Corporation) equipped with a dual λ absorbance detector (Waters 2487), binary HPLC pump (Waters 1525) and Cd (Te) detector equipped with a flow scintillation analyzer (Perkin Elmer). The sample was passed through a Millipore filter carefully and injected into the HPLC column (Amethyst C18-H, PN:320185-4615, 4.6 \times 150 mm 5 μm , SN:06060815870, Sepax Technologies, Inc.). The absorbance for UV detector was measured at 249 nm. The flow rate was adjusted to 0.8 mL min^{-1} after an injection volume of 10 μL tracer with solvents 0.1% TFA in H_2O (solvent A) and 0.1% TFA in CH_3CN (solvent B). The HPLC gradient system started with a solvent composition of 95% A and 5% B and reached 25% A and 75% B in 25 min. RHPLC analysis of the labeled compound was conducted using a Cd (Te) detector. The eluted radioactivity was monitored on line using a NaI probe and collected fractions were also measured by the gamma counter.

Characterization of ^{99m}Tc -PQQ

Determination of lipid–water partition coefficient of ^{99m}Tc -PQQ

Lipid–water partition coefficient of ^{99m}Tc -PQQ was measured by adding 1 mL phosphate-buffered saline (PBS, pH = 7.4) saturated by *n*-octyl alcohol and 1 mL *n*-octyl alcohol saturated by PBS (pH = 7.4) to centrifuge tube containing 200 μL of sample. The tube was capped and vortexed vigorously for 5 min at room temperature then standed for 5 min. After reaching equilibrium, the tube was centrifuged at 2000 r min^{-1} ($r = 6.0$ cm) for 10 min. 100 μL of the organic phase and water phase were pipetted out respectively and each phase was counted in the gamma counter. 500 μL of the water phase was pipetted out in another centrifuge tube and then followed by the addition of 500 μL of PBS (pH = 7.4) saturated by *n*-octyl alcohol

and 1 mL *n*-octyl alcohol saturated by PBS (pH = 7.4). The above procedure was repeated for six times. The partition coefficient was calculated as (cpm in organic phase)/(cpm in water phase).

Stability studies

The stability of the complex was assessed by carrying out the complex at 25 °C for 5 h. The amount of ^{99m}Tc -PQQ was determined by TLC using acetone as eluent and the radioactivity of ^{99m}Tc -PQQ was counted by using a NaI (TI) detector at various time points (0, 1, 3, 4, 5 h).

Blood kinetic studies

The blood clearance studies were performed in ICR (Institute for Cancer Research 7701 Burhelme Are., Fox Chase, Philadelphia, PA 1911 USA) mice ($n = 6$, 25 ± 3 g). 9.62 MBq of the ^{99m}Tc -PQQ (0.2 mL) was administered intravenously through the tail vein. At different time intervals (2, 5, 10, 15, 30, 60, 120, 180, 240, 360 min) after intravenous injection, about 10 μL blood samples were withdrawn from the tail vein and radioactivity was measured by the gamma counter. The data from the experiment were expressed as percentage of administered dose at each time interval. The weight of each blood sample was determined by weighing the microcentrifuge tube before and after blood collection. The concentrations of radioactivity in the blood were calculated as $\%ID\text{ g}^{-1}$.

Autoradiography of NMDAR *in vitro* by storage phosphor imaging

Disturbances of activity of the glutamatergic neurotransmitter system in the brain are present in many neuropsychiatric disorders. NMDAR is the most abundant receptor of the glutamatergic system. As a result, (semi)quantitative imaging of the NMDAR may help in the diagnosis of neuropsychiatric disorders. What's more, Elias Aizenman et al. [13] studied the interaction of PQQ with the NMDAR redox modulatory site. Visualization of ^{99m}Tc -PQQ binding to NMDAR was performed by autoradiography of brain sections by means of Cyclone Storage Phosphor System (Perkin Elmer).

The healthy Sprague–Dawley (SD) rat (body weight 290 g) was sacrificed. The brains were excised, quickly frozen and sliced into 20 μm horizontal sections using temperature cold box frozen section machine (See TECHNIK GMBH). Every one in two sections was pasted on a glass plate. The glass plates with brain sections were infused in new environmental alcohol fixative for 30 min. Then the plates were taken out from fixative and dried at room temperature. Before the experiment, the slides were

shaken at room temperature for 30 min in buffer containing 50 mM Tris–HCl (pH 7.4) and 120 mM NaCl by WZ-2A-based micro-oscillator (Electronic medical instrument factory of Beijing Haidian). The slides were divided into two groups. To determine the pharmacological specificity and selectivity of ^{99m}Tc -PQQ receptor binding, 0.3 mg (60 μL) of NMDAR redox sites regulator—EB [16] was added to the slide of control group. The experimental group was pretreated with normal saline. These brain slides were preincubated for 30 min in room temperature. After incubation, the slides were washed thrice for 30 min in ice-cold Tris–HCl buffer, rinsed in cold distilled water, and dried by cold air. The brain sections were exposed to storage phosphor screen for 30 min. The imaging plates were scanned in the bio-imaging analyzer (Perkin Elmer) for approximately 30 min. In line with earlier study, relatively high uptake ratios were found in areas that express a high availability of NMDAR binding sites such as hippocampus and cerebral cortex while thalamus showed relatively lower uptake than hippocampus and cerebral cortex [17]. So the hippocampus was chosen as a brain area with high specific binding of ^{99m}Tc -PQQ and the thalamus was chosen as an area representative of low ^{99m}Tc -PQQ uptake. The data were recorded as digital light intensity units (DLU) mm^{-2} , which were proportional to the radioactivity of the measured sample. The hippocampus/thalamus ratios were calculated for each brain section separately to correct the measurements for slight variations in section thickness. The data analysis was carried out with the software program of OptiQuantTM by region of interest (ROI)-analysis. The phosphor plate was erased for 5 min.

Biodistribution studies in mice

Thirty-five ICR mice (25 ± 3 g) were used in animal experiments. ^{99m}Tc -PQQ, specific activity 0.296 MBq mg^{-1} (0.198 mg PQQ/rat), was injected into the tail vein of the mice and groups of five animals were sacrificed at various time points (5, 15, 30, 60, 120, 180 and 360 min) after injection. The organs of interest, samples of liver, spleen, pancreas, stomach, intestine, femur, muscle, gonad, lung, kidney, heart, brain and blood samples were removed and weighed, and the specific radioactivity in each tissue sample was counted using the gamma counter. Mice were injected intravenously with ^{99m}Tc -PQQ and their blood and visceral organs were evaluated for ^{99m}Tc -PQQ uptake as percent of the injected dose per gram wet weight of each tissue ($\%ID\text{ g}^{-1}$). The percent dose per organ was calculated by a comparison of the tissue counts to the counts of a suitably diluted aliquot of the injected material. In addition, various tissues were compared with respect to $\%ID\text{ g}^{-1}$ of ^{99m}Tc -PQQ at 5, 15, 30, 60, 120, 180 and 360 min. The concentrations of radioactivity in the

blood were calculated as $\%ID\ g^{-1}$, and $\%ID\ g^{-1}$ at 5 min was normalized to 100%.

Statistical analysis

Comparison of the differences in biodistribution results was performed using the software (SPSS 16.0 for Windows). The statistic of $\%ID\ g^{-1}$.organ was determined by univariate analysis of variance. Probability values of 0.05 were considered statistically significant.

The experiments were carried out in compliance with national laws for the conduct of animal experimentation and were approved by the local committee for animal research.

Results

Structure characterization and purity assay of PQQ

^1H NMR data (500MHZ, DMSO) 7.202 (s, 1H, H-C(3)), 8.602 (s, 1H, H-C(8)), 13.259 (br, 1H, NH); ^{13}C NMR data (DMSO) 113.351, 124.472, 126.394, 127.633, 128.941, 133.960, 135.514, 146.920, 148.560, 160.631, 164.712, 168.672, 173.231, 177.955. MS spectra MS(m/z): (M + 1): 331, (M + Na): 353, 375, 397, 419, 441. HPLC chromatogram analysis showed formation of PQQ was eluted at a retention time of 11.770 min. The purity of PQQ was more than 99%.

Radiolabeling procedure

The amount of labeling precursor PQQ plays an important role in radiolabeling yields. The effect of PQQ on the radiolabeling yields was examined at 20, 50, 100, 200, 300, 400, 500, 600, 700 μL ($3.3\ \text{mg}\ \text{mL}^{-1}$), with yields of 67.3, 77, 85, 92.4, 93.1, 94, 94, 94 and 94%, respectively. The optimized labeling yields were obtained at the amount of 200–300 μL with a concentration of $3.3\ \text{mg}\ \text{mL}^{-1}$. The effect of pH on the radiolabeling yields was examined at pH values of 3, 4, 5, 6, 7, 8, 9 and 10. pH plays an important role in effecting the reducibility of SnF_2 . Radiochemical species such as hydrolyzed technetium, ^{99m}Tc -stannous colloids and free pertechnetate are formed especially in alkaline pH medium. The optimized labeling yields were obtained at pH 6. In general, SnF_2 is the most widely used reducing agent. The effect of SnF_2 concentration on radiolabeling was studied in the range of 5, 10, 20, 40, 60, 80, 100, 120 and 140 μL ($1\ \text{mg}\ \text{mL}^{-1}$). The amount of SnF_2 , which gave the highest labeling efficiency, was 30 μL . Therefore radiochemical impurity may be due to formation of tin colloids. The amount of EDTA-2Na was tested in the range of 5, 10, 20, 50, 100, 200, 400 and

600 μL ($10\ \text{mg}\ \text{mL}^{-1}$), and the optimized labeling yield for it was obtained at 50 μL ($10\ \text{mg}\ \text{mL}^{-1}$). The effects of total activity (1.85, 3.7, 7.4, 37, 55.5, 111, 185 and 370 MBq with the volume of 0.4 mL) and different volumes of $\text{Na}^{99m}\text{TcO}_4$ (0.1, 0.5, 1, 1.5 and 2 mL with the total activity of 18.5 MBq) were tested, respectively. There was no significant difference observed from 0.1 to 2.0 mL for the reaction volume. Coming with the optimized labeling yields at 0.05–0.1 mL of $\text{Na}^{99m}\text{TcO}_4$ (14.8–18.5 MBq). Finally, the time and temperature on labeling yields were studied. The complexation of ^{99m}Tc with PQQ was rapid and maximum labeling efficiency was achieved after 20 min at room temperature.

TLC

In paper chromatography using acetone as the solvent, ^{99m}Tc -PQQ moved towards the solvent front ($R_f = 0.7$ – 1) with average radiolabeling yield of $94 \pm 1\%$, while reduced/hydrolyzed ^{99m}Tc remained at the point of spotting ($R_f = 0$) in control group without PQQ.

Electrophoresis

The negative charge of complex was confirmed by paper electrophoresis which showed that the ^{99m}Tc -PQQ species moved to anode indicating that the compound exhibited anionic behavior.

RHPLC

RHPLC chromatogram analysis revealed formation of unlabeled PQQ was eluted at a retention time of 10.8 min at UV detector, whereas labeled PQQ was eluted at a retention time of 5.2 min, the control group without PQQ ($\text{R-}^{99m}\text{Tc}$) was eluted at 2.2 min and the free pertechnetate ($^{99m}\text{TcO}_4^-$) was eluted at 2.5 min at Cd (Te) detector. The chromatograms obtained for PQQ, ^{99m}Tc -PQQ, $\text{R-}^{99m}\text{Tc}$ and free pertechnetate are shown in Fig. 2.

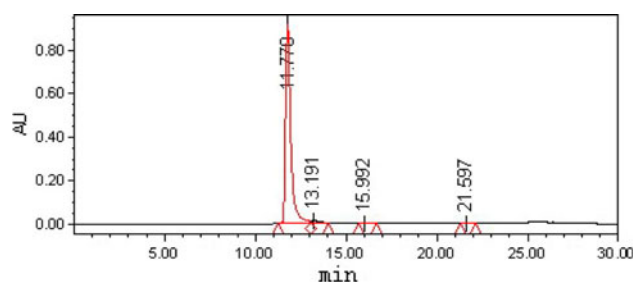


Fig. 2 HPLC chromatogram of PQQ

Determination of lipid–water partition coefficient of ^{99m}Tc -PQQ

The lipophilicity of ^{99m}Tc -PQQ is determined by partition coefficient, and the results are listed in Table 1 (Lipid–water partition coefficient, $\log P = -1.49 \pm 0.16$, $n = 5$).

Stability studies

The stability of the radiolabeled compound over time was also investigated. The radiochemical purity of ^{99m}Tc -PQQ is stable, remaining at the level about 90% up to hours (Fig. 3).

Blood kinetic studies

The blood clearance patterns of ^{99m}Tc -PQQ were simulated using software. A dual-exponential equation, $Y = b_1e^{-\alpha t} + b_2e^{-\beta t}$, was used. Here Y is %ID g^{-1} in blood; t is the time after injection; and b_1 , b_2 , α and β are constants. The distribution-phase half-time and elimination-phase

half-time ($t_{1/2\alpha}$ and $t_{1/2\beta}$) were calculated from the simulated dual-exponential curves as follows: $t_{1/2\alpha} = 18.16$ min and $t_{1/2\beta} = 100.45$ min. Equation was $Y = 10.88e^{-0.038t} + 5.21e^{-0.0069t}$ (Fig. 4, Table 2). The distribution and elimination are coincident with the results calculated by compartment model, and pharmacokinetics parameters of ^{99m}Tc -PQQ are as following (Table 2).

In vitro autoradiography

The regional in vitro distribution and accumulation of ^{99m}Tc -PQQ were measured in sections of rat brain. Autoradiograms of brain slides are shown in Fig. 5. Data were measured by ROI analysis on the obtained storage phosphor images and were presented for the experimental and EB pretreated group. The regions selected were all 3.3 mm^2 . The DLU mm^{-2} of ^{99m}Tc -PQQ in each target

Table 1 The lipid–water partition coefficient of ^{99m}Tc -PQQ

Organic phase (cpm)	Water phase (cpm)	Distribution ratio	Lipid–water partition coefficient $\log P$
97201.8	4301915.2	0.0226	−1.65
84039.6	2183392.9	0.0385	−1.41
46285.6	1070611.2	0.0432	−1.36
10272.6	238040.2	0.0432	−1.36
2540.1	119811.3	0.0212	−1.68

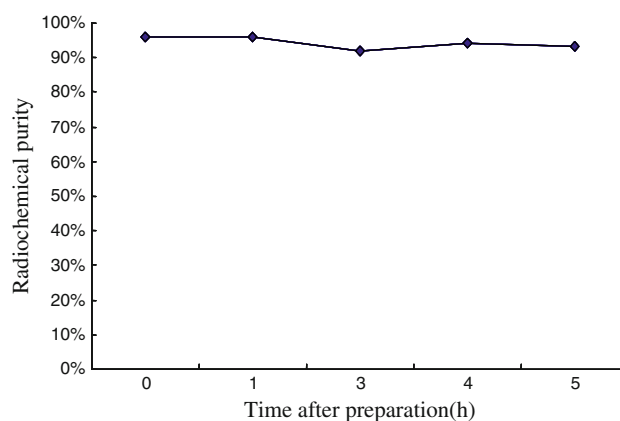


Fig. 4 Stability of ^{99m}Tc -PQQ at room temperature

Fig. 3 HPLC chromatogram (dash line) and RHPLC chromatograms (solid line) (PQQ $rt = 10.8$ min at UV detector $\lambda = 249$ nm) (^{99m}Tc -PQQ $rt = 5.2$ min, $\text{R-}^{99m}\text{Tc}$ $rt = 2.2$ min and $^{99m}\text{TcO}_4^-$ $rt = 2.5$ min at Cd (Te) detector)

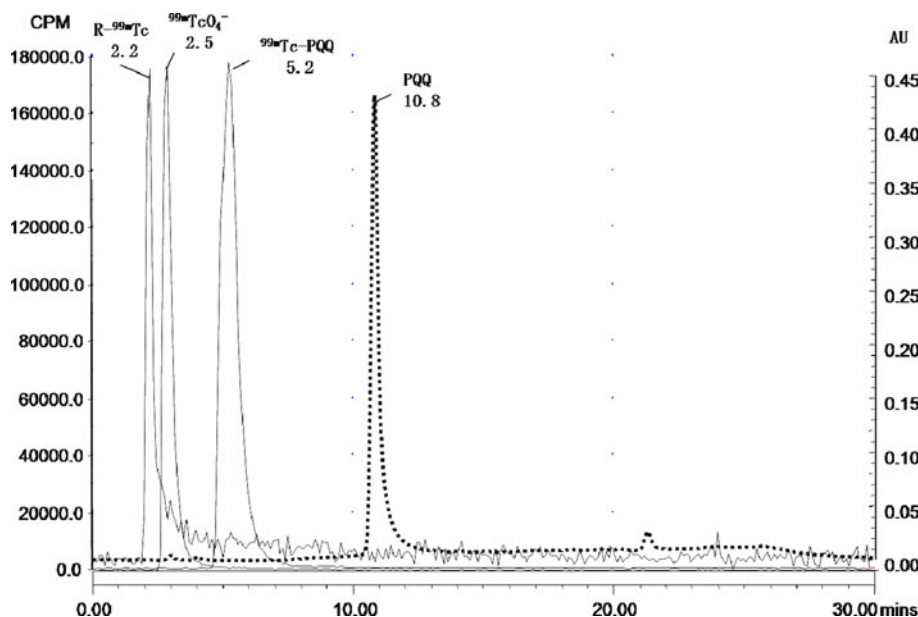
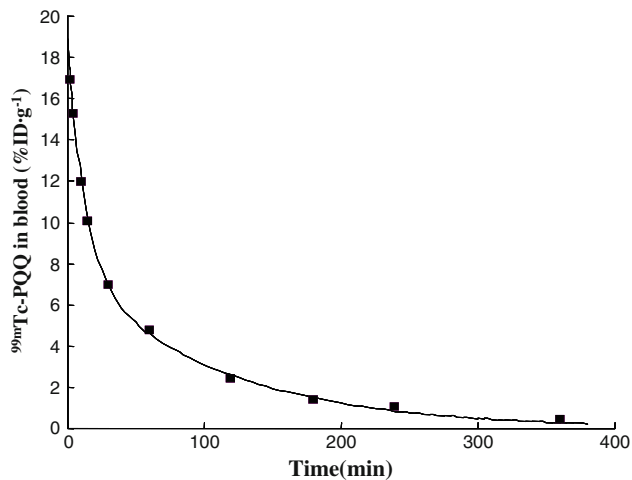


Table 2 Pharmacokinetics parameters of ^{99m}Tc -PQQ

Parameter	^{99m}Tc -PQQ
$t_{1/2a}$ (min)	18.16
$t_{1/2b}$ (min)	100.45
K_{12} (min^{-1})	0.013
K_{21} (min^{-1})	0.017
K_e (min^{-1})	0.016
AUC ($\% \text{ID g}^{-1} \text{ min}$)	1040.78
CL (mL min^{-1})	0.096

**Fig. 5** Time course of ^{99m}Tc -PQQ (closed square) in blood after intravenous administration of ^{99m}Tc -PQQ (9.9 mg kg^{-1}) to ICR mice ($n = 6$, mean $\% \text{ID g}^{-1} \pm \text{SD}$)**Table 3** The DLU/ mm^2 of ^{99m}Tc -PQQ in target region

	ID	Experimental group	Control group
Hippocampus	1	151779.0	107353.9
	2	123963.1	96932.7
Thalamus	3	96914.2	106355.9
	4	94419.8	92337.0

region was shown in Table 3. The hippocampus was chosen as the brain area of interest, since all obtained brain sections demonstrated the highest amount of radioactivity in this brain structure. Although slightly higher accumulation of ^{99m}Tc -PQQ was also found in the thalamus, the thalamus was chosen as the reference region. The hippocampus/thalamus ratios were 1.44 and 1.03 in experimental group and EB pretreated group, respectively. Obviously, the ^{99m}Tc -PQQ radioactivity accumulates predominantly in the hippocampus and cortex, areas known to have a high density of NMDAR. As has been shown, the accumulation of ^{99m}Tc -PQQ in several brain regions, such as the hippocampus and cortex, can be inhibited by EB (Fig. 6).

Biodistribution studies in mice

The change of ^{99m}Tc -PQQ uptake in various tissues over time is shown in Table 4. Smidt et al. have shown that 62% of [^{14}C] PQQ was absorbed in the lower intestine and 81% of the absorbed dose was excreted by the kidneys [14]. However, the distribution of radiolabeled PQQ in various tissues and the factors affecting this biodistribution had not been studied in detail. In our study, most of the activity was confined to stomach, liver and kidney tissue throughout the study ($\% \text{ID g}^{-1}$ value). Stomach tissue had a significantly greater uptake than all other tissues ($p < 0.05$), followed by liver, then by kidney. The lowest uptake was detected in the brain, muscle and skeleton. The major radioactivity may be metabolized by the hepatic and renal system. When the change in uptake over time was evaluated, the liver showed increasing uptake of ^{99m}Tc -PQQ from 5 min to 2nd hour with significant values ($p < 0.05$ for the 15, 30 min, 1st and 2nd hour vs. the 5 min). ^{99m}Tc -PQQ uptake in liver decreased in the 3rd and 6th hours of the study. The increase in uptake was also significant in stomach (15 vs. 5 min), small intestine (15, 30 vs. 5 min), ($p < 0.05$). ^{99m}Tc -PQQ uptake in brain, kidney, heart, spleen, lung, gonads, muscle and skeleton tissue remained almost decreased throughout the study. However, there was more ^{99m}Tc -PQQ uptake in kidney than in liver in the first 15 min ($p < 0.05$). The activity in brain tissue reached its maximum by the 5 min and decreased significantly thereafter ($p < 0.05$). The low uptake of ^{99m}Tc -PQQ in the mice brain is probably due to its negative charge and low lipophilicity (lipid–water partition coefficient $\log P = -1.49 \pm 0.16$). Further studies are needed to improve the uptake of ^{99m}Tc -PQQ in brain.

Discussion

As glutamate neurotransmitter plays an important role in numerous neuropsychic disorders, the NMDAR is the target for research to develop an appropriate ligand for non-invasive imaging. Many potential radiotracers for in vivo labeling of activated NMDAR have been evaluated previously. The interaction of unlabeled PQQ for the NMDAR was previously reported by Aizenman et al. [13].

Due to operational complexity and high cost, ^{14}C with long half-life is limited to the labeling with PQQ. Considering the moderate energy (140 keV), half-life (6 h) and variety of source, ^{99m}Tc is the most widely used radionuclide in nuclear medicine for imaging. The various complexes of ^{99m}Tc -PQQ may be formed by interaction between electron donor atoms in the structure of PQQ and reduced technetium. In order to form bonds with technetium, the labeled complex may be formed electron pairs of

Fig. 6 Autoradiography of NMDAR in vitro by storage phosphor imaging in vitro autoradiography of sagittal brain sections of rat shown the distribution of ^{99m}Tc -PQQ. (A, A': brain section without EB pretreated. B, B': control section of EB-pretreated, which displays a relatively low uptake of ^{99m}Tc -PQQ in the hippocampus.)

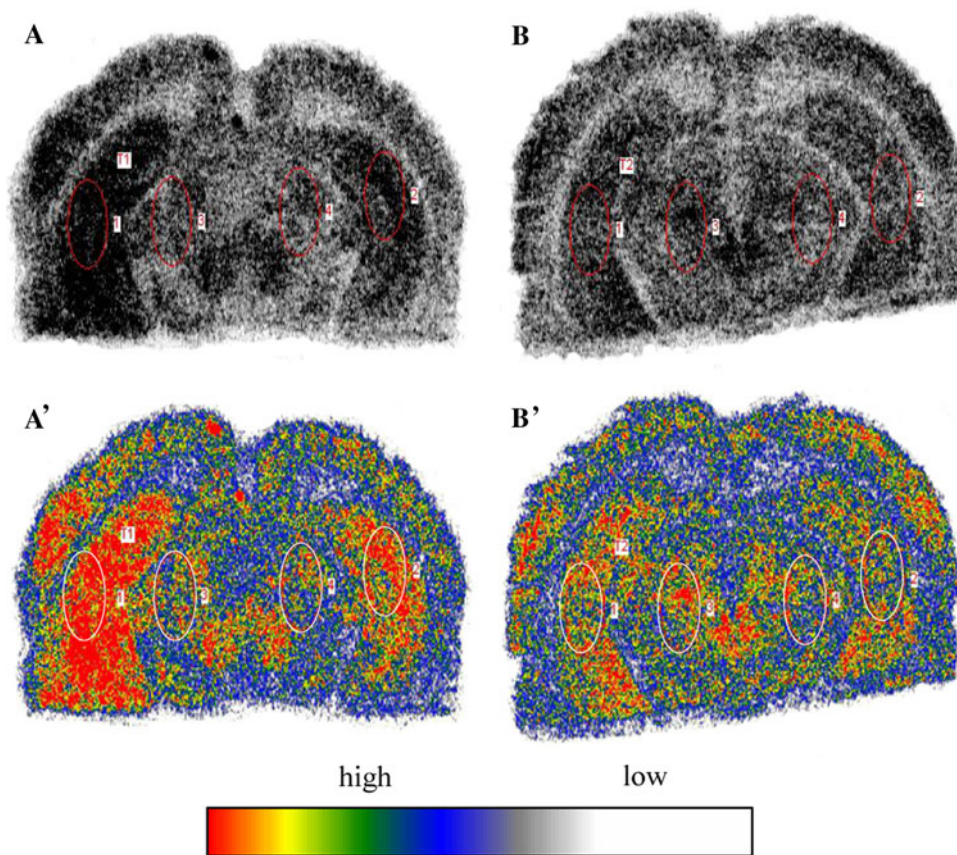


Table 4 Biodistribution of ^{99m}Tc -PQQ in mice (average \pm SD, $n = 35$, %ID g^{-1})

Time point							
Region	5 m	15 m	30 m	1 h	2 h	3 h	6 h
Heart	3.16 \pm 0.66	1.88 \pm 0.41	1.41 \pm 0.15	1.00 \pm 0.16	0.57 \pm 0.11	0.60 \pm 0.07	0.44 \pm 0.09
Liver	5.74 \pm 0.55	5.48 \pm 1.08	6.78 \pm 1.60	8.24 \pm 1.57	8.06 \pm 0.37	6.38 \pm 1.54	6.19 \pm 1.36
Spleen	3.25 \pm 0.82	2.11 \pm 0.61	1.86 \pm 0.11	1.44 \pm 0.25	1.17 \pm 0.22	1.38 \pm 0.88	0.72 \pm 0.21
Lung	7.49 \pm 0.69	4.91 \pm 0.82	4.11 \pm 0.63	2.88 \pm 0.87	2.33 \pm 0.74	1.82 \pm 0.85	0.90 \pm 0.32
Kidney	8.90 \pm 0.07	7.04 \pm 1.62	5.84 \pm 0.33	5.72 \pm 0.60	4.60 \pm 1.27	5.73 \pm 0.59	2.97 \pm 1.69
Stomach	13.19 \pm 2.85	16.46 \pm 3.67	15.11 \pm 4.52	16.25 \pm 2.65	10.70 \pm 3.53	11.80 \pm 3.00	9.45 \pm 3.86
Intestine	2.61 \pm 0.41	2.69 \pm 0.30	2.94 \pm 1.54	2.61 \pm 0.37	1.69 \pm 0.83	1.63 \pm 0.57	1.38 \pm 0.80
Gonads	1.12 \pm 0.17	1.69 \pm 0.55	0.96 \pm 0.43	0.90 \pm 0.15	0.61 \pm 0.30	1.27 \pm 0.38	0.21 \pm 0.07
Muscle	1.20 \pm 0.32	0.99 \pm 0.40	0.66 \pm 0.15	0.51 \pm 0.13	0.31 \pm 0.05	0.32 \pm 0.07	0.20 \pm 0.05
Skeleton	0.84 \pm 0.51	1.35 \pm 0.28	1.38 \pm 0.20	0.70 \pm 0.20	0.37 \pm 0.18	0.45 \pm 0.04	0.25 \pm 0.04
Blood	9.53 \pm 1.58	6.60 \pm 1.06	4.46 \pm 0.50	2.78 \pm 0.94	3.42 \pm 0.78	1.29 \pm 0.12	0.80 \pm 0.36
Brain	0.26 \pm 0.04	0.16 \pm 0.02	0.12 \pm 0.02	0.08 \pm 0.00	0.05 \pm 0.01	0.04 \pm 0.00	0.03 \pm 0.00

these atoms with reduced technetium that is +1 or +3 in the reduced states similar to other studies. The preparation of ^{99m}Tc -PQQ provided a convenient and promising way to investigate NMDAR. Smidt et al. [14] suggested that [^{14}C] PQQ was mainly excreted by kidneys. Our data of biodistribution in vivo displayed that the major radioactivity was metabolized by the hepatic and renal systems, what's

more, kidney was the main excretory organ in the first 15 min and liver was the primary excretory organ thereafter. Meanwhile, our study has shown that ^{99m}Tc -PQQ was quickly distributed from blood to another organs ($t_{1/2} = 18.16$ min), and had relatively favorable retention time in target organs ($t_{1/2\beta} = 100.45$ min). Although ^{99m}Tc -PQQ has a $\log P$ value of -1.49 ± 0.16 , it is able to

pass the blood–brain barrier in biodistribution study in vivo and it may be not so lipophilic that it would have prohibitively high nonspecific binding in brain. Due to the low lipophilicity, researches are carried out to improve the uptake of ^{99m}Tc -PQQ by our research team. We have performed a traditional blocking experiment with a pharmacological dose of another NMDAR antagonist (EB). The distribution of ^{99m}Tc -PQQ in the brain was consistent with the known region of NMDAR in autoradiography. In view of our results and the above considerations, ^{99m}Tc -PQQ appears to be a suitable ligand for in vivo imaging of changes in NMDAR in animals. Future studies should focus on the sensitivity of ^{99m}Tc -PQQ to detect pathological changes of NMDAR in animal models so as to be required in serial assessment of diagnosis and therapy in individual neuropsychiatric diseases.

Conclusions

A simple method for radiolabeling of PQQ with ^{99m}Tc has been developed and standardized. The most consistent and reliable formulation contained 0.99 mg of PQQ, 30 μg of SnF_2 , 0.5 mg of EDTA-2Na, $\text{Na}^{99m}\text{TcO}_4$ (0.1 mL, 17.5 MBq), a buffer with a pH value of 6. Reaction mixture volume used in all experiments was 1 ± 0.1 mL. As a result, PQQ was labeled with ^{99m}Tc with high labeling yield of $94 \pm 1.0\%$. The resulting complex of ^{99m}Tc -PQQ was quite stable, and radiochemical purity of more than 90% was maintained for up to 5 h at room temperature. Blood kinetic studies revealed the dual-exponential equation was $Y = 10.88e^{-0.038t} + 5.21e^{-0.0069t}$ with parameters of $t_{1/2\alpha} = 18.16$ min, $t_{1/2\beta} = 100.45$ min, $K_{12} = 0.013$ min^{-1} , $K_{21} = 0.017$ min^{-1} , $K_e = 0.016$ min^{-1} , $\text{AUC} = 1040.78$ $\text{ID}\%$ g^{-1} min and $\text{CL} = 0.096$ mL min^{-1} . The radiopharmaceutical potential of ^{99m}Tc -PQQ in animals was investigated and it was found that ^{99m}Tc -PQQ had high specificity and selectivity to bind to NMDAR according to autoradiography. For these reasons, ^{99m}Tc -PQQ might be a promising

candidate for the molecular imaging of NMDAR. Further studies are needed to apply ^{99m}Tc -PQQ for neurological and psychical disorders imaging.

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