Preparation of 99mTc-PQQE and preliminary biological evaluation for the NMDA receptor post-print

Authors: ZHOU Xingqin, KONG Yanyan, ZOU Meifen, ZHANG Jiankang, CAO Guoxian

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Abstract

The 4,5-dioxo-4,5-dihydro-1H-pyrrolo(2,3-f)quinoline-2,7,9-tricarboxylic acid 2-ethyl ester 7,9-dimethyl ester (PQQE) was synthesized on the basis of Pyrroloquinoline quinine (PQQ). 99mTc-PQQE was prepared using stannous fluoride (SnF2) as reducing agent. Biological characteristics of 99mTc-PQQE include lipophilic and the charge properties were compared to 99mTc-PQQ. The biodistributions of 99mTc-PQQE in mice and brain regional distribution were performed. In vivo distribution of 99mTc-PQQE in mice indicates that the concentration ratio of drug and blood increases steadily over time. The major radioactivity may be metabolized by the hepatic and renal system. The elimination-phase half-time (t1/2β) results indicate that the residence time of 99mTc-PQQE (203.92) in the body is twice as long as 99mTc-PQQ (100.45). The uptake of 99mTc-PQQE in brain was improved due to the ameliorating of charge and lipophilicity. The highest total regional brain uptake of 99mTc-PQQE was in the frontal lobe and hippocampus, where the NMDA receptor is very abundant. 99mTc-PQQE had a good target to nontarget ratio (hippocampus/cerebellum) which preserved a higher value (peak 4.0 at 120 min) from 60 min to 180 min after injection. In vitro autoradiographic results are in close agreement with the regional brain map. The enrichment can be blocked by N-methyl-D-aspartate receptor (NMDAR) redox modulatory site antagonists-ebselen (EB). This work suggests that 99mTc-PQQE has some specific targeting to the NMDA receptor.

Full Text

Preamble

Preparation of 99mTc-PQQE and Preliminary Biological Evaluation for the NMDA Receptor

ZHOU Xingqin*, KONG Yanyan, ZOU Meifen, ZHANG Jiankang, CAO Guoxian
Key Laboratory of Nuclear Medicine, Ministry of Health; Jiangsu Key Laboratory of Molecular Nuclear Medicine, Jiangsu Institute of Nuclear Medicine, Wuxi 214063, China

Abstract

The 4,5-dioxo-4,5-dihydro-1H-pyrrolo(2,3-f)quinoline-2,7,9-tricarboxylic acid 2-ethyl ester 7,9-dimethyl ester (PQQE) was synthesized based on pyrroloquinoline quinone (PQQ). 99mTc-PQQE was prepared using stannous fluoride (SnF2) as a reducing agent. Biological characteristics of 99mTc-PQQE, including lipophilicity and charge properties, were compared to those of 99mTc-PQQ. Biodistribution studies of 99mTc-PQQE were performed in mice, and brain regional distribution was examined. In vivo distribution in mice indicated that the drug-to-blood concentration ratio increased steadily over time. The major radioactivity appeared to be metabolized by the hepatic and renal systems. The elimination-phase half-time (t1/2β) results indicated that the residence time of 99mTc-PQQE (203.92 min) in the body is twice as long as that of 99mTc-PQQ (100.45 min). Brain uptake of 99mTc-PQQE was improved due to modifications in charge and lipophilicity. The highest regional brain uptake of 99mTc-PQQE occurred in the frontal lobe and hippocampus, where NMDA receptors are most abundant. 99mTc-PQQE exhibited a favorable target-to-nontarget ratio (hippocampus/cerebellum), maintaining a high value (peak 4.0 at 120 min) from 60 to 180 minutes post-injection. In vitro autoradiographic results were in close agreement with the regional brain distribution pattern. This enrichment could be blocked by the N-methyl-D-aspartate receptor (NMDAR) redox modulatory site antagonist ebselen (EB). This work suggests that 99mTc-PQQE exhibits specific targeting to the NMDA receptor.

Key words: PQQE, 99mTc-PQQE, NMDA receptor, biodistribution, in vitro autoradiography assay

Introduction

Previous studies reported that electron-rich aromatic and poly-anionic compounds show potential as therapeutic agents for mental disorders because they can inhibit the replication of PrP-res protein [1,2]. Representative compounds include anthracene helix (co-conjugate quinone) and Congo red (poly-anionic). Pyrroloquinoline quinone (PQQ) readily reacts with different functional groups, such as amino acids, alcohols, and nucleophiles [7,8].

Recently, PQQ and its derivatives have received attention for their ability to inhibit amyloid fibril formation [9] and protect the brain against oxidative damage [10,11]. Moreover, in human fibroblast cultures, PQQ and imidazolopyrroloquinoline, a PQQ derivative, enhance cell growth and proliferation when added at nmol/L concentrations [12]. 4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid is a redox cofactor found in plants and animal tissues

[3-6]. It shares very similar functional groups (conjugated quinone and anions) with the two aforementioned compound types. It is an aromatic heterocyclic anionic orthoquinone that can act as an electron donor or at the NMDAR redox reaction site, readily reacting with nucleophiles to form stable condensation products [13].

In the brain, the N-methyl-D-aspartate receptor (NMDAR) is considered among the most critical molecular regulators of cognition, memory, motor control, and synaptic plasticity. 99mTc-PQQ was previously prepared in our laboratory and found to potentially accumulate predominantly in the hippocampus and cortex, which have a high density of NMDAR [14]. However, 99mTc-PQQ is water-soluble, negatively charged, and possesses chelating groups that further limit its ability to cross the blood-brain barrier (BBB). Therefore, our research goal became to modify the structure of 99mTc-PQQ to enable BBB penetration. In this work, 4,5-dioxo-4,5-dihydro-1H-pyrrolo(2,3-f)quinoline-2,7,9-tricarboxylic acid 2-ethyl ester 7,9-dimethyl ester (PQQE) (Fig.1) was synthesized based on PQQ and labeled with 99mTc. Biological characteristics of 99mTc-PQQE, including lipophilicity and charge properties, were compared with those of 99mTc-PQQ. Biodistribution of 99mTc-PQQE in mice and brain regional distribution were studied, and in vitro autoradiography assay was further performed.

Fig.1 Chemical structures of PQQ (a) 4,5-dioxo-H-pyrrolo and PQQE (b) (2,3-f) quinoline-2,7,9-tricarboxylic 4,5-dioxo-4,5-dihydro-1H-pyrrolo quinoline-2,7,9-tricarboxylic acid 2-ethyl ester 7,9-dimethyl ester. (2,3-f)

2.1 Materials

The free ligand PQQE was synthesized in our laboratory. SnF2, EDTA-2Na, Na2HPO4, KH2PO4, and other reagents were of analytical grade. Ebselen (EB) was purchased from Sigma-Aldrich (USA). Polyamide thin-film was obtained from Taizhou Luqiao Sijia Bio-chemical Plastic Factory (China). 99mTc-pertechnetate obtained from a locally produced fission-based 99Mo/99mTc generator system was used for PQQE labeling.

2.2 Instruments

Instruments used included a Wizard 1470 γ-automatic counting device (Perkin Elmer, USA), AC22105 and AC2105-type analytical balances (Sartorius, Germany), J2-HS Centrifuge (Beckman, USA), quantitative bleeding vessel (Nantong Futura Medical Equipment Co., Ltd., China), C431200 Cyclone Storage Phosphor System (Perkin Elmer, USA), 99Mo-99mTc generator (Chengdu Nuclear Isotope Qualcomm Inc., China), and frozen section machine (Microtome-Cryostat MNT, Germany). High-performance liquid chromatography (HPLC) (600 system, Waters, USA) equipped with a UV detector (2487, Waters, USA) and radiomatic 610TR detector (Perkin Elmer, USA) was also employed.

2.3 Preparation of 99mTc-PQQE

PQQE was synthesized and characterized as previously reported in Ref.[15]. The purity of PQQE was assayed by HPLC, with content above 99.5%. 99mTc-PQQE was prepared using stannous fluoride (SnF2) as a reducing agent. Specifically, 50 L of EDTA-2Na solution (1 mg/mL), 50 L of SnF2 solution (1 mg/mL in 0.1 mol/L hydrochloric acid), 0.05 mL of PQQE (1 mg/mL), and 74 MBq of Na99mTcO4 were added into a penicillin vial, followed by addition of phosphate buffer (pH 6.5) to a total volume of 1 mL. The reaction vial was heated in boiling water for 30 minutes, then cooled to room temperature. Radiochemical purity of 99mTc-PQQE was tested by thin-layer chromatography (TLC) and HPLC equipped with a gamma-ray radiodetector and ultraviolet UV/Vis detector. 99mTc-PQQ was prepared as shown in our previous experiments [14].

2.4.1 TLC

The labeling yield of 99mTc-PQQE was assessed by TLC using polyamide thin-film as the stationary phase and acetone:N-hexane:triethylamine (3:1:1) as the mobile phase.

2.4.2 HPLC

The radiochemical purity (RCP) yield of 99mTc-PQQE was determined by reversed-phase HPLC performed on a C18 column (Lichropher, C18, 4.6$\times250mm, HanBang, China) using a flow rate of 0.8mL \cdot min^{-1}$ and UV detection at 249 nm (Fig.2). The solvent system consisted of 0.1% (v/v) trifluoroacetic acid (TFA) in acetonitrile (eluent A) and 0.1% (v/v) TFA in water (eluent B). The initial mobile phase composition was held at 5/95 (A/B) for 5 minutes, followed by a linear gradient to 75/25 (A/B) from 5 to 20 minutes, and then returned to 5/95 (A/B) from 20 to 25 minutes. Eluted radioactivity was monitored online using a NaI probe, and collected fractions were measured by the gamma counter. Data were collected and processed with Waters Empower software.

2.4.3 Stability Study

The stability of 99mTc-PQQE was studied at 25°C for 6 hours (Fig.3). The RCP of 99mTc-PQQE was determined by HPLC at various time points (0, 1, 2, 3, 4, 5, 6 h).

Fig.2 HPLC chromatograms of $99mTcO_4^-$ (a), 99mTc-PQQE (b), 99mTc-colloid (c) and PQQE (d). (PQQE tR = 14.1 min at UV detector λ=249 nm) (99mTc-PQQE tR=3.6 min, 99mTc-colloid tR=2.2 min and $[99mTcO_4]^-$ tR=2.5 min at Cd(Te) detector). (Lichropher, C18, 4.6$\times250mm, 0.8mL \cdot min^{-1}$, 249 nm. Eluent A: 0.1% TFA in acetonitrile, Eluent B: 0.1% TFA in water. 5/95 (A/B) for 5 min gradient to 75/25 (A/B) from 5 to 20 min and then to 5/95 (A/B) from 20 to 25 min.

2.5 Octanol/Water Partition Coefficients and Plasma Protein Binding

2.5.1 Octanol/Water Partition Coefficient

Octanol/water partition coefficients were measured by the following procedure: 1 mL of PBS (pH=7.4) saturated with 1-octanol and 1 mL of 1-octanol saturated with PBS were added to a centrifuge tube containing 0.2 mL of 99mTc-PQQE (0.37 MBq). The tube was capped and vigorously vortexed for 5 minutes at room temperature, then allowed to stand for 5 minutes. After reaching equilibrium, the tube was centrifuged at 2400 r/min for 10 minutes. Aliquots were taken from each phase and radioactivity was counted. The partition coefficient was calculated by dividing the radioactivity of the 1-octanol layer by that of the aqueous layer. The partition coefficient (logP) was calculated using the following equation: P = (activity in octanol layer - background activity) / (activity in aqueous layer - background activity). All experiments were performed in triplicate (Table 1). The charge of 99mTc-PQQE was determined by paper electrophoresis according to previously reported methods [14].

2.5.2 Plasma Protein Binding

A mixture of 0.1 mL of 99mTc-PQQE (1.55 MBq, 0.16 MBq, 0.016 MBq) in 0.2 mL plasma was incubated for 3 hours at 37°C. Following equilibrium, 1 mL of 15% trichloroacetic acid was added to each tube, followed by vortexing and centrifugation at 2400 r/min for 10 minutes. The supernatant was collected and the steps were repeated four times. Radioactivity of the supernatant and residue was counted using a gamma counter. Plasma protein binding was calculated by the relationship: plasma protein binding = [(residue counts)/(residue counts + supernatant counts)] × 100% (Table 1).

Table 1 Plasma protein binding and PO/W values of 99mTc-PQQ and 99mTc-PQQE

Test items	99mTc-memantine derivatives	99mTc-PQQ	99mTc-PQQE
Plasma protein binding (%)	Middle (0.16 MBq) Low (0.016MBq) High (1.55 MBq)	-1.46$±0.16|0.72±$0.11	
PO/W value		negative	neutral
Electric charge		electrode	

2.6 Biodistribution in Mice

The biodistribution of 99mTc-PQQE was studied in normal Kunming mice to evaluate pharmacokinetic properties. A volume of 0.2 mL of the purified radio-

tracer solution (~10 MBq) was injected into mice via the tail vein. Mice (n=5, 20$±$2 g) were sacrificed at 5, 15, 30, 60, 120, 180, 240, and 360 minutes after injection. Organs and brain regions of interest were dissected, weighed, and counted for radioactivity. The percentage of injected dose per gram (%ID/g) was calculated (Table 2 , Table 4) [16].

Table 2 Biodistribution of 99mTc-PQQE in mice ($\bar{x}$±SD, n=5, %ID · g^{-1})

Regions	5 min	15 min	30 min	60 min	120 min	180 min	240 min	360 min
Heart	3.20$±0.53	2.68±0.13	2.65±0.71	0.65±0.13	0.58±0.23	0.45±0.06	0.36±0.06	0.25±0.08
Liver	6.04±0.							

2.8 In Vitro Autoradiography

99mTc-PQQE (0.2 mL, 10 MBq) was injected through the tail vein into mice $(20\$\pm 2g, n = 5). At different times (2, 5, 10, 15, 30, 60, 120, 180, 240, and 360 minutes), 10 \mu L$ blood was collected fr
1}$). The data were analyzed using 3p87 pharmacokinetic software to fit the appropriate compartment model.

Visualization of 99mTc-PQQE binding to NMDAR was performed by autoradiography of brain sections using storage phosphor imaging. Brains were excised and sliced into 20 µm horizontal sections from healthy Sprague-Dawley (SD) rats $(250\$\pm 10g)$. The slides were redivided into two groups and processed as previously reported [14]. 0.01 mol/L EB was ada
which were proportional to the radioactivity of the measured samples.

3.1 Radiochemistry

99mTc-PQQE was characterized by HPLC (Fig.2). Under the selected chromatographic conditions, it was possible to separate peaks corresponding to the complex 99mTc-PQQE (tR=3.6 min) from 99mTc-colloid (tR=2.2 min) and $[TcO_4]^-$ (tR=2.5 min). In TLC tests, $99mTcO_4^-$ and 99mTc-PQQE moved toward the solvent front ($99mTcO_4^-$, Rf=1.0; 99mTc-PQQE, Rf=0.6–0.7), while 99mTc-colloid remained at the point of spotting (Rf=0) in the control group without PQQE.

3.3 Stability Studies

The stability of the radiolabeled compound was investigated over time. The radiochemical purity of 99mTc-PQQE remained above 95% at room temperature for 6 hours (Fig. 3 [Figure 3: see original paper]).

3.4 In Vivo Biodistribution

In vivo distribution of 99mTc-PQQE in mice is shown in Table 2. The stomach exhibited significantly greater uptake than all other tissues ($p<0.05$), followed by lung and then liver. Uptake of 99mTc-PQQE in liver significantly increased from

5 to 120 minutes and decreased from 180 to 360 minutes during the study. There was greater 99mTc-PQQE uptake in liver than in kidney over time, suggesting that major radioactivity may be metabolized by the hepatic rather than renal system.

Brain uptake of 99mTc-PQQE was improved due to amelioration of charge and lipophilicity (Table 1). As shown in Table 2, brain uptake of 99mTc-PQQE was 2.3 times that of 99mTc-PQQ at 5 minutes [14].

Fig.4 Blood kinetic curve of 99mTc-PQQE in mice
Fig.3 Stability of 99mTc-PQQE at room temperature in vitro.

3.2 Partition Coefficients and Plasma Protein Binding % (compared to 99mTc-PQQ)

One important factor determining whether a drug penetrates the blood-brain barrier is its lipid-water partition coefficient. Table 1 shows that 99mTc-PQQE is lipophilic, whereas 99mTc-PQQ is water-soluble. Electric charge and plasma protein binding values are also listed in Table 1.

The compartment model for 99mTc-PQQE was determined to be a two-compartment model by regression analysis. The blood kinetic curve is shown in Fig.4. A dual-exponential equation, $Y=38.68e^{-0\cdot027}+7.25e^{-0\cdot003}$, was used, where Y represents $\%ID\cdot g^{-1}$ in blood and t represents time after injection. The distribution-phase half-time ($t1/2\alpha$) and elimination-phase half-time ($t1/2\beta$) were calculated from the simulated dual-exponential curves. Pharmacokinetic parameters of 99mTc-PQQE are summarized in Table 3 . The $t1/2\alpha$ results show that 99mTc-PQQ (18.16 min) distributed slightly more rapidly in vivo than 99mTc-PQQE (25.443 min). Moreover, $t1/2\beta$ results clearly indicate that the residence time of 99mTc-PQQE (203.916 min) in the body is twice as long as that of 99mTc-PQQ (100.45 min). Both 99mTc-PQQ and 99mTc-PQQE distribute quickly in vivo; however, clearance of 99mTc-PQQ is much faster than that of 99mTc-PQQE. In vivo distribution of 99mTc-PQQE in mice indicates that the drug-to-blood concentration ratio increases steadily over time.

Table 3 Pharmacokinetic parameters of 99mTc-PQQE in mice (6.66 MBq/0.2 mL, n=5)

Parameter	99mTc-PQQE	99mTc-PQQ
$t1/2\alpha$ / min		
$t1/2\beta$ / min		
K12 / min^{-1}		
K21 / min^{-1}		
Ke / min^{-1}		
AUC / $ID\%\cdot g^{-1}\cdot min$		

Parameter	99mTc-PQQE	99mTc-PQQ
CL / mL · min^{-1}		

Table 4 Biodistribution of 99mTc-PQQE in brain of mice ($\bar{x}$±SD, n=5, %ID · g^{-1})

Regions	5 min	15 min	30 min	60 min	120 min	180 min	240 min	360 min
Striatum	0.26$±0.03	0.21±0.03	0.21±0.01	0.12±0.01	0.09±0.02	0.09±0.04	0.07±0.01	0.05±0.01
Hippocampus/Cerebellum								
Frontal/Cerebellum								

3.6 Brain Regional Distribution of 99mTc-PQQE

The brain regional distribution of 99mTc-PQQE was evaluated by measuring uptake in regions of interest including striatum, hippocampus, thalamus, cerebellum, and cortex (frontal, occipital, parietal, and temporal lobes). The frontal lobe and hippocampus were used as target regions due to their high density of NMDA receptors, while cerebellum served as a non-target region due to its low NMDA receptor density. Brain regional distribution and target-to-nontarget ratios for 99mTc-PQQE are shown in Table 4. The highest regional brain uptake of 99mTc-PQQE occurred in the frontal lobe and hippocampus, where NMDA receptors are most abundant. 99mTc-PQQE demonstrated a favorable target-to-nontarget ratio (hippocampus/cerebellum) that maintained a high value (peak 4.0 at 120 min) from 60 to 180 minutes post-injection. The frontal/cerebellum ratio reached 3.4 at 120 minutes. Significant abnormalities have been reported in the superior frontal gyrus in schizophrenia [17], and the prefrontal cortex has been demonstrated to play a crucial role in modulating cognitive functions [18].

Fig.5 Autoradiography of 99mTc-PQQE in rat brain (Coronal plane) by storage phosphor imaging
A: Brain section without pretreatment
B: Control section of EB-pretreated.

Table 5 DLU/mm^2 of 99mTc-PQQE in target regions

Regions	Experimental groups (Fig.5a)	EB-pretreated group (Fig.5b)
Cortex		
Hippocampus		
Thalamus		
cortex/Thalamus		
Hippocampus/Thalamus		

3.7 In Vitro Autoradiography

The regional distribution of 99mTc-PQQE in rat brain was measured by autoradiograms (Fig.5). The cortex and hippocampus were selected as brain areas of interest, while the thalamus served as the reference region. Data were measured by ROI analysis on the obtained storage phosphor images and presented for both experimental and EB-pretreated groups. Obviously, uptake of 99mTc-PQQE in the cortex of the experimental group was higher than in the EB-pretreated group. The hippocampus/thalamus ratios were calculated in Table 5. As shown, accumulation of 99mTc-PQQE in several brain regions, such as the hippocampus and cortex (areas known to have high NMDAR density), can be inhibited by EB.

4 Conclusion

99mTc-PQQ was prepared and the binding between 99mTc-PQQ and NMDAR in vitro and in vivo was characterized [14]. However, 99mTc-PQQ is water-soluble, negatively charged, and possesses chelating groups that limit its brain uptake. To resolve this problem, BBB opening by mannitol was used to enable 99mTc-PQQ to enter the brain [19]. Furthermore, 99mTc-PQQE was prepared to improve lipophilicity and charge properties (Table 1). Here we characterized 99mTc-PQQE and mapped its regional distribution in brain. It is clear that brain uptake of 99mTc-PQQE was significantly higher than that of 99mTc-PQQ, with levels 2.3 times greater at 5 minutes. Target tissues with abundant NMDAR, such as the hippocampus and cortex, also showed increased uptake and retention of 99mTc-PQQE. Furthermore, the AUC of 99mTc-PQQE was 3553. AUC represents the area under the curve of plasma concentration versus time, and the extent of drug absorption is proportional to the AUC. This result suggests that 99mTc-PQQE can be easily taken up by tissue, thus contributing to high-quality imaging of target tissue. Further studies are needed to apply 99mTc-PQQE for NMDA receptor imaging. The AUC of 99mTc-PQQ is much smaller (1040).

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Note: Figure translations are in progress. See original paper for figures.