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Preparation, quality control and physico-chemical properties of ^{99m}Tc-BAT-AV-45

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Abstract One novel styrylpyridine derivatives(AV-45) coupled with ^{99m}Tc complex was synthesized. ^{99m}Tc-BAT-AV-45 was prepared by a ligand exchange reaction employing sodium glucoheptonate, and effects of the amount of ligand, stannous chloride, sodium glucoheptonate and pH value of reaction mixture on the radiolabeling yield were studied in details. Quality control was performed by thin layer chromatography and high performance liquid chromatography. Besides the stability, partition coefficient and electrophoresis of ^{99m}Tc-BAT-AV-45 were also investigated. The results showed that the average radiolabeling yield was (95 ± 1%) and ^{99m}Tc-BAT-AV-45 with suitable lipophilicity was stable and uncharged at physiological pH.

Keywords Styrylpyridine derivatives · Bisaminobisthiol · Technium-99m · β-Amyloid Plaques · Single photon emission computed tomography (SPECT)

Introduction

Alzheimer's disease (AD) is a form of dementia with progressive memory loss, irreversible cognitive decline, language impairment, and behavioral changes. The accumulation of β-amyloid (Aβ) aggregates in the brain is a defining pathologic feature for this disease [1, 2]. Nowadays, clinical diagnosis of AD is primarily done by assessment of clinical symptoms, which is often difficult and unreliable, and the only definitive diagnosis is established by pathological examination of the postmortem staining of affected brain tissue. Single photoemission tomography (SPECT) or positron emission tomography (PET) imaging of β-amyloid plaques in the living human brain may serve as a helpful tool for the early diagnosis of AD and monitoring of therapeutic effects.

Technetium-99m ($t_{1/2}$ = 6 h, 140 keV) is the most commonly used radionuclide in routine clinical nuclear medicine. Because ^{99m}Tc can be easily prepared with a ^{99}Mo/^{99m}Tc generator, emits the medium γ-ray energy suitable for γ-camera detection and has the ideal physical half-life compatible with biological localization and residence time required for imaging [3]. New ^{99m}Tc-labeled Aβ imaging agents will have more widespread clinical applicability for detecting and eventually quantifying β-amyloid plaques in living brain tissue.

The successful development of [Tc-99m]TRODAT-1 already showed the probability of ^{99m}Tc imaging agent that can penetrate the blood–brain barrier(BBB) via a simple diffusion mechanism and localize at sites in the central nervous system [4–6]. Based on this success, many efforts have been made so far to create a suitable Aβ plaques ligand based on ^{99m}Tc, but no clinical study of them has been reported [7–16].

(E)-2-(2-(2-(2-[^{18}F]Fluoroethoxy)ethoxy)ethoxy)-5-(4-methylaminostyryl)pyridine ([^{18}F]AV-45) with styrylpyridine core, and high affinity for Aβ plaques (Ki = 2.87 nM), which binds specifically to fibrillar Aβ and has favorable pharmacokinetic properties [17]. Preliminary clinical studies in patients with diagnosed mild AD showed notable retention of [^{18}F]AV-45 in the cortex, known to contain large amounts of amyloid deposits in AD [18, 19]. To develop more useful ^{99m}Tc-labeled probes for clinical diagnosis, we synthesized AV-45 derivatives with bis-amino-bis-thiol (BAT).We selected BAT as chelation

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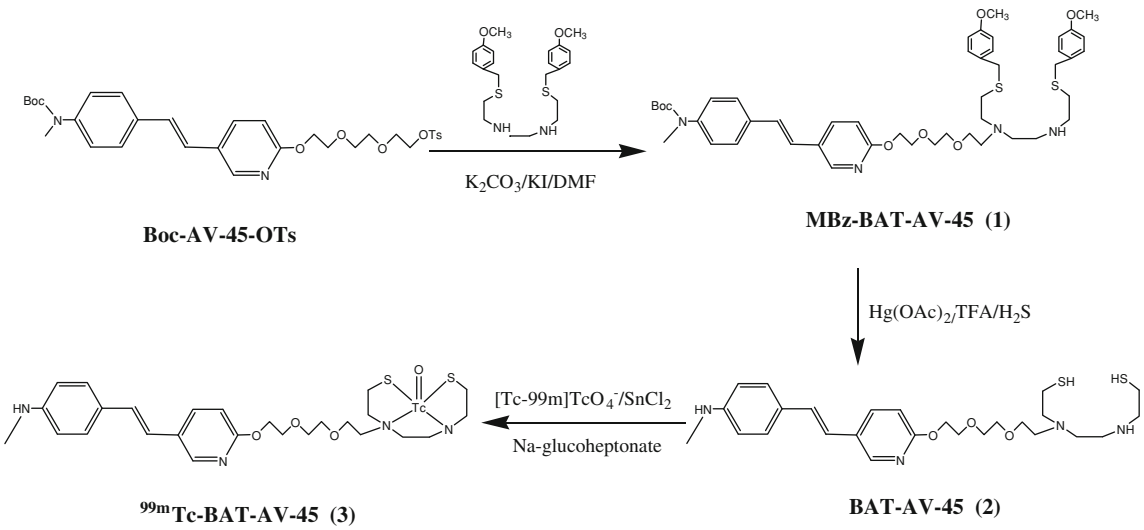

Scheme 1 Synthesis of AV-45 derivatives

ligands taking into consideration the permeability of BBB with forming an electrically neutral complex with ^{99m}Tc [19].

Here, we report the synthesis of the precursor for labeling by coupling styrylpyridine derivatives(AV-45) with N1,N2-[bis(4-methoxybenzyl) thioethyl] ethanediamine (MBz- BAT),the final free thiol ligands (BAT-AV-45) were obtained by deblocking the 4-methooxybenzyl protecting group with $Hg(OAc)_2$ (Scheme 1). Radiolabeling of BAT-AV-45 was carried outby using stannous chloride method. The quality control was determined by using thin layer chromatography and radio high performance liquid chromatography. The stability, partition coefficient and electrophoresis of ^{99m}Tc-BAT-AV-45 were investigated.

Experimental

Materials

All reagents were obtained commercially and used without further purification unless otherwise indicated.(E)-2-(2-(2-(5-(4-(tert-butoxycarbonyl(methyl)amino)styryl)pyridine-2-yloxy)-ethoxy)ethoxy)ethyl-4-methylbenzenesulfonate (Boc-AV-45-OTs) [20] and N1,N2-[bis(4-methoxybenzyl) thioethyl]ethanediamine(MBz-BAT) were self-synthesized. $Na^{99m}TcO_4$ was supplied by Jiangsu Institute of Nuclear Medicine. Electron spray ion(ESI) mass spectra was measured using a Waters Platform ZMD4000LC/MS. NMR spectra was obtained on a Bruker DRX-500 spectrometer, and the chemical shift value was given relative to the internaltetramethylsilane (TMS). Wizard 1470 automatic gamma counter equipped with a multi-channel analyzer (U.S. Perkin Elmer Company). RHPLC was performed on a Waters 600-type high-performance liquid chromatography (the United States Waters Corporation) equipped with a dural λ absorbance detector (Waters 2487), binary HPLC pump (Waters 1525) and Cd (Te) detector equipped with a flow scintillation analyzer (Perkin Elmer).

Synthesis of BAT-AV-45 ligand

(E)-2-(2-(2-(2-(N-(4-methoxybenzylthio)-(N-(N-(4-methoxybenzylthioethyl)aminoethyl))aminoe-thoxy)ethoxy)ethoxy)-5-(4-tert-butoxycarbonylmethylaminostyryl)pyridin (**MBz-BAT-AV-45, 1**).

Boc-AV-45-OTs (0.21 g, 0.4 mmol) and N1,N2-[bis(4-methoxybenzyl) thioethyl] ethanediamine(MBz-BAT) (0.19 g, 0.45 mmol) were dissolved in anhydrous DMF (10 mL), Potassium carbonate(0.12 g, 0.81 mmol) and Potassium iodide(0.07 g, 0.09 mmol) were added. The reaction mixture was stirred at 60 °C for 24 h. After the evaporation of the solvent, water was added. The mixture was extracted with ethyl acetate. The organic layers were combined and dried with Na_2SO_4. Evaporation of the solvent gave a residue, which was purified by silica gel chromatography(methanol/methylene chloride/ammonia water; 1:20:0.05;V/V/V) to give MBz-BAT-AV-45(0.18 g, 62%). ^{1}H-NMR (500 MHz, $CDCl_3$)δ:8.16(1H, s), 7.76(1H, d, d), 7.42(2H, d), 7.22(6H, m), 6.95(2H, s), 6.81(4H, m), 6.79(1H, d), 4.46(2H, m), 3.81(2H, m), 3.77(1H, m), 3.75–3.60(13H, m), 3.49(2H, t), 2.99(3H, s), 2.95(1H, br), 2.79(6H, br), 2.69(2H, t), 2.63(2H, t), 2.57(2H, t), 1.47(9H, s); MS(ESI):positive mode $m/z = 862([M + 1]^+)$.

(E)-2-(2-(2-(2-(N-mercaptoethyl)-(N-(N-mercaptoethyl aminoethyl))aminoethoxy)ethoxy)ethoxy)-5-(4-methylaminostyryl)pyridin(**BAT-AV-45, 2**).

MBz-BAT-AV-45(0.18 g, 0.2 mmol) was dissolved in TFA (10 mL) and anisole (0.05 mL, 0.46 mmol) at 0 °C, and $Hg(OAc)_2$ (0.39 g, 1.22 mmol) was added. The resulting mixture was stirred for 30 min and concentrated in vacuo to obtain a viscous oil that was dried in vacuo for 30 min. Dry ether (10 mL) was then added to the above oil, and the resulting suspension was sonicated for 5 min. The yellow solid that formed was collected by suction filtration, dried in vacuo for 20 min, and dissolved in absolute EtOH (10 mL). H_2S gas was passed through the solution for 20 min, and the reaction mixture was filtered through a pad of Celite. The filtrate was concentrated in vacuo to obtain the trifluoroacetate salts of BAT-AV-45, which were used for further reactions without additional purification. MS(ESI):positive mode $m/z = 521([M + 1]^+)$.

Radiolabeling procedure

In the labeling of ^{99m}Tc-BAT-AV-45, BAT-AV-45(50 μg) was dissolved in 100 μL ethanol in an evacuated nitrogen filled vial. To this solution, sodium glucoheptonate (10 mg) dissolved in 0.2 mL water, stannous chloride (50 μg) dissolved in HCl(0.1 N) were added respectively, pH value(5–6) of the solution was adjusted by using HCl or NaOH. After addition of all reagents, 0.1 mL of [Tc-99 m]pertechnetate solution(∼0.5 mCi) and additional some water were added into the vial. Reaction mixture volume used was about 0.9 mL. The vial was incubated at 90 °C in a water bath for 30 min. After cooling down to room temperature, 0.1 mL of injected sodium phosphate solution (0.1 M, pH 7.4) was added. A series of studies were performed to optimize labeling efficiency step by step, such as the amount of BAT-AV-45 ligand, reductant $SnCl_2$, sodium glucoheptonate and the pH value of the reaction mixture.

Quality control of ^{99m}Tc-BAT-AV-45

The labeling yield and radiochemical purity were determined by thin layer chromatographic method using polyamide strip. The reaction product was spotted on polyamide strips and developed in methanol/ethyl acetate(1:1; V:V) solution. After developing, the strips were dried at room temperature. Then, they were cut into 0.5 cm pieces and counted by using an automatic γ counter. Retention factor (Rf) and labeling yield were determined from TLC chromatogram data. For comparison, samples of $Na^{99m}TcO4$, ^{99m}Tc-GH and reduced/hydrolyzed ^{99m}Tc (RH-^{99m}Tc) were also run under identical conditions.

^{99m}Tc-BAT-AV-45 was further confirmed by radio-HPLC system. The flow rate was maintained at 0.8 mL/min after an injection 10 μL from the reaction mixture into analytical reversed-phase column (Amethyst C18, 4.6 × 150 mm 5 μm, Sepax Technologies, Inc.). The eluting solvent consisted of H_2O/acetonitrile (1:4;V:V),and the elution was monitored by observing the radioactivity profile. For comparison, samples of $Na^{99m}TcO4$, ^{99m}Tc-GH were also run under identical condition. The chromatograms for $Na^{99m}TcO4$, ^{99m}Tc-GH, ^{99m}Tc-BAT-AV-45 are showing in Fig. 2.

Stability of ^{99m}Tc-BAT-AV-45

The stability of freshly prepared ^{99m}Tc-BAT-AV-45 at physiological pH was measured at different times at room temperature (∼25 °C).

Partition coefficient

A 25 μL aliquot of ^{99m}Tc-BAT-AV-45 was added to a test tube containing 3 mL of 1-octanol and 3 mL of 0.02 M phosphate buffer pH 7.4. The test tube was vortexed at room temperature for 5 min and then centrifuged at 3,500 rpm for 10 min. A 100 μL aliquot was taken from the 1-octanol phase and a 100 μL aliquot from the aqueous phase, taking care to avoid cross contamination between the phases and weighed. The radioactivity of the aliquots was counted using an automatic γ counter and the partition coefficient P was calculated using the following equation:

$$p = \frac{\text{cpm/ml octanol}}{\text{cpm/ml bufffer}}$$

with cpm = counts per minute.

Experiments were performed at least in triplicate.

Electrophoresis

A 5 μL was spotted on a paper strip (3.5 × 13 cm, Whatman 1 chromatography paper) wetted with a mixture of 0.02 M phosphate buffer pH 7.4 and methanol (50:50, V/V). Electrophoresis was performed during 60 min using the described mixture as the electrolyte solution and an applied voltage of 300 V. After drying, the paper was cut into 0.5 cm strips and the activity on each strip was counted using an automatic γ counter.

Results and discussion

Synthesis and radiolabeling procedure

As shown in Scheme 1, the labeling precursor was synthesized by coupling styrylpyridine derivatives(AV-45)

with N1,N2-[bis(4-methoxybenzyl) thioethyl] ethanediamine (MBz- BAT), and then deblocking the protecting group. The key compound MBz-BAT-AV-45 was identified by ^{1}H NMR and MS. The labeling precursor BAT-AV-45 was not additionally purified because of its easy oxidation and just identified by MS.

^{99m}Tc-BAT-AV-45 was prepared by a ligand exchange reaction employing the precursor ^{99m}Tc-GH. Several reaction parameters were optimized by varying relative reaction parameter step by step, such as the amount of BAT-AV-45 ligand, reductant $SnCl_2$, sodium glucoheptonate and the pH value of the reaction mixture.

The effects of the amount of BAT-AV-45 ligand were summarized in Fig. 1. The data showed that the radiolabeling yield of ^{99m}Tc-BAT-AV-45 reached to 95.7 ± 1% at the optimum amount of ligand (50 μg). Below the amount of 50 μg, the labeling yield was low due to the substrate concentrations being insufficient to form complex with all of the reduced technetium.

The effects of the amount of stannous chloride were shown in Fig. 1. The data showed that the radiolabeling yield was dependent on the amount of stannous chloride, and the highest labeling efficiency was 95.4 ± 1% by using 50 μg of $SnCl_2$. Below the amount of 50 μg, stannous chloride was not sufficient for complete reduction of pertechnetate to form ^{99m}Tc-complex because of easy oxidation. Above the amount of 50 μg, the radiochemical yield was decreased. This may be due to the fact that the excess amount of stannous chloride leaded to the formation of stannous hydroxide colloid in basic medium or by oxidation and stannous hydroxide colloid adsorbed the free technetium-99 m or ^{99m}Tc-complex.

The effects of the amount of sodium glucoheptonate were summarized in Fig. 1. The data interestingly showed that large amount of sodium glucoheptonate (10 mg) was required for optimal preparation of ^{99m}Tc-BAT-AV-45 (RCP .95%), contrast to a minimal amount of ligand. A small amount of sodium glucoheptonate played a transitional ligand role as the form of ^{99m}Tc-GH in the radiolabeling procedure. While excess of glucoheptonate with a multi- hydroxyl moiety may facilitate the solubility of BAT-AV-45 ligand by dispersing the compound into reaction solution. Thus ^{99m}Tc-GH complex may transchelate more effectively with BAT-AV-45 ligand to form ^{99m}Tc-BAT-AV-45. Similar results were previously reported in the preparation of [Tc-99m]TRODAT-1 [21].

The effects of pH value were shown in Fig. 1. The data showed that the radiolabeling yield was dependent on the pH value, and the highest labeling efficiency was 95.5 ± 1% with the pH value (5–6). Above the optimum pH value, the radiochemical yield was drastically decreased because of forming RH-^{99m}Tc which is the main radiochemical impurities at alkaline medium.

Quality control of ^{99m}Tc-BAT-AV-45

In thin layer chromatographic method using methanol/ethyl acetate(1:1;V:V) as solvent, The Rf value of ^{99m}Tc-BAT-AV-45 was 0.7–1, and the Rf value of ^{99m}Tc-GH was 0–0.2, while RH-^{99m}Tc and free Technetium-99m remained at the

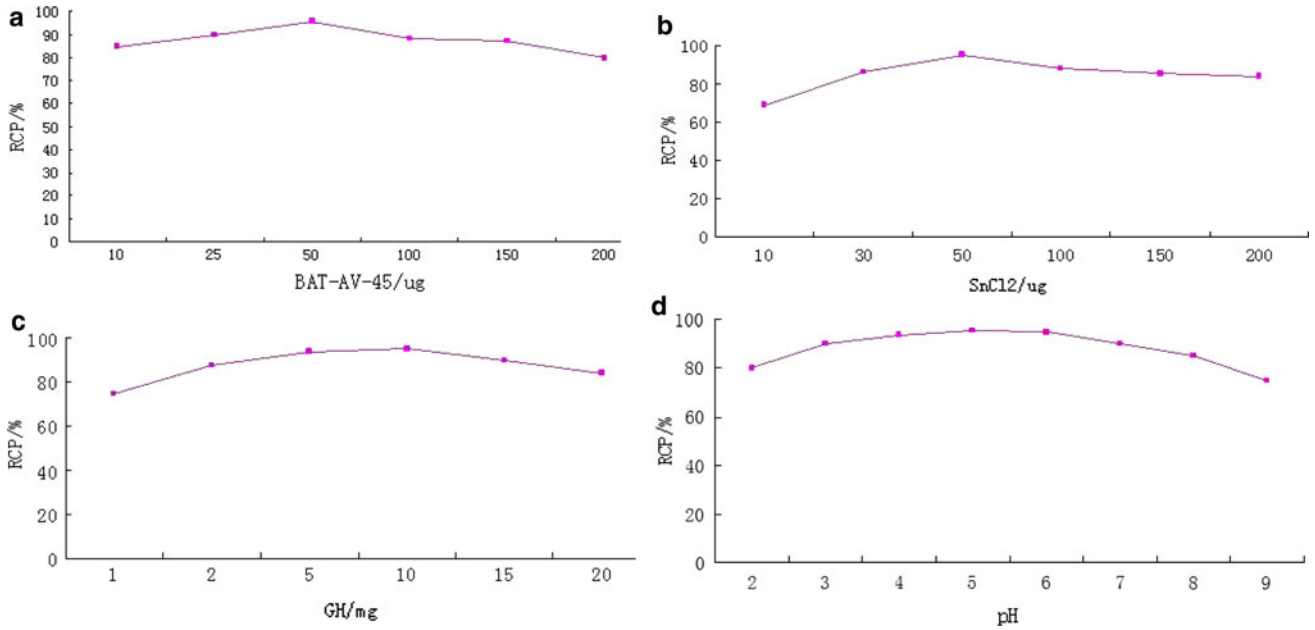

Fig. 1 Effects of reaction parameters on the labeling yield of ^{99m}Tc-BAT-AV-45: **a** the amount of ligand; **b** the amount of $SnCl_2$; **c** the amount of sodium glucoheptonate; **d** the pH value

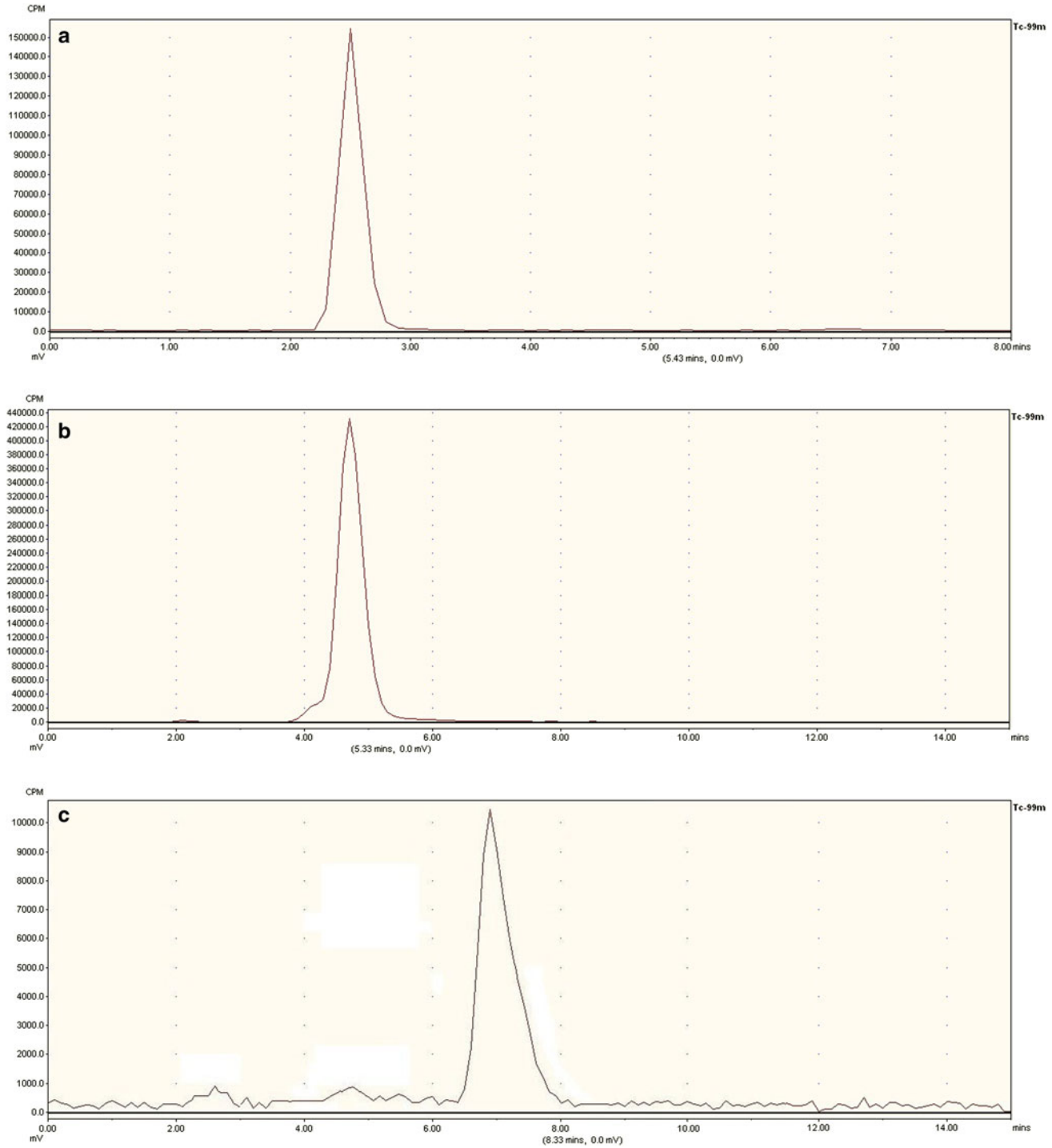

Fig. 2 RHPLC chromatogram: **a** $Na^{99m}TcO_4$; **b** ^{99m}Tc-GH; **c** ^{99m}Tc-BAT-AV-45

point of spotting (Rf = 0).The average radiolabeling yield was 95 ± 1%.

HPLC radiochromatograms of $Na^{99m}TcO4$, ^{99m}Tc-GH, ^{99m}Tc-BAT-AV-45 are presented in Fig. 2. The radio peak at 2.7 and 4.7 min represent the free $Na^{99m}TcO4$ and ^{99m}Tc-GH respectively. ^{99m}Tc-BAT-AV-45 gives one radio peak at 7 min of retention.

Stability of ^{99m}Tc-BAT-AV-45

To avoid the radio decomposed side products which may accumulate in non-target organs, the stability of ^{99m}Tc-BAT-AV-45 at physiological pH was studied in order to determine the suitable time for injection. The results of the stability of ^{99m}Tc-BAT-AV-45 are presented in Fig. 3.

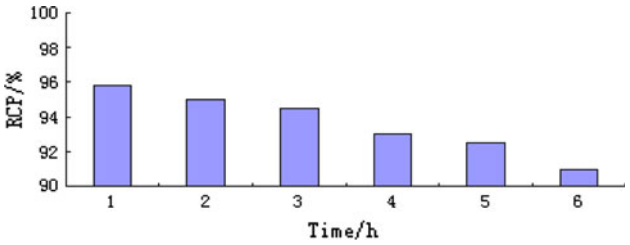

Fig. 3 Stability of ^{99m}Tc-BAT-AV-45 at physiological pH at different intervals

Table 1 The liquid–water partition coefficient of ^{99m}Tc-BAT-AV-45

Organic phase (cpm)	Water phase (cpm)	Distribution ratio	Log p
48014	348	146.4	2.17
49272	458	107.6	2.03
47776	466	102.5	2.01

The data clearly shows that ^{99m}Tc-BAT-AV-45 is stable (RCP. $> 90\%$) for up to 6 h which is suitable for nuclear medicine applications.

Partition coefficient

Previous studies suggested that the optimal lipophilicity for entry into the brain is obtained with log *P* values of between 1 and 3 [22]. ^{99m}Tc-BAT-AV-45 displayed suitable lipophilicity with the value of log $P(2.07 \pm 0.087)$ and the results are listed in Table 1.

Electrophoresis

In electrophoresis experiments the applied radioactivity of ^{99m}Tc-BAT-AV-45 remained at the spotted point, which indicates that the compounds is uncharged at physiological pH.

Conclusions

In conclusion, we successfully designed and synthesized novel styrylpyridine derivative conjugated with ^{99m}Tc. Satisfactory results were obtained by optimizing the radiolabeling conditions. ^{99m}Tc-BAT-AV-45 was prepared at an average yield of $95 \pm 1\%$ by mixing 50 μg of BAT-AV-45, 10 mg of sodium glucoheptonate, and stannous chloride (50 μg) dissolved in HCl (0.1 N) at pH value (5–6) at 90 °C for 30 min. ^{99m}Tc-BAT-AV-45 with suitable lipophilicity (log $P = 2.07 \pm 0.087$) was stable and uncharged at physiological pH. Further studies are needed to apply ^{99m}Tc-BAT-AV-45 for Aβ plaques imaging.

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