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ORIGINAL ARTICLE

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A new efficient route for synthesis of R,R- and S,S-hexamethylpropyleneamine oxime for labeling with technetium-99m

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ABSTRACT

[^{99m}Tc]Tc-Hexamethylpropylene amine oxime (HMPAO) is currently used as a regional cerebral blood flow imaging agent for single photon emission computed tomography (SPECT). The HMPAO ligand exists in two isomeric forms: d,l and meso showing different properties in vivo. Later studies indicated that brain uptake patterns of ^{99m}Tc-complexes formed from separated enantiomers differed. Separation of enantiomers is difficult by fractional crystallizations method. Usually, the substance is obtained in low chemical yield in a time-consuming procedure. Furthermore, the final product still contains some impurity. So we have developed new efficient route for synthesis of *R,R*- and *S,S*-HMPAO enantiomeric compounds in 6-steps. Nucleophilic substitution (S_N2) reactions of 2,2-dimethylpropane-1,3-diamine either with *S*- **(1a)** or R-methyl-2-chloropropanoate **(1b)** were performed to produce compounds *R,R*- **(2a)** or *S,S*-isomer **(2b)** derivatives protected with benzylchloroformate (Cbz), respectively. And then Weinreb amide and methylation reaction using Grignard reagent, oxime formation with ketone group and deprotectiion of Cbz group by hydrogenolysis gave *S,S*- **(7a)** or *R,R*-HMPAO **(7b)**, respectively. Entaniomeric compounds were synthesied with high yield and purity without any undesired product. The **7a** or **7b** kits containing 10 μg $SnCl_2$-$2H_2O$ were labeled with ^{99m}Tc with high radiolabeling yield (90%).

Key Word: HMPAO; Exametazime; SPECT; single photon emission computed tomography; cerebral blood flow; hexamethylpropylene amine oxime

Introduction

Various radiopharmaceuticals labeled with selenium-75 ([^{75}Se]di-(piperidinoethyl) selenide (PIPSE) and [^{75}Se] di-(morpholinoethyl)selenide (MOSE)) (1), iodine-123 ([^{123}I]p-iodo-N-isopropylamphetamine (IMP) (2) and [^{123}I] N,N-dimethyl-N'-(2-hydroxy-5-iodo-3-methylbenzyl)-1,3-propanediamine (HIPDM)) (3) or thallium-20l (^{201}Tl-diethyldithiocarbamate (DDC)) have been developed in the last decades for imaging of regional cerebral blood flow (rCBF) using single photon emission computed tomography (SPECT) (4). However, none of these radiopharmaceuticals is suitable for routine rCBF studies due to some drawbacks showing poor physical characteristics, high production costs, and limited availability.

Compared with other radioisotopes, technetium-99m (half-life of 6.02 h and γ-ray of 140.5 keV energy) does not have such drawbacks. Moreover, it is produced from ^{99}Mo/^{99m}Tc-generator which is readily and economically available in hospitals. Thus, many investigators have

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studied to develop new rCBF radiopharmaceuticals labeled with technetium-99m (5-7). The technetium complex should be relatively small (<500 Daltons), lipophilic, and neutral to satisfy the important requirement of rCBF—crossing the blood-brain-barrier (BBB).

The most well-known rCBF imaging agent hexamethyl propyleneamine oxime (HMPAO) has been first described by Novotnik et al. (8-13). As elucidated by Neirinckx et al. (10), HMPAO has two chiral centers producing the meso- (R,S), d- (R,R) and l- (S,S) diastereomers.

It was originally confirmed that technetium-99m complexes of these two HMPAO diastereomers (*meso*- and *dl* racemate) showed different properties in vivo both in rats (10) and in humans (14). Both of them rapidly passed across the BBB after intravenous injection. However, the dl-complex revealed superior brain uptake and retention (4.1% after 8 h post-injection) compared to the meso-complex (1.7%) and their stereoisomeric mixtures (1.9%) (14). Thus, the meso-diastereomer component decreased the radiopharmaceutical concentration in the brain. In addition, the later studies showed that brain uptakes of ^{99m}Tc-complexes formed from separated *d* (R)- and *l* (S)-enantiomers were different.

The previously reported procedure for separation of *d*-HMPAO and *l*-HMPAO from mixture of *dl*- and *meso*-HMPAO involves the repeated fractional co-crystallization of crude HMPAO with L- or D-tartaric acid from organic solvents (13). However, this method is time-consuming and affords many fractions of different crystallinity and diastereomeric composition. Hence, it results in the drastic reduction of the final yields of both *d*- and *l*-enantiomers, and also has the possibility of trace amount of *meso*–HMPAO as impurity (15).

The aim of this study was to develop new efficient route for synthesis of *R,R*- and *S,S*-HMPAO enantiomeric compounds, which would provide with the high yield and purity. We also studied formulation of the synthesized isomers for improved radiolabeling using stannous chloride solutions with various concentrations.

Materials and Methods

1. General

A ^{99}Mo/ ^{99m}Tc Generator was purchased from Enviro Korea (Samyoung Unitech, Korea). ^{1}H-NMR spectra were recorded on a 500 MHz Avance III (Bruker, German) NMR spectrometer. Chemical shifts (δ) were reported in ppm downfield from tetra methyl silane and multiplicities were indicated by s (singlet), d (doublet), t (triplet), m (multiplet), br (broad), and bs (broad singlet). Low Resolution-MS (LRMS) were acquired on a LTQ-Vellos ion trap spectrometer (Thermo Scientific, France). High Resolution-MS (HRMS) were acquired on a LTQ-Orbitrap Velos ion trap spectrometer (Thermo Scientific, France). Data were reported in the form of m/z versus intensity. Radio-thin layer chromatography (radio-TLC) was counted using a Bio-Scan AR-2000 System imaging scanner from Bioscan (Washington, DC, U.S.A.). Instant TLC-silica gel (ITLCSG) plates were purchased from Varian, Agilent Technologies (Lake Forest City, CA, U.S.A.). All chemicals were purchased from Tokyo Chemical Industry (Japan) and Sigma-Aldrich (St. Louis, MO, U.S.A.).

2. Chemical synthesis of S, S-HMPAO

Dimethyl 2,2'-((2,2-dimethyl propane-1,3-diyl)bis-(azanediyl)) (2S,2'S)-dipropionate ***(2a)***

50-mL 3-necked round-bottom flask was tightly fitted with a reflux condenser and dropping funnel and was

charged with 2, 2-dimethylpropane-1,3-diamine 1.38 g (1 eq), acetonitrile 15 mL, and potassium carbonate 11.2 g (6 eq). The above mixture was refluxed and stirred for 30 min. Meanwhile solution of methyl (*R*)-2-chloropropanoate **1a** 5.0 g (3 eq) in 15 mL acetonitrile were prepared and transfered to dropping funnel. After reflux, reagent solution was added dropwise with vigorous stirring. After complete addition, the slurry was stirred under reflux for 66 h. Reaction mixture was stopped and then cooled to room temperature. Solid in reaction mixture was removed by filteration using celite pad and concentrated in vacuo. The residue was dissolved in ethyl actate and extracted with 2 x 50 mL of purified water. The organic layer was treated with magnesium sulfate and then concentrated in vacuo. The resulting dark yellow oil residue was purified by silica gel column chromatography (Dichloromethane: Methanol) affording the title compound **2a** (2.0 g, 54%) as pale yellow oil: ^{1}H-NMR 500MHz ($CDCl_3$): δ3.71 (m, 6H), 3.28 (q, 2H, *J*= 3.0 Hz), 2.43 (d, 2H, *J*= 6.6 Hz), 2.27 (d, 2H, *J*= 6.9 Hz), 1.73 (brs, 2H), 1.29 (s, 3H), 1.27 (s, 3H), 1.27 (s, 3H), 0.89 (s, 6H). High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{13}H_{27}N_2O_4$ 275.19; found, 275.19.

Dimethyl 2,2'-(6,6-dimethyl-3,9-dioxo-1,11-diphenyl-2, 10-dioxa-4,8-diazaundecane-4,8-diyl)(2S,2'S)-dipropionate ***(3a)***

2.0 g (1 eq) of dimethyl 2,2'-((2,2-dimethylpropane-1,3-diyl)-bis (azanediyl))-(2S,2'S)-dipropionate **2a** and 3.68 g (5 eq) of sodium carbonate in 20 mL tetrahydrofuran (THF) was stirred for 10 min at 0°C. 2.37 mL (2.4 eq) of benzyloxycarbonyl chloride was added dropwise to above mixture for 15 min and stirred at room temperature for 12 h. The reaction mixture was extracted by ethyl actate and purified water. The organic layer was wahed 2 times with water, dried with magnesium sulfate and then concentrated in vacuo. The resulting residue was purified by silica gel column chromatography (hexane: ethyl acetate) affording the title compound **3a** (4.85 g, 92%) as transparent viscous oil: ^{1}H-NMR 500MHz ($CDCl_3$): δ 7.38-7.27 (m, 10H), 5.21-5.14 (m, 1H), 5.13-5.07 (m, 2H), 5.0 (d, 1H, *J*= 6.0 Hz), 3.83-3.62 (m, 5H), 3.42 (d, 4H, *J*= 9.0 Hz), 3.34-3.06 (m, 3H), 1.62-1.34 (m, 8H), 1.11-0.93 (m, 6H). High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{29}H_{38}N_2O_8$ 543.27; found, 543.27. $[M + Na]^+$: calculated for $C_{29}H_{38}N_2NaO_8$ 565.25; found, 565.25.

Dibenzyl (2,2-dimethylpropane-1,3-diyl)-bis (((S)-1-(methoxy- (methyl)-amino)-1-oxopropan-2-yl)-carbamate) ***(4a)***

Appropriate dimethyl 2,2'-(6,6-dimethyl-3,9-dioxo-1,11-diphenyl-2,10-dioxa-4,8-diazaundecane-4,8-diyl) (2S,2'S)-dipropionate 3a (1.0 g, 1 eq) was dissolved in 10 mL anhydrous THF in a 50 mL round-bottomed flask and cooled to -20 °C under nitrogen. The solution was treated with N,O-dimethylhydroxylamine hydrochloride (0.71 g, 4 eq) and 7.4 mL (8 eq) isopropylmagnesium chloride (2 M in THF) was then added dropwisely. The resulting mixture was stirred at -20 °C for 2 h then quenched with 20 mL satuated ammonium chloride solution and stirred for 30 min. The reaction mixture was extracted with ethyl acetate (3 X 30 mL). The combined organic extracts were washed with brine, dried over sodium sulfate and the solvent was removed in vacuo. Purification of the residue took place on a silica-gel column (hexane/ethyl acetate, 3:1) to give the desired Weinreb amide **4a** (0.95 g, 96%) as pale yellow oil: ^{1}H-NMR 500MHz ($CDCl_3$): δ 7.33-7.28 (m, 10H), 5.09 (q, 4H, *J*= 9.0 Hz), 4.60-4.42 (m, 2H), 3.72-3.69 (m, 3H), 3.35 (brs, 5H), 3.18-3.05 (m, 8H), 1.58-1.36 (m, 8H), 0.88 (brs, 6H). High resolution mass spectrometry

(HRMS) $[M + Na]^+$: calculated for $C_{31}H_{44}N_4NaO_8$ 623.30; found, 623.30.

Dibenzyl (2,2-dimethyl propane-1,3-diyl)-bis (((S)-3-oxobutan-2-yl)-carbamate) ***(5a)***

Dibenzyl (2, 2-dimethyl propane-1, 3-diyl)-bis-(((*S*)-1-(methoxy-(methyl)-amino)-1-oxopropan-2-yl)-carbamate) **4a** (0.95 g, 1 eq) was dissolved in 12 mL anhydrous THF and cooled to -20 ˚C under nitrogen atmosphere. 12.5 mL (20 eq) of methyl magnesium bromide (3 M in diethyl ether) was added slowly at -20 ˚C. The resulting solution was stirred at -20 ˚C for 3 h and then quenched with 20 mL saturated ammonium chloride solution and stirred for 30 min. The reaction mixture was extracted with ethyl acetate (3 X 30 mL). The combined organic extracts were washed with brine, dried over sodium sulfate and the solvent was removed in vacuum. Purification of the residue took place on a silica-gel column (hexane/ethyl acetate, 6:4) to give the desired product **5a** (0.68 g, 74%) as clear gummy oil: ^{1}H-NMR 500 MHz ($CDCl_3$): δ 7.38-7.28 (m, 10H), 5.13-5.06 (m, 4H), 3.56-3.13 (m, 6H), 2.22-1.92 (m, 6H), 1.50 (dd, 3H, *J*= 3 & 21 Hz), 1.37 (dd, 3H, *J*=3 & 27 Hz), 1.06 (d, 3H, *J*= 15 Hz), 0.99 (d, 3H, *J*=15Hz). High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{29}H_{39}N_2O_6$ 511.28; found, 511.27. $[M + Na]^+$: calculated for $C_{29}H_{39}N_2NaO_6$ 533.26; found, 533.25.

Dibenzyl (2,2-dimethylpropane-1,3-diyl)-bis(((S,E)-3-(hydroxyimino)-butan-2-y l)carbamate) ***(6a)***

0.427 g of dibenzyl (2,2-dimethyl propane-1,3-diyl)-bis(((S)-3-oxobutan-2-yl)-carbamate) **5a** was dissolved in 4 mL ethanol. 0.8 g of 50% hydroxyl amine in an aqueous solution and 0.225 g of acetic acid were added. The mixture was heated to 50 δ and stirred for 5 h, cooled to room temperature and concentrated under vacuum to remove the ethanol solvent. Residue was extracted with 3 mL purified water and 3 mL EA. The organic layer was washed with 3 mL purified water, dried on magnesium sulfate and then concentrated in vacuum to give the desired compound **6a** (0.38 g, 81%) as sticky oil. High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{29}H_{41}N_4O_6$ 541.30; found, 541.30. $[M + Na]^+$: calculated for $C_{29}H_{39}N_2NaO_6$ 563.28; found, 563.28.

(2E,2'E,3S,3'S)-3,3'-((2,2-dimethyl-propane-1,3-diyl)-bis-(azanediyl))-bis(butan-2-one) dioxime ***(7a)***

0.38 g of dibenzyl (2,2-dimethylpropane-1,3-diyl)-bis(((*S,E*)-3-(hydroxyimino)-butan-2-yl)carbamate) **6a** was dissolved in 4 mL of methanol and remove air using nitogen gas ballown. Added 0.165 g of 10% Pd/C was added and reacted with stirring for 10 h under a hydrogen atmosphere. The solution was filtered through celite pad to remove Pd/C and concentrated in vacuum to give yellow oil. Recystalization involved 2-steps. In the first step, the resulting compound was dissolved in dichloromethane and stirred under reflux for 2 h. The solution was cooled down to room temperature and stirred for 3 h until more solid obtained. The solid was filtered on Whatman filter paper. In the secound step, the solid was dissolved in ethyl acetate and stirred under reflux for 2 h. Slowly cool down to room temperature and stirred for 2 h until more solid obtained, filtered to give white crystaline product **7a** (0.08 g, 77%), m.p: 138-141°C. ^{1}H-NMR 300MHz (CD_3OD): δ 3.23 (q, 2H, *J*= 9 Hz), 2.26 (q, 4H, *J*= 12 Hz), 1.77 (s, 6H), 1.17 (d, 6H, *J*= 6 Hz), 0.88 (d, 6H, *J*= 3Hz). High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{13}H_{28}N_4O_2$ 273.30; found, 273.30.

3. Chemical synthesis of R, R HMPAO

Dimethyl2,2'-((2,2-dimethylpropane-1,3-diyl)bis-(azanediyl)) (2R,2'R)-dipropionate ***(2b)***

50-mL 3-necked round-bottom flask was tightly fitted with a reflux condenser and dropping funnel, and was charged with 2, 2-dimethylpropane-1,3-diamine 1.51 g (1 eq), acetonitrile 20 mL and potassium carbonate 12.32 g (6 eq). The above mixture was refluxed with stirring for 30 min. Meanwhile solution of methyl (S) -2-chloropropanoate **1b** 5.5 g (3 eq) in 15 mL acetonitrile was prepared and transferred to dropping funnel. After reaching to reflux, reagent solution was added dropwise with vigorous stirring. After complete addition, the slurry was allowed to stirring under reflux for 66 h. Reaction mixture was stopped and then cooled to room temperature. Solid in the reaction mixture was removed by filteration using celite pad and concentrated in vacuo. The residue was dissolved in ethyl actate and extracted with 2 x 50 mL of purified water. The organic layer was treated with magnesium sulfate and then concentrated in vacuo. The resulting dark yellow oil residue was purified by silica gel column chromatography (dichloromethane: methanol) affording the title compound **2b** (2.32 g, 58%) as pale yellow oil: ^{1}H-NMR 500MHz ($CDCl_3$): δ 3.71 (m, 6H), 3.28 (q, 2H, *J*= 3.0 Hz), 2.43 (d, 2H, *J*= 6.6 Hz), 2.27 (d, 2H, *J*= 6.9 Hz), 1.73 (brs, 2H), 1.29 (s, 3H), 1.27 (s, 3H), 1.27 (s, 3H), 0.89 (s, 6H). High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{13}H_{26}N_2O_4$ 275.19; found, 275.19.

Dimethyl 2,2'-(6,6-dimethyl-3,9-dioxo-1,11-diphenyl-2,10-dioxa-4,8-diazaundecane-4,8-diyl)(2R,2'R)-dipropionate ***(3b)***

2.32 g (1 eq) of dimethyl 2,2'-((2,2-dimethylpropane-1,3-diyl)-bis (azanediyl)) (2R,2'R)-dipropionate **2b** and 4.5g (5 eq) of sodium carbonate in 20 ml tetrahydrofuran (THF) was stirred for 10 min at 0°C. 2.9 mL (2.4 eq) of benzyloxycarbonyl chloride was added dropwise to above mixture for 15 min and stirred at room temperature for 12 h. The reaction mixture was extracted with ethyl actate and purified water. The organic layer was wahed 2 times with water, dried with magnesium sulfate and then concentrated in vacuo. The resulting residue was purified by silica gel column chromatography (hexane: ethyl acetate) affording the title compound **3b** (5.2 g, 90%) as transparent viscous oil: ^{1}H-NMR 500MHz ($CDCl_3$): δ 7.38-7.27 (m, 10H), 5.21-5.14 (m, 1H), 5.13-5.07 (m, 2H), 5.0 (d, 1H, *J*= 6.0 Hz), 3.83-3.62 (m, 5H), 3.42 (d, 4H, *J*= 9.0 Hz), 3.34-3.06 (m, 3H), 1.62-1.34 (m, 8H), 1.11-0.93 (m, 6H). High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{29}H_{38}N_2O_8$ 543.27; found, 543.27. $[M + Na]^+$: calculated for $C_{29}H_{38}N_2NaO_8$ 565.25; found, 565.25.

Dibenzyl (2,2-dimethyl propane-1,3-diyl)-bis(((R)-1-(methoxy-(methyl) amino)-1-oxopropan-2-yl)carbamate) ***(4b)***

2,2'-(6,6-dimethyl-3,9-dioxo-1,11-diphenyl-2,10-dioxa-4,8-diazaundecane-4,8-diyl)(*2R,2'R*)-dipropionate **3b** (1.2 g, 1 eq) was dissolved in 12 mL anhydrous THF in a 50 mL round-bottomed flask and cooled to -20°C under nitrogen. The solution was treated with *N*,O-dimethylhydroxylamine hydrochloride (0.852 g, 4 eq) and 8.88 mL (8 eq) isopropylmagnesium chloride (2 M in THF) was then added dropwisely. The resulting mixture was stirred at -20 °C for 2 h and then quenched with 20 mL satuated ammonium chloride solution and stirred for 30 min. The reaction mixture was extracted with ethyl acetate (3 X 30 mL). The combined organic extracts were washed with brine, dried over sodium sulfate and the solvent was removed in vacuo. Purification of the residue was performed by a silica-gel column (hexane/ethyl acetate, 3:1) to give the desired Weinreb amide 4b (1.27 g, 98%) as pale yellow oil: ^{1}H-NMR 500MHz ($CDCl_3$): δ 7.33-7.28 (m, 10H), 5.09 (q, 4H, *J*= 9.0 Hz), 4.60-4.42 (m, 2H), 3.72-

3.69 (m, 3H), 3.35 (brs, 5H), 3.18-3.05 (m, 8H), 1.58-1.36 (m, 8H), 0.88 (brs, 6H). High resolution mass spectrometry (HRMS) $[M + Na]^+$: calculated for $C_{31}H_{44}N_4NaO_8$ 623.30; found, 623.30.

Dibenzyl(2,2-dimethylpropane-1,3-diyl)-bis-(((R)-3-oxobutan-2-yl) carbamate) ***(5b)***

Dibenzyl (2,2-dimethyl propane-1,3-diyl)-bis(((R)-1-(methoxy-(methyl) amino)-1-oxopropan-2-yl)carbamate) **4b** (1.27 g, 1 eq) was dissolved in 16 mL anhydrous THF and cooled to -20 ˚C under nitrogen atmosphere. 16.7 mL (20 eq) of methyl magnesium bromide (3 M in diethyl ether) was added slowly at -20 ˚C. The resulting solution was stirred at -20 ˚C for 3 h and then quenched with 20 mL saturated ammonium chloride solution and stirred for 30 min. The reaction mixture was extracted with ethyl acetate (3 X 30 mL). The combined organic extracts were washed with brine, dried over sodium sulfate and the solvent was removed in vacuum. Purification of the residue was performed by a silica-gel column (hexane/ethyl acetate, 6:4) to give the desired product **5b** (0.81 g, 70%) as clear gummy oil: ^{1}H-NMR 500MHz ($CDCl_3$): δ 7.38-7.28 (m, 10H), 5.13-5.06 (m, 4H), 3.56-3.13 (m, 6H), 2.22-1.92 (m, 6H), 1.50 (dd, 3H, *J*= 3 & 21 Hz), 1.37 (dd, 3H, *J*=3 & 27 Hz), 1.06 (d, 3H, *J*= 15 Hz), 0.99 (d, 3H, *J*=15Hz). High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{29}H_{39}N_2O_6$ 511.28; found, 511.27. $[M + Na]^+$: calculated for $C_{29}H_{39}N_2NaO_6$ 533.26; found, 533.25.

Dibenzyl (2,2-dimethylpropane-1,3-diyl)-bis(((R,E)-3-(hydroxyimino) butan-2-yl)carbamate) ***(6b)***

0.726 g of dibenzyl (2,2-dimethyl propane-1,3-diyl)-bis(((R)-3-oxobutan-2-yl) carbamate) **5b** was dissolved in 6 mL ethanol. 1.36 g of 50% hydroxyl amine in an aqueous solution and 0.384 g of acetic acid were added. The mixture was heated to 50 δ and stirred for 5 h, cooled to room temperature, and concentrated under vacuum to remove the ethanol solvent. Residue was extracted with 3 mL purified water and 3 mL ethyl acetate. The organic layer was washed with 3 mL purified water, dried on magnesium sulfate and then concentrated in vacuum to give the desired compound **6b** (0.69 g, 92%) as sticky oil: High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{29}H_{41}N_4O_6$ 541.30; found, 541.30. $[M + Na]^+$: calculated for $C_{29}H_{39}N_2NaO_6$ 563.28; found, 563.28.

(2E,2'E,3R,3'R)-3,3'-((2,2-dimethylpropane-1,3-diyl)-bis-(azanediyl)) bis(butan-2-one) dioxime ***(7b)***

0.2 g of dibenzyl (2,2-dimethylpropane-1,3-diyl)-bis(((R,E)-3-(hydroxyimino)butan-2-yl)carbamate) **6b** was dissolved in 5 mL of methanol and remove air using nitogen gas ballown. 0.12 g of 10% Pd/C was added and reacted with stirring for 10 h under a hydrogen atmosphere. The solution was filtered through celite pad to remove Pd /C and concentrated in vacuum to give yellow oil. Recystalization involved 2-steps. In the first step, the resulting compound was dissolved in dichloromethane and stirred under reflux for 2 h. The solution was cooled down to room temperature and stirred for 3 h until more solid obtained. The solid was filtered on Whatman filter paper. In the secound step, the solid was dissolved in ethyl acetate and stirred under reflux for 2 h. Slowly cool down to room temperature and stirred for 2 h until more solid obtained, filtered to give white crystaline product **7b** (0.074 g, 80%), m.p: 135-136°C. ^{1}H-NMR 300MHz (CD_3OD): δ 3.23 (q, 2H, *J*= 9 Hz), 2.26 (q, 4H, *J*= 12 Hz), 1.77 (s, 6H), 1.17 (d, 6H, *J*= 6 Hz), 0.88 (d, 6H, *J*= 3Hz). High resolution mass spectrometry (HRMS) $[M + H]^+$: calculated for $C_{13}H_{28}N_4O_2$ 273.30; found, 273.30.

4. Chemical synthesis of meso HMPAO

4,8-diaza-3,6,6,9-tetra-methylundecane 3,8-diene-2, 10-diene bisoxime ***(2c)***

2, 3-Butanedione monoxime **1c** (4.66 g, 46.09 mmol)

was dissolved in benzene (20 mL) and the solution was brought to reflux in an apparatus fitted with a Dean and Stark trap, and under a nitrogen atmosphere. To this was added a solution of 2, 2-dimethyl-l, 3-propanediamine (2.0 g, 19.5 mmol) in benzene (40 ml) over a period of 5 h. The resulting yellow brown solution was refluxed for a further 16 h under nitrogen, and then allowed to cool to room temperature. The precipitated solid was removed under suction, and washed with a little cold (-40°C) acetonitrile, giving the product as a white powder. Drying under high vacuum for 2 h gave the 2c product: 1.38 g (60%). Low resolution mass spectrometry (LRMS) $[M + H]^+$: calculated for $C_{13}H_{25}N_4O_2$ 269.20; found, 269.25.

(RR, SS)-4, 8-diaza-3,6,6,9-tetra-methyl-undecane-2, 10-dione bisoxime (dl-HMPAO) ***(3c)***

The above bis-imine **2c** (1.38 g, 287 mmol) was slurried in 95% aqueous ethanol (13.0 mL) at 0°C. Sodium borohydride (0.195 g, 5.15 mmol) was added in portions over 30 min, and the mixture stirred at 0°C for 2 h. Water (10 mL) was added and the mixture was stirred well for further 2 h. The ethanol was removed and more water (5 mL) was added. The pH was adjusted to 11, and the resulting precipitate was removed by filtration, washed with a little water, dried giving the impure HMPAO: 0.65 g (47%). Double recrystallization from acetonitrile provided the diastereoisomeric mixture of HMPAO free from major impurities: 0.5 g (33%), m.p: 119-122°C. Fractional crystallization from ethyl acetate provided the dl-diastereoisomer **3c**, m.p: 125-127°C. ^{1}H-NMR 300 MHz (CD_3OD): δ 3.23 (q, 2H, *J*= 9 Hz), 2.26 (q, 4H, *J*= 12 Hz), 1.77 (s, 6H), 1.17 (d, 6H, *J*= 6 Hz), 0.88 (d, 6H, *J*= 3Hz). Low resolution mass spectrometry (LRMS) $[M + H]^+$: calculated for $C_{13}H_{29}N_4O_2$ 273.23; found, 273.83.

5. Kit formulation

R,R-HMPAO, *S,S*-HMPAO, *dl*-HMPAO, and *meso*-HMPAO kits were prepared for radiolabeling with ^{99m}Tc according to a previously published method. The kits were prepared in 10-mL vials containing 0.5 mg of precursor (*R,R- or S,S-* or *dl-*, *Meso*-HMPAO), 4.5 mg of sodium chloride (NaCl), various concentration of stannous chloride dihydrate ($SnCl_2 \cdot 2H_2O$) in 0.2 N hydrochloric acid (HCl) solution (16, 12, 10, 8, 4, 2, 1, and 0.5 μg) in each vial, and 25 μL of 0.1 N sodium hydroxide (NaOH) solution to adjust pH (~9.0-10.0). Vortex for 30 s to each step addition. The vials were sealed with aluminum cap before radiolabeling.

6. Radiolabeling

Sodium pertechnetate (^{99m}Tc) was eluted from ^{99}Mo/^{99m}Tc generator using normal saline solution. To label the precursor (*R,R-, S,S-, dl-,* or *meso*-HMPAO), 0.5 mL of ^{99m}Tc (2-4mCi/mL) was added into each vial. The mixture was shaken vigorously using vortex for uniform mixing of solution. The pH of the solution ranged 9~10. The radioactive solution was maintained at room temperature for 10 min. The radiolabeling efficiency was evaluated in three different conditions using ITLC-SG (10 × 100 mm) and Whatman No.1 paper as a stationary phase; methyl ethyl ketone (MEK), normal saline and 50% acetonitrile in water as a mobile phase.

Results

2.1. Chemical synthesis

S,S-HMPAO (**7a)** and R,R-HMPAO (**7b**) were synthesized by new 6 step pathways (**Scheme 1, 2**). New route starting from nucleophilic substitution (S_N2) of 2,2-dimethylpropane-1,3-diamine reacting either with methyl (S)-2-chloropropanoate **(1a**) or methyl (R)-2-chloropropanoate (1b) to produce compound R,R-isomer **2a** or S,S-isomer **2b**. By reacting the above prepared compound of **2a** or **2b** with an benzylchloroformate (Cbz), Cbz protected **3a** or **3b** were synthesized for easy handling

of the compounds to further steps.

Compound **3a** or **3b** containing methyl ester group reacting with 2.0 M isopropyl magnesium chloride in THF (i-PrMgCl) (Grignard reagent) and N, O-dimethyl hydroxylamine hydrochloride to form Weinreb amide derivative of **4a** or **4b**. Nucleophilic methylation of the Grignard reagent (3.0 M methyl magnesium chloride in diethyl ether) on Weinreb amide group **4a** or **4b** to give compound **5a** or **5b**. The oxime compound of **6a** or **6b** was produced when ketone group condensed with hydroxylamine hydrochloride (NH_2OH) in the presence of acetic acid as catalyst. Finally removing amine protecting group (Cbz group) through hydrogenolysis performed under the metal catalyst 10% Pd/C and hydrogen gas gave S,S-HMPAO (**7a**) or R,R-HMPAO (**7b**) entaniomeric compounds.

Recrystalization proceess of S,S-HMPAO (**7a**) or R,R-HMPAO (**7b**) was done in two steps. Repeated recrystalization of cold precursor in dichloromethane (step-1) and ethyl acetate (step-2) followed by reflux, slow cooling and finally filteration on Whatman paper afforded white crystaline product. All intermediate compounds were purified by silica gel column chromatography with >50 % to 95% yields. Melting point of final R,R-HMPAO (m.p: 135-136°C) and S,S-HMPAO (m.p: 138-141°C) enantiomers were checked and confirmed for desired isomeric product. All intermediates and the final compound were fully characterized by ^{1}H-NMR 500 MHz Avance III (Bruker, German), low resolution and high resolution mass spectroscopy ESI^+-MS (LTQ-Vellos, Thermo Scientific, France).

The synthesis route of HMPAO (meso and dl) according to published method (10) was also about comparative study with enantiomers (RR-, SS-HMPAO). Condensation of the starting material 2,2-dimethyl-1,3-propanediamine with two molecular equivalents of the 2,3-butanedione mono-oxime **1c** using Dean-Stark trap provides the diimine **2c** derivative in 60% yield. Reduction of this diimine groups with sodium borohydride provides HMPAO as an equal mixture of the two diastereoisomers (meso- and dl). Repeated crystallization from acetonitrile and ethyl acetate permits the separation of the two diastereoisomers and the dl-diastereoisomer product 3c was obtained. Melting point of final meso-HMPAO (m.p: 119-122°C), and dl-HMPAO (m.p: 125-127°C °C) diastereomers were checked and confirmed for desired isomeric product

2.2. Radiolabeling

The amount of active substance in the single kit vial was 0.5 mg, similar to the commercially available formulation (10, 16). R,R-HMPAO, S,S-HMPAO, dl-HMPAO, and meso-HMPAO kits (containing 0.5 mg of precursor (R,R-, S,S-, dl, or meso-HMPAO), 4.5 mg of sodium chloride, various concentration of $SnCl_2 \cdot 2H_2O$ in 0.2 N HCl solution (0.5-16 μg) in each vial, 0.1 N NaOH solution) were prepared. ^{99m}Tc (2.0 to 4.0 mCi) was added, kept at room temperature for 10 min. The radiolabeling efficiency was determined by using ITLC-SG (10 × 100 mm) and Whatman's No.1 (1 mm) paper as a stationary phase; MEK, normal saline and 50% acetonitrile in water as a mobile phase. The R_f value of ^{99m}Tc-HMPAO complex (primary) was found to be 1.0 by ITLC-SG/ MEK or 0.0 by ITLC-SG/ saline or 1.0 by Whatman's No.1 paper/ 50% acetonitrile in water. We found that under optimized conditions, 10 μg of $SnCl_2 2H_2O$ provides higher radiolabeling efficiency (90.8%, 85.2% respectively) for ^{99m}Tc-R,R HMPAO (**Figure 1**) and ^{99m}Tc-S,S HMPAO (**Figure 2**) than with other $SnCl_2 \delta 2H_2O$ concentration (**Table 1**). Based on our study, at lower $SnCl_2 2H_2O$ concentration (8 to 0.5 μg) resulted in decreased radiolabeling efficiency of ^{99m}Tc-HMPAO isomers (< 5%) with an increased percentage of free ^{99m}Tc (>90%). Whereas at higher $SnCl_2 2H_2O$ concentration (12 to 16 μg) also resulted in decreased radiolabeling efficiency (60 to 80%) with an increased percentage of secondary complex (between 5 to 20%) and hydrolysed form (between 5 to 20%). Therefore, the amount of $SnCl_2 2H_2O$ plays an

Table 1. Radiolabeling efficiency of ^{99m}Tc-*R,R* HMPAO and ^{99m}Tc-*S,S* HMPAO

$SnCl_2.2H_2O$ Conc	^{99mTc}R,R HMPAO		^{99mTc}S,S HMPAO	
	Primary complex (%)	Secondary complex (%)	Primary complex (%)	Secondary complex (%)
16 μg	67.9±1.46	19.1±0.82	82.1±1.38	4.9±0.80
12 μg	61.6±2.27	10.4±1.05	78.0±1.91	9.2±1.08
10 μg	90.8±0.87	0.16±0.06	85.2±1.65	4.5±1.39
8 μg	80.3±1.63	11.5±0.75	78.0±1.91	6.7±0.91
4 μg	72.8±1.11	17.0±1.10	71.0±2.03	12.8±1.56
2 μg	71.4±0.78	16.2±0.2	69.4±1.55	-
1 μg	8.8±0.87	-	4.9±0.91	-
0.5 μg	4.8±0.35	-	0.4±0.67	-

The values represents mean ± standard deviation (n = 3).

Table 2. Radiolabeling efficiency of ^{99m}Tc-*dl* HMPAO and ^{99m}Tc-*Meso*

$SnCl_2$-$2H_2O$ Conc	^{99m}Tc-*dl* HMPAO		^{99m}Tc-*Meso* HMPAO	
	Primary complex (%)	Secondary complex (%)	Primary complex (%)	Secondary complex (%)
8 μg	83.0±1.06	14.2±1.70	72.5±0.92	9.3±0.99
4 μg	81.0±1.27	10.5±0.85	84.1±1.48	4.6±0.35
2 μg	68.0±1.41	27.9±1.48	81.8±2.33	3.7±1.48
1 μg	71.2±2.69	15.5±1.13	54.6±2.26	-
0.5 μg	-	-	42.4±2.33	-

The values represents mean ± standard deviation (n = 2).

important role on ^{99m}Tc- HMPAO (**Figure 3**). We also compared radiolabeling effciency of ^{99m}Tc-R,R- or S,S-HMPAO with ^{99m}Tc-dl- HMPAO racemic mixture (70 – 80%) (**Figure 4**), ^{99m}Tc-meso HMPAO (40 – 75%) (**Figure 5**) with various $SnCl_2 2H_2O$ solution concentration (**Table 2**) and also compared with commercial available Ceretec™ dl-HMPAO kit (≤ 80%) (**Figure 6**).

Discussion

Technetium-99m exametazime (^{99m}Tc-HMPAO) has been introduced as a tracer for brain studies in humans as early as 1985 (8, 9) and its diagnostic utility has been very well documented (10). Currently, it is recommended by the European Association of Nuclear Medicine (EANM) and approved by FDA for brain perfusion studies using SPECT (11). It is used to diagnose abnormalities in the regional cerebral blood flow such as those occurring after a stroke (17, 18) and other cerebrovascular diseases including epilepsy (19, 20), Alzheimer's disease (17, 18) and other forms of dementia (21), transient ischemic attacks (22), migraine (23, 24) and brain tumors (25). Another application is the labelling of autologous leucocytes for infection and inflammation imaging (12).

The synthesis and radiolabeling of the active substance, ligand HMPAO have been previously reported and

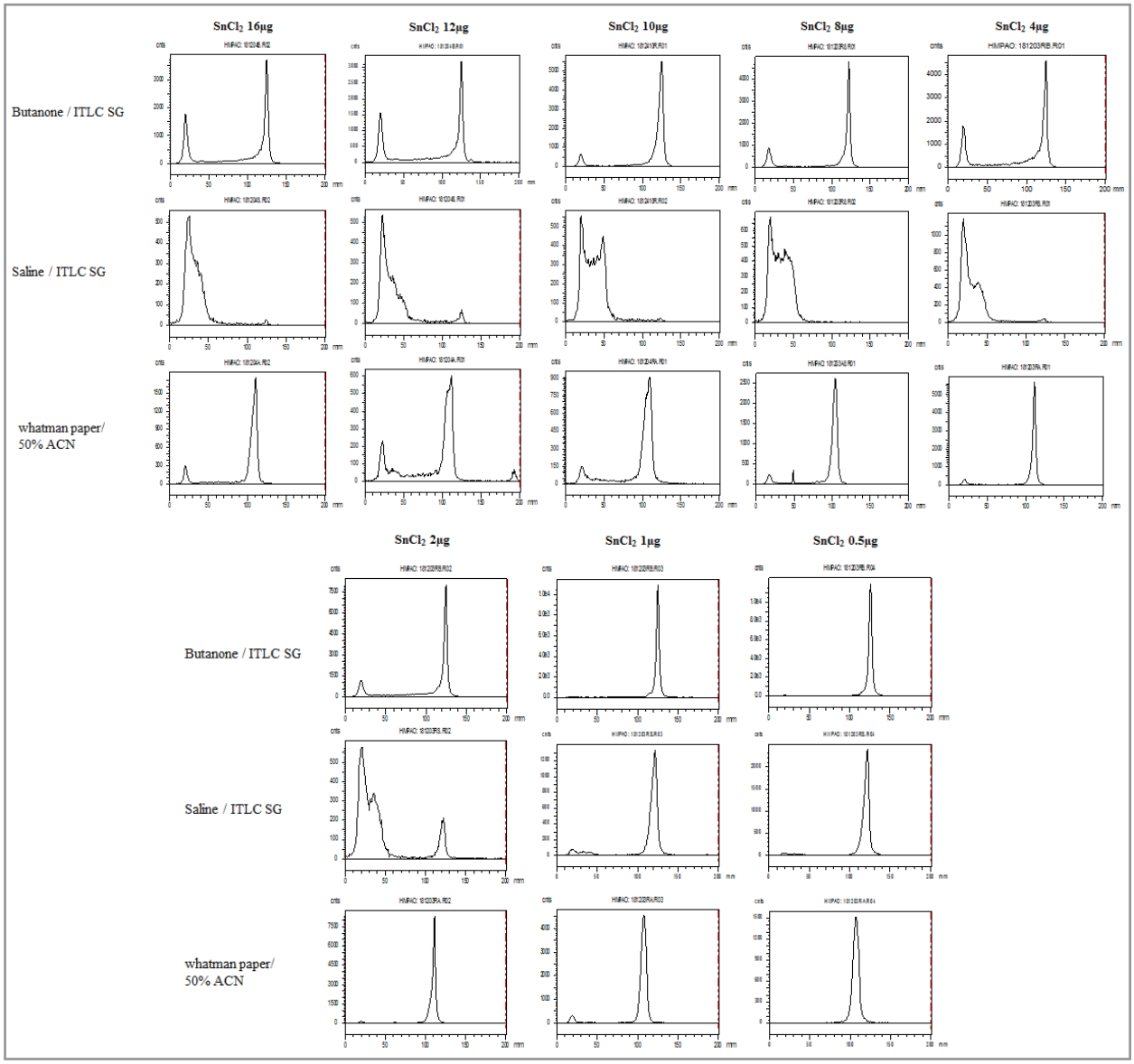


Figure 1. Radiolabeling efficiencies of ^{99m}Tc-*R,R* HMPAO with various concentration of $SnCl_2.2H_2O$ solution (0.5-16µg) were evaluated by ITLC-SG/Butanone, ITLC-SG/Saline and Whatman's No-1 paper/ 50% Acetonitrile in water. Radioactivity was detected by a Radio-TLC scanner.

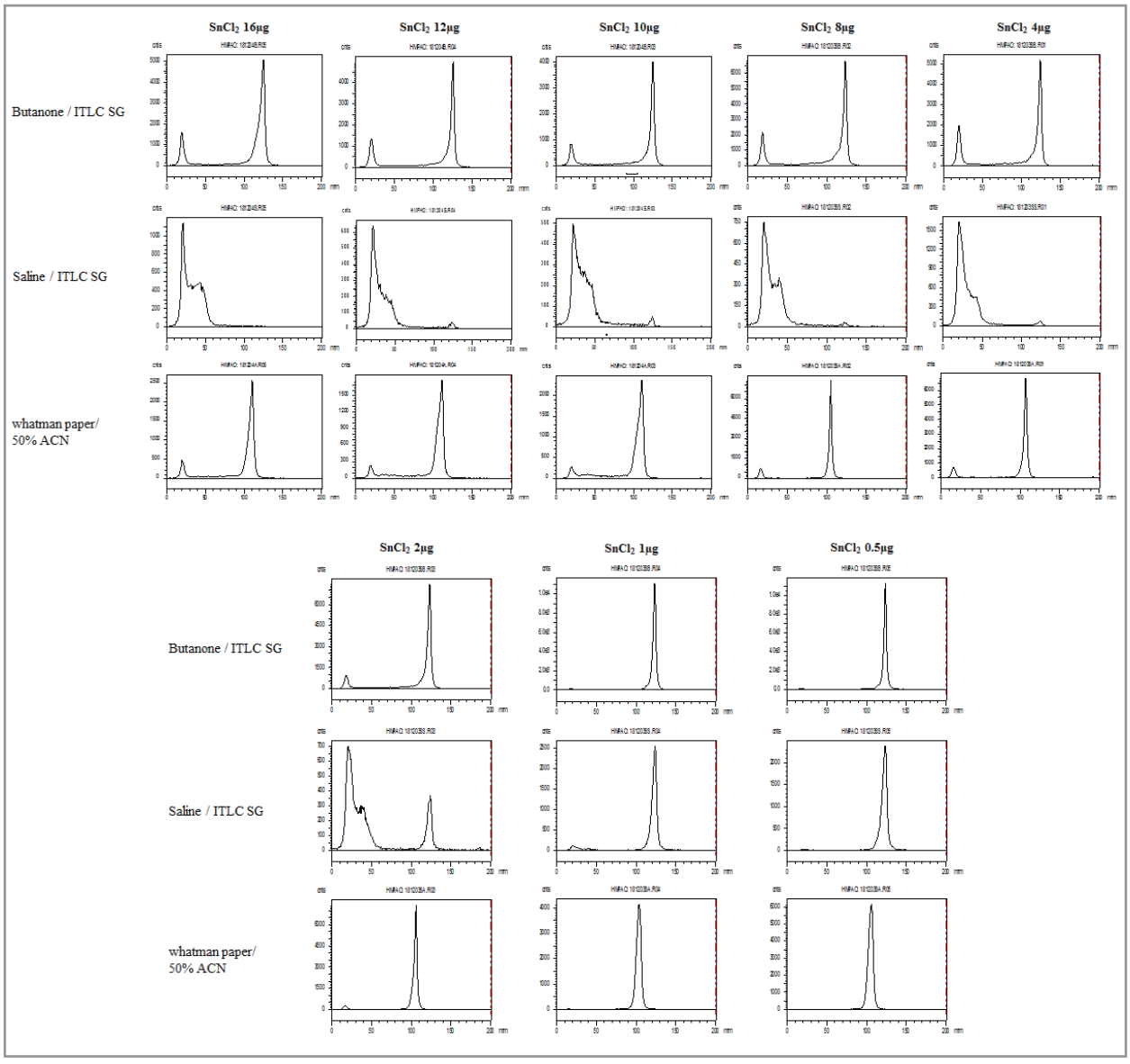


Figure 2. Radiolabeling efficiencies of ^{99m}Tc-*S,S* HMPAO with various concentration of $SnCl_2.2H_2O$ solution (0.5-16μg) were evaluated by ITLC-SG/Butanone, ITLC-SG/Saline and Whatman's No-1 paper/ 50% Acetonitrile in water. Radioactivity was detected by a Radio-TLC scanner

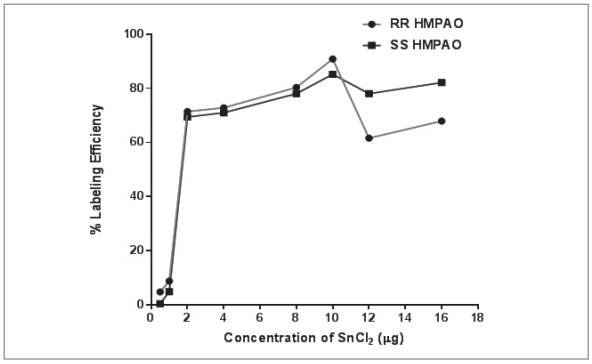


Figure 3. Labeling efficiency of ^{99m}Tc-*R,R* HMPAO and ^{99m}Tc-*S,S* HMPAO at various concentration of $SnCl_2.2H_2O$ solution

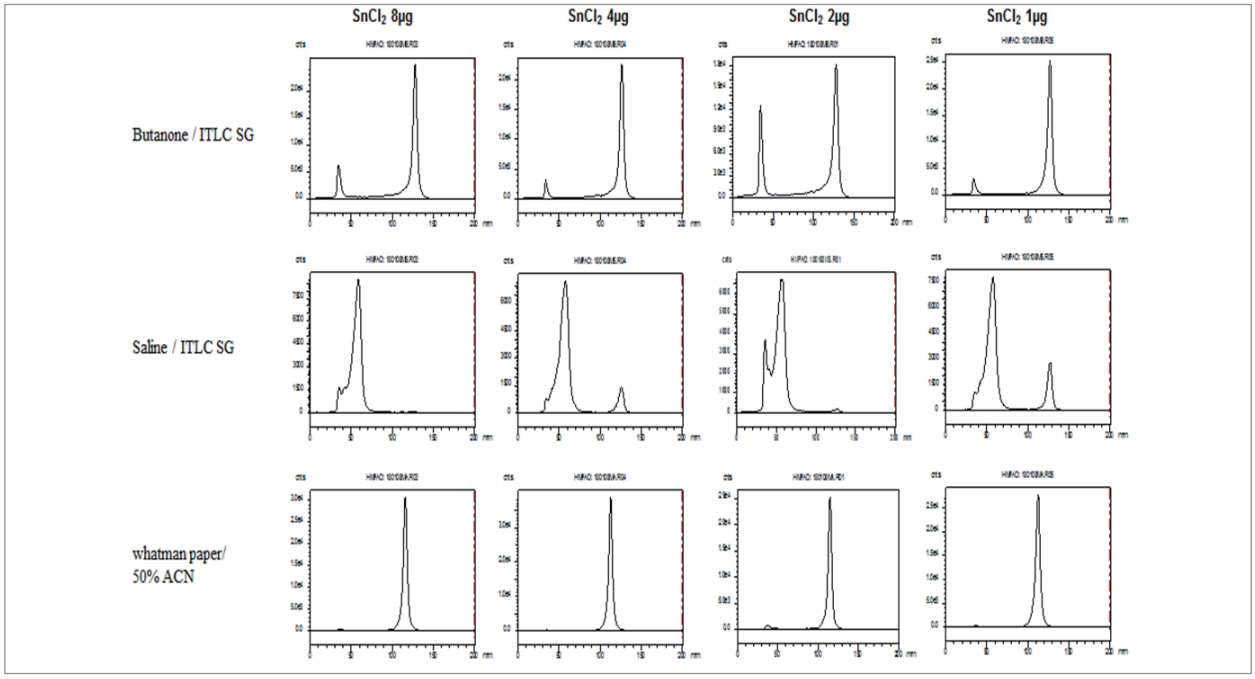


Figure 4. Radiolabeling efficiencies of ^{99}mTc-dl HMPAO with various concentration of SnCl2 (1.0-8.0 µg) were evaluated by ITLC-SG/Butanone, ITLC-SG/Saline and Whatman's No-1 paper/ 50% Acetonitrile in water. Radioactivity was detected by a Radio-TLC scanner.

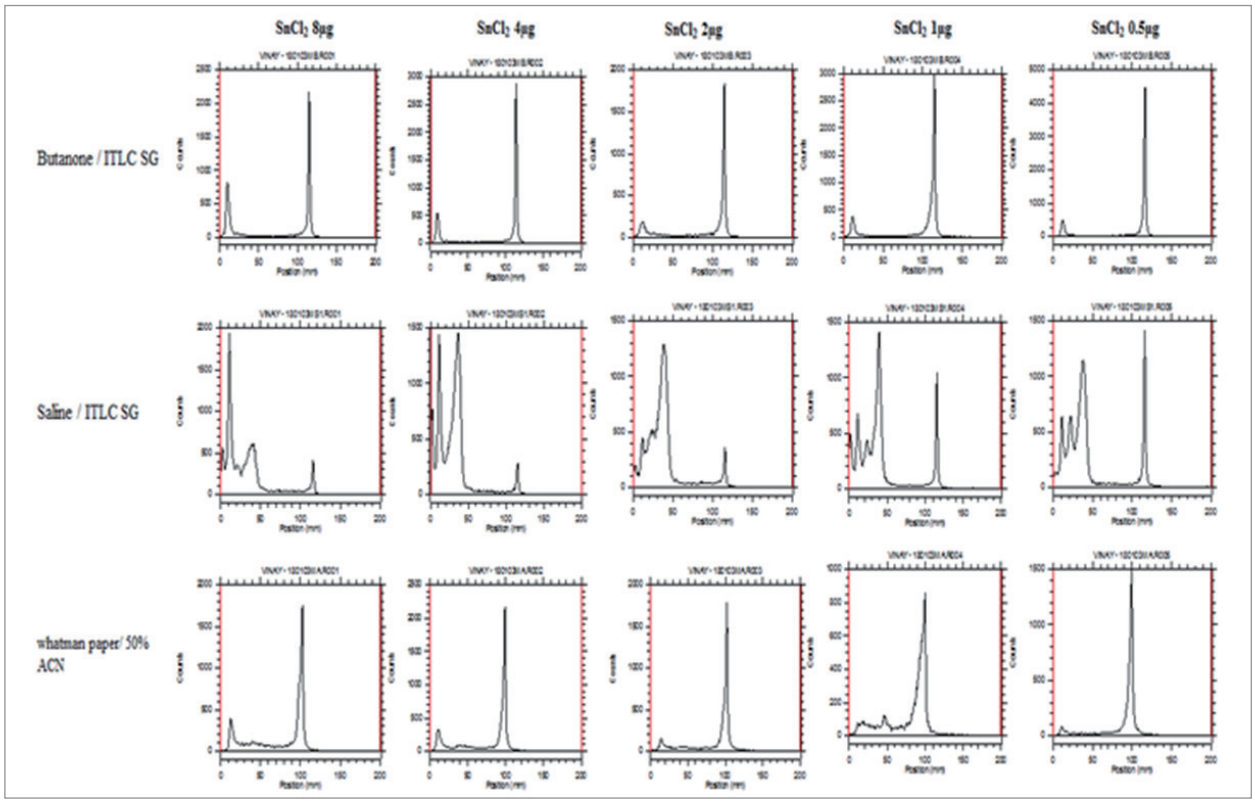


Figure 5. Radiolabeling efficiencies of ^{99}mTc-*Meso* HMPAO with various concentration of $SnCl_2.2H_2O$ solution (0.5-8.0 µg) were evaluated by ITLC-SG/Butanone, ITLC-SG/Saline and Whatman's No-1 paper/ 50%Acetonitrile in water. Radioactivity was detected by a Radio-TLC scanner.

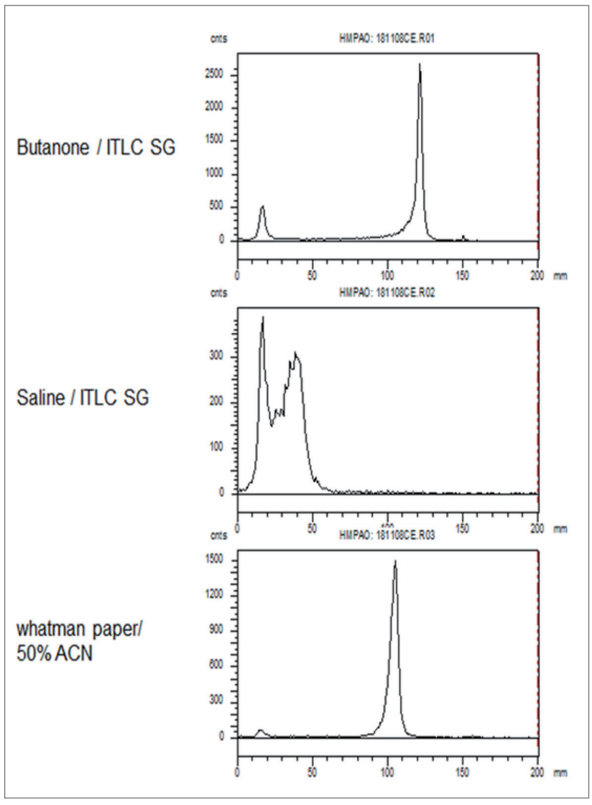


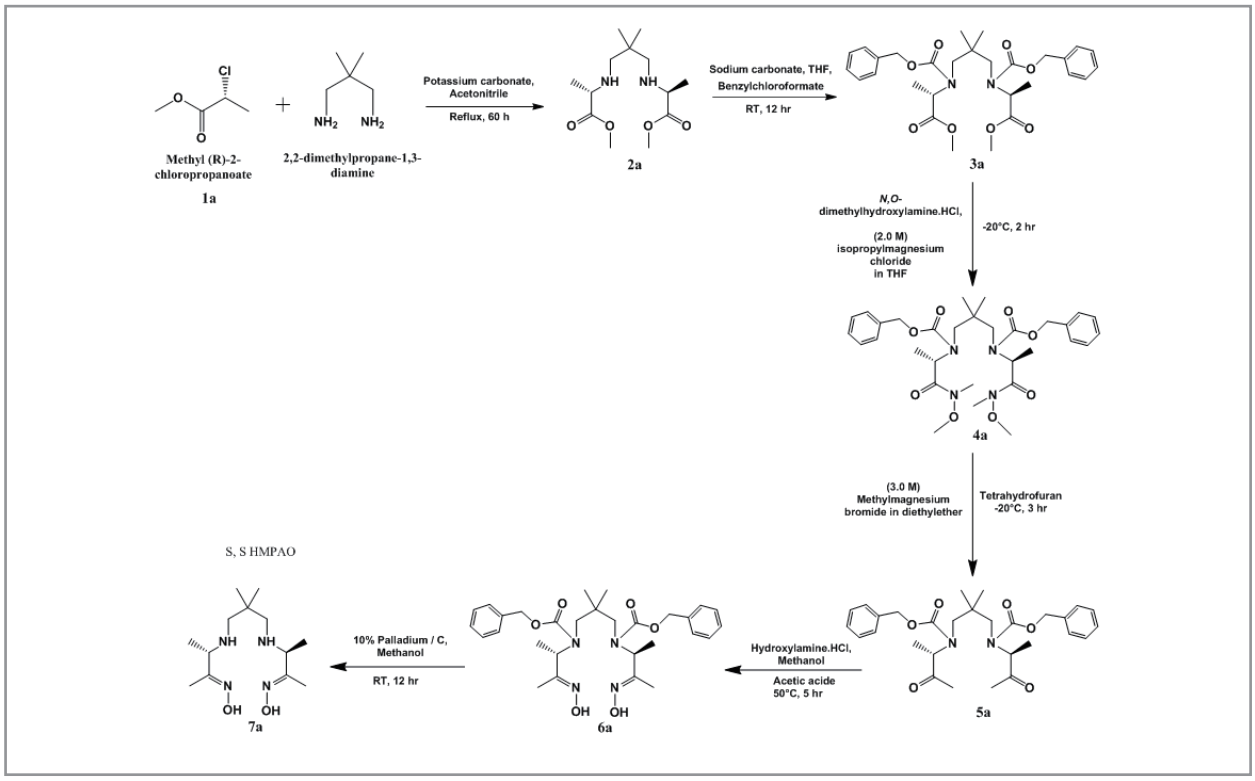
Figure 6. Radiolabeling efficiencies of ^{99m}Tc-dl HMPAO (Ceretec™) were evaluated by ITLC-SG/Butanone, ITLC-SG/Saline and Whatman's No-1 paper/ 50%Acetonitrile in water. Radioactivity was detected by a Radio-TLC scanner

evaluated as a brain imaging agent (8, 10). It was originally confirmed that ^{99m}Tc-complexes of HMPAO diastereomers (*meso*- and *dl* racemate) showed different pharmacokinetics properties *in vivo*. It rapidly diffused across the BBB at normal flow rates. However, the ^{99m}Tc dl-HMPAO revealed superior brain uptake and retention (4.1%) compared to the ^{99m}Tc meso-isomer (1.7%) and stereoisomeric mixtures (1.9%) after 8 h post injection (14). The reason for higher uptake and retention of *dl*-form in brain is due to rapid intracellular conversion rate of lipophilic to hydrophilic form. Whereas meso form gives 14-fold slower conversion rate than dl-form, resulted in decreased uptake and retention in the brain.

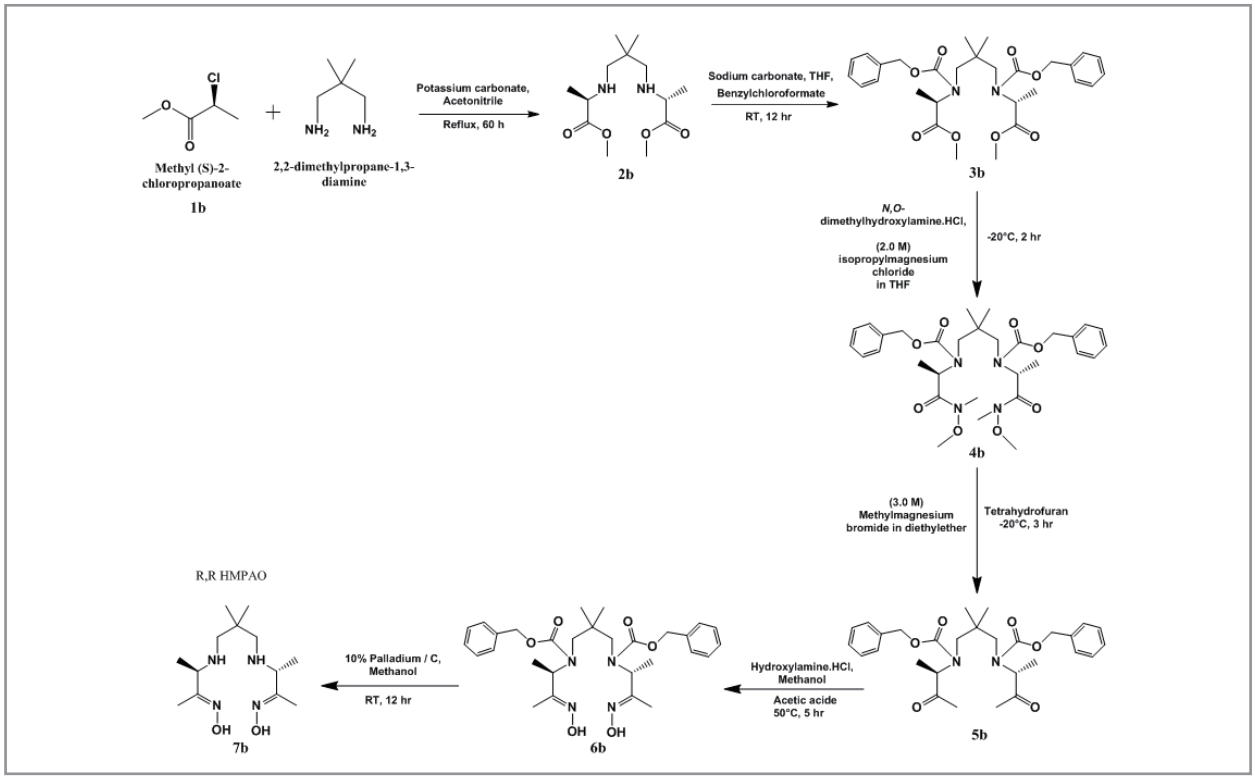
In addition, the later studies indicated that brain uptake of ^{99m}Tc-complexes formed from separated *d (R)* - and l (*S*)-enantiomers were different. Although ^{99m}Tc-*dl*-HMPAO have been widely studied, however less attention has paid to evaluate the efficacy and potency of enantiomers due to cumbersome and time-consuming fractional crystallizations procedures for separation of d *(R)*-HMPAO, l (*S*)-HMPAO from mixture of *dl*- and *meso* -HMPAO (13). Hence, it results in the drastic reduction of the final synthesis yield of *d(R)*- & *l(S)*-enantiomers and also still containing tracer amount of *meso* –HMPAO(15). There are some reported routes to synthesized d (*R*) - and l (*S*)-enantiomers (26).

However, it has some drawbacks such as difficult in synthesizing each step with lower yields, final product cannot be recrystallized due to minor impurity and high pressure liquid chromatograpy (HPLC) purification for final precursor that results in changing chemical properties because of 0.1% trifluroacetic acid (TFA) buffer as mobile phase was not suitable.According to our practical experience worked on many synthesis routes, we concluded HMPAO precursor is very sensitive and can easily decompose with acid.

To circumvent this problem, we developed new efficient route for synthesis of *S,S*-HMPAO and *R,R*-HMPAO enantiomeric compounds (Scheme 1, 2). All reaction steps involved in this designed scheme were straightforward with excellent yield between 50% to 95%, Final enantiomeric products with high purity and which does not contain any undesired product such as meso- and diastereomers as compared with other HMPAO routes. This route allows us to synthesize the final HMPAO in large scale for commercial applications. Previously published old route of HM-PAO (*meso, dl)* was also synthesized for comparative radiolabeling study with enantiomers (*RR-, SS*-HMPAO). In addition, the specific desired isomers which were developed from new route synthesis are confirmed with help of melting point test. The melting point is an important physical property of a compound. The melting point can be used to identify a substance and as an indication of its purity. From new route synthesis, we proved final specific enantiomers such as SS- (m.p: 138-141°C), RR- (m.p: 135-136°C) HMPAO



Scheme 1. Synthesis of S,S-HMPAO



Scheme 2. Synthesis of R,R-HMPAO

melting point results shown to be matched with previously reported enantiomers synthesis in old route (26). On other side synthesized final *dl*- (m.p: 125-127°C °C) and meso- (m.p: 119-122°C) HMPAO isomers melting points were also matched according to published method (10).

The cold precursor *SS*-, *RR*-, *dl*-, and *meso*-HMPAO isomers were formulated into kits with various concentration of $SnCl_2\delta2H_2O$ (0.5 -16 μg) in each vail. Addition of ^{99m}Tc to a HMPAO kits produce ^{99m}Tc-labeled HMPAO isomers. Combination of three chromatographic systems (ITLC-SG/ MEK, ITLC-SG/saline, and whatman No.1 (1mm) /50% acetonitrile in water) was used for complete characterization of the ^{99m}Tc-HMPAO radiochemical composition. The above TLC condition allows to characterize the desired ^{99m}Tc-lipophilic complex, free ^{99m}Tc, reduced-hydrolyzed technetium (TcO_2) and hydrophilic secondary complex.

Over decades, several groups followed same kind of radiolabeling procedure (8, 10) giving lower radiolabeling efficiency. The commercially available kits from Ceretec™, GE Healthcare (each vial contains 7.6 μg $SnCl_2.2H_2O$) give ≤ 80 % radiolabeling efficiency. Therefore we also optimized radiolabeling conditions, results with 10 μg of $SnCl_2.2H_2O$ provides higher radiolabeling efficiency for ^{99m}Tc-*R,R*-HMPAO and ^{99m}Tc-*S,S*-HMPAO than with other $SnCl_2.2H_2O$ concentration. Based on our study, at lower $SnCl_2.2H_2O$ concentration (8 to 0.5 μg) resulted in decreased radiolabeling efficiency of ^{99m}Tc-HMPAO isomers (< 5%) with an increased percentage of free ^{99m}Tc (> 90%). In particular, the increase of $SnCl_2.2H_2O$ concentration from 12 to 16 μg in the kit resulted in decreased radiolabeling purity with an increased percentage of secondary complex (between 5 to 20%) and hydrolysed form (between 5 to 20%). Therefore, the amount of $SnCl_2.2H_2O$ plays an important role on ^{99m}Tc-HMPAO. The radiochemical purity and biodistribution results were dependent on the content of $SnCl_2.2H_2O$ in the kit formulation.

Conclusion

We successfully synthesized pure isomers of HMPAO and could label them with ^{99m}Tc in high yield. These isomeric HMPAO might be helpful for brain perfusion imaging and inflammation imaging in the hospitals.

Acknowledgements

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