Synthesis, characterization and biodistribution of novel amine thiophene ^{99m}Tc labeled complex

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Abstract: ^{99m}Tc-labeled amine thiophene ligand might be a potential candidate for brain imaging. The purpose was to investigate the uptake of a radiolabeled drug in the brain. In this study, a tetradentate amine-thiophene-dione ligand was synthesized by the reaction of thiophene-2-carboxaldehyde with ethane-1,2-diamine and reducing with $NaBH_4$. The ligand system was characterized by elemental analysis, FTIR and ^{1}H NMR. Radiolabeling of the complex with ^{99m}Tc was performed by reducing with stannous ions. The radiochemical purity of the radiolabeled drug was determined by paper chromatography (PC) and instant thin layer chromatography (ITLC). Bioevaluation of the ^{99m}Tc complex was studied in rabbits. The yield of the final product was 4.42 g (60%) and the characterization data confirmed the synthesis of the final product. The efficacy of radiolabeling was >98%. A significant uptake was observed in the brain which retained significantly upto 4h. The data indicate that the proposed system may be suitable for brain imaging in future clinical applications.

Keywords: Amine-thiophene-dione, radiolabeling, brain imaging, biodistribution.

INTRODUCTION

^{99m}Tc-labeled diagnostic radiopharmaceuticals are common in use because of the superior medico-imaging characteristics of ^{99m}Tc ($t_{1/2}$ = 6.0h, E =140 keV) and the convenient availability of radionuclide (Jurisson *et al.,* 1999; Liu and Edwards, 1999). As the small size of the radiolabled complex has great importance for the retention of drug in organs (Jaouen *et al.,* 2000), one of the purposes of this investigation is to explore novel complexes for small size with multifunctional ligands possessing specific bioactivities.

Compounds with the structure –C=N- are called Schiff bases that are usually synthesized from the condensation of active carbonyl groups and primary amines. Schiff bases have significant importance in the field of pharmaceutics and medicine. The development of ^{99m}Tc brain perfusion imaging agents has been a subject of great interest in radiopharmaceutical research. These agents have to cross the intact blood-brain barrier and retain in the brain for a reasonable time to allow SPECT imaging (Nowotnik, 2002; Kung, 1990; Holman and Devous, 1992; Moretti *et al.,* 1995; Neirinckx *et al.,* 1986). The essential requirement of a technetium complex to be designed for neurologic imaging is that the compound should be neutral and lipophilic to cross the blood-brain barrier and possess an intrinsic mechanism, by which the complex which is diffused inside may not diffuse out (Neirinckx *et al.,* 1986). Several compounds such as amines labeled with selenium-75 (^{75}Se) (Kung and Blau, 1990; Kung *et al.,* 1990) and iodinated (with ^{123}I) amphetamine analogs (Kung *et al.,*1983; Winchell *et al.,* 1980; David *et al.,*1982) have been proposed in the past as possible brain radiotracers. However, due to certain drawbacks, these compounds have never gained worldwide application.

Recently, synthesis and study of diimino tetradentate Schiff bases and their complexes have gained particular attention (Costes *et al.,* 1982; Averill and Broman, 1978) because of their potential application in development of biological models, for understanding the structure of biomolecules and the related biological processes (Kovacic, 1967; Atkins *et al.,* 1985).

Amine-phenol ligands were previously synthesized by condensing two equivalents of aldehyde with one equivalent of triamines and reducing the resultant compound with sodium borohydrate. Tisato *et al.* (1988) reported the synthesis and characterization of ^{99m}Tc Schiff base of the similar pentadentate ligands. Like amine-phenol ligand, amine-thiophene ligand can also be prepared by using the same experimental pattern.

In the present study, we have synthesized the amine thiophene ligand by the reaction of thiophene-2-carboxaldehyde with ethane-1,2-diamine and reduced it with $NaBH_4$, followed the condensation with pentane-2,4-dione. Afterwards, the complexation of the resultant NNOS tetradentate was performed with ^{99m}Tc and its biodistribution was studied in rabbit. The synthesis, radiolabeling, protein binding and radioimaging are described herein.

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MATERIALS AND METHODS

Materials

Thiophene-2-carboxaldehyde and $SnCl_2.2H_2O$ were purchased from Aldrich, USA. Ethane-1,2-diamine, and molecular sieves 4^0A were purchased from Fluka, USA. Pentane dione was taken from Acros Organics, USA. Ethyl acetate was purchased from Panreac, France. Methanol and ethanol were purchased from Merck, Germany. Methyl ethyl ketone (MEK) was purchased from Fisher Scientific, UK. Acetonitrile was purchased from J. T. Baker, Holland. Trichloroacetic acid (TCA) was purchased from BDH Laboratory Reagents, England. ^{99m}Tc generator was taken from PINSTECH, Pakistan. Saline was purchased from Ostuka, Pakistan. Chromatograms were analyzed by using high pressure liquid chromatography - HPLC (Merck, Hitachi), with interface D-7000, system controller L-7200, pumps L-7100, diode-array detector L-7455, degasser L-7612, and separating column C18 (Vydac). Elemental analysis was performed on Elemental Analyzer MOD-1106 Carlo Erba. EM 360 MHz Nuclear Magnetic Resonance (NMR) spectrometer (Varian Int.) was used for identification of the compound and FT-IR spectra were recorded on Perkin-Elmer 882 spectrophotometer by using KBr disc technique. Quality control of the labeled compound was performed by using Whatmann No.3 MM chromatography paper. Radiopharmaceutical biodistribution was determined in rabbits following intravenous injection (111 MBq or 3 mCi in 300 µl) via the rear ear vein. Animal handling and all animal experiments were carried out in accordance with procedures approved by the local ethical and animal care committee.

Methodology

Whole process of synthesis of thiophene-2-carboxaldehyde ethylene diamine-N-dione was carried out in following three steps:

Reaction of thiophene-2-carboxaldehyde with ethane-1, 2-diamine

The complex was prepared by slowly adding 80 ml of ethanolic solution of 11.21 g (0.1 moles) of thiophene-2-carboxaldehyde during one hour to 36.06g (0.6 moles) of ethane-1,2-diamine. The resulting mixture was stirred with a magnetic bar for 30 min, followed by addition of 7g molecular sieve (4Å) and continued overnight stirring at room temperature. The solution was filtered and used for the next step without purification.

Reduction of azomethane (-C=N-) bond

7.56g (0.2 moles) of $NaBH_4$ was slowly added to the solution obtained from first step in small portions within 30 min at constant stirring in the ice bath while keeping the temperature below 10^oC. After addition of sodium borohydrate, the reaction mixture was stirred for 2h at the same temperature. The solvent was evaporated by rotary evaporator and 100 ml of distilled water was added in reaction mixture. The aqueous phase was extracted with dichloromethane (3 × 50 ml). Organic phase was dried over anhydrous $MgSO_4$ overnight, filtered and the solvent evaporated by rotary evaporator to obtain the product.

Condensation with dione

4.82g (31.3 mmoles) of the crude product from the previous reaction was dissolved in 31.3 ml of acetonitrile and mixed slowly with 6.43 ml (62.6 mmoles) of pentane-2,4-dione (thus making total volume 37.7 ml) and stirred with a magnetic bar for 3h at room temperature. The solvent was evaporated and a dark brown product was obtained.

Purification of the resultant ligand

For purification of thiophene-2-carboxaldehyde ethylene diamine-N-dione derivative, charcoal and silica gel were used. As an experimental procedure, 0.5g of the charcoal was added to the methanolic solution of the newly produced ligand and boiled for 2 min. to get the impurities on charcoal absorbed. The solution was filtered twice to remove the charcoal particles and evaporated to obtain the pure derived ligand. In case of silica gel, the ligand dissolved in 0.5 ml methanol was eluted on a column by using methanol and ethyl acetate (1:1) as eluting solvents. The outflow of the column was collected in a fraction collector; various fractions were combined and evaporated to get the pure tetradentate ligand which was obtained as viscous oil.

Radiopharmaceutical kit formulation of the compound

In order to label the newly synthesized compound with ^{99m}Tc, the standard procedure used for the formulation of a radiopharmaceutical kit, was adopted. Each kit of the ligand contained the following constituents: i) ligand (2 mg dissolved in 1 ml ethanol at continuous stirring), ii) 5 µl solution containing 25µg of stannous chloride-dihydrate (from a solution containing 50 mg stannous chloride-dihydrate dissolved in 500µl conc. HCl by boiling and diluted to 10 ml by using 0.1 N HCl), iii) pH of the resultant solution maintained at pH 8-8.5 and filtered through a 0.22 µm membrane filter.

Radiolabeling

The radiolabeling was performed by the addition of (< 1h) freshly eluted 500 µl saline solution of 740 MBq or 20 mCi sodium pertechnetate ($Na^{99m}TcO_4$) in the cold kit formulated in above step. The resultant radiolabeled mixture was shaken well and incubated at room temperature for 15 min. Radiochemical purity of the complex (amount of radiometal bound to ligand) was determined by quality control procedures.

Quality control of ^{99m}Tc-complex

Paper chromatography (PC) was used to perform the quality control. To determine the amount of free pertechnetate in the reconstituted kit, acetone or

Table 1: ^{1}H-NMR data of the ligand

Proton	Multiplicity	Chemical Shift (ppm)	No. of protons
-H at position 2	doublet, J = 2.01 Hz	6.83	1
-H at position 3	d, d; J = 3.0, 5.1 Hz	6.89	1
-H at position 4	doublet, J = 3.00 Hz	7.23	1
-H_2 at position 6	singlet	3.80	2
-H at position 7	broad peak	4.66	1
-H_2 at position 8, 9	doublet, J = 6.10 Hz	3.30	4
-H at position 10	broad peak	4.69	1
-H_3 at position 12	singlet	1.90	3
-H at position 13	singlet	4.95	1
-H_3 at position 16	singlet	2.10	3

The signal at δ 3.30 integrated four protons and was observed as doublet having j = 6.10 (4 protons at positions 8 and 9). The signal at δ 2.1 integrates for 3 protons.

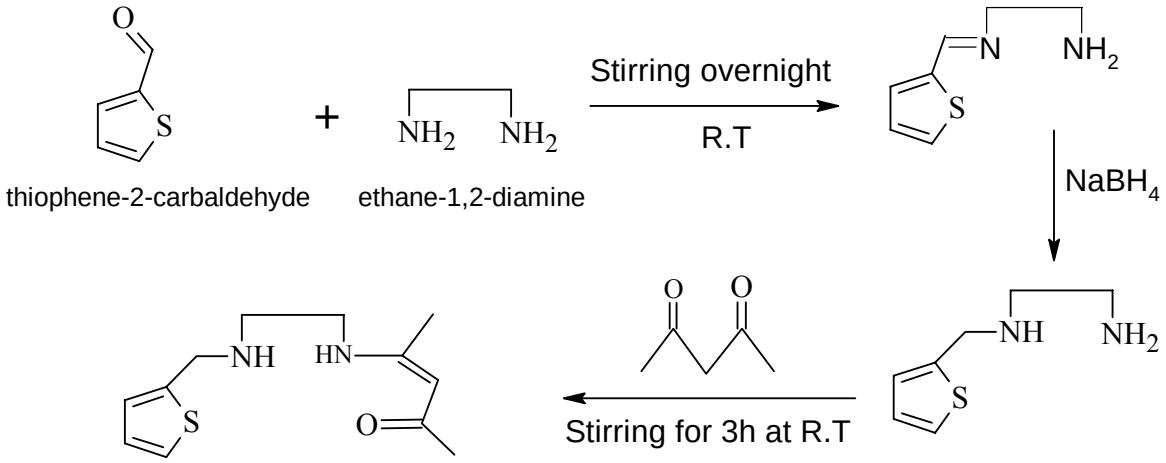

Fig. 1: Schematic diagram showing the formation of thiophene-2-carboxaldehyde ethylene diamine-N-dione.

methylethyl ketone (MEK) was used as the mobile phase on a Whatman No. 3 MM paper. The hydrolyzed or reduced activity was determined by using instant thin-layer chromatography (ITLC) in which strips of fiber glass coated with silica gel were used as the stationary phase and saline (0.9% NaCl) was used as the mobile phase. For determination of free and hydrolyzed fractions in the ^{99m}Tc-complex, small samples of the reconstituted kit were spotted on the respective strips. The strips, after elution, were cut in fractions of 1 cm and counted for radioactivity in a well type scintillation counter. In paper chromatography, free pertechnetate migrated with the solvent front while ^{99m}Tc-ligand and reduced or hydrolyzed $^{99m}TcO_2$-portions remained at the origin (A). In ITLC, ^{99m}Tc-ligand and free pertechnetate migrated with the solvent front while the reduced or hydrolyzed $^{99m}TcO_2$ portion remained at the origin (B). The radiochemical purity was calculated as 100 - (A + B) %.

Stability of ^{99m}Tc complex at room temperature

Stability of the ligand complex with ^{99m}Tc was studied at time intervals of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4h at room temperature. Change in stability of the radiolabeled complex was analyzed at each time interval by ITLC-SG and PC to determine any dissociation of the complex. The percentage of free pertechnetate (%) at a particular time presented percentage dissociation of the complex at that time. In case of significant loss of metal-complex stability, the radiopharmaceutical is usually not considered suitable for clinical applications.

Protein binding

5 ml of fresh blood (collected from a healthy volunteer) was taken in the tube. The kit was prepared and radiolabeled according to the procedure as mentioned before. A volume of 250 µl (3 mCi, 111 MBq) from the kit was added in the blood and incubated at room

temperature for 1h. The reaction was terminated by incubating the tube in water bath for 10 min pre-set at 37°C. Contents of the tube were centrifuged for 10 min, serum and blood cells were separated in two different layers. Equal volume of 10% trichloroacetic acid (TCA) solution was added into separated serum. TCA was used as a precipitating agent for the serum proteins. Tube containing serum and TCA solution was placed on the shaker for 10 min and then centrifuged at 4000 rpm for another 10 min time interval. Residues were separated from supernatant and counted for radioactivity.

Bioevaluation and pharmacokinetics

For evaluating the potential of the novel radiopharmaceutical as a feasible imaging agent (to study the uptake of radiopharmaceutical and its route of excretion), the acquisition study was carried out in healthy, New Zealand white rabbit models. The animals were originally obtained from a farm house and preserved in an animal house at INMOL, with a free access to green fodder and water. Ethical approval for the experiments was arranged according to Animal Ethics Principles of the Institute of Nuclear Medicine and Oncology (INMOL), Lahore. The kit was prepared under sterilized conditions, and after radiolabeling, 300 µl (3 mCi, 111 MBq) from it was injected intravenously to the rear ear vein of the rabbit. Images were taken at various time intervals upto 4h p.i. which were recorded by using a large field-of-view dual head SPECT gamma camera equipped with a low-energy, all-purpose collimator for acquisition (M/S Siemens, USA). As an experimental procedure, the imaging was started by dynamic study of 15 min followed by static views of 200 kcounts at 15 min., 30 min., 1h, 3h and 4h p.i. At each time point, the ventral and dorsal views of the animals were imaged. The whole study was performed at a matrix size 256 x 256 and a zoom of 1.0. Data processing was performed on the ICON8.5 Macintosh System interfaced with the camera.

RESULTS

Synthesis of the ligand

The synthesis reactions resulted in 60% (4.42g) yield of the final product. The success of the overall procedure was confirmed by characterization of the resultant product. The reaction scheme is shown in fig. 1 and proposed structure of ^{99m}Tc complex of thiophene-2-carboxaldehyde ethylene diamine N-dione is shown in fig. 2.

Characterization of the resultant ligand

The organic compound prepared was viscous oil which decomposed at 160 – 170°C. The predicted molecular formula of the ligand is $C_{12}H_{18}N_2OS$. Anal. calc'd for $C_{12}H_{18}N_2OS$: C, 60.22; H, 7.6; N, 11.43. Found: C, 60.5; H, 7.56; N, 11.76%. The FT-IR of the proposed tetradentate NNOS ligand (fig. 3) differs from the starting ligand, notably in the absence of the absorption band at 1634 cm^{-1}, assignable to the –C=N bond which was formed in the first step of the synthesis. In the second step, reduction with $NaBH_4$ converted –C=N- bond into ─CH─NH- bond, as a result, the spectrum exhibited a new FT-IR absorption at 3392.4 cm^{-1} for –N–H bond. In the third step, condensation of the crude product with pentane dione was performed and a new absorption at 1606.5 cm^{-1} for the conjugation (C=C-C=O) bond was observed instead of absorption of –C=O bond at 1700 cm^{-1}. Conjugation of –C=O bond with C=C bond lowered the absorption frequency which was detected at 1606.5 cm^{-1}. Tetradentate N_2OS ligand was also characterized by

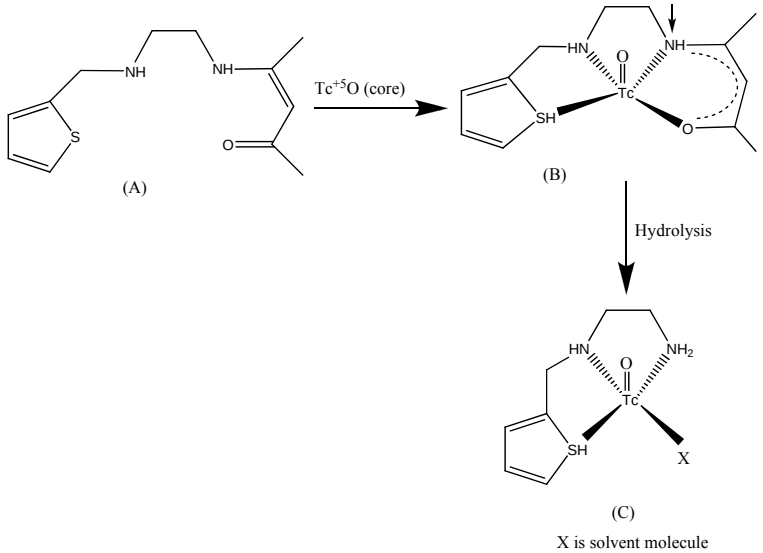

Fig. 2: Schematic diagram shows the probable sites of hydrolysis in the ligand thiophene-2-carboxaldehyde ethylene diamine-N-dione (A) and the technetium complex (B). The hydrolysis of (B) results in a compound of uncertain structure (C), with a vacant coordination site X, which may loosely coordinate solvent molecules.

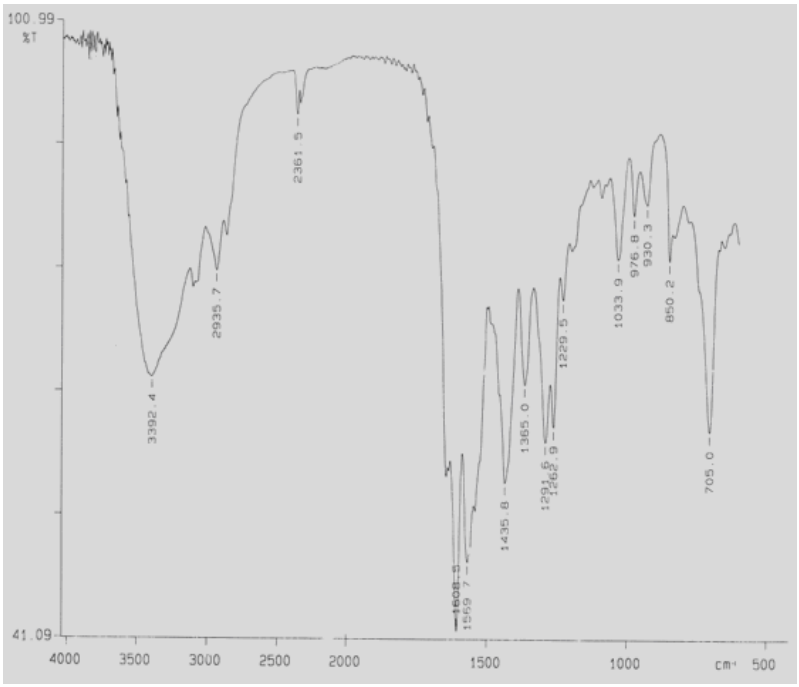

Fig. 3: FTIR spectra of thiophene-2-carboxaldehyde ethylene diamine-N-dione.

^{1}HNMR. The spectra consisted of three distinct regions: doublet, singlet and broad peak. –CH resonance (proton present in the ring) appeared as multiplet in the region at 6.83-7.23 ppm, and -CH_2 proton resonance appeared as a singlet at 3.80 ppm. Because of the slow exchange of the secondary N–H bond, the amino proton has been partly decoupled and a broad spectrum resulted at 4.66 ppm. The signal at δ 3.30 integrated four protons and was observed as doublet having j = 6.10 (4 protons at positions 8 and 9). The signal at δ 2.1 integrates for 3 protons. The possible structure of the ligand is shown in fig. 4 and the analytical data of ^{1}HNMR of the ligand is summarized as: ^{1}HNMR ($CDCl_3$) δ 1.9-2.1(s) (6H, 2 x –CH_3), δ 3.3(d) (4H, 2 x –CH_2), δ 3.8(s) (2H, 1 x -CH_2), δ 4.66-4.69(b) (2H, 2 x –NH), δ 4.95(s) (1H, =CH), δ 6.83-7.23(m) (3H, thiophene hydrogens). These data are summarized in table 1.

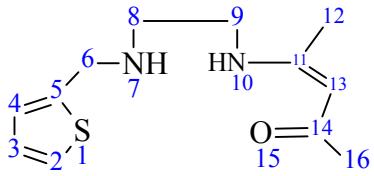

Fig. 4: Structure of ligand.

HPLC was performed by using an isocratic system. Mobile phase consisted of acetonitrile/water in 50:50 ratios. Reverse phase C-18 column was used at a flow rate of 0.8 ml/min. UV/VIS detector was employed to obtain the chromatogram. The retention time of the thiophene carboxaldehyde ethylene diamine-N-dione Schiff base was at 11.6 min.

Radiolabeling

In PC, $^{99m}TcO_4^-$ had an *Rf* of 0.8 – 0.9, while the ^{99m}Tc-ligand complex and the reduced or hydrolyzed $^{99m}TcO_2$ appeared at *Rf* = 0.00 – 0.01. The reduced or hydrolyzed fraction was separated from ^{99m}Tc-ligand complex by using saline, in which case the ^{99m}Tc-ligand complex and free $^{99m}TcO_4^-$ appeared at *Rf* = 0.9 – 1.0, and reduced or hydrolyzed fraction ($^{99m}TcO_2$) was detected at *Rf* = 0.00 – 0.01. The overall labeling yield of ^{99m}Tc-ligand complex, as calculated by these methods, was more than 98.1 ± 1.2%.

Stability and protein binding

As far stability data are concerned, the ligand remained intact with the radio-metal in complex form and no significant change was observed in the percentage binding till 4h at room temperature. These data indicate that the radiometal complex should be quite suitable for its further evaluation as a potential scintigraphic agent. For protein binding, the data indicated that the 85.7% radiolabeled complex showed binding with the proteins and 14.3% of the labeled drug was retained in the supernatant. As the protein binding of the complex was high so it was retained in the brain.

Bioevaluation

Consequently, the potential of the novel radiopharmaceutical as a feasible imaging agent was tested in a rabbit model. It was observed that the radiolabeled complex formed a blood pool with heart, brain and liver (fig. 5A-B), that was excreted from the heart and liver within 1h. Delayed static images of brain showed high tracer uptake in brain (fig. 5C-E). There is

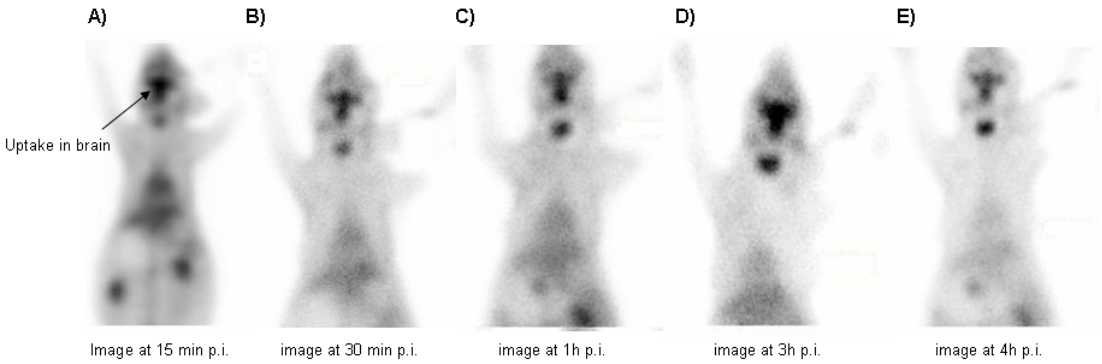

Fig. 5: Pharmacokinetic concentration-time profile of the first 4 hours after intravenous administration.

no sign of excretion through the hepatobiliary route. Both kidneys were visualized and activity was collected in the urinary bladder. These findings confirmed that complex was excreted through the urinary system and that the compound got rapidly extracted and retained in the brain which may be used in future, as a feasible candidate for imaging of regional blood flow in brain.

DISCUSSION

The reaction scheme has two intermediate products – the first one is actually an azomethine and the second is its reduced form. The second intermediate product was further condensed with a dione to get the final product that was radiolabeled and used in all subsequent studies. The reduction of azomethine (-C=N-) bond in the resultant reaction mixture was performed to achieve the condensation with dione.

Three species were formed during labeling of the diamine-dione ligand with ^{99m}Tc - the bound ^{99m}Tc complex, free pertechnetate ($^{99m}TcO_4^-$) and reduced or hydrolyzed $^{99m}TcO_2$, which were separated by PC and ITLC, respectively. The overall labeling yield of ^{99m}Tc-ligand complex, as calculated by these methods, was more than 98.1 ± 1.2%.

The radiolabeling study was done under physiological conditions (Kumari *et al.,* 2004). Complexation of newly synthesized compound with ^{99m}Tc produce sufficiently stable complex under physiological conditions.

The binding of blood proteins with radiolabeled drug is an essential parameter for the measurement of the effectiveness of chelating moiety to coordinate the radiometal. Generally, the reaction involves the transchelation of radiometal to plasma proteins, particularly, albumin. So, before studying the uptake of the drug in an organism, binding with plasma protein was studied by an *in vitro* method. The interaction between plasma proteins affects different pharmaco kinetic parameters such as metabolism, volume of distribution, excretion of the drug, and dosage (Rolan, 1994) we examined the protein binding of ^{99m}Tc-ligand as an *in vitro* procedure in human blood. High binding with plasma proteins generally suggests the slow clearance of radiopharmaceutical from the blood, and its high accumulation in the organ.

Bioevaluation of a radiocomplex is important because it gives an idea about the *in vivo* biodistribution, pharmacokinetic behavior of the radiopharmaceutical and its excretion from the body. The absence of radioactivity in the stomach shows the absence of free pertechnetate which indicates *in vivo* stability of the complex.

The most probable route of excretion of this present drug and metabolites was through kidneys and bladder. This is in accordance with findings described by (Huang *et al.,* 2004).

CONCLUSION

The novel amine thiophene ^{99m}Tc labeled complex was successfully synthesized, characterized and biologically evaluated in a rabbit model. The preliminary studies with this ligand encourage further *in vivo* experiments to study the uptake in brain and develop it as a potential brain imaging agent.

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