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STUDIES ON ^{99m}Tc COMPLEXES OF FUNCTIONALISED PROPYLENE AMINE-OXIME (PnAO) LIGANDS

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SUMMARY

Three functionalised PnAO ligands, 3,3,9,9-tetramethyl-6-R-4, 8-diazaundecane-2, 10-dionedioxime (R= benzyl, p-nitrobenzyl and p-aminobenzyl) were synthesized. ^{99m}Tc complexes of these ligands were prepared at both tracer levels and carrier added levels with $^{99m}TcO_4^-$ and with $^{99}TcO_4^-$. Complexation yields were estimated by thin layer chromatography, HPLC and solvent extraction studies. The complexes were formed in high yields; the resultant complexes were neutral, highly lipophilic (log P ~ 2.3-2.5) and elute as single peak on a reverse phase HPLC column. Complexes with all the three ligands showed excellent stability over 24 h period of storage at room temperature. Biodistribution studies of the ^{99m}Tc complexes of the benzyl and nitrobenzyl ligands in Wistar rats showed brain uptake of $1.2 \pm 0.25\%$ and $0.88 \pm 0.10\%$ at 1 min post injection, respectively. The blood clearance was rapid in both the cases while the main excretory route was the hepatobiliary system. The aminobenzyl derivative is a bifunctional ligand which could be used for labeling antibodies and peptides with radiometals.

Keywords : Amine-oxime, ^{99m}Tc-complexes, brain imaging agents, bifunctional chelating agents

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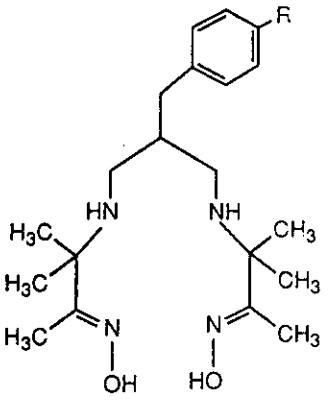
INTRODUCTION

Earlier studies by Troutner et al. (1) and Volkert et al. (2) showed that the ligand propylene amine-oxime (PnAO) forms neutral and lipophilic complexes with technetium which are highly stable under normal storage conditions. ^{99m}Tc complexes of this ligand's derivatives such as the hexamethyl propylene amine-oxime (HMPAO) have found application as a brain blood flow imaging radiopharmaceutical (3). Among this class of tetradentate amine-oxime ligands, ^{99m}Tc complexes of PnAO showed much higher stability than those of the complexes of HMPAO, though they differ in structure only slightly. We were interested in the development of bifunctional chelating agents based on an amine-oxime mainly for labeling antibodies with radiometals, more specifically with the radioisotopes of technetium and rhenium. The amine-oxime ligands form mononuclear complexes with ^{99}Tc (4,5) and hence should be suitable as bifunctional chelating agents. The higher stability of ^{99m}Tc complexes of the PnAO ligand as compared to that of the other derivative of the hexamethyl propylene amine-oxime ligand, led us to modify the PnAO ligand by introducing an additional functional group (6). Maecke and coworkers have also reported the use of PnAO ligand for the preparation of bifunctional chelating agents (7-9). This paper describes the synthesis and characterisation of three new tetradentate amine-oxime ligands and their radiochemical studies with technetium. The ligands are 3,3,9,9-tetramethyl-6-benzyl-4, 8-diazaundecane-2,10-dionedioxime (benzyl PnAO); 3,3,9,9-tetramethyl-6-(p-nitrobenzyl)-4,8-diazaundecane-2,10-dionedioxime (nitrobenzyl PnAO); and 3,3,9,9-tetramethyl-6-(p-aminobenzyl)-4,8-diazaundecane-2,10-dionedioxime (aminobenzyl PnAO). These ligands are henceforth referred to as L1, L2 and L3, respectively (Fig. 1). L3 is a bifunctional ligand and L2 is an intermediate in the synthesis of L3. L1 was synthesized for studying the non-specific protein binding of the complexes through sites other than the functional group.

MATERIALS AND METHODS

All organic chemicals needed for synthesis were purchased from Aldrich Chemical Company. Sodium borohydride and stannous tartrate were from Sigma Chemical Company. Flexible silica gel plates (7.5 x 2.5 cm) for thin layer chromatography were obtained from J.T. Baker Chemical Co. Whatman 3 mm chromatography paper (34 x 2.5 cm) was used for paper electrophoresis. HPLC grade solvents were used for liquid



L1 R = H

L2 R = NO_2

L3 R = NH_2

Figure 1. Ligands synthesised

chromatographic separations. All other reagents and solvents used were of analytical reagent grade.

$^{99m}TcO_4^-$ was eluted from a Mallinckrodt ^{99}Mo-^{99m}Tc generator after 24 h growth or from a solvent extraction generator with MEK as solvent. The activity ranged from 50 to 75 MBq/mL. ^{99}Tc as NH_4TcO_4 was obtained from New England Nuclear.

^1H NMR spectra were obtained on a JEOL 90Q spectrometer or a 500 MHz Brucker NMR spectrometer.

Syntheses

The synthetic scheme of L2 and L3 are given in Fig. 2 (10). L1 was also synthesized by similar procedure by starting with 2-benzyl-1,3-propane diamine.

2-p-Nitrobenzyl-1,3-propanediamine

2-benzyl-1, 3-propane diamine, the precursor for the synthesis of L1, was prepared according to the method of Whitmore and Eisenberg (11). 2-benzyl-1,3-propanediamine (2.68 g, 0.016 mole) was placed in a 25 mL round bottom flask. Glacial acetic acid (5.0 mL) and acetic anhydride (10.0 mL) were added to the diamine and stirred for 16 h. The excess glacial acetic acid and acetic anhydride were removed by rotary evaporation.

Conc. HNO_3 (6.0 mL) and conc. H_2SO_4 (5.2 mL) were added to the protected diamine residue and allowed to react for approximately 2 h after which the reaction was quenched by pouring the solution over ice. The nitration product was isolated by extracting the aqueous solution with 3 x 100 mL aliquots of $CHCl_3$. The organic layers were combined,

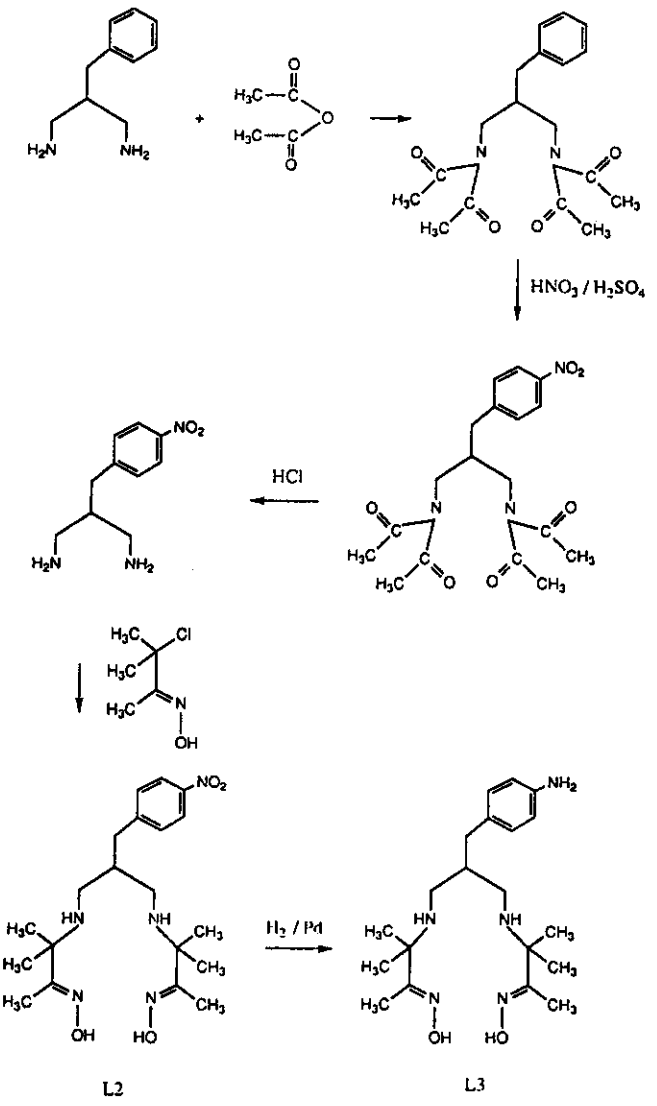


Figure 2. Synthetic scheme of L3

dried with anhydrous $MgSO_4$ and filtered. After filtration, the $CHCl_3$ was removed by rotary evaporation and the yellow oil obtained was refluxed with 70 mL of conc. HCl for 6 h. The excess HCl was removed by rotary evaporation. The residue was then dissolved in 45 mL of water. This aqueous solution was washed with a 70 mL aliquot of $CHCl_3$. 10 M sodium hydroxide was added to the resulting solution until it was extremely basic (pH 14). The diamine was recovered by extraction with 4 x 75 mL aliquot of $CHCl_3$. The organic layers were combined and dried with anhydrous $MgSO_4$, followed by removal

of the solvent. The reddish oil remaining was dissolved in absolute ethanol to which conc. HCl (5.0 mL) was added to form the hydrochloride salt of the free amine. The ethanol was removed by rotary evaporation to give a mixture of the ortho- and para- nitro substituted compounds. The para compound was separated from the mixture by fractional crystallization. The mixture was dissolved in a minimal volume of methanol, and benzene vapours were then allowed to diffuse into methanol until the para product crystallized. These crystals were recovered by filtration and dried in a vacuum desiccator. The yield was 1.31 g (28.5%). ^{1}H NMR [$CDCl_3$] (δ) 2.2-2.6 (m, CH, 1 H), 2.75 (s, the first half of the doublet of the benzyl-CH_2, 1H), 2.8-3.1 (m, CH_2 and the second half of the doublet of the benzyl-CH_2 proton, 5 H), 7.2- 8.1 (m, aromatic 4 H).

Benzyl PnAO (L1)

Chloroxime was synthesized as per the reported procedure (12). Chloroxime (4.5 g, 33 mmol) was dissolved in 60 mL of methanol. This solution was stirred at 0°C. When all of the chloroxime was dissolved, 2-benzyl-1,3-propane diamine (2.4 g, 15 mmol) dissolved in methanol was added dropwise. This reaction mixture was stirred for 5 days and then refluxed for 24 h. The solvent was removed by rotary evaporation leaving behind a yellow oily residue. The oily residue was dissolved in ~60 mL of 0.2 M HCl and filtered to remove any insoluble material. The filtrate was washed with 2 x 30 mL aliquots of $CHCl_3$ to remove any uncharged organic materials. 2 M NaOH was added to raise the pH of the solution to approximately 8. A small amount of solid appeared. The aqueous solution was made very basic and then extracted with 3 x 50 mL aliquots of $CHCl_3$.

The organic layers were combined, dried over anhydrous $MgSO_4$ and the solvent was removed under vacuum. The oily residue was reconstituted in approximately 10 mL of acetonitrile. This solution was added slowly to a solution of chloroxime (3.0 g, 22 mmol) in approximately 20 mL of acetonitrile at room temperature. After an hour a white solid had formed. This white solid was filtered and vacuum dried yielding 3.1 g (53% based on the original amount of 2-benzyl-1,3-propanediamine). ^{1}H NMR [D_2O] (δ) 1.3 (s, CH_3, 12 H), 1.85 (s, CH_3, 6 H), 2.5-3.0 (m, benzyl- CH_2, CH and CH_2, 7 H), 7.2-7.5 (m, aromatic 5 H).

2-p-Nitrobenzyl PnAO (L2)

The hydrochloride salt of 2-para-nitrobenzyl-1,3-propane diamine (1.29 g, 45.6 mmol) was dissolved in 2 M NaOH solution and extracted with 2 x 60 mL aliquots of CH_2Cl_2. The organic layers were combined, dried with anhydrous $MgSO_4$ and the solvent removed by rotary evaporation. The oily residue was dissolved in approximately 50 mL of CH_3CN. This solution was added dropwise to chloroxime (1.95 g, 14.4 mmol) in CH_3CN (~50 mL). The mixture was allowed to react for 3 days. A solid precipitate formed which was recovered by filtration and dried, yielding 0.56 g of product. This quantity of precipitate did not represent the amount of product that should have formed; therefore, the filtrate was further reacted.

The solvent was removed from the filtrate and the remaining oil dissolved in 2 M NaOH solution. This aqueous solution was extracted with 3 x 30 mL aliquots of $CHCl_3$. The organic layers were combined, dried with anhydrous $MgSO_4$ and the solvent removed by rotary evaporation. An oil (0.55 g) remained and was dissolved in approximately 20 mL of CH_3CN. Chloroxime (1.01 g, 7.5 mmol) dissolved in approximately 20 mL of CH_3CN was added to the above solution. This reaction mixture was stirred for approximately 3 h, after which the solvent was removed by rotary evaporation. The oil was dissolved in distilled water (~35 mL) and washed with 2 x 60 mL of CH_2Cl_2. The aqueous solution was made basic by slow addition of 20 mL of 1 M NaOH solution. A white precipitate formed which was recovered by filtration and dried to yield 0.83 g of product.

A portion of the above solid was recrystallized in MeOH and analysed by FAB mass spectrometry. The pseudo molecular ion 408.2 was observed which was consistent with $[M+H]^+$ ion for the 2-para-nitrobenzyl PnAO, free base molecule. ^1H NMR [acetone-d_6] (δ) 1.1 (s, CH_3, 12 H), 1.6 (S, CH_3, 6 H), 2.1 (b, CH_2, 4 H), 2.6-2.8 (d, benzyl-CH_2, 2 H), 7.3-8.2 (m, aromatic 4 H), 10.5 (s, oxime, 2 H).

P-aminobenzyl PnAO (L3)

2-p-nitrobenzyl PnAO (0.24 g, 0.58 mmol) was dissolved in ~25 mL of absolute ethanol and placed in a container with side arm inlet for H_2 gas. PtO_2 (0.032 g) was introduced into the container. The solution was placed under 1 atmosphere of hydrogen gas and was allowed to react until 38 mL of H_2 gas was taken up (~2 h). The reaction mixture was

then filtered to remove the solid PtO_2 catalyst. The solid residue remaining after removal of solvent weighed 0.17 g (77% yield). ^1H NMR (acetone-d_6) (δ) 1.2 (s, CH_3, 12 H), 1.75 (s, CH_3, 6 H), 1.9-2.1 (m, CH, 1 H), 2.3-2.6 (m, CH_2 and benzyl-CH_2, 6 H), 6.45-7.2 (m, aromatic, 4 H), 8.0 (s, oxime, 2 H).

Complexation Studies

The complexation reactions were carried out in pH 9 bicarbonate buffer. 0.1 mL of an ethanolic solution of the ligand (5×10^{-3} M), 0.5 mL of 0.5 M $NaHCO_3$ (pH 9), 4.0 mL of 0.9% NaCl solution and 0.2 mL of $^{99m}TcO_4^-$ (10-15 MBq) were placed in a 20 mL scintillation vial. Complexation was achieved by adding 0.2 mL of a saturated solution of stannous tartrate. The final ligand concentration in the reaction was 10^{-4} M. Quality control tests were done at 1 h and 24 h after complexation using TLC, paper electrophoresis and solvent extraction.

Thin Layer Chromatography

Silica gel TLC was run using 0.9% saline as the eluent. 5 µL portion of the complex was applied 1 cm from one end of the strip and was developed to the top. The strips were dried, cut into 10 equal segments and counted in a NaI(Tl) scintillation well counter.

Paper Electrophoresis

Paper electrophoresis was run in a Gelman Deluxe Chamber and power supply unit for 1 h at 300 V in 0.01 M $NaHCO_3$ (pH 9) solution. Samples were spotted midway between the two electrodes. Following the run, the strips were dried, cut into 1-cm segments and analysed for their activity.

Solvent Extraction

Solvent extraction involved vortexing one mL of the reaction mixture (aqueous) with 1 mL of $CHCl_3$ for about 2 min. Equal volume aliquots of the aqueous and organic layers were withdrawn and assayed for radioactivity. The geometry of the counter was suitably adjusted such that the count rates were low enough to minimize dead time loss. 0.5 mL of the organic layer was back extracted with 0.5 mL of 0.05 M bicarbonate buffer to estimate the distribution ratio.

HPLC

Liquid chromatography separations were carried out using a Beckman series 332 dual pump gradient elution system equipped with a radiation detector connected to a strip chart recorder and an integrator. A Hamilton PRP-1 reverse phase column (150 mm, 5 μM) was used for separation with the mobile phase consisting of a mixture of CH_3CN and H_2O, at varying concentrations. Flow rate was maintained at 1 mL/min. The gradient elution was initiated at 2 minute, with 90% H_2O and 10% CH_3CN followed by a linear ramp to 20% H_2O and 80% CH_3CN in two minutes. This concentration was maintained for 20 minutes.

Biodistribution Studies

Biodistribution studies were carried out in Wistar rats weighing 150-250 g. The complexes (0.1 to 0.2 mL) were injected into the tail vein and the rats were sacrificed by cervical dislocation at 1, 5, 30 and 120 min after injection. The organs were excised, blotted to remove blood and assayed for radioactivity. Biodistribution studies in Swiss mice were also done similarly. (All the animal experiments were carried out in compliance with the relevant national laws relating to animal experimentation.)

RESULTS AND DISCUSSION

The chemical shifts observed in the ^1H NMR spectra were consistent with the structure of the compounds (L1, L2, L3) expected.

Complexation yields were analysed using the combination of four different analytical techniques: solvent extraction (Tables 1 and 2), TLC-SG, paper electrophoresis and HPLC. The yields obtained using these methods were consistent. The complexes formed are highly lipophilic and extract into $CHCl_3$ with log P ranging from 2.3-2.5 in a $CHCl_3$/ 0.05 M bicarbonate buffer (pH 9.0) system (Table 3); hence extraction of the chelates into excess $CHCl_3$ could be used for estimating the complexation yields. The complexes remained at origin in TLC using normal saline as eluent and hence this technique was used for the estimation of free TcO_4^- impurity (R_f = 0.9-1) in the complexes (Table 1). TcO_2 impurity was not directly measured, but was minimal, as inferred from the observation that the complexation yields estimated by solvent extraction when added to the TcO_4^- impurity accounted for 97 to 99% of the total activity in the complex. Paper

Table 1. Results of NCA ^{99m}Tc complexation studies with PnAO derivatives

Ligand	% extraction[a]		$^{99m}TcO_4^-$[b]	
	1 h	24 h	1 h	24 h
L1	98.7	96.4	0.8	2.6
L2	96.0	96.2	1.0	2.4
L3	96.3	95.0	0.8	1.7

[a]into $CHCl_3$
[b]TLC-SG, developed with 0.9% saline

electrophoresis results were consistent with the complexes being neutral as they remained at the origin.

The results of the complexation studies using solvent extraction are summarised in Table 1. The complexation yields were >96% for all the three NCA ^{99m}Tc chelates and the radiochemical purity did not significantly change over a 24 h period at room temperature. The $^{99m}TcO_4^-$ impurity was less than 1% in all the three complexes 1 h after complexation and did not increase significantly even after 24 h. Complexation of L1 and L2 was studied at different concentrations of Tc, using $^{99}TcO_4^-$ as carrier. Complexation yield was higher for L1 compared to L2 when carrier Tc was used. When the ligand:Tc ratio was 1:1, L1 gave 73% while L2 gave 43% complex yield (Table 2).

Table 2. Complexation yields, at different concentrations of ^{99}Tc, at pH 9 measured by extraction into $CHCl_3$

[Tc]	NCA		10^{-7}		10^{-6}		10^{-5}		10^{-4}	
Ligand	1 h	24 h	1 h	24 h	1 h	24 h	1 h	24 h	1 h	24 h
L1	98	95	97	92	96	95	93	91	73	68
L2	96	96	94	94	94	93	81	81	43	31

Table 3. Results of HPLC and partition ratio studies using ^{99m}Tc complexes with L1, L2 and L3

Ligand	HPLC Retention time (min)	Partition ratios ($CHCl_3$/buffer[a])
L1	13.0	322 ± 48
L2	12.6	203 ± 85
L3	10.6	223 ± 68

[a]0.05 M bicarbonate buffer

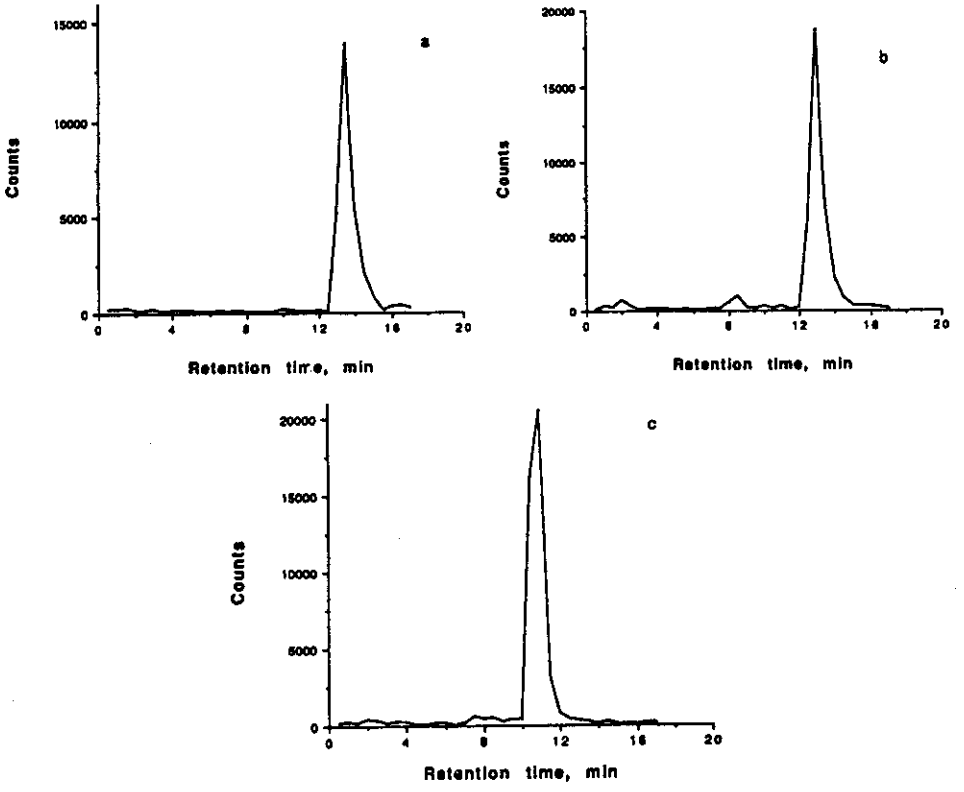


Figure 3. Liquid chromatographic pattern of the complexes (a) ^{99m}Tc-L1, (b) ^{99m}Tc-L2 and (c) ^{99m}Tc-L3

The HPLC chromatograms of the complexes given in Fig. 3 a-c show that the complexes are essentially eluted as single peaks, with retention times varying from 10.6 min to 13.0 min (Table 3). The ^{99m}Tc-PnAO has a retention time of 10.5 min under identical conditions (13).

Table 4. Organ update of ^{99m}Tc complex of benzyl PnAO (Tc-L1) in Wistar rats.

Organ	% Administered dose, Mean (std. deviation). n=3			
	1 min	5 min	30 min	120 min
Brain	1.2 (0.26)	1.36 (0.16)	0.88 (0.29)	1.0 (0.45)
Heart	1.1 (0.29)	0.93 (0.03)	0.88 (0.23)	0.96 (0.44)
Blood	8.9 (2.0)	5.0 (0)	4.4 (0.89)	4.86 (1.6)
Liver	5.8 (0)	20.0 (3.0)	12.4 (0.32)	9.16 (2.46)
Lungs	1.7 (0.29)	1.36 (0.24)	0.94 (0.23)	1.1 (0.5)
Kidneys	2.0 (0.41)	2.0 (0.14)	1.3 (0.24)	1.4 (0.51)
Intestine	5.75 (0)	16.0 (3.5)	60.0 (1.28)	80.3 (3.4)
Stomach	0.84 (0.32)	1.55 (0.14)	1.4 (0.14)	2.9 (2.4)

The results of the biodistribution studies in rats are given in Tables 4 and 5. Both the ^{99m}Tc complexes of L1 and L2 showed fast clearance from blood despite their high Log P values. About 1% of the injected activity was found in the brain at 1 min p.i., which was subsequently washed out due to lack of intracellular trapping of the activity in brain, as was also observed with ^{99m}Tc-PnAO. Consistent with the lipophilicity and blood clearance rate, both the complexes were extracted by the liver and excreted through the hepatobiliary system in to the intestines (Table 4 and 5). The complex Tc-L1 showed better hepatobiliary clearance than Tc- L2, but the renal involvement was comparable for both the complexes. Despite being a neutral complex Tc-L1 also showed an uptake of about 1% in heart without any significant washout for at least 2 h. ^{99m}Tc-L1 was also evaluated in mice and indicated similar *in vivo* distribution and hepatobiliary clearance characteristics (Table 6). Minimal uptake of activity in the stomach in both animal models indicated absence of $^{99m}TcO_4^-$ formation *in vivo*.

CONCLUSIONS

The tetradentate amine-oxime ligands (L1, L2 and L3) form neutral, lipophilic and highly stable complexes with ^{99m}Tc. The uptake of the complexes in brain occurs but is lower than ^{99m}Tc- PnAO, ^{99m}Tc-d,l-HMPAO or ^{99m}Tc-ECD (2,3,14);. The excellent stability of the ^{99m}Tc complex of L3 suggests that this ligand could be used as a bifunctional chelating agent for the labeling of biomolecular targeting agents with ^{99m}Tc. The utility

Table 5. Organ update of ^{99m}Tc complex of nitrobenzyl PnAO (Tc-L2) in Wistar rats.

Organ	% Administered dose, Mean (std. deviation). n=3			
	1 min	5 min	30 min	120 min
Brain	0.88 (0.1)	0.57 (0.09)	0.19 (0.15)	0.18 (0.03)
Heart	1.2 (0.05)	0.75 (0.04)	0.42 (0.04)	0.29 (0.08)
Blood	10.2 (0.94)	3.9 (0.55)	3.9 (0.92)	3.1 (0.53)
Liver	5.6 (1.3)	25.0 (5.8)	22.0 (1.0)	12.6 (1.15)
Lungs	2.5 (0.45)	1.2 (0)	0.63 (0.8)	0.41 (0.03)
Kidneys	2.2 (0.98)	2.6 (0.15)	1.8 (0.17)	1.1 (0.15)
Intestine	5.5 (1.6)	14.9 (1.0)	33.0 (3.0)	67.1 (2.4)
Stomach	1.12 (0.17)	1.5 (0.25)	1.36 (0.32)	1.0 (0.17)

Table 6. Organ update of ^{99m}Tc complex of benzyl PnAO (Tc-L1) in Swiss mice.

Organ	% Administered dose Mean (std. deviation). n=3		
	1 min	5 min	60 min
Blood	4.5 (0.85)	5.1 (0.6)	5.1 (0.51)
Liver	30.9 (1.5)	22.0 (0.68)	20.6 (0.57)
Kidneys	1.7 (0.05)	1.6 (0.29)	1.2 (0.15)
Stomach	1.12 (0.17)	1.5 (0.25)	1.36 (0.32)
Intestine & gall bladder	9.4 (0.55)	38.0 (5.0)	55.6 (0.7)

of our approach is borne out by the success shown in the case of hypoxia imaging agent TcO(PnAO-1-2-nitroimidazole) [BMS 181321] by Linder et al. (15). This ligand may also be useful for labeling antibodies or peptides with other radionuclides like $^{186/188}$Re. In addition, they also may find application for labelling monoclonal antibodies with ^{105}Rh(III) as reported by Venkatesh et al. (16).

Acknowledgments

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