SYNTHESIS AND CHARACTERIZATION OF LIGANDS AND BIFUNCTIONAL CHELATING AGENTS BY MODIFICATION OF CYSTEINE FOR COMPLEXATION STUDIES WITH ^{99m}Tc

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The synthesis of four novel ligands using the amino-acid cysteine and its ethyl carboxylate derivative is described. The synthetic method involves a two-step procedure, wherein the intermediate Schiff base formed by the condensation of the amino group of the cysteine substrate and salicylaldehyde is reduced to give the target ligands. The intermediates and the final products are characterised by high resolution NMR spectroscopy. Complexation studies of the ligands with ^{99m}Tc are standardised using stannous tartrate as the reducing agent at varying reaction conditions. The complexes are characterised using standard quality control techniques such as TLC, paper electrophoresis and PC. Lipophilicities of the complexes are estimated by solvent extraction into chloroform. Substantial changes in net charge and lipophilicity in the ^{99m}Tc complexes are observed on substituting the carboxylic acid residue in ligand I and II with the ethyl carboxylate groups (ligands III and IV). All the ligands formed complexes in high yield. While the complexes of ligand I and II are observed to be hydrophilic in nature and are not extractable into $CHCl_3$, ligands III and IV gave neutral and lipophilic complexes. Though the distribution ratios of the complexes of ligands III and IV in $CHCl_3$/saline system are observed to be very high, considerable differences in lipophilicities are also observed as evidenced by the difference in their respective extractabilities in chloroform. On storage, the complex of ligand III exhibit a tendency to get converted to a hydrophilic and non-extractable species. The bio-distribution of the complexes of ligands I and II showed that they have predominantly renal clearances whereas the complexes of ligands III and IV exhibited a significant hepatobiliary uptake and did not show much uptake in brain in spite of its favourable properties such as neutrality, lipophilicity and conversion into a hydrophilic species.

1. INTRODUCTION

Several reports describe the use of amino acids having sulphydryl groups in complexing ^{99m}Tc as a novel approach to the designing of radiopharmaceutical agents [1-4]. Considerable interest has been focused on the development of ^{99m}Tc labelled N_2S_2 type complexes incorporating functional moieties which enable metabolic trapping in the brain on crossing the blood brain barrier [5]. This has lead to the development of ^{99m}Tc-ECD (ethylcysteinate dimer) as a brain perfusion agent [6]. A few other radiopharmaceutical agents having N_2S_2 and N_3S_2 donor atom sets such as ^{99m}Tc labelled EC (ethylene dicysteine), MAG_3 (mercaptoacetyl triglycinate) respectively have also been developed for renal function imaging [7-8]. Prior to the use of ^{99m}Tc complexes of iminodiacetic acid derivatives, cysteine coupled with bromosulphonaphthalein has also been used [9] as a hepatobiliary agent.

The amino acid cysteine has been used without modification [10] for complexation with ^{99m}Tc in the development of new radiopharmaceuticals. In this direction, the designing of novel synthetic derivatives of cysteine as models for the radiopharmaceutical preparation of ^{99m}Tc mercaptide complexes constitute an interesting study. Cysteine offers the possibility of synthetic modification at the amino, carboxylic acid and the sulphydryl terminal. This structural versatility can be exploited to offer a variety of ligands which could be explored as potential radiopharmaceutical agents. Alterations in the biological functions of the complexes obtained from these ligands can be effected by suitable modification of the carboxylic acid group in designing either neutral and lipophilic or charged and hydrophilic complexes.

In the pursuit of novel ligands for use as diagnostic radiopharmaceuticals, Pillai et al. have reported the syntheses and radiochemical studies of a wide variety of amine phenol ligands [11-13]. These ligands were found to form neutral, lipophilic ^{99m}Tc complexes in high yields. It was therefore felt pertinent to synthesise novel ligands incorporating an amine phenol unit in the cysteine moiety. The apparently simple synthetic strategy which has been employed earlier could be envisaged in the present case also. However, the presence of reactive groups such as the carboxylate and the sulphydryl groups could possibly call for standardisation of the usual reaction conditions[11]. In the present study, we report the syntheses of four novel ligands, wherein cysteine and ethyl cysteinate have been condensed with salicylaldehyde to give the corresponding imines (I, III) and subsequently their reduced products (II, IV) (Fig.1).

Ligands I and II could also be used as bifunctional chelating agents in covalent linking of the free carboxylate substituent with suitable functional groups of receptor specific substrates or antibodies for development of receptor or antibody based radiopharmaceuticals. The addition of more chiral centres to the molecule is possible by alteration of the aromatic moiety from salicylaldehyde to 2-hydroxy acetophenone. Lipophilicity of the resultant complex can also be varied thus or by substitution of other suitable groups. Such synthetic manipulations open up the possibility of arriving at a spectrum of ^{99m}Tc complexes that could be explored as potential radiopharmaceutical agents.

^{99m}Tc complexes of all the four ligands were synthesised by reducing TcO_4^- in the presence of ligand with stannous tartrate at optimum pH. The yields of complexation were estimated by TLC, PC and paper electrophoresis. The lipophilicities of the complexes were estimated by solvent extraction into $CHCl_3$.

Bio-distribution studies of the complexes were carried out in Swiss mice.

2. MATERIALS

L-Cysteine, salicylaldehyde and sodium borohydride were purchased from Aldrich Chemical Company, U.S.A. L-Ethylcysteinate hydrochloride was purchased from Lancaster, England. Stannous tartrate was obtained from Sigma Chemical Company St. Louis, MO, U.S.A. $^{99m}TcO_4^-$ was eluted from a ^{99}Mo-^{99m}Tc column generator prepared in our laboratory [14]. $NH_4{}^{99}TcO_4$ was obtained from New England Nuclear and diluted in 0.9% saline solution to the required concentration. Flexible silica gel plates IB-F(7.5 x 2.5 cm) were from J.T. Baker Chemical Company, N.J. Whatman 1 chromatography paper (30 x 2.5 cm) was used for paper electrophoresis.

FT-IR spectra were recorded in Nicolet Magna IR Spectrometer 550. Proton NMR spectra were obtained using a Bruker 500 spectrometer, 500 MHz for ^{1}H and Varian VXR 300S, 300 MHz for ^{1}H using d_6-DMSO and $CDCl_3$ as solvents and TMS as the internal reference. Elemental analyses were done by Desert Analytics, Tucson, Arizona.

3. METHODS

3.1. Synthesis and spectroscopic characterisation of the ligands

3.1.1. Ligand I : The synthesis of ligand I was achieved via a two-step procedure (Fig. 1). 5 g (0.04 mol) of L-Cysteine and 5.0 g (0.04 mol) of salicylaldehyde were refluxed for 4 hours in ethanol (200 mL). The solution turned light yellow and precipitation was observed during reaction and on cooling. The pale yellow solid was collected by filtration, washed with cold water and dried to give 9.0 g (95 %) of the imine.

IR (KBr, ν cm^{-1}) : 3434 (OH), 2585, 2696 (SH), 1630 (C=N), 1597,1578 (Ar);

^1H NMR (d_6 DMSO, δ ppm): 3.44 (1H dd, J=12.0, 8.2 Hz) $-C\underline{H}_AH_BSH$, 3.21 (1H dd, 12.0, 8.2 Hz) $-CH_A\underline{H}_BSH$, 3.83 (1H dd, J=10.8, 8.3 Hz) $-C\underline{H}(N)(CH_2SH)$, 5.65, 5.84 (1H s) Ar-C$\underline{H}$=N-(geometrical isomers), 6.73-6.83 (2H m) Ar, 7.03-7.16 (1H m) Ar, 7.28-7.36 (1H m) Ar.

Elemental analysis: %Calculated for $C_{10}H_{11}NO_3S$: C 53.33 H 4.89 N 6.22

%Found : 52.97 4.99 6.30

3.1.2. Ligand II : To a stirred solution of 3.6 g (0.02 mol) of the imine (I) in 200 mL dry ethanol was added 0.3 g of sodium borohydride in instalments (0.05 g) when the solid slowly dissolves. After 30 min the solution was gently heated for complete dissolution. An additional 0.7 g of sodium borohydride was added in two portions and stirring continued for two hours. The reaction mixture was concentrated under vacuum and 100 mL water was added and pH of the solution adjusted to approximately 5 when a precipitate was observed. The product could not be extracted with chloroform and hence the aqueous solution was filtered and concentrated *in vacuo* to give a white precipitate (3.3 g, 92 %). The crude product was recrystallized from ethanol: water: acetone (1:1:0.2) mixture.

IR (KBr, ν cm^{-1}) : 3460 (OH), 3151 (NH), 2539 (SH), 1624 (acid >C=O), 1564 (NH), 1597(Ar); ^1H NMR (d_6 DMSO, δ ppm): 2.92 (1H dd , J = 16.6, 5.8 Hz) $-C\underline{H}_AH_BSH$, 2.80 (1H dd, J = 16.6, 7.3 Hz) $-CH_A\underline{H}_BSH$, 3.38 (1H broad t, J = 6.5 Hz) - $C\underline{H}(NH)(CH_2SH)$, 3.56-3.60 (1H m) $-CH(N\underline{H})(CH_2SH)$, 4.0 (2H d, J = 16Hz) Ar-$C\underline{H}_2(NH)$, 6.76-6.85 (2H m) Ar, 7.13-7.25 (2H m) Ar.

Elemental analysis:				
%Calculated for $C_{10}H_{13}NO_3S$:	C 52.86	H 5.73	N 6.17
%Found :	51.80	5.67	6.00

3.1.3. Ligand III : L-cysteine ethyl ester was generated by basification (pH 8-9, 1M NaOH) of an aqueous solution of the hydrochloride salt (5 g in 5 mL water), extracted with chloroform, dried over anhydrous sodium sulphate and solvent removed to give 3.3 g of L-ethyl cysteinate. Freshly generated L-ethyl cysteinate (3.4 g, 0.02 mol) was reacted with 3 mL of salicylaldehyde (0.03 mol) in 20 mL dry ethanol at 0-5°C for 15 min. The reaction was monitored to completion using TLC (1 % methanol in chloroform). (It may be mentioned that under the refluxing condition employed initially the reaction mixture showed decomposition). The crude reaction mixture was concentrated *in vacuo* and 100 mL water was added. The product was extracted with chloroform (4 x 10 mL) after saturation with brine. The organic extracts were pooled, washed with brine (2 x 10 mL), dried over anhydrous sodium sulphate and evaporated in vacuum to yield the crude product. The crude product (0.7 g) was purified by column chromatography on silica gel using 0.3 % methanol in chloroform as the eluate to give (0.13 g, 19 %) of the pure imine as a pale yellow solid.

IR (KBr, ν cm^{-1}) : 2575 (SH), 1744 (COOEt), 1627 (C=N), 1583,1490 (Ar);

^1H NMR ($CDCl_3$, δ ppm): 1.31, 1.32 (3H a pair of triplets, J=7.1 Hz) $-CO_2CH_2C\underline{H}_3$ (geometrical isomers), 1.59 (1H broad singlet) $-CH_2S\underline{H}$, 3.19-3.24 (1H m), $-C\underline{H}_AH_BSH$, 3.40-3.46 (1H m) $CH_A\underline{H}_BSH$, 4.13 (1H dd, J = 5.5, 7.5 Hz) $-C\underline{H}(COOEt)(CH_2SH)$, 4.27, 4.28 (2H q, J = 7.3 Hz) $-CO_2C\underline{H}_2CH_3$ (geometrical isomers), 5.62, 5.92 (1H s) Ar-$C\underline{H}$=N-(geometrical isomers), 6.79-7.12 (2H m) Ar, 7.16-7.25 (2H m)Ar.

3.1.4. Ligand IV : To a stirred solution of 0.13 g (0.5 mmol) of ligand III in 5 mL dry ethanol at 0°C was added 0.04 g of sodium borohydride in small portions and stirring continued for three hours. The reaction mixture was rotary evaporated and water (20 mL) was added. The product was extracted in chloroform (4 x 5 mL) after saturation with brine. The organic extracts were pooled, dried over sodium sulphate to give 0.07 g of the pure amine obtained as a viscous product and observed as a single spot on TLC (5 % methanol in chloroform). IR (KBr, ν cm^{-1}) : 3315 (NH), 2558 (SH), 1742 (ester >C=O), 1492,1591 (Ar); ^1H NMR ($CDCl_3$, δ ppm): 1.32 (3H, a pair of triplets J = 7.14 Hz) $-CO_2CH_2C\underline{H}_3$, 1.45 (1H t, J = 8.6 Hz) $CH_AH_BS\underline{H}$, 2.89 (2H dt, J = 2.65, 5.6 Hz) $C\underline{H}_A\underline{H}_BSH$, 3.53 (1H t, J = 5.4 Hz) $-C\underline{H}(NH)(CH_2SH)$, 4.1 (2H t, J = 13.7 Hz) Ar-$C\underline{H}_2(NH)$, 4.21-4.30 (2H m) $-CO_2C\underline{H}_2CH_3$ diastereomeric, 6.76-6.82 (2H m) Ar, 7.15-7.22 (2H m) Ar.

3.2. Complexation studies with ^{99m}Tc

Complexation studies of ^{99m}Tc with the ligands were carried out using stannous tartrate as the reducing agent. Ligands I and II were prepared in 0.05 M bicarbonate buffer and ligands III and IV were prepared in methanol. Solutions of the ligands (0.1 mL), 0.5 mL of 0.5 M bicarbonate buffer of pH 9 and 1.0 mL of $^{99m}TcO_4^-$ (10 - 20 MBq/mL) was made up to a volume of 4.8 mL with nitrogen purged saline. Saturated solution of stannous tartrate (0.2 mL) was added to the above solution. The resultant complexes were characterised by PC, TLC, paper electrophoresis and solvent extraction.

Reaction conditions were optimised by varying the parameters such as ligand concentration, pH (buffers- acetate pH 3, phosphate pH 5 and 7 and bicarbonate pH 9 and 12) and time. Complexation yields were also studied using varying concentrations of $^{99}TcO_4^-$.

3.2.1. Thin layer chromatography

TLC was performed using flexible silica gel plates. A 5 µL aliquot of the reaction mixture was spotted 1 cm from the lower end of the plate. The strips were developed using solvents such as saline or acetonitrile, dried, cut into equal segments of 0.5 cm width and measured for radioactivity in a NaI(Tl) well counter.

3.2.2. Paper electrophoresis

Samples were spotted on Whatman 3 chromatography paper at 10-12 cm from the cathode and paper electrophoresis was carried out at 10 volts/cm for 1.5 hour in 0.02 M phosphate buffer, pH 7.5. The strips were dried, cut into 1 cm segments and measured for radioactivity.

3.2.3. Solvent Extraction

Solvent extraction was performed by mixing 1 mL of the reaction mixture with 1 mL of chloroform on a vortex mixer for about a minute. Equal aliquots of the aqueous and the organic layers were withdrawn and measured for radioactivity. The organic extract was back extracted repeatedly with saline to estimate the distribution ratio.

3.3. Biodistribution

Swiss mice weighing 20-25 g were injected through the tail vein with 0.1-0.3 mL of the complex solution containing ~370-550 kBq of activity. Tissues and organs excised from the mice following sacrifice at 10 min, 30 min 2 h and 4 h, post injection were weighed and the radioactivity estimated in a NaI(Tl) well counter. The percent injected dose in each tissue was calculated from the above data. The % ID in blood was calculated by measurement of the activity in 0.5 to 1 g of blood withdrawn by cardiac puncture immediately after sacrifice and assuming the whole blood volume as 6.5% of the body weight.

4. RESULTS AND DISCUSSION

4.1. Characterisation of the ligands

The formations of the imine ligands were indicated preliminarily by the observation of an IR band at ~1630 cm^{-1} for ligands I and III. The spectral characteristics, peak multiplicities and integrations of the ^{1}H NMR spectra of the ligands were consistent with the expected features. The presence of the geometrical isomers in case of the imine ligands I and II were reflected in the appearance of a pair of singlets at δ 5.65, 5.84 for ligand I and δ 5.62 and 5.92 for ligand III corresponding to the imine proton. In case of the reduced products, the appearance of the NH band at 3151 and 3315 cm^{-1} in IR corresponding to the amine ligands II and IV respectively, indicated the reduction of the imine bond. The SH bands at 2539 and 2558 cm^{-1} IR spectra also confirmed that the thiol group remained unaffected during the course of the reaction. The formation of the diastereomeric products emerging from non-stereospecific reduction was evident in the ^{1}H NMR spectra of the reduced products. The results of elemental analyses for the imine ligands provide additional evidence to support the formation of the ligands.

TABLE I. COMPLEXATION YIELDS (%) WITH VARYING AMOUNTS OF $^{99}TcO_4^-$

		$^{99}TcO_4^-$ (M/L)			
Ligands	NCA*	10^{-7}	10^{-6}	10^{-5}	10^{-4}
I	93	93	93	93	30
II	90	88	83	50	30
III	90	90	90	48	25
IV	96	97	96	94	28

* No carrier added

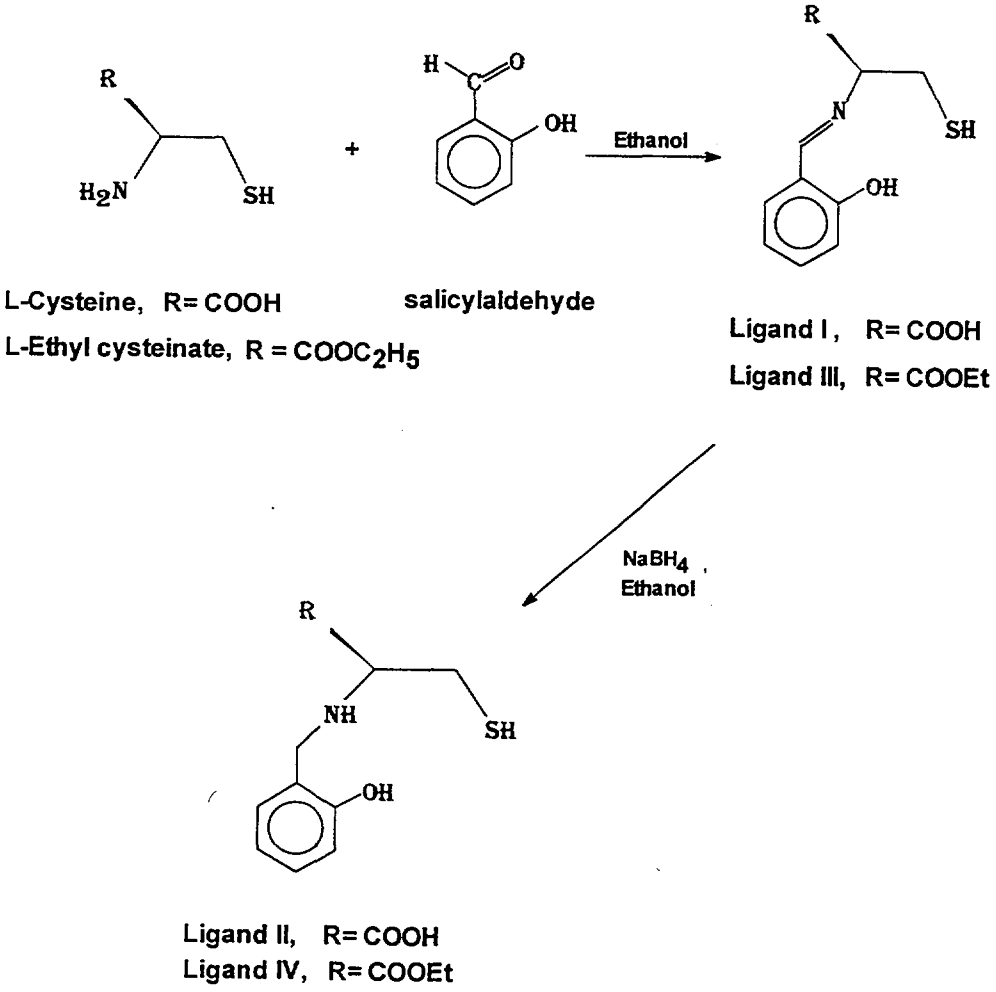


Fig.1: Scheme for synthesis of the ligands

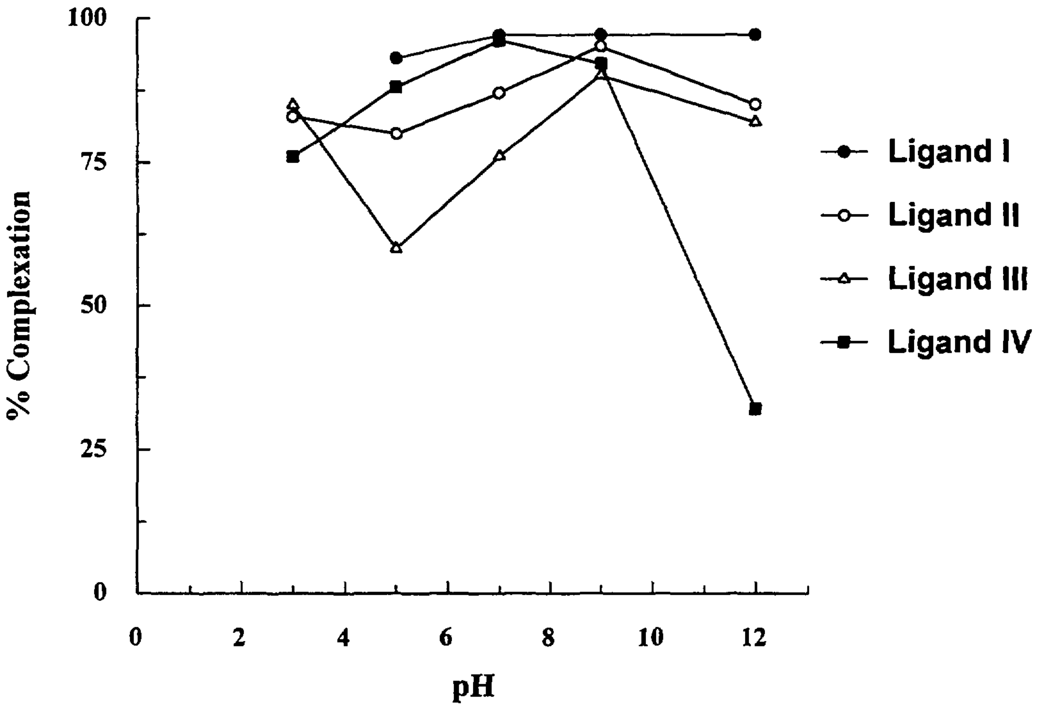


Fig. 2: Complexation yields at varying pH using different buffers (acetate pH 3, phosphate pH 5 and 7, bicarbonate pH 9, 12)

4.2. Radiochemical studies

The results of complexation studies with the ligands I - IV at various pH is shown in Fig. 2. While ligand I was found to complex with ^{99m}Tc in high yields at both alkaline as well as acidic pH, ligand II and III showed maximum complexation at pH 9. The complexation yields for ligand IV was observed to be maximum at pH 7 with a drastic reduction at pH 12. At the respective optimum pH, maximum complexation was achieved at concentrations of 5 mM for ligands I and II, 0.1 mM for ligand III and 0.5 mM for ligand IV.

Multiple quality control techniques were essential to obtain the complexation yields in the case of these ligands. The complexes of ligands I and II were found to be non-extractable in chloroform and were characterised by PC in saline and TLC in acetone (Fig. 3A and 3B). In PC/saline the complexes of ligands I and II were found to move

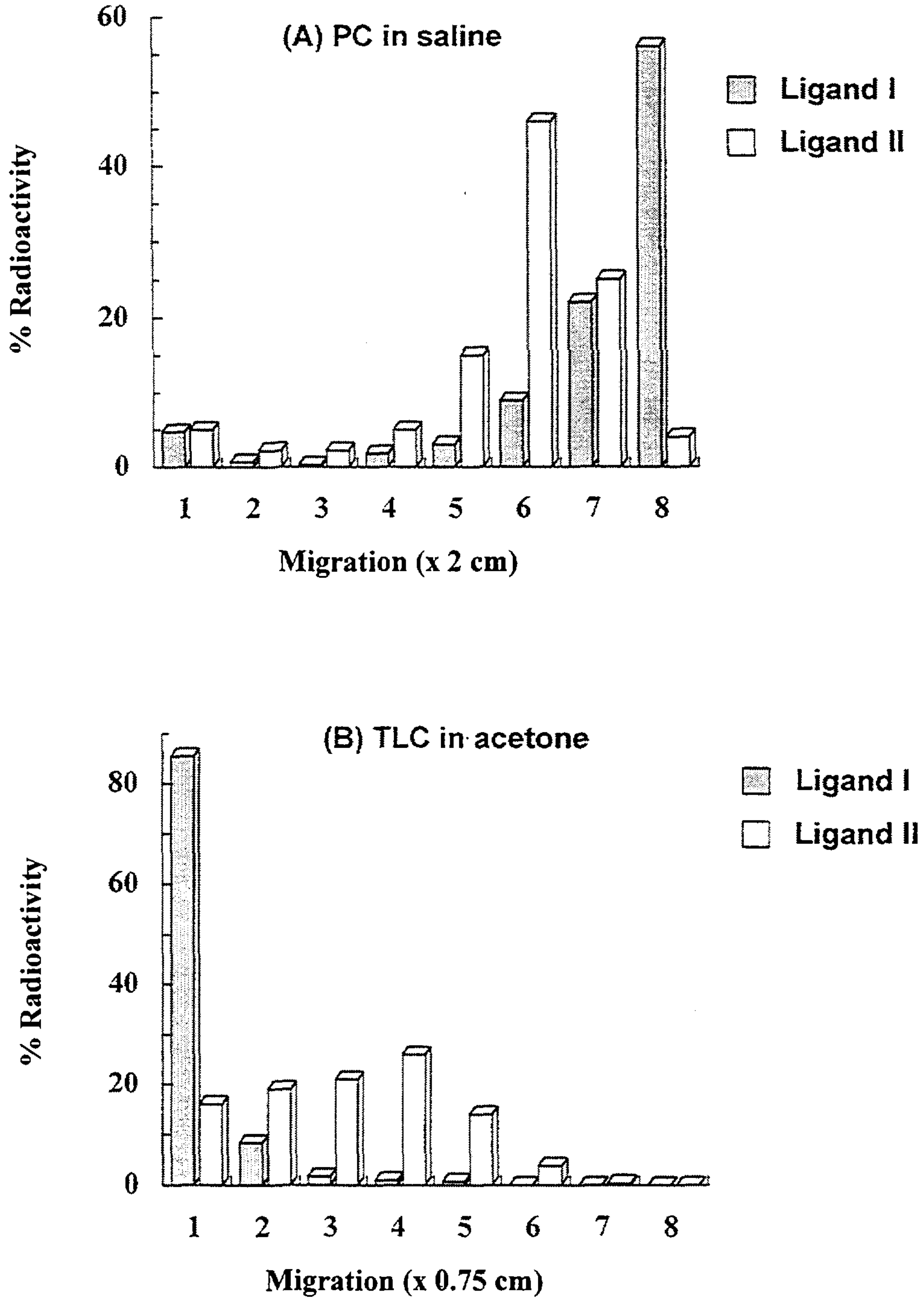
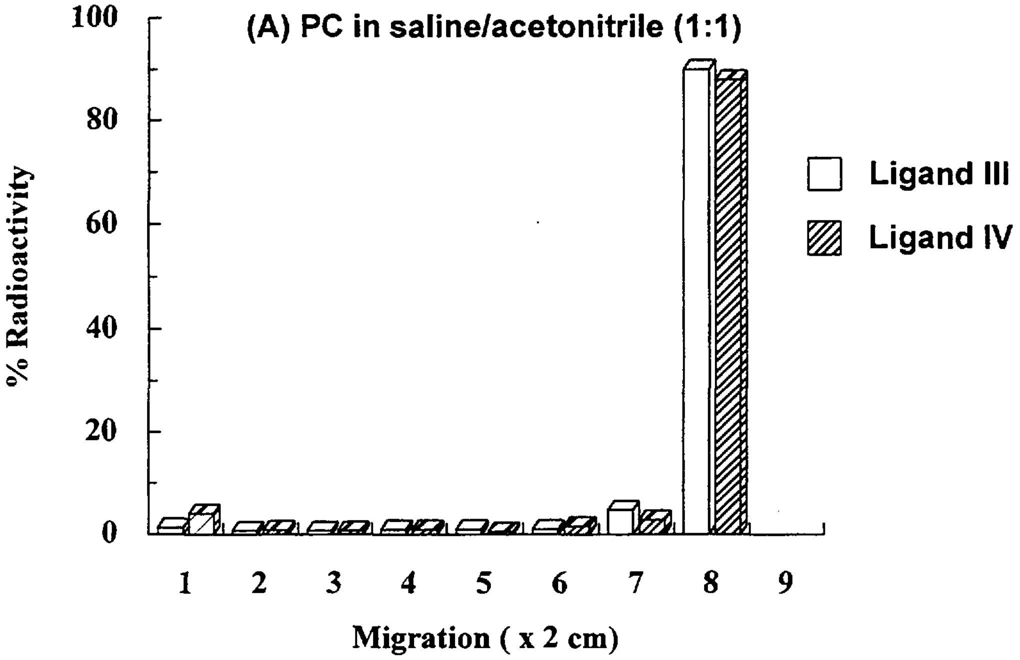


Fig. 3: Characterisation of ^{99m}Tc-complexes of Ligands I and II by **(A)** Paper chromatography in saline and **(B)** TLC in acetone



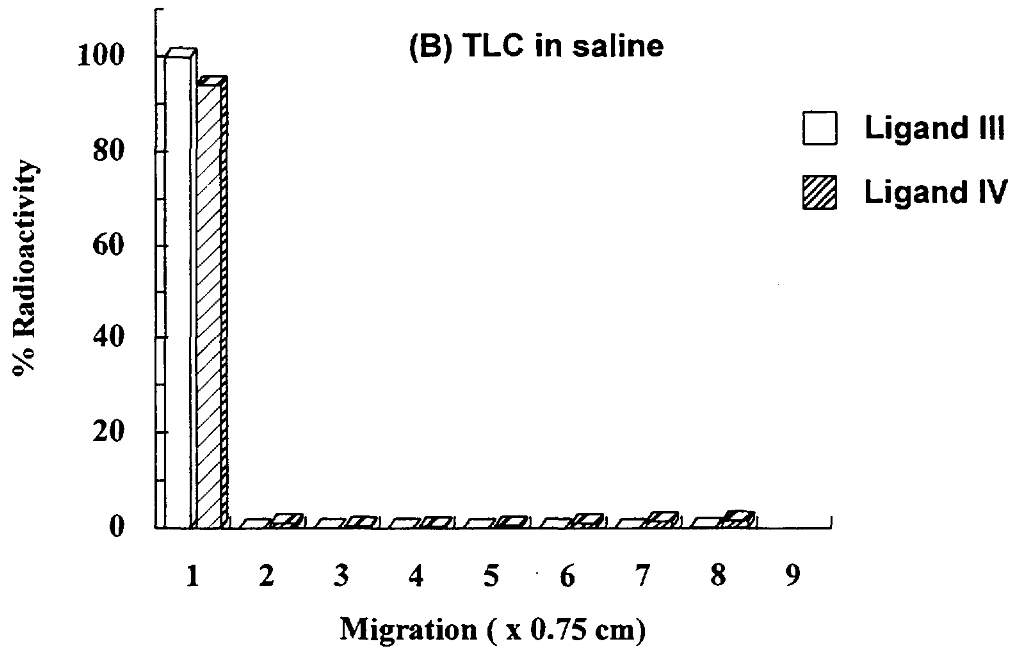


Fig. 4: Characterisation of ^{99m}Tc-complexes of Ligands III and IV by **(A)** Paper chromatography in saline: acetonitrile (1:1) and **(B)** TLC in saline

with the solvent front whereas in TLC/acetone, the complex of ligand I remained at the point of spotting but in case of the complex of ligand II, the activity was distributed on the chromatography plate. The above observations suggest that the complexes are hydrophilic and the presence of pertechnetate and TcO_2 in appreciable quantities in the reaction mixture are also ruled out. These results can be substantiated by considering the presence of carboxylic acid groups which results in the formation of charged hydrophilic complexes as observed earlier by Baldas et al [15]. The hydrophilic nature of the complexes of ligands I and II were inferred from their movement on paper electrophoresis. The complexes of ligand I and II moved towards anode, whereas complexes of ligand III and IV remained at the point of spotting. The complexes of both the ligands III and IV were extractable into chloroform. In PC/ saline:acetonitrile 1:1 (Fig.4A), the complexes moved towards the solvent front whereas in TLC/saline (Fig.4B), they did not exhibit any migration. The lipophilicity of the ligands were

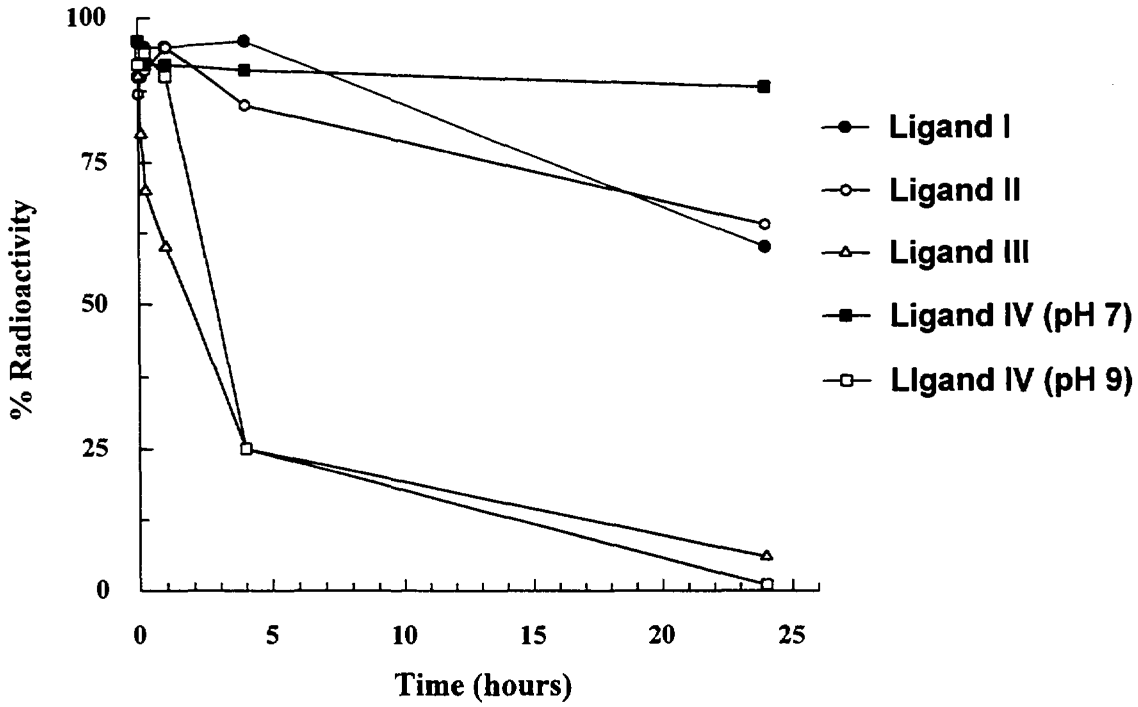


Fig. 5: Stability of ^{99m}Tc-complexes of Ligands I - IV as a function of time

estimated by determination of the distribution ratio of the ligands. Complexes of ligands I and II showed poor extractability (<1%) whereas those of ligands III and IV were observed to be 90 and 95%, respectively. The $CHCl_3$/saline distribution ratios were 99±8 and 70±7 for complexes of ligand III and IV, respectively.

To establish the equivalence of chemistry occurring at ^{99}Tc and at no carrier added ^{99m}Tc (~10^{-7} M) concentrations, $^{99m}TcO_4^-$ was added as a tracer in $^{99}TcO_4^-$ and complexation studies were carried out with the respective optimum concentration of the ligands. It is evident from the studies (Table I) that upto 10^{-5} M concentration of ^{99}Tc, there is no change in the complexation yield for ligands I and IV whereas for ligands II and III there is significant reduction as ^{99}Tc concentration is increased to 10^{-5} M.

The stability of the complexes with time was studied by the standardised quality control techniques and the results are given on Fig. 5. While the complex of ligand I exhibited remarkable stability upto 4 hours , that of ligand II showed slight reduction in stability with time. The complex of ligand IV showed an appreciable reduction in stability with time when prepared at pH 9 but was found to be stable when prepared at pH 7. Complex of ligand III was found to be significantly unstable and underwent rapid interconversion to a hydrophilic species as estimated by solvent extraction yields. The simultaneous estimation of TcO_4^- and TcO_2 by chromatographic techniques also confirmed the above observation.

4.3. Bio-distribution

The complexes of all the ligands showed rapid blood clearance. Results of the renal excretion and hepatobiliary uptakes for the complexes are given in Fig 6 and 7, respectively. The uptake in the liver for complexes of ligands I and II were significantly

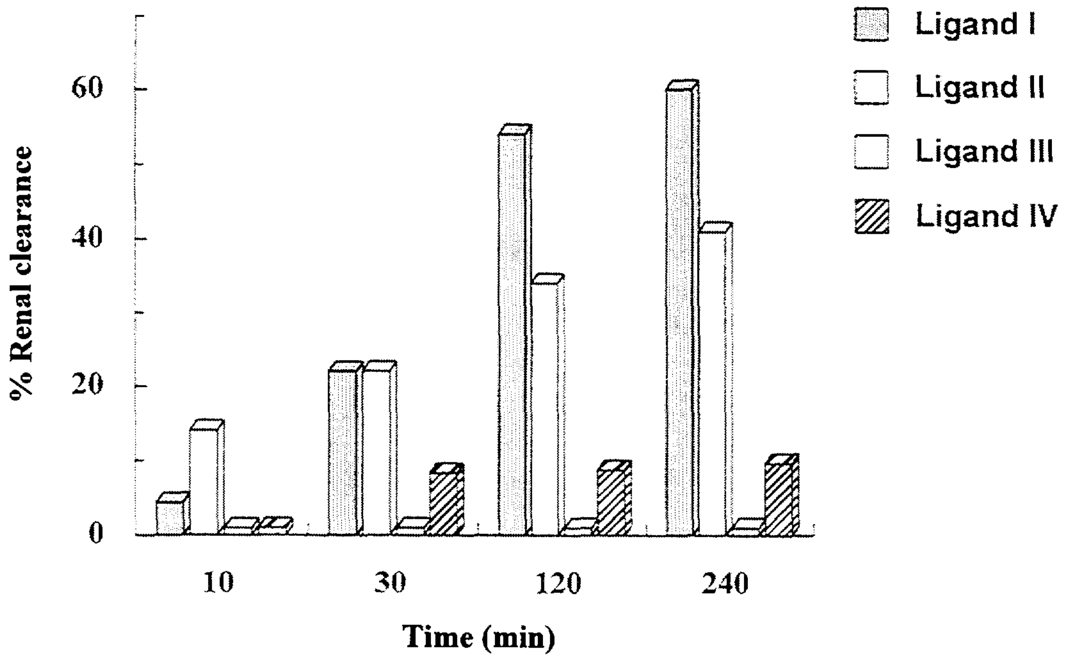


Fig. 6: Renal clearances of the ^{99m}Tc-complexes of Ligands I-IV :

10, 30, 120 and 240 minutes post injection

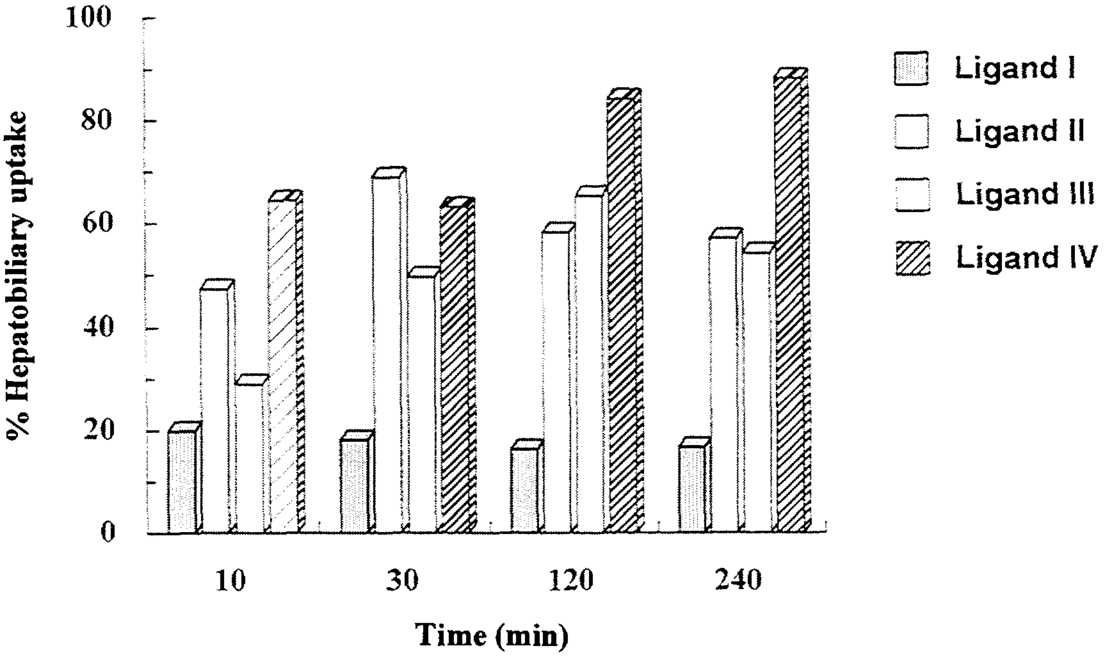


Fig. 7: Hepatobiliary clearances of the ^{99m}Tc-complexes of Ligands I-IV :

10, 30, 120 and 240 minutes post injection low being 9 % and 16 % respectively. Histograms of the urinary clearances (Fig.6) at 120 and 240 minutes show that complex of ligand I exhibits predominantly renal excretion. The complex of ligand II though hydrophilic, exhibit both hepatobiliary as well as renal clearance. The rates of renal clearances for complexes of ligand III and IV were found to be negligible. The above results could be attributed to the presence of carboxylic acid groups in I and II imparting a negative charge to the complex thereby augmenting renal excretion properties. This observation is in concurrence with the results of Eshima et al. [16]. Esterification of the carboxylic acid residue increases the lipophilicity of the complexes of ligand III and IV and an increase in the hepatobiliary clearance could thus be expected. These observations are reflected in Fig. 7. However, it has also been documented earlier that increase in the lipophilicity decreases the rate of renal clearance at the expense of an increase in the slow hepatocellular transit times [17]. This has been observed in the present case wherein initial liver uptake of the complexes of ligands III and IV were found to be significant with relatively slower rates of hepatic clearances. Criteria such as rapid uptake by the liver, low renal excretion and slow transition time therefore contribute to possibility that the complex of ligand IV could be used as a liver-function evaluating agent. On the other hand, complex of ligand III exhibiting an initial lower liver uptake and a faster clearance rate compared to that of the complex of ligand IV therefore offers potential as a intra hepatic bile-duct imaging agent.

CONCLUSION

Four novel cysteine based ligands of varying lipophilicities were synthesised and characterised. The radiochemical studies of these ligands with $^{99m}TcO_4^-$ were standardised and the complexes were characterised by established quality control techniques. The complexes of the ligands containing the free carboxylic acid groups exhibited predominant renal clearances. A significant increase in the lipophilicity of the complexes was achieved on preparing the corresponding ester derivatives and this was found to be reflected in the difference in the excretory patterns of the latter to hepatobiliary modes. The free carboxylic acid residue in two of the ligands could be derivatized for use as bifunctional chelating agents.

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