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Radiolabelling characteristics of novel iminophosphorane ligands with technetium-99m

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Abstract

A variety of unconventional phosphorus-nitrogen (P-N) backbone ligands have been synthesized with a unique, chemically flexible heterobifunctional ligand system that has the capability to complex with a radioactive metal ion and to link a bioactive peptide. We approach the synthesis of the iminophosphorane ligands (P-N) through the reaction of silylaminophosphines, T_2NPR_2 ($T=SiMe_3$). The P-N compounds which are derived from the applications of these silylaminophosphine reactions with a diversity of organic substrates, have the capability to attach ^{99m}Tc by means of a covalent bond. A further advantage of these type of chelating agents is their ability to selectively react with ^{99m}Tc in a +7 oxidation state and in the absence of reducing agent. This type of ligand presents the possibility of forming a metal-nitrogen σ bond with a high oxidation-state early transition metal via the elimination of Me_3SiCl. The properties of the resulting chelates with these ligands can be readily modified by introducing a variety of substituents on the P or N atoms (R or R'). Several studied P-N ligands have been shown to hold the potential for preparing stable ^{99m}Tc-complexes with high labelling yield under the mild

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labelling conditions and with and without reducing agents. Furthermore, the labelled P-N compounds showed a high stability both when incubated with plasma and when challenged with excess amount of cysteine.

Key Words : Phosphorus-Nitrogen bifunctional ligands, ^{99m}Tc-iminophosphoranes, labelling with ^{99m}Tc, radiolabelling with and without reducing agents.

Introduction

The introduction of several new high oxidation state compounds of Tc and Re has created a renew interest in nuclear medicine (1-9). The wide acceptance of these type of complexes as radiopharmaceuticals is due to the fact that some of the Tc (VII) and Re (VII) complexes can be prepared under relatively mild labelling conditions in high radiolabelling yields and form complexes in 1:1 ligand-to-metal ratios. A further advantage of this type of chelation system is the ability to selectively react with Tc-99m or 186/188-Re in the +7 oxidation state and in the total absence of reducing agents. This is particularly important for radiolabelling of peptides, since reducing agents may cleave the disulphide bonds of the proteins and certain peptides thus influencing their biological activity (10). For example, in an effort to prepare ^{99m}Tc-octreotide, the direct labelling via disulphide reduction leads to the cleavage of the disulphide bond in the peptide resulting in the reduced somatostatin receptor affinity (11-12).

In recent years, radiolabelled peptides have emerged as a new, useful class of radiopharmaceuticals for diagnosis of a variety of endocrine tumors and other disease states. Although clinical data with ^{111}In-labelled and ^{123}I-labelled peptides have been promising (13-16), extensive efforts have been made to prepare and evaluate peptides labelled with ^{99m}Tc (17-22). Technetium-99m is the radionuclide of choice for peptide labelling by virtue of its low cost, availability, high specific activity and favourable imaging characteristics. Two approaches , direct labelling and bifunctional chelates methods have been described for radiolabelling peptides with ^{99m}Tc (11). Peptides that contain

appropriate amino acid sequence can be directly radiolabelled with ^{99m}Tc by pertechnetate reduction in the presence of the peptide without the loss of function and specificity. Direct labelling usually generates endogenous free sulphydryl groups by reduction of disulphide bridges in the peptides, and the thiol group of cysteine is a key functional group for ^{99m}Tc-binding (18,23-25). But certain peptides which do not contain cyclized cysteine residues must be labelled with ^{99m}Tc via bifunctional chelating agents. The advantage of this method is that it can be used to label all peptides, with or without cysteine bridge (26). A number of different bifunctional chelating agents have been used for radiolabelling peptides with ^{99m}Tc (11,20-21,27-30). Despite the successful applications of some of the currently used bifunctional chelating agents, development of new bifunctional chelates which are capable to selectively chelating ^{99m}Tc under mild labelling conditions is important for labelling of ^{99m}Tc-peptides and for improving the *in vivo* behaviour (e.g., clearance of radioactivity from non-target tissues) of the labelled compounds.

The aim of this work is to develop ^{99m}Tc-labelled peptides for imaging of cancer by using the phosphorus-nitrogen (P-N) backbone bifunctional ligands. A multitude of conventional, carbon backbone ligands have dominated in radiochemical studies of different transition metal radionuclides. However, the use of other main group elements such as phosphorus to link chelating functional groups have not been extensively used for radiopharmaceutical applications. These type of ligands may hold enormous potential for achieving suitable beneficial structure activity relationships because of their unusual steric and electronic characteristics (31). Several different phosphorus-nitrogen ligands have been developed by us and have been shown to hold the potential for preparing stable ^{99m}Tc-complexes with and without reducing agents. These ligands are derived from developments of the silylaminophosphine reactions that have the capability to attach with Tc-99m and Re-186 by means of a covalent bond.

The newly made P-N compounds evaluated in this study have been previously shown to complex unlabelled Re (VII) in a highly oxidized chemical state (32). This paper

summarizes the labelling characteristics of P-N compounds with ^{99m}Tc in the presence and absence of reducing agent and evaluating their potential as bifunctional ligands. The best P-N compound(s) with respect to radiolabelling characteristics, would serve as a model template to be chemically modified to incorporate the second functionality to enable linking to a bioactive peptide.

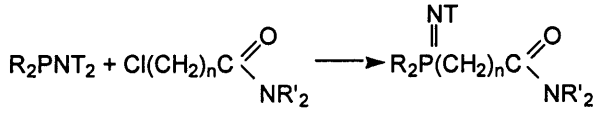
Materials and Methods

Materials

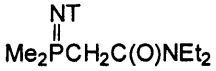
The reagents, stannous chloride dihydrate and sodium potassium tartrate were purchased from Sigma Chemical Co. All other chemicals were of reagent grade and were used without further purification unless otherwise indicated. Sodium pertechnetate ($^{99m}TcO_4^-$) was supplied by the Edmonton Radiopharmaceutical Centre (ERC).

Chemical Synthesis of P-N Ligands

All iminophosphorane or phosphinimine ligands evaluated in this study were synthesized through the reaction of silylaminophosphines, T_2NPR_2 (T = $SiMe_3$), with functionalized organic chlorides and related compounds. The reaction of dimethylcarbamyl and 2-chloro-N,N-diethylacetamide with silylaminophosphines goes through an addition-elimination process to yield N,N-dialkylamidoiminophosphoranes:



n = 0 or 1
R = Me, Et, i-Pr, Ph
R' = Me, Et



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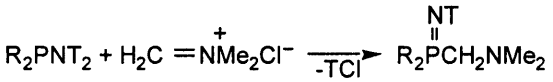
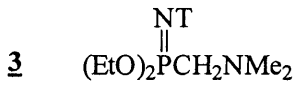
The reaction of silylaminophosphines with N,N-dimethylmethylene ammonium chloride proceeds similarly, eliminating trimethylsilylchloride, to yield structurally varied iminophosphoranes :

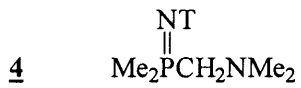


$R_2PNT_2 + H_2C{=}\overset{+}{N}Me_2Cl^- \xrightarrow[-TCl]{} R_2P(NT)CH_2NMe_2$

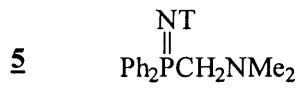
R = Me, Et, i-Pr, EtO, etc.



3 $(EtO)_2P(NT)CH_2NMe_2$

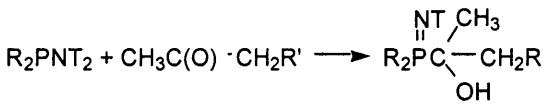


4 $Me_2P(NT)CH_2NMe_2$



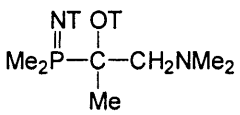
5 $Ph_2P(NT)CH_2NMe_2$

The reactions of silylaminophosphines with ketoamines and ketoamides are addition-oxidation process, again yielding iminophosphoranes:



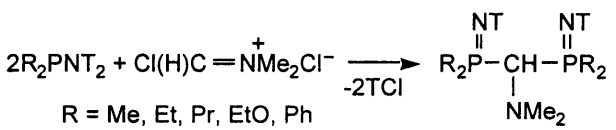
$R_2PNT_2 + CH_3C(O)\cdot CH_2R' \longrightarrow R_2P(NT)C(CH_3)(OH)-CH_2R$

R = Me, Et
R' = NMe_2 or $CONH_2$



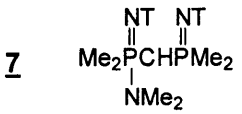
6 $Me_2P(NT)-C(OT)(Me)-CH_2NMe_2$

With (chloromethylene)dimethylammonium chloride the reaction goes by elimination of two moles of trimethylsilylchloride to form bis-iminophosphoranes:



$2R_2PNT_2 + Cl(H)C{=}\overset{+}{N}Me_2Cl^- \xrightarrow[-2TCl]{} R_2P(NT)-CH(NMe_2)-P(NT)R_2$

R = Me, Et, Pr, EtO, Ph



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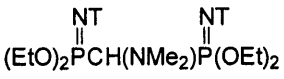

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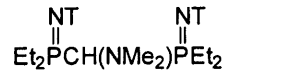

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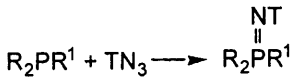
Simple oxidation of selected phosphines with trimethylsilylazide yields additional N-silyliminophosphoranes:



R= Ph, EtO ; R' = NT_2 ; R,R' = Et_2NCH_2

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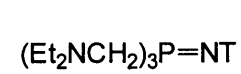

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Full synthetic details will be published separately.

Labelling of P-N compounds with ^{99m}Tc

Labelling of newly made P-N compounds with ^{99m}Tc can be achieved either in the presence of stannous tartrate or by the direct addition of pertechnetate ($^{99m}TcO^-_4$) to the P-N ligand. Stock solutions of all the P-N compounds were prepared in ethanol prior to radiolabelling. For labelling experiments: 0.1 mg to 20 mg of the ligand was mixed with 2 mg of tartrate, 50 μL $SnCl_2$ solution (20 mg $SnCl_2.2H_2O$ dissolved in 5 mL of 0.05N HCl) and 0.1 to 0.3 mL $^{99m}TcO^-_4$ (1 to 5 mCi) in saline. The labelling mixture was incubated

at room temperature for 30 min prior to radio-TLC analysis. Radiolabelling with and without the reducing agents of some of the P-N compounds with ^{99m}Tc was also performed in the presence of 0.1 mL of citrate-phosphate buffer 0.1M (pH 2 to 12) in order to observe the effect of pH on labelling. In some experiments, the reaction mixture was also heated at 80°C in a water-bath for 15 min to promote the radiolabelling yield.

Radiochemical Purity

Paper chromatography is one of the accepted tools for the analysis of radiochemical impurities in ^{99m}Tc-radiopharmaceuticals (33). The radiochemical purity of the reaction mixture was analyzed by radio-TLC using Paper Chromatography (*Whatman* Chromatography Paper No.1). Acetone and saline (0.9% NaCl) were used as TLC mobile phase solvents. Acetone was used to determine the free or unbound ^{99m}Tc while saline was used to determine the hydrolyzed and reduced ^{99m}Tc that occurs only in the reduced ^{99m}Tc radiopharmaceuticals. When using acetone, the labelled P-N complex remained at the origin, while it migrated with the solvent front when saline was the mobile phase. Free ^{99m}Tc migrated with solvent front when using acetone whereas reduced and hydrolyzed ^{99m}Tc remained at the origin in the saline system. Hence labelling efficiency can be determined by the following formula (34) :

Labelling Efficiency (%) = 100% - (% free ^{99m}Tc + % reduced and hydrolyzed ^{99m}Tc)

Co-Labelling with Tc-99m and P-32

In order to determine the co-migration of ^{32}P and ^{99m}Tc, one of the P-N compounds under study was first irradiated in the SLOWPOKE nuclear reactor to produce ^{32}P labelled P-N compound, and then labelled with ^{99m}Tc to determine co-migration of the two radioisotopes .

Aliquots of the P-N compound to be activated were weighed into nitric acid washed polyethylene microcentrifuge tubes which were encapsulated in standard 7.0 mL

polyethylene irradiation vials. Dihydrogen ammonium phosphate was also prepared in order to determine the behavior of free phosphorus on TLC. For the production of ^{32}P, the samples were irradiated in the University of Alberta SLOWPOKE II Nuclear Reactor at a nominal thermal neutron flux of $1x\ 10^{12}$ n cm^{-2} s^{-1}. Experiments were performed using a short irradiation time to minimize bond breakage of the P-N compound by recoil. Radiochemical analysis in the dual labelling experiment was performed sequentially in order to investigate and compare the migratory pattern of ^{99m}Tc- labelled complex on TLC first with the radiochromatographic scanner. The TLC strips were then cut into segments and each segment placed in a separate counting vial. The ^{99m}Tc activity in each TLC segment was measured using a gamma scintillation counter. After the subsequent decay of ^{99m}Tc, the ^{32}P β^- activity of the same fractions were measured by liquid scintillation counting.

In vitro Stability in plasma

^{99m}Tc-labelled P-N ligands were incubated (in triplicate) with human plasma at 37°C for 18 hr. Subsequently, the stability of the labelled compound was determined by TLC using acetone and saline as the developing solvents. The radiochemical purity of labelled P-N compound alone was also determined without plasma at 30 min and 18 hr after the radiolabelling.

In vitro transchelation

The strength of binding of the radionuclide to the P-N compounds and eventually their peptide derivatives can be checked by transchelation studies. For transchelation, ^{99m}Tc labelled P-N compound was mixed with cysteine in saline at the following molar ratios of labelled P-N compound to transchelator; 1:50 and 1:500. The reaction mixture was incubated at 37°C for up to 4 hr. One and 4 hours after the incubation, the mixture was analyzed by radio-TLC and the percentage radioactivity transchelated was determined.

Results and Discussion

The studied P-N ligands can be divided into four groups according to their chemical structure namely : dialkylamidoiminophosphoranes (1 , 2), iminophosphoranes (3 , 4 , 5 & 6), bis-iminophosphoranes (7 , 8 , 9) , and N-silyliminophosphoranes (10 , 11).

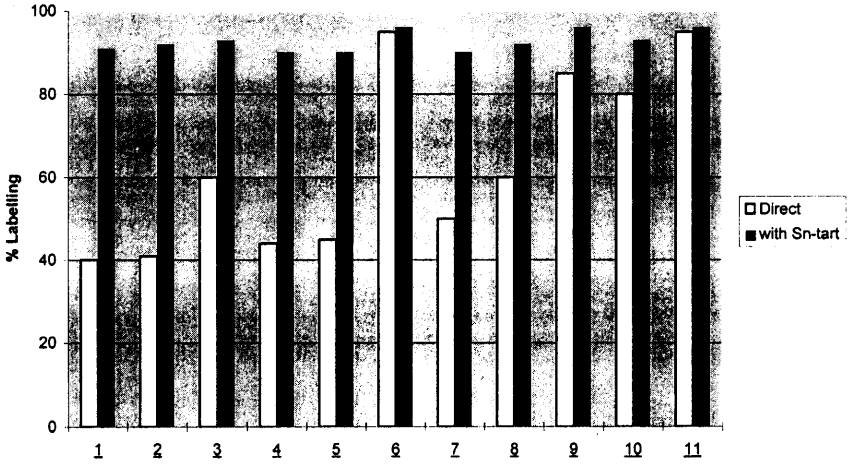
Labelling with ^{99m}Tc

Results of the labelling experiments are summarized in Figure 1.

Both dialkylamidoiminophosphorane ligands 1 and 2 showed a poor labelling efficiency (up to 40%) when labelling with ^{99m}Tc was performed directly without using any reducing agent. However, labelling yield improved significantly (up to 90%) for both dialkylamidoiminophosphorane ligands when labelling was carried out in the presence of stannous tartrate.

In iminophosphorane ligands (3 , 4 , 5), diethyl, dimethyl and diphenyl substituents are attached to phosphorus of the ligands 3 , 4 , 5 respectively, furthermore, all these ligands also contain an α dimethylamino groups in their backbone. These ligands exhibited almost similar labelling characteristics (greater than 90%), when labelled with ^{99m}Tc under reducing conditions. In the direct labelling method, however, ligand 3

Figure 1. Labelling of P-N ligands with Tc-99m under non-reducing and reducing conditions.



showed relatively higher labelling efficiency (up to 60%) compared to the other ligands 4 and 5 (up to 45%), when these ligands were labelled with ^{99m}Tc in the absence of reducing agent. The other iminophosphorane ligand 6 which contain a β dimethylamino group and a trimethylsilyloxy moiety in its structure hold a good potential to complex with ^{99m}Tc and exhibited superior labelling characteristics (greater than 95%) regardless of the labelling method.

The P-N ligands 7 , 8 , 9 which belong to the bis-iminophosphorane family (and in which dimethyl, diethoxy and diethyl groups respectively, attached to each phosphorus of the ligand), showed similar labelling efficiency (greater than 90%) when radiolabelling was carried out in the presence of stannous tartrate. The P-N ligands 7 and 8 showed variable radiolabelling yields (up to 50% and 60% respectively), under non-reducing labelling condition. Radiolabelling efficiency was equally good (up to 85%) when the third bis-iminophosphoranes ligand 9 was labelled directly with ^{99m}Tc and in the absence of reducing agent.

The P-N ligands 10 and 11 which belong to the N-silyliminophosphoranes family showed a high radiochemical purity, greater than 80% and 95% respectively, when labelling with ^{99m}Tc was performed directly. The radiochemical yields of both N-silyliminophosphoranes ligands increased to a maximum of 98% when radiolabelling was performed under reducing condition.

For each ^{99m}Tc-P-N complex prepared, two radiolabelling methods were used in this study i.e., direct labelling without using any reducing agent and, labelling in the presence of a suitable reducing agent. Reducing agents are commonly used to reduce ^{99m}Tc from its highest oxidation state to a lower state to allow the formation of a complex with a chelating ligand and to minimize the formation of radiochemical impurities. Although a high labelling efficiency can often be achieved with reducing agents, sometimes these agents may results in the alteration of the peptide as well as formation of labelled impurities (35). In order to avoid these problems, the direct labelling method without reducing agents was employed in this study.

Since no reducing agent was used in the direct labelling method, it was assumed that in these type of complexes ^{99m}Tc binds to the phosphine-containing (P-N) ligands at the high oxidation state (+7). Although it is well described that, phosphines act both as ligand and as coordinating reducing agent towards ^{99m}Tc radiopharmaceuticals (1,36-38), but the real potential of phosphine as a intrinsic reducing agent in labelling with ^{99m}Tc is still not very clear. Nevertheless, the radiochromatographic analysis (Figure 2) indicated that for some of the P-N ligands, a single ^{99m}Tc-species is formed by simply mixing the P-N ligand in ethanolic solution with the eluate of a generator containing ^{99m}Tc (1 to 5 mCi) in the form of sodium pertechnetate. Since labelling is carried out without any reducing agent, it is hypothesized that in this type of ^{99m}Tc-P-N complex, ^{99m}Tc remains in the high oxidation state. However, it seems that in the formation of such ^{99m}Tc-P-N-complexes, phosphine often behaves as a coordinating reducing agent. P-N complexes produced under non-reducing conditions represented some inconsistencies in terms of labelling efficiency, perhaps due to the weak or irregular reducing and coordinating property of

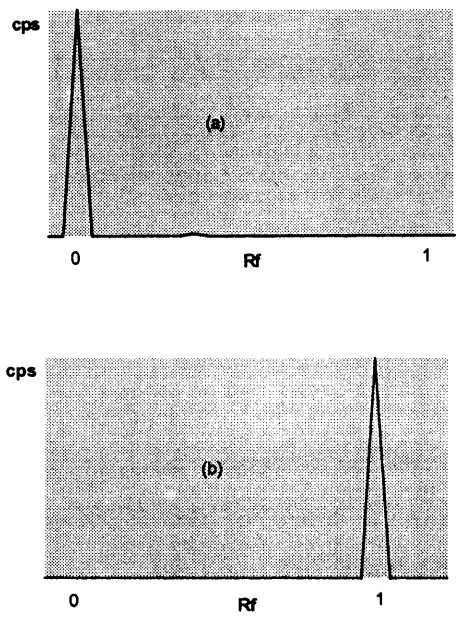


Figure 2. TLC-radiochromatograms of ^{99m}Tc-P-N complex.
These TLC strips were developed with: (a) acetone (b) saline.

phosphine. Thus, relatively weak ^{99m}Tc-complexes are produced under direct labelling condition.

On the contrary, ^{99m}Tc-P-N-complexes formed in the presence of sodium tartrate and stannous chloride were usually more stable and consistent. This may be due to the fact that the stannous chloride is a strong reducing agent. It reduces $^{99m}TcO_4^-$ (oxidation state = +7) to a lower oxidation state and in the reduced state, ^{99m}Tc forms an intermediate labile complex with tartrate. This ^{99m}Tc-tartrate complex has a weak chelating properties and likely withdraws ^{99m}Tc from its weak ^{99m}Tc-tartrate complex to form the more stable ^{99m}Tc-P-N complex.

The labelling conditions for preparing ^{99m}Tc-labelled P-N compounds were designed to maximize the labelling yields while minimizing the formation of labelled impurities. This can be accomplished by controlling pH and concentration of the ligand. Time and temperature are also critical radiolabelling parameters.

Labelling vs. Concentrations

In order to observe the effect of varying amounts of the P-N ligands on labelling efficiency, 0.1 mg to 20 mg of the P-N compounds were labelled with ^{99m}Tc under reducing conditions. For most of the P-N compounds evaluated in this study, it was observed that the optimal radiolabelling efficiency was obtained when 0.1 mg to 1.0 mg of the P-N ligand was used for radiolabelling with ^{99m}Tc both under reducing and non-reducing conditions (Table 1). High concentrations of these P-N ligands usually reverse the effect on labelling yield. Perhaps, at the low concentrations of P-N ligand, all the ^{99m}Tc atoms bind well with the ligand resulting in high labelling efficiency. On the other hand, higher amount of the ligand results in a high amount of ^{99m}Tc-colloids and unreacted $^{99m}TcO_4^-$ yielding in a low radiochemical purity .

^{99m}Tc-P-N	0.1 mg	0.5 mg	1 mg	5 mg	10 mg	20 mg
1	92%	92%	91%	80%	71%	29%
2	93%	90%	89%	81%	73%	45%
3	94%	93%	91%	88%	83%	81%
4	91%	92%	87%	61%	50%	31%
5	94%	86%	77%	65%	39%	30%
6	95%	96%	94%	90%	87%	83%
7	92%	90%	89%	63%	49%	44%
8	91%	89%	86%	85%	83%	83%
9	95%	91%	80%	73%	64%	40%
10	94%	94%	90%	87%	75%	60%
11	96%	92%	83%	68%	50%	38%

Table 1. % Radiolabelling yield of ^{99m}Tc- P-N ligands at different concentrations of the P-N ligand.

Labelling vs. pH

In order to observe the influence of pH on labelling yield, radiolabelling with and without the reducing agent of the P-N compounds with ^{99m}Tc was also performed in the presence of 0.1 mL of citrate-phosphate buffer 0.1M (pH 2 to pH 12). It has been noted that for most of the P-N ligands, a more stable complex with ^{99m}Tc and a high radiochemical purity can often be achieved when labelling with ^{99m}Tc was performed at an acidic pH.

Stability in plasma

Since the efficacy of a diagnostic test largely depends on the stability of the radiopharmaceuticals used, one of the important factors was to determine the stability of the P-N compounds in plasma. The results of these experiments (Table 2) demonstrated that all the studied ^{99m}Tc-P-N complexes remained stable and did not show any significant breakdown when incubated *in vitro* with human plasma at 37°C for 18 hr. A maximum of 5% of dissociated ^{99m}Tc activity was found when compared with the radiochemical yield of the original labelled P-N ligand.

^{99m}Tc-P-N	Without Plasma		With Plasma	
	30 min	18 hr	30 min	18 hr
1	92%	90%	92%	88%
2	91%	90%	90%	89%
3	94%	92%	93%	91%
4	90%	89%	90%	88%
5	91%	89%	89%	88%
6	95%	91%	92%	91%
7	91%	89%	90%	89%
8	92%	90%	89%	88%
9	94%	91%	94%	90%
10	93%	90%	91%	90%
11	95%	93%	94%	92%

Table 2. Percentage of ^{99m}Tc- P-N ligands alone and after the incubation with human plasma at 30 min and 18 hr after labelling.

Cysteine Challenge

The results of transchelation experiments are presented in Table 3. Transchelation of ^{99m}Tc from peptides or proteins to endogenous cysteine is known to occur *in vivo* (39). Following the incubation of radiolabelled P-N ligand with a 50-fold and 500-fold molar excess of cysteine with respect to the P-N ligand at 37°C for 1 hr and 4 hr. Periodic samples were analyzed by TLC and examined for any displacement of ^{99m}Tc activity from the labelled P-N compound. It was found that after 1 hr of incubation, a maximum of 20% of the radioactivity was transchelated when these ligands were challenged with a 500-fold molar excess of cysteine, whereas radiochromatographic analysis for the same samples after a 4 hr incubation indicated that for some of the P-N ligands, a maximum of 30% of the ^{99m}Tc activity was dissociated when compared with the labelling efficiency of the original P-N compound (Table 3).

^{99m}Tc-P-N	^{99m}Tc-P-N (% RCP)	1h at 37°C		4h at 37°C	
		1 : 50	1 : 500	1 : 50	1 : 500
1	93%	90%	81%	78%	72%
2	94%	89%	80%	77%	70%
3	97%	95%	94%	92%	90%
4	90%	88%	89%	80%	78%
5	91%	87%	80%	76%	70%
6	98%	88%	81%	82%	81%
7	93%	92%	92%	91%	90%
8	95%	94%	95%	85%	84%
9	96%	92%	91%	84%	79%
10	95%	93%	93%	92%	92%
11	96%	90%	88%	88%	87%

Table 3. Percentage of ^{99m}Tc-P-N ligands before and after incubation with 50 and 500 fold molar excess of cysteine for 1hr and 4 hr at 37°C.

Co-labelling with ^{32}P and ^{99m}Tc

In the dual labelling experiment, the radiochromatographic analysis indicated that there was no correlation with respect to the migratory pattern when one of the P-N compound was first irradiated with neutrons to produce ^{32}P prior to radiolabelling with ^{99m}Tc under non-reducing conditions. It is, however, noted that a comparable co-migratory pattern was observed on TLC when ^{32}P irradiated ligand was labelled with ^{99m}Tc in the presence of stannous tartrate (Table 4), suggesting the potential of the P-N ligands to label both with beta and gamma emitters.

Conclusion

Several P-N ligands examined in this study have been shown to hold the potential for preparing stable ^{99m}Tc-complexes under the mild labelling conditions. Radiolabelling of these ligands with ^{99m}Tc and with and without reducing agents make these ligands particularly attractive. The properties of phosphine containing P-N ligands to act both as

		Labelling under reducing conditions		Direct labelling	
		^{99m}Tc	^{32}P	^{99m}Tc	^{32}P
Acetone	Bottom	99.0%	86.0%	4.0%	52.0%
	Top	1.0%	14.0%	96.0%	48.0%
Saline	Bottom	13.0%	19.0%	7.0%	25.0%
	Top	87.0%	81.0%	93.0%	75.0%

Table 4. Radio-TLC migratory pattern of ^{99m}Tc and ^{32}P-labelled P-N complex. Labelling with ^{99m}Tc was performed under reducing & non-reducing conditions. After the subsequent decay of ^{99m}Tc, the same TLC fractions were measured for ^{32}P activity.

ligands and as coordinating reducing agents towards ^{99m}Tc radiopharmaceuticals have made them a useful family of ligating frameworks in diagnostic radiopharmaceutical applications. The properties of the resulting chelates with these ligands can be readily modified by introducing a variety of substituents on the P or N atoms (R or R'). Moreover, all the studied P-N ligands have shown a high stability both when incubated with plasma and when challenged with excess of cysteine.

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