JOURNAL OF LABELLED COMPOUNDS AND RADIOPHARMACEUTICALS
J Label Compd Radiopharm 2005; **48**: 1003–1011.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jlcr.1012

Research Article

Preparation, characterization and biological evaluation of $^{99m}Tc(CO)_3$-labelled cyclic polyamines

H. P. Vanbilloen[1], K. Eraets[2], N. Evens[2], C. Terwinghe[1], D. Rattat[2], G. Bormans[2], L. Mortelmans[3] and A. M. Verbruggen[2,*]

[1] *Radiopharmacy, U.Z. Gasthuisberg, Belgium*
[2] *Laboratory of Radiopharmaceutical Chemistry, University of Leuven, Belgium*
[3] *Nuclear Medicine, U.Z. Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium*

Summary

Three cyclic polyamines, namely 1,4,7-triazacyclononane, 1,4,7,10-tetraazacyclododecane (cyclen) and 1,4,8,11-tetraazacyclotetradecane (cyclam), were evaluated as potential ligands for complexation of a technetium(I) tricarbonyl core. They can be used as bifunctional chelating agents for labelling bioactive compounds. Each of the three ligands forms a positively charged technetium tricarbonyl complex in high yield but heating is required to promote complex formation. The charge of the ^{99m}Tc(I)tricarbonyl labelled derivatives was confirmed using electrophoresis, and radio-LC–MS supports their proposed chemical identity. After i.v. injection in mice, the compounds were rapidly cleared from the blood by the hepatobiliary or urinary pathway depending on their lipophilicity.

Key Words: Technetium-99m-tricarbonyl; cyclic polyamines; reversed phase HPLC; IsoLink®; Radio-LC–MS

Introduction

It is well known that cyclic polyamines such as 1,4,8,11-tetraazacyclotetradecane (cyclam) have the ability to complex metals such as nickel, copper and manganese.[1–5] A positively charged ^{99m}Tc(V)dioxo cyclam complex has been described[6] and characterized[7,8] more than 20 years ago. In this complex a central Tc(V) dioxo core is bound to the cyclam ligand via the four amine nitrogen atoms without deprotonation, resulting in a monocationic complex. The cyclam ligand allows derivatization of one or more amines and has been proposed as a bifunctional chelating agent for labelling a variety of

*Correspondence to: Alfons Verbruggen, Radiopharmacy, UZ Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. E-mail: Alfons.verbruggen@uz.kuleuven.be

Received 14 July 2005
Revised 30 August 2005
Accepted 4 September 2005

compounds such as antibodies, nitroimidazole derivatives and other compounds of biological interest.[9–13]

A few years ago, Alberto *et al.* developed a convenient new method to prepare Tc(I) tricarbonyl complexes.[14] This opened a field of new ^{99m}Tc-complexes with distinct properties and the potential to produce an up to now unexplored class of ^{99m}Tc-tricarbonyl labelled bioactive molecules with attractive characteristics such as high stability and specific activity.[15–17] Labelling with this method became much easier since Tyco-Mallinckrodt introduced the IsoLink® kit to prepare the technetium tricarbonyl precursor.[18] In this study we have investigated the suitability of cyclic polyamines such as cyclam for complex formation with a Tc(I)tricarbonyl moiety and compared the biological properties of the resulting $^{99m}Tc(I)(CO)_3$ polyamines with those of the ^{99m}Tc(V) dioxo cyclam analog.

Materials and methods

Labelling

Cyclam, cyclen and triazacyclononane were obtained from Fluka (Sigma-Aldrich, Bornem, Belgium) and were used as such without further purification. The precursor $[^{99m/99}Tc(CO)_3(OH_2)_3]^+$ was prepared by adding 1 ml generator eluate (Ultra-Technekow ^{99}Mo/^{99m}Tc generator, Tyco Healthcare, Petten, The Netherlands) containing up to 4 GBq ^{99m}Tc-pertechnetate to an IsoLink® kit and heating the vial for 10 min in a waterbath at 100°C. If a higher content of technetium was necessary for mass spectrometry analysis an additional 1.5 μg of technetium-99 in the form of ammonium pertechnetate in 0.1 ml H_2O was added before the heating step. For 'standard' preparation of the technetium tricarbonyl complexes with cyclic polyamines, 0.1 ml of technetium tricarbonyl precursor prepared from an IsoLink® kit was added to 0.5 ml of a solution of the cyclic polyamine (1 mg/ml) adjusted to pH 11 by addition of 0.5 ml of a 0.5 M phosphate buffer pH 11 and the mixture was heated in a waterbath at 100°C for 15 min. When the influence of pH or temperature of the reaction mixtures on the labelling of cyclam was studied, 0.5 ml of a 0.5 M phosphate buffer of different pH (7, 9, 11) was added and labelling was performed at different temperatures (room temperature, 50, 70, 100°C). $^{99m}Tc(V)O_2$-cyclam was prepared by adding consecutively 0.5 ml of a 0.5 M phosphate buffer pH 11, 100 μg $SnCl_2.2H_2O$ (4 mg/ml in HCl 0.05 M) and 0.5 ml generator eluate (20–40 MBq $^{99m}TcO_4^-$) to a solution of 0.5 mg cyclam (1 mg/ml in H_2O). The mixture was then incubated for 10 min at room temperature.

High performance liquid chromatography (HPLC)

The HPLC equipment consisted of a Merck-Hitachi ternary gradient pump (model L-6200 intelligent pump, Merck, Haasrode, Belgium), a Valco N6

injector (Alltech, Laarne, Belgium) and a polymeric reversed phase PRP-1 (10 μm) cartridge (4.1 mm × 250 mm) (Alltech) eluted with gradient mixtures of 0.1 M ammonium acetate adjusted to pH 10 and acetonitrile (0–90% acetonitrile in 20 min) at a flow rate of 1 ml/min. The column effluent was monitored for radioactivity using a 2-inch NaI(Tl) scintillation detector coupled to a single channel analyser and a Rachel analysis program (version 1.40, Lablogic, Sheffield, UK).

Electrophoresis

Electrophoresis was performed on Whatman 1 paper (3.5 cm × 13 cm) by application of a potential of 300 V (Elvi 22 power supply, Milan, Italy) for 30 min with methanol – 0.025 M phosphate buffer pH 3, 7 or 11 (50:50 v/v) as the electrolyte solution. Thioflavin-T, a yellow coloured quaternary ammonium salt, was used as internal standard.

Radio-LC–MS analysis

Radio-LC–MS was carried out using a Waters Alliance 2690 separations module, an XTerra MS RP18 3.5 μm column (2.1 mm × 50 mm) (Waters, Milford, MA, USA) eluted with gradient mixtures of 0.1% ammonium formate and acetonitrile (from 20 to 80% acetonitrile (v/v) in 20 min at a flow rate of 300 μl/min, a 3-inch NaI(Tl) detector connected to a radiation analyser module (The Nucleus, Oak Ridge, USA) and a time-of-flight mass spectrometer (Micromass, Manchester, UK) equipped with an orthogonal electrospray ionization probe (ESI) in positive mode (ES+). Acquisition and processing of data were performed with Masslynx software (version 3.5). A volume of 10 μl of the labelling reaction mixtures was injected.

Biodistribution studies

The experiments in mice were carried out in compliance with the national laws relating to the conduct of animal experimentation. The biodistribution of HPLC isolated peaks of the ^{99m}Tc-complexes was studied in male NMRI mice 10 and 30 min after injection ($n = 4$ at each time point). The activity in each organ was expressed as a percentage of the injected dose. Blood was supposed to be 7% of the body weight[19–21] and muscles 40%.[20]

Results and discussion

Preparation of $^{99m}Tc(CO)_3$-complexes has been significantly facilitated by the availability of IsoLink® kits, which allow one to obtain the $^{99m}Tc(CO)_3(OH_2)_3^+$ precursor rapidly and without the necessity to bubble the solution with CO gas. The IsoLink® kit makes use of an internal CO source (sodium boranocarbonate, which also acts as reducing agent) and in

this way the technetium tricarbonyl precursor can be efficiently made by adding pertechnetate to the vial and heating. Technetium is rapidly reduced to an oxidation state +1 and converted to the technetium tricarbonyl precursor. In this study, we have used IsoLink® kits for labelling of three cyclic polyamines with a ^{99m}Tc-tricarbonyl moiety (Figure 2). For preparation of the final complex, an aliquot of the technetium tricarbonyl precursor was added to a solution of the cyclic polyamine, the mixture was adjusted to pH 11 and the vial was heated in a waterbath at 100°C for 15 min. Polyamines are supposed to be among the best ligands for binding of a technetium tricarbonyl core.[22] In contrast with aliphatic polyamines, which can be labelled at room temperature and neutral pH, labelling of cyclic polyamines demands more drastic conditions (pH 11 and 100°C, Figure 1). A high pH is always favourable, as at lower pH protons compete with the technetium tricarbonyl precursor for binding to the amines. Aliphatic polyamines are flexible and the amines can interact very easily with the technetium atom in the technetium tricarbonyl precursor and replace the water molecules. Cyclic polyamines are much more rigid and require a distortion of the bonds in the technetium tricarbonyl core to take the position of the three water molecules. Therefore the labelling process is more energy demanding. However, under the more drastic conditions high labelling yields were obtained ($\geqslant$90%) and once the complex formation is completed the formed complexes are stable. No significant decomposition of the ^{99m}Tc(CO)$_3$-polyamines was observed in the reaction mixtures up to 2 h after formation.

In HPLC analyses using acidic or neutral mobile phases the studied compounds are characterized by a short retention time, indicative of a high polarity, probably due to protonation of the amines in the cyclic polyamine ligand.

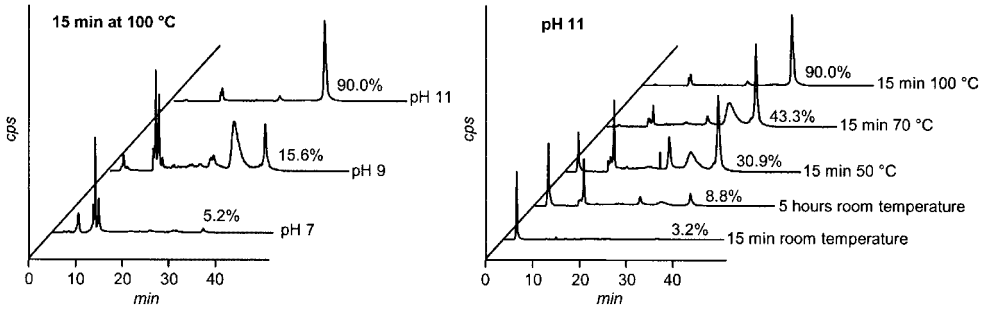

Figure 1. Influence of pH and temperature of the reaction mixtures on the labelling of cyclam with a ^{99m}Tc-tricarbonyl moiety (elution with gradient mixtures of 0.1 M ammonium acetate adjusted to pH 10 and acetonitrile (0–90% acetonitrile in 20 min) at a flow rate of 1 ml/min)

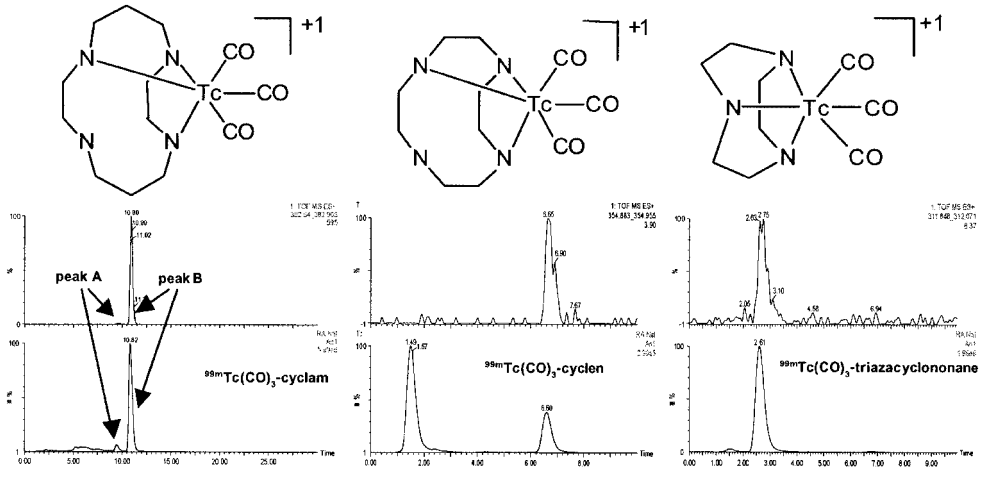

Figure 2. Proposed structure, single ion mass chromatogram and radiometric signal of the radio-LC–MS analysis of ^{99m}Tc(CO)$_3$-cyclam, ^{99m}Tc(CO)$_3$-cyclen and ^{99m}Tc(CO)$_3$-triazacyclononane

Table 1. Retention times of the ^{99m}Tc(I)tricarbonyl precursor, the different ^{99m}Tc(I)-tricarbonyl cyclic polyamine complexes and ^{99m}Tc(V)dioxo-cyclam on RP-HPLC (conditions HPLC see text)

Technetium-99m labelled compound	Retention time (min)
$[^{99m}Tc(I)(CO)_3(OH_2)_3]^+$	3′30″
^{99m}Tc(I)(CO)$_3$-triazacyclononane	10′10″
^{99m}Tc(I)(CO)$_3$-cyclen	14′10″
^{99m}Tc(I)(CO)$_3$-cyclam	32′30″
^{99m}Tc(V)O$_2$-cyclam	10′10″

Therefore, an HPLC system was developed with an alkaline mobile phase (gradient of acetonitrile and ammonium acetate 0.1 M pH 10) and a polymer reversed phase (PRP) column. A PRP column was chosen as this polymer packing is very resistant to high pH.

By comparison to the technetium(V)dioxo cyclam complex, the newly formed technetium tricarbonyl complex of cyclam is much more lipophilic (Table 1). This means that introduction of three CO groups significantly increases the lipophilicity. This could be a disadvantage when the Tc(CO)$_3$-polyamines are used as bifunctional chelates to label polar compounds, because this would affect the biological behaviour of the resulting compounds.

To determine the charge of the newly formed complexes electrophoresis experiments were performed. Thioflavine-T, a lipophilic yellow coloured

cation, was used as internal standard. Methanol was added to the solvent to avoid precipitation of the lipophilic complexes on the application point.

On a theoretical basis, $^{99m}Tc(I)(CO)_3$ complexes with polyamines can be expected to be monocationic, with a technetium(I) tricarbonyl core bound to three amines of one ligand molecule. As expected, in alkaline medium the studied technetium tricarbonyl complexes showed migration to the cathode comparable to that of the monocationic reference compound thioflavine-T. At lower pH the migration distance was higher, due to further protonation of the amines (Table 2).

The studied technetium complexes were analysed using radio-LC–MS. The molecular ion mass of the main peaks (382.8750, 354.9160 and 311.9625 Da, respectively, for $^{99m}Tc(CO)_3$-cyclam, $^{99m}Tc(CO)_3$-cyclen and $^{99m}Tc(CO)_3$-triazacyclononane) is in accordance with the calculated mass of a positively charged complex in which a technetium tricarbonyl core is bound to three amine nitrogens of one cyclic polyamine molecule. The retention times of the main peak in the corresponding single ion mass chromatogram matched the retention time of the main peak in the radiometric channel for each of the complexes (Figure 2).

For ^{99m}Tc-cyclam, the mass spectrum of a small peak eluting before the main peak in the radiometric channel shows the same molecular ion mass as the main peak. This suggests that both compounds on HPLC are diastereomers in which the propylene bridges in the cyclam ring may be oriented either in the same or in the opposite direction. One of both conformations is clearly preferential.

The biological behaviour of $^{99m}Tc(CO)_3$-cyclam corresponds to what can be expected for a lipophilic cationic complex. This means rapid clearance from the blood mainly by the hepatobiliary system, and to a lesser degree via the urinary pathway and a small but consistent uptake in the heart. The activity sticks to the kidneys and the excretion to the urine is low (Table 3).

$^{99m}Tc(CO)_3$-cyclen is more hydrophilic and in contrast with $^{99m}Tc(CO)_3$-cyclam, $^{99m}Tc(CO)_3$-cyclen is rapidly cleared from the blood mainly via the

Table 2. Migration distance to the cathode during electrophoresis (300 V, 30 min) of the different ^{99m}Tc(I)tricarbonyl cyclic polyamine complexes as a function of pH

	Migration to the cathode (cm)		
	$^{99m}Tc(I)(CO)_3$-cyclam	$^{99m}Tc(I)(CO)_3$-cyclen	$^{99m}Tc(I)(CO)_3$-triazacyclononane
pH 11	2.3	2.3	2.0
pH 7	3.4	4.0	4.7
pH 3	5.5	9.0	7.5

Table 3. Biodistribution of $^{99m}Tc(I)(CO)_3$-cyclam, $^{99m}Tc(I)(CO)_3$-cyclen, $^{99m}Tc(I)(CO)_3$-triazacyclononane and $^{99m}Tc(V)O_2$-cyclam 10 and 30 min post injection in mice ($n = 4$)

	$^{99m}Tc(I)(CO)_3$-cyclam		$^{99m}Tc(I)(CO)_3$-cyclen		$^{99m}Tc(I)(CO)_3$-triazanonane		$^{99m}Tc(V)O_2$-cyclam[23]	
	% of injected dose ± standard deviation							
Organ	10 min	30 min	10 min	30 min	10 min	30 min	10 min	30 min
Kidneys	34.0 ± 5.6	28.4 ± 0.3	9.9 ± 1.4	6.7 ± 3.6	11.4 ± 0.2	7.0 ± 3.3	3.4	1.1
Urine	2.0 ± 1.5	4.3 ± 1.2	37.5 ± 2.3	54.3 ± 4.3	38.9 ± 3.8	52.3 ± 1.0	56	76
Liver	30.2 ± 6.9	28.7 ± 1.5	15.7 ± 2.1	14.3 ± 2.0	21.8 ± 1.2	13.9 ± 1.6	10.7	2.4
Intestines	25.3 ± 1.2	31.1 ± 2.6	11.7 ± 3.4	13.7 ± 1.6	17.3 ± 3.1	18.5 ± 3.3	7.9	8.9
Stomach	0.7 ± 0.1	0.8 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.7 ± 0.1	0.7 ± 0.2		
Lungs	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0		
Spleen	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		
Pancreas	0.9 ± 0.3	0.9 ± 0.4	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.7 ± 0.1		
Blood	0.8 ± 0.1	0.7 ± 0.1	3.4 ± 0.6	2.0 ± 0.4	1.2 ± 0.1	0.8 ± 0.3	2.4	0.8
Muscle	3.9 ± 0.6	3.6 ± 1.5	6.3 ± 0.8	2.9 ± 1.6	3.9 ± 0.4	2.6 ± 2.0		
Heart	0.14 ± 0.02	0.16 ± 0.02	0.11 ± 0.01	0.06 ± 0.01	0.06 ± 0.00	0.03 ± 0.00		
Cerebrum	0.10 ± 0.15	0.02 ± 0.01	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00		
Cerebellum	0.04 ± 0.03	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00		

urinary system and to a lesser degree through the hepatobiliary pathway. Also heart uptake was lower.

The biological behaviour of $^{99m}Tc(CO)_3$-triazacyclononane is comparable to that of $^{99m}Tc(CO)_3$-cyclen. This means a rapid clearance from the blood mainly via the urinary system and to a lesser degree through the hepatobiliary pathway. The biological behaviour of this triaza compound and $^{99m}Tc(CO)_3$-cyclen is more closely related to that of the classical $^{99m}Tc(V)$-dioxo cyclam[23] which has a lipophilicity comparable to that of both of the former compounds. Lipophilicity therefore seems to be an important parameter in the way these compounds are behaving *in vivo*. No significant uptake in the stomach was observed for any of the new complexes, proving that there is no important *in vivo* decomposition to pertechnetate.

Conclusion

Three cyclic polyamines were labelled in high yields with a $Tc(CO)_3$ core to form cationic complexes in which a technetium tricarbonyl core is bound to one ligand molecule. As cyclic polyamines are rigid, the complex formation requires a distortion of the technetium tricarbonyl core and this necessitates more drastic labelling conditions as compared to those applied for the labelling of aliphatic poly-amines. As a result, the studied ligands can only be used as bifunctional chelating agent for labelling via tricarbonyl chemistry of a biomolecule which is resistant to rather drastic labelling conditions.

Acknowledgements

The authors thank Tyco-Healthcare for supplying them with IsoLink© kits.

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