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Research Article

Preparation and animal biodistribution of ^{166}Ho labeled DOTA for possible use in intravascular radiation therapy (IVRT)

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Summary

Owing to its favorable decay characteristics ($T_{1/2} = 27$ h, $E_{\beta(\max)} = 1.85$ MeV, $E_{\gamma} = 81$ keV) and its availability with a specific activity of 3.7–4.4 GBq/mg from a moderate flux reactor, ^{166}Ho can be considered as a potential radionuclide for intravascular radiation therapy (IVRT) using liquid-filled balloons. In the present work, studies on the use of ^{166}Ho labeled 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) as a possible agent for IVRT for the prevention of restenosis has been initiated. ^{166}Ho was obtained by irradiating natural Ho_2O_3 powder and DOTA was synthesized by a multistep procedure. The optimum protocol of radiolabeling of DOTA with ^{166}Ho was achieved by varying different reaction parameters. The complex was found to retain its stability for 7 days at room temperature. Bioevaluation studies carried out in Wistar rats showed that $>95\%$ of the injected activity was excreted within 3 h p.i. with almost no retention in any major organ. Both radiochemical and biological studies showed that ^{166}Ho labeled DOTA can be further explored as a potential agent for IVRT. Copyright © 2002 John Wiley & Sons, Ltd.

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Introduction

Restenosis is the major drawback of percutaneous transluminal coronary angioplasty (PTCA) and occur within 6 months in 40–60% cases.[1,2] Several studies have demonstrated effective inhibition of arterial neointimal proliferation by the delivery of sufficiently high dose of ionizing radiation to coronary vessel wall.[1,3–5] High-dose-rate irradiation can be delivered from radioactive wires or seeds and liquid-filled balloon catheters. The most attractive of these techniques is the use of liquid-filled balloons containing short-lived high-energy β^- emitting radioisotope to ensure the most uniform and homogeneous dose delivery to the arterial wall.[6,7] However, the major concern in adopting this technique is the risk of balloon rupture resulting in the release of radioactivity in the circulation. Therefore, it is important that the radioactive species used inside the balloon should exhibit rapid renal excretion so as to minimize the dose to critical organs.[7,8]

In connection with our work on the development of possible IVRT agents, we have evaluated the potential of several radioisotopes for their applicability to complex with chelating agents.[8–12] The prime factors which require consideration prior to the choice of the radioisotopes emerge from their ease of production with adequate radionuclide purity as well as specific activity and decay characteristics ($E_{\beta\max}$, $T_{1/2}$). The potential candidates in liquid-filled balloons are short-lived high-energy β^- emitters like ^{188}Re, ^{186}Re, ^{90}Y and ^{166}Ho (Table 1). ^{188}Re is the preferred choice owing to its suitable decay characteristics and availability in carrier free state from a ^{188}W–^{188}Re generator installed at the hospital radiopharmacy.[7,13–16] However, the main drawback for setting up a ^{188}W–^{188}Re generator may be the limited availability of ^{188}W, the parent radionuclide, since it requires a very high neutron flux

Table 1. Typical radionuclides for intravascular radionuclide therapy

Radionuclide	Half life	E_β(max.) (MeV)	E_γ (keV)	Maximum soft tissue penetration (mm)	Production route
^{188}Re	17 h	2.1	155(15%)	11.0	^{188}W–^{188}Re generator
^{186}Re	90 h	1.07	137(9%)	5.5	^{185}Re (n,γ)
^{90}Y	64 h	2.3	—	12.0	^{90}Sr–^{90}Y generator
^{166}Ho	26.9 h	1.8	81(6%)	8.5	^{165}Ho (n,γ)

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($>5\times 10^{14}$ n/cm^2/s) as well as isotopically enriched WO_3 to obtain reasonable quantities of ^{188}W with adequate specific activity.[10,17] The alternate method of production of ^{188}Re is by thermal neutron bombardment on enriched ^{187}Re target. However, the comparatively higher cost of enriched ^{187}Re target than natural Ho (100% ^{165}Ho) makes production of ^{166}Ho cheaper than that of ^{188}Re using (n,γ) activation. ^{90}Y is also a practical choice as it is feasible to make a ^{90}Sr–^{90}Y generator which can be housed in hospitals.[18,19] Radionuclidic contamination of ^{90}Sr ($T_{1/2} = 28.3$ year) in the eluted ^{90}Y is a crucial factor and so far there is no commercially available ^{90}Sr–^{90}Y generator. ^{166}Ho is an ideal alternative to ^{188}Re, especially where ^{188}W–^{188}Re generator is not easily available, owing to the similar decay characteristics and feasibility of producing ^{166}Ho in high radionuclidic purity and moderate specific activity by irradiating natural Ho target in reactors with medium thermal neutron flux (^{165}Ho has 100% natural abundance and thermal neutron capture cross section of 66 barns).[10]

The choice of the carrier molecule for radioisotope to be used in liquid-filled balloons for IVRT is guided by its chelating ability with the radioisotope of choice as well as its rapid clearance from biological system.[20–26] In case of any accidental release of the agent in the blood circulation due to balloon rupture, the dissociation of the radiometal from the metal chelate could eventually be favored due to its very low concentration.[27] The loss of the radiometal from the chelate could result in its accumulation in vital organs. Hence, the carrier should form a metal chelate with high thermodynamic stability and kinetic inertness. A careful review of the literature has shown that metal complexes with macrocyclic chelators are much more kinetically inert as compared to their acyclic counterparts. In particular, extremely high solution stability of lanthanide complexes with polyazamacrocycles such as DOTA (1,4,7,10-tetraazacyclodocane-1,4,7,10-tetraacetic acid) has already been reported.[27] In the present paper we report the synthesis of DOTA, its complexation studies with ^{166}Ho and the study of its pharmacological behavior in Wistar rats.

Results and discussion

Characterization of ligand

The synthesized ligand was characterized by FT-IR and ^{1}H-NMR spectroscopy. FT-IR (KBr, v cm^{-1}): 3443, 3096, 2966 2853, 1682, 1460

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^{1}H-NMR, D_2O (δ ppm): 2.44, 2.67 (16 H, broad singlets, –N–CH_2–CH_2–N–), 3.12 (8 H, singlet, –CH_2–COOH).

Production of ^{166}Ho

^{166}Ho was produced in moderate specific activity and moderate radionuclidic purity by irradiating natural Ho_2O_3. 3.7–4.4 TBq/g (100–120 Ci/g) of ^{166}Ho activity was obtained after radiochemical processing. γ-ray spectrum showed the major photopeak at 81 keV (6%) and minor peaks at 1379, 1581 and 1662 keV. All these peaks correspond to photopeaks of ^{166}Ho.[28] The moderate specific activity and radionuclidic purity of ^{166}Ho could be attributed to the fact that natural Ho is mono-nuclidic and has reasonably good neutron capture cross section ($\sigma = 66$ b). It is pertinent to note that neutron bombardment on natural Ho also produces ^{166m}Ho ($T_{1/2} = 1200$ year) as a radionuclidic impurity.[7,10] In our studies, the γ-ray spectrum recorded 24 h post-EOB did not show any of the photopeaks (184, 280, 410, 529, 711 keV) corresponding to that of ^{166m}Ho.[28] However, when the γ-ray spectrum of a 20 days old sample of ^{166}Ho was recorded the activity of ^{166m}Ho was found to be $\sim$3.7 kBq which is only 10^{-5}% of the total activity produced after EOB. This is expected as the radioactivity of ^{166m}Ho will be insignificant after 7 days irradiation owing to its very long life, though the cross sections of formation of both ^{166}Ho and ^{166m}Ho by the thermal neutron irradiation on natural Ho are nearly comparable.

Characterization of the complex

The complex was characterized by paper chromatography using normal saline and paper electrophoresis techniques. It was found that in paper chromatography using normal saline as eluting solvent, ^{166}Ho–DOTA complex moved along with the solvent front ($R_f = 0.8-1.0$) while $^{166}HoCl_3$ remained at the point of spotting ($R_f = 0$). In paper electrophoresis using phosphate buffer, the complex moved towards anode and $^{166}HoCl_3$ under identical conditions did not show any movement from the point of application.

Optimization studies

In order to obtain the optimum protocol for maximum complexation, several experiments were carried out varying reaction parameters such

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as ligand concentration, pH, reaction time and carrier Ho concentration. Figure 1 shows the variation of the complexation yield with ligand concentration. While 60% complexation was obtained with 0.5 mg of DOTA, an increase of complexation to > 99% was observed with 2 mg of the ligand. Complexation studies carried out at different pH (2–11) indicated that maximum yield was obtained at pH 7. Estimation of the complexation yield in the reaction mixture at various time intervals showed that 15 min incubation at room temperature is adequate to achieve > 99% complexation.

Stability studies carried out by using paper chromatography and paper electrophoresis techniques indicated that the complex was highly stable at room temperature, as it did not show any appreciable degradation even after storage for 7 days.

Complexation yields were determined with different concentrations of carrier Ho maintaining the ligand concentration at 2 mg/ml. The complexation yield was found to be > 99% when no carrier was added and it remained unaltered up to the addition of 400 μg of carrier. Table 2 shows the variation of complexation yield with different ligand:metal ratio.

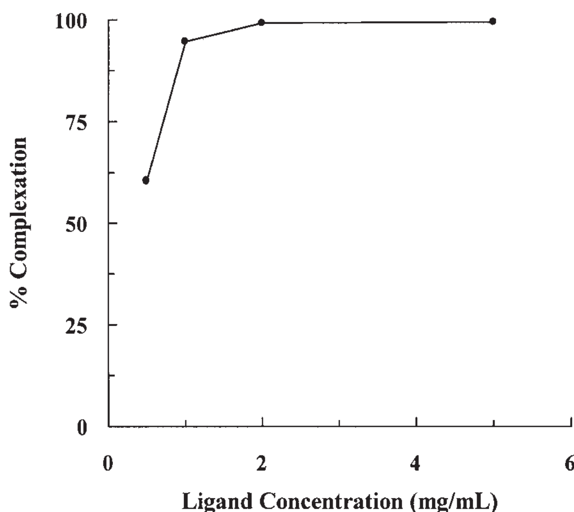

Figure 1. Effect of ligand concentration on complexation yield of ^{166}Ho–DOTA complex. The complexation was carried out by incubating the reaction mixture containing 100 μg of carrier Ho at pH 7 for 15 min at room temperature

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Table 2. Complexation yields of ^{166}Ho–DOTA complex at various ligand: metal ratios

Ligand:metal	Complexation yield (%)
10:1	99.8
5:1	99.6
2.5:1	99.1
1.3:1	96.8
1:1	88.4

Table 3. Biodistribution pattern of ^{166}Ho–DOTA complex in Wistar rats

Organ	% ID/Organ *% ID/g of Organ*		
	10 min	30 min	3 h
Blood	8.46 (0.52)	4.36 (1.21)	0.00 (0.00)
	0.46 (0.08)	*0.23 (0.11)*	*0.00 (0.00)*
Liver	2.30 (0.21)	2.23 (0.41)	0.14 (0.05)
	0.18 (0.06)	*0.16 (0.00)*	*0.01 (0.00)*
Intestine	2.89 (0.01)	2.80 (1.02)	2.03 (0.67)
	0.11 (0.03)	*0.10 (0.03)*	*0.08 (0.01)*
Kidneys	5.07 (0.51)	5.28 (2.86)	0.67 (0.27)
	1.86 (0.25)	*1.69 (0.69)*	*0.25 (0.06)*
Stomach	0.46 (0.01)	0.31 (0.05)	0.10 (0.03)
	0.10 (0.02)	*0.06 (0.02)*	*0.03 (0.01)*
Heart	0.31 (0.11)	0.18 (0.03)	0.00 (0.00)
	0.24 (0.13)	*0.13 (0.04)*	*0.00 (0.00)*
Lungs	0.63 (0.06)	0.36 (0.07)	0.02 (0.01)
	0.33 (0.04)	*0.17 (0.03)*	*0.00 (0.00)*
Bone	2.48 (1.13)	1.10 (0.22)	0.00 (0.00)
	0.13 (0.03)	*0.07 (0.02)*	*0.00 (0.00)*
Muscles	13.05 (1.76)	6.92 (2.36)	0.00 (0.00)
	0.11 (0.01)	*0.07 (0.04)*	*0.00 (0.00)*
Spleen	0.06 (0.00)	0.04 (0.01)	0.00 (0.00)
	0.07 (0.01)	*0.03 (0.01)*	*0.00 (0.00)*
Excretion[a]	64.31 (3.67)	73.82 (4.23)	96.35 (1.30)

Note: Figures in the parentheses show standard deviations ($n = 3$).
[a] Excretion is inferred from the difference of the injected activity and the activity accounted in all the organs.

Biodistribution studies

The results of biodistribution studies carried out in Wistar rats are given in Table 3. Results showed that >95% of the injected activity was excreted via urinary pathway with almost no accumulation of activity in any of the major organs at 3 h p.i. At 10 min p.i. 64.3% of injected activity was cleared with significant accumulation in blood (8.46%), muscle (13.05%), liver (2.30%) and bone (2.48%). However, with the

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progress of time, initially accumulated activities in the major organs showed rapid renal clearance and within 3 h p.i. there are almost no retention anywhere except ~2% of injected activity in the intestine. The constant presence of >2% of the injected activity in intestine throughout the time period studied may be due to the consumption of husks and cage paper which become contaminated by animal excretion. Figure 2 shows the comparative renal clearance and major organ uptake of the complex at different time p.i.

A comparative study among the ^{166}Ho based possible IVRT agents e.g. ^{166}Ho–EC,[10] ^{166}Ho–DTPA,[11] ^{166}Ho–EDTA and ^{166}Ho–DOTA studied by our group shows the superiority of ^{166}Ho–DOTA over the other agents in terms of the retention of the injected activity in different major organs notably in bone. The bone accumulation observed in case of ^{166}Ho–DTPA (0.66% ID/g at 30 min p.i. in Swiss mice), ^{166}Ho–EDTA (1.51% ID/g at 30 min p.i. in Wistar rats) and ^{166}Ho–EC (0.73% ID/g at 30 min p.i. in Wistar rats) complexes may be due to their comparatively lesser *in vivo* stability as all the chelators are acyclic in nature thereby releasing some radionuclide in the blood stream and it is well known that lanthanides have a tendency to be accumulated in the bone.

Experimental

Holmium oxide (spectroscopic grade, >99.99% pure) was obtained from American Potash Inc. Triethylenetetramine, *p*-toluene sulfonyl

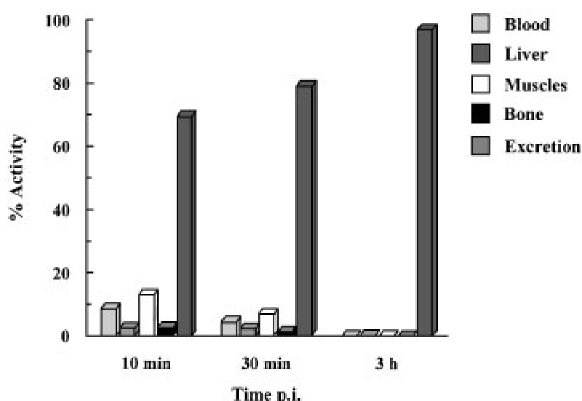

Figure 2. Comparative renal clearance and major organ uptake of ^{166}Ho–DOTA complex in Wistar rats at different time p.i.

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chloride, 1,2-dibromoethane and chloroacetic acid were obtained from Aldrich Chemical Company, MO, USA. All other chemical used in the studies were of AR grade and supplied by reputed chemical manufacturers. Whatman 3 MM chromatography paper was used for paper chromatography and paper electrophoresis studies.

All radioactivity measurements were made by using NaI(Tl) scintillation counter after adjusting the baseline at 50 keV and window to 100 keV. Radionuclidic purity of the processed ^{166}Ho was ascertained by high-resolution γ-ray spectrometry using a HPGe detector coupled to a 4 K multichannel analyzer (MCA) system.

For characterization of DOTA, IR and proton NMR spectra were recorded in a JASCO FT/IR-420 spectrometer and a 300 MHz Varian VXR 300S NMR spectrometer, respectively.

Synthesis of DOTA

DOTA was synthesized by a four-step reaction using triethylenetetramine as the starting material. In the first step, triethylenetetramine (3.3 g, 0.027 mol) was tosylated using *p*-toluene sulfonyl chloride (26.3 g, 0.14 mol) in acetonitrile (125 ml) in the presence of triethylamine (16 ml, 0.115 mol). The reaction was carried out by portionwise addition of *p*-toluene sulfonyl chloride to the magnetically stirred reaction mixture at room temperature for 1 h and stirring was continued for another 4 h. Solvent was removed under vacuum to yield a white semisolid. To this, double distilled water was added followed by extraction with chloroform. The pooled organic extracts were dried over Na_2SO_4 and then dried under vacuum whereby tetratosylated triethylenetetramine was obtained as a white solid (19.7 g, 97%).

In the second step, cyclization of tetratosylated triethylenetetramine was effected by condensation with 1,2-dibromoethane in dimethylformamide as the solvent and K_2CO_3 as the base. In a typical reaction, a magnetically stirred mixture of tetratosylated triethylenetetramine (10 g, 0.013 mol) and anhydrous K_2CO_3 (4.5 g, 0.032 mol) in freshly distilled dry dimethylformamide (70 ml) under nitrogen atmosphere was stirred for 1.5 h at 30°C, when a white precepitate was observed. 2.4 g (0.013 mol) of 1,2-dibromoethane in 30 ml dry dimethylformamide was dropwise added to the reaction mixture and the reaction was continued for 30 h at an elevated temperature of 100°C, following which dimethylformamide was removed by vacuum distillation. The work-up involved addition of 50 ml of double distilled water followed by

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extraction in chloroform. The removal of solvent resulted in 8 g (78%) of tetratosylated cyclen.

In the third step, detosylation was effected by allowing tetratosylated cyclen (6.2 g, 0.008 mol) to react with concentrated H_2SO_4 (35 ml) for 28 h at 115°C. The reaction mixture was allowed to cool to room temperature and the pH adjusted to ~ 12 under cooling (0–5°C) and extracted in dichloromethane after saturation with brine. The pooled organic extracts were washed with brine and the solvent removed to yield 0.46 g (33%) of cyclen (1,4,7,10-tetraazacyclododecane).

In the final step, a mixture of cyclen (0.36 g, 0.0021 mol) and chloroacetic acid (0.9 g, 0.009 mol) in 25 ml of double distilled water was stirred for 10 min. The pH of the solution was adjusted to 9.5–10 by dropwise addition of 5 M NaOH solution and the resulting solution was heated to 70°C. The progress of the reaction was indicated by the decrease in the pH of the reaction, which was maintained at approximately 10 throughout the reaction time (30 h). The pH of the reaction mixture was brought down to 2 using concentrated HCl on attaining room temperature. The reaction mixture is concentrated to dryness in vacuum to yield the crude DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid).

The purification of DOTA was carried out by column chromatography on silica gel. The solid was dissolved in 0.1% trifluoroacetic acid in water. The elution was carried out by using 0.1% trifluoroacetic acid in water and subsequently by 0.1% trifluoroacetic acid in acetonitrile. The solvents from the eluted fractions were removed by vacuum distillation to give the pure product as a white solid (0.75 g, 88% yield, literature melting point 286.2°C, observed melting point 283°C).

The total synthetic scheme for the synthesis of DOTA is given in Figure 3.

Production of $^{166}HoCl_3$

5–6 mg of Ho_2O_3 was weighed and sealed in a quartz ampoule and irradiated at a thermal neutron flux of 3×10^{13} n/cm^2/s for 7 days and cooled for 6 h. Irradiated Ho_2O_3 powder was dissolved in 5 ml of 0.1 M HCl by gentle warming. The resultant solution was evaporated to near dryness and reconstituted in 5 ml of double distilled water.

Radionuclidic purity was ascertained from the γ-ray spectrum of an appropriately diluted sample recorded using a HPGe detector coupled to the 4 K MCA. Radioactive concentration was also measured with the

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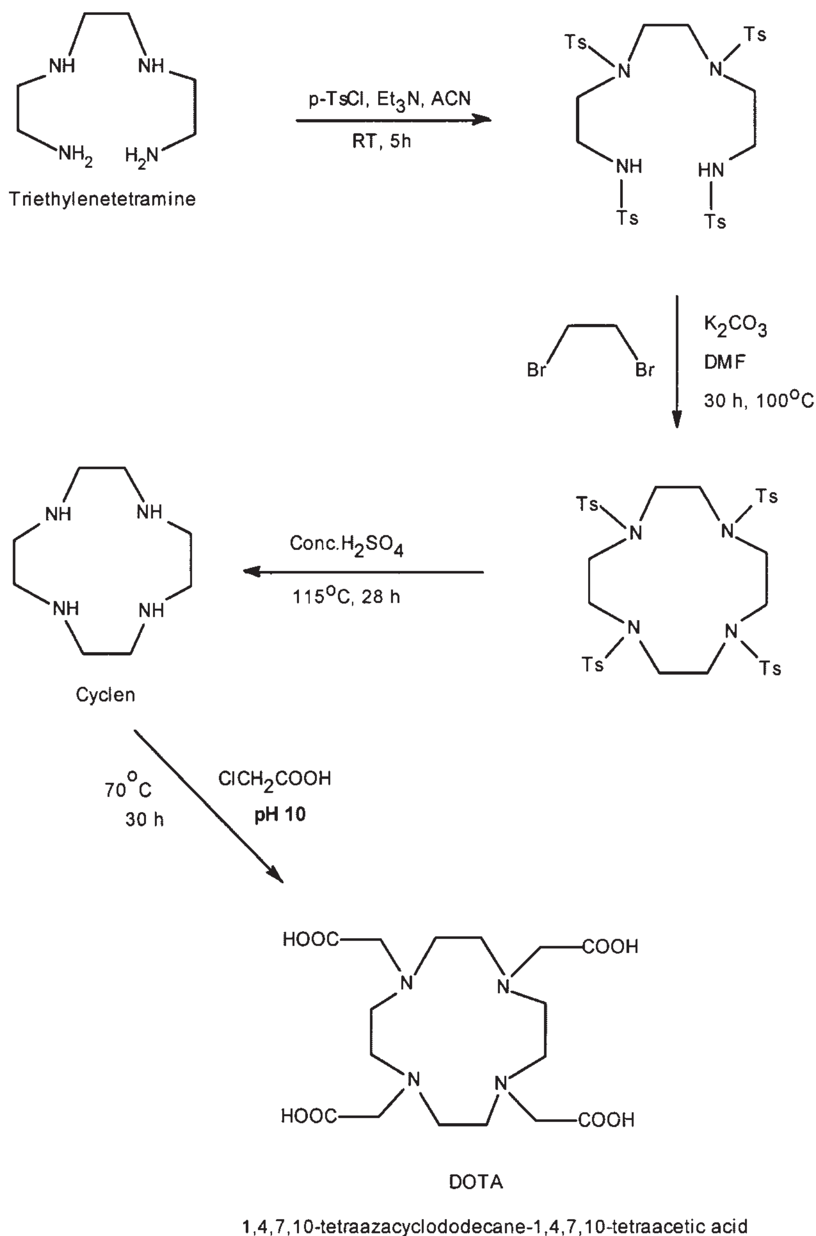

Figure 3. Synthetic scheme of DOTA

same system after efficiency calibration with a standard ^{152}Eu source. Radioactivity assay of high activity samples was carried out by measuring the ionization current obtained when an aliquot of the batch was placed inside a precalibrated well-type ion chamber.

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Preparation of ^{166}Ho–DOTA complex

2 mg of DOTA (5 mM) was dissolved in 0.2 ml of bicarbonate buffer (0.5 M, pH 9) in a glass vial. ^{166}Ho activity (20–30 MBq) as $^{166}HoCl_3$ and 100 μl of $^{165}HoCl_3$ carrier solution (100 μg, 0.6 mM) were added, pH of the reaction mixture adjusted to ~7 and the volume made upto 1 ml with normal saline. The reaction mixture was incubated at room temperature for 15 min.

Various parameters such as ligand concentration, pH of the reaction mixture, carrier Ho concentration, reaction time and temperature were optimized to achieve maximum complexation yield. The optimized protocol in order to get maximum complexation yield is given in Table 4.

Characterization of the complex

Paper chromatography. 5 μl of the test solutions were applied 1.5 cm from one end of Whatman 3 MM chromatography paper strips (12 × 2 cm). The strips were developed in normal saline until the solvent reached the top of the strip. The strips were dried, cut into 1 cm segments and activity measured.

Paper electrophoresis. 5 μl of the complex solution prepared was spotted on pre-equilibrated Whatman 3 MM (35 × 2 cm) chromatography paper at 15 cm from the cathode. Paper electrophoresis was carried out for 1 h under a voltage gradient of 10 V/cm using 0.025 M phosphate buffer, pH 7.5. The strips were dried, cut into 1 cm segments and activity counted.

Biodistribution studies

Biodistribution studies of ^{166}Ho–DOTA was performed in Wistar rats weighing 200–300 g. 0.1 ml (2–3 MBq) of the complex was injected

Table 4. Optimum protocol for labeling of DOTA with ^{166}Ho

Reagents	Volume	Concentration
Ligand (2 mg)	—	5 mM
0.5 M $NaHCO_3$ buffer, pH 9	0.2 ml	0.1 M
Normal saline	0.7 ml	—
^{166}Ho activity	0.02 ml	20–30 MBq
$^{165}HoCl_3$ carrier (1 mg Ho/ml)	0.1 ml	0.6 mM

pH of the reaction mixture is adjusted to 7 and incubated for 15 min at room temperature

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through the tail vein and the animals were sacrificed at the end of 10 min, 30 min and 3 h post-injection by cervical dislocation. Three rats were used for each time point. The tissues and the organs were excised and activity measured in an NaI(Tl) scintillation counter. Distribution of the activity in different organs was calculated as percentage of injected activity per organ as well as activity per gram of the organs. Total blood and muscle activity were calculated assuming blood and muscle weight as 6.5 and 40% of the total body weight, respectively.[29] All the biodistribution studies were carried out in compliance with the national laws related to the conduct of animal experiments.

Conclusion

The present studies indicate that ^{166}Ho complex of DOTA could be prepared in high yield (99.5%) and the complex was found to retain its stability for 7 days at room temperature. Biodsitribution studies carried out in Wistar rats showed that more than 95% of the injected activity cleared through urinary excretion within 3 h post-injection with almost no retention in any of the major organ. These results confirm that ^{166}Ho–DOTA complex could be a potential agent and needs to be explored further for use in liquid-filled balloon for prevention of restenosis.

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