Original Article

Production and Quality Control of ^{166}Ho-Chitosan for Therapeutic Applications

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(Received 25 October 2010, Revised 6 November 2010, Accepted 14 November 2010)

ABSTRACT

Introduction: In this research, [^{166}Ho]Holmium chitosan complex production is described in details, followed by determination of complex radiochemical purity, stability and biodistribution (after intra-articular injection) in wild-type male rats. Finally a Ho-166 based chitosan kit for ultimate radiosynovectomy as well as radiotherapy applications was developed.
Methods: ^{166}Ho-chitosan complex was prepared using chitosan concentrations and $^{166}HoCl_3$ followed by intra-articular injection and biodistribution studies in wild-type rats including and excluding injected knee.
Results: The [^{166}Ho]Holmium chitosan complex was prepared with high radiochemical yield (>95 %) in the optimized condition (35mg/3ml of chitosan in %1 AcOH, pH. 3, >98%, ITLC) was injected to wild-type rats followed by the biodistribution studies of the compound among the tissues excluding the injected knee data. Intra-articular injection of [^{166}Ho]holmium chitosan complex to male wild-type rats and investigation of leakage of activity in the body showed that most of injected dose has remained in injection site 144 h after injection .
Conclusion: Successful development and formulation of ^{166}Ho-chitosan kit is described. This kit has the potential for use in clinical setting namely for radiosynovectomy and cancer radiochemotherapy.

Keywords: Chitosan, Holmium-166, Hepatic cancer, Radiosynovectomy, Biodistribution

Iran J Nucl Med 2010;18(2):1-8

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INTRODUCTION

Many beta-emitters such as Sm-153, Lu-166 and Ho-166 can be produced in reasonable amounts using (n, gamma) reactions. Holmium-166 (E_{β^-} max = 1.84 MeV, $T_{1/2}$ = 26.8 hr) is one of the most interesting radionuclides for targeted therapy modalities. ^{166}Ho-radiopharmaceuticals have been developed for therapeutic purposes. Holmium-166 particles and microspheres are widely used for the treatment of liver malignancies (1) radiosynovectomy (RSV) (2) and cancer therapy (3). Chitosan, a natural and biodegradable polysaccharide with wide range applications in bio-pharmaceutics, agriculture and water treatment purposes (4), is an excellent molecule for intra-cavital therapy (Figure 1). Ho-166 chitosan and Sm-153 chitosan complexes are reported for internal radiation therapy for treatment of diseases such as hepatocellular carcinoma and rheumatoid arthritis (5, 6). The effectiveness of Ho-166 chitosan complex (^{166}Ho-CHITO) for malignancies such as gliomas (7) and prostate cancer (8) has been successfully described.

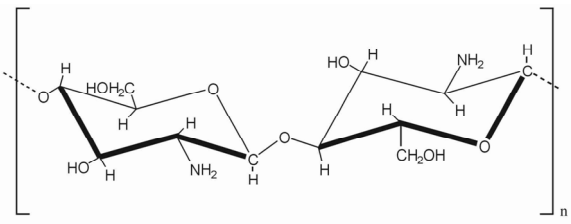

Figure 1. Chemical formula for chitosan.

We have recently reported the development of another chitosan complex, ^{153}Sm-Chitosan (9), however no human study data is yet available for this agent in the literature, while as mentioned earlier, clinical trials are already available for ^{166}Ho-CHITO which is now also available to Iranian Medical Community for the purpose of RSV and/or cancer therapy. In this research, the production of ^{166}Ho-CHITO complex is described in details, followed by determination of complex radiochemical purity, stability and biodistribution (after intra-articular injection) in wild-type male rats. Finally a Ho-166 based chitosan kit for ultimate radiosynovectomy applications is developed and introduced.

METHODS

Production of ^{166}Ho was performed at the Tehran Research Reactor (TRR) using ^{nat}Ho (n, gamma)^{166}Ho nuclear reaction. Natural holmium nitrate with purity of >99.99% was obtained from Aldrich Co. Chitosan (medium molecular weight, MW=400 kDa, DDA= %85) was obtained from Fluka (Bucks, Switzerland). Chromatography paper, Whatman No. 1 was obtained from Whatman (Maidstone, UK). Radio-chromatography was performed by using a bioscan AR-2000 radio TLC scanner instrument (Bioscan, Washington, DC, USA). A high purity germanium (HPGe) detector coupled with a Canberra™ (model GC1020-7500SL) multichannel analyzer and a dose calibrator ISOMED 1010 (Dresden, Germany) were used for counting distributed activity in mice organs. All other chemical reagents were purchased from Merck (Darmstadt, Germany). Calculations were based on the 81 keV peak for ^{166}Ho. All values were expressed as mean ± standard deviation (Mean± SD) and the data were compared using student T-test. Statistical significance was defined as $P<0.05$. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn. All of rats were purchased from Pasteur Institute of Iran, weighing 180-220 g (n=5) and were kept at routine day/night light program and were kept under common rodent diet pellets.

Production and quality control of $^{166}HoCl_3$ solution

Holmium-166 was produced by neutron irradiation of 100 µg of natural $^{nat}Ho(NO_3)_3$ (^{165}Ho, 99.99%) according to reported procedures (10) in the Tehran Research Reactor at a thermal neutron flux of 4-5×10^{13} n.cm^{-2}.s^{-1}. Specific activity of the produced ^{166}Ho was 5GBq/mg after 20h of irradiation. The irradiated target was dissolved in 200 µl of 1.0 M HCl, to prepare $^{166}HoCl_3$ and diluted to the appropriate volume with ultra pure water, to produce a stock solution. The mixture was filtered through a 0.22 µm biological filter and sent for use in the radiolableing step. The radionuclidic purity of the solution was tested for the presence of other radionuclides using beta spectroscopy as well as HPGe spectroscopy for the detection of various interfering beta and gamma emitting radionuclides. The radiochemical purity of the $^{166}HoCl_3$ was checked using 2 solvent systems for ITLC (A: 10mM DTPA pH.4 and B: ammonium acetate 10%:methanol (1:1)).

Synthesis of ^{166}Ho chitosan complex

[^{166}Ho]Holmium chitosan complex was prepared by dissolving of chitosan (35 mg) in 3.5 ml of 1% acetic acid aqueous solution following the addition of ascorbic acid (15 mg) and the mixture was stirred at room temperature till a transparent solution was formed. To the above mixture 2-5 mCi (in 0.5 ml) of [^{166}Ho]holmium chloride was added followed by stirring for 5 minutes and standing for 30 minutes at room temperature. For sterlisation, the 0.22 micron sterile filteration was not successful due to viscosity of the solution. Thus the radiolabeled mixture was autoclaved for 20 min at 120ºC. The effect of the autoclave on the stability of the complex was also studied using ITLC. For kit preparation, the starting chitosan, ascorbic acid and acetic acid solution containing preservative amounts of benzyl alcohol was lyophilized under sterile conditions and kept in fridge up to 60 days.

Stability testing of the radiolabeled compound in final formulation

Stability of ^{166}Ho-chitosan in final preparation was determined by storing the final solution at 4° and 25ºC for 2 days and performing frequent ITLC analysis to determine radiochemical purity. The stability of lyophilized ^{166}Ho-chitosan kit stored at 5°C for more than 60 days was also investigated. ITLC analysis of the conjugated product was performed to monitor for degradation products or other impurities. After subsequent ^{166}Ho-labeling of the stored product, both labeling efficiency and radiochemical purity were determined.

Stability testing of the radiolabeled compound at 37°C

Final ^{166}Ho-CHITO solution (200µCi) was incubated in presence of freshly prepared human serum (300 µl) and kept at 37ºC for 2 days. The complex stability was assessed by size exclusion chromatography on a Sepharose column (1 x 30 cm). The column was equilibrated with PBS and eluted at a flow rate of 0.5 mL/min at room temperature; 1 mL fractions were collected and their activities were determined in a dose calibrator, also the presence of serum proteins in each fraction was determined by Folin-Colciteau® method. The control samples were applied to the column separately, including Ho^{3+} cation, ^{166}Ho-CHITO complex and human serum sample for retention time determination.

Quality control

For measuring radiochemical purity and radiolabeling yield, a 1µL sample of the [^{166}Ho]holmium chitosan complex was spotted on a chromatography paper (Whatman No. 1), and developed in a mixture of methanol/water/acetic acid

(4:4:2) as the mobile phase. The R_f values of free Ho-166 and [^{166}Ho]holmium chitosan complex were 0.9 and 0.0 respectively.

Biodistribution of $^{166}HoCl_3$ in male wild-type rats

To determine the biodistribution of free $^{166}HoCl_3$, in case of any radioisotope leak from the injection site, the cation in normal saline solution was administered to wild-type rats. A volume of $^{166}HoCl_3$ stock solution (50-100 µl,) was evaporated at 50°C and N_2 flow. To the residue, sterilized normal saline solution (according to appropriate specific activity) was added. For each animal, appropriate amount of $^{166}HoCl_3$ activity (150±5 µCi in 50-100 µl,) was injected intravenously to rats through their tail vein. The animals were sacrificed at the exact time intervals (2, 3, 4, 24 and 48 h), and the specific activity of different organs was calculated as percentage of injected dose per gram using an HPGe detector.

Biodistribution of radiolabeled chitosan wild-type rats after intra-articular administration

To determine the accumulation of radiolabeled chitosan in the intra-articular cavity radiolabeled chitosan solution was carefully administered to wild-type rats. A volume (50 µl) of final radiolabeled chitosan solution containing 60 ± 2 µCi radioactivity was injected intravenously to rats through their tail vein. The animals were sacrificed at exact time intervals (4, 24, 48, 120 and 144 h). The specific activity of different organs was calculated as percentage of urea under the curve of 80 keV peak per gram using an HPGe detector.

RESULTS AND DISCUSSION

Production and quality control of ^{166}Ho

The radionuclide was prepared in a research reactor according to regular methods with a range of specific activity 3-5MBq/mg for radiolabeling use, after counting the samples on an HPGe detector for 5 h, two major photons (5.4% of 0.081 MeV and 0.9% of 1.38 MeV) were observed (Figure 2).

The radioisotope was dissolved in acidic media as a starting sample and was further diluted and evaporated for obtaining the desired pH and volume followed by sterile filtering.

The radiochemical purity of the Ho-166 solution was checked in two solvent systems, in 10mM DTPA, Ho^{3+} cation is complexed to more lipophilic HoDTPA form and migrates to higher R_f, while small radioactive fraction remains at the origin which could be related to other Ho ionic species, not forming HoDTPA complex, such as $HoCl_4$, etc. and/or colloids.

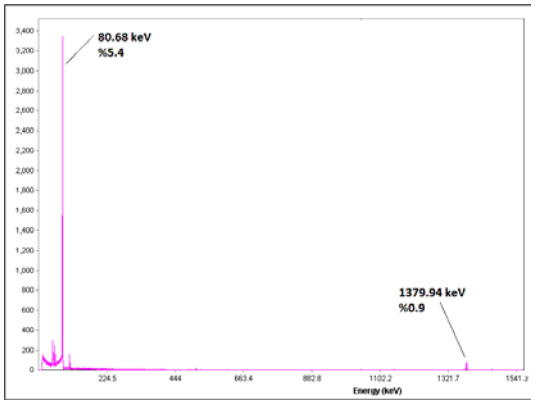

Figure 2. Gamma-ray spectrum for Ho-166 chloride solution used in this study.

Table 1. Chromatographic data for ^{166}Ho-$HoCl_3$ and ^{166}Ho-chitosan

Chemical spieces	Solvent systems			Radiochemical purity (%)
	DTPA solution (pH 5)	methanol: water: acetic acid (4:4:2)	10% ammonium acetate:methanol (1:1)	
^{166}Ho-$HoCl_3$	0.9	0.9	0.05	99
^{166}Ho-chitosan	0.01	0.1	0.05	95

On the other hand, ammonium acetate: methanol mixture was also used for the determination of radiochemical purity. The fast eluting species was possibly the ionic Ho-166 cation other than Ho^{3+} and the remaining fraction at R_f.0 was a possible mixture of Ho^{3+} and/or colloids. Due to existence of 1% impurity in both cases the existence of colloids is unlikely (Table 1).

Proparation of [^{166}Ho]holmium chitosan complex

The effect of various factors on the labeling yield of [^{166}Ho]holmium chitosan were studied. In higher concentration no significant deference exist on labeling yield for added [^{166}Ho]holmium chloride activity (30 mCi). The chitosan which had a molecular weight of 400 kDa was used to investigate of effect of chitosan concentration on labeling yield at pH=3.5.

Labeling yield increased with increasing chitosan concentration and reached above 98% when the concentration reached 35 mg/3 ml. The highest labeling yield was achieved at pH=2.8-3.2 while decreased beyond this range. The labeling yield of 99% was achieved after 30 minutes. The effect of absence and presence of ascorbic acid (at various concentrations) as a complex stabilizer were also studied.

ITLC using a mixture of methanol, water and acetic acid showed that the complex is majorly prepared in 30 min with 99% radiochemical purity; the remaining 1% is possibly attributed to other Ho ionic species which can not react with chitosan (Table 1).

Based on the obtained results, the optimal procedure for the preparation of [^{166}Ho]holmium chitosan complex with a high labeling yield is as follows. 35mg of chitosan (MW=400 kDa) was dissolved in 3.5 ml of 1% acetic acid aqueous solution. The acidity of obtained solution was adjusted to pH=3 by addition of 0.5 M NaOH solution and followed by addition of [^{166}Ho]holmium chloride solution. Finally the total volume was adjusted to 4 ml by addition of deionized water.

Stability studies of [^{166}Ho]Holmium chitosan complex

The stability of prepared [^{166}Ho]holmium chitosan complex was checked up to 48 hours after preparation. The complex was stable in acidic media (pH=3.5) and it's radiochemical purity was above 98% even 48 hours after preparation. The radiochemical purity of [^{166}Ho]holmium chitosan complex was decreased to 93% after 72 hours in phosphate buffer solution. Also the stability of the complex was determined at 37ºC for 48 h and the data were almost consistent with the final solution stability.

Although the complex is injected intra-articularly and is not in direct contact with serum, stability test was developed for the complex in presence of human serum at 37ºC. the mixture was then passed through a

sephadex column followed by the elution with PBS and each fraction was checked for radioactivity and presence of serum proteins by colorimetric method.

Biodistribution studies for free Ho^{3+} cation in rats

The animals were sacrificed by CO_2 asphyxiation at selected times after injection (2, 3, 4, 24 and 48h). Dissection began by drawing blood from the aorta followed by removing heart, spleen, muscle, brain, bone, kidneys, liver, intestine, stomach, lungs and skin samples.

The tissue uptakes were calculated as the percent of area under the curve of the related photo peak per gram of tissue (% ID/g) (Figure 3).

The biodistribution of Ho^{3+} cation was determined in wild-type animals for better comparison for 2-48 h post injection. The liver uptake of the cation is comparable to many other radio-metals mimicking ferric cation accumulation, about 3.5% of the cation accumulates in the liver. The transferin metal uptake and final liver delivery looks the possible route of accumulation. The blood content is low at all time intervals and this shows the rapid removal of activity in the circulation. Brain, muscle and also skin do not demonstrate significant uptake while it is in accordance with other cations accumulation. A 0.5-1% bone uptake is observed for the cation which remains almost constant up to 24 h. Spleen also has significant uptake possibly related to reticulo-endothelial uptake.

Biodistribution studies after intra-articular administration of Ho^{3+} cation in rats

The distribution of injected dose in rat organs up to 144 h after intra-articular injection of [^{166}Ho]holmium chloride (60 µCi/100ul) solution was determined for control studies. Based on these results, it was concluded that the most portion of injected activity of [^{166}Ho]holmium chloride was extracted to blood circulation and distributed in rat organs which was consistent with free Ho^{3+} distribution while administered intravenously (data not shown).

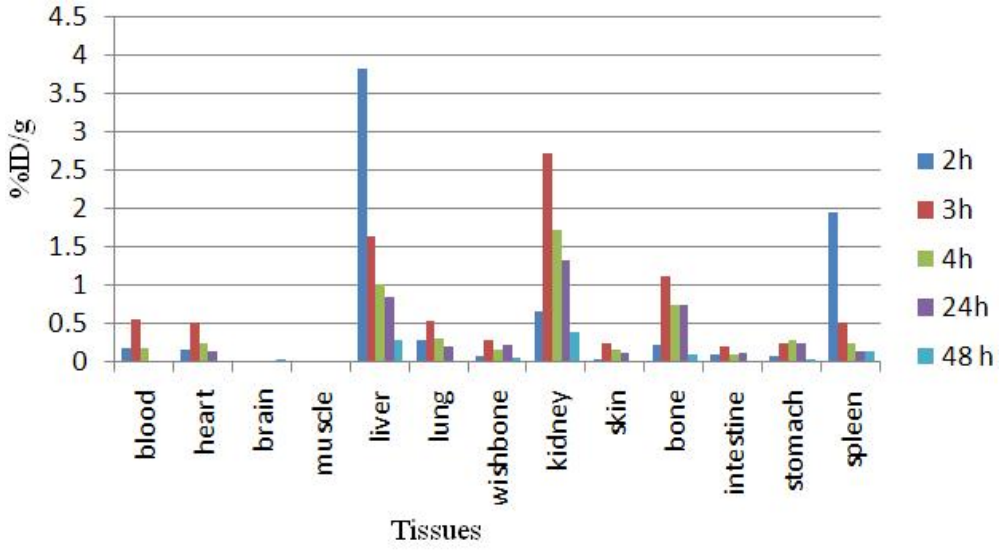

Figure 3. Percentage of injected dose per gram (ID/g %) of $^{166}HoCl_3$ in wild-type rat tissues at 2, 3, 4, 24 and 48 h post injection.

Biodistribution studies after intra-articular administration of ^{166}Ho-chitosan cation in rats

Figure 4 presents the distribution of injected dose in the rat organs at various time intervals after intra-articular injection of 60 µCi/100µl of [^{166}Ho]holmium chitosan complex as percentage of injected dose. In case of any leak from the jont, the complex would accumulate in reticuluendothelial (RE) system due to high molecular weight of the complex, unless the complex would dissociate at serum pH and Ho^{3+} cation would be formed.

Almost no detectable amounts of activity was observed in spleen and lung, which are two important RE organs, showing no complex leak has occurred. Very negligible liver and kidney uptakes are observed which is possibly caused by Ho-166 cation release from the injected joint and not the radiolabeled complex uptake.

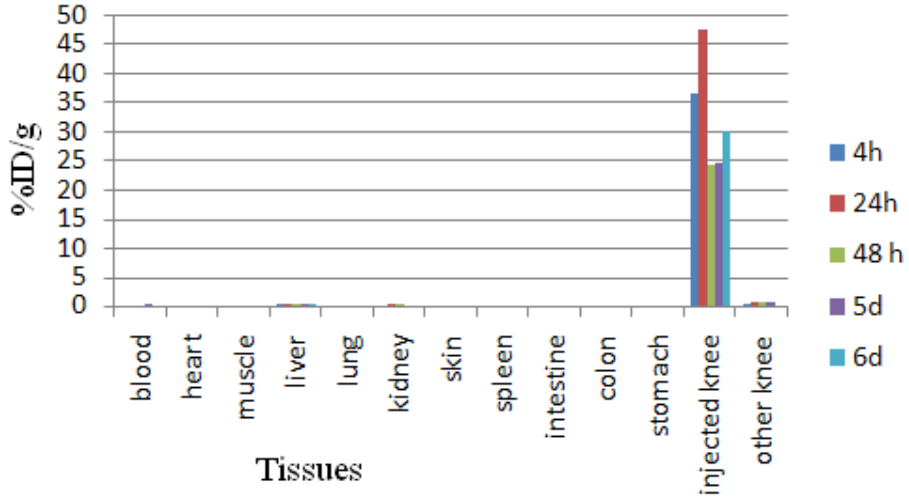

Figure 4. Distribution of [^{166}Ho]-chitosan in wild-type male rats, 4, 24, 48, 120h and 144h after intra-articular injection of 60 µCi of compound. %ID-percentage of injected dose. Each bar presents mean± SD (n=3).

Figure 5 demonstrates the biodistribution of the compound among the tissues excluding the injected knee data in order to better understand the biodistribution of the leaks from the knee.

The distribution of the radioactivity among tissues after removing knee joint accunulation data demonstrates a typical Ho^{3+} cation biodistribution among the tissues. It is believed that free Ho cation is the only radiochemical spieces escaping from knee joint and not ^{166}Ho-CHITO complex is found in circulation.

CONCLUSION

The [^{166}Ho]Holmium chitosan complex was prepared with high radiochemical yield (>99 %) in the optimized condition; 35mg/3ml of chitosan concentration in diluted acetic acid solution (pH=3). The prepared complex was stable in the final solution at room temperature, 37ºC and presence of human serum, and can be used even 24 hours after preparation.

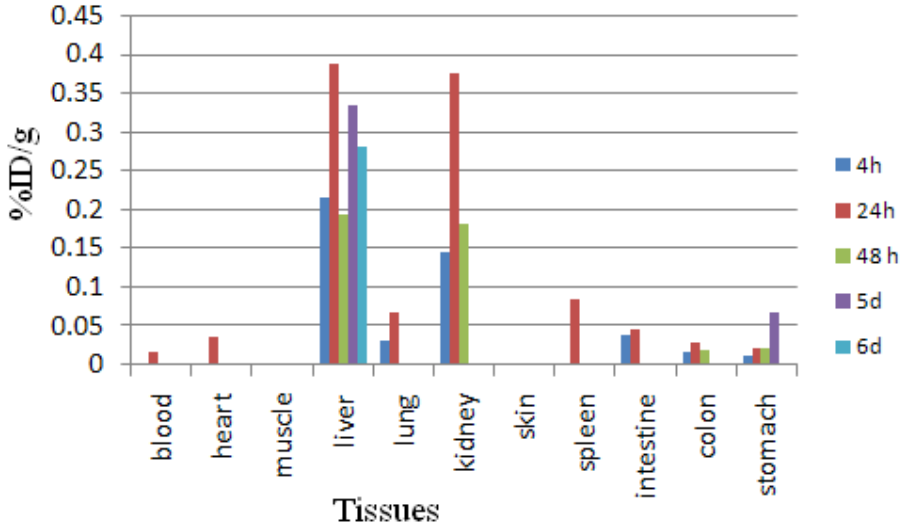

Figure 5. Distribution of [^{166}Ho]-chitosan in wild-type male rats excluding injected knee data at 4, 24, 48, 120h and 144h after intra-articular injection of 60 µCi of compound. %ID-percentage of injected dose. Each bar presents mean± SD (n=3).

Intra-articular injection of [^{166}Ho]holmium chitosan complex to male wild-type rats and investigation of leakage of activity in the body showed that most of injected dose has remained in injection site 144 h after injection. Finally a kit formulation was developed for the *in-situ* preparation of the radiopharmaceutical in clinical centers.

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