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Synthesis and Preclinical Studies of [^{61}Cu]-*N*-(2-hydroxyacetophenone)glycinate as a Possible PET Radiopharmaceutical

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Abstract

[^{61}Cu]-*N*-(2-hydroxyacetophenone)glycinate ([^{61}Cu]NHAG) was prepared using in house-made NHAG ligand and [^{61}Cu]$CuCl_2$ produced *via* the ^{nat}Zn(p,x)^{61}Cu (180μA proton irradiation, 22MeV, 3.2h) and purified by a ion chromatography method. [^{61}Cu]NHAG radiochemical purity was >98% and >99.9% by RTLC and HPLC methods respectively after purification by SPE. [^{61}Cu]NHAG was administered into normal and tumor bearing mice followed by biodistribution studies up to 180 minutes. The best tumor accumulation was observed by animal sacrification after 120 min (tumor/muscle and tumor/blood ratios were 25.6 and 3.4 respectively). [^{61}Cu]NHAG is a potential PET radiotracer for tumor imaging.

Keywords

Radiopharmaceuticals • Copper-61 • NHAG • Biodistribution • Fibrosarcoma

Introduction

There has been tremendous research activity on the preparation and biological evaluation of metal complexes as antiproliferative, antiviral, and enzyme inhibition agents. Thiosemicarbazone copper complexes have shown interesting anti-proliferative activity *in vitro* and *in vivo* [1, 2]. Various radiocopper thiosemicarbazones have been developed for creating possible radiopharmaceuticals as well as tracers with great interest in nuclear medicine and some have already demonstrated putative application in clinical studies, such as Cu-ATSM, accumulating avidly in hypoxic cells [3, 4] and Cu-PTSM as a blood perfusion among tumors as well as vital organs [5, 6].

Copper offers a unique selection of radioisotopes (^{60}Cu, ^{61}Cu, ^{62}Cu, ^{64}Cu, and ^{67}Cu) with half-lives ranging from 9.8 minutes to 61.9 hours, suitable for imaging and/or radiotherapy [7]. Copper-61 is a positron emitter ($T_{1/2}$=3.33 h, β^+: 62%, E.C: 38%), with excellent potentials for application in positron emission tomography (PET) method and molecular imaging [8]. PET is an amazing new tool in the molecular imaging research that has already shown to be superior to many other medical imaging modalities, while technological advances have evolved this technique to more sophisticated fusion systems such as PET/MRI and PET/CT [9].

There has been not many reports of the production and biological evaluation of radiocopper non-thiosemicarbazone complexes to our knowledge for radiopharma-ceutical purposes. However, antitumor property of the ligand N-(2-hydroxy acetophenone) glycinate (NHAG) and some of its complexes has been studied [10]. The non-toxic nature, water solubility and strong coordination behavior of NHAG has been under research for some time [11]. The synthesis; structure, antitumor property, toxicity and glutathione (GSH) depletion property *in vivo* of the NHAG copper complex has also been reported previously [12]. Regarding these properties development of a possible copper tracer using this complex seemed interesting to us based on our recent experiences on radiocopper production *via* ^{nat}Zn(p,x)^{61}Cu [13] and developing important Cu-radiopharmaceuticals in our research group [14, 15].

In continuation of this research project, we hereby report the radiosynthesis, quality control and biodistribution studies of [^{61}Cu]NHAG as a possible tumor imaging agent in normal and fibrosarcoma-bearing mice.

Results and discussion

Copper-61 production

For 76 min bombardment of the ^{nat}Zn target with 22 MeV proton, 150 μA , the resulting activity of ^{61}Cu was 222 GBq (6.0 Ci) at the end of bombardment (E.O.B.) and the production yield was 440 MBq/μAh. Yield from the radiochemical separation was more than 95%. Quality control of the product was performed in two steps. Radionuclidic control showed the presence of 67.41(4.23%), 282.96(12.2%), 373(2.15%), 511(122.9%), 656(10.77%), 1186(3.75%) keV γ-rays from ^{61}Cu and showed a radionuclidic purity higher than 99% (E.O.S.). The rest of activity was attributed to ^{60}Cu (0.23%). In order to check the chemical purity, concentration of zinc (from target material) and gold (from target support) were determined using visible colorimetric assays. The presence of zinc cations was

checked by visible colorimetric assays. Even at 1 ppm of standard zinc concentration, the pinkish complex was visible by naked eye, while the test sample remained similar to the blank. The colorimetric assay demonstrated that the zinc cation concentration was far below the maximum permitted levels, i.e. 5 ppm (less than 1 ppm zinc). The gold concentration was less than 0.9 ppm.

Preparation of NHAG-K and Cu-NHAG compounds

The ligand NHAG was prepared in aqueous media by an imine formation reaction which was controlled by TLC the formation of the ligand was checked by ^{1}H NMR, Mass and IR spectroscopic methods. The aromatic, CH_2 and CH_3 protons were are resolved for the expected product as reported in the experimental parts. The removal of C=O ketone peak and appearance of COOH carbonyl peak in IR spectroscopy were also detected.

Production of the labeled compound

[^{61}Cu]NHAG is a neutral, lipophillic compound which can penetrate all the cells with perfusion, however the structure changes due to the reduction of Cu^{2+} cation to Cu^{+} cation in many cells containing thiol compounds results in hypoxic cell accumulation. Figure 1, shows the synthesis and radiolabeling procedure in this work.

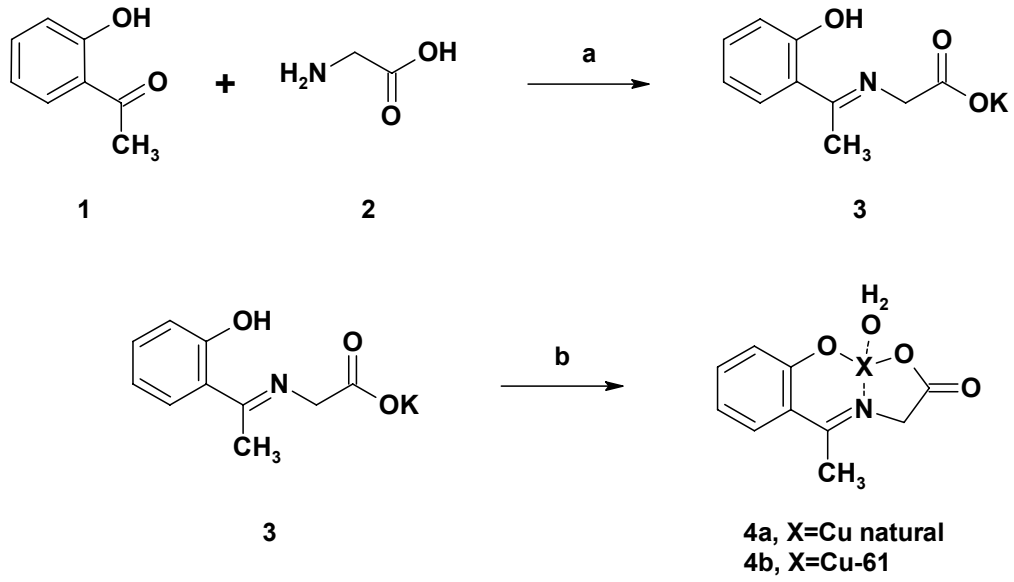

Fig. 1. Synthesis of precursor, K^{+}NHAG **3**, and Cu-NHAG **4a,b**;
a: KOH, 50ºC, Ethanol; b: [^{61}Cu]acetate, 25ºC, 30 min, C_{18} Sep-Pak purification.

In TLC studies, the more polar un-complexed NHAG and free copper fractions, correlate to smaller R_fs (R_f =0.1-0.2) (Fig. 2), while the [^{61}Cu]NHAG complex migrates at the higher R_f (R_f=0.9). In all radiolabeling runs (n=5) (Fig. 5), the integral ratio of the two peaks were constant (97:3), showing the high radiochemical purity and consistency of the labeling method.

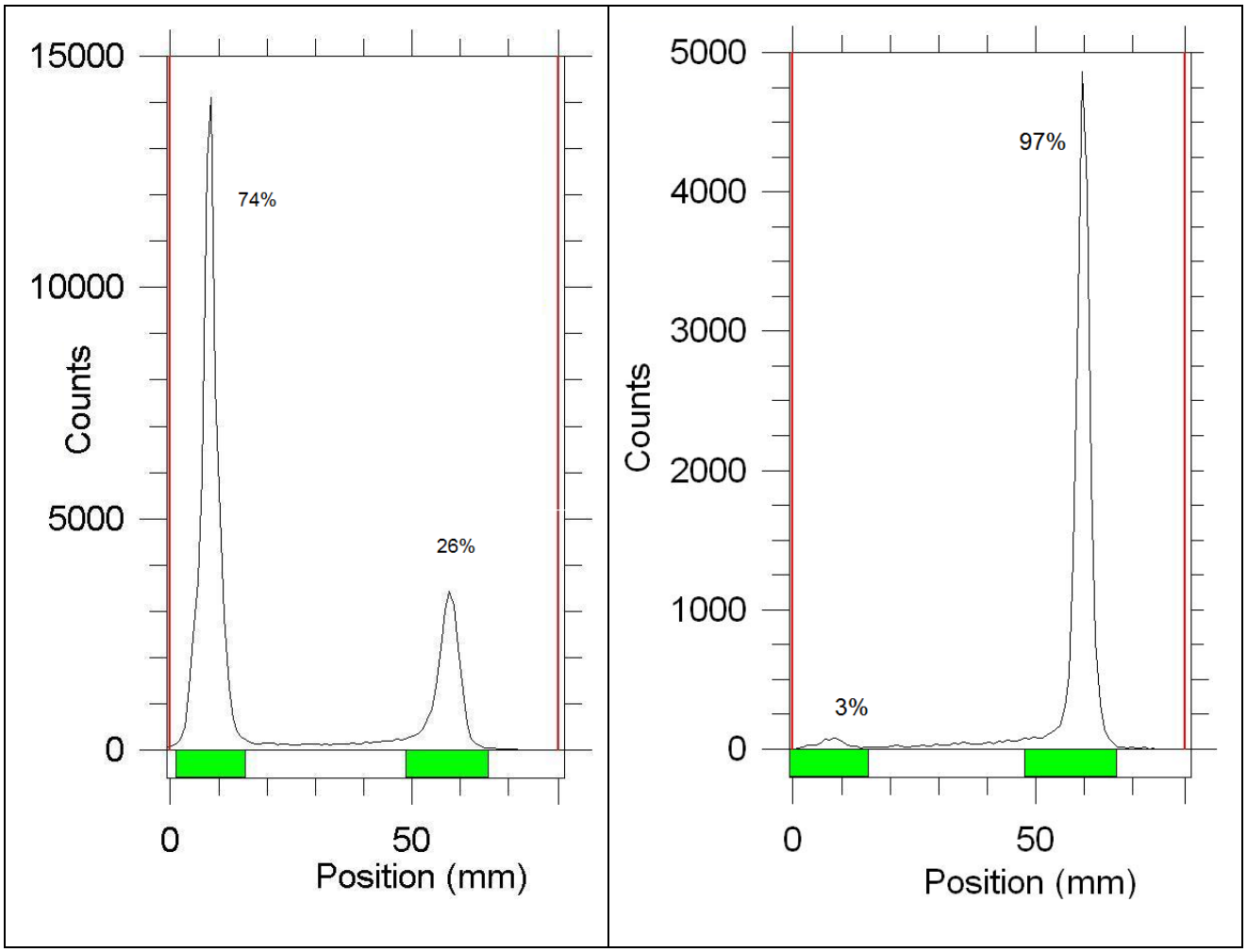

Fig. 2. RTLC of the labeling reaction mixture of [^{61}Cu]NHAG (left) and purified [^{61}Cu]NHAG (right) after C_{18} Sep-Pak purification

Because of the engagement of several polar functional groups in its structure, labeling of NHAG with copper cation affects its chromatographic properties and the final complex is highly lipophilic. Thus free copper and unlabeled NHAG can easily be separated using solid phase C_{18} Sep-Pak column. Fig.2 shows the radiochemical of labeled mixture and the purified post-column fraction. The purity changed from 26 to 97% yielding a suitable labeled compound for further biological tests. In order to minimize the dilution of the eluted fractions from SPE column and minimize the time for obtaining a fraction with the best activity concentration, a precise elution study was performed to choose the best fraction and as it is shown in Fig.3 fractions 2 and 3 were of the highest specific activity which were concentrated to 100–200 µL using gas flow with gentle heat.

In order to obtain the best labeling reaction conditions, the complex formation was studied for temperature dependence. Heating the reaction mixture to 50°C not only did not increase the yield but also yielded in various unknown radiochemical impurities with complex behaviors. Thus 25°C was considered as the best temperature.

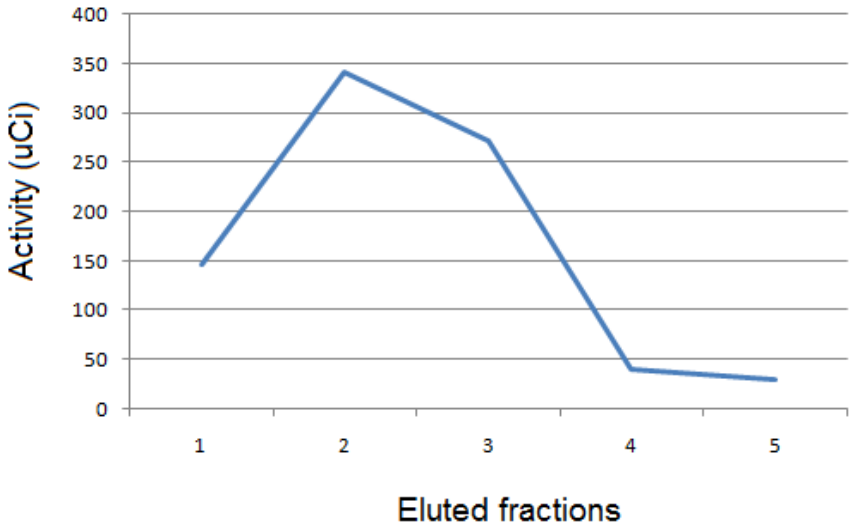

Fig. 3. Radioactivity of eluted 1 mL-ethanol fractions from C_{18} (n=5)

In HPLC studies the fast eluting compound was shown to be hydrophilic [^{61}Cu]Cu^{2+} cation (2.01 min), while the lipophillic [^{61}Cu]NHAG complex was eluted couple of minutes later (6.22).

In various studies, n=9, the purity of both radiochemical spieces were shown to be almost 100% as shown in Fig. 4.

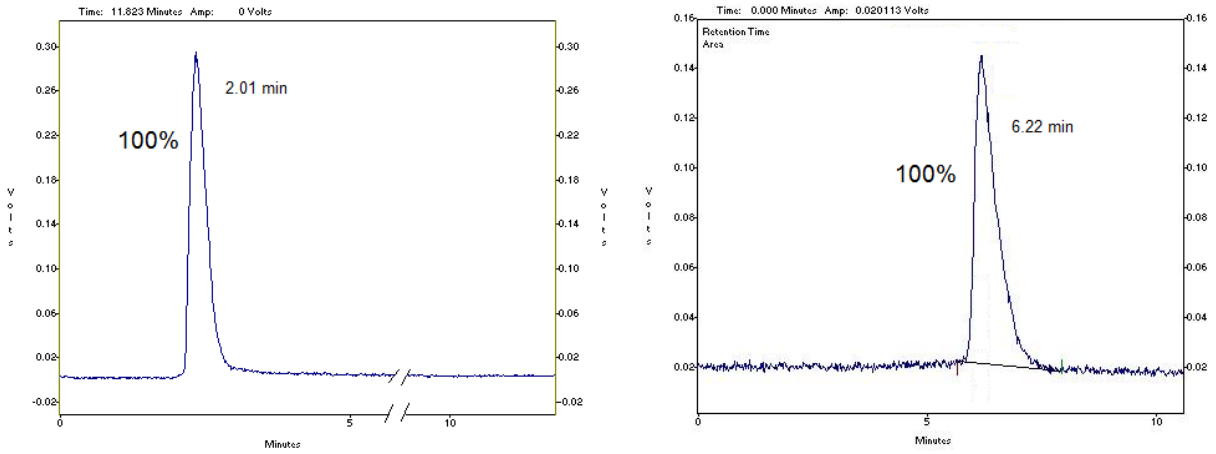

Fig. 4. HPLC diagram for [^{61}Cu]Cu^{2+} (in acetate and chloride form, left) and [^{61}Cu]NHAG (right) using a reverse phase column with a mixture of H_2O:CH_3CN (1:1) as eluent

The final radiolabelled complex diluted in normal saline was then passed through a 0.22 micron (Millipore) filter for sterilization. Due to its thermal instability, [^{61}Cu]NHAG preparation could totally be degraded and left detectable amounts of free copper after autoclaving.

Serum stability of the labeled compound

Incubation of [^{61}Cu]NHAG in freshly prepared human serum at 37°C showed no loss of ^{61}Cu from the complex at least for 2.5 hours.

Radio thin layer chromatography was performed to control the radiochemical purity of the product, using 10 mM DTPA solution or 10% ammonium acetate:methanol (1:1) mixture or of dry ethyl acetate as mobile phases.

Biodistribution of the labeled compound in normal rats

In order to investigate biodistribution of [^{61}Cu]NHAG in our animal models we had to obtain the biodistribution data for free copper cation in our hands, thus after injection of 40 microcurries of the [^{61}Cu]$CuCl_2$ pre-formulated by the normal saline (pH. 6.5–7) through the tail vein of adult mice the bidistribution of the cation was checked in various vital organs. Copper is partly accumulated in liver as a reservoir for many metals esp. by serum ceruloplasmin. GI accumulation especially in the first hour is expressed as a result of liver secretion *via* hepatobilliary excretion, while it is not significant after 2 hours. The major content of copper is washed out by kidneys and consequently urinary tract due to high water solubility of the cation. The uptakes of rest of the tissues are not significant. The biodistribution of copper cation in normal animals up to 2 hours is demonstrated in Fig 5.

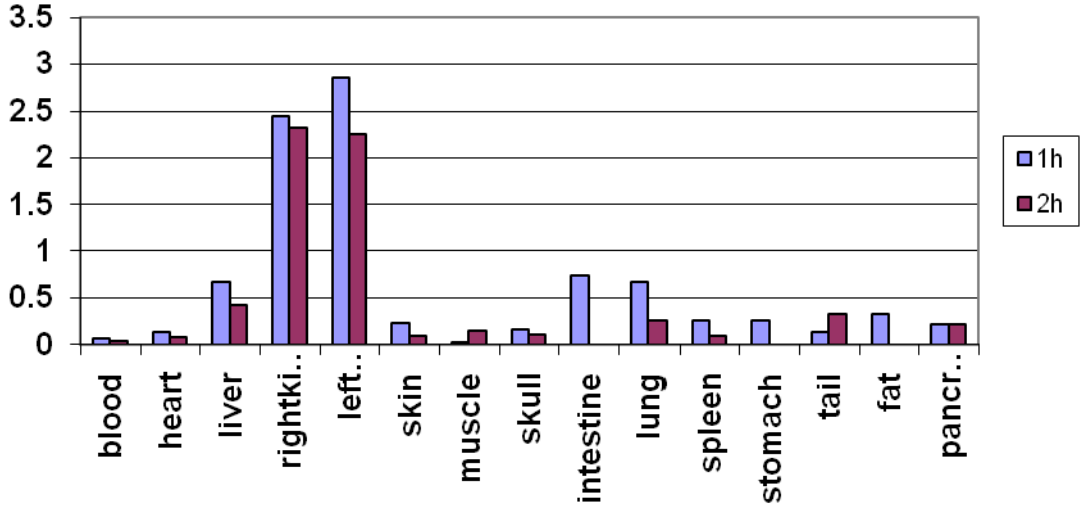

Fig. 5. Calculated ID/g% of [^{61}Cu]$CuCl_2$ 60–120 minutes post I.V. injection in normal mice

The uptake of free copper cation must be checked in fibrosarcoma-bearing animals in order to validate the real [^{61}Cu]NHAG uptake and not the released ^{61}Cu cation from radiolabeled complex in case of biodegradation. The tumor uptake in various parts of the tumor were less than 0.1% in all time intervals. While kidney and liver demonstrate the excretion after 3 hours (Fig. 6). in order to be sure of tumor local accumulation due to inconsistency of blood circulation, the tumor tissue was cut into pieces and all were separately counted.

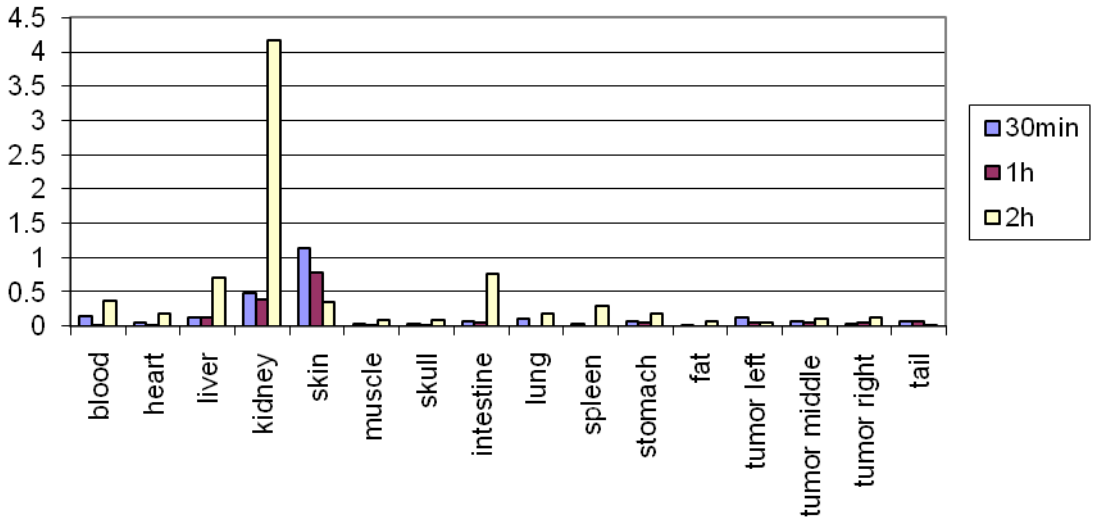

Fig. 6. Calculated ID/g% of [^{61}Cu]$CuCl_2$ 30–120 minutes post injection of 40 μCi IV of the tracer in fibrosarcoma-bearing rats

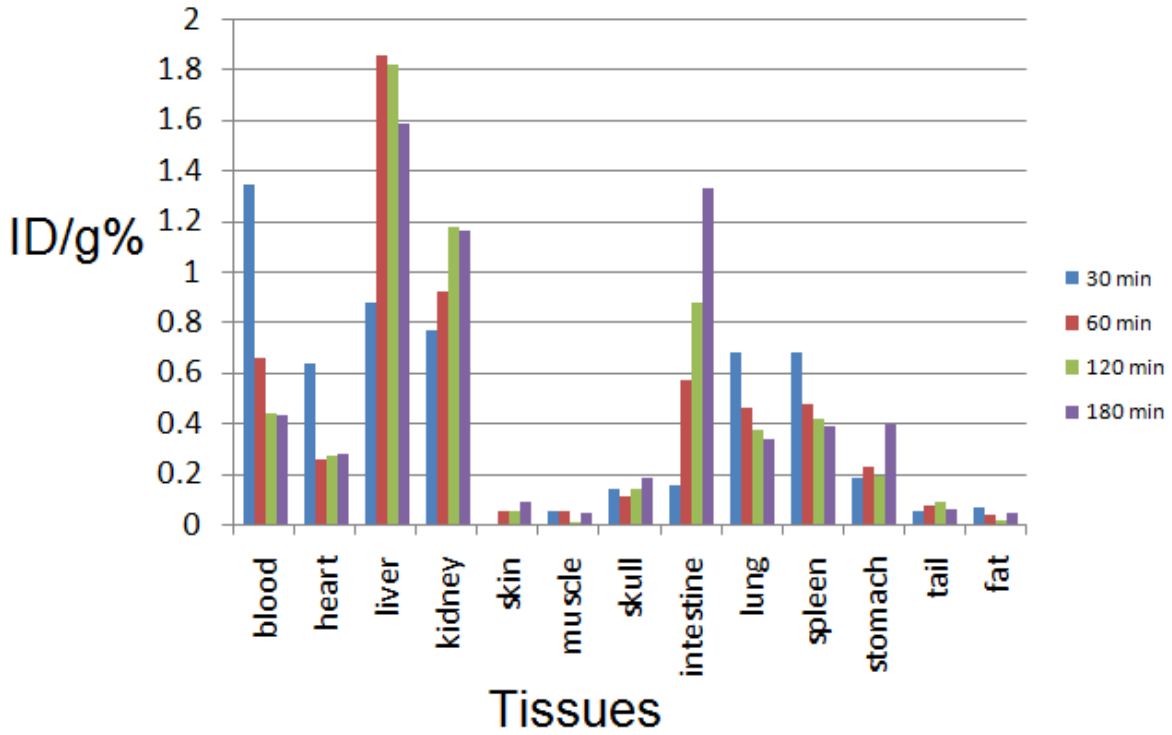

Fig. 7. Calculated ID/g% of [^{61}Cu]-NHAG 30–180 minutes post injection of 40 μCi IV of the tracer in normal mice

As shown in Fig 6, [^{61}Cu]NHAG, it is washed out from blood stream while easily penetrates into phosphor-lipid bi-layer of cells. The compound has significant liver uptake which is common with most of metal complexes.

Along with decrease in blood stream, the complex is washed out through kidneys due to its small size and solubility.

Following liver uptake, the hepatobilliary excretion takes place since the activity in intestine increases. The fat tissue uptake is low which is in accordance to our preliminary hypothesis regarding the hydrophilicity of the complex.

With an overall look into the reticuluendothelial system (RES), spleen, lung, liver and even kidneys, we can hypothesize that the complex has possibly targetted RES, with an unknown mechanism.

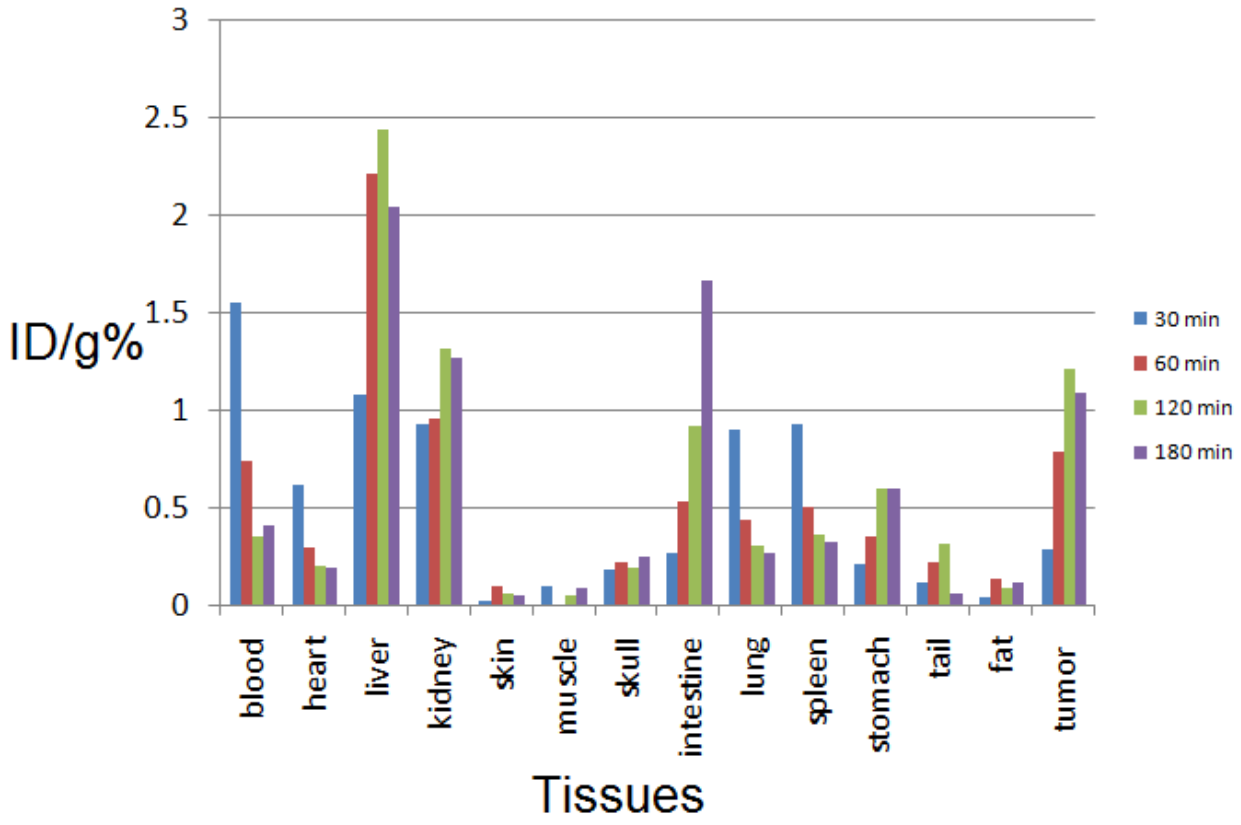

Fig. 8. Calculated ID/g% of [^{61}Cu]NHAG 30–180 minutes post injection of 40μCi IV of the tracer in fibrosarcoma-bearing mice

On the other hand, considering the lung as a metabolic organ, the presence of various reductases and especially matrix metalloproteinases in this tissue can be a cause of retention of the tracer since the Cu^{2+}/Cu^{+} reduction can produce $Cu(I)NHAG^{-}$ in charged form, without the ability of crossing the phospholipid bilayer of the cell membrane [16]. The penetration of the tracer is less than 0.2% in brain that can be explained by its hydrophilic properties.

The administration of the tracer to fibrosarcoma-bearing mice showed high uptake in liver, stomach, kidney, lung, spleen and intestine as discussed in normal animal distribution.

The tumor uptake is high at all time intervals and almost stayed constant above one percent after 120 min. It seems that the tracer is trapped in tumor cells possibly *via* copper-thiosemicarbazones retention mechanisms [17] (Fig. 8).

The significantly high tumor/muscle and tumor/blood ratios were 25.64 and 3.43, two hours post injection, proposes suitable targeting properties of the tracer. Liver, kidney and intestine are the major dose-bearing organs in animal models.

Experimental

Production of ^{61}Cu was performed at the Agricultural, Medical and Industrial Research School (AMIRS), 30 MeV cyclotron (Cyclone-30, IBA). Natural zinc chloride with a high purity of more than 98% was provided commercially (Merck chemical company, Darmstadt, Germany). Mass spectra were recorded by a Finnigan Mat TSQ-70 Spectrometer. ^{1}H NMR spectra were run at 500 MHz using a Bruker machine. Infrared spectra were taken on a Perkin-Elmer 781 spectrometer (KBr disks). All chemicals were purchased from Sigma-Aldrich Chemical Co. U.K. Radio-chromatography was performed by counting of polymer-backed silica gel paper thin layer sheets using a thin layer chromatography scanner, Bioscan AR2000, Paris, France. Analytical HPLC to determine the specific activity was performed by a Shimadzu LC-10AT, armed with two detector systems, flow scintillation analyzer (Packard-150 TR) and UV-visible (Shimadzu) using Whatman Partisphere C-18 column 250 x 4.6 mm, Whatman Co. NJ, USA. Eluent, H_2O:CH_3CN (1:1), FR=1 ml/min. the ID/g% of various organs determined by counting the dissected organs on an HPGe detector, Canberra, NJ, USA. All calculations and RTLC counting were based on 283 keV peak. 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 experiments in this work were conducted in conformity with the guidelines approved by the Iranian Ministry of Health, and were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edn.

Targetry and bombardment

The target was a layer of natural zinc, electroplated on copper plate which was coated with a 50-μm gold layer to prevent interference of the backing copper during radiochemical separation. Cross section calculations by ALICE nuclear code [18] showed that the best proton energy range for ^{nat}Zn(p,x)^{61}Cu reaction is 22–12 MeV. The target had to be thick enough to reduce the proton energy from 22 MeV to about 12 MeV. The targets were irradiated in a glancing angle of 6° to achieve higher production yield. SRIM code [19] was run to determine the best target thickness in the energy range.

Gold and Zinc electrodeposition

A gold containing bath was prepared according to Ref. [20] with slight modifications. As the 6° glancing angle reduces the required target thickness by 10 fold, electroplating a 75-μm thick target is good enough. The target was irradiated by 22 MeV (150 μA) protons with for 76°minutes.

Chemical Separation

Chemical separation was carried out in no-carrier-added form. The irradiated target was dissolved in 10 M HCl (15 mL, H_2O_2 added). The solution was passed through a cation exchange resin (AG 50W, H^+ form, mesh 200-400, 1.3 x 10cm) which had been preconditioned by passing 25 mL of 9 M HCl. The column was then washed by 25 mL of 9M HCl with a rate of 1 mL/min to remove copper and zinc ion contents. To the eluent 30 mL water (30 mL) was added to about 100 mL of a 6 M HCl solution. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 100-200 mesh, 25x1.7 cm) pretreated with 6 M HCl (100 mL). Finally, ^{61}Cu was eluted using 2 M HCl (50 mL) in form of $[^{61}Cu]CuCl_2$. The whole process took about 60 min [21].

Quality control of the product

Gamma spectroscopy of the final sample was carried out by an HPGe detector coupled with a Canberra™ multi-channel analyzer. The peaks were observed and the area under curve was counted for 1000 seconds. The formation of colored dithizone-zinc complex was measured using visible spectroscopic assay to determine zinc cation concentrations [22] using dithizone organic reagent (0.002% in CCl_4). The amount of gold cation in the final solution was checked using color formation of acidic rhodamine B reagent reacting with gold dilutions based on a previously reported colorimetric method [23].

Synthesis of Potassium N-*(2-hydroxyacetophenone) glycinate (K-NHAG) [= Potassium (*E*)-N-[1-(2-Hydroxyphenyl)ethylidene]glycinate]*

Potassium *N*-(2-hydroxyacetophenone) glycinate was prepared as reported previously with slight modifications [12]. A solution of KOH (100 mg, in 5 mL, 1.8 mmol) was stirred in an ice bath for 10 minutes followed by the addition of a cold aqueous solution of glycine (138 mg in 4 mL,1.8 mmol) and the mixture was stirred in an ice bath (10 minutes) followed keeping at room temperature (10 minutes). A mixture of 2-(hydroxy)acetophenone (250 mg, 1.8 mmol) in ethanol (5 mL) was added drop wise to above mixture using a syringe. The mixture turned yellow and stirring was continued for 15 h at room temperature. The progress of the reaction was checked by TLC using a mixture of chloroform:methanol (1:1) as mobile phase. The solvent was removed by rotary evaporator followed by washing the solid on a filter paper with diethylether and chloroform. The residue was recrystallised from methanol to yield K-NHAG. (83)%, m.p. 262–264ºC. ^1H NMR (d_6-DMSO) 7.52 (dd, 1H, H_6 benzene), 7.16 (m, 1H, H_2 benzene), 6.58 (d, 1H, H_3 benzene), 6.43 (t,1H, H_4 benzene), 3.94 (s, 2H, CH_2), 2.33 (s, 3H, N=C-CH_3). MS: m/z (%) 230 (M^+, 55), IR (KBr, cm^{-1}) ν_{max} 2400, 1710, 1596, 1470, 1275.

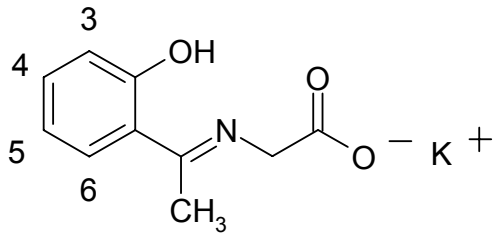

Fig. 8. Molecular structure of NHAG-K

Preparation of copper N-*(2-hydroxyacetophenone)glycinate (Cu-NHAG) [= Aqua[*N-*{1-[2-(hydroxy-κ*O*)phenyl]ethylidene}glycinato(2-)-κ^2*N,O]copper]*

The copper complex was prepared according to the reported method [12] in order to obtain a cold authentic sample for chromatographic studies. The ligand, K-NHAG (758 mg, 2.28 mmol) was dissolved in 5 ml anhydrous ethanol while stirred at room temperature till a transparent solution was formed. The ligand sample was added dropwise to a stirring mixture of $CuSO_4.5H_2O$ (680 mg, 2.7 mmol) dissolved in 4 ml of DDH_2O through a PE-syringe. The deep-green colored mixture was stirred for 2 hours at 50ºC and finally filtered through filter paper and the precipitate was heated at 80–90ºC followed by keeping under vacuum overnight. Yield 35–45%, m.p.241–244ºC.

*Preparation of [^{61}Cu]-*N*-(2-hydroxyacetophenone)glycinate ([^{61}Cu]NHAG) [= Aqua[*N-*{1-[2-(hydroxy-κ*O*)phenyl]ethylidene}glycinato(2-)-κ^2*N,O](*^{61}*Cu)copper*

NHAG was prepared starting from H_2NHAG and copper cation [24]. Briefly, [^{61}Cu]$CuCl_2$ (10 mCi) dissolved in acidic medium obtained above (about 2 ml) was transferred to a 5 ml-vial containing 0.1M (0.5 ml) sodium acetate buffer to prepare a [^{64}Cu]copper acetate solution. A mixture of NHAG (1 mg) in anhydrous ethanol (1 ml) was added to the copper solution and vortexed at room temperature for 30 min. The progress of the reaction was checked by RTLC. The mixture (about 1.5 ml) was then rapidly injected into a C_{18} Sep-Pak column pretreated with 5ml of ethanol and 2 ml of water. The column was washed with water (4 ml) and purged with a stream of dry N_2. The labeled compound was finally eluted using 1 ml-portions of absolute ethanol and the fractions were counted in a dose calibrator. The vial containing the maximum radioactivity was concentrated using a flow of N_2 at 50ºC to reach a volume of 100–150 µL. The alcoholic residue was diluted to a 5% solution by addition of normal saline. The active solution was checked for radiochemical purity by polymer-backed silica gel layer chromatography using dry ethyl acetate as mobile phase as well as HPLC. The final solution was then passed through a 0.22 µm filter and pH was adjusted to 5.5–7 by the addition of 3 M sodium acetate buffer.

Quality control of [^{61}Cu]-NHAG

Radio thin layer chromatography: A 5 µl sample of the final fraction was spotted on a chromatography Si sheet paper, and developed in a mixture of 10% ammonium acetate:methanol (1:1) and/or ethyl acetate as the mobile phase. Alternatively, 10 mM DTPA solution can be used as another mobile phase to discriminate free copper from radiolabeled compound.

High performance liquid chromatography: HPLC was performed on the final preparation using a mixture of water:acetonitrile 1:1(v/v) as the eluent (flow rate: 1 ml/min, pressure: 120–140 kgF/cm^2) for 10 min, in order to elute low molecular weight components. Radiolabeled compound was eluted using reverse stationary phase.

Stability of [^{61}Cu]-NHAG complex in the final product

Stability tests were based on previous studies performed for radiolabeled copper complexes [25]. A sample of [^{61}Cu]NHAG (1 mCi) was kept at room temperature for 3 hours while checked by RTLC every half an hour. A micropipet sample (5 µl) was taken from the shaking mixture and the ratio of free radiocopper to [^{61}Cu]NHAG was checked by radio thin layer chromatography (eluent: dry ethyl acetate).

Serum Stability Studies

To 36.1 MBq (976 μCi) of [^{61}Cu]NHAG was added 500μl of freshly prepared human serum and the resulting mixture was incubated at 37°C for 3 hours, Aliquots (5-μl) were analyzed by radio-TLC after 0, 0.25, 0.5, 1, 2 and 3 hours of incubation to determine the stability of the complex.

Biodistribution of [^{61}Cu]cation and [^{61}Cu]NHAG in normal and fibrosarcoma-bearing mice

[^{61}Cu]cation in acetate buffer and [^{61}Cu]NHAG were administered to separate normal mice groups. A volume (50 μl) of [^{61}Cu]NHAG or [^{61}Cu]cation solutions containing 40±2 μCi radioactivity were injected intravenously to mice via their tail veins. The animals were sacrificed at exact time intervals (1 and 2h for [^{61}Cu]cation and 30–180 minutes for [^{61}Cu]NHAG), and the ID/gr % of different organs was calculated as percentage of injected dose (based on area under the curve of 283 keV peak) per gram using an HPGe detector.

Conclusion

The method used in this research for the production and chemical separation of ^{61}Cu was quite simple and cost effective. Total labeling and formulation of [^{61}Cu]NHAG took about 40 minutes, with a radiochemical purity of higher than 98%. No labeled by-products were observed upon RTLC/HPLC analysis of the final preparations after SPE purification. The radio-labeled complex was stable in aqueous solutions for at least 3 hours and no significant amount of other radioactive species were detected by RTLC, 3 hours after labeling. Trace amounts of [^{61}Cu]copper acetate (≈2%) were detected by RTLC/HPLC which showed that radiochemical purity of the [^{61}Cu]NHAG was higher than 97%. The biodistribution of tracer was checked in normal and tumor-bearing mice up to 3 hours and a significant accumulation took place in lungs, liver and kidneys, while significant fibrosarcoma uptake was observed in all animals. Tumor/muscle and tumor/blood ratios were 25.64 and 3.43 respectively in tumor-bearing mice 120 minutes after administration of the tracer.

[^{61}Cu]NHAG is an interesting PET tracer for tumor imaging with an intermediate half life, and our experiments on this compound have shown satisfactory quality, suitable for future PET studies in experimental models.

Acknowledgements

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Authors' Statements

Competing Interests

The authors declare no conflict of interest.

Animal Rights

The institutional and (inter)national guide for the care and use of laboratory animals was followed. See the experimental part for details.

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