Preparation, quality control and biodistribution studies of [61Cu]-oxinate for PET tumor imaging

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Abstract. Targeting apoptosis is an interesting issue in molecular imaging and various modalities have been presented. However, recent experiences in nuclear pharmacy demonstrated the application of small tracer molecules is more desired. This work was conducted for production of a radiolabeled copper complex, i.e. ^{61}Cu-oxinate as a potential PET tracer for apoptosis imaging in oncology. Cu-61 was prepared by natural zinc target irradiation with 22 MeV protons (150 µA) via the ^{nat}Zn(p,xn)^{61}Cu nuclear reaction with a yield of 3.33 mCi/µAh. In order to obtain the best labeling method, optimization reactions were performed for pH, temperature and concentration followed by solid phase extraction. Biodistribution of the tracer was studied in wild-type and fibrosarcoma bearing mice. Under the optimized conditions, radio-thin-layer chromatography (RTLC) and HPLC showed radiochemical purities of 99.99% and 97% respectively (with a minimum specific activity of 16 Ci/mM). Biodistribution of the tracer in fibrosarcoma bearing mice demonstrated a significant tumor uptake after 3 h. Tumor:blood and tumor:muscle ratios were 2.0 and 6.0 after 3 h, respectively.

Key words: copper-61 • 8-hydroxy quinoline • positron emission tomography • fibrosarcoma

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Introduction

According to our previous works on the radiosynthesis and evaluation of non-fluorine PET radiopharmaceuticals [5, 13], we have been interested in the production and application of Cu-61 tumor seeking radiopharmaceuticals [6]. ^{61}Cu is a positron emitter ($t_{1/2}$ = 3.33 h, β$^+$: 62%, EC: 38%), with excellent potentials for application in PET method and molecular imaging. Few production methods of copper-61 have been reported for radiolabeling of biomolecules and other applications [9, 10] leading to the development of small molecules [3, 8] for various diagnostic purposes.

Copper oxinate complex is considered an antineoplastic compound with various mechanisms. Some reports demonstrate anti-proteasome and apoptosis inducing properties for this molecule [1]. These interesting biological activities are not reported for free copper cation as well as oxine ligand by any research group independently.

Studies concerning the anti-proteasome activity demonstrated that the Cu-oxinate complex forms an unknown complex with proteasome and, finally, quinoline moiety of the complex inactivates the proteasome by an oxido-redox mechanism. Such a mechanism has
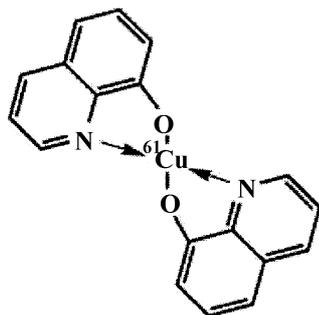
Fig. 1. Chemical formula of [^{61}Cu]-oxinate.

been reported to induce apoptosis in leukemic tumor models *in vivo* and *in vitro* [2].

The use of direct administration of oxine complexes in the nuclear medicine appeared to be limited to the direct intratumoral injection of tracers such as ^{111}In-oxine [15], however, according to our knowledge, there are no reports on systemic administration of any radiolabeled oxinate using SPECT and/or PET radionuclides.

Based on the above Cu-oxinate complex properties and our recently reported routine ^{61}Cu production method [14], we were interested in developing a potential PET imaging agent, i.e., [^{61}Cu]-oxinate for apoptosis phenomenon (Fig. 1).

We hereby report the preparation, purification, quality control, stability tests and biodistribution studies of [^{61}Cu]-oxinate in wild-type and tumor bearing mice.

Experimental

Materials

Production of ^{61}Cu was performed at the Agricultural, Medical and Industrial Research School (AMIRS), 30 MeV cyclotron (Cyclone-30, IBA). Natural zinc chloride of high purity (> 98%) was provided commercially (Merck Chemical Company, Darmstadt, Germany). Other chemicals were purchased from Sigma--Aldrich Chemical Co. UK. Radio-chromatography was performed by counting 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, a flow scintillation analyzer (Packard-150 TR) and UV-visible (Shimadzu) using a Whatman Partisphere C-18 column 250 × 4.6 mm, Whatman Co. NJ, USA. Eluent, $H_2O:CH_3CN$ (1:1), flow rate = 1 ml/min. Biodistribution data were acquired by counting normal saline washed tissues after counting on a Canberra™ high purity germanium (HPGe) detector (model GC1020-7500SL). Radionuclidic purity was checked with the same detector. For activity measurement of the samples, a CRC Capintech Radiometer (NJ, USA) was used. All calculations and tissue counting were based on the 283 keV peak. Animal studies were performed in accordance with the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, 2nd ed. Purification of the final compound was performed with C$_{18}$ Sep-Pak short columns, which were purchased from waters.

Procedures

Targetry and bombardment

The target was a layer of natural zinc, electroplated on a copper plate which was coated with a 50-μm gold layer to prevent interference in the backing copper during radiochemical separation. Cross-section calculations by ALICE nuclear code showed that the best proton energy range for the $^{nat}Zn(p,x)^{61}$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 was run to determine the best target thickness in the energy range.

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 × 10 cm) which was preconditioned by passing 25 mL of 9 M HCl. The column was then washed with 25 mL of 9 M 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 up 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, 25 × 1.7 cm) pretreated with 6 M HCl (100 mL). Finally, ^{61}Cu was eluted using 2 M HCl (50 mL) in the form of [^{61}Cu]CuCl$_2$. The whole process took about 60 min.

Preparation of copper-61 bis (8-hydroxyquinoline) ([^{61}Cu]-oxinate)

[^{61}Cu]CuCl$_2$ obtained from natural zinc irradiation on a gold-plated support in a two-step cation exchange chromatography was prepared according to the reported method [14]. [^{61}Cu]CuCl$_2$ (5 mCi) dissolved in acidic medium obtained above (0.5–2 ml) was transferred to a 2 ml-vial and the mixture was evaporated by slight warming under a nitrogen flow. A 50 μl-volume of oxine solution (1.5 mg/ml) in anhydrous ethanol was added to the above vial and vortexed immediately and kept at 25°C for 60 min. Due to half-life consideration and reaching a maximum radiochemical purity of 75–80% in labeling procedure, solid phase purification was employed.

The labeling solution was passed through a C$_{18}$ Sep--Pak column pre-conditioned with ethanol (2 ml) and DDH$_2$O (double-distilled water) (2 ml) subsequently, and purged with a flow of N$_2$ gas. The loaded column was washed with DDH$_2$O (2 ml) followed by eluting the column using ethanol portions (5 × 1 ml) and the fractions were counted in a dose calibrator. The fraction containing the maximum activity (in our case elution no. 1) was concentrated to the volume of 0.1 ml under a flow of N$_2$ at 40–45°C. The vial mixture was diluted by the addition of 0.9% saline (2 ml) to bring the solution to ≈ 5% ethanol. pH was adjusted to 5.5–7 and the final solution was then passed through a 0.22 μm filter.

Quality control of ^{61}Cu-oxinate

Radio-thin-layer chromatography: A 5 µl sample of the final fraction with the highest activity was spotted on aluminium backed silica gel layers, and developed in a mixture of 10% ammonium acetate:methanol (1:1) as the mobile phase. The formation of [^{61}Cu]-oxinate with less polarity at R_f 0.8 was compared with the remaining ^{61}Cu cation at the base (R_f 0.0) in each measurement.

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 40 min.

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

A sample of [^{61}Cu]-oxinate (0.5 mCi) was kept at room temperature for 24 h while checked by RTLC. A micropipet sample (5 µL) was taken from the shaken mixture and the ratio of free radio-copper to [^{61}Cu]-oxinate was checked by radio-thin-layer chromatography (eluent: 10% NH$_4$OAc and methanol 1:1).

Serum stability studies

To 36.1 MBq (976 µCi) of [^{61}Cu]-oxinate 500 µl of freshly prepared human serum was added and the resulting mixture was incubated at 37°C for 5 h. Aliquots (5-µl) were analyzed by radio-TLC after 0, 0.25, 0.5, 1, 2 and 3 h of incubation to determine the stability of the complex (RTLC: eluent: 10% NH$_4$OAc and methanol 1:1).

Determination of partition coefficient

Partition coefficient (log P) of [^{61}Cu]-oxinate was calculated following the determination of P (P – the ratio of specific activities of the organic and aqueous phases).

A mixture of 1 ml of 1-octanol and 1 ml of isotonic acetate-buffered saline (pH = 7) containing approximately 3.7 MBq of the radiolabeled copper complex at 37°C was vortexed for 1 min and left for 5 min. Following centrifugation at > 1200 g for 5 min, the octanol and aqueous phases were sampled and counted in an automatic well-type counter.

A 500 µl sample of the octanol phase from this experiment was shaken again two to three times with fresh buffer samples to ensure that traces of hydrophilic ^{61}Cu impurities did not alter the calculated P values. The reported log P values are the average of the second and third extractions from three to four independent measurements.

Induction of fibrosarcoma tumors in mice

Tumor induction performed with the use of polyaromatic hydrocarbon injection in rodents as reported previously [3]. For tumor model preparation, 10 µl of 3-methyl cholanthrene solution in extra-virgin olive oil (4 mg/ml) was injected subcutaneously (SC) to the dorsal area of the mice. After 14–16 weeks, the tumor weighed 0.2–0.4 g and was not grossly necrotic. Tumor tissues of some random animals were sent for pathological tests and were diagnosed as fibrosarcoma.

Biodistribution of [^{61}Cu]CuCl$_2$ and [^{61}Cu]-oxinate in wild-type and fibrosarcoma bearing animals

[^{61}Cu]CuCl$_2$ and [^{61}Cu]-oxinate were administered to separate wild-type and tumor-bearing mice groups. A volume (50 µl) of [^{61}Cu]-oxinate or [^{61}Cu]CuCl$_2$ solutions containing radioactivity (40±2 µCi for mice) were injected intravenously via their tail veins. The animals were sacrificed at exact time intervals (1, 2, 3 and 4 h for [^{61}Cu]CuCl$_2$ and 1, 2 and 3 h for [^{61}Cu]-oxinate), and the injected dose (ID)/g% 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.

Results

Because of the engagement of oxine molecule N and O atoms in the coordination of copper cation, it was assumed that the polarity of radiolabeled complex was greatly different from copper cations and the final complex is possibly a lipophilic species. In Si-TLC studies, the free copper(II) fraction corresponded to smaller R_f (R_f = 0.0), while the oxinate complex migrates to the higher R_f (R_f = 0.8) (Fig. 2). The radiolabeling in the best conditions afforded the radiolabeled compound with a radiochemical purity of 76–80%. Thus, due to the polarity difference, solid phase extraction (SPE) was employed to remove remaining portions of ^{61}Cu cations using C$_{18}$ Sep-Pak in order to increase the radiochemical purity as already reported for other copper complexes [7]. Figure 2 demonstrates the radiochromatograms of the radiolabeling mixture before and after SPE purification.

It can be observed that the radiochemical purity reaches the highest possible lovel (99.99%) as shown by RTLC, however HPLC was also employed for a better judgment.

In the HPLC studies reverse phase column was used in water/acetonitrile mixture as an eluent. The fast eluting component (2.26 min) were shown to be free ^{61}Cu^{2+}, the cationic fraction was eluted faster than any complex species. The radiolabeled compound was finally washed out in 19.68 min however, the radiopeak was not sharp in our methods (Fig. 3).

The best radiolabeling conditions were shown to be pH 5, 25°C for an hour using 50–75 µl of a 1.5 mg/ml ethanolic solution of oxine for an activity range of 2.5–8.6 mCi.

The final radiolabeled complex diluted with normal saline was then passed through a 0.22 µm (Cativex) filter for sterilization to avoid possible thermal instability of the complex at autoclave temperatures. The chemical stability of [^{61}Cu]-oxinate was high enough to perform further studies. RTLC of the final product showed no change in stability and the pattern for [^{61}Cu]-oxinate in aqueous solutions at pH = 5.5–7 was not changed during 24 h at room temperature.

[^{61}Cu]-oxinate was incubated in freshly prepared human serum for 5 h at 37°C. The aliquots of the resulting mixtures were analyzed to determine the kinetic stability of the radiolabeled conjugate. No decomposition of ^{61}Cu from the complex was observed during the course of the studies, and the radiochemical purity of the complex remained > 99% for 5 h under physiological conditions.
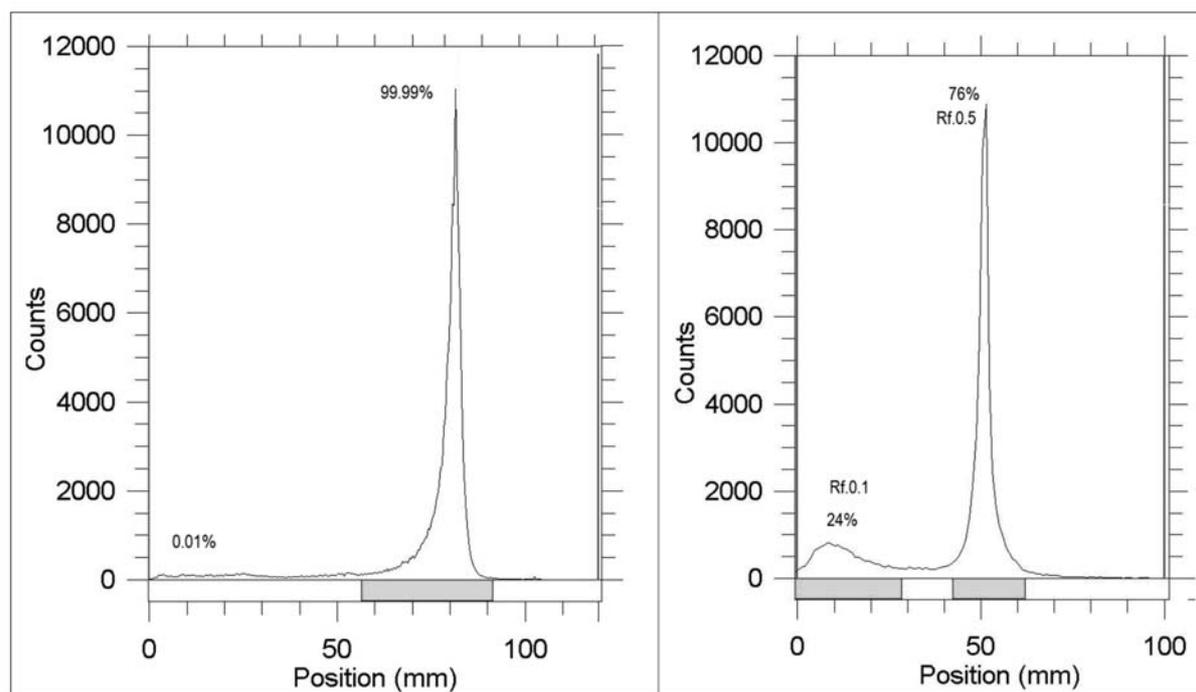
Fig. 2. RTLC of final [^{61}Cu]-oxinate solution before (right) and after (left) SPE purification (*n* = 5).

As expected, the lipophilicity of the compound was rather medial. The measured octanol/water partition coefficient, *P*, for the ^{61}Cu-complex was found to be 1.65 at pH 5.5.

In order to investigate biodistribution of [^{61}Cu]-oxinate in the animal models we had to obtain the biodistribution data for free copper cation in our hands, thus after injection of 40 µCi of the [^{61}Cu]CuCl$_2$ pre-formulated by the normal saline (pH 6.5–7) through the tail vein of adult mice the biodistribution of the cation was checked in various vital organs. The major content of copper is washed out by the kidneys and consequently urinary tract due to high water solubility of the cation. The uptakes of rest of the tissues are not significant. Copper is also partly accumulated in the liver as a reservoir for many metals transferred by serum ceruloplasmin (data not shown).

The radioactivity decreased in the blood through the course of study while the uptake took place in the liver as a metabolic organ. In response to the decrease of blood activity, part of the activity is excreted via the kidneys due to the small size of the molecule. The stomach uptake is the highest among the determined tissues and this must be an outcome of the gastrointestinal (GI) readsorption after liver transformation (Fig. 4).

The uptake of free copper cation must be checked in fibrosarcoma-bearing animals in order to validate the real [^{61}Cu]-oxinate uptake and not the released ^{61}Cu cation from oxine complex in case of biodegradation. The tumor uptake in various parts of the tumor were less than 0.1% in all time intervals, while the kidney and liver demonstrate the excretion after 3 h (data not shown).

Figure 5 demonstrates the tracer uptake in tumor-bearing animals. The amount of the tracer in blood diminishes in 3 h possibly by tissue uptake and/or physical decay. The major excretory organ seems to be the liver in the first 3 h however, due to the small size and readsorption from the GI it can be excreted by the urinary tract as well. These observations are in agreement with the formerly reported copper-oxinate biodistribution in
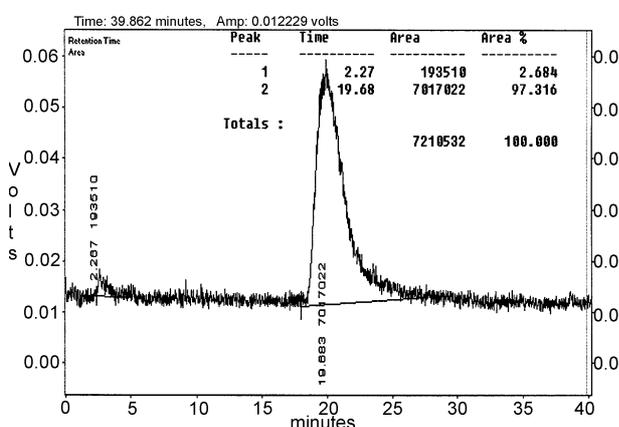
Fig. 3. HPLC of final [^{61}Cu](II)oxinate solution after Sep-Pak purification in water/acetonitrile solution as mobile phase using reverse stationary phase.
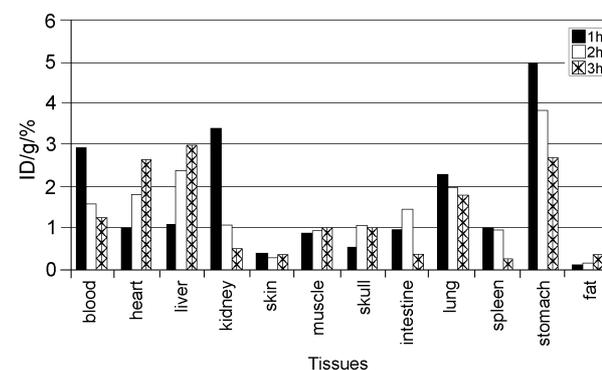
Fig. 4. Calculated ID/g% of [^{61}Cu]-oxinate 60–180 min post injection of 40 µCi IV of the tracer in wild-type mice.
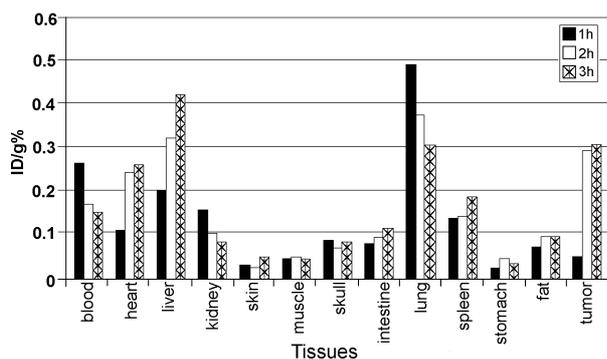
Fig. 5. Calculated ID/g% of [61Cu]-oxinate 60–180 min post injection of 40 µCi IV of the tracer in fibrosarcoma-bearing mice.

mammals [11]. The lung has also an interesting uptake, which can be explained by the lipophilic nature of the complex, although the uptake is not constant and there are flactuations due to the enzymatic and/or chemical degradation of the complex and the release of copper into systemic circulation and/or physical decay. There is also an intersting myocardial uptake which cannot be explained.

The tracer is significantly absorbed in tumors within 2–3 h which has been already reported in some mice tumors [12], so in our case this accumulation can or cannot be related to apoptosis.

An lethal dose, 50% (LD50%) of 32.12 mg/kg, was calculated for Cu-oxine complex in mice according to reported toxicological data [4]. Regarding the use of a 1.5 mg/ml solution of oxine (50 µL), the amount of the oxine used in each animal was 1.36 µg (injection of 100 µl of a labeled solution diluted with 2000 µl of normal saline solution). Thus, the injected amount of the oxine in our tracer was calculated to be 23,600 times less than LD50%.

Discussion

The method used in this research for the production and chemical separation of 61Cu was quite simple and cost effective. Total labeling and formulation of [61Cu]-oxinate took about 90 min, with a yield higher than 97% and/or 99% (determined by HPLC and RTLC, respectively). The radiolabeled complex was stable in aqueous solutions for at least 24 h and no significant amount of other radioactive species were detected by RTLC. The biodistribution of tracer was checked in wild-type and tumor-bearing animals up to 4 h and a significant accumulation took place in the liver and kidneys, while significant fibrosarcoma uptake was observed in all animals after 3 h. The tumor:blood and tumor:muscle ratios were 2 and 6, respectively. [61Cu]-oxinate can be a PET tracer with an intermediate half-life, and our experiments on this tracer have shown a satisfactory quality, suitable for future PET studies on apoptosis. However, further experiments in apoptotic models are

required for the efficacy of this tracer in the detection of apoptosis.

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