Original Article

Production, Quality Control and Imaging of ^{64}Cu-ATSM in Healthy Rabbits for Clinical Applications

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ABSTRACT

Introduction: [^{64}Cu]diacetyl-*bis*(N^4-methylthiosemicarbazone) ([^{64}Cu]ATSM) is a well-established hypoxia imaging tracer with reproducible production and significant specifity. In this work the high yield production and quality control as well as imaging studies in healthy rabbits is reported.
Methods: Copper-64 produced *via* the ^{68}Zn(p,αn)^{64}Cu nuclear reaction (30 MeV protons at 180 μA) was used for the preparation of [^{64}Cu]diacetyl-bis(N4-methylthiosemicarbazone)([^{64}Cu]ATSM). Followed by quality control and administration to healthy rats as well as healthy rabbits for biodistribution and imaging studies respectively.
Results: $^{64}Cu^{2+}$ (500 mCi, separation yield> 95%, radionuclide purity>96%) was used for [^{64}Cu]ATSM production (radiochemical purity>99%, specific activity of 300 Ci/mmol) followed by administration to healthy rabbits and coincidence imaging demonstrating uptake in liver, kidney and bowel as shown by other reports in various rodents and human.
Conclusion: [^{64}Cu]ATSM radiopharmaceutical is produced and now available in large quantities for research and/or clinical trials in the country.

Keywords: Radiolabeling, Quality control, [^{64}Cu]ATSM, Rabbits, Biodistribution, Coincidence imaging

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INTRODUCTION

Copper-64 (half-life=12.7 h; β^+ 655 keV [19%]; β^- 573 keV [40%]; E.C. [41%]) is an attractive radionuclide for PET imaging and targeted therapy of cancer (1). This radionuclide is mainly produced *via* ^{64}Ni(p,n)^{64}Cu reaction at medical cyclotrons (2, 3), although it can also be prepared in lower yields by ^{68}Zn(p,αn)^{64}Cu reaction (4, 5).

Hypoxia is an important determinant for biological behavior of malignant solid tumors. In vitro and in vivo studies have shown that tumor hypoxia is associated with an increased likelihood of local recurrence and distant metastasis, as well as resistance to radiation therapy and certain types of chemotherapy (6). Tumor uptake of copper(II)-diacetyl-bis(N4-methylthiosemicarbazone) (copper-ATSM) (7, 8), a hypoxia-targeting radiopharmaceutical, as assessed by PET, has been confirmed in several human cancers. Copper-ATSM PET has been shown to distinguish patients likely and unlikely to respond to conventional therapies for cancers of the lung (9), uterine cervix (10) and rectum (11). Also various data insist on the therapeutic potentials of [^{64}Cu]ATSM (12) and the influence of MDR1 protein expression on the tumor uptake (13).

Due to interesting imaging properties of ^{64}Cu-ATSM, routine copper-64 production *via* ^{68}Zn(p,αn)^{64}Cu reaction at 30MeV cyclotron (14) and successful reports of production and biological evaluation of [^{61}Cu]ATSM in the country (8, 15) and various ongoing human trials using this tracer at phase II, III and IV around the world (16, 17), we developed interest in large scale production and imaging properties of [^{64}Cu]-ATSM as a PET hypoxia tracer, having good potential for future research and possible clinical trials in our country.

METHODS

Production of ^{64}Cu was performed at the Agricultural, Medical and Industrial Research School 30 MeV cyclotron (Cyclone-30, IBA). The zinc-68 oxide used had a high purity of more than 95%. Other chemicals were purchased from Aldrich Chemical Company (Germany). All exchange resins were provided commercially (Bio-Rad Laboratories, Canada). ^{1}H-NMR spectrum was obtained on a Bruker FT-80 (80 MHz) instrument with tetramethylsilane as the internal standard. Infrared spectrum was taken on a Perkin–Elmer 781 (KBr disks). Mass spectrum was recorded using a Finnigan Mat TSQ-70 spectrometer. Radio thin layer chromatography (RTLC) was performed on polymer-backed silica gel (F 1500/LS 254, 20×20 cm, TLC Ready Foil, Schleicher & Schuell®, Germany). High purity ethyl acetate and normal saline were used for labeling. Radio-chromatography was performed by counting 5 mm-slices of polymer-backed silica gel paper using a Canberra™ high purity germanium (HPGe) detector (model GC1020-7500SL). Radionuclide purity was checked by the same detector. All calculations and RTLC counting were based on 511 keV peak.

Production and quality control of Copper-64 chloride

The ^{68}Zn target was electroplated on a gold-coated copper backing plate and irradiated at an angle of 6 degrees toward the proton beam, in order to achieve a higher production yield. The optimum energy for the production of ^{64}Cu *via* ^{68}Zn(p,αn)^{64}Cu reaction is 35-20 MeV (4), but the highest available proton energy is 30 MeV at the AMIRS cyclotron. Therefore, the target had to be thick enough to reduce the energy of the incident protons from 30 MeV to about 20 MeV (18) which showed 100 μm of the target material was electroplated on the

copper backing. For this purpose, ^{68}ZnO was dissolved in 0.05 N HCl to prepare a zinc cation-containing solution at the optimized conditions electrodeposition was performed and resulted in a 100 μ zinc layer on the gold-coated copper backing after 3.5 hours. In order to prepare the gold supporting layer, a gold containing bath was prepared according to a previously reported method with slight modifications (19). The irradiated target was dissolved by 10 N HCl (15 ml, 20 μl of H_2O_2 added) and the solution was passed through a cation exchange resin (AG 50 W×8, H+ form; mesh 200-400) (h:10 cm, Ø:1.3 cm) that was preconditioned by passing 25 ml of 9 N HCl. The column was then washed by 25 ml of 9 N HCl at a rate of 1 ml/min to elute copper and zinc ion contents. To the latter elute was added 30 ml of DDH_2O. The mixture was passed through another cation exchange resin (Dowex 1X8, Cl^- form; mesh: 100-200) (h:25 cm; Ø:1.7 cm), preconditioned with 100 ml of 6 N HCl. In order to elute copper-64 ions, the column was washed by 50 ml of 2 N HCl. The column was finally eluted by 0.05 N HCl (150 ml), in order to recover of precious zinc-68 contents. The whole chemical separation process took about 105 min. Gamma spectroscopy of the final sample was carried out using an HPGe detector coupled to a Canberra™ multi-channel analyzer for 1000 seconds. The presence of zinc and copper cations were checked by polarographic methods. The resulting high-purity [^{64}Cu]$CuCl_2$ solution was used directly in the labeling step. For chemical purity, the formation of colored dithizone-zinc complex was measured using visible spectroscopic assay to determine zinc cation concentration (20). The amount of gold cation in the final solution was checked using color formation with acidic rhodamine B reagent reacting with gold dilutions based on a previously reported colorimetric method.

Preparation and quality control of [^{64}Cu]diacetyl-bis(N^4-methylthiosemicarbazone)

H_2ATSM was prepared according the method for the production of thiosemicarbazones starting N^4-methylthiosemicarbazide and diacetyl, which was consistent with results of the previous study reported by Gingras *et al.* in 1962 (21).

Preparation of [^{64}Cu] diacetyl-bis(N^4-methylthiosemicarbazone) was accomplished according to a formerly reported method with slight modifications (22) (Figure 1).

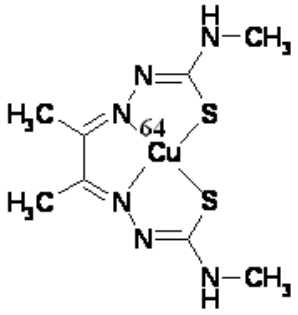

Figure 1. Structure formula for [^{64}Cu]ATSM

[^{64}Cu]$CuCl_2$ (5-50 mCi) dissolved in acidic medium obtained above (about 2 ml) was transferred to a 5 ml-vial containing 3M (4 ml) sodium acetate to prepare a [^{64}Cu]copper acetate solution. A mixture of ATSM (4 μg) in anhydrous DMSO (0.1 ml) was added to the copper acetate solution and vortexed at 25°C for 5 min.

The mixture (about 5 ml) was then cooled in an ice bath, and 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 0.2 ml-portions of absolute ethanol and the fractions were counted in HPGe detector (Figure 2).

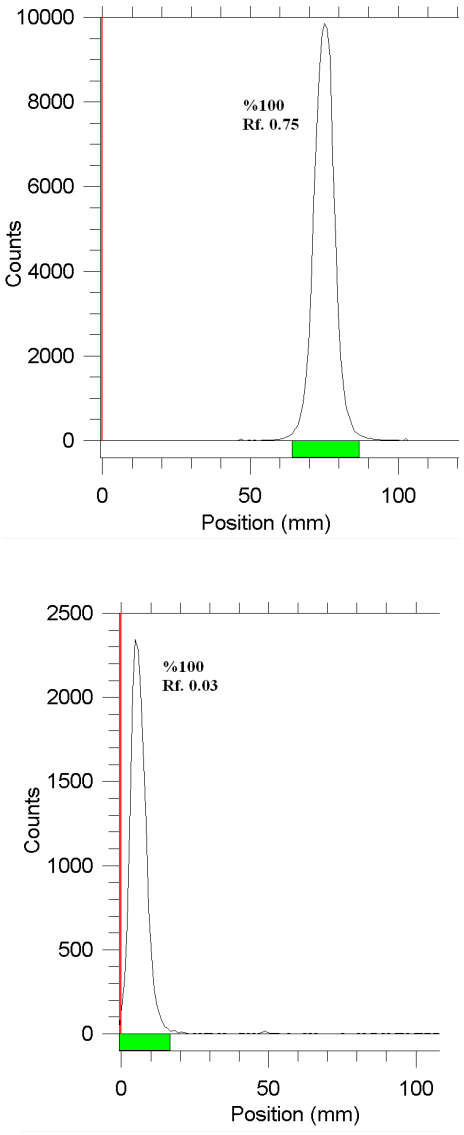

Figure 2. RTLC chromatograms for [^{64}Cu]Cu^{2+} (in acetate and chloride form, below) and [^{64}Cu]ATSM (above) on silica gel papers using ethyl acetate as eluent.

The vial containing the maximum radioactivity 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 and also HPLC using reverse phase. The final solution was then passed through a 0.22 μm filter and pH was adjusted to 5-7 by the addition of 3 M sodium acetate buffer. Stability tests were based on previous studies performed for radiolabeled copper complexes (23) in final formulations and presence of human serum while checked by radio thin layer chromatography (eluent: dry ethyl acetate) every half an hour.

The partition coefficient of the [^{64}Cu]ATSM was measured following 1 min of vigorous vortex mixing of 1 ml of 1-octanol and 1 ml of isotonic acetate-buffered saline (pH=7) with approximately 3.7 MBq (100 μCi) of the radiolabeled copper complex at 37°C.

Biodistribution of [^{64}Cu]ATSM in normal rats

Mature male NMRI rats (Razi Institute, Iran) weighing 150–250g (n=10) were used in experiments. The animals were kept in groups of ten, in cages under constant temperature (24ºC) and 12 h light/dark schedule. They had free access to standard rat diet and water except during the experiment. On the day of the experiment, animals were randomly transferred to individual cages and allowed to acclimatize for 30 min before injection of tracer and free copper cation. [^{64}Cu]ATSM was administered to separate normal rat groups. A volume (50 μL) of [^{64}Cu]ATSM solution containing 37MBq radioactivity were injected intravenously to rats via their tail veins.

The animals were sacrificed at exact time intervals (30-210 minutes), and the ID/gr % of different organs was calculated as percentage of injected dose (based on area under the curve of 511 keV peak) per gram using an HPGe detector.

Co-incidence imaging studies

The final [^{64}Cu]ATSM solution of 18.5 MBq activity (0.1 mL) was injected into the ear vein of a healthy rabbit. The animals were relaxed by halothane and fixed in a suitable probe. The total amount of radioactive material injected into each rabbit was measured by counting the 1-mL syringe before and after injection in an activity meter with fixed geometry. Images were taken 3 hours after administration of the radiopharmaceutical in coincidence mode of a Dual-Head SPECT system (SMV, France, Sopha DST-XL). The useful field of view (UFOV) was 540 mm × 400 mm. The spatial resolution in the coincidence mode was 10 mm full width at half maximum (FWHM) at the central field of view (CFOV), and sensitivity was 320 Kcps/MBq/mL. Sixty four projections were acquired for 30 seconds per view with a 64 × 64 matrix.

RESULTS AND DISCUSSION

The availability of Zn-68 in many countries due to high natural abundance and lower price proposes a very interesting production route for precious Cu-64 radioisotope compared to Ni-64 as the target material. ^{64}Cu is the most frequently used copper radioisotopes serving as a dual source of therapeutic beta particles as well as positrons for PET imaging.

Using (p,αn) nuclear reaction significant amounts of ^{64}Cu can be prepared and using a two step ion chromatography technique both Ga-67 and Cu-64 can be obtained from the same target simultaneously. This can be interesting for centers producing Ga-67 routinely to launch the copper-64 radionuclide as a new product. For carrier-free production, the use of a gold-layered copper backing was employed; interestingly no detectable copper and gold cations were detected in range of our detection. Another option for gold-layered backings is application of a natural Ni layer serving as a protecting layer with undetectable acid dissolution properties.

Various copper-64 compounds such as ^{64}Cu-ATSM, ^{64}Cu-PTSM, ^{64}Cu-ETS as well as many other tracers can be prepared using this radionuclide. The hypoxic targeting properties of ^{64}Cu-ATSM can be evaluated in many tumor models and diseases as already performed in other works.

The starting ligand can be prepared easily in a regular chemistry lab, and applying appropriate crystallization techniques in ethanol would yield an almost pure sample (more than 99%), appropriate for radiolabeling.

The quality control of compound can also be performed using ITLC and/or RTLC methods in a radiopharmacy/radiochemistry lab even in clinics, since HPLC data were consistent with ITLC/RTLC data with a high precision.

The activity obtained by our method was 500 mCi ^{64}Cu at the end of bombardment (E.O.B.) and the production yield was 1.01 mCi/μAh. Because of the engagement of several polar functional groups in its structure, labeling of ATSM with copper cation greatly affects its chromatographic properties and the final complex is highly lipophillic. Thus the labeled and unlabeled ATSM can easily be separated using solid phase C_{18} Sep-Pak column.

In TLC studies, the more polar un-complexed ATSM and free copper fractions, correlate to smaller R_fs (R_f =0.01-0.03), while the ATSM complex migrates at the higher R_f (R_f=0.75). In all radiolabeling runs (n=9) (Figure 3), the integral ratio of the two peaks were constant, showing the high radiochemical purity and consistency of the labeling method.

In HPLC studies the fast eluting compound was shown to be hydrophilic [^{64}Cu]Cu^{2+} cation (2.01 min), while the lipophillic [^{64}Cu]ATSM complex was eluted couple of minutes after (6.22).

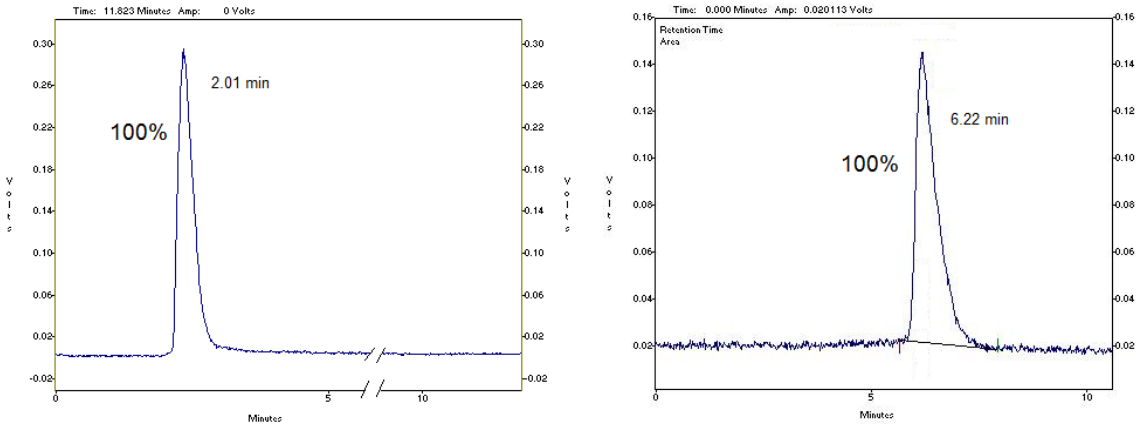

Figure 3. HPLC diagram for [^{64}Cu]Cu^{2+} (in acetate and chloride form, left) and [^{64}Cu]ATSM (right) using a reverse phase column with a mixture of H_2O:CH_3CN (1:1) as eluent.

In various studies, n=9, the purity of both radiochemical spieces were shown to be 100% as shown in Figure 3.

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, [^{64}Cu]ATSM preparation could totally be degraded and left detectable amounts of free copper after autoclaving. The chemical stability of [^{64}Cu]ATSM was high enough to perform further studies.

Incubation of [^{64}Cu]ATSM in freshly prepared human serum for 12 hours at 37°C showed no loss of ^{64}Cu from the complex during the course of studies, and the radiochemical purity of the complex remained higher than 99% for 12 hours under physiologic conditions.

As expected from the RTLC behavior, the lipophilicity of [^{64}Cu]ATSM compound was rather high. The octanol/water partition coefficient, *P*, of the ^{64}Cu-complex was found to depend somewhat on the pH of the preparation. At pH=7 (final formulation) the lipophilicity was in agreement with the previous report given in the literature.

In our experiment freshly prepared Cu-64 was labeled by the addition of minimum amounts of ATSM resulting in high specific activity ^{64}Cu-ATSM for better therapeutic effects. Using high activity samples for radiolabeling is possible since the radiotracer can be further purified using C_{18} SPE columns. Automated and/or semi-automated systems seem mandatory for production of therapeutic scales of ^{64}Cu-ATSM radiopharmaceutical. While at diagnostic scales the tracer can be even manually prepared for 1-3 patients by an experienced operator or at research centers. ^{64}Cu-ATSM prepared using this method must be used in hypoxic and normoxic animal tumor models for understanding possible limitations as reported in the literature.

As shown in Figure 4, due to lipophilicity of [^{64}Cu]ATSM, it is rapidly washed out from blood stream while easily penetrates into phospholipid bilayer of cells. The compound has a slight liver uptake which is common with most of Cu-thiosemicarbazones.

Interestingly [^{64}Cu]ATSM is highly accumulated in the lungs starting from 30 minutes up to 210 minutes (Figure 4).

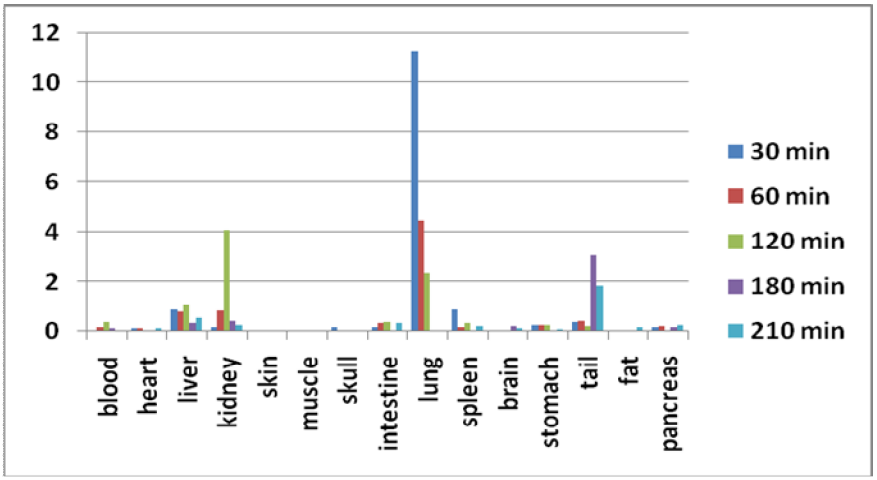

Figure 4. Calculated ID/g% of [^{64}Cu]-ATSM in norrmal rats (n=5)

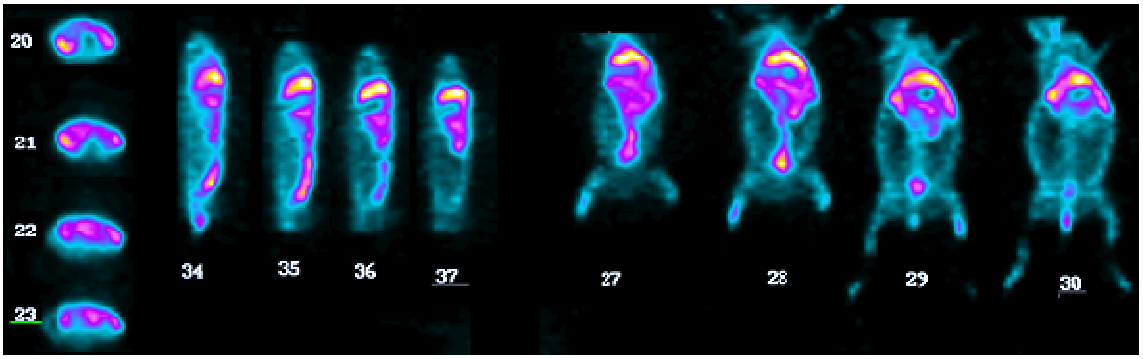

Figure 5. The images of [^{64}Cu]ATSM (18.5 MBq) in healthy male rabbits 3 hours post injection using coincidence system, 20-23 (transaxial), 34-37 (sagital) and 27-30 (coronal)

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)ATSM- in charged form, without the ability of crossing the phospholipid bilayer of the cell membrane (24).

In order to visualize the tissue uptake in the administered rabbit's co-incidence images was taken at 3 hours post injection. The images clearly demonstrate the tracer uptake in the liver, bowel, kidneys and also bladder, which is consistent with the previous reports from large rodents (13), primates and humans.

Also the compound and/or the metabolites are excreted from kidneys due to water soluble metabolites and/or small molecule filtration. Interestingly a right thigh muscle uptake was observed in the scans (Figure 5), which was consistent with the tightly fastened holder belt at the time of scanning, possibly due to the regional hypoxia occurring in the fatigued muscle as reported in the literature (25).

CONCLUSION

Copper-64 in the form of $^{64}Cu^{2+}$ (500 mCi, separation yield> 95%, radionuclide purity>96%) was used for [^{64}Cu]ATSM production (radiochemical purity>99%, specific activity of 300 Ci/mmol) in 10 min. No other labeled by-products were observed upon RTLC/HPLC analysis of the final preparations after solid phase extraction (SPE) purification. The radio-labeled complex was stable in aqueous solutions for at least 12 hours and no significant amount of other radioactive species were detected by RTLC 12 hours after labeling. The biodistribution of the tracer in normal rats up to 210 min demonstrated similar biodistribution to the other reports for [^{64}Cu]ATSM. Intravenous administration of the tracer to healthy rabbits and coincidence imaging demonstrated uptake in liver, kidney and bowel as shown by other reports in various rodents and human. [^{64}Cu]ATSM, is PET radiotracer with a long half life, and the high yield, large scale production and stability of this radiopharmaceutical make it a accessible diagnostic agent for research and clinical trial initiation in the country.

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