

Production of Copper-64 Diacetyl-bis (N4-methylthiosemicarbazone) for Therapeutic Purposes

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Abstract

Copper-64 produced by the $^{68}\text{Zn}(p,n)^{64}\text{Cu}$ nuclear reaction was used in the preparation of [^{64}Cu]diacetyl-bis(N4-methylthiosemicarbazone)(^{64}Cu -ATSM) using in-house ATSM ligand. After a proton irradiation of an electroplated Zinc-68 layer by 30 MeV protons at 180 μA for 1.1 hours, ^{64}Cu was recovered by two-step chromatography using a cation and an anion exchange column. About 200 mCi of $^{64}\text{Cu}^{2+}$ was obtained with a radiochemical separation yield of more than 95% and a radionuclide purity of better than 96%. Colorimetric methods showed that traces of chemical impurities in the product were below the accepted limits. The ^{64}Cu -ATSM production was optimized for reaction conditions (buffer concentration and temperature) with a radiochemical yield of higher than 80%, radiochemical purity of better than 98% and a specific activity of about 246 Ci/mmol. The produced ^{64}Cu -ATSM was injected to fibrosarcoma-bearing rats and showed a good retention in hypoxic tumors.

Key words: Copper-64, ATSM, Hypoxia imaging

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Introduction

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 min to 61.9 h, suitable for imaging and/or radionuclide

therapy(1). 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 (2). Copper-64 has been widely used in the labeling of peptides like octreotide (3,4), bombesin analogs (5), integrin (6) and vasoactive intestinal peptide (7). This radionuclide is mainly produced via $^{64}\text{Ni}(p,n)^{64}\text{Cu}$ reaction at medical cyclotrons (8,9), although it can also be prepared in lower yields by $^{68}\text{Zn}(p,\alpha n)^{64}\text{Cu}$ reaction(10,11). Based on the interesting therapeutic/imaging properties of ^{64}Cu -PTSM and possibility of copper-64 production via $^{68}\text{Zn}(p,\alpha n)^{64}\text{Cu}$ reaction as a by-product of ^{67}Ga at our 30MeV cyclotron, we were interested in the production and yield optimization of ^{64}Cu -ATSM as a possible PET tracer/therapeutic agent for hypoxic tissues (12).

Materials and Methods

Production of ^{64}Cu was performed at the 30 MeV cyclotron (Cyclone-30, IBA) of Agriculture, Medicine and Industry Research School (AMIRS). 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). ^1H -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 (RTL) was performed on polymer-backed silica gel (F 1500/LS 254, 2020 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 RTL counting were based on 511 keV peak.

Production of Copper-64

Production of ^{64}Cu was carried out using the $^{68}\text{Zn}(p,\alpha n)^{64}\text{Cu}$ nuclear reaction through the following steps.

Targetry of zinc-68: 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 target was cooled by a flow of 18°C distilled water at a rate of 50 L/min. The optimum

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energy for the production of ^{64}Cu via $^{68}\text{Zn}(p,an)^{64}\text{Cu}$ reaction is 35-20 MeV(13), 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. The best target thickness in this energy range was determined using SRIM nuclear code (14) which showed that the best target thickness was 984 μm , but the target angle of 6° reduced the required target thickness by 10 fold. Therefore, only 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. The mass of zinc ions in the cell had to be twice that of the electrodeposited layer. Hydrazine dihydrochloride (2 ml) was added as the reducing agent. Electrodeposition was performed at pH=2.5-3, with a cell volume of 480 ml and a current density of 35 mA/cm². Platinum was used as the anode material and resulted in a 100 μm zinc layer on the gold-coated copper backing after 3.5 hours.

Gold electrodeposition: In order to prepare the gold supporting layer, a gold containing bath was prepared according to a previously reported method with slight modifications (15). Briefly, a high purity (99.99%) gold sample (3.00 g) was dissolved in *aqua regia* (60 ml) and heated at 50°C to obtain an almost dried residue. The residue was re-dissolved in concentrated HCl (25 ml) and heated at 100°C to obtain an almost dried residue followed by the addition of distilled water (30 ml) and consequent evaporation. The residue was re-dissolved in distilled water (30 ml) and 25% ammonia (1 ml) to obtain a brown precipitate. Solid KCN (1-2 g) was added portion wise to the residue until a colorless transparent liquid was obtained. The latter mixture was added to a stirring mixture of KH_2PO_4 (45g), citric acid (45g) and KCN (30g) in distilled water (100ml) and the total volume brought up to 450 ml. pH was adjusted to 6.0 by the addition of a solution of KOH (0.1 M). The obtained bath solution was used for gold electroplating over copper backings (cathode) using platinum as the anode material. Electrodeposition was performed with a cell volume of 450 ml and a current density of 1.2 A/dm² at 60°C to give a 50 μm gold layer on the copper backing after 20 hours.

Separation of copper-64 from radiogallium and zinc: Ion exchange chromatography was employed in the separation process. After the target bombardment, chemical separation was carried out in no-carrier-added form. 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 W8, H⁺ form; mesh 200-400) (h:10 cm, O :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; O :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 precious zinc-68 contents. The whole chemical separation process took about 105 min. The resulting high-purity [^{64}Cu]CuCl₂ solution was used directly in the labeling procedure.

Quality control of the product

Control of Radionuclide purity: Gamma spectroscopy of the final sample was carried out using an HPGe detector coupled to a CanberraTM multi-channel analyzer for 1000 seconds.

Chemical purity control: The presence of zinc and copper cations were checked by polarographic methods. Even at 1 ppm of standard zinc and copper concentrations, the area under the curve of the polarogram of the experimental samples were lower than the standard limits.

Labeling and quality control

Preparation diacetyl-bis(*N*⁴-methylthiosemicarbazone) (3): H_2ATSM was prepared following the method for the production of thiosemicarbazones starting *N*⁴-methylthiosemicarbazide and diacetyl. The results were consistent with those of the previous study reported by Gingras *et al.* in 1962(16). Briefly, to a transparent stirring mixture of *N*⁴-methylthiosemicarbazide (210 mg, 2 mmol) (2) in 5% acetic acid at 50°C, was added drop wise freshly distilled 2,3-butanedione (86 mg, 1 mmol) (1) during 5 min. The mixture was stirred for another 30 min at 50°C. The reaction mixture was cooled down in an ice bath and the precipitate was filtered. The precipitate was washed with water (10 ml), ethanol (20 ml) and finally dried in oven at 70-80°C for at least 8 hours. The residue can be further purified by refluxing the mixture of the precipitate in 80% acetic acid at 50-70°C for 10-14 hours. The filtered mass was heated in an oven at 80°C and finally crystallized from hot ethanol to give a light yellow powder (60%) ¹H NMR($\text{D}_6\text{-DMSO}$) $\delta(\text{ppm})$ 10.14(s, 2H, NH-N₂), 8.33(m, 2H, NH-N₄), 3.06(s, 3H, CH₃-C=N), 3.00 (s, 3H, CH₃-C=N), 2.20 (s, 6H, C-NCH₃). IR (CHCl_3) λ_{max} 3600, 3385 3184(N-H), 1457(C=N), 1161(C=S). Mass (electrospray) 260.1(14%) M⁺, calculated; 260.

Preparation of [^{64}Cu]diacetyl-bis(*N*⁴-methylthiosemicarbazone) (4): Preparation of [^{64}Cu] diacetyl-bis(*N*⁴-methylthiosemicarbazone) (3) was accomplished according to a formerly reported method with slight modifications(17). [^{64}Cu]CuCl₂ (3 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 50°C for 1 min. The mixture (about 5 ml) was then cooled in an ice bath, and rapidly injected into a C₁₈ 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 N₂. The labeled compound was finally eluted using 0.2 ml-portions of absolute ethanol and the fractions were counted in HPGe detector (Figure 2). 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. 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 of [^{64}Cu]ATSM complex in the final product: Stability tests were based on previous studies performed for radiolabeled copper complexes(18). A sample of

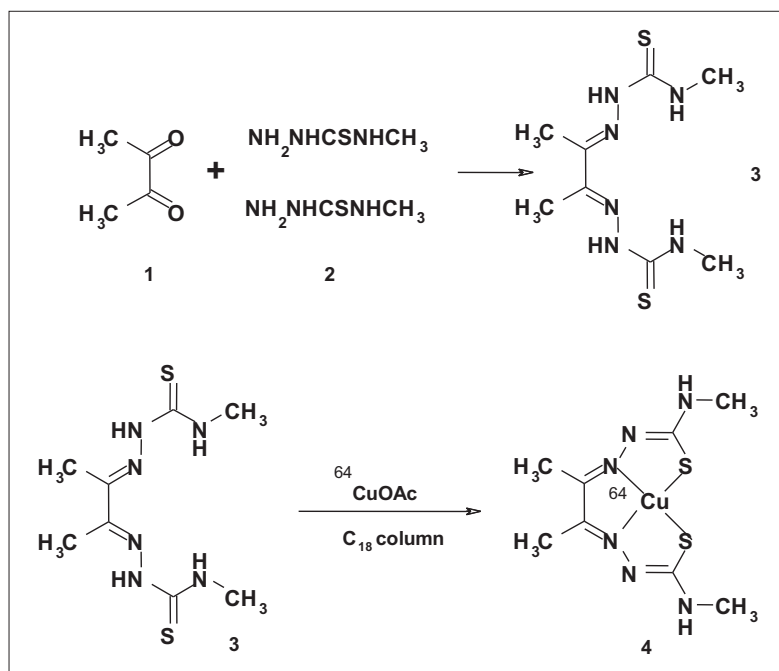


Figure 1 radiosynthesis of [^{64}Cu]ATSM

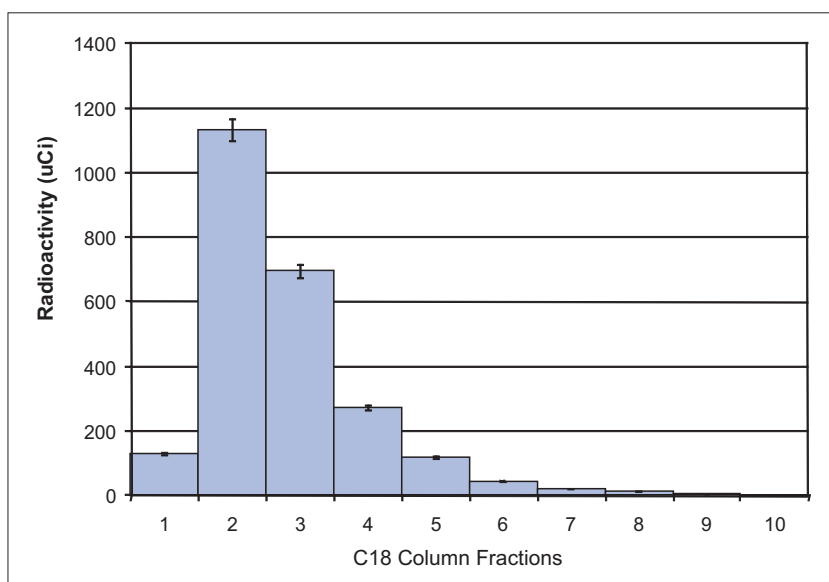


Figure 2. Radioactivity of eluted ethanol fractions from C_{18}

[^{64}Cu]ATSM (5 mCi) was kept at room temperature for 5 hours while checked by RTLC every half an hour. A micropipette sample (5 μl) was taken from the shaking mixture and the ratio of free radio-copper to [^{64}Cu]ATSM was checked by radio thin layer chromatography (eluent: dry ethyl acetate).

Serum Stability Studies: To 36.1 MBq (976 μCi) of [^{64}Cu]ATSM was added 500 μl of freshly prepared human serum and the resulting mixture was incubated at 37°C for 5 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.

Determination of Partition coefficient: 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. Following further incubation for 5 min, the octanol and aqueous phases were sampled and counted in an automatic well counter. A 500 μl sample of the octanol phase from this partitioning was repartitioned two to three times with fresh buffer to ensure that traces of hydrophilic ^{64}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, $\log P$ values represent the mean (standard deviation) of five measurements.

Results

Targetry & irradiation: Various nuclear reactions have been suggested for the production of ^{64}Cu . Since we used a

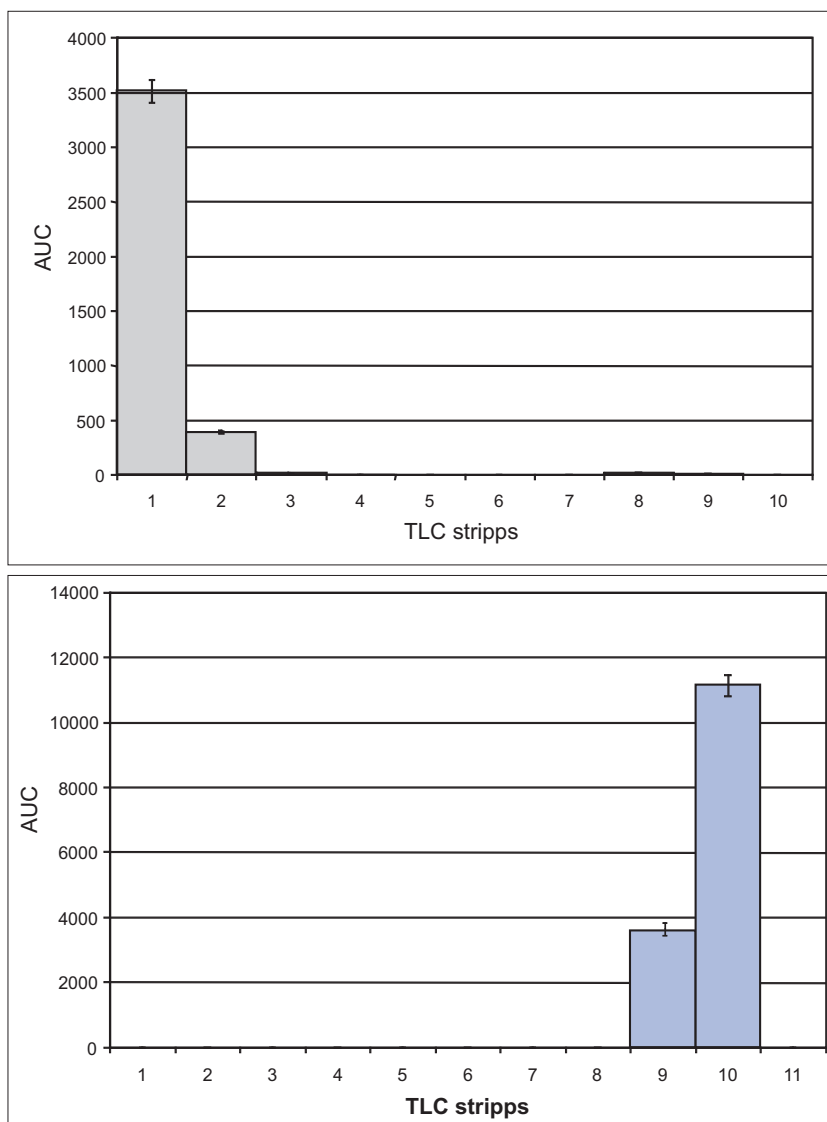


Figure 3. RTLC of the starting [⁶⁴Cu] CuOAc used for radiolabeling (Above) and final [⁶⁴Cu]ATSM product after column chromatography (Below)

proton accelerator in the energy range of 15-30 MeV with a maximum current intensity of 220 microamperes, the only available reactions were ⁶⁴Ni(p,n)⁶⁴Cu and ⁶⁸Zn(p, n)⁶⁴Cu. Among the mentioned reactions, ⁶⁸Zn(p, n)⁶⁴Cu was selected according to the availability of enriched zinc-68 at our institution. One major problem was the use of copper backings for bombardment, which might be dissolved in target dissolution process, introducing carrier copper into ⁶⁴Cu solution. For this reason, a gold layered (thickness ~50 μ) copper backing was used as the target substrate. Radionuclide impurities such as zinc and gallium were easily separated by chemical processes.

Many research groups have reported that the proton energy range between 35-20 MeV is best for the production of ⁶⁴Cu with a minimum amount of radioactive impurities(10,11), but 20-30 MeV proton energy was chosen in order to achieve the maximum possible production yield, according to our present available energies.

[⁶⁴Cu]CuCl₂ was prepared by 30 MeV proton bombardment of an electroplated enriched 0.0714 g/cm², ⁶⁸Zn-target at the angle of 6 in our 30 MeV cyclotron (Cyclone-30, IBA). The

target was bombarded with a current intensity of 180μA for about 1.1 h (200μAh). The chemical separation process was based on a no-carrier-added method. The resulting activity of ⁶⁴Cu was 202 mCi at the end of bombardment (E.O.B.) and the production yield was 1.01 mCi/μAh.

Labeling:

ATSM was prepared with high purity starting from the appropriate ketone and thiosemicarbazide followed by a 3-step purification procedure (Figure 1). The formed precipitate was filtered and thoroughly washed with water, cold ethanol and finally 10% acetic acid followed by overnight reflux in 5% acetic acid.

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 lipophilic. Thus the labeled and unlabeled ATSM can easily be separated using solid phase C₁₈Sep-Pak column (Figure 2).

In TLC studies, the more polar un-complexed ATSM and free copper fractions, correlate to smaller R_fs (R_f=0.1-0.2), while the ATSM complex migrates at the higher R_f (R_f=0.8).

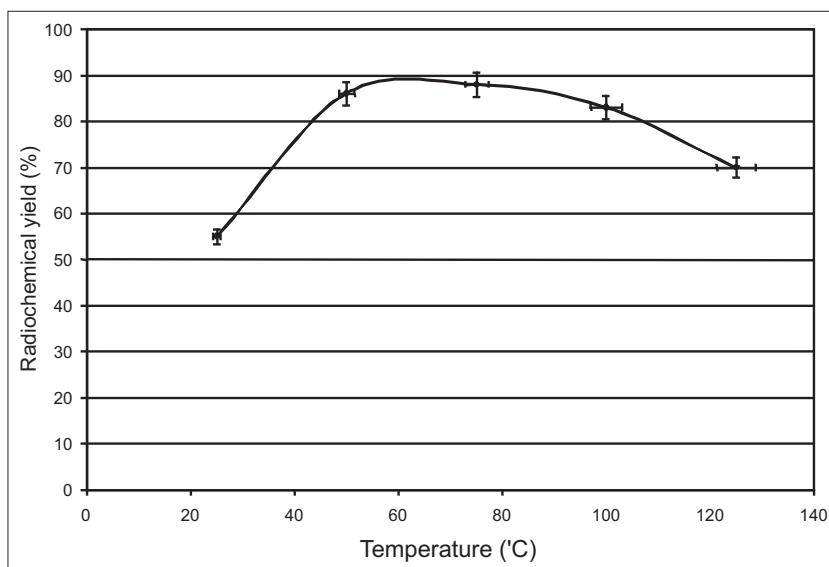


Figure 4. Temperature effect on the radiochemical yield of $[^{64}\text{Cu}]\text{ATSM}$ in 3M buffer

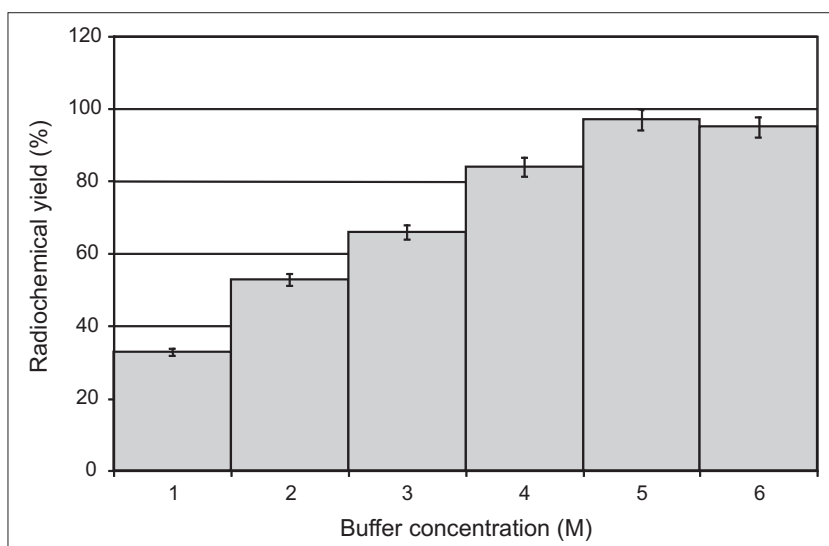


Figure 5. Effect of acetate buffer concentration on the radiochemical yield of $[^{64}\text{Cu}]\text{ATSM}$ at 50°C using 3.7 MBq (100 Ci) copper-61 samples

In all radiolabeling runs ($n=9$) (Figure 3), the integral ratio of the two peaks were constant (98:2), showing the high radiochemical purity and consistency of the labeling method.

In order to obtain the best labeling reaction conditions, the complex formation was studied for temperature dependence. Heating the reaction mixture to 50°C increased the yield from 50% to 88%. Heating to more than 90°C produced some degradation products (Figure 4). Thus 50°C was considered as the best temperature.

Various acetate buffer concentrations were used in order to investigate the best pH for $[^{64}\text{Cu}]\text{OAc}$ formation yield in similar reactions. The complexation reaction yields were measured as the criteria for the optimized buffer conditions, in order to determine the best results. As shown in the Figure 7, the optimized buffer concentration for a given radioactivity was not significantly different between 5-7 M,

while less than 4 M did not give satisfactory results.

The final radiolabeled complex diluted in normal saline was then passed through a 0.22 micron (Millipore) filter for sterilization. Due to its thermal instability, $[^{64}\text{Cu}]\text{ATSM}$ preparation could totally be degraded and left detectable amounts of free copper after autoclaving. The chemical stability of $[^{64}\text{Cu}]\text{ATSM}$ was high enough to perform further studies.

Incubation of $[^{64}\text{Cu}]\text{ATSM}$ in freshly prepared human serum for 3 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 at 99% for 3 hours under physiologic conditions.

As expected from the RTLC behavior, the lipophilicity of $[^{64}\text{Cu}]\text{ATSM}$ 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 (12). Radio thin layer chromatography was performed to control the radiochemical purity of the product, using a mixture of dry ethyl acetate as the mobile phase for both pre-column and post column fractions. The radio-chromatogram showed a major and distinct radio peak at the R_f of 0.90, using an in-house made radio-chromatogram scanner coupled with a HPGe detector. The step motor was installed to count 0.4 cm-pieces each for 30 seconds through the slot of a shielded chamber. Uncomplexed ^{64}Cu eluted at $R_f=0.0$. The radiochemical yields (higher than 98% in each case, $n=9$) were determined by comparison of the uncomplexed ^{64}Cu and the major radio peak at $R_f=0.80$. RTLC of the final product showed no change in stability and the patterns for trace $[^{64}\text{Cu}]\text{CuOAc}$ and $[^{64}\text{Cu}]\text{ATSM}$ were un-changed during 5 hours.

Conclusion

Total labeling and formulation of $[^{64}\text{Cu}]\text{ATSM}$ took about 10 min, with a yield of 97-98%. A suitable specific activity product was formed *via* insertion of $[^{64}\text{Cu}]$ copper cation. No unlabelled and/or labeled by-products were observed upon RTLC analysis of the final preparations after solid phase extraction (SPE) purification. The radio-labeled complex was stable in aqueous solutions for at least 5 hours and no significant amount of other radioactive species were detected by RTLC 12 hours after labeling. Trace amounts of $[^{64}\text{Cu}]$ copper acetate (2%) were detected by RTLC. The radiochemical purity of the $[^{64}\text{Cu}]\text{ATSM}$ is higher than 98%. $[^{64}\text{Cu}]\text{ATSM}$, is a therapeutic/PET radiotracer with an intermediate half life, and the high chemical stability of this radiopharmaceutical makes it a very suitable diagnostic/therapeutic agent to be sent to clinic.

References

- Blower PJ, Lewis JS, Zweit J. Copper radionuclides and radiopharmaceuticals in nuclear medicine. *J Nucl Med Biol* 1996;23:957-980
- Cutler CS, Lewis JS, Anderson CJ. Utilization of metabolic, transport and receptor-mediated processes to deliver agents for cancer diagnosis. *Advanc Drug Deliver Rev* 1999; 37:189-211.
- Maa D, Lua F, Overstreet T, Milenica DE, Brechbiela MW. Novel chelating agents for potential clinical applications of copper. *J Nucl Med Biol* 2002; 29:91-105.
- Anderson CJ, Pajean TS, Edwards WB, Sherman ELC, Rogers BE, Welch MJ. In vitro and in vivo evaluation of copper-64-octreotide conjugates. *J Nucl Med* 1995; 36: 2315-2325.
- Rogers BE, Manna DD, Safavy A. In vitro and in vivo evaluation of a ^{64}Cu -labeled polyethylene glycol-bombesin conjugate. *Cancer Biother Radiopharm* 2004; 19: 25-34.
- Chen X, Liu S, Hou Y, Tohme M, Park R, Bading JR, Conti PS. Micro PET imaging of breast cancer alpha V-integrin expression with ^{64}Cu -labeled dimeric RGD peptides. *Mol Imaging Biol* 2004; 6: 350-359.
- Hou X, Jacobsen U, Jorgensen JC. Separation of no-carrier-added ^{64}Cu from a proton irradiated ^{64}Ni enriched nickel target. *Appl Radiat Isot* 2002; 57:773-777.
- Zweit J, Smith AM, Downey S, Sharma HL. Excitation Functions for Deuteron Induced Reactions in Natural Nickel: Production of No-carrier-added ^{64}Cu from Enriched ^{64}Ni Targets for Positron Emission Tomography. *Appl Radiat Isot* 1991; 42:193-197.
- Szelecsényi F, Blessing G, Qaim SM. Excitation Functions of Proton Induced Nuclear Reactions on Enriched ^{61}Ni and ^{64}Ni : Possibility of Production of No-carrier-added ^{61}Cu and ^{64}Cu at a Small Cyclotron. *Appl Radiat Isot* 1993; 44(3):575-580.
- Hilgers K, Stoll T, Skakun Y, Coenen HH, Qaim SM. Cross-section measurements of the nuclear reactions $^{nat}\text{Zn}(d,x)^{64}\text{Cu}$, $^{66}\text{Zn}(d,\alpha)^{64}\text{Cu}$ and $^{68}\text{Zn}(p,\alpha n)^{64}\text{Cu}$ for production of ^{64}Cu and technical developments for small-scale production of ^{67}Cu *via* the $^{70}\text{Zn}(p,\alpha)^{67}\text{Cu}$ process. *Appl Radiat Isot* 2003; 59: 343-351.
- Boothe TE, Tavano E, Munoz J, Carroll S. Coproduction of Copper-64 and Copper-67 using protons on Zinc-68. *J Label Comp Radiopharm* 1991; 30: 108.
- Jason SL, Laforest R, Buettner TL, Song SK, Fujibayashi Y, Connett JM, Welch MJ. Copper-64-diacetyl-bis(N4-methylthiosemicarbazone): An agent for radiotherapy. *Proc Natl Acad Sci U.S.A.* 2001; 98: 1206-1211.
- Hilgers K, Stoll T, Skakun Y, Coenen H.H, Qaim S.M. Cross-section measurements of the nuclear reactions $^{nat}\text{Zn}(d,x)^{64}\text{Cu}$, $^{66}\text{Zn}(d,\alpha)^{64}\text{Cu}$ and $^{68}\text{Zn}(p,\alpha n)^{64}\text{Cu}$ for production of ^{64}Cu and technical developments for small-scale production of ^{67}Cu *via* the $^{70}\text{Zn}(p,\alpha)^{67}\text{Cu}$ process. *Appl. Radiat. Isot* 2003; 59: 343-351.
- Ziegler J.F, Biersack J.P, Littmark U. The stopping and range of ions in matter (SRIM Code). Version 2000 XX.
- Weisberg AM, Gold plating, 9th Ed, ASM International, U.S.A, 1990, p. 247-250.
- Gingras BA, Suprunchuk T, Bayley CH. The preparation of some thiosemicarbazones and their copper complexes, Part III. *Can J Chem* 1962; 40:1053-1057.
- Zweit J, Goodal R, Cox M, Babich JW, Potter GA, Sharma HL, Ott RJ. Development of a high performance zinc-62/copper-62 radionuclide generator for positron emission tomography. *Eur J Nucl Med* 1992; 19: 418-425.
- Packard AB, Kronauge JF, Barbarics E, Kiani S, Treves ST. Synthesis and biodistribution of a Lipophilic ^{64}Cu -labeled monocationic Copper(II) complex. *Nucl Med Biol* 2002; 29:289-294.