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Preparation and evaluation of [^{61}Cu]-thiophene-2-aldehyde thiosemicarbazone for PET studies

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Abstract

BACKGROUND: [^{61}Cu]Thiophene-2-aldehyde thiosemicarbazone ([^{61}Cu]TATS) (4) was prepared according to an analogy of carrier copper compound with antitumor activity, for eventual use in PET.

MATERIAL AND METHODS: [^{61}Cu]TATS was prepared using copper-61 acetate and in-house made ligand (TATS) in one step. ^{61}Cu was produced via the ^{nat}Zn(p,x)^{61}Cu nuclear reaction (180 μA, 22 MeV, 3.2 h) followed by a two-step chromatography method (222 GBq of $^{61}Cu^{2+}$). [^{61}Cu]TATS preparation was optimized for reaction conditions (buffer concentration and temperature). The tracer was finally administered to normal rats for biodistribution studies.

RESULTS: Total radiolabelling of the tracer took 30 minutes with a radiochemical purity of more than 90% (using HPLC and RTLC) and specific activity of about 250–300 Ci/mmol. The complex was stable in the presence of human serum for an hour. The biodistribution of copper cation and the tracer was checked in wild-type rats for up to 2 hours with significant spleen and lung uptake of the tracer.

CONCLUSIONS: The production of ^{61}Cu via the ^{nat}Zn(p,x)^{61}Cu is an efficient and reproducible method with high specific activity leading to the production and preliminary evaluation of [^{61}Cu]TATS, a potential PET tracer, was reported.

Key words: copper-61, radiolabelling, biodistribution, thiophene-2-aldehyde thiosemicarbazone

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Introduction

In the last 30 years the biological properties of thiosemicarbazones and their metal complexes have been studied extensively [1]. Copper (II) and iron (III) complexes have been shown to extensively modify the biological activity of related thiosemicarbazones [2, 3], as was found for other thiosemicarbazone complexes [4].

Inhibition of ribonucleotide reductase activity [5] and inhibition of DNA synthesis and mitochondrial respiration [6, 7] are major suggested mechanism for metal thiosemicarbazone complex biological activity.

Most copper (II) complexes penetrate into cells while, due to the reducing properties of interacellular thiols, produce the Cu (I) species which activate the molecular oxygen to hydroxyl radicals or superoxide anions [8]. These species attack and break the DNA backbone and damage the cell membrane or interact with proteins [4, 9, 10].

Analytical data on the copper (II) complex of thiophene-2-carbaldehyde thiosemicarbazone ligand has been already reported [11, 12], and the cytotoxic activity of thiophene-2-carbaldehyde thiosemicarbazone copper (II) complex (Cu-TATS) in Friend leukaemia and B16 melanoma cells has been demonstrated [13].

Copper-61 is a positron emitter ($T_{1/2}$ = 3.33 h, β^+: 62%, E.C: 38%), for which a few production methods have been reported for radiolabelling of biomolecules and other applications [14, 15], while the tomographic images obtained using ^{61}Cu are superior to those obtained using ^{64}Cu [16]. In continuation of our recent work on the production and application of this radionuclide [17–19], and re-

cent works on the development of radiocopper based radiotracers and radiopharmaceuticals [20, 21], we were interested in the development of [^{61}Cu]-thiophene-2-aldehyde thiosemicarbazone for its possible PET applications.

Material and methods

Production of ^{61}Cu was performed at the Agricultural, Medical, and Industrial Research School (AMIRS), using a 30 MeV cyclotron (Cyclone-30, IBA). Natural zinc chloride with a purity of more than 98% was provided commercially (Merck chemical company, Darmstadt, Germany). All chemicals were purchased from Sigma-Aldrich Chemical Co. U.K. All samples for NMR spectra were dissolved in d_6-DMSO. ^{1}H NMR spectra were run at 500 MHz, and chemical shifts (δ) are reported in ppm relative to tetramethylsilane (δ 0.0, internal standard). Mass spectra were recorded on a Finnigan Mat TSQ-70 Spectrometer. 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, 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), FR = 1 ml/min. All calculations and RTLC counts were based on 283 keV peaks. All values were expressed as mean ± standard deviation (Mean ± SD) and the data were compared using student T-test. Statistical significance was defined as $P < 0.05$. All animal studies were performed in accordance with the United Kingdom Biological Council Guidelines on the Use of Living Animals in Scientific Investigations, 2nd edition.

Targetry and bombardment

The target was a layer of natural zinc, electroplated onto 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 [22] 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 at a glancing angle of 6° to achieve higher production yield. SRIM code was run to determine the best target thickness in the energy range.

Gold and Zinc electrodeposition

A gold-containing bath was prepared according to Weisberg AM [24] 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 × 10 cm) which had been preconditioned by passing 25 mL of 9 M HCl. The column was then washed with 25 mL of 9 M HCl at a rate of 1 mL/min to remove copper and zinc ion contents. To the eluent, 30 mL of 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, 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 minutes [25].

Quality control of the product

Gamma spectroscopy of the final sample was carried out using 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 coloured dithizone-zinc complex was measured using visible spectroscopic assay to determine zinc cation concentrations [26] using dithizone organic reagent (0.002% in CCl_4). The amount of gold cation in the final solution was checked using colour formation of acidic rhodamine B reagent reacting with gold dilutions based on a previously reported colorimetric method [27].

Preparation of thiophene-2-aldehyde thiosemicarbazone (3)

TATS was prepared according the method for production of thiosemicarbazones starting with thiosemicarbazide and thiophene-2-aldehyde, which was consistent with the results of the previously-reported study [28]. Briefly, to a transparent stirring mixture of thiosemicarbazide (1 mmol) (2) in 5% acetic acid at 50°C, was added drop wise freshly distilled thiophene-2-carbaldehyde (1 mmol) (1) for 5 minutes. The mixture was stirred for another 30 minutes 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) and 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 (70%) ^{1}H NMR(D_6-DMSO) δ(*ppm*) 11.45 (s, ^{1}H, N=C-H), 8.24 (bs, ^{1}H, NH-N_1), 8.20 (bs, ^{1}H, NH-N_1), 7.64(d, 1H, H_5 thiophene), 7.54 (bs, 1H, NH-N_3), 7.44 (d, 1H, H_3 thiophene), 7.10 (m, 1H, H_4 thiophene). IR ($CHCl_3$) λ_{max} 3250, 3002 (N-H), 1575, 1537, (C = N), 1278 (C = S). Mass (electrospray) 185.1 (16%) M^+, calculated; 185.

Preparation of thiophene-2-aldehyde thiosemicarbazonate copper complex (4a)

The copper complex was prepared according to the reported method [9] with slight modifications. Compound 4a was prepared by addition of solid thiosemicarbazone (0.37 g; 2.0 mmol) to an aqueous solution (40 ml) of copper (II) chloride (0.19g, 1.1 mmol). A 5 M solution of sodium hydroxide was slowly added in order to reach pH = 6–7. The suspension was slightly heated and stirred for 6 h. The greenish-brown precipitate was filtered off, washed with water, and dried under a vacuum. The complex was soluble in DMSO and acetone, less soluble in ethanol, but not in water. It was recrystallized in diethyl ether, giving rise to brown crystals suitable for X-ray studies. (72%) m.p. 287°C. ^{1}H NMR(D_6-DMSO) δ(ppm) 8.75 (bs, 1H), 8.45 (bs. 1H), 6.92–7.77 (m, 12H, NH and thiophene protons).

Preparation of [^{61}Cu]-thiophene-2-aldehyde thiosemicarbazone (4b)

Preparation of [^{61}Cu]-thiophene-2-aldehyde thiosemicarbazone (4b) was accomplished according to our previously reported method [29]. The [^{61}Cu]$CuCl_2$ (5 mCi) dissolved in the acidic medium obtained above (about 2 ml) was evaporated until dry using a flow of N_2 and mild heat (50°C). To the vial, 3 M sodium acetate solution (1 ml) was added to prepare a [^{61}Cu] copper acetate solution. A mixture of TATS (4 μg) in anhydrous ethanol (50 μl) was added to the copper acetate solution and vortexed at room temperature for 30 minutes. The active solution was checked for radiochemical purity by HPLC and RTLC (Figure 1–3). The final solution was then passed through a 0.22 μm filter and the pH was adjusted to 5.5–7 by the addition of 3 M sodium acetate buffer.

Quality control of ^{61}Cu-TATS

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) as the mobile phase. Alternatively, 10 mM DTPA solution can be used as another mobile phase to discriminate free copper from the radiolabelled compound.

High performance liquid chromatography (HPLC)

High performance liquid chromatography 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: 130 kgF/cm^2) for 20

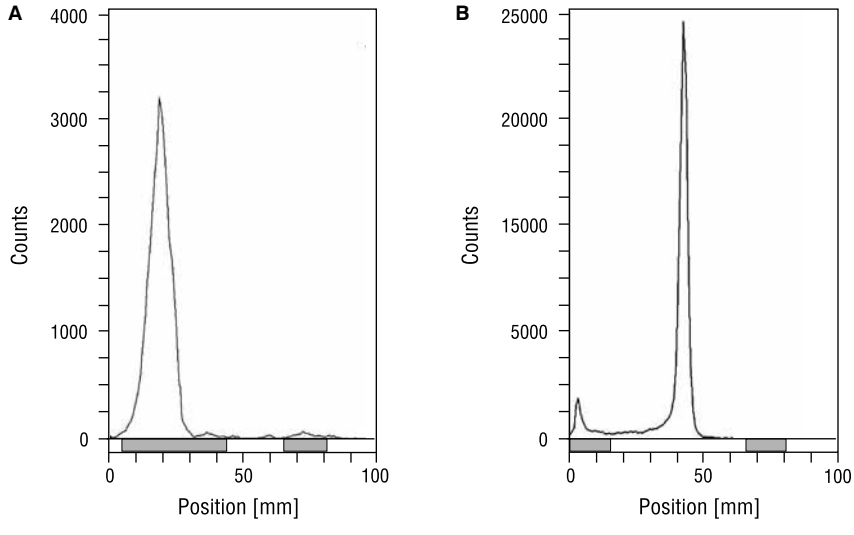

Figure 1. RTLC of the starting [^{61}Cu]CuOAc (**A**) and [^{61}Cu]TATS (**B**).

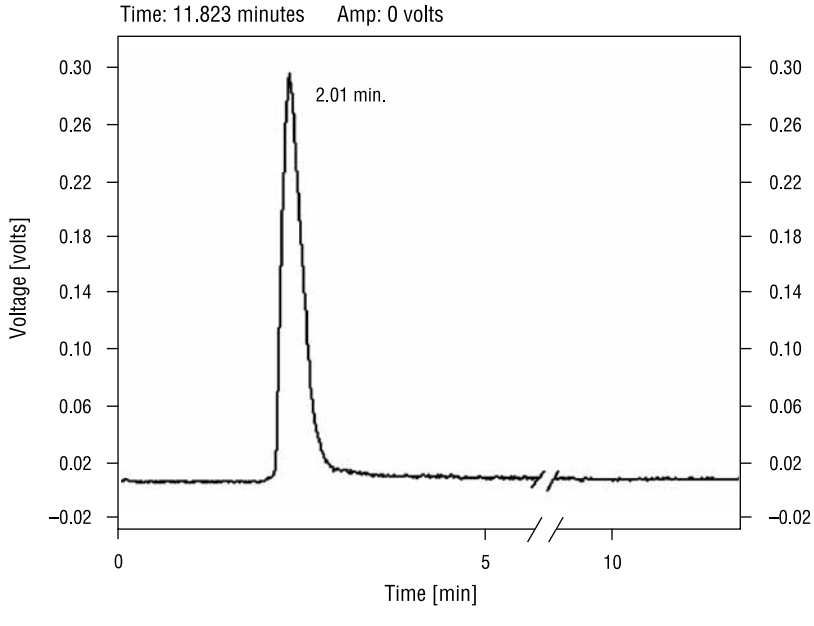

Figure 2. HPLC diagram for [^{61}Cu]Cu^{2+} (in acetate and chloride form) used in production of [^{61}Cu]TATS using a reverse phase column with a mixture of H_2O:CH_3CN (1:1) as eluent.

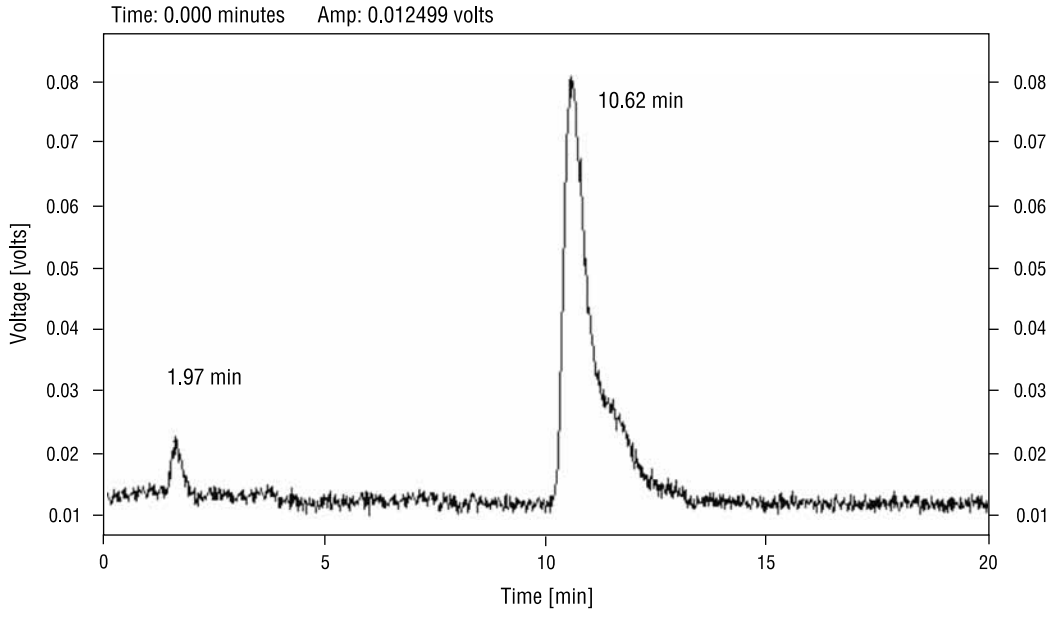

Figure 3. HPLC diagram for [^{61}Cu]TATS prepared from [^{61}Cu]$CuCl_2$ using a reverse phase column with a mixture of H_2O:CH_3CN (1:1) as eluent.

minutes, in order to elute low molecular weight components. The radiolabelled compound was eluted using the reverse stationary phase. Any remaining free Cu^{2+} cations with chloride or acetate counter ions are eluted at the same time.

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

Stability tests were based on previous studies performed for radiolabelled copper complexes [30]. A sample of [^{61}Cu]TATS (5 mCi) was kept at room temperature for 5 hours and was checked by RTLC every half hour. A micropipette sample (5 μl) was taken from the shaking mixture and the ratio of free radiocopper to [^{61}Cu]TATS was checked by radio thin layer chromatography (eluent: 10 mM DTPA solution or ammonium acetate:methanol 1:1).

Serum stability studies

To 36.1 MBq (976 μCi) of [^{61}Cu]TATS, 500 μl of freshly prepared human serum was added and the resulting mixture was incubated at 37°C for 3 hours. Aliquots (5-μl) were analyzed by radio-TLC after 0, 0.5, 1, 2, and 3 hours of incubation to determine the stability of the complex using 10 mM DTPA solution as eluent.

Determination of partition coefficient

The partition coefficient of the [^{61}Cu]TATS was measured following 1 minute 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 radiolabelled copper complex at 37°C. Following further incubation for 5 minutes, 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 ^{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; log *P* values represent the mean (standard deviation) of five measurements.

Biodistribution of [^{61}Cu]TATS in normal rats

To determine its biodistribution, [^{61}Cu]TATS was administered to wild-type rats. A volume (50 μl) of final [^{61}Cu]TATS solution containing 40 ± 2 μCi radioactivity was injected subcutaneously into the rats. The animals were sacrificed at exact time intervals (1 and 2 hours), and the ID/gr % of different organs was calculated as the percentage of injected dose (based on the area under the curve of the 283 keV peak) per gram using an HPGe detector (Figure 4).

Results and discussion

Targetry & irradiation

For 76 minutes 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.

The 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%), and 1186 (3.75%) keV γ-rays from ^{61}Cu and showed a radionuclidic purity higher than 99% (E.O.S.). The rest of the activity was attributed to ^{60}Cu (0.23%). In order to check the chemical purity, the concentrations of zinc (from the target material) and gold (from the 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 to the naked eye, whereas the test sample remained similar to the blank. The colorimetric assay demonstrated that the zinc cation concentration was far below the maximum

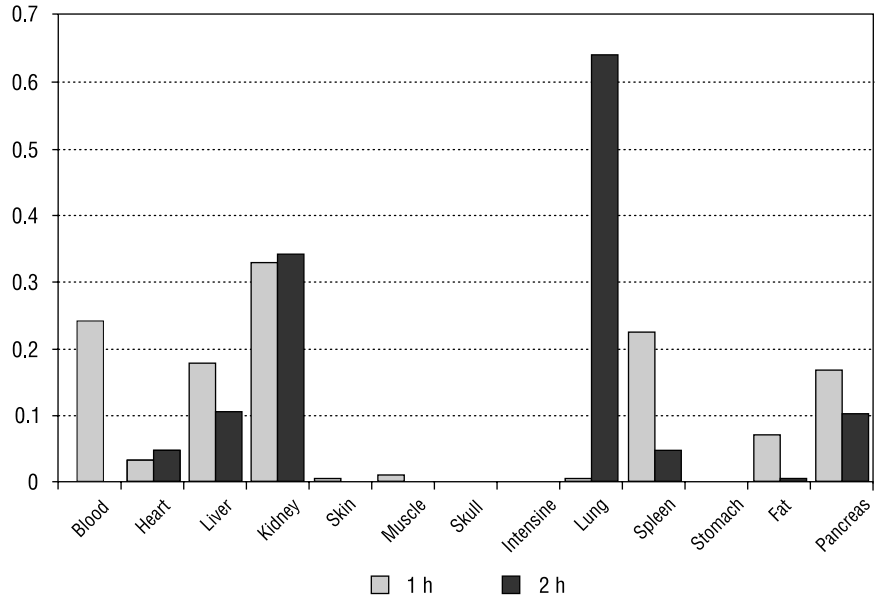

Figure 4. Biodistribution (ID/g%) of ^{61}Cu-TATS in saline, 1 and 2 hours post SC injection of 40 uCi of the tracer in wild-type rats.

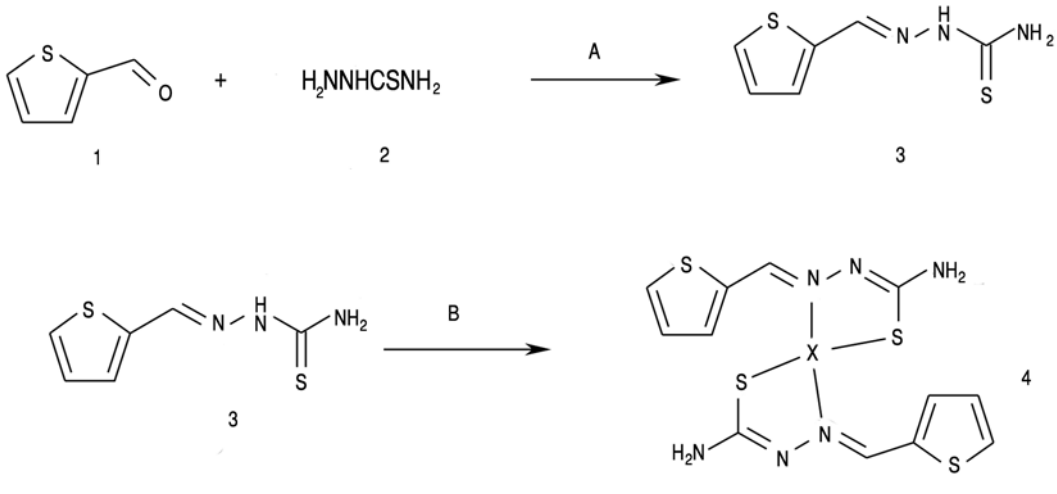

Figure 5. Schematic diagram of the preparation method for TATS 3, Cu-TATS (X=^{nat}Cu, 4a) and [^{61}Cu]TATS (X=^{61}Cu, 4b). A — 5% AcOH, 50°C; B — [^{61}Cu]CuOAc.

permitted levels, i.e. 5 ppm (less than 1 ppm zinc) [26]. The gold concentration was less than 0.9 ppm.

Preparation and structure confirmation of the ligand

Thiophene-2-aldehyde-thiosemicarbazone, not commercially available, was prepared according to the procedure reported previously [9]. The reaction was performed in 5% acetic acid solution containing N4-methyl thiosemicarbazide. Figure 5 shows the preparation method for the ligand and labelled compound.

In TLC studies, the more polar un-complexed TATS and free copper fractions correlate to smaller Rfs (Rf = 0.1–0.2), while the TATS complex migrates at the higher Rf (Rf = 0.9). In all radiolabelling runs (n = 9), the integral ratio of the two peaks were constant (90:10), showing the high radiochemical purity and consistency of the labelling method.

In order to obtain the best labelling 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 behaviours. Thus 25°C was considered the best temperature.

Various acetate buffer concentrations were used in order to investigate the best pH for [^{61}Cu]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. The optimized buffer concentration for a given radioactivity was not significantly different between 5 and 7 M, while less than 4 M did not give satisfactory results.

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]TATS preparation could totally

be degraded and left detectable amounts of free copper after autoclaving.

Incubation of [^{61}Cu]TATS in freshly prepared human serum for 3 hours at 37°C showed a loss of ^{61}Cu from the complex during 30–60 minutes.

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. The radiochromatogram showed a major and distinct radio peak at an R_f of 0.90, using an in-house made radiochromatogram scanner. Uncomplexed ^{61}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 ^{61}Cu and the major radio peak at $R_f = 0.90$.

Interestingly, due to subcutaneous injection of the tracer no detectable amount is found in the blood after 60 minutes, while most of the activity is excreted from the kidneys. For unknown reasons, the compound has significant uptake in the spleen, pancreas, and liver, showing the compound uptake in the reticuluendothelial system. After 120 minutes, however, the tracer is metabolized with the release of free copper cations, possibly leading to a release of activity in the blood, again in the kidneys, and in the lungs.

Conclusions

The method used, in this research, for the production and chemical separation of ^{61}Cu was quite simple and cost effective. The thick target yield, radiochemical separation yield, and level of impurities obtained in this study were comparable with the previous reports given in the literature, which reported a yield of 90 MBq/μAh for ^{60}Ni(d,n)^{61}Cu reaction [6, 25]

Total labelling and formulation of [^{61}Cu]TATS took about 40 minutes, with a yield of more than 90%. A significant specific activity (9.1 TBq/mmol or 246 Ci/mmol) was formed via insertion of ^{61}Cu cations. No unlabelled and/or labelled by-products were observed upon RTLC analysis of the final preparations after SPE purification. The radiolabelled complex was stable in aqueous solutions for at least one hour. Trace amounts of [^{61}Cu] copper acetate were detected by RTLC, which showed that the radiochemical purity of the [^{61}Cu]ATSM was higher than 90%.

[^{61}Cu]TATS can be a PET tracer with an intermediate half-life, and our experiments on this radiopharmaceutical and preclinical properties have shown it to have satisfactory quality. The DNA biding properties of the Cu-TATS as well as optimized production of ^{61}Cu-TATS can offer an interesting probe for DNA proliferation studies.

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