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Production and tumour uptake of [^{64}Cu]Pyruvaldehyde-*bis* (N^4-methylthiosemicarbazone) for PET and/or therapeutic purposes

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Abstract

BACKGROUND: Copper-64 ($T_{1/2}$=12.7°h) is an important radionuclide used both in PET imaging and therapy. [^{64}Cu]-pyruvaldehyde-*bis*(N^4-methylthiosemicarbazone) ([^{64}Cu]-PTSM) has already been used in the detection of cerebral and myocardial blood flow. In this study, a simple production method and tumour accumulation of [^{64}Cu]-PTSM in fibrosarcoma-bearing mice were reported.

MATERIAL AND METHODS: Cu-64 was produced *via* the ^{68}Zn(p, αn)^{64}Cu nuclear reaction. [^{64}Cu]-PTSM was prepared using in-house made PTSM ligand and [^{64}Cu]cuprous acetate and injected to fibrosarcoma-bearing mice.

RESULTS: Copper-64 was prepared in chloride form (≈ 200 mCi, > 95% chemical yield at 180° μA for 1.1 h irradiation, radionuclidic purity > 96%, copper-67 as impurity). The solution of ^{64}Cu-PTSM was prepared in > 80% radiochemical yield and more than 98% radiochemical purity. A significant tumour uptake was observed 2 hours post injection in tumour-bearing mice (tumour/muscle: 9, tumour/blood: 6).

CONCLUSION: [^{64}Cu]-PTSM was prepared on a radiopharmaceutical scale using readily available zinc-68, with high quality and was shown to possess application in the therapy and/or imaging of fibrosarcoma.

Key words: Copper-64, PTSM, PET, targeted therapy, fibrosarcoma

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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 radiotherapy [1]. Copper-64 (half-life = = 12.7 h; β^+ 653 keV [17.4%]; β^- 573 keV [39%]; E.C. [43.6%]) is an attractive radionuclide for PET imaging and targeted therapy of cancer [2]. Copper-64 has been widely used in the labelling of peptides like octreotide [3, 4], bombesin analogues [5], integrin [6] and vasoactive intestinal peptide [7]. Some of these compounds have already been used in PET imaging [8–11]. [^{64}Cu]-diethylenetriaminepentaacetic acid ([^{64}Cu]-DTPA) was used for the differential investigation of disorders in cerebrospinal fluid (CSF) transit and absorption [12].

[^{64}Cu]-Pyruvaldehyde-bis (N^4-methylthiosemicarbazone) ([^{64}Cu]--PTSM) was prepared for use in internal radiation therapy and imaging in the late 1980's [13]. Since then, this complex has been used in the determination of regional blood flow and renal perfusion [14––16] and ischemia [17, 18]. Figure 1 shows the production of PTSM and [^{64}Cu]-PTSM. This radionuclide is mainly produced *via* ^{64}Ni(p,n)^{64}Cu reaction in medical cyclotrons [19, 20]; however, expensive nickel-64 is not readily available in most of developing countries. Alternatively, it can prepared in lower yields by ^{68}Zn(p,αn)^{64}Cu reaction [21, 22].

Based on the interesting therapeutic/imaging properties of ^{64}Cu and the possibility of copper-64 production via ^{68}Zn(p, n)^{64}Cu reaction as a by-product in routine production of ^{67}Ga for national use, we were interested in the production and yield optimization of [^{64}Cu]-

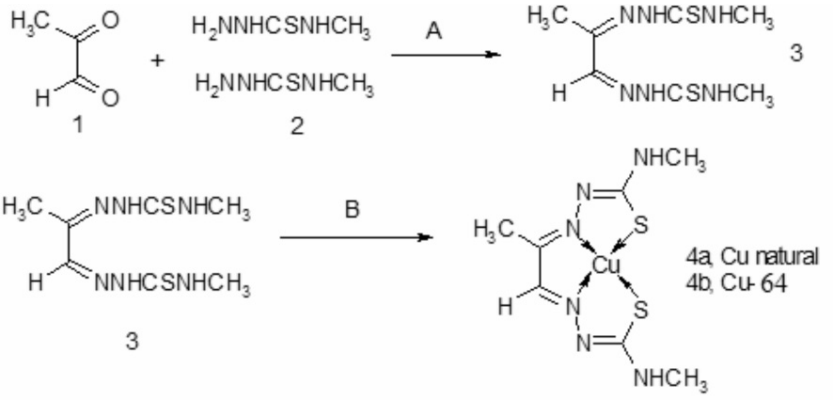

Figure 1. Schematic diagram of the preparation method for PTSM (3) and [^{64}Cu]PTSM (4b), A: ethanol, 50°C, B: [^{64}Cu]CuOAc, C_{18} Sep-Pak.

PTSM as a possible PET tracer/therapeutic agent (Figure 1) in a fibrosarcoma-bearing model.

Material and methods

Chemicals were purchased from Aldrich Chemical Company (U.K.). Thin layer chromatography (TLC) was performed on polymer-backed silica gel (F 1500/LS 254, 20 × 20 cm, TLC Ready Foil, Schleicher & Schuell®, Germany). Ethyl acetate and normal saline used for labelling were of high purity. ^{1}H-NMR spectra were obtained on a FT-80 (80MHz) Varian instrument with tetramethylsilane as the internal standard. Infrared spectra were taken on a Perkin-Elmer 781 instrument (KBr disc). Production of ^{64}Cu was performed in the NRCAM 30 MeV cyclotron (IBA, Cyclone-30). Enriched Zn-68 with a purity of more than 98% was provided by the Ion Beam Application Department, NRCAM, Karaj, Iran. 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 TLC counting were based on 511 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 edition.

Targetry of zinc-68

An electroplated ^{68}Zn target on a gold-coated copper backing plate was irradiated at an angle of 6 degrees toward the proton beam in order to achieve higher production yield. The target was cooled by a flow of 18°C distilled water with a rate of 50 Lit/min. The optimum energy for the production of ^{64}Cu *via* ^{68}Zn(p, n)^{64}Cu reaction is 35–20 MeV, but the highest available proton energy was 30 MeV. The target had to be thick enough to reduce the energy of the incident protons from 30 MeV to about 20 MeV. The SRIM nuclear program [23] was run in order to determine the best target thickness in the above energy range. Results of the SRIM program showed that the best target thickness was 984 μm, but the target angle of 6° reduced the required target thickness by 10 fold. Thus, we only needed to electroplate about 100 μm of the target material 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 accurate density of 35 mA/cm^2. Platinum was used as the anode material and resulted in a 100 μ zinc layer on the gold-coated copper backing after 3.5 hours.

Separation of copper-64 from radiogallium and zinc

Ion exchange chromatography was employed in the separation process. After the target bombardment process, 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 W × 8, H^+ form; mesh 200–400) (h: 10 cm, ϕ: 1.3 cm) which had been preconditioned by passing 25 ml of 9 N HCl. The column was then washed with 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 passed through another cation exchange resin (Dowex 1X8, Cl^- form; mesh: 100–200) (h: 13 cm; ϕ: 1.6 cm), preconditioned with 100 ml of 6N HCl. In order to elute copper-64 ions, the column was eluted by 50 ml of 2 N HCl. For the recovery of precious zinc-68 contents, the column was finally eluted with 0.05 N HCl (150 ml). The whole chemical separation process took about 105 min. The resulting high-purity [^{64}Cu]$CuCl_2$ solution was used directly in the labelling step.

Radionuclide purity

The gamma spectroscopy of the final sample was carried out using an HPGe detector. The peaks were observed and the area under the curve was counted for 1000 seconds.

Preparation of pyruvaldehyde-bis(N^4-methyl-thiosemicarbazone)

PTSM was prepared according to the reported method for the production of thiosemicarbazones [24]. A mixture of N^4-methylthiosemicarbazide (210 mg, 2 mmol) in acetic acid solution (5%, prepared with 99% AcOH and MilliQ-H_2O) was heated at 50°C with stirring until a transparent solution was formed. Then freshly distilled pyruvaldehyde (115 mg, 2 mmol) diluted (1:3)

with 5% acetic acid was added drop-wise to the mixture during 5 min under a blanket of N_2. The mixture was stirred for 3–4 h at 50°C. The hot reaction mixture was filtered off through two layers of Whatman No.2 filter paper. The filtered mass was washed with MilliQ-H_2O (50 ml), rectified ethanol (25 ml) and was finally heated in a vacuum oven overnight at 75°C. The dried powder was refluxed in 80% acetic acid (prepared with MilliQ-H_2O) for 2 h. The hot mixture was filtered, and the precipitate was washed with MilliQ-H_2O (50 ml) rectified ethanol (25 ml) and was heated in a vacuum oven overnight at 75°C. Alternatively, the powder could be crystallized from hot ethanol to give a brilliant white powder (60%) m.p. 241–243°C. ^{1}H NMR (D_6-DMSO) δ (*ppm*) 11.74 (s, 1H, NH-N_2), 10.33 (s, 1H, NH-N_2), 9.43 (m, 2H, NH-N_4), 7.68 (s, 1H, H-C = N), 3.31 (s, 3H, CH_3-C = N). IR ($CHCl_3$) λ_{max} 3208, 3132 (N-H), 1429 (C = N), 1111 (C = S). Mass (electrospray) 246.1 (14%) M^+, 215 (7), 172 (4), 157.1 (76), 130 (65), 115.8 (98), 73.8 (100), 56.9 (68).

Preparation of [^{64}Cu]pyruvaldehyde-bis(N^4-methylthiosemicarbazone)

[^{64}Cu]$CuCl_2$ (3 mCi) dissolved in the 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 pyruvaldehyde-bis(N^4-methylthiosemicarbazone) (3 μg) in absolute ethanol (0.1 ml) [8] was added to the copper acetate solution and vortexed at 50°C for 3–5 min. The mixture (about 5 ml) was then cooled in an ice bath, and rapidly injected into a C_{18} Sep-Pak column pre-treated with 5 ml 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 labelled compound was finally eluted using 0.2 ml-portions of absolute ethanol and the fractions were counted in an HPGe detector. The vial containing the maximum radioactivity was diluted to a 5% solution by the 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 the pH was adjusted to 5.5–7 by the addition of 3 M sodium acetate solution.

Chemical purity

The formation of coloured dithizone-zinc complex was measured using visible spectroscopic assay to determine Zn cation concentrations according to the literature [25] using dithizone organic reagent (0.002% in CCl_4). Briefly, the presence of the pinkish colour of zinc-dithizone complex was checked for the test sample, 1, 5, 10 ppm standards and finally a blank solution (1 ml each). The colour of the test tube must be less than that of the standard.

Radiochemical purity

Radio thin layer chromatography was performed using a mixture of dry ethyl acetate as the mobile phase for both pre-column and post-column fractions using an in-house made radiochromatogram scanner equipped with an HPGe detector. A step motor was installed to count 0.4 cm-pieces each 30 second through the slot of a shielded chamber. Radiochemical yields were determined by comparison of un-complexed ^{64}Cu (R_f = 0.0) and the major radio peak at R_f = 0.80.

Stability of [^{64}Cu]PTSM complex in the final product

Stability studies were based on the previous studies performed for radiolabelled copper complexes. A sample of [^{64}Cu]PTSM (0.5 mCi) was kept at room temperature for 5 hrs while being checked by RTLC every half hour. A micropipette sample (5 μl) was taken from the shaking mixture and the ratio of free radiocopper to [^{64}Cu]PTSM was checked by radio thin layer chromatography (eluent: dry ethyl acetate).

Stability of [^{64}Cu]PTSM complex in the presence of serum

A mixture of five parts of serum and one part radiopharmaceutical (0.2 mCi) was shaken in a 37-degree incubator under a nitrogen atmosphere. A micropipette sample (5 μl) was taken from the shaking mixture every 30 minutes. The ratio of free radiocopper (R_f = 0) to [^{64}Cu] PTSM (R_f = 0.8) was checked by radio thin layer chromatography (eluent: dry ethyl acetate).

Determination of partition coefficient

The partition coefficient of the [^{64}Cu]PTSM 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 radiolabelled 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.

Cell line formation

Cell line of murine fibroblastoma was employed for this investigation. Cultures of 1–2 × 10^4 cells of murine fibroblastoma were seeded into a 75 cm^3 flask containing 20 ml of medium supplemented with 10% fetal bovine serum and 1% glutamine. Cells were incubated at 37°C in 5% CO_2 and were maintained in exponential growth phase and passaged twice per week.

Animal studies

Fibrosarcoma cells (about 10^4) were injected subcutaneously to the dorsal area of Balb C mice weighing 20–25 g. After 14 days, the animals were sacrificed and tumour tissues which were not grossly necrotic weighed to a suitable amount (0.7 ± 0.05 g). The distribution of [^{64}Cu]$CuCl_2$ and [^{64}Cu]PTSM in tissues were determined for untreated mice and for mice with fibrosarcoma. A volume (0.1 ml) of final [^{64}Cu]PTSM solution containing 15 ± ± 3 μCi radioactivity (in 50 μL) was injected into the dorsal tail vein. The total amount of radioactivity injected into each mouse was measured by counting the 1-ml syringe before and after injection in a curie meter with a fixed geometry. The animals were sacrificed by ether asphyxiation at selected times after injection (1 and 2 hours), stool and tissues (blood, heart, lung, spleen, intestine, skin, bladder, kidneys, liver, muscle and bone and tumour) were weighed and their specific activities determined with a HPGe detector.

Results and discussion

Targetry and irradiation

Various nuclear reactions used for the production of ^{64}Cu have been suggested. Since we used a proton accelerator in the energy range of 15–30 MeV with a maximum current intensity of 220 microamperes, the only available reactions were ^{64}Ni(p,n)^{64}Cu and ^{68}Zn(p, αn)^{64}Cu. Among the mentioned reactions, ^{68}Zn(p, αn)^{64}Cu was selected according to the availability of enriched zinc-68 at our institution. The other problem was the use of copper backing for the bombardment, which might be dissolved in the target dissolution process, introducing carrier copper into the ^{64}Cu solution. For this reason, a gold layered (thickness ~50 μ) copper backing was used as the target substrate. Radioisotope 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 ^{64}Cu with a minimum amount of radioactive impurities [24, 25], but 20–30 MeV proton energy was chosen to achieve the maximum possible production yield, according to our present available energies.

[^{64}Cu]$CuCl_2$ was prepared by 30 MeV proton bombardments of an electroplated enriched 0.0714 g/cm^2, ^{68}Zn-target at an angle of 6° in our 30 MeV cyclotron (Cyclone-30, IBA). The target was bombarded with a current intensity of 180 μA for about 1.1 h (200 μAh). The chemical separation process was based on a no-carrier-added method. The resulting activity of ^{64}Cu was 202 mCi at the end of bombardment (E.O.B.) and the production yield was 1.01 mCi/μAh.

Preparation and structure confirmation of the ligand

In order to prepare, pyruvaldehyde-bis(N^4-methylthiosemicarbazone) that was not commercially available, we tried the general procedure of thiosemicarbazone preparation [26]. The reaction was performed in absolute ethanol containing N^4-methyl thiosemicarbazide.

Radionuclidic purity

Gamma spectroscopy of the final product showed a radionuclidic purity higher than 96%, showing the presence of 511, 1346 keV gamma energies, all of which resulted from ^{64}Cu.

Chemical purity

In order to check the chemical purity, the concentration of Zn (from the target material) was determined using colorimetric assay. 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).

Radiolabelling of pyruvaldehyde-bis(N^4-methyl-thiosemicarbazone)

The freshly eluted copper-64 chloride solution was changed into copper acetate using a 3M sodium acetate solution, keeping a suitable pH between 5.5–7 for complex formation. The ligand 3, dissolved in absolute ethanol, was then added to the buffered solution so that a final 2% concentration of ethanol was obtained. This procedure was superior to the former labelling procedure using DMSO as the ligand solvent [11]. The mixture was then vortexed in a tube shaker and left at room temperature for 5–10 minutes.

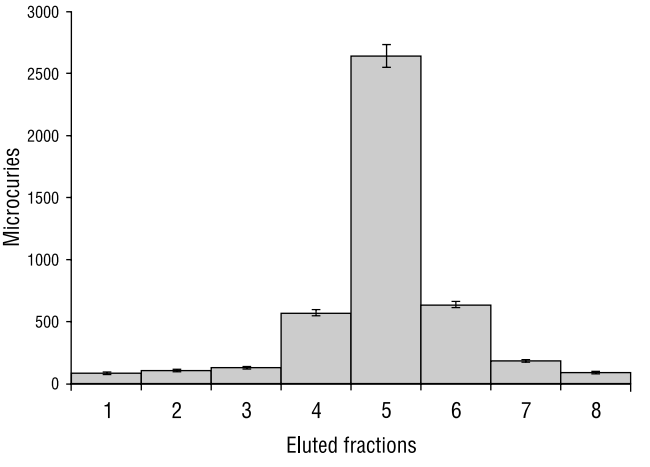

Figure 2. Radioactivity of eluted ethanol fractions from C_{18}.

The solid phase separation of the ^{64}Cu-PTSM from free copper cations was performed to obtain a better purity and then the lipophilic complex was eluted using 0.2-ml absolute ethanol fractions (Figure 2).

Radiochemical purity

Labelling of PTSM with copper cation affects its chromatographic properties and the final complex is highly lipophilic. Thus free copper, and the labelled PTSM, can easily be separated using solid phase C_{18} Sep-Pak column. In the TLC studies, the more polar uncomplexed PTSM and free copper fractions correlate to smaller R_fs (R_f = 0.0), while the complexed PTSM migrates at the higher R_f (R_f = 0.8). In all radiolabelling runs (n = 9), after solid phase extraction of the labelled mixture, the integral ratio of the two peaks were constant (98:2), showing the high radiochemical purity. The RTLC diagrams of the final tracer are shown before (Figure 3) and after (Figure 4) solid phase purification of the tracer.

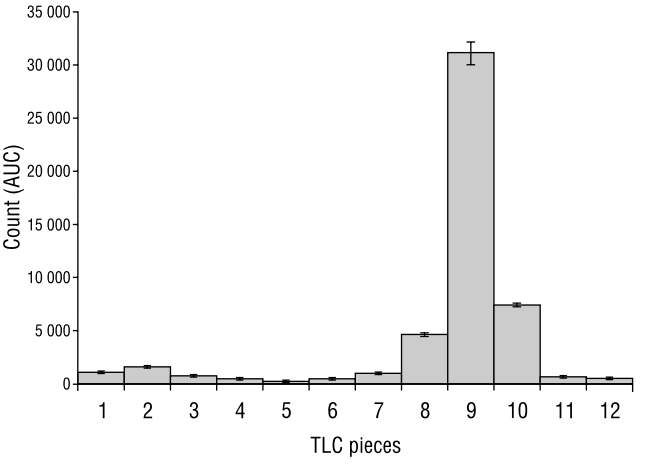

Figure 3. RTLC of the radiolabelling vial before injection into C_{18} Sep-Pak. AUC — area under curve.

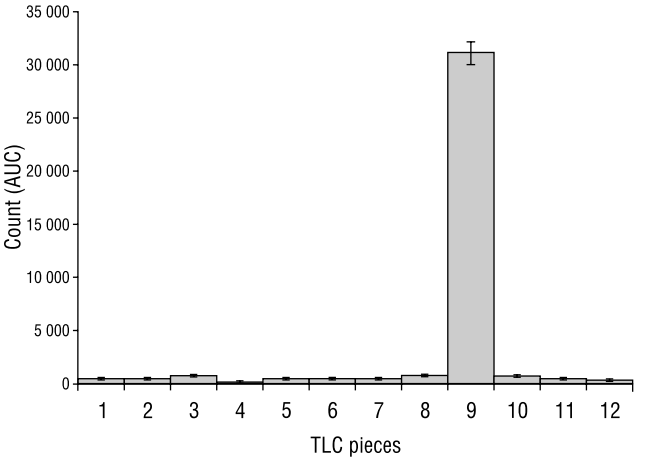

Figure 4. RTLC of final fraction after injection into C_{18} Sep-Pak. AUC — area under curve.

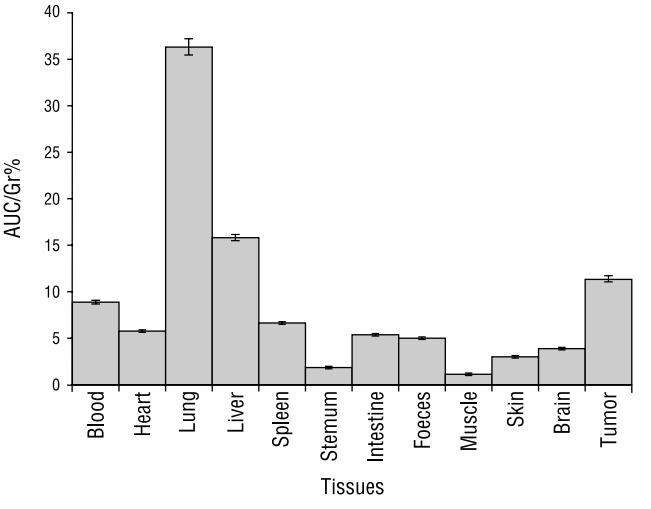

Figure 5. Biodistribution of [^{64}Cu]PTSM in fibrosarcoma-bearing rats 1 h post-injection of 15 μCi of the tracer. AUC — area under the curve of 511 keV peak.

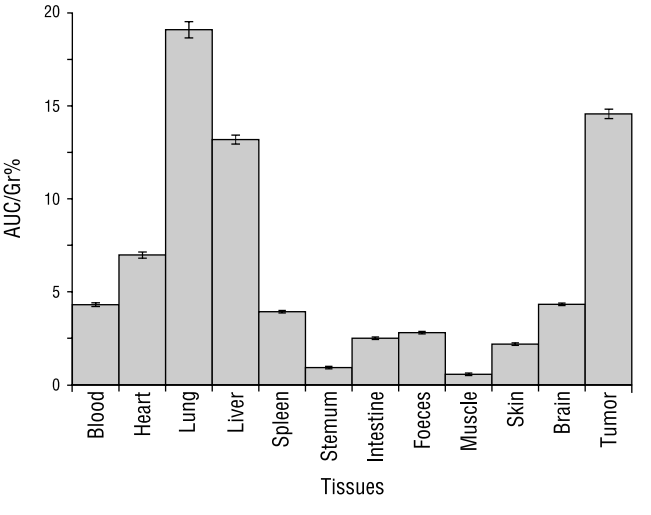

Figure 6. Biodistribution of [^{64}Cu]PTSM in fibrosarcoma-bearing rats 2 h post-injection of 15 μCi of the tracer. AUC — area under the curve of 511 keV peak.

Condition optimization

In order to obtain the best labelling reaction conditions, the complex formation was studied for temperature. Heating the reaction mixture to 50°C did not change the yield, while some degradation products were obtained via TLC and RTLC. Therefore, we continued the labelling procedure at ambient temperature.

Formulation and stability

The final radiolabelled complex diluted in normal saline, was then passed through a 0.22 μm filter (Millipore) when filtration was used to sterilize the product. Due to its thermal instability, [^{64}Cu]PTSM preparation could be totally degraded and left detectable amounts of free copper after autoclaving. The chemical stability of [^{64}Cu]PTSM was high enough to perform further studies. The final product RTLC showed no change in stability, and the patterns for trace [^{64}Cu]CuOAc and [^{64}Cu]PTSM were not changed during 5 hrs.

Partition coefficient

As expected from the RTLC behaviour, the lipophilicity of [^{64}Cu]PTSM 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.

Biodistribution studies

The biodistribution studies were performed 1 and 2 hours post injection of the tracer in normal and tumour bearing mice. After 2 hours a significant tumour uptake was observed as well as lung uptake. Post-mortem studies on the animals showed that the high lung uptake was mainly caused by tumour metastasis from dorsal injection site. Tumour to muscle uptake ratio was showed to be at least 9.0 and tumour to blood uptake ratio was 6.0. From previous human studies, it was already shown that the tracer accumulates in the liver, spleen and heart and, to a lesser extent, in the brain [28]. This was in agreement with our observations in this study (Figures 5, 6).

Conclusion

Total labelling and formulation of [^{64}Cu]PTSM took about 10 min, with a yield of 97–98%. A suitable specific activity product was formed via insertion of [^{64}Cu]copper cation. No unlabelled and/or labelled by-products were observed upon RTLC analysis of the final preparations after solid phase extraction (SPE) purification. The radiolabelled 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 labelling. Trace amounts of [^{64}Cu]copper acetate (≈ 2%) were detected by RTLC. The radiochemical purity of the [^{64}Cu]PTSM was higher than 98%. [^{64}Cu]PTSM is a therapeutic/PET radiotracer with an intermediate half life, and the high chemical stability of this radiophar-

maceutical makes it a very suitable diagnostic/therapeutic agent to be sent to clinics. This study also demonstrates the possibility of fibrosarcoma tumour therapy and/or imaging using [64Cu]PTSM.

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