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Evaluation of a [^{67}Ga]-Thiosemicarbazone Complex as Tumor Imaging Agent

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Abstract

[^{67}Ga]labeled 2-acetylpyridine 4,4-dimethylthiosemicarbazone ([^{67}Ga]-[$APTSM_2$]$_2^+$) was prepared using freshly prepared [^{67}Ga]$GaCl_3$ and 2-acetylpyridine 4,4-dimethylthiosemicarbazone ($APTSM_2$) for 30 min at 90°C (radiochemical purity: >97% ITLC, >98% HPLC, specific activity: 15–20 Ci/mmol). Stability of the complex was checked in human serum for 37°C. The biodistribution of the labeled compound in vital organs of normal and fibrosarcoma bearing mice were compared with that of free Ga^{3+} cation up to 24h. Initial SPECT images and biodistribution results showed significant tumor uptake in fibrosarcoma-bearing mice after 2 hour post injection.

Keywords

^{67}Ga-complexation • Thiosemicarbazone • Biodistribution • SPECT • Fibrosarcoma

Introduction

The interesting physical properties and availability of gallium-67 make it an interesting nuclide for radiopharmaceutical research [1]. The increasing trend in the production and use of PET radionuclides in nuclear medicine has offered new opportunities for researchers to focus on the production of new Ga-radiopharmaceuticals for feasibility studies using Ga-67 for their future PET gallium homologs. Table 1. demonstrates the most important Ga radionuclide physical properties.

Tab. 1. Nuclear properties of Ga radionuclides

Properties	^{67}Ga	^{68}Ga	^{66}Ga
Gamma energy (keV)	3 185 300	511(β+)	511(β+) 834 1039 2752
β-/β+ energy	84 92	1900(β+)	4153(β+)
Mode of decay	EC to ^{67}Zn	10% EC to ^{68}Zn 90% β+	43% EC to ^{66}Zn 57% β^+
Nuclear reaction	^{68}Zn(p,2n)^{67}Ga	^{68}Ge Daughter ^{66}Zn(α,2n)^{68}Ge	^{66}Zn(p,n)^{66}Ga
Half-life	78 h	68 min	9.6 h
Natural abundance(%)	(18%)	(28%)	(28%)
possible contaminations	^{66}Ga, ^{65}Zn	^{68}Ge	^{65}Zn
Proton energy(MeV)	12–22	12–22	6–15

Thiosemicarbazone gallium complexes have shown interesting anti-proliferative activity in vitro and in vivo [2]. The most studied compounds are pyridine-based compounds [3–6], this is possibly due to their resemblance to pyridoxal metabolites that attach to co-enzyme B_6-dependant enzymes and cause enzyme inhibition [7].

Due to the importance of pyridine thiosemicarbazones in anti-neoblastic activity and the necessity of gallium complexation in most of these compounds for enhancement of their activity, the idea of developing a possible tumor imaging agent using SPECT by incorporating ^{67}Ga into a suitable chelate, i.e. $APTSM_2$ was investigated (Fig. 1).

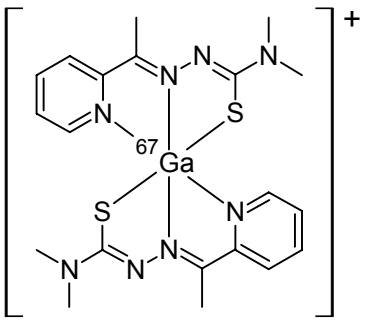


Fig. 1. Structure of $[^{67}Ga][APTSM_2]_2^+$

Results and discussion

Synthesis of cold compounds

The reaction of 2-acetylpyridine 4,4-dimethylthiosemicarbazone (HL) [2] with $GaCl_3$ in absolute ethanol in 1:1 molar ratio has yielded the complex $[GaL_2]^+ = [^{67}Ga][APTSM_2]_2^+$.

The crystal structure of the gallium(III) complex has already been determined by X-ray diffraction as well as other spectroscopic methods [2]. Interestingly, the cytotoxicity assay in several human cancer cell lines (SW480, SK-BR-3 and 41M) suggested that the gallium(III) complex might be endowed with promising antitumour properties [2].

Incorporation of radiogallium into HL moiety was interesting to us due to our background in production and evaluation of radio-gallium ligands for diagnosis of malignancies [8].

Production

Gallium-67, in form of $GaCl_3$, was prepared by 24 MeV proton bombardment of the ^{68}Zn target at Cyclone-30 on a regular basis. The target was bombarded with a current intensity of 170 μA and a charge of 1400μAh. The chemical separation process was based on a no-carrier-added method.

Radiochemical separation was performed by a two-step ion exchange chromatography method with a yield of higher than 95%. Quality control of the product was performed in two steps. Radionuclidic control showed the presence of 93 (40%), 184 (24%), 296 (22%), 378 (7%) keV gamma energies, all originating from ^{67}Ga and showed a radionuclidic purity higher than 99% (E.O.S.). The concentrations of zinc (from target material) and copper (from target support) were determined using polarography and shown to be below the internationally accepted levels, i.e. 0.1 ppm for Zn and Cu [9, 10].

Radiolabeling

Because of the engagement of several polar functional groups in its structure, labeling of $APTSM_2$ with gallium cation affects its chromatographic properties and the final complex is more lipophilic. The free gallium remains at the origin of the paper as a single peak.

In case of radiolabeling the more lipophil complex was tried using various conventional mobile pahses, however none were able to distinguish $^{67}Ga^{3+}$ from the radiolabled complex, that might be explained by the fact that the complex itself is a cationic species with polarity. The suitable solvent used was methanol and other methanol mixtures (Figures 2, 3).

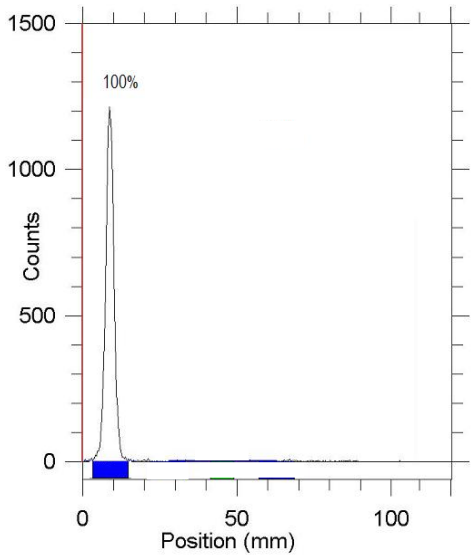


Fig. 2. ITLC of [^{67}Ga]$GaCl_3$ in pure methanol as mobile phase on Whatman No.2 papers.

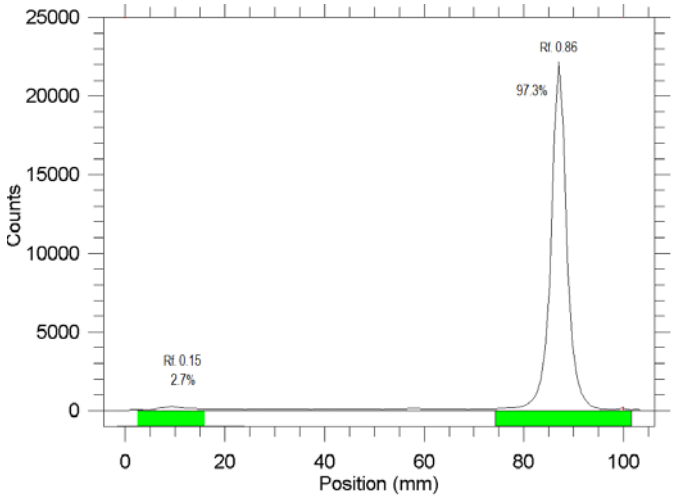
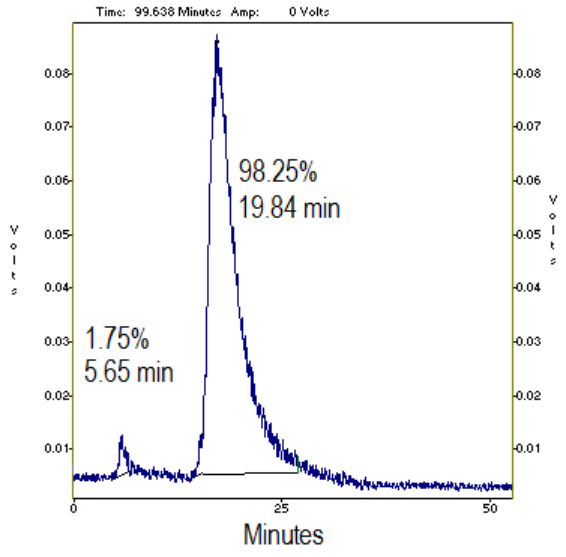


Fig. 3. ITLC of $[^{67}Ga][APTSM_2]_2^+$ in pure methanol as mobile phase on Whatman No.2 papers.



[1]

Fig. 4. HPLC chromatogram of $[^{67}Ga][APTSM_2]_2$+ solution on a reversed phase column using acetonitrile + 0.1% TFA/water + 0.1% TFA, 90:10

The cationic nature of the complex was a major obstacle in HPLC radioanalysis and a cationic column was preferable. However we used a reverse phase column in our settings and it worked with a tolerable difference in the retention times, enough for analytical measurements. Free Ga eluted at 5.65 minutes while the complex was eluted at 19.84 minutes demonstrating a radiochemical purity of higher than 98 percent using optimized conditions without further purifications (Figure 4).

When a $^{67}GaCl_3$ batch was older than 2 days, the radiochemical yield drastically dropped and a more polar complex with an R_f of 0.25–0.30 was detected that was not stable enough in normal saline as well as serum. In case of using 4-day old radiogallium, no detectable labeled compound was observed in ITLC at the R_f of 0.86, and the only radioactive component seemed to be free Ga^{3+}. This behavior might be explained by the

formation of non-radioactive zinc components produced by Ga-67 decay to Zn-67, since Zn-N,N-dimethyl-thiosemicarbazonato complexes have been reported to be very stable complexes [11]. So freshly prepared batches of Ga-67 were favored over older ones. Rapid complexation of gallium with 4,4-dimethylthiosemicarbazones in alcoholic solutions has been reported [2], therefore we tried this approach in alcoholic media. The labeling was not satisfactory when water was present in the solvent. At room temperature no detectable complex was formed. The best temperature was found to be 85–90°C. At this temperature, when freshly prepared gallium-67 was used, all the radio-gallium was inserted into the complex. Although in the reported method of $[^{67}Ga][APTSM_2]_2^+$ preparation [2], the complex was formed at room temperature at molar scales, at the radiochemical scales (nanomoles), the reaction was not fast enough to be performed at room temperature. While heating the reaction mixture over 100°C or for more than 1h, the radiochemical yield dropped. The final radiolabeled complex in alcoholic media was diluted in normal saline to a 2–4% solution. The solution was stable at room temperature up to 4 days post-formulation, allowing performance of biological experiments. Before experiments, the solution passed through a 0.22µm filter (Millipore).

Stability

The chemical stability of $[^{67}Ga][APTSM_2]_2^+$ was high enough to perform further studies. Incubation of $[^{67}Ga][APTSM_2]_2^+$ in freshly prepared human serum for 2 days at 37°C showed no loss of ^{67}Ga from the complex. The radiochemical purity of complex remained at 98% for 2 days under physiologic conditions.

Biodistribution

For better comparison a biodistribution was performed for free Ga^{3+} as well. The ID/g% data are summarized in Fig. 5.

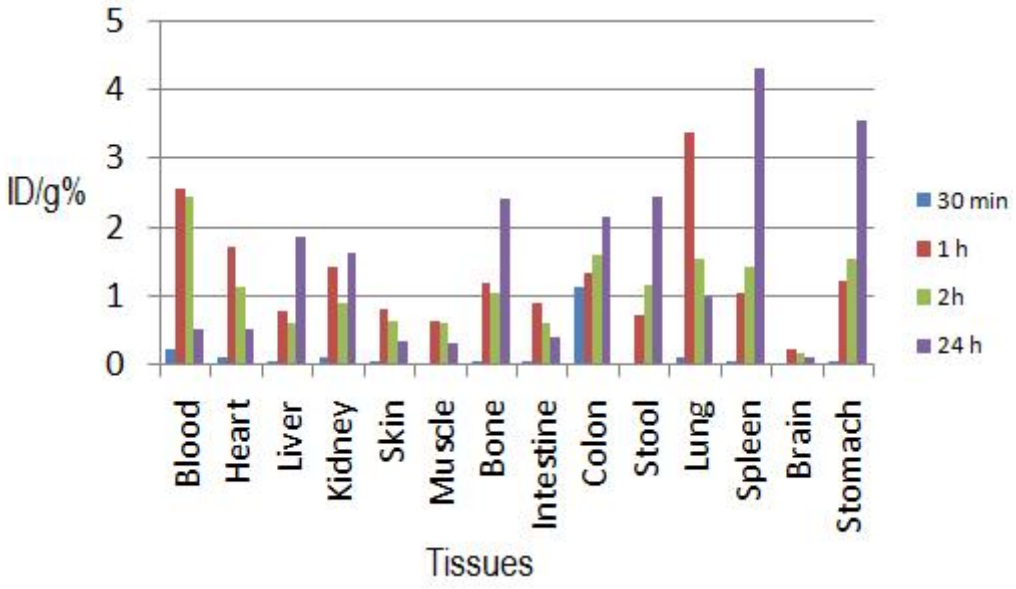


Fig 5. Biodistribution of $[^{67}Ga]GaCl_3$ (1.85 MBq, 50µCi) in normal mice 0.5–24h after iv injection via tail vein (ID/g%: percentage of injected dose per gram of tissue calculated based on the area under curve of 184 keV peak in gamma spectrum) (n=5)

Two hour post-injection the radioactivity enhanced in the kidneys (Fig. 6). This pattern stayed constant up to 24 hours. The radioactivity of intestine as well as stomach was high at 2h which could be due to the metabolism of the radiolabelled complex in the liver. The pattern for bone and brain remained almost unchanged.

In case of brain no penetration of the complex is observed due to the hydrophilic nature of the complex (GaL^+). The concentration of the tracer in blood decreases almost linearly. Interestingly the amount of the radioactivity was increasing in the heart muscle even without considering decay correction. This might be due to the accumulation of the monocationic metal complexes in myocardium like that of [^{99m}Tc]-MIBI$^+$ [12]. The mechanism of myocardium accumulation needs to be studied further. The tracer is possibly accumulated in the liver as seen after 2h, and possibly degraded to release free Ga^{3+}. A clear accumulation occurred in the spleen and the reticuloendothelial system after 24h possibly due to the biodistribution of free Ga^{3+}.

The biodistribution data for radiolabeled compound in fibrosarcoma mice is presented in Figure 6. The tumor uptake is not significant at least an hour post-injection however after 2 hours a significant radioactivity was accumulated in the tumor mass. But after 24 hours the tumor uptake fades. This is an interesting result since compared to the Ga^{3+} cation usually the tumor uptake increases after 24–48 hours due to various mechanisms suggested such as transferrin receptors and/or acidity of the tumor cells [13].

However, this is not a residualizing tracer as would be desirable for tumor diagnosis while using Ga-67, while the use of Ga-68 can be a better option. Also the target/non-target ratios were not suitable.

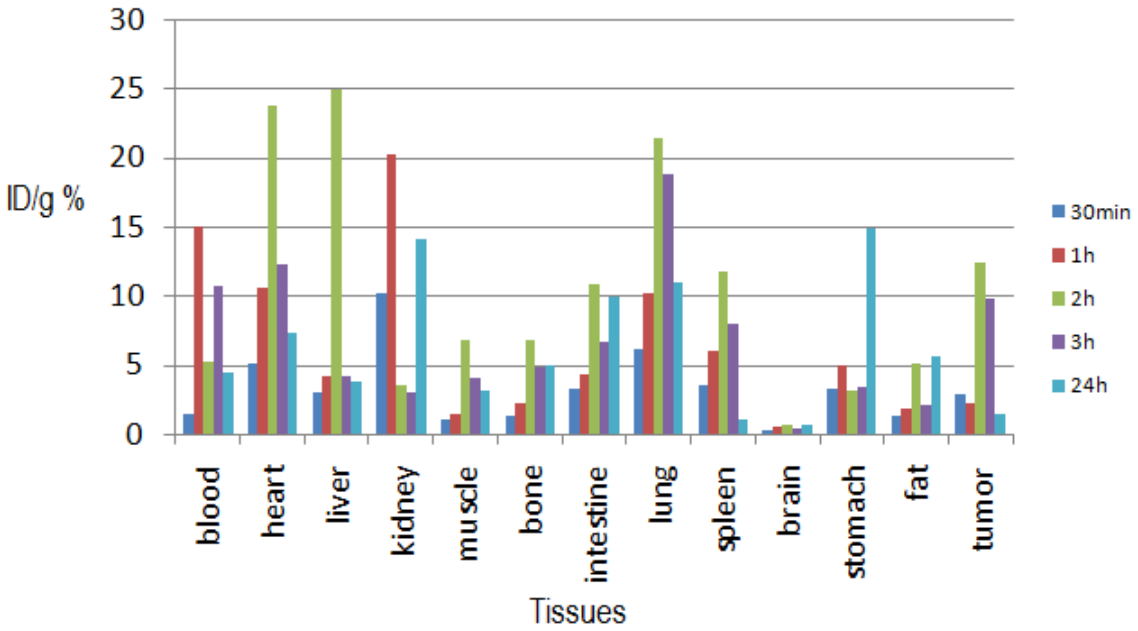


Fig 6. Biodistribution of [^{67}Ga][$APTSM_2$]$_2^+$ (1.85 MBq, 50μCi) in fibrosarcoma-bearing mice 0.5–24h after iv injection via tail vein (ID/g%: percentage of injected dose per gram of tissue calculated based on the area under curve of 184 keV peak in gamma spectrum) (n=5)

Imaging of fibrosarcoma-bearing mice

[^{67}Ga][APTSM$_2$]$_2^+$ imaging in the fibrosarcoma-bearing mice showed a distinct accumulation of the radiotracer in the tumor tissue around the lumbar region (Fig. 7) 2 h post injection where a visible lump can be diagnosed. The retention of the radioactive material in the target organ was further investigated by anatomical study of the tumor position after imaging. Tumor accumulation is not due to Ga^{3+} free cation (like that of Ga-citrate), since gallium scans usually result in positive tumor uptake at longer times.

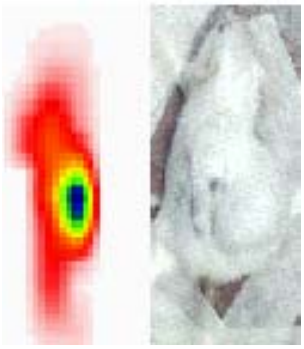


Fig. 7. SPECT images of [^{67}Ga][APTSM$_2$]$_2^+$ (90 MBq, 22 μCi) in fibrosarcoma bearing mice 2h post injection

Experimental

Enriched zinc-68 chloride with a purity of more than 95% was obtained from Ion Beam Separation Group at Agricultural, Medical and Industrial Research School (AMIRS). Production of ^{67}Ga was performed at the Nuclear Medicine Research Group (AMIRS) 30 MeV cyclotron (Cyclone-30, IBA). Other chemicals were purchased from the Aldrich Chemical Co. (Gemany); and the ion-exchange resins from Bio-Rad Laboratories (Canada). NMR spectra were obtained on a FT-80 Varian instrument (80MHz) with tetramethylsilane as the internal standard. Infrared spectrum was measured on a Perkin-Elmer 781 spectrometer using a KBr disc. Mass spectrum was recorded by a Finnigan Mat TSQ-70 Spectrometer. Thin layer chromatography (TLC) for cold compounds was performed on polymer-backed silica gel (F 1500/LS 254, 20 × 20 cm, TLC Ready Foil, Schleicher & Schuell®, Germany). Normal saline and sodium acetate used for labeling were of high purity and had been filtered through 0.22 μm Cativex filters. Instant thin layer chromatography (ITLC) was performed by counting Whatman No. 2 papers using a thin layer chromatography scanner, Bioscan AR2000, Bioscan Europe Ltd. (France). Analytical high performance liquid chromatography (HPLC) used 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 Whatman Partisphere C-18 column 250×4.6 mm, Whatman, NJ (USA). Analytical HPLC was also used to determine the specific radioactivity of the labeled compound. A standard curve was generated to calculate the mass of the final solution. Biodistribution data were acquired by counting normal saline washed tissues after weighting 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 ITLC counting were

based on the 184 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 edn.

Production of 67Ga

$^{68}Zn(p,2n)^{67}Ga$ was used as the best nuclear reaction for the production of ^{67}Ga. Other impurities could be removed in the radiochemical separation process. After the target bombardment process, chemical separation was carried out in no-carrier-added form. The irradiated target was dissolved in 10 M HCl (15 ml) and the solution was passed through a cation exchange resin (AG 50W, H+ form, mesh 200-400, h:10 cm, Ø:1.3 cm) which had been preconditioned by passing 25 ml of 9 M HCl.

The column was then washed by 25 ml of 9M HCl at a rate of 1 ml/min to remove copper and zinc ions. To the eluent 30 ml water plus about 100 ml of a 6 M HCl solution was added. The latter solution was loaded on another exchange resin (AG1X8 Cl^- form, 100–200 mesh, h: 25 cm, Ø: 1.7 cm) pretreated with 6 M HCl (100 ml). Finally, the gallium-67 was eluted as $[^{67}Ga]GaCl_3$ using 2 M HCl (50 ml); the whole process took about 60 min.

Quality control of the product

Control of Radionuclide purity:

Gamma spectroscopy of the final sample was carried out counting in an HPGe detector coupled to a Canberra™ multi-channel analyzer for 1000 seconds.

Chemical purity control:

This step was carried out to ensure that the amounts of zinc and copper ions resulting from the target material and backing in the final product are acceptable regarding internationally accepted limits. Chemical purity was checked by differential-pulsed anodic stripping polarography. The detection limit of our system was 0.1 ppm for both zinc and copper ions.

Preparation of 2-acetylpyridine 4,4-dimethylthiosemicarbazone **[N,N-*dimethyl*-N′-*(1-pyridin-2-ylethylidene)carbamohydrazonothioic acid*]**

This compound was prepared with slight modifications to the reported method [2]. (60%) m.p. 148–150°C. ^1H NMR ($CDCl_3$) δ (ppm) 10.2 (s, 1H, NH), 8.64 (m, 1H, H_6 pyridine), 7.73 (m, 2H, H_5 & H_3 pyridine), 7.33 (m, 1H, H_4 pyridine), 3.42 (s, 6H, $N(CH_3)_2$), 2.51 (s, 3H, CH_3-C=N). IR ($CHCl_3$) λ max 3532, 3440, 2905, 1469, 1343, 1256. Mass (electrospray) 221.8 (M^+) (100%), 206.8 (74%), 121 (66%), 106 (72), 87.9 (88%), 78 (74%), 51 (44%).

Synthesis of Ga-[APTSM$_2$]$_2^+$ **[*(OC-6-33′)-Bis{*N,N-*dimethyl*-N′-*[1-(pyridin-2-yl-κ*N)*ethylidene]-carbamohydrazonothioato-κ^2*N′,S}gallium(1+) tetrachlorogallate(1-)*]**

The synthesis of the complex was according to the reported method with slight modifications and more supportive analytical data [2]. To 2-acetylpyridine 4,4-dimethyl-thiosemicarbazone (214 mg, 1 mmol) in dry ethanol (7 ml) under vigorous stirring was added drop wise over 10 min $GaCl_3$ (170 mg, 1 mmol) in dry ethanol (2 ml) under N_2

atmosphere. After 30 min, the product was filtered off, washed with dry ethanol and dry ether and finally re-crystallised from dry ethanol. Yield 62.0%; yellow plates suitable for spectroscopy; m.p. 238°C. ^{1}H NMR (DMSO-d_6) 8.92 (d, 1H, H_1 pyridine), 8.37 (d, 1H, H_4 pyridine), 8.22 (m, 2H, two $H_{3,4}$ pyridine), 8.12 (m, 2H, two H_4 pyridine), 7.91 (m, 2H, two H_1 pyridine),7.84 (m, 2H, two H_2 pyridine), 7.57 (m,1H, H2' pyridine), 3.30 (s, 12H, $N(CH_3)_2$), 2.84 (s, 3H, =C-CH_3), 2.62 (s, 3H, =C-CH_3). ^{71}Ga NMR (D_2O): 248.95 ppm ($^{71}GaCl_3$ considered as 0.00 ppm).

Preparation of [^{67}Ga][APTSM$_2$]$_2^+$
[(OC-6-33')-Bis{N,N-dimethyl-N'-[1-(pyridin-2-yl-κN)ethylidene]-carbamohydrazonothioato-κ^{2}N',S}(^{67}Ga)gallium(1+) tetrachlorogallate(1-)]

The optimized radiolabeling of the tracer has been reported previously by our group [14]. The acidic solution (2 ml) of [^{67}Ga]$GaCl_3$ (111 MBq, 3 mCi) was transferred to a 5 ml-borosilicate vial and heated to dryness using a flow of N_2 gas at 50-60°C. Fifty microlitres of 2-acetylpyridine 4,4-dimethylthiosemicarbazone in absolute ethanol (1 mg/ml ≈173 nmoles) was added to the gallium-containing vial and vortexed at 90°C for 30–60 min. The mixture was then cooled to room temperature. The vial mixture was diluted by the addition of normal saline (4.5 ml). The active solution was checked for radiochemical purity by ITLC and HPLC. The final solution was then passed through a 0.22 µm filter and pH was adjusted to 5.5–7.

Quality control of [^{67}Ga][APTSM$_2$]$_2^+$

Radio thin layer chromatography:

A 5 µl sample of the final fraction was spotted on a chromatography whatman No. 2 paper, and developed in methanol as the mobile phase.

High performance liquid chromatography:

HPLC was performed with a flow rate of 1 ml/min, pressure: 130 kgF/cm^2 for 20 min. Radiolabeled compound was eluted using a mixture of two solutions (A: acetonitrile + 0.1% TFA/water + 0.1% TFA, 90:10) using reversed phase column Whatman Partisphere C_{18} 4.6 × 250 mm.

Stability tests

The stability of the complex was checked according to the conventional ITLC method [15]. A sample of [^{67}Ga][APTSM$_2$]$_2^+$ (37 MBq) was kept at room temperature for 2 days while being checked by ITLC at time intervals in order to check stability in final product using above chromatography system. For serum stability studies, to 36.1 MBq (976 µCi) of [^{67}Ga][APTSM$_2$]$_2^+$ was added 500µl of freshly prepared human serum and the resulting mixture was incubated at 37°C for 5 h, Aliquots (5-µl) were analyzed by ITLC.

Biodistribution in normal mice

The distribution of the radiolabelled complex among tissues was determined for normal mice immediately after imaging. The total amount of radioactivity injected into each mouse was measured by counting the 1-ml syringe before and after injection in a dose calibrator with fixed geometry. The animals were sacrificed by CO_2 asphyxiation at selected times after injection (2 to 24h), the tissues (blood, heart, lung, brain, intestine, faeces, skin,

stomach, kidneys, liver, muscle and bone) were weighed and rinsed with normal saline and their specific activities were determined with a HPGe detector equipped with a sample holder device as percent of injected dose per gram of tissues.

Induction of fibrosarcoma tumors in mice

Tumor induction performed by the use of poly aromatic hydrocarbon injection in rodents as reported previously [16]. For tumor model preparation, 10µl of 3-methyl cholanthrene solution in extra-virgin olive oil (4 mg/ml) was injected 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 in tumor-bearing animals

The distribution of ^{67}Ga-APTSM$_2$ among tissues was determined for fibrosarcoma-bearing mice. A volume (0.1 ml) of the final [^{67}Ga][APTSM$_2$]$_2^+$ solution containing 50 µCi activity (≤ 2 µg APTSM$_2$ in 100 µl) was injected into the dorsal tail vein. The total amount of activity injected into each mouse was measured by counting the 1-ml syringe before and after injection in an activity meter with fixed geometry. The animals were sacrificed by CO_2 asphyxiation at selected time intervals post injection, the tissues were weighed and their specific activities were determined by HPGe detector as a percentage of the injected dose per gram of tissues.

Imaging of [^{67}Ga][APTSM$_2$]$_2^+$ in tumor bearing mice

Fibrosarcoma-bearing mice were used for tumor imaging when tumors reached a size of 0.2–0.4 g, 14–16 weeks after its induction. Images were taken 1, 2 and 3 hours after administration of the tracer by a dual-head SPECT system. The mouse-to-high energy septa distance was 12 cm. The useful field of view (UFOV) was 540 mm×400 mm. The spatial resolution was 10 mm FWHM at the CFOV. Sixty four projections were acquired for 30 seconds per view with a 64×64 matrix.

Conclusion

Total labeling and formulation of [^{67}Ga][APTSM$_2$]$_2^+$ took about 70 minutes, with a radiochemical purity >98% and a specific activity of ≈15–20 Ci/mmol. No unlabelled and/or labeled by-products were observed.

No unlabelled and/or labeled by-products were observed by ITLC/HPLC analysis of the final preparations. The radio-labeled complex was stable in aqueous solution and in human serum at 37°C, for at least 2 days, and no significant amount of other radioactive species were detected by ITLC. Biodistribution of the complex in fibrosarcoma bearing mice demonstrated significant tumor uptake at 2h post injection. The SPECT images also showed the specific accumulation in tumors at 2h post injection. All the tests were compared with that of $^{67}GaCl_3$ in tumor bearing rodents showing the different pattern of accumulation of the tracer. It is suggested that [^{67}Ga][APTSM$_2$]$_2^+$ could be a possible SPECT tracer, however considering the fast tumor uptake, the short half life gallium-68 can be a better candidate for tumor imaging applications and future [^{68}Ga]-PET studies and less imposed radiation doses to patients.

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Authors' Statements

Competing Interests

The authors declare no conflict of interest.

Animal Rights

The institutional and (inter)national guide for the care and use of laboratory animals was followed. See the experimental part for details.

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