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The development of radiogallium-acetylacetonate bis(thiosemicarbazone) complex for tumour imaging

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Abstract

BACKGROUND: Various radiometal complexes have been developed for tumour imaging, especially Ga-68 tracer. In this work, the development of a radiogallium bis(thiosemicarbazone) complex has been reported.

MATERIAL AND METHODS: [^{67}Ga]acetylacetonate bis(thio-semicarbazone) complex ([^{67}Ga]AATS) was prepared starting with [^{67}Ga]Gallium acetate and freshly prepared acetylacetonate bis(thiosemicarbazone) (AATS) for 30 min at 90°C. The partition co-efficient and stability of the tracer was determined in final solution (25°C) and the presence of human serum (37°C) for up to 24 hours. The biodistribution of the labelled compound in wild-type and fibrosarcoma-bearing rodents were determined for up to 72 hours.

RESULTS: The radiolabelled Ga complex was prepared to a high radiochemical purity (> 97%, HPLC) followed by initial biodistribution data with the significant tumour accumulation of the tracer at two hours, which is far higher than free Ga-67 cation, while the compound wash-out is significantly faster.

CONCLUSION: The above-mentioned pharmacokinetic properties suggest an interesting radiogallium complex prepared by the PET Ga radioisotope, ^{68}Ga, in accordance with the physical half life, for use in fibrosarcoma tumours and possibly in other malignancies.

Key words: Gallium-67, acetyl acetone bis(thiosemicarbazonate), biodistribution, fibrosarcoma

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Introduction

The physical properties and availability of gallium-67 make it an interesting nuclide for radiopharmaceutical research [1]. A growing increasing trend in the production and use of PET gallium nuclides in nuclear medicine has offered new opportunities for researchers to focus on the production of new ^{67}Ga-radio-pharmaceuticals for feasibility studies for their future PET gallium homologues.

The auger electrons emitted by ^{67}Ga possess potent cytotoxicity pointing towards potential therapeutic applications of the radionuclide [2], while the positrons emitted by ^{68}Ga may also have therapeutic applications in the prevention of restenosis by intracoronary radiation therapy [3].

Thiosemicarbazone gallium complexes have shown interesting anti-proliferative activity *in vitro* and *in vivo* [4]. The most studied compounds are nitrogen-containing heterocycles [5]; this is possibly due to their resemblance to the pyridoxal metabolites that attach to co-enzyme B_6-dependant enzymes and cause enzyme inhibition [6]. Various gallium-based radiotracers have been reported by Green et al. [7–9], including an acetoacetate gallium-67 complex as a potential radiopharmaceutical [10].

Table 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	43% EC to ^{66}Zn
	90% β+	57% β+	
Nuclear reaction	^{68}Zn(p,2n)^{67}Ga	^{68}Ge Daughter	^{66}Zn(p,n)^{66}Ga
		^{66}Zn(b,2n)^{68}Ge	
Natural abundance (%)	(18%)	(28%)	(28%)
Possible contaminations	^{66}Ga, ^{65}Zn	^{68}Ge	^{65}Zn
Proton energy (MeV)	12–22	12–22	6–15

However, there are rare examples of gallium bis thiosemicarbazones, according to our knowledge, while copper analogues of bis thiosemicarbazones have been extensively studied [11, 12]. Their biological activity and structure-activity have been well reported and the retention mechanisms in hypoxic and normoxic tumours are cited.

Usually, traditional bis thiosemicarbazones such as ATSM, PTSM, etc. do not form complexes with gallium due to many chemical and molecular orbital considerations. However, in our experiments we were able to detect interesting radiolabelling properties for acetyl acetoacetate bis thiosemicarbazone homologues. Such complexes have not been reported by others, while information on the copper and nickel complexes have recently been reported [13].

Recently we have reported the radiosynthesis of ^{67}Ga-labelled pyridine-based thiosemicarbazone (^{67}Ga-APTSM$_2$) [14] as well as its evaluation in fibrosarcoma-bearing mice using scarification and SPECT studies [15].

Due to the interesting anti-neoblastic activity of gallium thiosemicarbazones and the possibility of developing a new series of ^{67}Ga-labelled acetyl acetate bis-thiosemicarbazonate complexes as possible tumour imaging agents using SPECT, the production, purification, and biodistribution studies of [^{67}Ga]-AAPS were investigated (Figure 1).

Materials and methods

Enriched zinc-68 chloride with a purity of more than 95% was obtained from the Ion Beam Separation Group at the Agricultural, Medical, and Industrial Research School (AMIRS). Production of ^{67}Ga was performed using the Nuclear Medicine Research Group (AMIRS) 30 MeV cyclotron (Cyclone-30, IBA). Other chemicals were purchased from the Aldrich Chemical Co. (Germany) 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). The normal saline and sodium acetate used

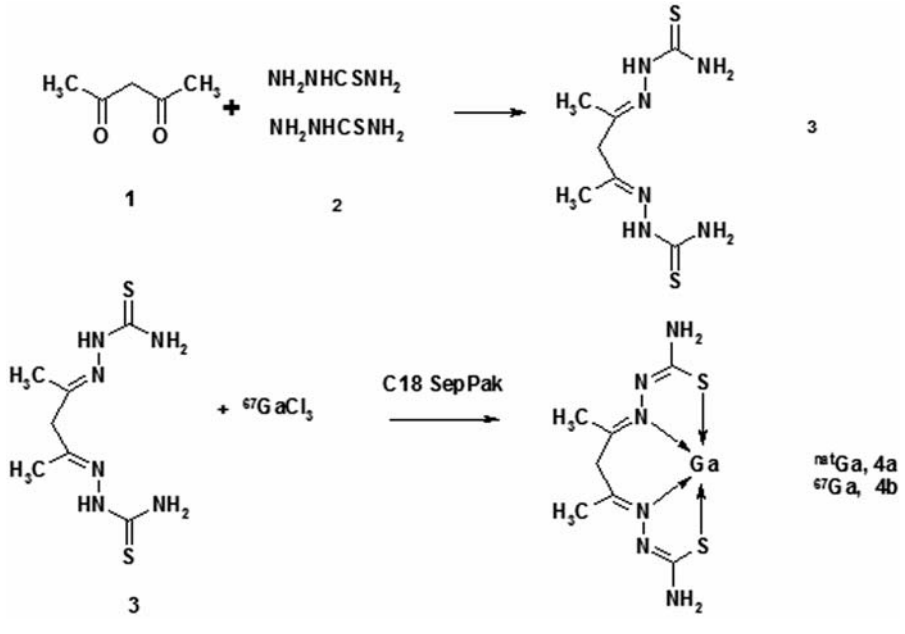

Figure 1. Synthesis of [^{67}Ga]AATS.

for labelling 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 a Whatman Partisphere C-18 column 250 × 4.6 mm, Whatman, NJ (USA). 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 counts 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 edition.

Production of ^{67}Ga

^{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, ∅: 1.3 cm) which had been preconditioned by passing 25 ml of 9 M HCl.

The column was then washed with 25 ml of 9M HCl at a rate of 1 ml/min to remove copper and zinc ions. To the eluent, 30 ml of 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, ∅: 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

The presence of zinc cation was detected by visible colorimetric assays. Even at 1 ppm of standard zinc concentration, the pinkish complex is visible to the naked eye, while the test sample remained similar to the blank [16]. The amount of copper cation was checked in the final solution using colour formation with dithizone reagent [17].

Production of acetylacetonate bis(thiosemicarbazone) (AATS) [3]

This compound was prepared with slight modifications to the reported method [18]. Briefly, to a transparent stirring mixture of thiosemicarbazide (2 mmol) (2) in 5% acetic acid at 50°C, was added drop wise freshly distilled acetyl acetone (1 mmol) (1) during 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 (60%) m.p. 148–150°C. ^{1}H NMR ($CDCl_3$) δ (*ppm*) 9.19 (s, 1H, NH), 7.91 (s, 2H, -CH_2-), 2.86 (d, 6H, CH_3-C=N). IR ($CHCl_3$) λ max 3535, 3129, 2956, 2495, 2361, 1563, 1399, 1215, 1041, 661, 556. Mass (electrospray) 190.1 (M^+).

Preparation of [^{67}Ga]acetylacetonate bis(thiosemicarbazone) complex ([^{67}Ga]AATS):

The acidic solution (2 ml) of [^{67}Ga]$GaCl_3$ (111 MBq, 3 mCi) was transferred to a 5 ml-borosilicate vial containing 0.5 ml of acetate buffer pH.5.5. Fifty microlitres of acetylacetonate bis(thiosemicarbazone) (AATS) in absolute ethanol (1 mg/ml ≈ ≈ 260 nmoles) was added to the gallium-containing vial and vortexed at 80–90°C for 30 minutes. 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. In case of high free gallium content presence, the mixture (about 5 ml) was cooled in an ice bath and rapidly injected into a C_{18} Sep-Pak column pretreated with 5ml of ethanol and 2 ml of water. The column was washed with water (4 ml) and purged with a stream of dry N_2. The 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 followed by passing through a 0.22 μm filter, and the pH was adjusted to 5.5–7.

Quality control of [^{67}Ga]AATS

Radio thin layer chromatography

A 5 μl sample of the final fraction was spotted on a chromatography Whatman No. 2 paper, and developed in 10% ammonium acetate:methanol (1:1) mixture 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. Radiolabelled 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 of [^{67}Ga]AATS complex in the final product

Stability tests were based on previous studies performed for radiolabelled metal complexes [19]. A sample of [^{67}Ga]AATS (5 mCi) was kept at room temperature for 4 days while being checked by RTLC every half an hour. A micropipette sample (5 μl) was taken from the shaking mixture, and the ratio of free radiogallium to [^{67}Ga]AATS was checked by instant thin layer chromatography.

Serum stability studies

To 36.1 MBq (976 μCi) of [^{67}Ga]AATS was added 500 μl of freshly prepared human serum, and the resulting mixture was incubated at 37°C for 2 days. Aliquots (5-μl) were analyzed by ITLC 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 [67Ga]AATS was measured following 1 minute of vigorous vortex mixing of 1 ml of 2-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 ^{67}Ga 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.

Induction of fibrosarcoma tumours in rodents

Tumour induction was performed by the use of poly aromatic hydrocarbon injection in rodents, as reported previously [20].

For tumour model preparation, 10 μl of 3-methyl cholanthrene solution in extra-virgin olive oil (4 mg/ml) was injected SC into the dorsal area of the mice. After 14–16 weeks the tumour weighed 0.2–0.4 g and was not grossly necrotic. Tumour tissues of some random animals were sent for pathological tests and were diagnosed as fibrosarcoma.

Biodistribution in wild-type and fibrosarcoma-bearing animal tissues

The distribution of the radiolabelled complex among tissues was determined for normal rats immediately after imaging. The total amount of radioactivity (35 $\pm$ 2 μCi) injected into each rat 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 (0.5, 1, 24, and 48), 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 an HPGe detector equipped with a sample holder device, as the percentage of injected dose per gram of tissue.

Results and discussion

Although there are reports on the reaction of acetyl acetone and thiosemicarbazides in the literature showing that the reaction is complicated by the possible formation of pyrazoline forms [21], it has fortunately been shown that these compounds, in the presence of mild acidic conditions (pH = 4–6) and metallic ions, afford in the metallic bis-thiosemicarbazonate complexes [22]. These studies are mostly performed in presence of copper and nickel cations although it is very possible that a similarity would be observed for Ga cations as well. A major drawback in copper complexes has proven to be the oxidation of the methylene backbone of the complex, while in 15 hours the oxidized species can be observed [17], leading to the formation of copper cations. Although we did not have any proof regarding the oxidation of the Ga complexes, the whole radiolabelling procedure was performed under N2 atmosphere to avoid possible oxidation. According to our knowledge, there are no reports of other Ga oxidation states in the literature.

Production

Gallium-67, as $GaCl_3$, 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 1400 μAh. The chemical separation process was based on a no-carrier-added method.

Radiochemical separation was performed by two-step ion exchange chromatography method with a yield 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%), and 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 visible colorimetric assays and were shown to be below the internationally accepted levels, i.e. 1 ppm Zn [14] and 5 ppm for Cu [15].

Radiolabelling of [^{67}Ga]AATS

Because of the engagement of several polar functional groups in its structure, labelling of AATS with a gallium cation affects its chromatographic properties, and the final complex is more lipophilic. Thus free gallium remains at the origin (Rf. 0.0), while the radiolabelled complex migrates to a higher Rf (0.86) (Figure 2).

The lipophilic nature of the complex was a major reason in HPLC distinct radio-analysis, and a reverse phase column was preferable. Free Ga eluted at 1.99 minutes while the complex was eluted at 18.57 minutes, demonstrating a radiochemical purity of 93 percent using optimized conditions without further purification (Figure 3).

Optimization

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. While heating the reaction mixture to over 100°C or for more than 1h, the radiochemical yield dropped. The final radiolabelled complex in alcoholic media was diluted in normal saline to a 5% solution.

The solution was stable at room temperature for up to 4 days post-formulation, allowing performance of biological experiments. Before experiments, the solution passed through a 0.22 μm filter (Millipore).

Stability

The chemical stability of [^{67}Ga]AATS was high enough to perform further studies. Incubation of [^{67}Ga]AATS in freshly prepared human serum for 2 days at 37°C showed no loss of ^{67}Ga from the complex. The radiochemical purity of the complex remained at 95 $\pm$ 3% for 4 days under physiologic conditions.

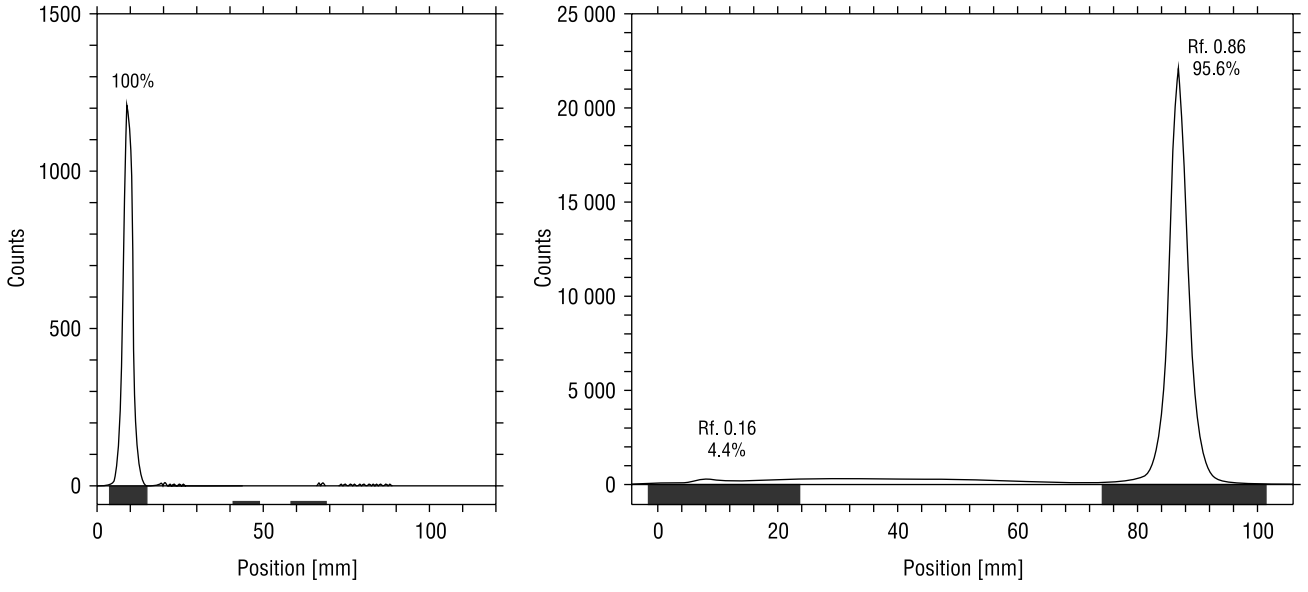

Figure 2. ITLC of [^{67}Ga]$GaCl_3$ (left) and [^{67}Ga]AATS (right) on Whatman No. 2 paper developed in 10% ammonium acetate:methanol (1:1).

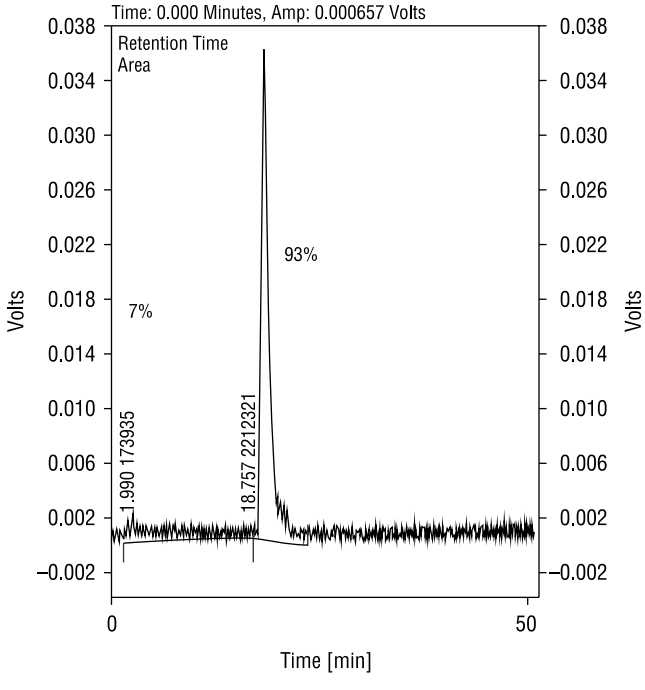

Figure 3. HPLC chromatogram of [^{67}Ga]AATS solution on a reversed phase column using acetonitrile + 0.1% TFA/water + 0.1% TFA, 90:10.

Biodistribution

One hour post-injection, the radioactivity was enhanced in the kidneys (Figure 4). This pattern rapidly dropped after two hours. The radioactivity of intestine as well as GI tract was high at two hours, which could be due to the metabolism of the radiolabelled complex in the liver. The pattern for sternum, skin, and brain remained almost unchanged. The major excretion route for the tracer is the urinary tract, as shown in Figure 4. A clear accumulation occurred in the spleen and the reticuloendothelial system after 24 hours, possibly due to the biodistribution of free Ga^{3+}.

The biodistribution data for radiolabelled compound in fibrosarcoma-bearing mice is presented in Figure 5. The tumour uptake is not significant for at least one hour post-injection; however, at two hours, major activity content (6–7%) accumulated in the tumour mass. Nevertheless, after 48 hours the tumour uptake faded. This is an interesting result since compared to the Ga^{3+} cation usually the tumour uptake increases after 24–48 hours due to various suggested mechanisms such as transferrin receptors and/or acidity of the tumour cells [23]

Conclusions

Total labelling and formulation of [^{67}Ga]AATS took about 40 minutes, with a radiochemical purity higher than 93% (HPLC). A significant specific activity (9.1 TBq/mmol or 246 Ci/mmol) was formed via insertion of ^{67}Ga cations. The radiolabelled complex was stable in aqueous solutions for at least 4 days, and 2 days in the presence of human serum, and no significant amount of other radioactive species were detected by ITLC 12 hours after labelling. Trace amounts of [^{67}Ga]gallium cation (≈ 4%) were detected by ITLC, which showed that radiochemical purity of the [^{67}Ga]AATS was higher than 96%; in HPLC studies a radiochemical purity of 93% was detected. The biodistribution of the tracer in wild-type rats demonstrated that the major route of excretion is the urinary tract. The tracer afforded significant tumour uptake (7%) after 24 hours in fibrosarcoma-bearing mice. [^{67}Ga]AATS can be a potential SPECT radiotracer for malignancy imaging. Further investigations on other tumour models and trapping mechanisms are required, while production of ^{68}Ga homologue can be of great interest for PET studies.

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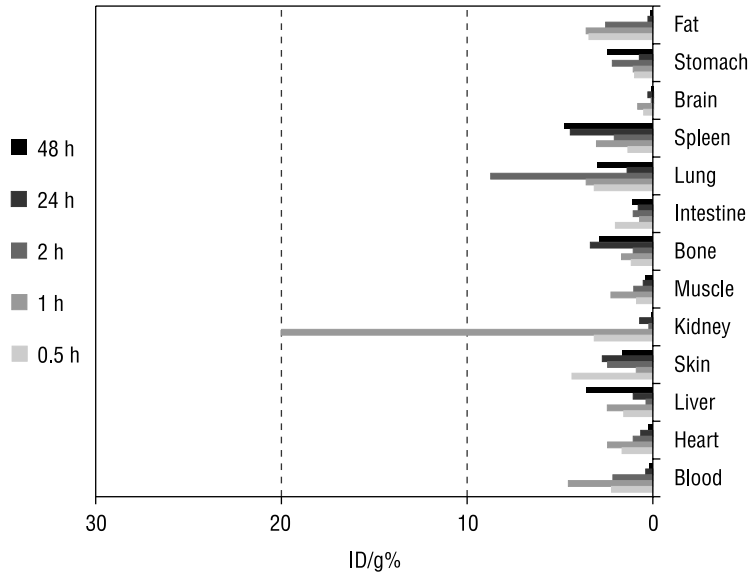

Figure 4. Biodistribution of [^{67}Ga]AAPS (1.85 MBq, 35 ± 2 μCi) in wild-type mice 0.5–48 hours after IV injection via the tail vein (ID/g%: percentage of injected per gram calculated using area under curve of 184 keV peak in gamma spectrum).

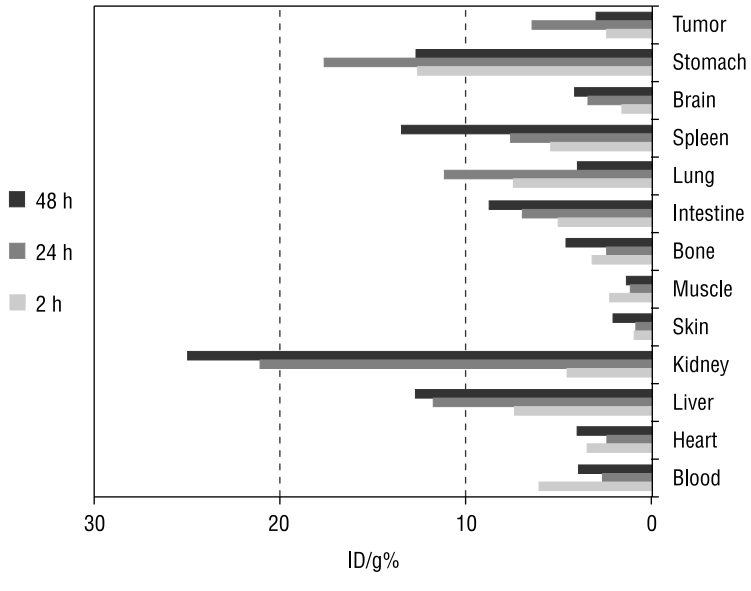

Figure 5. Biodistribution of [^{67}Ga]AAPS (1.85 MBq, 38 μCi) in fibrosarcoma-bearing mice 2–48 hours after IV injection via the tail vein (ID/g%: percentage of injected per gram calculated using area under curve of 184 keV peak in gamma spectrum).

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