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^{99m}Tc-Tricarbonyl-(2-amino-5,10-dioxide-7(8)-fluorophenazine) as Probe for Mammary Hypoxic-tumors Imaging

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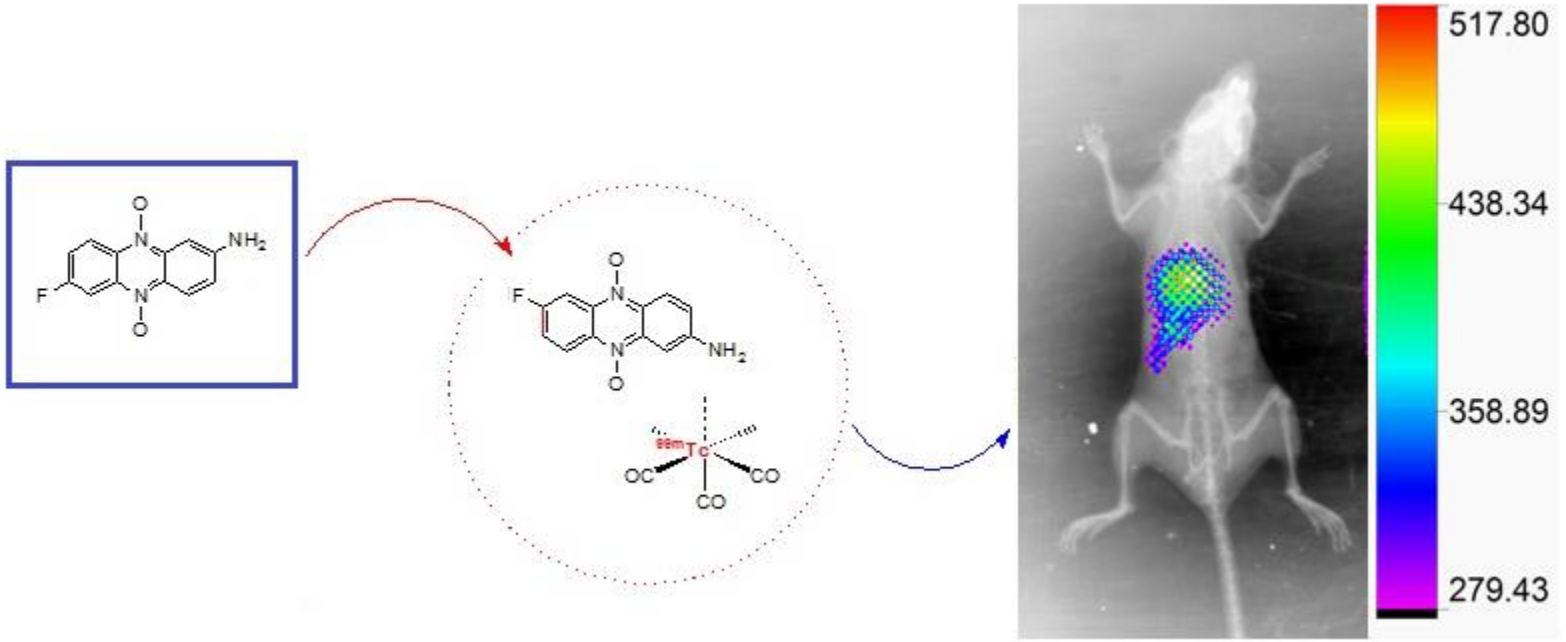


Abstract:
Our group has previously reported potential new radiopharmaceuticals, i.e. ^{99m}Tc-tricarbonyl-(2-amino-5,10-dioxide-7(8)-fluorophenazine), **Tc-FZ**, with the ability to detect hypoxic tumoral tissues. Previously, this probe was used to detect solid tumors, employing a model of lung carcinoma generated by inoculation of 3LL Lewis murine cells in C57BL/6 mice. The results revealed an adequate tumor/muscle ratio, 3.8 at 2 h post-injection (p.i.), with lower tumor/blood ratio, i.e. 0.6 at 2 h p.i..
Due to some models of breast cancer also being characterized by hypoxic areas, herein we decided to analyze the behavior of **Tc-FZ** in BALB-c mice bearing mammary tumor induced with 4T1-mouse tumoral cell line.
The studied ratios revealed to be slightly a little more unfavorable than in the lung tumor-porting animals, being tumor/muscle and tumor/blood ratios, at 2 h p.i., of 3.0 and 0.23, respectively.
Additionally, animals *in vivo* images showed that the liver masked tumor signal.

Keywords: Biomarkers; Biosensors; Imaging techniques

Introduction

Breast cancer is still one of the most prevalent cancers in women and its early detection may present advantages in decreasing its mortality rate and the disease burden in the general population. It occurs when a malignant tumor develops in some type of breast tissue, such as lobules and ducts. There are some susceptible genes which may hold a relation with the tumor's presence, which are called BRCA1 and BRCA2. Breast cancer is also considered a solid tumor, which means that there are some areas in it that are hypoxic, meaning that no oxygen gets to them (Klevos *et al*, 2017).

Due to this cancer's importance, it is essential to find ways in which this tumor could be diagnosed. That is why the development of novel radiopharmaceuticals for its diagnosis and therapy is a major task to focus on. When considering this, the use of technetium-99m coordinated to a molecule serves as a good way to detect tumors.

For specifically detecting solid tumors, technetium-99m combined with phenazine *N,N'*–dioxides are an efficient way because these molecules have the capacity to be bioreduced under hypoxic conditions (Rey *et al*, 2015).

Introduction

In this sense, we have been previously described two potential radiopharmaceuticals that combine this bioreductive system and ^{99m}Tc radionuclide. One of them was ^{99m}Tc-tricarbonyl-(2-amino-5,10-dioxide-7(8)-fluorophenazine), **Tc-FZ**, that displayed adequate tumor/non-tumor ratios in a model of lung carcinoma (Rey *et al*, 2015).

The 4T1 represents a mouse invasive cell line, which was the one used in the current work to induce the tumors to the Balb-c mice (Salafzoon *et al*, 2017).

The 4T1 mammary carcinoma cell line was originally isolated at the Karmanos Cancer Institute (Miller *et al*, 2000). Its use has increased in recent years due to its high tumorigenic and invasive character.

We waited five days for implantation of primary tumor to use as a model for the study of late stage breast cancer. The growth and metastasis of 4T1 cancer cells in Balb-c mice are similar to the growth of breast cancer in a human organism (Kim *et al*, 2011), that's why this model was the one chosen.

Materials and methods

The first step in the present work was to synthesise the bioreductive fluorophenazine. This was done according to the procedure established by María Laura Lavaggi *et al* in 2015.

Synthesis of the coordinated FNZ with radionuclide ^{99m}Tc

After the fluorophenazine was synthesised, this had to be coordinated with the radionuclide ^{99m}Tc. This was done using commercial kit Isolink Kit. In this process, the $^{99m}Tco4^-$ was used to radiolabel the trycarbonil first. So, the ^{99m}Tc complex precursor with the tricarbonyl ($^{99m}Tc(CO)_3(H_2O)_3$) was synthesised. For this, the mixture had to be agitated for 1 minute, heated up at 100 C for 30 mintes, and, after cooling, its pH had to be adjusted using HCl 0,1 M to make it 7-8. After having checked the purity of this complex, through HPLC analysis, the complex of interest was formed. This required mixing up a fluorophenazine solution with a concentration of 1mg/mL and then heating at 70 ^oC during 30 minutes. Again, the product of this reaction was tested through HPLC analysis, in Whatman 1 and in alumina thin layer.

Materials and methods

Stability

Once the complex's presence was confirmed, stability was studied. This implied an injection of 25 µL of product in the HPLC at the times 1, 2, 3, 4 and 24 hours after the finalization of its formation.

Log P

In this assay the complex of interest is added to a mix of *n*-octanol and PBS. Then, we proceeded to separate the two phases: the organic phase (*n*-octanol) and the aqueous phase (PBS). Then the counts per second of each of the phases were obtained using an Ortec Counter. This assay was done in triplicate. The Log P assay allowed us to see whether the molecule was more inclined to be soluble in a hydrophilic or in a hydrophobic phase and if the molecule was able to go through plasmatic membranes.

The next two assays explained were performed using a sample taken when the HPLC profile for the radioabelled fluorophenazine was being obtained i.e. one eppendorf of that radiolabelled sample was obtained specifically in the retention time that had previously been identified.

Materials and methods

Competition binding assay

The competition assay was performed using the amino acid Histidine. This was to evaluate, from a certain point of view, the complex's stability. Histidine has an imidazole in its structure and this functional group is able to link the ^{99m}Tc, therefore competing for it with the fluorophenazine. Two solutions at two different Histidine concentrations were used: one at 1 mM and the other at 0,1 mM. This assay was performed using 180 µL of ^{99m}Tc-fluorophenazine and 20 µL of the two concentration-solutions. After this, both eppendprfs' activities were measured using the Ortec Counter and then they were incubated at 37 C for 1 and 3 hours. The HPLC profiles for both concentrations were obtained. For both, the assay was performed in triplicate.

Bovine serum assay

In this, the molecule of interest was added and mixed with 250 µL of bovine serum. This was done in triplicate for each time considered. the Then an HPLC profile was obtained after 1 and 3 hours of incubation at 37 C had taken place. After the time had passed, 500 µL of acetonitrile were added to each eppendorf of the mixture. Later on, they were all centrifuged at 1500 rpm and the corresponding pellets and supernatants' counts per 10 seconds were obtained using the Ortec Counter.

Materials and methods

Biodistribution assay

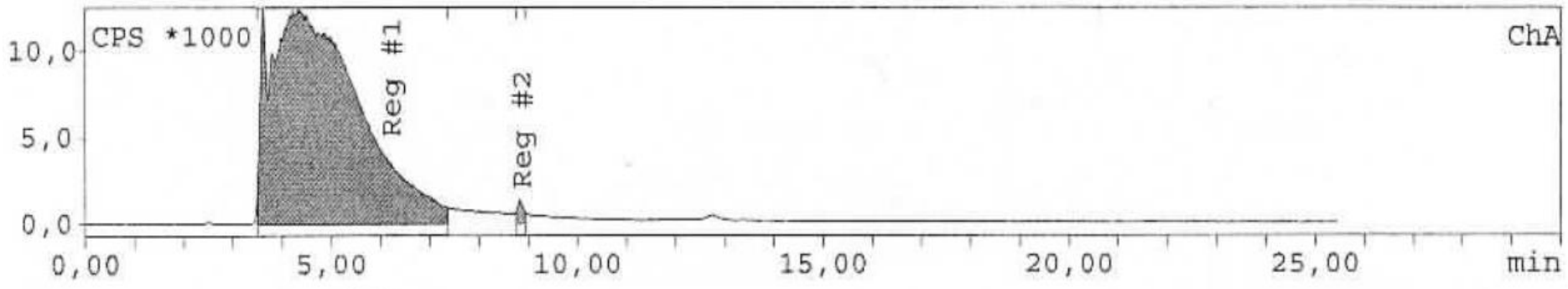
The 4T1 bearing mice were sacrificed by cervical dislocation at 30 minutes (*n*=4), 1 h (*n*=4) and 2 h (*n*=4) after the injection of ^{99m}Tc-fluorphenazine. Biodistribution of fluorophenazine complexed with ^{99m}Tc was determined after weighing and measuring the radioactivity in organs and tissues in a solid scintillation counter NaI (Tl) 3"x3" crystal detector associated with an ORTEC single channel analyzer at fixed time intervals. The percentage mean uptake per tissue was calculated and uptake ratios were generatedto later get average % activities and % activities/g. The organs and tissues whose activities were measured were blood, liver, heart, lungs, spleen, kidneys, thyroid, muscle, bone, stomach, gut, urine and bladder, carcass and the induced tumor.

In vivo imaging

Images were taken in 4T1 bearing mice. These were to see how the ^{99m}Tc-fluorophenazine distributed in the mouse's body. After the impantation of tumors, the complex diluted in PBS was injected into the mice and sacrificed after 30 minutes (*n*=4), 1 h (*n*=4) and 2 h (*n*=4) post inyection to take the image in an *in vivo FX PRO camera*.

Results and discussion

Firstly, the tricabonyl-^{99m}Tc complex's radiochemical purity (RCP) was obtained and it was of approximately 99,3 % (Figure 1). Its retention time was of approximately 4 minutes.

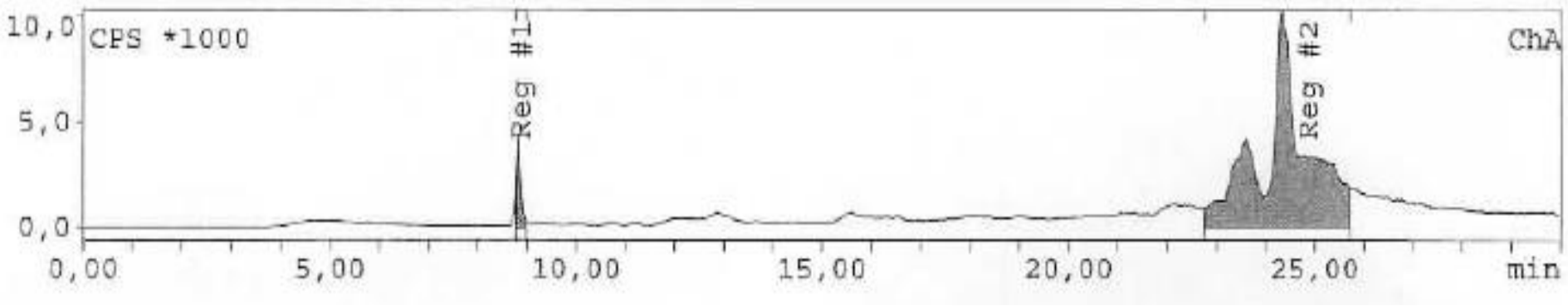


ChA

Substance	R/T min	Type	Area Counts	%Area %
Reg #1	4,33	DD(	1601864	99,28
Reg #2	8,82	DD(	11666	0,72
Sum in ROI			1613530	
Area			1820079	
Ext. BKG			0,00 CPS	

Figure 1. HPLC profile obtained for the complexation of the ^{99m}Tc and the tricarbonyl and its relative percentage, which informs about the radiochemical purity (RCP).

Results and discussion



ChA

Substance	R/T min	Type	Area Counts	%Area %
Reg #1	8,82	DD(	28043,7	4,60
Reg #2	24,33	DD(	581270,2	95,40
Sum in ROI			609314,0	
Area			1291121,4	
Ext. BKG			0,00 CPS	

Figure 2. HPLC profile obtained for the complexation of the fluorophenazine and ^{99m}Tc and its relative percentage, which informs about the RCP.

Results and discussion

The formation of the complex between $Tc(CO)_3(H_2O)_3$ and fluorophenazine showed a radiochemichal performance of 95,7 % which is considerably higher than the 90 % required for radiopharmaceuticals to be injected, this being a positive outcome. Its retention time was of 24,3 minutes.

The stability assay showed that the RCP after 1 h of synthesis of the complex formed between fluorophenazine and $Tc(CO)_3(H_2O)_3$ was of approximately 95 %. Then, its RCP after 2 h was of 92 %, as was the stability obtained at 3 and 4 h. And finally, the stability obtained 24 h after the synthesis was of 88 %. All these results point towards a considerable stability as far as the complex of interest is concerned. It is clearly a good property that this complex possesses, it is stable in the reaction medium, as shown in Table 1.

Results and discussion

Time elapsed since radiolabelling (h)	Radiochemical purity (RCP,%)
1	95,1
2	92,4
3	92,3
4	92,3
24	87,8

Table 1. Results of RCP obtained for the stability assay of the complex between fluorophenazine and ^{99m}Tc.

Results and discussion

The competition assay performed showed that after 1 h of incubation and using a solution at 1 mM of histidine the radioactivity of the interest peak diminished to 66,5 %. After 1 h but using a solution 0,1 mM in histidine, the radioactivity was of 75,8 %. Afterwards, when the assay was performed for 3 h of incubation, the relationship established between the solution's concentration and the RCP was inverted. The RCP we got for 3 h with histidine 1 mM was of 56,8 % while the one with the concentration of 0,1 mM was of 47,4 %.

The Log P assay showed that the complex of interest had a subtle preference towards the hydrophilic phase (PBS). The calculated Log P was of -0,15, which implies that this molecule is permeable to cell's membranes, because its value is between -0,5 and 2.

Results and discussion

After the biodistribution assay was performed, relationships between the tumor's activity/g % and the blood's, muscle's and bone's were obtained. These showed also a clear preference towards the liver as well. Although this happened here too, by analysing the data obtained there is an improvement of these ratios towards the 2 hours after the radiopharmaceutical was injected (p.i.).

After 30 minutes p.i. the tumor/muscle ratio was clearly favourable towards the tumor, but the tumor/blood and tumor liver ratios remained unfavourable. After 1 hour p.i., the ratio that improved mildly was the tumor/liver ratio, which went from a 0.03 to a 0.05, which is a positive outcome, because it means that the liver's captation became relatively lower. And finally, after 2 hours p.i., the three ratios became more favourable, which denotes a very good outcome in this assay. These data can be seen in Table 2.

Results and discussion

Time (h)/Body parts ratios	Tumor/muscle	Tumor/blood	Tumor/liver
0,5	2.15	0.18	0.03
1	1.47	0.15	0.05
2	3.00	0.23	0.06

Table 2. The relationships between the tumor and some body parts is shown, considering the time elapsed since the mice were injected and sacrificed.

Results and discussion

The results obtained in this assay show that after 30 minutes the level of % activity/g located in the liver is considerably high, being this of approximately 35 %. Although it is a high percentage, it became lower as the hours went by. When it was 1 h p.i., this percentage became 20 %, which is a considerable change taking into consideration the initial percentage. This could mean that at 1 h p.i., a major portion of the molecule of interest that had been excreted through the liver is not anymore. And by the time 2 h p.i. had passed it became a 29 %. Although it is higher than the previous percentage, it is still lower than the first one, which makes us consider that after 2 h there still is a portion of the molecule of interest which is passing through the liver while being excreted at the same time (Figures 3 and 4).

The fact that so much of the molecule of interest's activity was concentrated in the liver could be because of the tumor not being in the optimal stage while the assay was being performed. These studies could be done in an optimal tumor's stage, in which the necrotic center is bigger and clearer.

Results and discussion

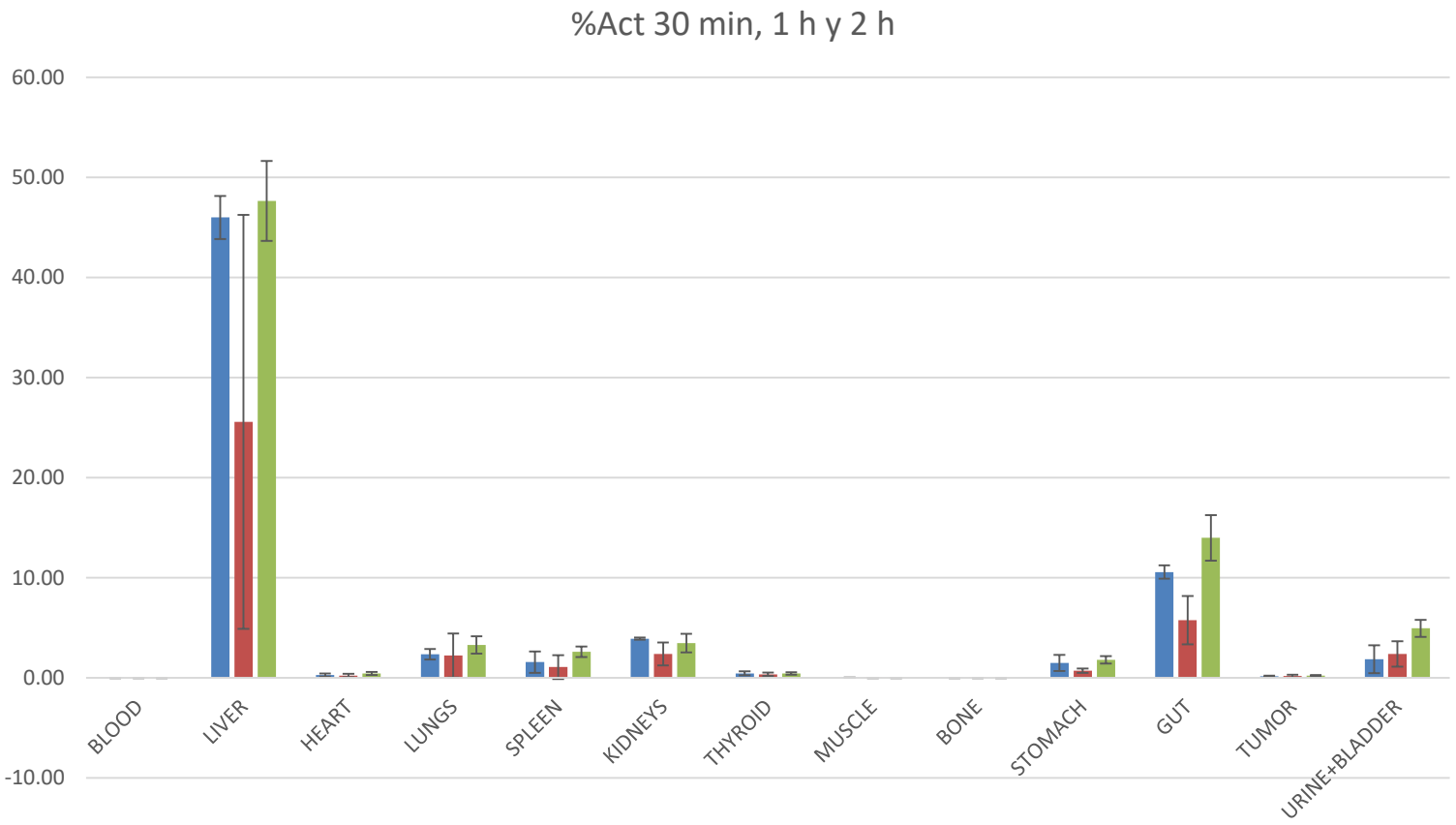


Figure 3. Percentage of activity after 30 minutes (in blue), 1 (in red) and 2 (in green) h of injection.

Results and discussion

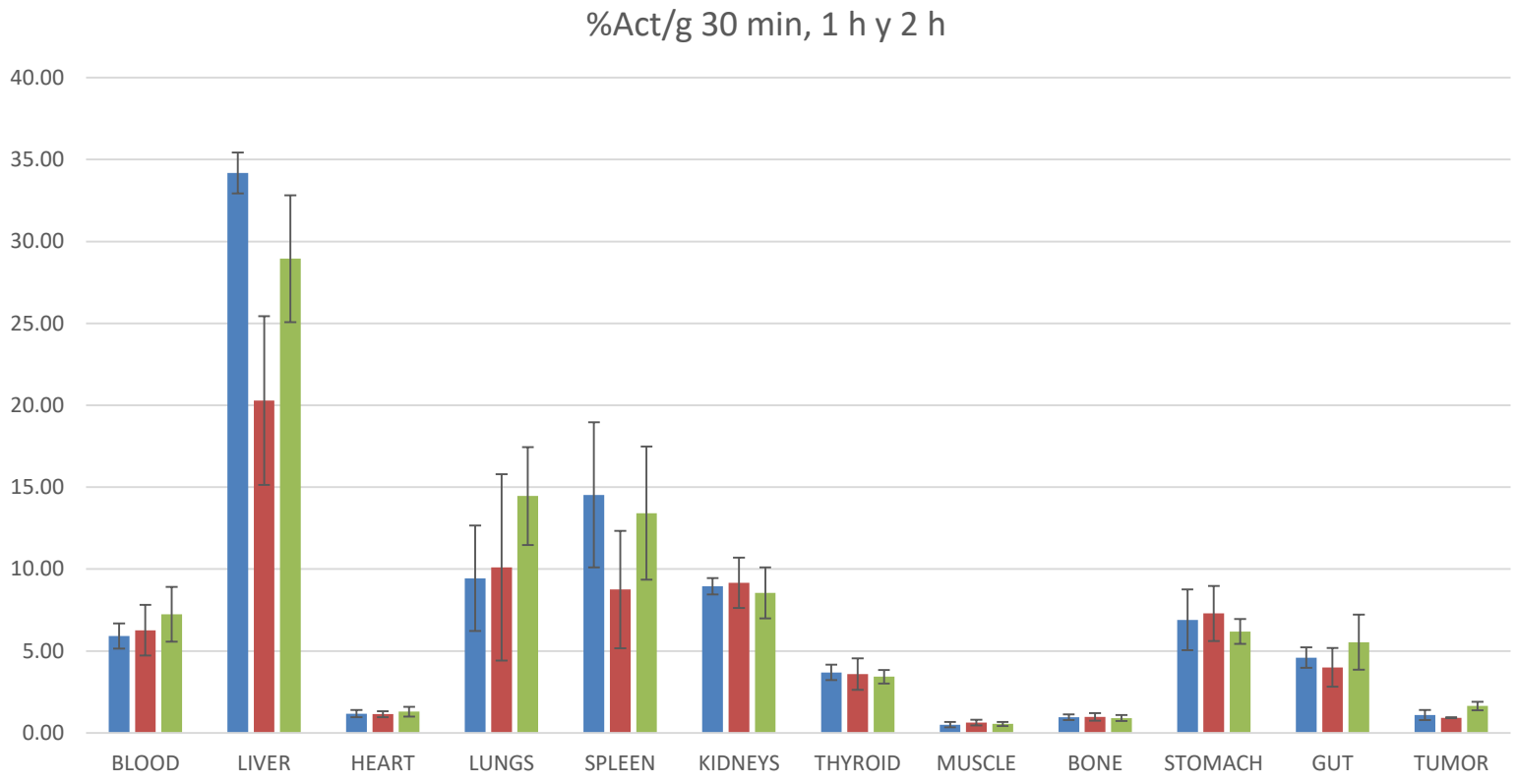


Figure 4. Percentage of activity/weight of organ (g) after 30 minutes (in blue), 1 (in red) and 2 (in green) h of injection.

In vivo imaging of 99mTc-FNZ in BALB/c mice bearing 4T1-tumor at 30 minutes post inyection

The complex diluted in PBS was injected to the mice and sacrificed after 30 minutes (*n*=4), 1 h (*n*=4) and 2 h (*n*=4) post inyection to take the image in an *in vivo FX PRO camera*.

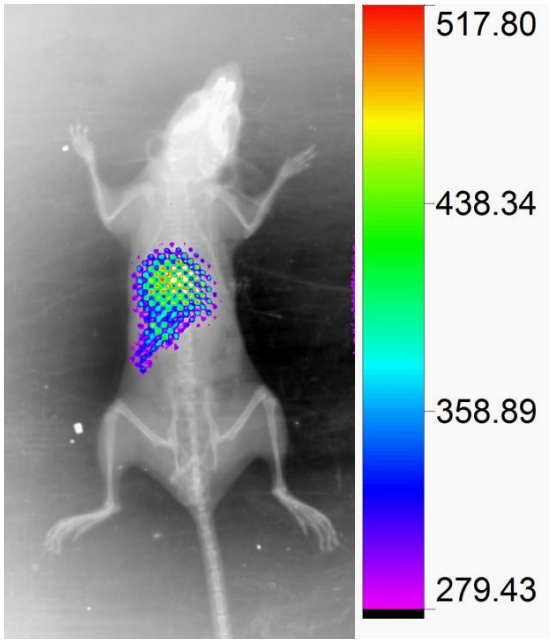


Figure 5. The picture shown is of a Balb-c mouse which was 30 minutes p.i. of the ^{99m}Tc-fluorophenazine. The unit scale shown is arbitrary.

Conclusions

- The Tc-FZ complex was formed successfully, with a radiopharmaceutical purity of 95,7 %.
- The stability, in the reaction medium, of that complex after 1, 2, 3, 4 and 24 h barely changed, meaning the complex itself is clearly stable.
- The complex presented a mild preference towards the hydrophilic phase.
- The competition assay performed with histidine showed that the complex's stability can be clearly affected *in vivo*.
- The molecule by itself delivered a picture in which the liver was very visible, which was coherent with the results obtained through the biodistribution assay.
- The biodistribution assay showed that after 2 hours p.i. the tumor/muscle ratio, the tumor/blood ratio and the tumor/liver ratio improved.

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