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COMPARATIVE PHARMACOKINETICS AND BIODISTRIBUTION STUDIES OF ^{99m}Tc-ANNEXIN V PRODUCED BY DIFFERENT RADIOLABELING METHODS

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ABSTRACT

The use of radiolabeled annexin A5 (ANXA5) to detect cell death *in vivo* has increased in the last years. Several ^{99m}Tc-labeling techniques were reported using different cores, such as $[^{99m}Tc{=}O]^{+3}$, $[^{99m}Tc]$HYNIC, $[^{99m}Tc{\equiv}N]^{+2}$ and $[Tc(CO_3)]^{+1}$. The goal of the present work was to evaluate the influence of ^{99m}Tc cores in the biological behavior of radiolabeled ANXA5 in *Swiss* mice using $[^{99m}Tc{=}O]^{+3}$, $[^{99m}Tc]$HYNIC cores. Ethylenedicysteine (EC) was applied to obtain $[Tc{=}O]^{+3}$ core, N,N,N',N'-tetramethyl(succinimide) uranium tetrafluoroborate (TSTU) was employed to transfer the carboxyl group to their corresponding hydroxysuccinimide ester and HYNIC-ANXA5 was provided by National Cancer Institute-Frederick. ITLC-SG and HPLC analysis were applied to determine non-desirable products and the stability of preparations was evaluated after incubation at room temperature, 4° C and in human serum at 37º C. *In vivo* biodistribution and kinetics studies were performed after the intravenous injection of ^{99m}Tc-HYNIC-ANXA5 and ^{99m}Tc-EC-ANXA5 and pharmacokinetic parameters were calculated using Biexp software. ANXA5 was radiolabeled at room temperature with high yield (> 95%). The results of biodistribution in mice showed, as expected, higher renal uptake of ^{99m}Tc-HYNIC-ANXA5 and higher liver uptake of ^{99m}Tc-EC-ANXA5. The percent injected activity per gram (% IA/g) in liver at 0.5 hours were 6.52 and 1.09 and in kidneys were 1.59 and 32.2 for ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-HYNIC-ANXA5, respectively. The results of radioactivity in blood showed that both HYNIC- and EC- radiolabeled ANXA5 presented fast blood clearance. In this study two ^{99m}Tc-ANXA5 obtained from three different available radiolabeling methods presently were investigated. Each labeling method possesses unique advantages and disadvantages.

INTRODUCTION

The increasing in nuclear medicine techniques application is probably due to the development of more specific radiopharmaceuticals which take to better image quality. In the development of a new ^{99m}Tc-based radiopharmaceutical several factors have to be considered to satisfy the clinical requirements. Firstly, the new radiopharmaceutical has to demonstrate its biological efficacy (high uptake in the target organ, low background radiation and

favorable pharmacokinetics). Second, the new radiopharmaceutical should have high radiochemical purity (> 95%) and high solution stability with a shelf life preferable higher than 6h. Finally, the ^{99m}Tc-labeling should be accomplished in 10-30 minutes, preferably at room temperature [1].

Annexin A5 (ANXA5) is an intracellular human protein of 36 kDa that binds to phosphatidylserine (PS) in the presence of calcium. The affinity of ANXA5 to PS remains a ligand-receptor system and this interaction occurs during apoptosis. As a result, ANXA5 binds to apoptotic cells *in vivo* and *in vitro*. This protein is folded into a planar cyclic arrangement with a unique N-terminal region followed by four homologous repeats of approximately 70 amino acids, each one composed by five alpha-helices segments. ANXA5 target site to the PS is attributed to the N-terminal domain. ANXA5 has been targeted to several radionuclides such as ^{99m}Tc, ^{18}F, ^{64}Cu and $^{123/124}$I, and these radiopharmaceuticals have been used to detect cell death *in vivo* by both Single Photon Emission Computer Tomography (SPECT) and Positron Emission Tomography (PET) imaging [2,4,6].

Most of the radiolabeling methods of ANXA5 have been developed with technetium-99m, probably due to its extremely favorable physical characteristics, availability and cost. ^{99m}Tc has been coupled to ANXA5 using either direct or indirect methods. Direct methods usually apply Sn^{+2} as reducing agent after the introduction of a chelator in the amino acid sequence (annexin A5 mutant). The indirect procedure can be developed via bifunctional chelating agents (BFA), such as 4.5-bis(thioacetamido)pentanoyl group (^{99m}Tc-Apomate), n-1-imino-4-mercaptobutyl group (^{99m}Tc-i-ANXA5), hydrazinonicotinamide (^{99m}Tc-HYNIC-ANXA5), ethylendedicysteine (^{99m}Tc-EC-ANXA5) or mercaptoacetyl-glycyl-glycine (^{99m}Tc-MAG3-ANXA5) [3,5,7,9].

The pharmacokinetic profile of ^{99m}Tc-ANXA5 is not only determined by the annexin A5, but also by the chelator and the radiolabeling method applied. The chelator can provide a significant impact in ^{99m}Tc-ANXA5 lipophilicity, biodistribution and stability. The ANXA5 compelled to HYNIC chelator and radiolabeled with ^{99m}Tc (^{99m}Tc-HYNIC-ANAXA5) is known to be the most promising tracer for apoptosis imaging because of its success in preclinical studies, phase II/III trial in patients and commercial availability. In addition, L,L-ethylenedicysteine (EC) is an example of N_2S_2 chelator which has been successfully applied and widely studied [8,9].

Although ANXA5 has been largely studied and its radiolabeling with technetium-99m has been already developed, further studies have to be done in order to evaluate the chelator and radiolabeling methods influence in ANXA5 biological behavior. This work presents the comparative results of syntesis and pharmacokinetic studies in *Swiss* mice for ANXA5 compelled to HYNIC and EC chelators and radiolabeled with ^{99m}Tc.

MATERIALS AND METHODS

Synthesis of EC-ANXA5

Ethylenedicysteine (EC) was conjugated to ANXA5 using the N,N.N',N'-tetramethyl(succinimide) uranium tetrafluoroborate (TSTU). TSTU was employed to transfer the carboxyl group into the corresponding hydroxysuccinimide esters. Briefly, TSTU

(0,1mmol) was added to an EC solution (0,05mmol, pH 10) and the mixture was heated at 60°C for 30 min. Then, 80 μg of ANXA5 was added to this mixture and the reaction proceeded at 4-8° C for more 24 hours. At the end of this time, the mixture was dialyzed at 4-8° C for 48 hours and EC-ANXA5 was fractioned and stored at -20°C.

Radiolabeling of EC-ANXA5 with ^{99m}Tc

Radiolabeling of EC-ANXA5 with technetium-99m was performed by adding ^{99m}Tc-pertechnetate (185 – 259 MBq) and $SnCl_2$ (100μg) into a vial containing EC-ANXA5, derivatized as described earlier. Radiochemical purity was determined by Instant Thin Layer Chromatography in Silica Gel plates (ITLC SG, PALL Life Sciences) using 1 $mol.L^{-1}$ ammonium acetate:methanol (4:1) as eluent.

2.3 Radiolabeling of HYNIC-ANXA5

ANXA5 derivatized with HYNIC was from the kit formulation of the National Cancer Institute-Frederick. ^{99m}Tc-HYNIC-ANXA5 was prepared in a sterile vial containing HYNIC-ANXA5 and ^{99m}Tc-pertechnetate (1.11 to 1.85 GBq) was introduced followed by the addition of a stannous/tricine mixture obtained from a second lyophilized vial reconstituted with saline solution. The preparation was incubated for 15 minutes at room temperature. The radiochemical purity was determined by ITLC-SG, using acid citrate dextrose (ACD) buffer.

2.4 Stability study

2.4.1 Time-course stability

The stability of the ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 was assayed by measuring the radiochemical purity different times (0.5, 1, 2, 4 and 6 hours) after storing at room temperature or 4-8° C.

2.4.2 Time-course stability in human serum

^{99m}Tc-EC-ANXA5 (100 μL) and ^{99m}Tc-HYNIC-ANXA5 (100 μL) were added individually to 1 mL of fresh human serum and incubated at 37° C. After different time intervals (1, 2, 4 and 6 hours) the radiochemical purity was determined as described earlier.

2.5 Binding affinity measurement

The affinity of radiolabeled protein for PS was assessed in human prostate cell line (PC-3) with radiation-induced apoptosis. The cells were washed twice with phosphate buffered saline (PBS), ressuspended in binding buffer containing the radiolabeled protein and incubated at 25° C for 1 hour. At the end of incubation, the cells were centrifuged at 1397 g for 5 min and washed with PBS (three times). The radioactivity associated to the cells was measured. Induction of apoptosis in PC-3 cells by radiation was confirmed by MTS and differential staining assays.

2.6 Partition coefficient determination

The partition coefficient (CP) of the ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 was determined in n-octanol/water system, previously saturated for 24 hours. The radiolabeled protein (25 μL) was partitioned between 3 mL of water and 3 mL of n-octanol by vortexing

the tube for 1 hour at room temperature. An aliquot of each phase was collected and the associated activity in each one was determined. The partition coefficient was determined by the function: Partition coefficient = $\log_{10}$ (counts in n-octanol phase / counts in water phase). The experiments were performed in triplicate.

2.7 Biodistribution studies

Normal *Swiss* mice (20–25 g weight) were used in invasive biodistribution studies. The animals were intravenously injected with the radiolabeled protein (3.7 MBq in 100 μL) via the tail vein. At 0.5, 1, and 2 hours after injection, mice were sacrificed by cervical dislocation and selected organs were removed, weighed and counted in a well type gamma detector. The radioactivity of the tissue samples was expressed as percentage of injected dose per gram of tissue (% ID/g).

2.8 Pharmacokinetics

Kinetics studies were performed by measuring ^{99m}Tc-HYNIC and EC-ANXA5 in blood. Blood samples were collected 5, 10, 30, 60 and 120 minutes after the intravenous injection in the caudal vein and their radioactivity were measured in a gamma counter (Packard). The pharmacokinetics parameters were determined using Biexp software. This software normalizes the experimental data to a bicompartimental biodistribution model with two components: a central or vascular and an extravascular component, described by equation below:

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t}$$

where C(t) is the concentration at any given time, A and B are the intercepts at the Y-axis, of the fast a and slow b slopes, and e is the base of the natural logarithms. By least square regression analysis the program calculates points A and B on the Y-axis and the constants α (rapid distribution constant) and β (slow elimination constant). With these four data, BIEXP calculates all the other parameters according to conventional equations for a two-compartment biodistribution such as half-live of the rapid distribution ($T_{1/2\alpha}$) and slow elimination ($T_{1/2\beta}$), mean residence time, transfer constants (k_{12}, k_{21}, k_{00}), distribution volume and elimination constant (kss).

RESULTS AND DISCUSSION

Radiolabeling of ANXA5

The coupling of EC to the N-terminal groups of ANXA5 was performed by activation of the carboxylic acids during the reaction with TSTU in alkaline medium. The procedure of radiolabeling EC- and HYNIC-ANXA5 was performed in a short time and at room temperature and high radiochemical yields were obtained (Table 1).

Table 1: Radiochemical yields (%) of the radiolabeling of EC- and HYNIC-ANXA5 with technetium-99m.

Radiochemical yields (%)	
^{99m}Tc-EC-ANXA5	^{99m}Tc-HYNIC-ANXA5
98.4 ± 0.4	92.7 ± 0.6

3.1 **Stability studies**

3.2.1. Time-course stability

The results of time-course stability of ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 are show in Fig. 1. The radiolabeled annexins exhibited high stability at room temperature and after storing at 4-8° C. However, ^{99m}Tc-EC-ANXA5 presented higher stability than ^{99m}Tc-HYNIC-ANXA5, showing radiochemical purity higher than 90 % for more than 360 minutes at both storage conditions.

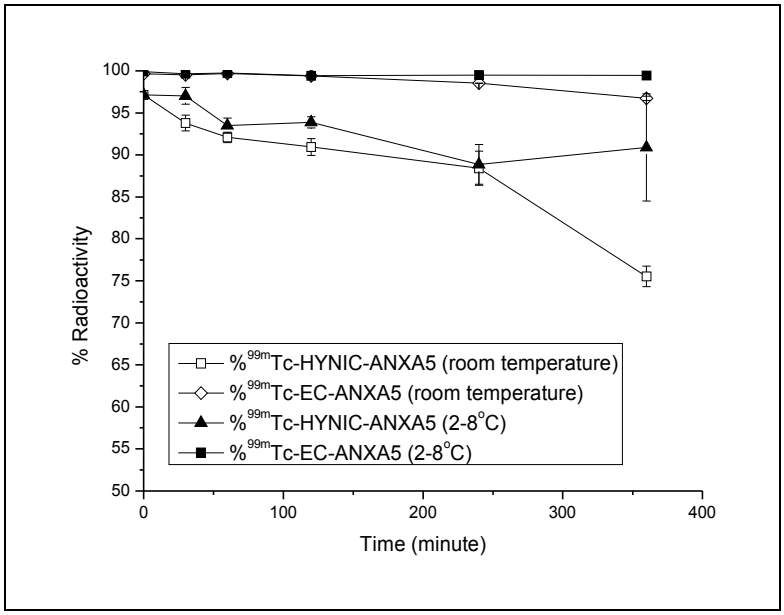

Figure 1. Stability of ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 after storing at room temperature and 4-8° C for different times.

3.2.2. Time-course stability in human serum

After 240 min of incubation in human serum at 37° C, 90 and 91% of ^{99m}Tc-EC-ANX and ^{99m}Tc-HYNIC-ANXA5 remained intact, respectively. The results indicate *in vitro* stability of radiolabeled ANXA5 under metabolism by human serum enzymes, as shown in Fig. 2.

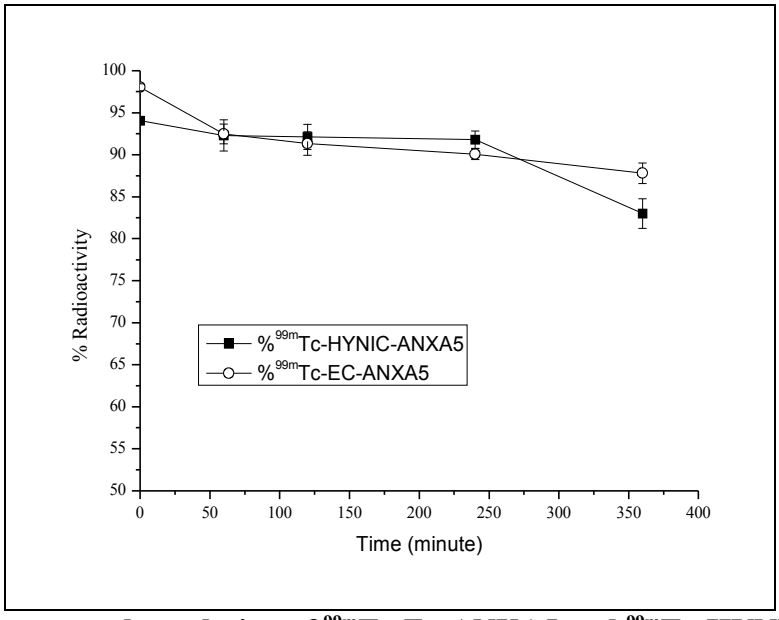

Figure 2. Time-course degradation of ^{99m}Tc-Ec-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 by human serum enzymes at 37° C.

3.3. Binding affinity measurement

The biological activity *in vitro* of ^{99m}Tc-HYNIC-ANXA5 and ^{99m}Tc-EC-ANXA5 was evaluated by the binding to PC-3 cells with radiation-induced apoptosis (Table 2). The results showed that ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-EC-ANXA5 bind specifically to apoptotic cells, but ^{99m}Tc-EC-ANXA5 was the radiolabeled protein with higher specific binding. The apoptosis detected by radiolabeled annexins was confirmed by both MTS and differential staining assays.

Table 2. Specific binding of ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 to apoptotic PC-3 cells *in vitro*.

Percentage of specific binding (%)	
^{99m}Tc-EC-ANXA5	**^{99m}Tc-HYNIC-ANXA5**
50.50	23.21

3.4 Partition coefficient determination

Partition coefficients of ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 are shown in Table 3. Both studied compounds exhibited low lipophilicity, but ^{99m}Tc-EC-ANXA5 is less lipophilic than ^{99m}Tc-EC-ANXA5. These results are in accordance to the literature data, which show that EC chelator is more hidrophilic than HYNIC chelator.

Table 3: Partition coefficients of ^{99m}Tc-Ec-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 (n=3).

Partition Coefficient		
	^{99m}Tc-EC-ANXA5	^{99m}Tc-HYNIC-ANXA5
Log P	-1.43 ± 0.05	-0.53 ± 0.32

3.5 Biodistribution studies

Results from biodistribution studies using the ^{99m}Tc-labeled annexins performed with *Swiss* mice are presented in Table 4 and 5 as the percentage of injected dose per gram of organ (% ID/g). Appreciable radioactivity could be detected in the kidneys and liver until 2 hours post injection of ^{99m}Tc-HYNIC-ANXA5 and ^{99m}Tc-EC-ANXA5, indicating radiopeptides primarily excretion by renal and hepatic pathway, respectively. Kidneys and liver may be the critical organs for dosimetry.

Stomach uptake is commonly assumed as a control of technetium-labeled compounds stability in *in vivo* assays. This tissue actively uptakes free technetium, being a good indicator of radiochemical purity, mainly at the initial time. Stomach uptake of ^{99m}Tc-labeled annexins was negligible, confirming no contamination of free technetium in the preparation.

Table 4. Biodistribuition of ^{99m}Tc-HYNIC-ANXA5 in *Swiss* mice.

Organs	% ID/g		
	0.5 h.	1 h.	2 h.
Liver	1.09 ± 0.18	0.81 ± 0.03	0.73 ± 0.15
Lung	0.74 ± 0.15	0.32 ± 0.08	0.25 ± 0.04
Stomach	0.32 ± 0.06	0.20 ± 0.04	0.20 ± 0.04
Kidney	32.20 ± 8.28	23.08 ± 5.59	22.24 ± 1.24
Heart	0.43 ± 0.16	0.24 ± 0.09	0.24 ± 0.04
Spleen	1.81 ± 0.28	1.87 ± 0.52	1.34 ± 0.46
Small intestine.	0.27 ± 0.09	0.18 ± 0.05	0.18 ± 0.03
Large intestine	0.12 ± 0.02	0.08 ± 0.02	0.09 ± 0.02
Muscle	0.14 ± 0.06	0.07 ± 0.02	0.05 ± 0.01

Table 5. Biodistribuition of ^{99m}Tc-EC-ANXA5 in *Swiss* mice.

Organs	% ID/g		
	0.5 h.	1 h.	2 h.
Liver	6.52 ± 1.23	5.09 ± 0.58	4.39 ± 1.11
Lung	0.79 ± 0.12	0.52 ± 0.07	0.58 ± 0.27
Stomach	0.58 ± 0.12	0.54 ± 0.10	0.36 ± 0.12
Kidney	1.59 ± 0.39	1.36 ± 0.17	1.14 ± 0.33
Heart	0.49 ± 0.08	0.37 ± 0.04	0.19 ± 0.04
Spleen	4.79 ± 0.96	4.67 ± 0.90	2.46± 0.73
Small intestine	0.23 ± 0.07	0.21 ± 0.03	0.26 ± 0.09
Large intestine	0.12 ± 0.02	0.11 ± 0.04	0.09 ± 0.02
Muscle	0.13 ± 0.01	0.11 ± 0.03	0.07 ± 0.01

3.6 Farmacokinetics

The blood clearance of ^{99m}Tc-EC-ANXA5 and ^{99m}Tc-HYNIC-ANXA5 are show in Fig. 3, where there is an extremely rapid blood clearance rate for fast slope.

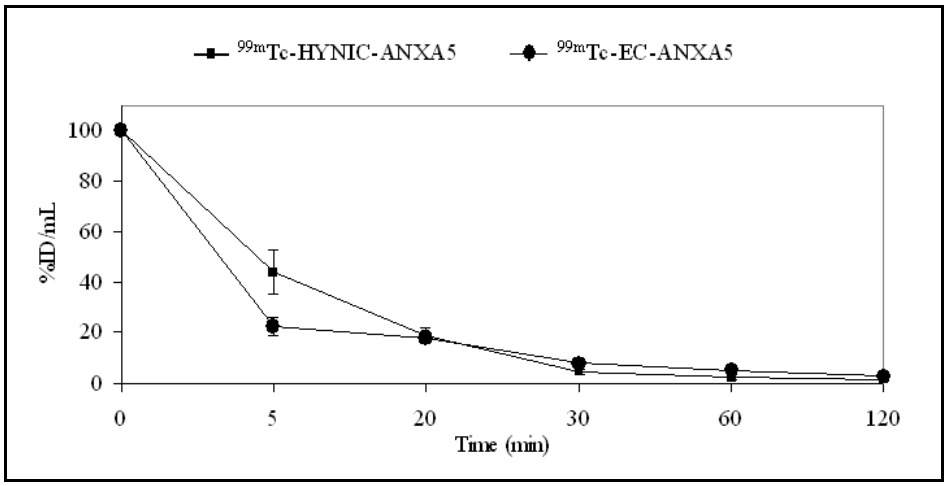

Figure 3. Curve of blood clearance of ^{99m}Tc-Ec-ANXA5 and ^{99m}Tc-HYNIC-ANXA5

The equations 1 and 2 express the concentration of the radiopharmaceuticals (C), dependent of the time (t) for ^{99m}Tc-HYNIC-ANXA5 and ^{99m}Tc-EC-ANXA5, respectively.

$$C(t) = 95{,}186.96e^{-11{,}06t} + 4{,}975.45e^{-0{,}58t} \quad (1)$$
$$C(t) = 67{,}940.63e^{-13{,}06t} + 9{,}090.68e^{-0{,}56t} \quad (2)$$

The pharmacokinetic data are shown in Table 7, where one can appreciate that the radiolabeled annexins are distributed and eliminated quickly. The mean residence time in the central compartment was calculated with the elimination constant k_{10} with a 0.9 h for ^{99m}Tc-HYNIC-ANXA5 and 1.36 h for ^{99m}Tc-EC-ANXA5.

Table 7. Radiopharmacokinetic data for radiolabeled ANXA5

Parâmetros farmacocinétos	^{99m}Tc-HYNIC-ANXA5	^{99m}Tc-EC-ANXA5
$T_{1/2\alpha}$ (h)	0.06	0.05
$T_{1/2\beta}$ (h)	1.18	1.23
K_{12} (h^{-1})	4.69	7.97
K_{21} (h^{-1})	1.11	2.04
K_{10} (h^{-1})	5.85	3.61
Residence time (h)	0.90	1.36
K_{ss} (h^{-1})	1.12	0.74

CONCLUSIONS

The results indicated that ^{99m}Tc-EC-ANXA5 may be an attractive alternative to ^{99m}Tc-HYNIC-ANXA5 for the *in vivo* imaging of apoptosis and phosphatidylserine receptors.

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