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Study of the automated synthesis of the radiopharmaceutical [^{18}F]fluoroestradiol

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ABSTRACT

Breast cancer is the second leading cause of cancer death among women worldwide, with an incidence increase of 25 % per year. Approximately 75 % of breast cancer cells express estrogen receptors. The 16α-[^{18}F]fluoro-17β-estradiol, [^{18}F]FES, is a radiopharmaceutical that binds to estrogen receptors applied in PET-CT molecular images for non-invasive diagnosis of primary and metastatic breast cancer. The objective of this work was to study the synthesis of the [^{18}F]FES in the GE TRACERlab® MXFDG module, using the Chemical Kit and the ABX® disposable cassette. Moreover, to determine the process yield and the analytical parameters to be used in the routine production of this radiopharmaceutical. Automated synthesis took place in 75 minutes and included percolation of [^{18}F]fluoride ($^{18}F^-$) in an anion exchange cartridge, elution of the cartridge, azeotropic drying in 3 steps, labeling of the precursor 3-methoxymethyl-16β,17β-epiestriol-O-cyclic sulfone (MMSE) and a hydrolysis step. The product was purified in the module by solid-phase extraction (SPE) cartridges. The radiochemical yield was reproductive, despite initial [^{18}F]fluorine activity, and the results of quality control tests suggest that the radiopharmaceutical meets the acceptance criteria established in official monographs for other radiopharmaceuticals labeled with 18-fluor. *In vivo* biodistribution studies in healthy mice and mice bearing MCF7 tumors showed the specific uptake on breast tumor cells.

Keywords: [^{18}F]FES, Automated synthesis, quality control, biodistribution.

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1. INTRODUCTION

According to the National Cancer Institute, breast cancer is the second cause of death globally, and the incidence rate has increased 25% each year. About 75% of cases express estrogen receptors and they are called positive estrogen receptor (ER +) cancers [1,2,3].

Receptor-specific radiopharmaceuticals have been used in clinical studies to diagnose and treat different types of cancer with gamma radionuclides or positron emitters for diagnosis or beta minus and alpha emitters are used for diagnosis therapy with gamma or positron emitters radionuclides for diagnosis or beta minus and alpha emitters for therapy.

The radiopharmaceutical 16α-[^{18}F]fluoro-17β-estradiol, [^{18}F]FES, has an in vivo lipophilic characteristic, similar to estradiol, and binds to estrogen receptors that are over expressed in breast cancer. Thus, they are an alternative to ER positive metastases, especially when biopsy from multiple foci becomes unfeasible [4,5,6].

The radiopharmaceutical [^{18}F]FDG ([^{18}F]fludesoxyglucose) is currently used in PET-CT, [7] being a non-specific tumor marker, since glucose metabolism is increased in tumor cells compared to non-tumor cells [8].

The development of the radiopharmaceutical [^{18}F]FES by IPEN/CNEN represents a new tool for the receptor-specific diagnosis of breast cancer using ~~the~~ PET and PET-CT.

Knott et al., 2011, Huang et al. 2017 e Bispo et al., 2019 [9,10,11] describe a similar automated synthesis of [^{18}F]FES, using the same reagents and process employed in this work. A great advantage of this synthesis approach is using disposable purification cartridges in place of purification using HPLC, as described by OH et al., 2007 [5].

The high quality of the produced batches is the first requirement for obtaining the marketing authorization from National Health Surveillance Agency (ANVISA). The studies of quality control are the assays of radionuclide purity and identity, radiochemical purity by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC), chemical impurity, including TBA-HCO3 and residual solvent (acetonitrile and ethanol), pH, bacterial endotoxins and sterility. The quality attributes for the product were defined and evaluated based on monographs of other [^{18}F]fluorine radiopharmaceuticals, since there are no monographs in official compendiums for the product [^{18}F]FES.

In addition, for the registration of a new radiopharmaceutical in Brazil, the manufacturer must demonstrate the efficacy and safety of the product based on pre-clinical and clinical studies. [12]. Conducting pre-clinical studies in healthy animals and in tumor models suitable for the radiopharmaceutical under study leads to important results that should precede clinical studies.

The results of this work support the implementation of the routine production and quality control of the radiopharmaceutical [^{18}F]FES and determine aspects of efficacy and safety in an animal model.

2. MATERIALS AND METHODS

2.1 Synthesis

The [^{18}F]fluoride ($^{18}F^-$) was obtained as a result of the bombardment of ^{18}O (enriched water, Rotem, Israel) by accelerated protons, through the nuclear reaction $^{18}O(p,n)^{18}F$ in the cyclotron IBA 18 MeV (IBA Cyclotron Solutions, Belgium), from IPEN / CNEN. [11] The synthesis of [^{18}F]FES, (16α-[^{18}F]fluoro-17β-estradiol) was performed in the automated GE TRACERlab™ MXFDG® module. Kits of reagents from ABX (Germany), consisting of anhydrous acetonitrile for drying, the precursor 3-methoxymethyl-16β,17β-epiestriol-O-cyclic sulfone (MMSE) to be diluted with acetonitrile, 40% ethanol solution, 95% ethanol solution, 0.075 M tetrabutylammonium bicarbonate eluent (TBA-HCO3), and syringes with water for washing and a syringe with ethanol/sulfuric acid solution [13].

The diagram of the module and the automated synthesis process of [^{18}F]FES is shown in Figure1.

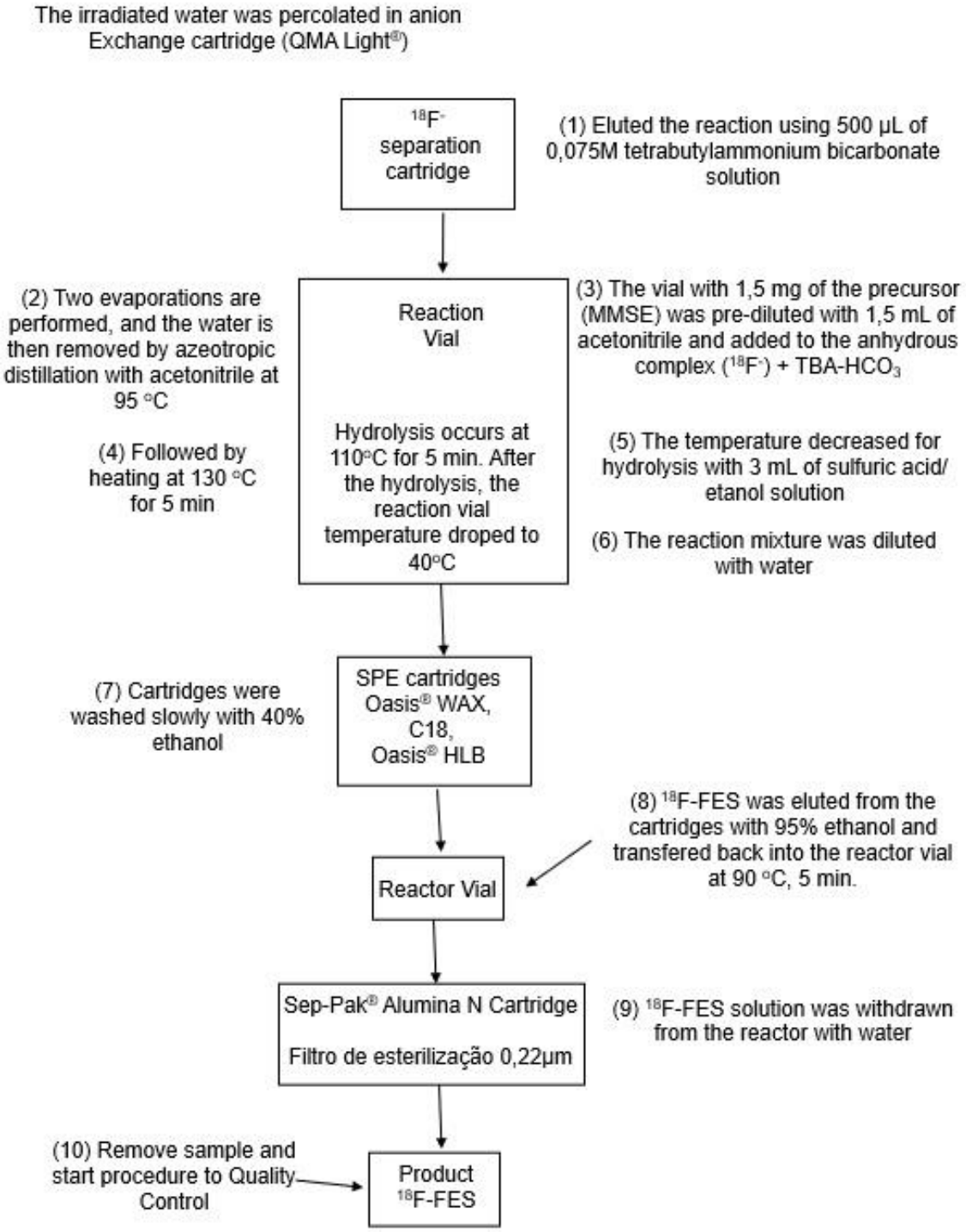


Figure 1 - *Steps of the production of the [^{18}F]FES in the GE TRACERlab™ MXFDG module. Source. Balieiro, 2019 [14]*

2.2 Quality Control

The radionuclide identity was performed with the determination of the physical half-life of [^{18}F]fluorine in a sample of the radiopharmaceutical [^{18}F]FES. The activity of an aliquot of the radiopharmaceutical was measured (Capintec, USA) three times, every 10 minutes, and a semi-logarithmic decay curve was constructed. The radionuclide purity of the radiopharmaceutical [^{18}F]FES was analyzed using a germanium hyper pure (HPGe) detector (Canberra Inc., USA), with a multichannel analyzer system in [^{18}F]FES samples (0.37 MBq/1mL), packed in glass vials, for 30 minutes.

The radiochemical purity was determined by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) techniques.

HPLC was performed using Shimadzu equipment (Japan) C18 reverse phase column, Phenomenex (Phenomenex, USA) with 5 µm particle, 4.6 x 250 mm, eluted with an isocratic mixture of acetonitrile:water (40:60), a flow rate of 1mL/minute, with UV (280 nm) and radioactivity detectors.

The TLC was performed in aluminum strips covered with silica gel 60 (TLC-SG, Merck) of 1,5 x 12,5 cm and a mobile phase of acetonitrile:water 95:5. After running, the strips were cut into 1 cm segments and radioactivity was detected with a Gamma Counter(Packard, USA). The percent of radiochemical purity was determined as follows:

$$\% \text{ Radiochemical Purity} = \frac{\Sigma \text{ (segments 8, 9 and 10 cm)}}{\Sigma \text{ total de segments}} \times 100 \quad (1)$$

The chemical impurity TBA-HCO_3, which may result from the production process, was assayed by TLC-SG, using the same TLC-SG strip and methanol: ammonium hydroxide (30% in water) 9:1, v / v, as mobile phase. It was applied on a 4.5 cm wide (3x1.5 cm) and 12.5 cm long strip, 5 µL of the $TBAHCO_3$ standard (2.6 mg / V, V being the volume of 20 mL), 5 µL of the product sample to be evaluated and 5 µL of water for injections as a negative control. Ascending chromatography was developed, the strips were dried at room temperature and stained with iodine vapor (iodine crystals) to reveal the impurity (TBA-HCO_3).

The determination of residual solvents acetonitrile and ethanol was performed using gas chromatography. Under the conditions for analysis, the samples were placed in the gas chromatograph injection system (Shimadzu, Japan) and diluted at 10:1 ratio and the flame at 250 °C. A column of 30 m x 0.25 mm of fused silica G16, coated with 0.50 µm particles, was employed and helium gas flow of 2 mL/minute. The chromatograph was initially programmed at a temperature of 40 °C for 2 minutes, and the temperature was increased at a rate of 20 °C per minute to 130 °C and maintained at 130 °C for 3 minutes. The injection port and detector temperatures were maintained at 250 ° C and 300 ° C, respectively.

For the bacterial endotoxin assay, equal volumes of LAL reagent (The Charles River Endosafe, USA) and radiopharmaceutical [^{18}F]FES (0.1 mL) were added in three test tubes. The mixture was homogenized and incubated in a water bath at 37 ºC for one hour. Then, the tubes were removed from the bath and inverted 180 degrees to observe any gel formation.

The pH of the product was determined using Merck indicator strips from 0 to 14.

Sterility assay of the [^{18}F]FES was performed using the direct inoculation method described in the Brazilian Pharmacopeia, using fluid thioglycolate broth medium for anaerobic and aerobic bacteria, and the appropriate casein soy medium (TSB) for fungi and aerobic bacteria.

The stability of the [^{18}F]FES was evaluated at room temperature for 6 hours after synthesis, in one hour intervals, using radiochemical purity tests (TLC-SG). After 6 hours, all quality assays for the final product were performed. The stability study aimed to determine the shelf life of the radiopharmaceutical.

2.3 Biodistribution

The studies in healthy animals were carried out in female *BALB-c* mice, weighing between 20 - 24 g. The animals were separated into three groups of 5 animals, which received the administration of the activity of 6,5 MBq / 100 µL (150 µCi / 100 µL) by caudal intravenous route. They were euthanized at the times of 30, 60 minutes and 2 hours after administration.

After euthanasia and dissection, blood and organ/tissue samples of interest were subsequently taken, washed, weighed and the radioactivity in each organ or tissue was determined in a gamma counter (Cobra, Packard Instrument).

The percentage of the activity injected per gram of the organ / tissue or mL of blood, (% AI / g), was determined as follows (where cpm means counts per minute):

$$\%AI/g = \frac{\text{cpm organs}}{\text{weight organ (g)} \times (\text{cpm standard} - \text{cpm caudal})} \times 100 \quad (2)$$

Biodistribution studies were performed in the tumor breast MCF-7 cells xenograft model using immunodeficient female mice (*BALB-c/Scid*) weighing between 20 -24 g.

MCF-7 cell line, as previously described [15], is interesting because it maintains a series of characteristics like the human mammary epithelium and being one of the few strains that express estrogen receptors. ATCC® HTB-22™ strains were grown at 37 ºC and 5 % de CO_2 in DMEM-High Glucose culture medium (Life Technologies, NY, USA) enriched with 10% fetal bovine serum (SFB) (Life Technologies, NY, USA), 100 mg/mL of penicillin/streptomycin antibiotic mixture (Gibco Life Technologies, NY, USA) and 44 nM sodium bicarbonate (Synth, SP, Brazil).

Mice received hormonal supplementation orally with 5 mg of estradiol (manipulated as a solution), offered for free demand with weekly change. The mice were inoculated with $5x10^6$ tumor cells on the upper left flank and the hormonal supplementation continued for 13 days. Approximately 17 days after injection, when the tumor reached the desired size, palpable and not measurable, the animals were prepared for the biodistribution, following the same conditions proposed for the biodistribution study at 30 and 60 minutes, in healthy animals, and at 60 and 120 minutes with receptor blocked.

The blocking group received 5 mg of injectable hormone E.C.P.® (estradiol cypionate) 2 mg/mL (Zoetis), intramuscularly, 24 hours before the biodistribution study.

2.4 PET/CT Image

A PET / CT image with the radiopharmaceutical [^{18}F]FES was performed in female *BALB-c/Scid* mice bearing MCF7 tumors. When the tumor reached the desired size, palpable and not measurable, the animals received a dose of 6.5 MBq / 100 μL (150 μCi) of the radiopharmaceutical [^{18}F]FES. Immediately after dose administration, sequential images were acquired in the Albira micro-PET / CT equipment from the IPEN / CNEN for 90 minutes. The images were reconstructed

by the Albira software and analyzed by the PMOD software (PMOD Technologies, version 8.1), mainly observing the uptake in the tumor and the elimination of the compound.

3. RESULTS AND DISCUSSION

Eight syntheses were carried out, the irradiation time and the production activity of $^{18}F^-$ during the experiments ranged from 60 to 140 minutes and 74 and 159.1 GBq (2.0 and 4.3 Ci), respectively, and all irradiations were carried out in the low volume target holder (2.6 mL) synthesis.

The software provided by ABX for [^{18}F]FES synthesis, had routine adapted in the step corresponding to the final transfer of the product, because in the production facility of IPEN, the module was installed in a cell and the sterilizing filtration was performed in another cell. The need to adapt the production program had already been previously reported, but without presenting details of the adjustment performed [11].

Thorough analysis was performed of all components and materials that are provided by ABX. The cassettes are sterile for single use, which avoids the risk of cross contamination between different radiopharmaceuticals. Different productions are carried out in the same automated module.

The synthesis module was easy to operate, presenting the advantages related to the use of a reagent kit and a disposable cassette, which have a presentation as recommended by good manufacturing practices, for single use, avoiding possible contamination between processes.

The average yield for the syntheses synthesis from high activity labeling (159.1 GBq) was 12.1%, practically equal to the average yield from low activity (74 GBq), given 11.9%. Thus, the starting activity of [^{18}F]fluorine did not interfere in the synthesis yield. The acceptance criterion adopted for synthesis yield (10-20%) was based on literature, considering the chosen synthesis route. Some authors [5,9,16,17,18] described yields of 20% without correction, but the yields found in this work was the same as those of Bispo et al. [11] and higher than those described by Huang et al. [10]

Although the synthesis showed a low radiochemical yield, the final activity of the radiopharmaceutical batch (148 GBq) may result in numerous individual doses, depending on their calibration.

The TLC assay provided results in about 25 minutes and proved to be a good method to separate the product from its impurity. Furthermore, the volume of solvent used for TLC was much smaller than that used in HPLC assay and at a low cost.

All used methods in the quality control assays demonstrated suitability for evaluating the [^{18}F]FES. The results of TLC and HPLC final product chromatograms are shown in figure 2 and 3, respectively. The HPLC chromatogram shows some impurities, although not identified yet. However, the peak area did not exceed the impurities limit of 5%. The average results obtained in all tests performed for the [^{18}F]FES batches are shown in Table 1. The concentrations obtained of acetonitrile are below the limit of quantification (LOQ). Therefore, the values fully met the acceptance criteria adopted, established in the Brazilian, American and European Pharmacopoeias [19,20,21].

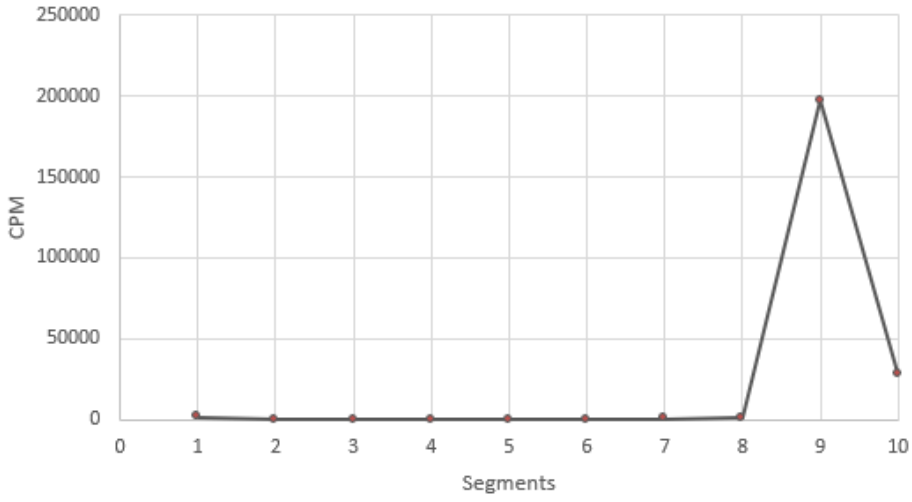


Figure 2 – *CCD-SG Chromatographic profile of [^{18}F]FES. Rf. 0.9 – 1.0.*

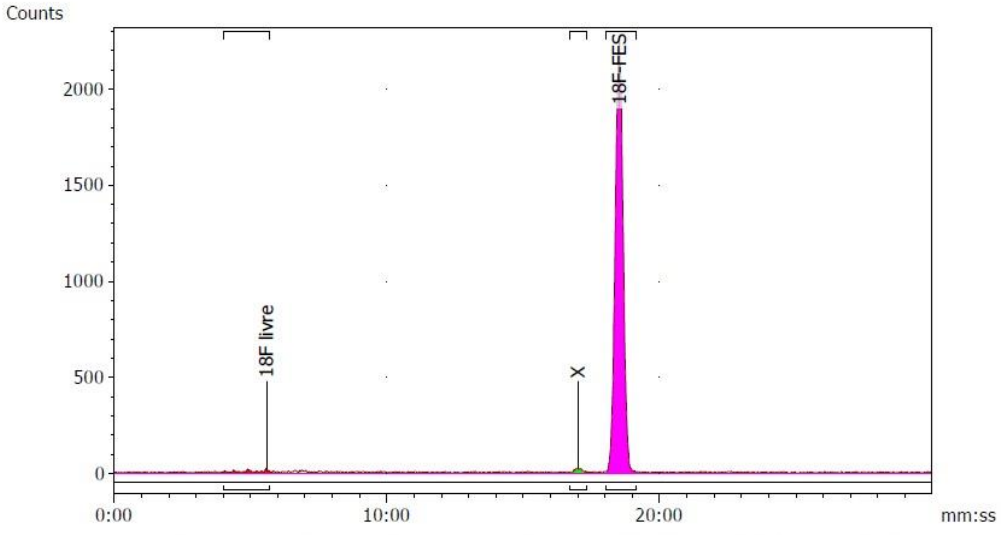


***Figure 3** – HPLC Chromatographic profile of [^{18}F]FES.*

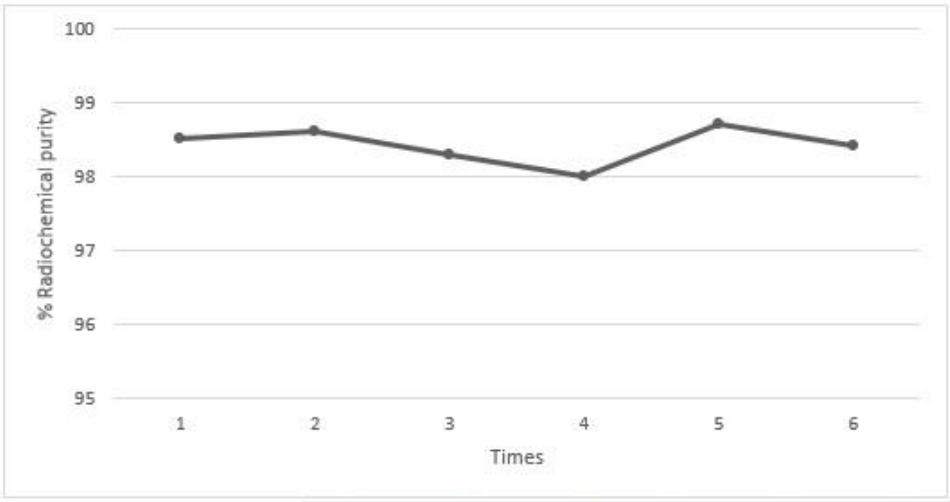
Table 1 - Average results of synthesis yield and quality control assays for 8 batches [^{18}F]FES.

Teste	Result	Medium deviation	Acceptance criteria	Reference criteria
Yield	11.9	± 1.63	10 - 20%	Literature
Radionuclide Identity	109.5	± 0.50	105 – 115	FB - fludesoxiglicose (18F)
Radionuclide Purity	100 %	0	> 99.5 %	
Description	Limpid e colorless	N.A.	Limpid e colorless	
Radiochemical purity (CCD)	98.6 %	± 0.47	≥ 95%	
Chemical Impurity	< 2.6 mg/V	N.A.	< 2.6 mg/V	FE - fluorodopa (18F)
pH	6.5	± 0.5	4.0 – 7.5	USP - Draft fluoroestradiol (18F)
Acetonitrile	LOQ	LOQ	< 410 ppm	FB - fludesoxiglicose (18F)

Ethanol	4.1 %	± 1.25	< 10%	FE - fluorodopa (18F)
LAL	12.5 UE mL $^{-1}$	N.A.	< 175 UE/mL	FB - fludesoxiglicose (18F)
Sterility	Negative	N.A.	Negative	FB-fludesoxiglicose (18F)

FB = Brazilian Pharmacopoeia; FE = European Pharmacopoeia

The study of the stability of [^{18}F]FES, carried out with a dose of 18.4 GBq (498 mCi) at room temperature demonstrated the radiochemical stability of the product for 6 hours (figure 4).



	1	2	3	4	5	6
Rf	0.9	1.0	0.9	1.0	1.0	1.0
% Radiochemical purity	98.5	98.6	98.3	98.0	98.7	98.4

***Figure 4** – Radiopharmaceutical stability of [^{18}F]FES at room temperature.*

The results of the biodistribution of [^{18}F]FES in animals, in %AI/g of organ/tissue and mL of blood (Figure 5), showing that the radiopharmaceutical is rapidly cleared from the blood.

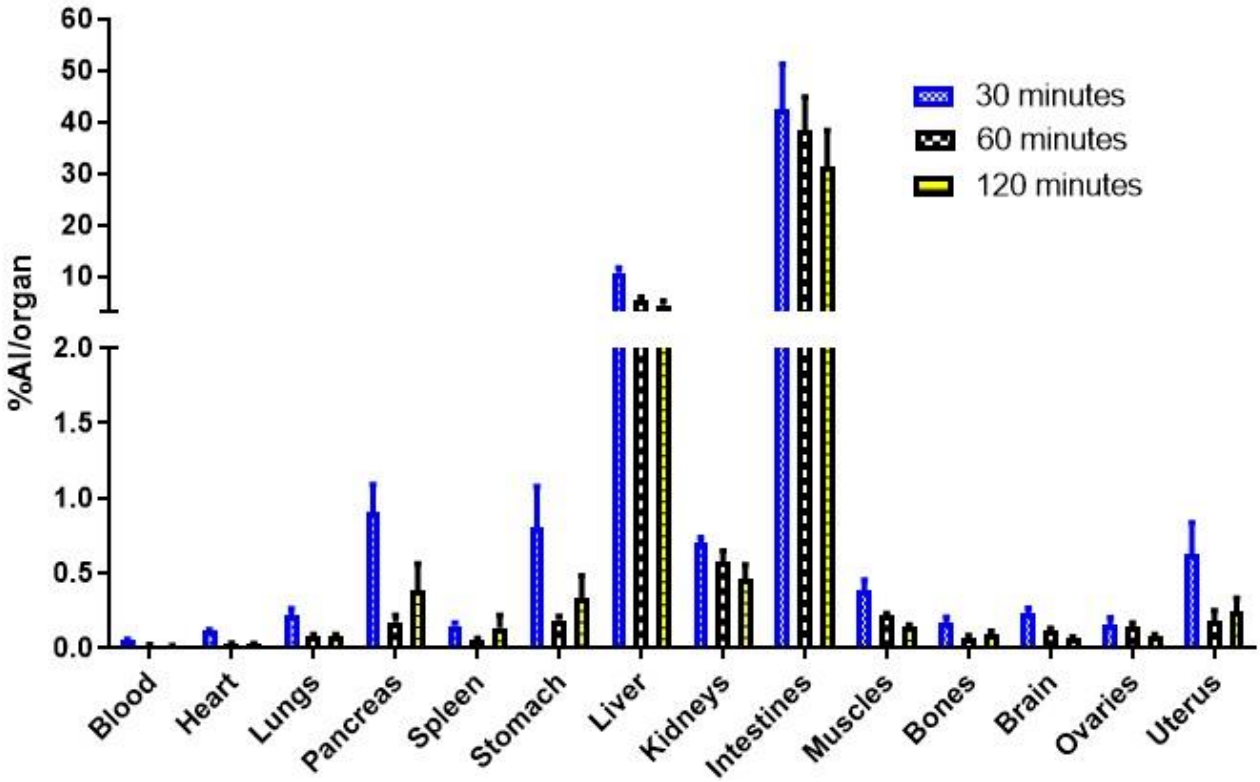


***Figure 5** - Percentage of injected activity of [^{18}F]FES per gram (% AI / g) of organ/tissue and mL of blood in BALBc mice at different times after administration.*

The uptake of [^{18}F]FES in all analyzed organs, including those with estrogen receptors, was greater at thirty minutes post-injection, especially in the uterus and ovaries. Significant uptake in the liver and intestines was observed, possibly related to preferential excretion via the intestinal tract, with a metabolic profile like estradiol. In the experimental protocol, the intestines were removed with content. Renal uptake was compatible with elimination from the urinary tract.

The [^{18}F]FES uptake data in the organs and tumors of the *BALB-c/Scid* mice are presented in %AI/g of organ/tissue or mL of blood in Figure 6.

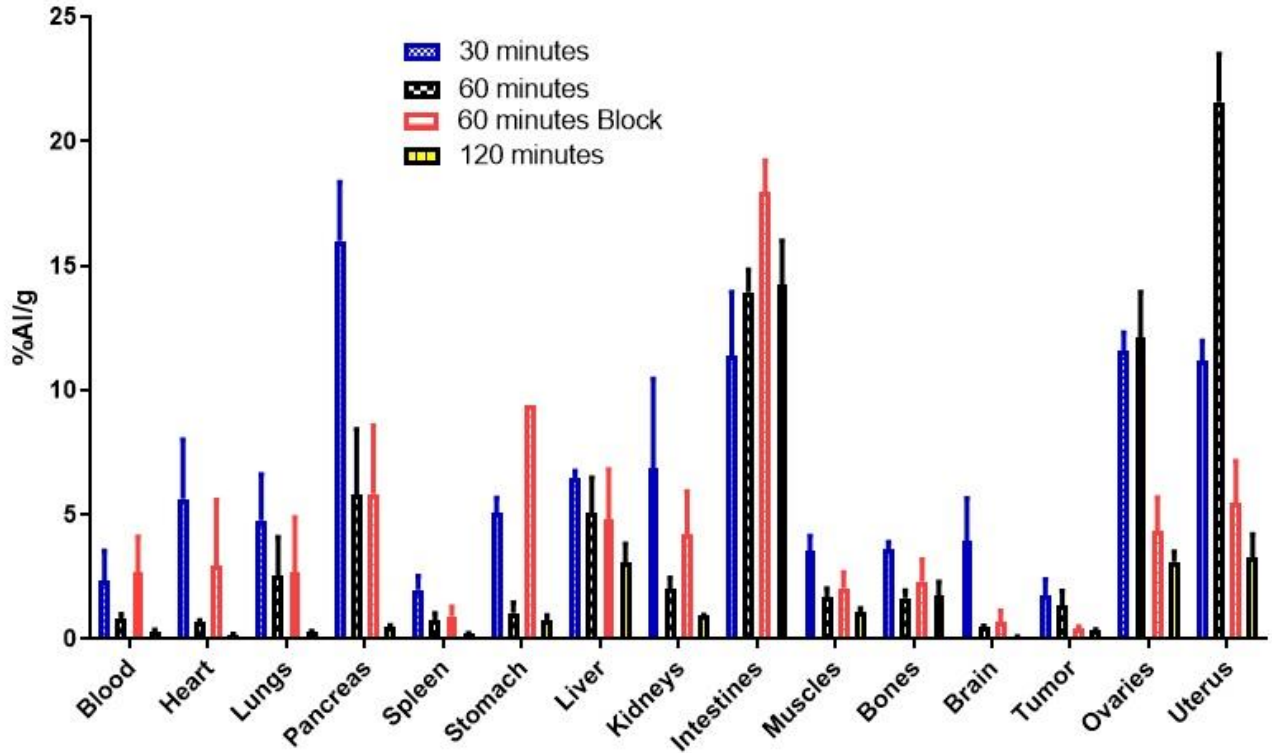


Figure 6 - *Percentage of the injected activity of [^{18}F]FES per gram (%AI/g) of organ / tissue and mL of blood, in BALBc/Scid mice with tumor different times after administration.*

There is no significant difference in the uptake of [^{18}F]FES in the periods of 30 and 60 minutes in the tumor (p-value of 0.55) and the ovaries (p-value of 0.76). However, the greater uptake of [^{18}F]FES was observed in the uterus in 60 minutes (p-value 0.03), and this organ has ER + estrogen receptors.

The group that received hormonal blocking of estradiol 24 hours before the [^{18}F]FES administration showed a good response. A decreased uptake of the radiopharmaceutical [^{18}F]FES was observed in the receptors, in tumor cells, and organs such as the uterus and ovaries. Therefore, it was confirmed the specificity of the radiopharmaceutical binding to the estrogen receptor. In the group with the blockade, greater blood uptake was observed, suggesting a decrease in blood clearance, in addition to greater uptake by excretory organs, compatible with the literature. [15,22,23].

The image showed in the Figure 7 represents the PET biodistributio of the [^{18}F]FES, with intense uptake in the digestive system, compatible with estradiol biodistribution. The arrow

indicates the area of localization and uptake in the tumor on the upper left flank, and an area also delimited in red representing the VOI (volume of interest).

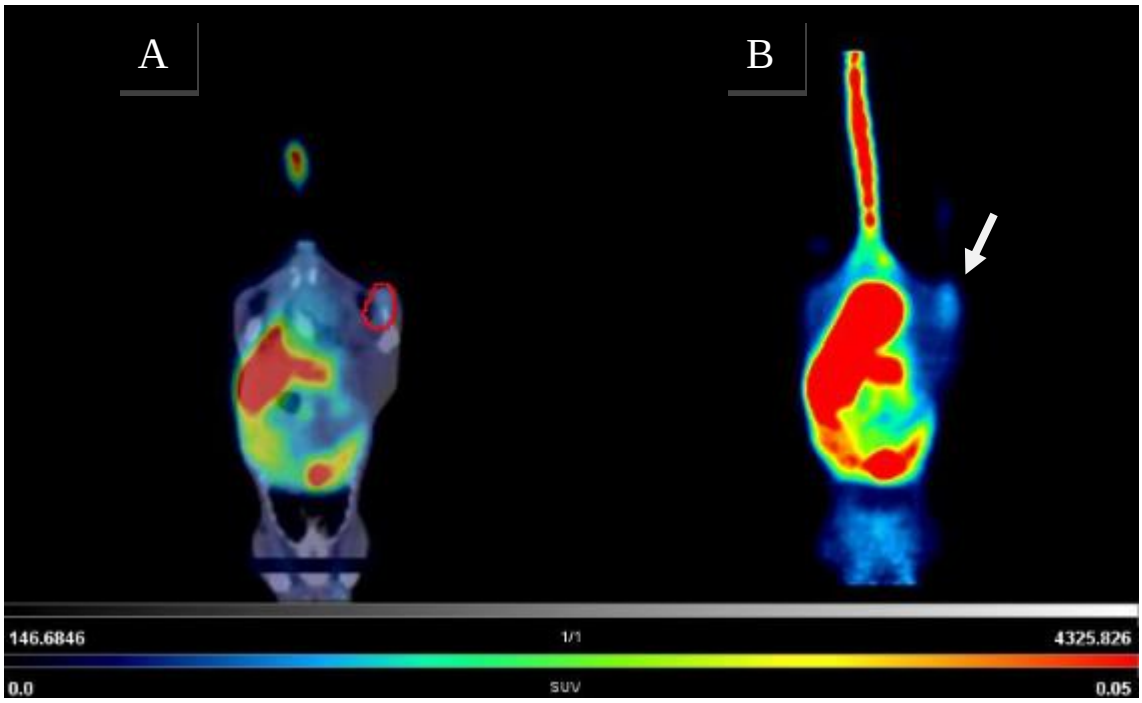


Figure 7 - *Longitudinal prone position of micro-PET / CT of female BALBc/Scid mice after administration of the radiopharmaceutical [^{18}F]FES. Fig 5A represents the fusion of PET and CT techniques, delimiting the VOI area in red. Fig 5B represents the PET image, cumulative of 60 minutes; the arrow indicates the location of the tumor.*

4. CONCLUSION

The synthesis of the radiopharmaceutical 16α-[^{18}F]fluoro-17β-estradiol ([^{18}F]FES), performed in 75 minutes, showed reproductive yields independent of the [^{18}F]fluorine activity used for labeling, within the limits studied.

The quality control analysis showed that the [^{18}F]FES meets all the acceptance criteria, in addition to remaining stable for 6 hours after the synthesis.

Biodistribution studies in healthy mice have shown the metabolism of [^{18}F]FES in female *BALB*-c, demonstrating the radiopharmaceutical's affinity for organs that have alpha estrogen receptors and their elimination compatible with endogenous estradiol.

The biodistribution study in *BALB-c/Scid* mice bearing MCF7 tumors showed uptake in the tumor in the periods of 30 and 60 minutes. When comparing the time of 60 minutes with or without the blockade, the difference in uptake is statistically noted, showing that the blockage of estrogen receptors worked and decreased the uptake of the radiopharmaceutical, confirming the affinity of [^{18}F]FES for the estrogen receptors.

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