Scientific Research Publishing

Advances in Chemical Engineering and Science, 2017, 7, 23-33
http://www.scirp.org/journal/aces
ISSN Online: 2160-0406
ISSN Print: 2160-0392

Comparison between Two Ethanolic Solutions for 3'-Deoxy-3'-[^{18}F]Fluorothymidine Elution

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How to cite this paper: Nascimento, L.T.C., Silveira, M.B., Ferreira, S.M.Z.M.D. and Silva, J.B. (2017) Comparison between Two Ethanolic Solutions for 3'-Deoxy-3'-[18F] Fluorothymidine Elution. *Advances in Chemical Engineering and Science*, **7**, 23-33.
http://dx.doi.org/10.4236/aces.2017.71003

Received: August 30, 2016
Accepted: December 18, 2016
Published: December 21, 2016

Abstract

3'-Deoxy-3'-[^{18}F]Fluorothymidine—[^{18}F]FLT is a Positron Emission Tomography (PET) tracer which has been used for noninvasive assessment of proliferation activity in several types of cancer. During the past few years, some novel approaches for [^{18}F]FLT synthesis have been developed, mainly focused on optimization of reaction conditions and purification methods. The present study reports the use of two different eluents in the final step of [^{18}F]FLT production and the evaluation of its effect on radiochemical yield and product quality. The first eluent evaluated was water: ethanol (90:10, v/v), commercially available, and the second was NaCl 0.9% (saline): ethanol (92:8, v/v). The mean of the corrected radiochemical yields corresponded to 27% ± 7% for elution with water and ethanol and to 23% ± 3% for elution with saline and ethanol, which could indicate that the eluent solutions have similar elution strength. Besides, quality control results were in accordance with the requirements and demonstrated that there was no significant difference between both formulations. Considering that pharmaceutical preparations containing ethanol should be preferentially diluted with saline to avoid hemolysis, the eluent saline:ethanol (92:8, v/v) was chosen for [^{18}F]FLT extraction and final formulation.

Keywords

[^{18}F]FLT, Synthesis, Quality Control, Formulation, Elution, Radiopharmaceutical

1. Introduction

The usefulness of Positron Emission Tomography/Computed Tomography (PET/CT) imaging has been established for diagnosis and response assessment of various diseases, especially in oncology [1]. [^{18}F]Fluorodeoxyglucose ([^{18}F]FDG) is the most commonly used PET radiopharmaceutical for tumor imaging with a wide field of application. However, it has some limitations for evaluating malignancies in organs with physiological uptake such as central nervous system tissues, heart and bladder [2] [3]. Besides, [^{18}F]FDG is not a highly selective tracer for tumor imaging and cannot distinguish be-

tween inflammatory processes and cancer, for example [4].

Efforts have been applied to develop new PET imaging agents to quantify specific metabolic processes such as cell proliferation, receptor density and function, and other characteristics [5]. 3'-deoxy-3'-[^{18}F]fluorothymidine ([^{18}F]FLT) is a radioactive analogue of the nucleoside thymidine which has been proposed for imaging of cellular proliferation [6]. Clinical studies have demonstrated that this radiopharmaceutical is a promising radiotracer for detection and therapy response assessment in several types of cancer, including breast, lung, colorectal and brain cancers [7] [8] [9], or other disturbs such as inflammation in arthritis [10].

[^{18}F]FLT stable analogue ([^{19}F]3'-deoxy-3'-fluorothymidine, FLT) was developed for the first time in 1969 intending to be an antitumoral agent [11]. Later, in the 90's it was used as the anti-retroviral agent named alovudine [12]. In both cases, it showed toxicity incompatible with therapeutic applications [13] [14]. The ^{18}F-labelled thymidine was synthesized for the first time in 1991 to study the anti-retroviral mechanism [15]. The molecule was used for PET for the first time in 1996 and from this year to now, different synthesis routes were investigated to improve its radiochemical yield and radiochemical purity [16] [17] [18].

The [^{18}F]FLT formulations generally are constituted by an ethanolic solution containing up to 10% of this solvent [19] [20] [21] [22]. The use of ethanol is required in the purification step when high yields are desired. According to the International Conference on Harmonization [23], the concentration of ethanol should be lower than 0.5% for human use as it is a class 3 solvent, unless specifically justified. However, commercially available drugs, as paricalcitrol, may contain up to 20% of ethanol [24]. Radiopharmaceuticals such as [^{18}F]fluoromisonidazole and [^{18}F]fluoroethyltyrosine also contain a high concentration of ethanol (up to 10% and 20% respectively) in their final formulation [25].

However, whenever possible, it is desirable that have radiopharmaceutical formulations containing as low ethanol content as possible to reduce the risk of hemolysis. Another reason is that children, women during pregnancy and lactation, persons with alcoholism liver disease and/or epilepsy, could experience some toxic effects due to injection of higher ethanol concentrations [26].

This article presents a comparative study between two different eluents: water:ethanol (90:10, v/v), commercially available, and saline:ethanol (92:8, v/v) solution. Both were compared in order to determine the effect on the radiochemical yield and final formulation properties.

2. Material and Methods

2.1. General

The reagents kits and sterile cassettes used for production of [^{18}F]FLT were purchased from ABX Advanced Biochemical Compounds (Radeberg, Germany). Fluorothymidine standard was also supplied by ABX. Enriched ^{18}O water was purchased from Center of Molecular Research (Moscow, Russia). The reference standards of stavudine and 3'-chloro-3'-deoxy-thymidine (chlorothymidine) were acquired from U. S. Pharmacopeia (Rockville, USA). Other chemicals and solvents were of analytical grade and were ob-

tained from conventional chemical suppliers. PTS™ cartridges were supplied by Charles River Laboratories (Wilmington, USA). Soybean-Casein digest and fluid thioglycolate media were acquired from Newprov (Paraná, Brazil).

SepPak® Light Accell Plus QMA, Oasis® WAX, Oasis® HLB, SepPak® Alumina N and Chromabond® PS-H$^+$ cartridges were conditioned before use: QMA with 0.5 M carbonate solution (10 mL), water (15 mL) and dried with air; HLB, Alumina and WAX with ethanol (5 mL), water (10 mL) and dried with air; and PS-H$^+$ with ethanol (2 mL), water (4 mL) and dried with air.

The water:ethanol (90:10, v/v) solution was used without any modifications from ABX (Radeberg, Germany). Ethanol acquired from J.T. Baker (Phillipsburg, USA) was used to prepare the 8% (v/v) solution of ethanol in 0.9% sodium chloride for injection (saline) which was purchased from Sanobiol (Pouso Alegre, Brazil).

2.2. Automated Synthesis of [^{18}F]FLT

[^{18}F]Fluoride (11 to 135 GBq) was produced on a 16.5 MeV Cyclotron PETtrace® (GE Healthcare Technologies, Waukesha, USA) via the ^{18}O(p,n)^{18}F nuclear reaction. The solution containing [^{18}F]fluoride was delivered from the cyclotron to a TracerLab® MX_{FDG} module (GE Healthcare, USA), adapted for the production of [^{18}F]FLT as shown in Figure 1. Firstly, the [^{18}F]fluoride was trapped in an anion exchange column (SepPak

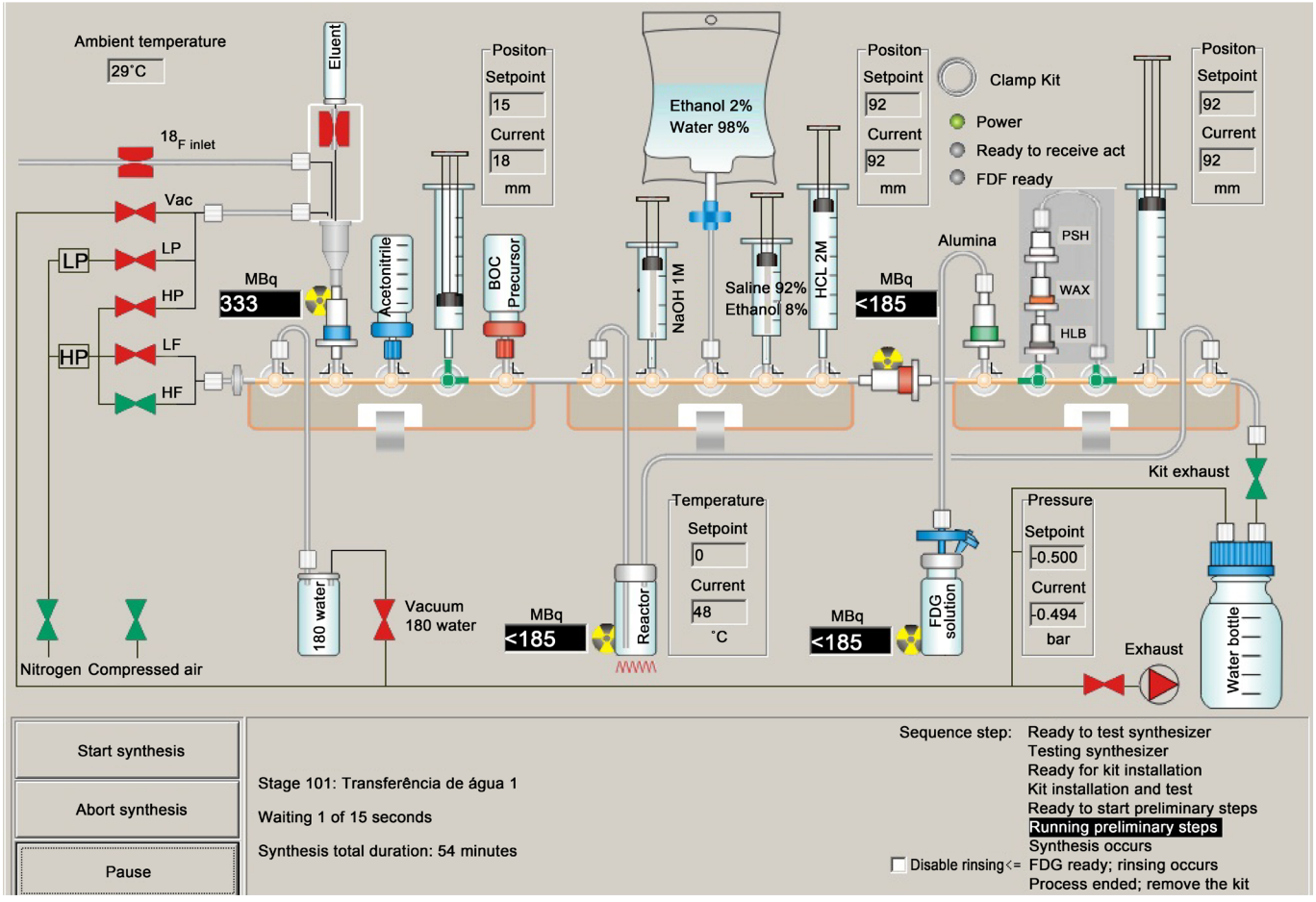

Figure 1. TracerLab MX_{FDG} configuration, accessories and reagents used for the production of [^{18}F]FLT.

Light Accell Plus QMA). Then, it was eluted with 0.75 mL of 0.075 M tetrabutylammonium bicarbonate to the reaction vessel where was dried by azeotropic distillation at 95˚C using three successive additions of anhydrous acetonitrile (80 µL) and low flow of nitrogen (40 mL/min). Once [^{18}F]fluoride-tetrabutylammonium was obtained, 25 mg of the [^{18}F]FLT precursor (3-N-BOC-5-O-DMTr-3-O-nosyl-lyxothymidine) solubilized in anhydrous acetonitrile was added to the dried residue in the reactor vial. Nucleophilic substitution was carried out at 100˚C for 5 minutes, followed by hydrolysis step with 2 M HCl (1.5 mL) at 85˚C for another 5 minutes. The mixture was allowed to evaporate at the same temperature. Then, it was cooled and neutralized with 1 M NaOH (2.4 mL) and passed through PS-H$^+$, WAX and HLB cartridges for purification. Cartridges were washed with ethanol 2% (v/v) (approximately 90 mL). [^{18}F]FLT was eluted from the HLB cartridge with a saline:ethanol (92:8, v/v) or a water:ethanol (90:10, v/v) solution (both 14 mL). The product was passed through an Alumina N short cartridge and sterilized through a 0.22 µm membrane filter. Total synthesis time was 54 minutes.

2.3. Quality Control of [^{18}F]FLT

The pH, radionuclidic identity and purity, residual solvent (acetonitrile), ethanol determination, radiochemical identity and purity, chemical purity (tetrabutylammonium, thymine, thymidine, stavudine, chlorothymidine), bacterial endotoxins, and sterility of each final formulation of [^{18}F]FLT were assessed. The pH was measured using micro pHmeter (Mettler Toledo, USA). Radionuclidic identity was confirmed by measuring the half-life of the final product in a CRC 25R dose calibrator (Capintec, USA) at 3 time points. Radionuclidic purity of [^{18}F]FLT was determined on a gamma-ray spectrometer with an ultrapure Ge detector (Canberra Multichannel Analyzer, USA).

The amount of ethanol and residual solvent (acetonitrile) in [^{18}F]FLT was determined by gas chromatography. Analyses were carried out in a Varian 3900 GC equipment (Santa Clara, USA), equipped with split/splitless injector inlet and a flame ionization. An Agilent CP-Wax 52 CB column (30 m × 0.53 mm; 1 µm) was used as the stationary phase. Helium at a flow rate of 1.5 mL/min was employed as the carrier gas. The GC oven temperature was programmed as follows. Initially the temperature was maintained at 45˚C for 4 minutes. Then the temperature was increased to 80˚C at a rate of 30˚C/min, and maintained at 80˚C for 0.83 min. The injector port and detector were maintained at 140˚C and 250˚C, respectively. The injector was operated in a split mode of 70:1.

Both products were tested for chemical and radiochemical purity by analytical HPLC. Analyses were performed using an Agilent 1200 Liquid Chromatograph (Santa Clara, USA), equipped with a UV detector (adjusted at 267 nm) and a Raytest radioactive detector (Straubenhardt, Germany). A Supelcosil LC-18 column (250 × 4.6 mm; 5 µm) was used for separation. The mobile phase A was ethanol 10% in water, while mobile phase B was ethanol 100%, used as a gradient according to Table 1. The mobile phase flow rate was 1.2 mL/min. The presence of non-radioactive impurities (thymine, thymidine, stavudine and chlorothymidine) was also checked under the same HPLC conditions. The method was validated in terms of quantification/detection limits, precision, specificity, accuracy, robustness and linearity.

Table 1. Mobile phase composition in HPLC analysis.

Time (minutes)	% mobile phase A	% mobile phase B	Condition
0 to 10	100	0	Isocratic
10 to 13	90	10	Gradient
13 to 18	90	10	Isocratic
18 to 21	100	0	Gradient

A spot test was used to evaluate tetrabutylammonium cation in [^{18}F]FLT [27]. A sample of the radiopharmaceutical was applied to a thin layer silica plate, alongside two another standard solutions of tetrabutylammonium bicarbonate 100 and 50 μg/mL (1 μL). Thereafter the plate was placed on an iodine chamber for 4 minutes. The size and intensity of the spot obtained from the test solution were compared to that obtained from the standard.

Bacterial endotoxins were quantified by LAL kinetic chromogenic method, using the Endosafe® - Portable Test System (Charles River, USA). Sterility test was performed by direct inoculation of [^{18}F]FLT into soybean-casein digest and fluid thioglycolate media.

3. Results and Discussion

Although various precursors have been proposed for the radiosynthesis of [^{18}F]FLT, in this work, 5'-O-DMTr-3'-O-nosyl-lyxothymidine was used, as several reports indicated high incorporation of [^{18}F]fluoride. A wide range of radiochemical yield and chemical purity for the synthesis of [^{18}F]FLT have been described in literature, mainly because it may be affected by many factors, such as: phase-transfer agent, precursor amount, synthesis module, purification method, etc. Although semipreparative HPLC purification is described by some authors [22] [28] [29], it has been reported as time consuming. In the present study, purification was performed by means of solid-phase extraction cartridges, a simple, rapid and efficient purification method suitable for the TracerLab MX_{FDG} synthesis module [20].

Quality control tests, product specifications and results obtained for [^{18}F]FLT are presented in **Table 2**. All parameters were in agreement to quality criteria confirming that [^{18}F]FLT, eluted with both solution, was suitable for use. A solution without ethanol was previously evaluated (data not shown), as proposed by Pascali and co-workers [20]. However, the uncorrected radiochemical yields were lower than 7% and the concentration of impurities such as thymidine was higher than 100 μg/mL.

A syringe with 20 mL of water:ethanol (90:10, v/v) is included in the reagent kit used in this work. Tests using different volumes of eluent solution were performed and the results (not shown) indicated that the yield was the same when 14 mL was used. Smaller volumes make feasible further dilutions, reducing ethanol final concentrations.

Since no brazilian pharmacopoeic monograph is current available for [^{18}F]FLT, the quality control parameters were established according the European Pharmacopoeia 8.0 and related literature [20] [30]. The impurities (thymine, thymidine, stavudine, chlorothymidine and unknown ones), must be found in concentrations lower than 6.7 μg/mL, considering the maximum volume (V) of 15 mL. In this work their concentra-

Table 2. Comparative results between both proposed [^{18}F]FLT formulations and Alovudine (^{18}F) Injection European Pharmacopoeia 8.0 monograph criteria [30].

	Quality Control Tests	Ph.Eur 8.0	Ethanol 10% in water for injections (n = 6)	Ethanol 8% in saline (n = 11)
	Corrected yield (%)	Not applicable	27± 7	23 ± 3
	Specific activity in the end of synthesis (GBq/μmol)	Not applicable	>117.1	>178.6
	pH	4.5 to 8.5	4.54 to 7.03	4.57 to 7.06
	Residual Solvents (Acetonitrile) (%V/V)	<0.04	0.00012 to 0.00193	0.00096 to 0.0067
	Ethanol Determination (%V/V)	<10% or 2.5 g per administration	6.15 to 8.99	5.81 to 8.07
	Radiochemical Purity (%)	>95%	98.43 to 99.40	98.43 to 99.19
	Radionuclidic Identity (min)	105 to 115	109.56 to 110.70	109.30 to 113.08
	Radionuclidic Purity (%)*	>99.9%	99.86 to 99.99	99.70 to 100.00
Chemical Purity	Tetrabutylammonium (μg/mL)	<2.6 mg/V**	<50	<50
	Thymine (μg/mL)	Any other impurities, for each one < 0.1 mg/V**	0.089 to 0.898	0.081 to 0.146
	Thymidine (μg/mL)		0.129 to 0.176	0.096 to 0.176
	Chlorothymidine (μg/mL)		0.132 to 0.532	0.148 to 0.425
	Stavudine (μg/mL)	<0.1 mg/V**	0.023 to 0.061	0.019 to 0.040
	Fluorothymidine (μg/mL)	<0.1 mg/V**	0.178 to 0.466	0.057 to 0.509
	Bacterial Endotoxin (EU/mL)	<175/V**	<5.00	<5.00
	Sterility	Sterile	Pass	Pass

*Radionuclidic Purity, according to the Alovudine's monograph, should present results higher than 99.9%. However, ^{18}F based radiopharmaceuticals monographs, like USP (>99.5% [31]) or the International one (>99.0% of ^{18}F [32], both from [^{18}F]FDG monographs) presents different criteria. **V = Maximum recommended dose in milliliters.

tion were assessed and both formulations meet the requirements established in European Pharmacopoeia 8.0.

It is known that thymidine and thymine are endogenous substances. Their concentrations in the formulation (Table 2) are meaningless for toxicity purposes. However, as thymidine is a [^{18}F]FLT competitor for the intracellular accumulation [33] [34], it should have the lower concentration as possible in order to avoid lack of efficiency. Viertl and co-workers discovered that pretreatment with a low-dose of 5-fluoro-2'-deoxyuridine, a thymidine synthesis inhibitor, increased uptake of [^{18}F]FLT in different tumor xenografts [35], being a promising tool to increase [^{18}F]FLT's sensitivity in humans.

As thymidine, fluorothymidine and chlorothymidine differs only in the carbon 3 ligand (hidroxide, fluoride and chloride, respectively) in their molecular structure, it is hard to believe that chlorothymidine will not affect [^{18}F]FLT cellular's uptake, considering its atomic configuration and chemical properties. As far as we know, only Pascali and co-workers reports its presence in the final product [20].

Stavudine, which is used as an anti-retroviral agent, is often prescribed 40 mg orally twice a day to generate a maximal therapeutic plasma concentration of 0.5 ± 0.2 μg/mL [36]. As small amount of stavudine was detected in both [^{18}F]FLT formulations, any toxic effect due to its presence is very unlikely.

A typical HPLC chromatogram obtained for the [^{18}F]FLT obtained by elution with saline:ethanol (92:8, v/v) is shown in Figure 2. It was possible to verify the presence of already described [20] and unknown impurities. The peak corresponding to [^{18}F]FLT was visualized using the radioactive detector (Figure 2(b)). Radioactivity detection also revealed three minor peaks corresponding to unidentified radiochemical impurities; however, their sum never exceeded 2%. The retention time of the main radioactive peak observed in the chromatogram of [^{18}F]FLT test solution was similar to that of the fluorothymidine reference standard.

The GC analysis of [^{18}F]FLT revealed that it contains a maximum of 8.75% ethanol and 0.00236% of residual acetonitrile (Figure 3) for the saline:ethanol (92:8, v/v) eluent and a maximum of 8.99% of ethanol and 0.00193% of acetonitrile in the water:ethanol (90:10, v/v) eluent, both in accordance with the acceptance criteria. In [^{18}F]FLT final product, the ethanol is not a residual solvent, unlike [^{18}F]FDG, being added purposely to have better yields.

4. Conclusion

[^{18}F]FLT was successfully produced using the synthesizer TracerLab® MX$_{FDG}$. The final

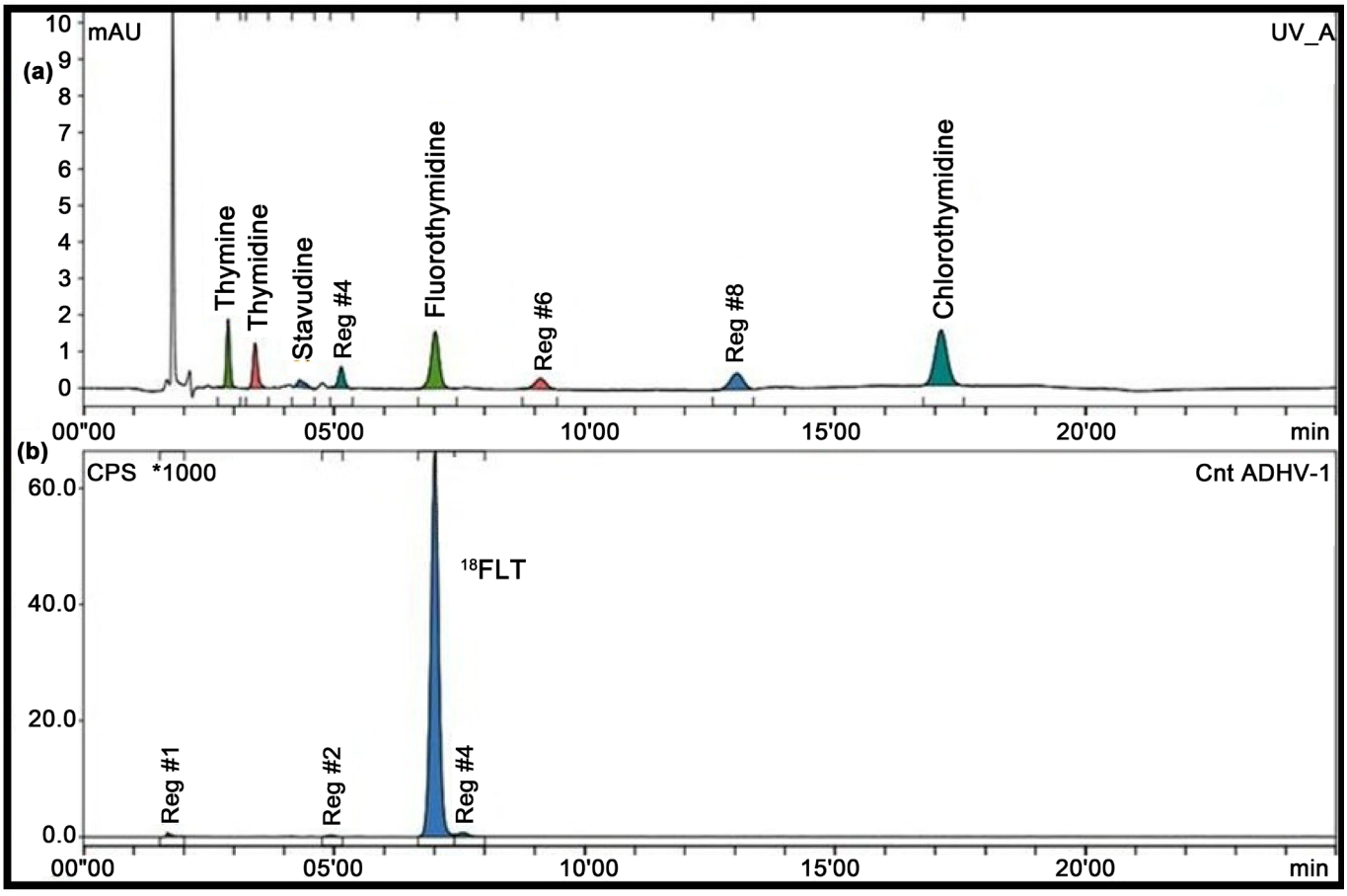

Figure 2. [^{18}F]FLT typical chromatogram of the saline:ethanol (92:8, v/v) formulation obtained with simultaneous detection by an (A) UV absorbance detector (267 nm) and (B) NaI(Tl) scintillation detector. Conditions: see Table 1. Concentration of known substances: thymine = 0.130 μg/mL; thymidine = 0.183 μg/mL; stavudine = 0.053 μg/mL; fluorothymidine = 0.429 μg/mL; chlorothymidine = 0.217 μg/mL. CPS = counts per second. mAU = mili absorbance unit.

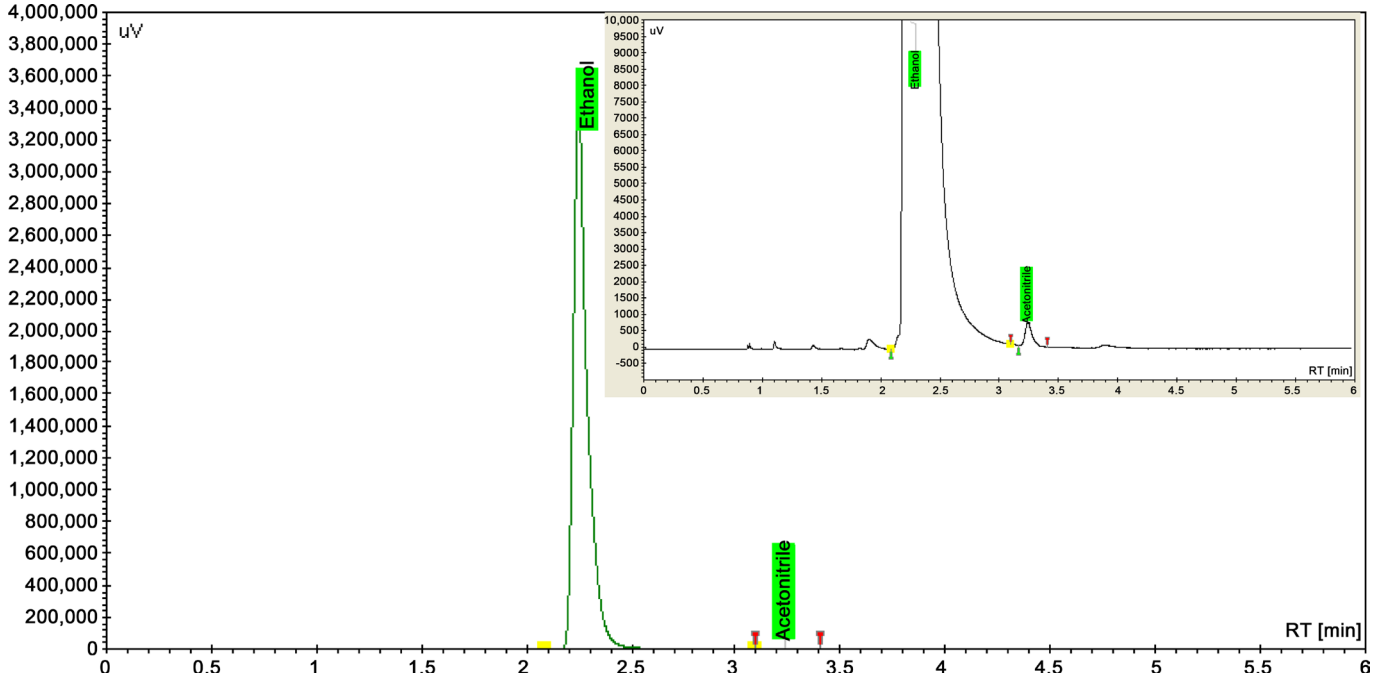

Figure 3. GC chromatogram of saline:ethanol (92:8, v/v) [^{18}F]FLT's eluent. Acetonitrile's peak is magnified on the upper right side of the figure. Conditions: Agilent CP-Wax 52 CB column (30 m × 0.53 mm; 1 μm) eluted with Helium at a flow rate of 1.5 mL/min. Injector port and flame ionizing detector at 140˚C and 250˚C respectively. Oven was submitted to a temperature gradient, varying from 45˚C to 80˚C. RT = retention time. uV = microvolts.

step of the synthesis of [^{18}F]FLT using two different eluent solutions led to similar final radiopharmaceuticals formulations. Saline:ethanol (92:8, v/v) was chosen as our standard, considering that satisfactory yields were achieved and quality control results were in accordance with the requirements. Finally, this radiopharmaceutical can be consistently produced and controlled at Centro de Desenvolvimento da Tecnologia Nuclear.

Acknowledgements

This work was supported by Centro de Desenvolvimento da Tecnologia Nuclear (CDTN, CNEN), Fundação de Amparo à Pesquisa (FAPEMIG APQ-04740-10), INCT de Medicina Molecular (CNPq 573646/2008-2; CBB-APQ-00075-09) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 573646/2008-2). The authors would like to thank the whole group of the Unidade de Pesquisa e Produção de Radiofármacos of CDTN for technical support on production and quality control. We are also grateful to Fundação Ezequiel Dias (FUNED).

Conflict of Interests

The authors declare that there is no conflict of interests.

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