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VALIDATION OF AN HPLC METHOD FOR DETERMINATION OF CHEMICAL PURITY OF [^{18}F]FLUOROMISONIDAZOLE ([^{18}F]FMISO)

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ABSTRACT

[^{18}F]Fluoromisonidazole ([^{18}F]FMISO) is a nitroimidazole derivative labelled with fluorine-18 that selectively binds to hypoxic cells. It has been shown to be a suitable PET tracer for imaging hypoxia in tumors as well as in noncancerous tissues. [^{18}F]FMISO was prepared using a TRACERlabMX$_{FDG}$® module (GE) with cassettes, software sequence and reagents kits from ABX. In this work, we aimed to develop and to validate a new high performance liquid chromatography (HPLC) method for determination of chemical purity of [^{18}F]FMISO. Analyses were performed with an Agilent chromatograph equipped with radioactivity and UV detectors. [18F]FMISO and impurities were

separated on a C18 column by gradient elution with water and acetonitrile. Selectivity, linearity, detection limit (DL), quantification limit (LQ), precision, accuracy and robustness were assessed to demonstrate that the HPLC method is adequate for its intended purpose. The HPLC method showed a good precision, as all RSD values were lower than 5%. Robustness was evaluated considering a variation on parameters such mobile phase gradient and flow rate. Results evidenced that the HPLC method is validated and is suitable for radiochemical purity evaluation of [^{18}F]FMISO, considering operational conditions of our laboratory. As an extension of this work, other analytical methods used for [^{18}F]FMISO quality control should be evaluated, in compliance with good manufacture practice.

Keywords: [^{18}F]Fluoromisonidazole, chemical purity, HPLC

1. INTRODUCTION

Hypoxic regions in tumors are a condition in which the oxygen supply of the tissue is below the levels provided by the bloodstream. Tumor hypoxia is indicative of more aggressive tumor progression and is associated with angiogenesis and also contributes to metastasis [1]. In addition, hypoxia decrease the effectiveness of chemotherapy due to poor diffusion of drugs from blood vessels to the tumor, and also increase radio-resistance due to lower concentration of free radicals from oxygen compared to normally to normally oxygenated tumor tissue [2].

Due to the inherent disadvantages of invasive techniques to detect hypoxia, nuclear medicine imaging is considered an interesting alternative. Positron Emission Tomography (PET) offers the possibility to detect hypoxia non-invasively *in vivo* [3]. [^{18}F]Fluoromisonidazole ([^{18}F]FMISO) is a nitroimidazole derivative labelled with fluorine-18 that selectively binds to hypoxic cells. It has been shown to be a suitable PET tracer for imaging hypoxia in tumors as well as in noncancerous tissues.

The quality requirements of [^{18}F]FMISO are established in the European Pharmacopeia. According to that reference, quality control of [^{18}F]FMISO should include the evaluation of various parameters, such as: appearance, pH, radionuclidic identity and purity, radiochemical identity and purity, chemical purity, residual solvents, bacterial endotoxins and sterility [4].

Although Good Manufacturing Practice (GMP) guidelines states that analytical methods which are included in pharmacopeia do not need to be validated, the suitability of all testing methods used should be verified under actual conditions of use and documented [5]. [^{18}F]FMISO was prepared at Centro de Desenvolvimento da Tecnologia Nuclear (CDTN) using a TRACERlabMX$_{FDG}$^{TM} module (GE) with cassettes, software sequence and reagents kits from ABX.

In this context, the aim in this work was to develop and to validate a new high performance liquid chromatography (HPLC) method for determination of chemical purity of [^{18}F]FMISO and to verify its suitability for routine quality control in CDTN.

2. EXPERIMENTAL

2.1. Chemicals

The reference standard of [^{18}F]FMISO was obtained from the manufacturer ABX. ^{18}O-enriched water was acquired from Center of Molecular Research. Reagent kits containing eluent solution, acetonitrile, ethanol, HCl solution 2.0 M, buffer solution, water for injections and precursor for [^{18}F]FMISO dissolved in 2 ml acetonitrile synthesis were purchased from ABX. Acetonitrile used in HPLC mobile phase was purchased from Merck. The Luna® C18 column (4.6 mm x 250 mm) was acquired from Phenomenex.

2.2. Preparation of [^{18}F]FMISO

[^{18}F]FMISO was synthesized in Centro de Desenvolvimento da Tecnologia Nuclear (CDTN) using an automated synthesis module TRACERlab MX FDG (General Eletric, USA) adapted for production of [^{18}F]FMISO based on previous work, using Solid Phase Extraction exclusively as purification method [6].

2.3. Instrumentation and chromatographic conditions

[^{18}F]FMISO and impurities were analysed by HPLC equipped with UV/radioactive detectors (Agilent 1200 series/Raytest) at 320 nm on a reverse phase column (Luna® 5µm C18, 4.6x 250 mm) and mobile phase acetonitrila/water (5:95). The volume of the injection was 20 µL and flow rate of the mobile phase was set at 1.0 mL/min, with a total run of 30 minutes.

2.4. Method

Stock standard solution of FMISO

A stock solution was prepared with 10 mg FMISO standard and diluted with 1 mL ultrapure water. Working standard solution was obtained by serial dilution of standard stock solution to reach five concentration levels ranging from 0.2 to 1 ppm.

Stock standard solution of precursor NITTP

A stock solution was prepared with 10 mg precursor NITTP standard and diluted with 1 mL ultrapure water. Working standard solution was obtained by serial dilution of standard stock solution to reach five concentration levels ranging from 0.2 to 1 ppm.

Stock standard solution of desmethylmisonidazole

A stock solution was prepared with 10 mg desmethylmisonidazole standard and diluted with 1 mL ultrapure water. Working standard solution was obtained by serial dilution of standard stock solution to reach five concentration levels ranging from 0.2 to 1 ppm.

2.5 Validation

Selectivity

Selectivity was assessed to verity if HPLC method was able to discriminate [^{18}F]FMISO in the presence of impurities and excipients. It was evaluated by comparing the chromatograms, in triplicate, from solution containing FMISO standard (10 ppm), NITTP precursor (10 ppm), desmethylmisonidazole (10 ppm), ethanol (10%), saline solution and tetrabutylammonium (50 ppm) with standard solution without impurities. Peak resolution was determined on a mixture of FMISO spiked with its impurity in order to verify the separation of these peaks.

Detection limit and Quantification limit

To determine the detection limit (DL) and the quantification limit (QL) was considered the standard deviation of the y-axis intercept of calibration curve (S intercept) and the average of your angular coefficient (slope of the line).

The DL and QL values were expressed in concentration and were based on the ratio of three times the noise baseline for DL, and ten times to QL, as shown in the following equations (1 and 2):

$$DL = \frac{3\ x\ \text{S intercept}}{slope\ average} \quad (1)$$

$$QL = \frac{10\ x\ S\ intercept}{slope\ average} \quad (2)$$

Linearity

Linearity was determined to check if HPLC results were directly proportional to the amount of FMISO in the sample. Five solutions with different concentration of FMISO (0.2 to 1 ppm) were analyzed in triplicate on HPLC. A linear adjust was utilized to fit the experimental data; the equation (y=ax+b) and the coefficient of determination (R^2) were obtained for curve.

Accuracy

The accuracy was determined by the standard addition method. Amounts of 0.4; 0.6; 0.8 ppm of the FMISO standard were added to the samples. The recovery experiments were performed in triplicate for each concentration.

Precision

To determine the precision of the chromatographic method, FMISO was injected as the standard FMISO in three concentration levels (low, medium and high) with three authentic replicates each one. With the evaluation of the obtained chromatograms, determined the average of peak area and retention time then calculated the relative standard deviation of the values.

Repeatability

Repeatability was performed under the same operating conditions on the same day, and by the intermediate precision, comparing the results obtained in three different days and by three different analysts.

The precision is quantified as the coefficient of variation expressed as percent:

$$CV(\%) = \frac{s}{\bar{X}} \times 100\% \quad (3)$$

Where s is the standard deviation and $\bar{X}$ is the sample mean.

Robustness

Robustness was evaluated considering a variation on parameters such as flow rate and mobile phase gradient. HPLC analysis of [^{18}F]FMISO were performed, in triplicate, with a higher (1.05 mL/min) and a lower (0.95 mL/min) flow rates. The mobile phase gradient was also modified to 8% of acetonitrile in water and 3% of acetonitrile in water.

3. RESULTS AND DISCUSSION

Validation of the chemical purity test of [^{18}F]FMISO was carried out according to Brazilian Health Surveillance Agency (ANVISA) requirements (RDC 899/2003) and ICH Q2 (R1) guidelines. The parameters of the validation were: selectivity linearity, precision, detection limit (DL), quantification limit (QL) and robustness [5,7].

3.1 Selectivity

The selectivity of the method was confirmed by the resolution between consecutive peaks. No interference from the matrix at each retention time of analyte peaks was observed. A chromatogram obtained (Figure 1) corresponding to desmethylmisonidazole, FMISO standard and NITTP precursor showed how well chromatographic peaks are separated/resolved, indicating the high specificity of the method.

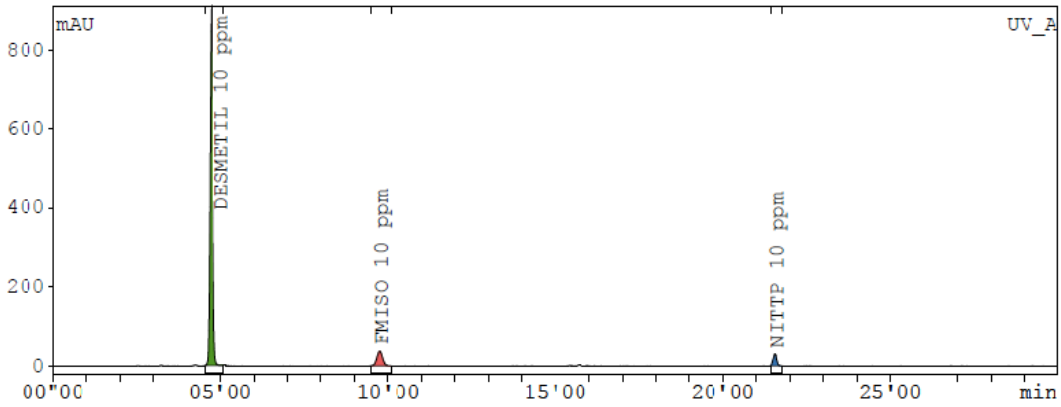

Figure 1. HPLC chromatogram of desmethylmisonidazole, FMISO standard and NITTP precursor. (Isocratic mobile phase of acetonitrile:water (5:95 v/v) flow rate at 1 mL.min-1, Luna® 5µm C18, 4.6x 250 mm, UV detector at 320 nm).

The resolution between two successive elution peaks was higher than 1.0, which meets the acceptance criteria [8]. The analyzes obtained for the substances showed a symmetry factor close to 1 and a high number of theoretical plates being preferred for unequivocal quantification of the analytes [8,9]. The values and required parameters are listed in table 1.

Table 1. Chromatographic parameters for method selectivity: resolution, symmetry, theoretical plates and retention time.

Parameters	UV detector			Reference
	Desmethylmisonidazole	FMISO	NITTP	
Resolution	11.12	25.06	25.06	> 1 [8]
Symmetry	0.97	0.98	0.90	As close as possible to 1.0 [8, 9]
Theoretical plates	14450	17373	249398	Highest possible value [1,2]
Retention time (min)	4.33	9.50	21.34	-

3.2 Detection limit and quantification limit

For [^{18}F]FMISO, DL and QL were respectively 0.10 and 0.33 µg/mL, with a relative standard deviation lower than 10% (Table 2).

Table 2. The values of the DL and QL for [^{18}F]FMISO.

	Slope	Intercept	DL	QL
Curve 1	36.72	-2.55	0.10	0.33
Curve 2	36.64	-0.28		
Curve 3	37.54	-0.56		
	(mean±S.D.,n=3)	(mean±S.D.,n=3)		
	36.96±0.50	-1.13±1.24		

3.3. Linearity

The analytical curve for FMISO standard was constructed by plotting the area under the curve (mAU) of the main peak versus concentration (Figure 2). It was found to be linear over a wide concentration range (0.2 - 1 µg.mL^{-1}) with a correlation coefficient of 0.9999. The straight line equation obtained from the experimental results was found to be (Equation 4):

$$y = 36.968x - 0.3673 \quad (4)$$

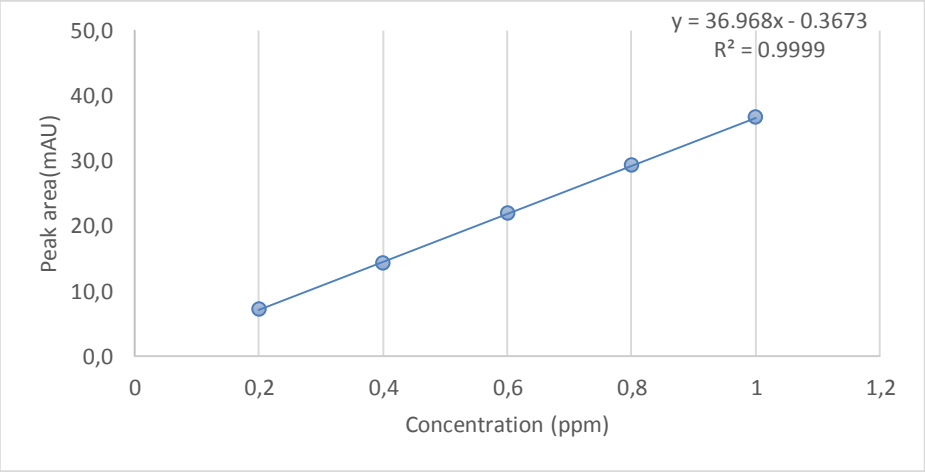

Figure 2. Assessment of the linearity of the method

Thus, this HPLC method can be considered adequate linearity in the concentration studied for quantitative analysis of FMISO under the experimental conditions described.

3.4 Accuracy

Accuracy is one of the most important parameters of an analytical methodology and it can be expressed as the percent recovery of known amounts of standard added to a sample. The recoveries were determined by adding known amounts of the standard FMISO (0.4; 0.6; 0.8 ppm). The final concentrations of the fortified solutions were 0.9, 1.1 and 1.3 ppm de FMISO. The results presented in Table 3 refer to the average of three assays for each concentration. The results are in good agreement with acceptable values for the validation of an analytical procedure (recovery = 80-120 %).

Table 3. Analytical recovery of FMISO standard solution added to sample.

Amount added	Recovery		
	Final concentration	Mean	DPR%
0.4 ppm	0.9 ppm	106%	6.7
0.6 ppm	1.1 ppm	102%	7.6
0.8 ppm	1.3 ppm	101%	8.7

3.5 Precision

The precision refers to the variability of the results in repeated analyses of the sample under identical experimental conditions. The method was validated by evaluating the intra- and inter-day precision. The intra- and inter-day precision assays were expressed as relative standard deviation (RSD). The method was considered precise obtaining coefficients of variation RSD between 0.07% and 0.53% for intra-day and 1.3% and 4.19% for the inter-day precision, indicating that this method presents a good precision. RSD < 5% is considered an acceptable value [7]. The detailed precision data are shown at Table 4.

Table 4. Precision of the method by HPLC.

	Theorical Concentration (ppm)		Area (mean)	Retention Time (s) (mean)	RSD(%)
Intra-day	low	0.2	7.19	593	0.46
	Medium	0.6	22.09	593	0.53
	High	1	33.92	592	0.07
Inter-day	low	0.2	7.72	585	4.19
	Medium	0.6	23.02	593	2.01
	High	1	38.20	592	1.31

3.6 Robustness

The robustness was evaluated calculating the coefficient of variation of peaks areas and retention time of FMISO (Tables 5 and 6). Although the retention times were displaced after variations in mobile phase gradient as well as the variation of flow rate, peaks kept well resolved and the method showed coefficient of variation below 5%. These results indicate that small variations of the parameters do not alter the separation between the peaks.

Table 5. Robustness: Mobile phase flow rate change, (0.95-1.05 mL/min).

Flow rate of mobile phase	Area (mean±S.D.,n=3)	Retention time (s) (mean±S.D.,n=3)	%CV (n=3)
0.95 mL/min	36.17±0.31	628.33±0.58	0.87
1.0 mL/min	34.08±0.31	594.33±1.53	0.92
1.05 mL/min	33.68±0.163	568.33±0.58	0.48
	Area (mean±S.D.,n=9)	Retention time (mean±S.D.,n=9)	%CV (n=9)
	34.64 ± 1.3365	597.0± 0.55	3.85

Table 6. Robustness: Mobile phase gradient change.

mobile phase gradient (H20:ACN)	Area (mean±S.D.,n=3)	Retention time (s) (mean±S.D.,n=3)	%CV (n=3)
92:8	34.28±0.21	604.67±29.16	0.62
95:5	34.07±0.31	593.33±1.53	0.92
97:3	33.07±0.87	572.33±0.58	2.41
	Area (mean±S.D.,n=9)	Retention time (mean±S.D.,n=9)	%CV (n=9)
	34.81±1.0973	590.0 ± 0.48	3.15

4. CONCLUSION

Results evidenced that the proposed HPLC method is suitable for radiochemical purity evaluation of [^{18}F]FMISO considering operational conditions of our laboratory, owing to its adequate selectivity, accuracy, linearity, precision, robustness and detection and quantification limits.

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