Validation of chromatographic analytical methods, TLC and HPLC, to determine the radiochemical purity on the radiopharmaceutical [177Lu]-PSMA I&T

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Abstract

The Energy and Nuclear Research Institute (IPEN) is studying the production of the radiopharmaceutical [177Lu]-PSMA I&T, in accordance with the good manufacturing practices recommended by ANVISA, to be used in the therapy of prostate cancer. This work aims to validate chromatographic methods, TLC and HPLC, to determine the radiochemical purity of the product. The entire validation process of this work was based on ANVISA's RDC 166, 2017 and the Guide 10, version 1, 2017, guided the statistical treatments adopted. The selectivity study found that the presence of impurities or excess excipients did not interfere with product quantification. The proposed methods were linear with linear correlation coefficients (r) above 0.99. The precision and repeatability presented relative standard deviation values lower than specified (RSD < 5 %). The small controlled variations in the method suggested for the robustness test also did not affect the radiochemical purity of the product. In view of the results and in accordance with the criteria established by the National Health Surveillance Agency (ANVISA), the two chromatographic methods were validated in accordance with RDC 166, 2017, proving to be selective, precise, linear and robust. The validation of TLC and HPLC methods enables their application in the batch release routine of the new radiopharmaceutical at Radiopharmacy Center of IPEN.

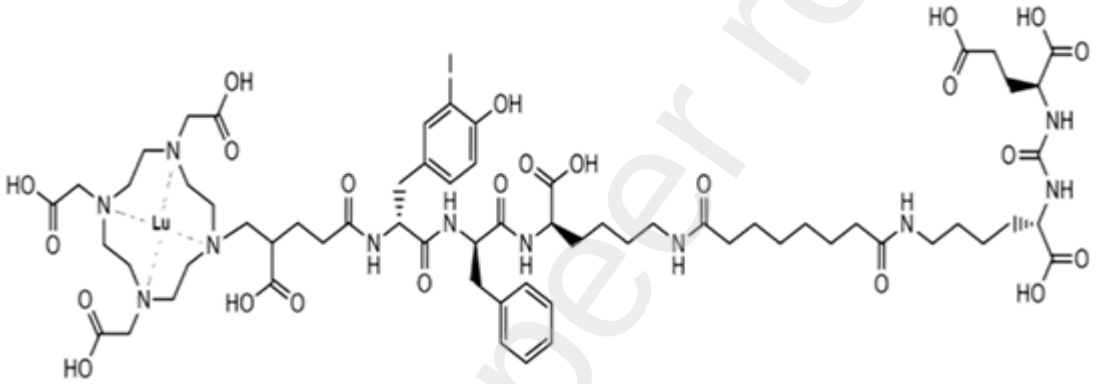
1. Introduction

The PSMA (*Prostatic Specific Membrane Antigen*), a type II glycoprotein, is an excellent target for new radiopharmaceuticals used in nuclear medicine imaging, playing an important role in therapeutic monitoring, as it is overexpressed in virtually all prostate neoplasms (HILLIER et al., 2009; GHOSH et al., 2004). The Institute of Energy and Nuclear Research (IPEN), the main producer of radiopharmaceuticals in Brazil, is studying the production, quality control, and stability of the radiopharmaceutical PSMA I&T radiolabeled with lutetium-177, show in figure 1, to be applied in the radionuclide therapy of metastatic and castration-resistant prostate cancer. However, to ensure that pharmaceutical products have the required characteristics of structure, identity, purity, concentration, potency, and safety for their use, it is necessary that they be produced and controlled according to Good Manufacturing Practices (GMP), recommended by the National Health Surveillance Agency (ANVISA), as per RDC 658 of March 30, 2022 (BRAZIL, 2022). In this sense, all analytical methods not described in official compendia require the conduct of analytical validation, according to parameters established in RDC 166 of July 24, 2017, which must demonstrate that the analytical method produces reliable results and is suitable for its intended purpose, in a documented manner and based on objective criteria (BRAZIL, 2017). The parameters tested in the validation are: Specificity; Accuracy; Precision; Limit of detection; Limit of quantification; Linearity; Range of application; and Robustness (DE BARROS, 2002).

The thin-layer chromatography system with silica gel (TLC-SG) is the most sensitive method for detecting and quantifying radiochemical impurities in the free cationic form (177Lu+3) or complexed with DTPA. However, it is not capable of separating stereoisomers and molecule degradation products. On the other hand, high-performance liquid chromatography (HPLC) is able to separate and quantify all forms of radiochemical impurities, including peptide degradation products.

Therefore, both methods will be used complementarily to quantify the radiochemical purity of the product ([177Lu]-PSMA I&T). Since the studied radiopharmaceutical does not yet have a monograph, the specifications for the assay limits of radiochemical purity and impurity (%RP ≥ 95% (HPLC); %Impurity ≤ 3% (TLC)), for method validation, were based on the monograph of [68Ga]-PSMA 11 and [18F]-PSMA-1007, as d escribed in the European Pharmacopoeia (EP, 2023).

Figure 1. Molecular structure of the [177Lu]-PSMA I&T (WIECZOREK, 2020).



2. Experimental

2.1. Materials and Methods

HPLC

High-performance liquid chromatography, HPLC, is the analytical method used to separate, detect, and quantify a large number of compounds, being the most widely used separation technique among all others. Separations are achieved by partition, adsorption, ion exchange, size exclusion, or stereochemical interactions, depending on the type of stationary phase used.

For HPLC analysis, 100 µL of the sample with a concentration of 37 MBq/mL was injected into the chromatographic system with a C18 column (250 mm x 4.6 mm, 5 µm), reverse phase, using the following mobile phase: water + 0.1% TFA (solvent A) and acetonitrile + 0.1% TFA (solvent B) for improved separation, the following gradient mode was employed: 0-8 minutes; 76% (A); 8-10 minutes 60% (A) and 10-18 minutes 60% (A), using a flow rate of 0.6 mL/minute at a constant temperature of 24°C. Attached to this unit is the gamma radiation detector - Flow-RAM Radio HPLC Detector

TLC

Thin-layer chromatography is a very important technique for the separation and quantification of small amounts of material. This technique consists of a stationary phase fixed on a plate (glass or aluminum) and a mobile phase, which is composed of a solvent or solvent

combination, called the eluent. The sample components are separated based on their affinity with the phases

Using a micropipette, a 2 µL aliquot of the radiolabeled product, with a radioactive concentration of 37 MBq/mL, was applied onto the TLC strips (1.5 x 12.5 cm) at a distance of 1.5 cm from the base of the strip. As the mobile phase of the chromatographic system, a 0.1 M sodium citrate buffer solution (pH 5.5) was used. The strips were brought into contact with the mobile phase contained in the chromatographic tanks. At the end of chromatography, the strips were dried in an oven (approximately 60 °C), cut into 1 cm segments, and the radioactivity reading corresponding to each segment was performed using an automatic well-type gamma counter (Hidex). The percentage of radiochemical impurity was calculated from the ratio of the sum of the activities of the impurity segments (Rf impurity = 0.5 – 1.0) to the total activity of the strip.

2.2. Validation Study

The analytical parameters commonly encountered for validation of separation methods are: selectivity; linearity and range of application; precision; accuracy; limit of detection; limit of quantification; and robustness. However, ANVISA, through RDC 166 of 2017, defines the assays according to the method category (identification, impurity testing, and assay) and the acceptance criteria for each parameter evaluated in validation, as shown in table 1.

Table 1. Parameters and acceptance for the validation of the HPLC and TLC methods. Abbreviations: RSD: relative standard deviation, R^2: correlation coefficient.

HPLC Method	
Parameter	**Acceptance Criteria**
Selectivity	Resolution ≥ 2.0
Intermediate Precision	RSD < 5%
Repeatability	RSD < 5%
Linearity	R^2 ≥ 0.99
Robustness	RSD < 5%
TLC Method	
Parameter	**Acceptance Criteria**
Selectivity	Resolution ≥ 2.0
Intermediate Precision	RSD < 5%
Repeatability	RSD < 5%
Linearity	R^2 ≥ 0.99
Robustness	RSD < 5%
LOQ	< 0,185 MBq/mL (lowest concentration of the analytical curve)

2.2.1 Selectivity

The selectivity test was carried out by evaluating whether the presence of impurities and excipients alter the retention factor (Rf) of the product.

2.2.2 Intermediate Precision/Repeatability

Repeatability, defined by agreement between results within a short period of time with the same analyst and instrumentation, was assessed by preparing 6 independent samples with a radioactive concentration of 37 MBq/mL. Intermediate precision was conducted in the same manner as repeatability, however, the samples were prepared by another analyst.

2.2.3 Linearity

The analytical curve was obtained from the radiolabeled product by preparing 6 solutions with different radioactive concentrations.

2.2.4 Robustness

Robustness is defined as the measure of a method's ability to withstand small and deliberate variations in analytical parameters. In the TLC method, the proposal was to vary the radioactive concentration and the sample volume. In the HPLC method, the proposal was to vary the acidic concentration of the mobile phase, as show in table 2.

Table 2. Variables of the robustness test

TLC Method		**HPLC Method**	
Robustness test variables		Robustness test variables	
Radioactive concentration	Sample volume	%TFA/water (A)	
44,4 MBq/mL (A)	5 µL (B)	%TFA/acetonitrile (B)	
18,5 MBq/mL (a)	2 µL (b)	HPLC analysis	
TLC analysis		Solution 1	0,05% (A) + 0,10% (B)
Solution 1	A + B	Solution 2	0,10% (A) + 0,05% (B)
Solution 2	A + b	Solution 3	0,05% (A) + 0,05% (B)
Solution 3	a + b	Solution 4	0,15% (A) + 0,15% (B)

2.2.5 Limit of Quantitation (LOQ)

The limit of quantification (LOQ) is the lowest amount of the analyte in a sample that can be reliably and accurately determined under the established experimental conditions. The limits of quantification were determined based on the standard deviation of 10 readings of the sample blank.

3. Determination of radiochemical purity – method HPLC

The percentage of radiochemical purity (%RP) is calculated from the ratio of the peak area of the product to the sum of the areas of all peaks found in the chromatogram, as per equation below.

$$\%\ RP = \frac{Product\ peak\ area}{\Sigma\ all\ peaks\ area\ in\ the\ chromatogram}\ x\ 100$$

4. Determination of radiochemical impurity – method TLC

Impurities may represent free lutetium-177 (in cationic form 177Lu+3) or in the form of lutetium oxide [177Lu (OH)3] or as lutetium-177 bound to the chelating agent DTPA (excipient of the product) and eventual degradation products of the radiopharmaceutical. Impurities can result from inadequate radiolabeling, peptide decomposition, pH change, or exposure to reducing or oxidizing agents (SHARP et al., 2005).

The percentage of radiochemical impurity was calculated from the ratio of the sum of the activities of the impurity segments (Rf = 0.5 – 1.0) to the total activity of the strip.

$$\%\ Impurity = \frac{\Sigma\ activies\ (Rf\ 0{,}5 - 1{,}0)}{Total\ activity\ of\ the\ strip}\ x\ 100$$

5. Results and Discussion

Selectivity was performed with the analysis of test solutions, where the retention factor (TLC) and retention time (HPLC) of the product ([177Lu]-PSMA I&T) and the impurity ($177LuCl_3$) was evaluated in the presence of excess excipient (DTPA), show in figure 2 and 3.

Figure 2. Chromatographic profile of the product and radiochemical impurities in the TLC method

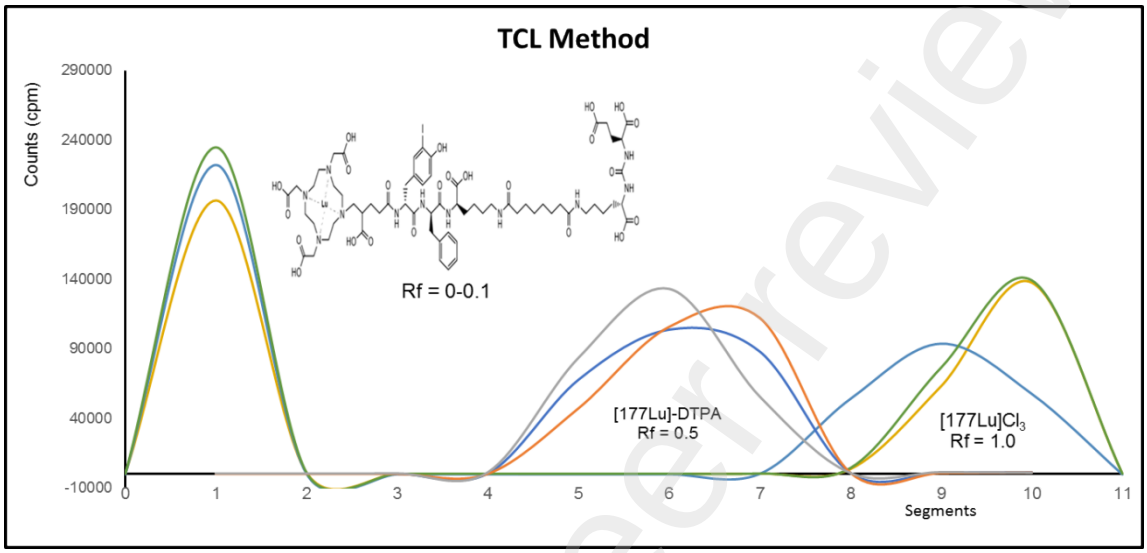
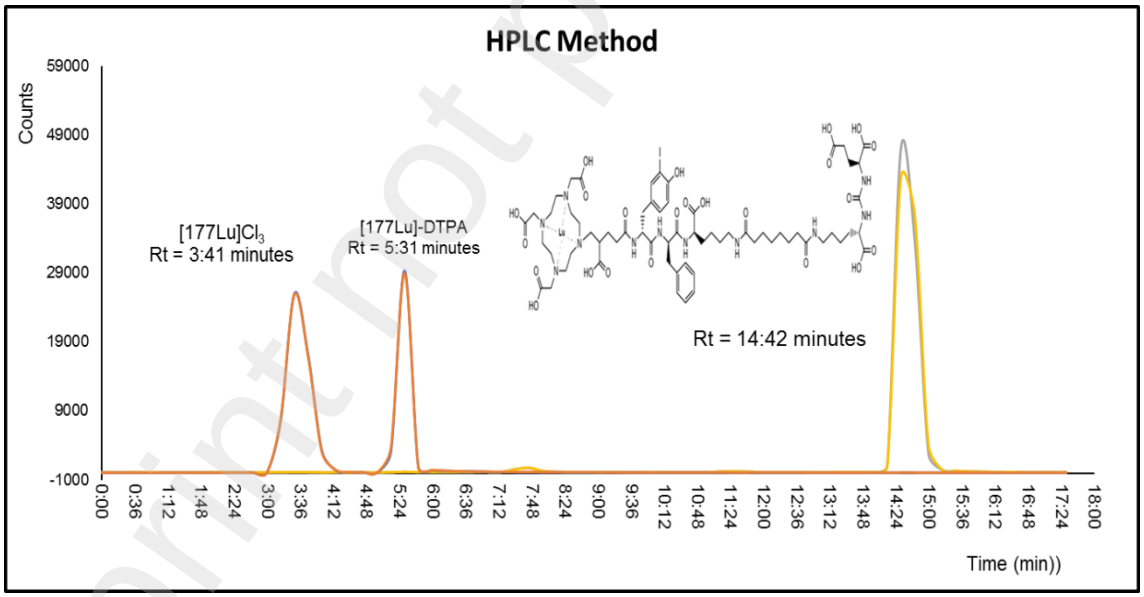


Figure 3. Chromatographic profile of the product and radiochemical impurities in the HPLC method.

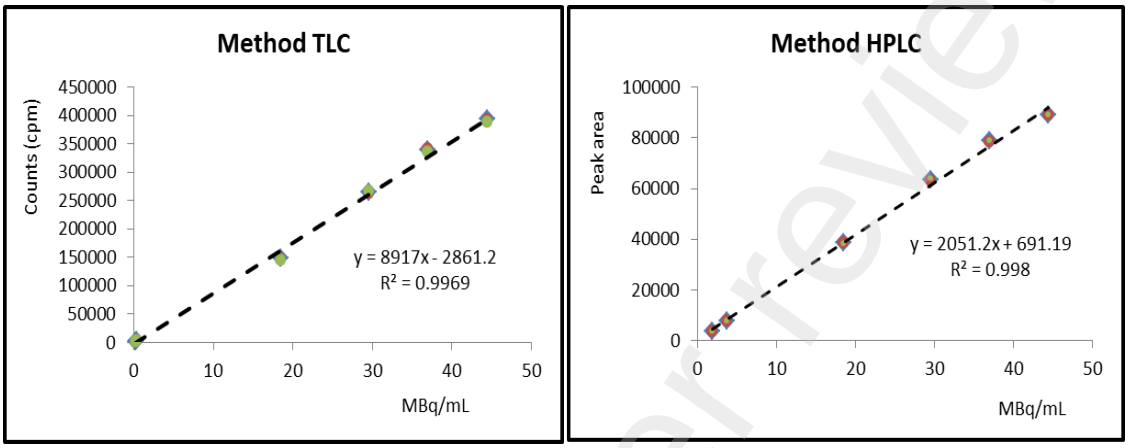


Both chromatographic methods proved to be selective and suitable for the separation of the radiochemical species present in the sample.

The linearity test was performed by obtaining the analytical curve, using the radiolabeled product as a standard to prepare 6 solutions with different radioactive concentrations, as show in table 3.

Table 3. Table with data to obtain the analytical curve graph - linearity test

TLC Method				HPLC Method			
MBq/mL	Counts (cpm)			MBq/mL	Peak area (counts*min)		
0.185	1835	1660	1840	1.85	3874	3933	3975
0.370	3377	3262	3723	3.70	7867	7948	7767
18.5	148978	147300	145536	18.5	38919	38528	38372
29.6	264774	263956	266459	29.6	63355	63258	63900
37.0	339764	340457	336528	37.0	78726	78287	78870
44.4	393083	391168	388453	44.4	89152	89214	88919

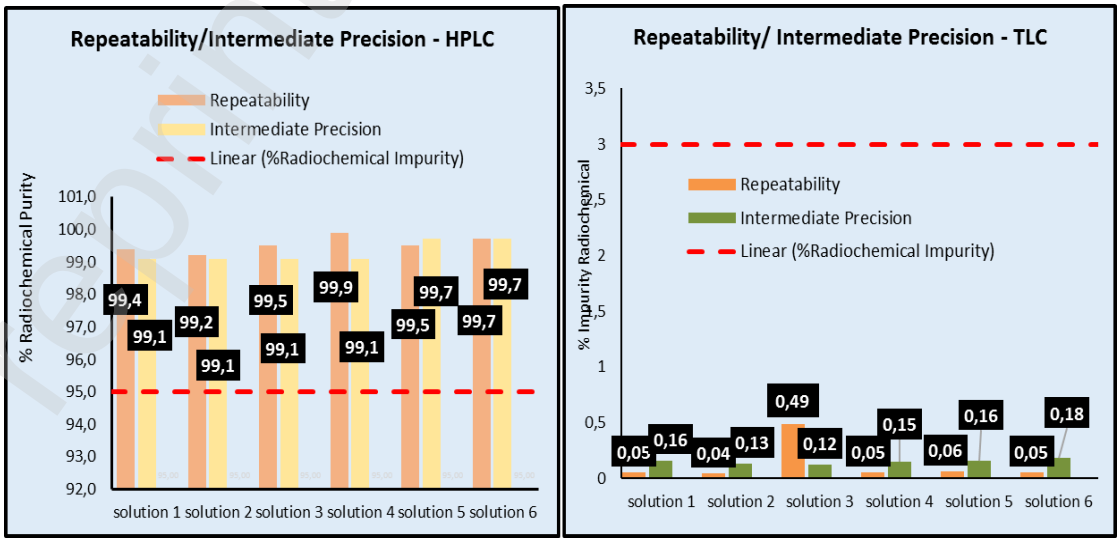


All statistical tests were applied (Cochran test, Grubbs test, F test, and Shapiro-Wilk test) to assess the linearity conformity, as per RDC 166/17 (ANVISA).

The limits of detection and quantification were determined based on the standard deviation of 10 readings of the sample blank. The limits of quantification (LOQ) were calculated 0.039 MBq/mL.

The repeatability and intermediate precision assays in the TLC method showed radiochemical impurity values lower than the specified limit (< 3%) and relative standard deviations of 1.51% and 0.17%, respectively. The HPLC method presented radiochemical purity values above the specified limit (>95%) in the repeatability and intermediate precision tests, with relative standard deviations of 0.22% and 0.32%, respectively, as shown in the in figure 4.

Figure 4. Determination of radiochemical purity and impurity values of repeatability and intermediate precision tests



All proposed robustness tests yielded satisfactory radiochemical purity values (PR > 95%) in the HPLC method, with relative standard deviations within the established limit (≤ 5%). The radiochemical impurities assays determined by the TLC method also presented satisfactory values (< 3%) with relative standard deviations within the established limit (≤ 5%), as show in the figure 5.

Figure 5. Determination of radiochemical purity and impurity values of robustness test.

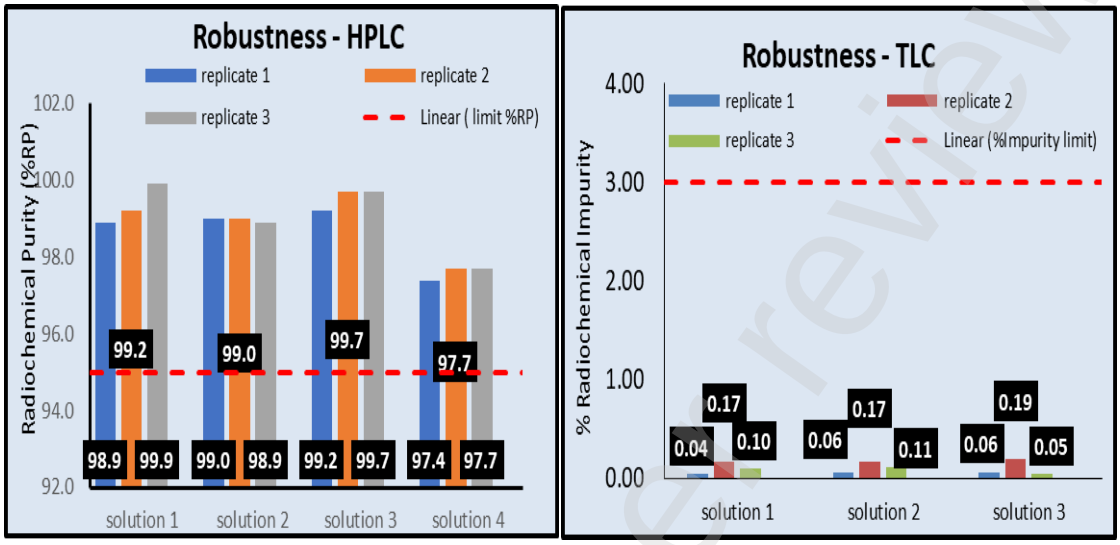


Table 3 presents all the results and parameters evaluated in the validation of the analytical methodologies.

Table 3. Parameters and results of the validation

HPLC Method		
Parameter	**Acceptance Criteria**	**Results**
Selectivity	Resolution ≥ 2.0	10.5
Intermediate Precision	RSD < 5%	0.32%
Repeatability	RSD < 5%	0.22%
Linearity	$R^2 \geq 0.99$	0,998
Robustness	RSD < 5%	0.52%, 0.03%, 0.29% and 0.18%
TLC Method		
Parameter	**Acceptance Criteria**	**Results**
Selectivity	Resolution ≥ 2.0	3.2
Intermediate Precision	RSD < 5%	0.17%
Repeatability	RSD < 5%	1.51%
Linearity	$R^2 \geq 0.99$	0.997
Robustness	RSD < 5%	0.14%, 0.06% and 0.33%
LOQ	< 0,185 MBq/mL (the lowest concentration of the analytical curve)	0,039 MBq/mL

6. Conclusion

The use of two chromatographic methodologies, in a complementary and unpublished, for determining the radiochemical purity of the product [177Lu]-PSMA I&T, yielded reliable results, proving to be suitable for the intended purpose and under control. All parameters were evaluated according to RDC 166/2017 of ANVISA, and statistical treatments were performed in the linearity test as per Guide 10/2017 (ANVISA). Therefore, they have been validated and made available for quality control use.

7. Acknowledgments

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