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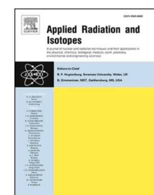

Validation of chromatographic methods, TLC and HPLC, to quantify known radiochemical impurities and the radiochemical purity the radiopharmaceutical [^{177}Lu]Lu-PSMA I&T

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A B S T R A C T

The Energy and Nuclear Research Institute (IPEN) is studying the production of the radiopharmaceutical [^{177}Lu]Lu-PSMA I&T, in accordance with the good manufacturing practices recommended by the National Health Surveillance Agency (ANVISA), to be used in the therapy of prostate cancer. This work aims to validate chromatographic methods, Thin-Layer Chromatographic (TLC) and high-performance liquid chromatography (HPLC), to determine the radiochemical purity (RCP) 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. With the selectivity study we can observe that the presence of impurities or excess excipients in the sample does not interfere with the quantification of the product. 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 (HILLER et al., 2009; GHOSH and Heston, 2004). In Brazil, prostate cancer is the second most common type of cancer among men, second only to non-melanoma skin cancer. For the period 2023–2025, the National Cancer Institute (INCA) projects approximately 71,730 new cases annually (INCA, 2020). In light of this situation, 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, shown in Fig. 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 ANVISA, as per RDC 658 of March 30, 2022 (BRASIL - National Health Surveillance Agency of the Ministry of Health, 2022). In this sense, all analytical methods that do not have a monograph in any pharmacopoeia 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, for a documented manner and based on objective criteria (BRASIL - National Health Surveillance Agency of the Ministry of Health, 2017). Therefore, the objective of this article is to present a study that validates the methodology used to quantify the radiochemical purity of the radiopharmaceutical [^{177}Lu]Lu-PSMA I&T, evaluating the validation parameters established by RDC 166, 2017. This is one of the processes required by ANVISA for the regulation of the production and commercialization of new radiopharmaceuticals. 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; N GILLINGS et al., 2020).

The TLC-SG method was used to quantify the radiochemical

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impurities such as ($^{177}Lu^{+3}$) in free cationic form or when complexed with DTPA ([^{177}Lu]Lu-DTPA). However, it cannot separate stereoisomers or degradation products from the molecule. In contrast, high-performance liquid chromatography (HPLC) effectively separates and quantifies all forms of radiochemical impurities, including those resulting from peptide degradation.

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

2. Experimental

2.1. Materials and methods

2.1.1. TLC-SG method

Thin-layer chromatography is a very important technique for the separation and quantification of small amounts of material. The technique offers advantages such as being easy to perform, providing quick results, low cost, and versatility. On the other hand, caution is needed when determining the Rf, and it has low reproducibility. 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. Chromatographic conditions, such as the strip dimensions, the concentration, and the type of solvent used in the mobile phase, were optimized during the development and refinement of the analytical method.

Using a micropipette, a 2 μL aliquot of the radiolabeled product (74 kBq), with a radioactive concentration of 37 MBq/mL, was applied onto the TLC strips (1.5 × 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. strips were placed in dedicated eluent and after eluent reached the top, strips were dried at 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), with energy window calibrated at 70–320 keV. The radiochemical yield 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 (specify: Radiochemical yield ≤3.0%).

2.1.2. HPLC method

High-performance liquid chromatography (HPLC) is a widely used analytical technique for separating, detecting, and quantifying a broad range of compounds. It is the most prevalent separation method due to its versatility. Separations are accomplished through partitioning, adsorption, ion exchange, size exclusion, or stereochemical interactions, depending on the type of stationary phase employed.

To assess the impurities generated by radiolysis and molecular degradation in the HPLC method, the sample was stored in a climate chamber for 48 h at 30 °C and 35% relative humidity. The goal was to induce the formation of degradation products and evaluate the method's ability to detect these impurities.

For HPLC analysis, 100 μL of the sample with a concentration of 37 MBq/mL was analyzed in the chromatographic system with a C18 column (250 mm × 4.6 mm, 5 μm), reversed phase, using the following mobile phase: water + 0. 1% TFA (solvent A) and acetonitrile + 0.1% TFA (solvent B).

The following gradient mode was employed: 0–8 min; 76% (A); 8–10 min 60% (A) and 10–18 min 60% (A), using a flow rate of 0.6 mL/min at a constant temperature of 24 °C. Attached to this unit is the gamma radiation detector - Flow-RAM Radio HPLC Detector.

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 criteria for the validation of the HPLC and TLC methods. Abbreviations: RSD: relative standard deviation, R: determination coefficient.

TLC-SG Method		
Parameter	Evaluated	Acceptance Criteria
Selectivity	+	Resolution ≥2.0
Intermediate Precision	+	RSD <5%
Repeatability	+	RSD <5%
Linearity	+	R ≥ 0.99
Robustness	+	RSD <5%
Limit of Quantification (LOQ)	+	<0,185 MBq/mL (lowest concentration of the analytical curve)
Limit of Detection (LOD)	+	Not applicable
HPLC Method		
Parameter	Evaluated	Acceptance Criteria
Selectivity	+	Resolution ≥2.0
Intermediate Precision	+	RSD <5%
Repeatability	+	RSD <5%
Linearity	+	R ≥ 0.99
Robustness	+	RSD <5%
Limit of Quantification (LOQ)	–	Not applicable
Limit of Detection (LOD)	–	Not applicable

+ normally evaluated.
- not evaluated.

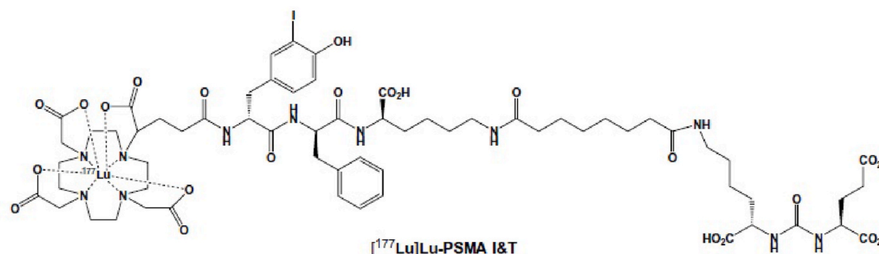

Fig. 1. Molecular structure of the [177Lu]Lu-PSMA I&T (WIECZOREK VILLAS BOAS, 2020).

2.2.1. Selectivity

The selectivity refers to the ability of an analytical method to accurately differentiate and quantify the analyte of interest in the presence of other components, such as impurities, degradation products, or matrix elements, without interference. The test was carried out by evaluating whether the presence of impurities and excipients alter the retention factor (Rf) of the product. Selectivity was evaluated by analyzing three independent test solutions: [^{177}Lu]Lu-PSMA I&T product, impurities ($^{177}LuCl_3$), and [^{177}Lu-DTPA]. A 0.1 mL aliquot from each solution was mixed in a glass vial and the mixture was applied to a TLC strip and injected into the HPLC.

2.2.2. Precision - repeatability/intermediate precision

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

In precision and robustness tests of the TLC method for the determination of radiochemical impurities, if known impurities are absent or present in concentrations lower than the specified limit (<3%), the sample must be enriched with known concentrations of the impurity standard, as per described by ANVISA's RDC 166/2017. Therefore, for these analyses, a sample with low radiochemical purity (88–90%) was used.

2.2.3. Linearity

The analytical curve was obtained from the radiolabeled product by preparing 6 solutions with different radioactive concentrations. The assessment of the linearity of the methods was carried out with statistical tests, as described below.

2.2.3.1. Cochran test. During linearity testing, multiple replicates at different concentration levels are often analyzed. The Cochran test can be applied to the variance of the replicate measurements at each concentration level to ensure the assumption of homoscedasticity (equal variance) holds. Homoscedasticity is an important assumption for linear regression models, including those used in linearity studies.

2.2.3.2. F-test. The F-test is a statistical test used to compare the variances of two populations or to test hypotheses about the overall significance of a model in regression analysis. It plays a crucial role in several statistical analyses, including the analysis of variance (ANOVA) and regression models.

2.2.3.3. Shapiro-Wilk test. The Shapiro-Wilk test calculates a test statistic [W] based on the correlation between the data and the corresponding normal distribution values. The test statistic [W] ranges between 0 and 1, with values close to 1 indicating that the data is likely normally distributed.

2.2.3.4. Grubbs test. The Grubbs test is a statistical test used to detect outliers in a dataset. Specifically, it is used to identify whether the smallest or largest value in a dataset is an outlier. The Grubbs test is also known as the maximum normalized residual test.

2.2.4. Robustness

Robustness refers to the ability of an analytical method to remain unaffected by small, deliberate variations in method parameters such as temperature, pH, flow rate, or instrument settings, demonstrating its reliability during normal usage. Robustness ensures that the method can produce reliable and accurate results under a variety of conditions.

In the TLC method, the proposal was to vary the radioactive concentration and the sample volume (2 e 5 μL). In the HPLC method, the proposal was to vary the acidic concentration, Trifluoroacetic Acid (TFA), of the mobile phase, as show in Table 2.

2.2.5. Limit of quantification (LOQ) and limit of detection (LOD)

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 limit of detection (LOD) must be demonstrated by obtaining the smallest quantity of the analyte present in a sample that can be detected, however, not necessarily quantified, under the established experimental conditions. The limits of quantification and detection were determined based on the standard deviation (σ) of 10 readings of the sample blank divided by the slope of the linearity test curve, as per equation below.

$$LOQ = \frac{10\ x\ \sigma}{Slope} \quad LOD = \frac{3,3\ x\ \sigma}{Slope}$$

3. Determination of radiochemical purity – method HPLC

The percentage of radiochemical purity (%RCP) 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.

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

4. Determination of radiochemical impurity – method TLC

Only impurities such as free lutetium-177 (in the cationic form $^{177}Lu^{+3}$) or in the form of lutetium oxide [$^{177}Lu\ (OH)^3$] or as lutetium-177 bound to the chelating agent DTPA can be determined by the TLC method.

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.

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

5. Results and discussion

To accelerate the formation of degradation products, the sample was subjected to a temperature of 30 °C and 35% relative humidity in a climate chamber for 48 h. Figs. 2 and 3 show the chromatograms of the sample before and after exposure to the climate chamber.

Chromatogram of the sample before exposure to the climate chamber, with an %RCP value greater than 99%.

Chromatogram of the sample before exposure to the climate

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	Activity TLC strip	Robustness 1	0.05% TFA (A) + 0.10% TFA (B); pH 2.3
Robustness 1	A + B (222 kBq)	Robustness 2	0.10% TFA (A) + 0.05% TFA (B); pH 2.0
Robustness 2	A + b (88.8 kBq)	Robustness 3	0.05% TFA (A) + 0.05% TFA (B); pH 1.8
Robustness 3	a + b (37 kBq)	Robustness 4	0.15% TFA (A) + 0.15% TFA (B); pH 2.3

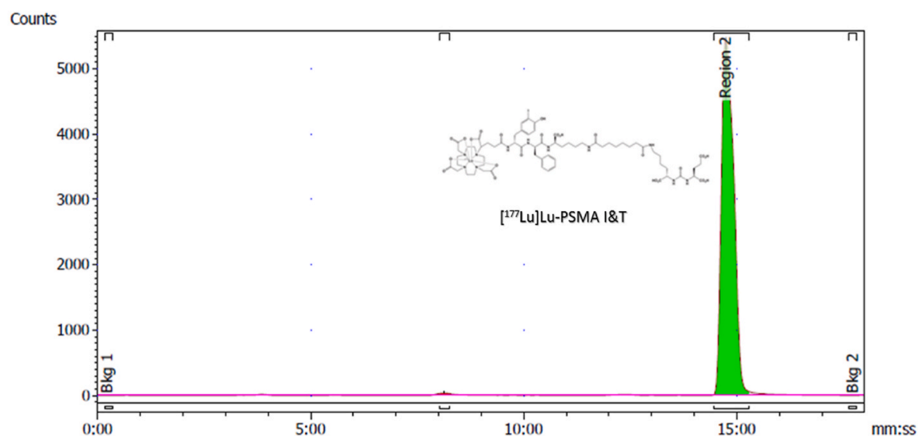

Fig. 2. Chromatogram obtained immediately after the product's radiolabeling process.

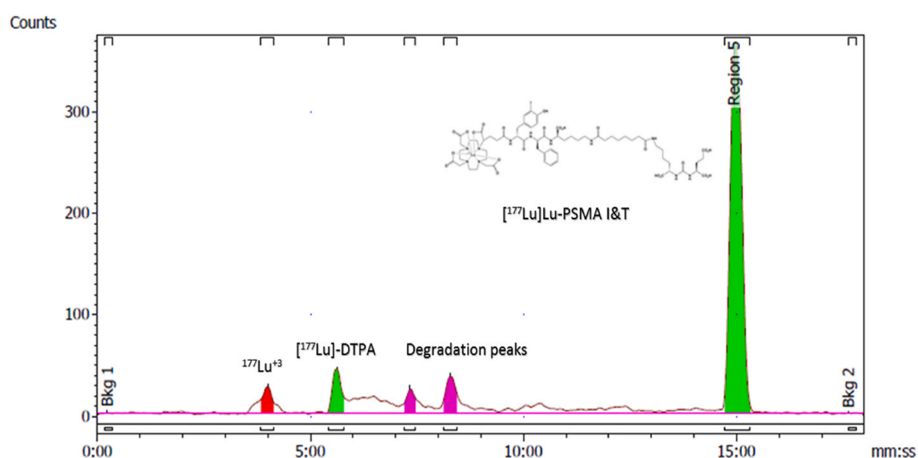

Fig. 3. Chromatogram obtained the sample was stored in a climate chamber for 48 h at 30 °C and 35% relative humidity.

chamber, with an % RCP value below 80%.

5.1. Selectivity

The analyses were performed in triplicate, and the retention factor (TLC) and retention time (HPLC) of both the product and impurities were assessed in the presence of excess excipient (DTPA), as shown in Figs. 4 and 5.

Three distinct samples, product and impurities($^{177}Lu^{+3}$ and ^{177}Lu-DTPA) were prepared separately, then combined into a single vial and analyzed with the aim of determining whether the method could separate all species. The analyses were performed in triplicate.

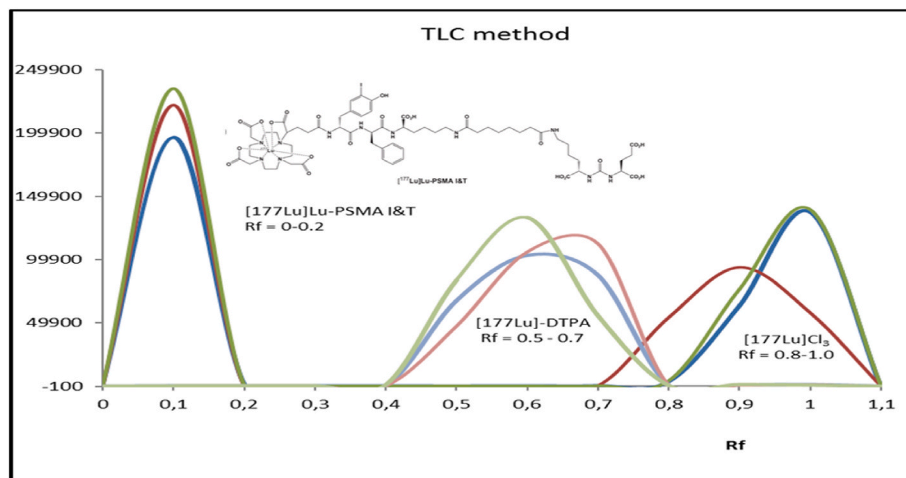

Fig. 4. Chromatographic profile of the product and radiochemical impurities in the TLC method.

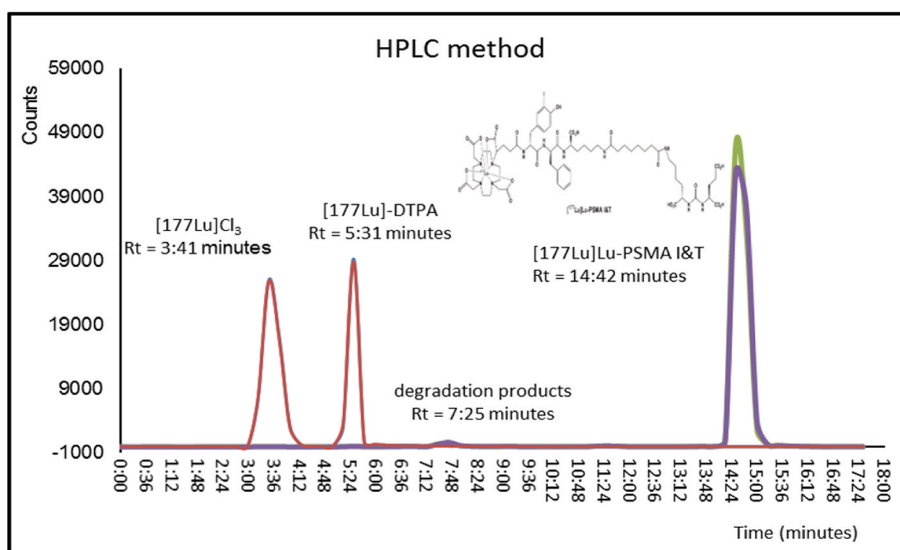

Fig. 5. Chromatographic profile of the product and radiochemical impurities in the HPLC method.

According to the graph in Fig. 4, we can see that the method adequately separates the product from impurities and that the excess excipient (DTPA) in the sample does not change the Rf of the product.

Both chromatographic methods proved to be selective and suitable for separating the radiochemical species present in the sample. Using the HPLC method we can observe product degradation peaks generated from radiolysis.

5.2. Linearity

For the linearity test of the TLC method, the analytical curve was obtained from the dilution of the radioactive sample, with a concentration of 1.85 GBq/mL, preparing 6 solutions with different radioactive concentrations, according to Table 3, and measuring the counts, in triplicate, of each solution in the well-type gamma counter (Hidex).

For the linearity test of the HPLC method, the analytical curve was obtained from the dilution of the radioactive sample, with a concentration of 1.85 GBq/mL, preparing 6 solutions with different radioactive concentrations, according to Table 3, and measuring the area of the product peak (Rt 14min42s) in triplicate.

The Cochran test assessed that the data were homoscedastic in both analytical curves, therefore the ordinary least squares method (OLS) was used to determine linear regression parameters.

The F-test confirmed that there is variance between the variables, that is, as the radioactive concentration of the product increases (independent variable) the response signal increases (dependent variable). The Shapiro wilk test assessed that the residues of the analytical curve follow a normal distribution and, accordingly, the Grubbs test verified that there is no presence of outliers.

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

5.3. Limit of quantification (LOQ) and limit of detection (LOD)

The limits of detection and quantification was determined based on the standard deviation of 10 readings of the sample blank, as show Table 4.

The limits of quantification (LOQ) were calculated:

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

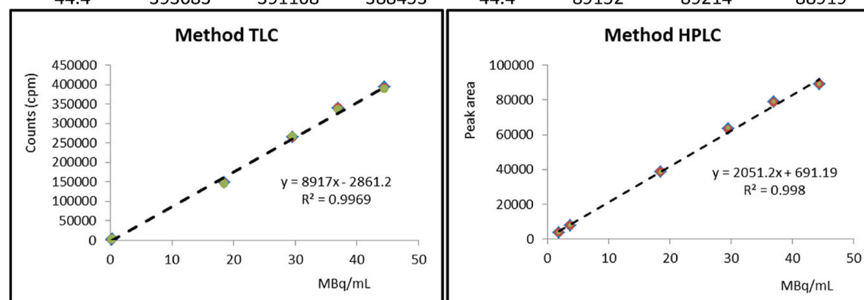

Table 4
Blank sample values to determine the limit of quantification.

Gama Counter - Hidex		
Sample	Counts (cpm)	Standard deviation (σ)
Blank	300	34.76
	320	
	290	
	315	
	245	
	300	
	295	
	290	
	240	
	360	

$$LOQ = \frac{10 \text{ x } \sigma}{Slope} = \frac{10 \text{ x } 34.76}{8917} = 0.039 \text{ MBq} \Big/ \text{mL}$$

$$LOD = \frac{3,3 \text{ x } \sigma}{Slope} = \frac{3,3 \text{ x } 34.76}{8917} = 0.013 \text{ MBq} \Big/ \text{mL}$$

The limits of detection and quantification are not evaluated in a dosing method, such as HPLC, as established in Table 1.

5.4. Precision – repeatability/intermediate precision

The repeatability and intermediate precision assays in the TLC method showed 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 Fig. 6.

5.5. Robustness

All proposed robustness tests yielded satisfactory radiochemical purity values (RCP >95%) in the HPLC method, as show in Fig. 7, with relative standard deviations within the established limit (≤5%), robustness 1 (RSD 0.52%), robustness 2 (RSD 0.06%), robustness 3 (RSD 0.18%), robustness 4 (RSD 0.29%).

The radiochemical impurity assays determined by the TLC method also presented satisfactory values with relative standard within the established limit (≤5%), robustness 1 (RSD 1.1%), robustness 2 (RSD 2.3%), robustness 3 (RSD 2.1%).

Robustness test 4, using the HPLC method, showed a decrease in the radiochemical purity of the product, which may be attributed to the acid hydrolysis of the peptide, caused by the mobile phase. However, with values still within the specified range for the relative standard deviation between measurements, the method proved to be robust.

Table 5 presents all the results and parameters evaluated in the validation of the analytical methodologies.

6. Conclusion

The use of two chromatographic methodologies, for determining the radiochemical impurity and radiochemical purity of the product [^{177}Lu]Lu-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 October 2017 (ANVISA). Therefore, they have been validated and made available for quality control use.

CRediT authorship contribution statement

Joel M. Santos: Writing – review & editing, Writing – original draft,

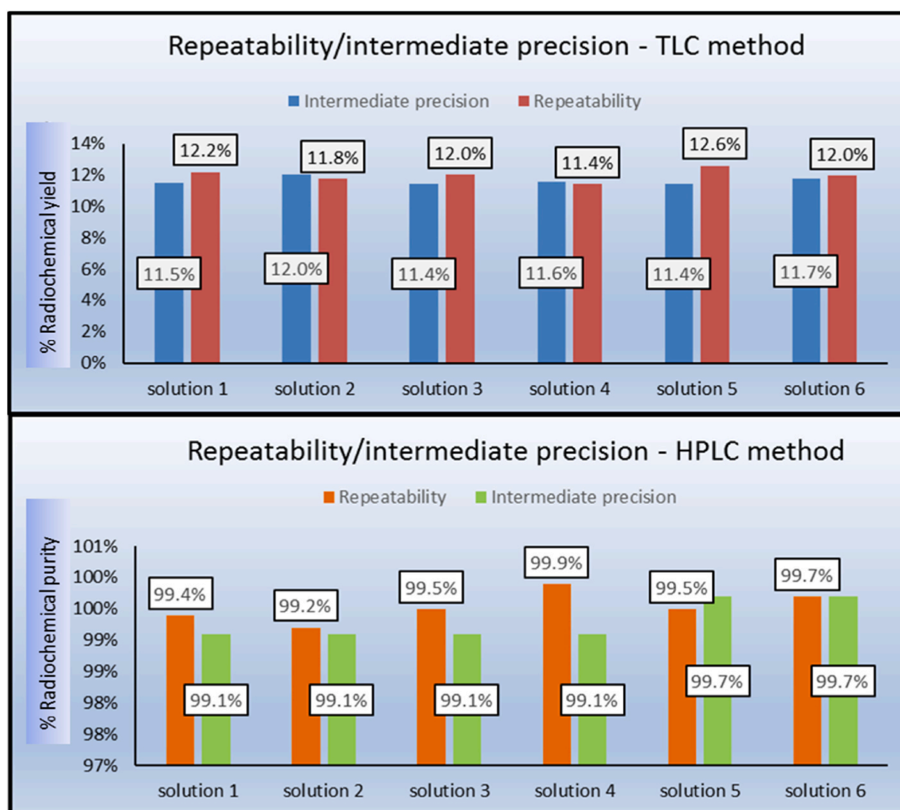

Fig. 6. Determination of radiochemical impurity (TLC method) and radiochemical purity (HPLC method) values of repeatability and intermediate precision tests.

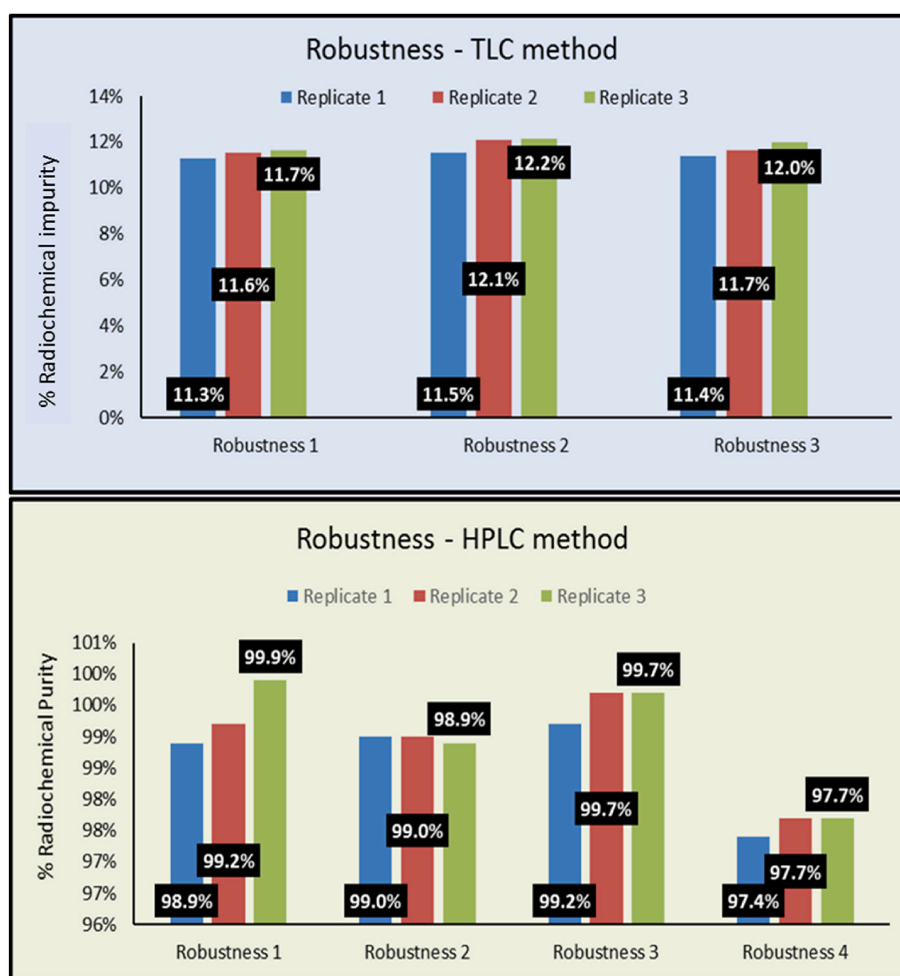

Fig. 7. Determination of radiochemical impurity (TLC method) and radiochemical purity (HPLC method) values of robustness test.

Table 5
Parameters and results of the validation.

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 ≥ 0.99	0,997
Robustness	RSD <5%	1.1%, 2.3% and 2.1%
LOQ	<0,185 MBq/mL (the lowest concentration of the analytical curve)	0,039 MBq/mL
LOD	–	0,013 MBq/mL

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 ≥ 0.99	0.998
Robustness	RSD <5%	0.52%, 0.06%, 0.18% and 0.29%

Data curation. **Luiza M. Balieiro:** Methodology. **Rodrigo S. Santos:** Resources. **Margareth M.N. Matsuda:** Writing – review & editing, Supervision, Methodology. **Elaine B. Araújo:** Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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