2015 International Nuclear Atlantic Conference - INAC 2015
São Paulo, SP, Brazil, October 4-9, 2015
ASSOCIAÇÃO BRASILEIRA DE ENERGIA NUCLEAR - ABEN
ISBN: **978-85-99141-06-9**

OPTIMIZATION OF LABELLING PSMA-HBED-CC PEPTIDE WITH ^{68}GA

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ABSTRACT

Early detection of metastases or recurrent prostate cancer (PC) lesions is of clinical relevance in terms of clinical staging, prognosis and therapy management. When PC is not treated, it is potentially lethal. Clinical methods for diagnosis of PC include the dosage of prostatic specific antigen (PSA) and the rectal touch. Unfortunately, these initial procedures are not specific for PC detection. The level of PSA, in about 20 to 30% of the cases is high, due to benign pathologies, that result in false positive and unneeded biopsy. The prostatic specific membrane antigen (PSMA) is a type II transmembrane glycoprotein and differs from the PSA that is a free protein in blood. High levels of PSMA are observed in almost all prostatic pathologies and low levels were observed in brain, kidneys, salivary glands and small intestine. This fact stimulated the development of PSMA inhibitor molecules that could be used as a vector for imaging tumor agents and that could perfuse in the tumor microvasculature. Recent studies suggest that the chelator HBED-CC contributes intrinsically for the labelling of the PSMA inhibitor peptide based in urea - Glu-urea-Lys (Ahx) – to the pharmacophore group. This work describes the study of labelling conditions of PSMA-HBED-CC with ^{68}Ga and determined the ideal conditions to obtaining the high radiochemical purity (≥ 95%) and stability, without final purification, and stimulates the *in vitro* and *in vivo* evaluation to determine the potential of the radiopharmaceutical for clinical application.

1. INTRODUCTION

The prostate cancer (PC) is the second most common among men in Brazil and the six most frequent malignancy in the world. The main risk factor for prostate cancer is age, since 62% of diagnoses in the world account for men aged 65 or more [1] and rarely affects men under the age of 40 years. As early the detection of PC occurs, better is the prognostic for the patient. About 100% of men with initial diagnosis are free from the disease in five years after the treatment. On the other hand, if PC is not treated, it is potentially lethal [2].

Clinical methods for diagnosis of PC include the dosage of prostatic specific antigen (PSA) and the rectal touch [3]. Unfortunately, these initial procedures are not specific for PC detection, especially the PSA dosage. The level of PSA is high, in about 20 to 30% of the benign pathologies that results in false positive and unneeded biopsy [4]. However, new methods for diagnosis of PC are needed.

The prostatic specific membrane antigen (PSMA) is a type II transmembrane glycoprotein, also called glutamate carboxypeptidase II (GCPII) and differs from the PSA that is a free

protein in blood. High levels of PSMA are observed in almost all prostatic pathologies and low levels were observed in brain, kidneys, salivary glands and small intestine [4, 5, 6].

The exact function of PSMA in prostatic cells is still unknown. In neuronal cells, PSMA has NAALADase activity, which is responsible for metabolizing the neurotransmitter N-acetyl-aspartyl-glutamate, and in intestine acting as carboxypeptidase and folate hydrolase, allowing the free folate absorption. The PSMA exists in monomers and dimers, and this is the necessary conformation for its enzymatic activity on the cell surface, and has the characteristic of being internalized, but it is not known what triggers this movement [7].

The high levels of PSMA in prostatic pathologies stimulated the development of PSMA inhibitor molecules that could be used as a vector for imaging tumor agents and that could perfuse in the tumor microvasculature [8]. Urea-based inhibitors of the PSMA are low-molecular-weight peptides, comprising two amino acids linked for urea groups R-amino [9].

For radiolabeling of biomolecules with a metal radionuclide a bifunctional chelator (BFC) linked covalently to the target molecule is required, either directly or through a ligand pharmacokinetic modifier (PKM), and strongly coordinate with the radiometal [10].

The choice the ideal BFC can promote great improvement in radiolabeling kinetic, increasing in the stability of the compound and can also influence the pharmacokinetics of the labeled compound, since it has a significant impact on the biological properties of the radiometal [10, 11].

The ^{68}Ga is an attractive radioisotope for PET imaging technology. It has a half-life of 68 minutes and decays 89% by positrons of 1.92 MeV. It is obtained from a ^{68}Ge/^{68}Ga-generator based on alumina, titanium or dioxide tin column, and the ^{68}Ga is eluted with 0.1M HCl [12].

The combination of the acyclic metal chelator HBED-CC and urea-based PSMA inhibitor has shown higher efficiency when compared to other chelators. This combination introduces an aromatic moietie that promotes the interaction with the hydrophobic pocket of the PSMA receptor S1 binding site. This combination introduces higher lipophilicity when compared with DOTA chelator, with the reduction of unspecific binding and increased internalization in LNCaP cells that result in better images of PET. When compared to ^{18}F-cholina, has also shown advantages, since it offers higher contrast and better detection of recurrent prostate cancer and metastatic lesions [13].

However, in contrast to other radiometal chelators, the complexation of gallium with HBED-CC forms three distinguishable diasteroisomers (RR, SS and RS configurations at the amine nitrogen atoms), that are influenced by the temperature and pH [6, 14, 15]. Recent studies suggest that these diasteroisomers has no influence on in vivo binding of the molecule to the receptor [6].

2. MATERIALS AND METHODS

The labelling of PSMA-HBED-CC with ^{68}Ga was studied to optimize the radiochemical yield of the final product with high specific activity.

The influence of the final labelling volume was investigated. For this study, 5µg of PSMA-HBED-CC in 1 or 2 mL of 0.2M sodium acetate buffer pH 4 was labelled with 740 MBq of [^{68}Ga]$GaCl_3$ eluate in 0.02M HCl in acetone ultrapure. The resulting solution was incubated for 7 min at 95 ºC. Alternatively, labeling was performed in 0.1M HEPES buffer pH 7.0.

The influence of the time reaction (2 min – 10 min) and temperature (25 ºC - 95 ºC) were investigated using sodium acetate buffer and the stability of the preparation was analyzed for labeling performed using 7 minutes as reaction time.

The influence of pH (3.5 and 5) in radiochemical purity and in the formation of the diasteroisomers was investigated.

To determine the maximum specific activity for the radiopharmaceutical, 5µg of the peptide was labelled with 0.37 – 11.1 GBq of [^{68}Ga]$GaCl_3$. The resulting solution was incubated for 7 min at 95 ºC.

The PSMA-HBED- ^{68}Ga was storage under refrigeration (2-8 ° C) or at ambient temperature (15-35 °C) for 4 hours to determine the stability of the preparation under these conditions**.**

In all the studies, the radiochemical purity was evaluated by thin layer chromatography in silica gel 60 (TLC-SG) using methanol/1M ammonium acetate solution pH 8.5 as mobile phase and reversed-phase high performance liquid chromatography (HPLC-Agilent) (column C18, 150 x 4,0 mm, 5µm – Waters) and flow rate of 1 mL/minute.

Statistical analysis was performed using the GraphPad Prism 5.00® statistical program (GraphPad Software, Inc., San Diego, CA, USA). The parametric data were expressed as mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

The ^{68}Ga-PSMA-HBED-CC was previously investigated and proved to be favorable for the interaction with the PSMA active binding site resulting in better acquisition of PET images [6, 14, 16].

The Gallium-68 is a positron emitting radioisotope with a short half life of approximately 68 minutes. Therefore, all the synthetic process must be optimized to minimize the maximum loss of activity by radioactive decay.

Before submitting the radiolabelling at high temperatures, the possible loss of [^{68}Ga]$GaCl_3$ by oxidation in the evaporation process was evaluated. The $^{68}GaCl_3$ solution was evaporated to final volume of 10µL, without change of radiochemical purity (TAB. 1).

Table 1: Radiochemical purity (TLC-SG) before and after [^{68}Ga]$GaCl_3$ evaporation (n = 3).

[^{68}Ga]$GaCl_3$ without evaporating	[^{68}Ga]$GaCl_3$ after evaporation
99,92% ± 0,0002	99,97% ± 0,0003

When using 1 mL of sodium acetate buffer, the pH of the preparation reduced to 3.5 after the introduction of 10µL of the [^{68}Ga]$GaCl_3$ acid solution. This problem was not observed employing 2 mL of buffer, resulting in higher radiochemical purity, despite both preparations showed stability over time (FIG. 1).

Based on previously published studies [6, 11, 14, 18] two different buffers were evaluated on the radiolabeling of PSMA-HBED-CC with [^{68}Ga]$GaCl_3$ using different reaction times.. Although both, sodium acetate and HEPES buffer resulted in high radiochemical purity of the preparations (Table 2), sodium acetate buffer was chosen for other procedures because HEPES buffer represents an impurity for intravenous administration in patients, and should be removed from the preparation before the administration [17].

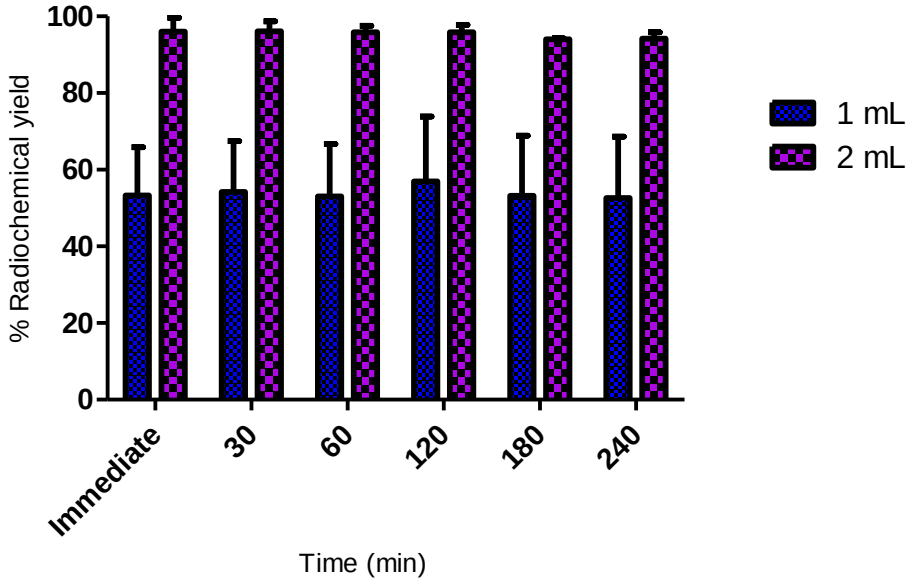

Figure 1: Radiochemical purity (TLC-SG) in the volume 1 mL or 2 mL (n = 2).

Table 2: Radiochemical yield (TLC-SG): influence of buffer and reaction time (n = 3)

Reaction time (minutes)	Sodium Acetate buffer 0.2 M pH 4	HEPES buffer 2.1 M pH 7.5
	Radiochemical Yield (%)	
2	87.05 ± 0.03	95.31 ± 0.03
5	95.54 ± 0.01	95.54 ± 0.04
7	96.45 ± 0.01	94.34 ± 0.05
10	95.59 ± 0.05	94.22 ± 0.05

After 2 minutes of reaction, as expected, radiolabeling with HEPES showed a higher radiochemical purity (> 95%), while for the sodium acetate buffer 5 minutes was necessary to obtain similar radiochemical yield. Both preparations remained stable after 4 hours, showing radiochemical yield of 1.07% and 1.82%, for sodium acetate buffer and HEPES buffer, respectively.

To study the influence of time and temperature, 5 µg of the peptide were labelled with 740 MBq of [^{68}Ga]$GaCl_3$ without shaking in sodium acetate buffer 0.2M pH 4. The highest radiochemical purity was obtained when peptide was radiolabelled with 95 °C for 7 minutes (FIG. 2). This result did not corroborate the literature which showed higher radiochemical yield for labelling performed at 25 ^{o}C [13, 14].

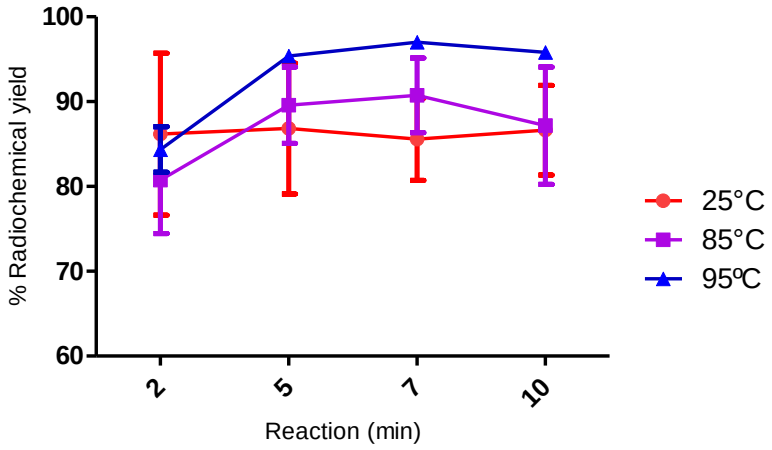

Figure 2: The influence of time and temperature on radiochemical yield (TLC-SG).

The formation of diasteroisomers can be influenced by the temperature of the reaction [13]. The HPLC profile of labelling performed at different temperatures are presented on Figure 3.

It is observed that the less thermodynamically stable specie changed its conformation resulting in the most thermodynamically stable specie, when comparing the percent of each specie in the total of radiochemical purity, immediately and 4 hours after labelling. No significant change was observed between the temperatures 85 °C and 95 °C, and the

relationship between the decrease in the formation of less stable diasteroisomers of 2.7% and 2.6% respectively. The radiolabeling at room temperature showed more pronounced variation, with 4.71%.

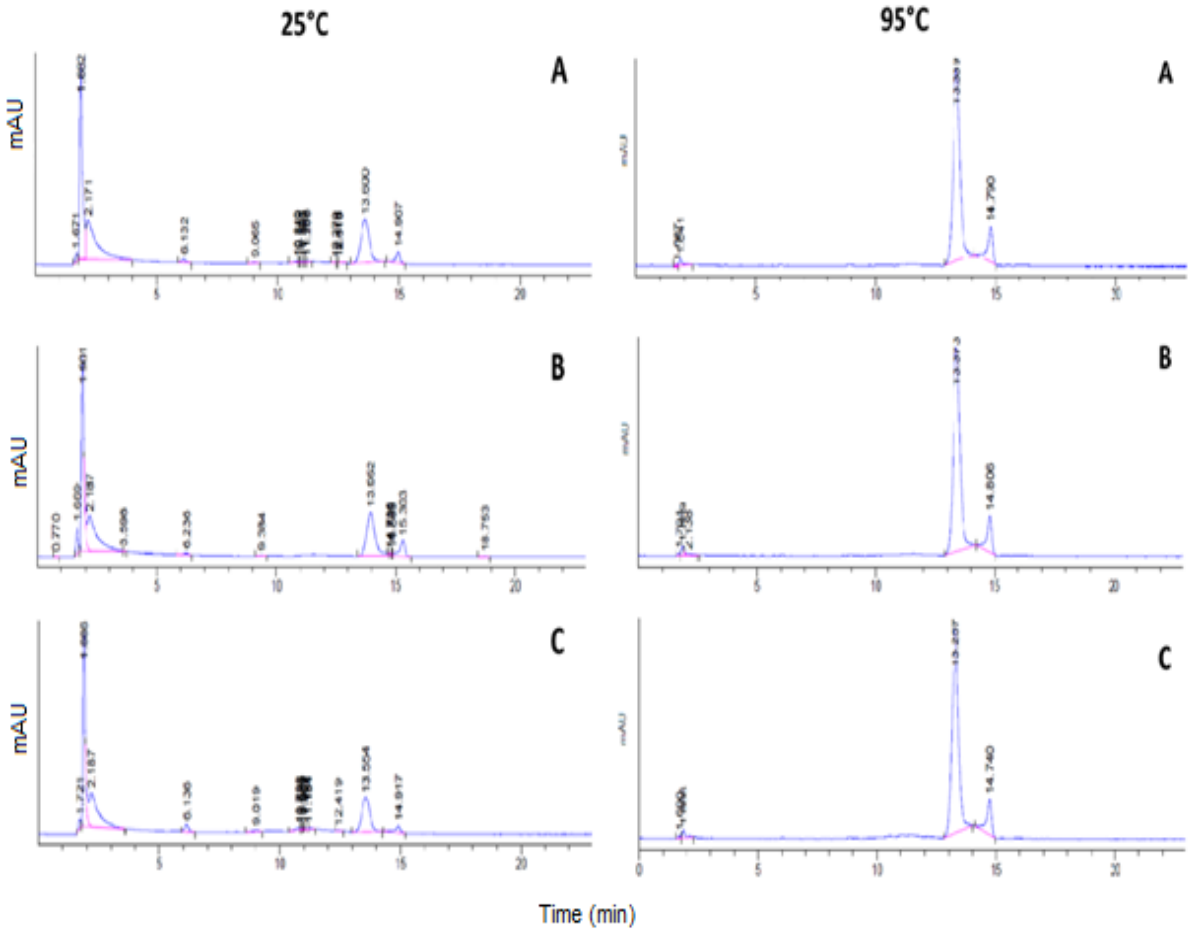

Figure 3: HPLC profile of ^{68}Ga-PSMA-HBED-CC preparation: the production of diasteroisomers at 25 ° C and 95 ° C, immediately after labelling (A), 30 min (B) and 4 hours after labelling(C).

As described above the volume of 2 mL of buffer proved effective to maintain the pH of the solution stable throughout the experimental period. The pH of the reaction is seen as a critical factor in the formation of isolated diasteroisomers and to obtain high radiochemical yield [6].

The pH was previously adjusted with 0.2 M acetic acid and verified before and after the reaction and throughout the period of stability study (4 hours). The results obtained are shown in FIG. 4.

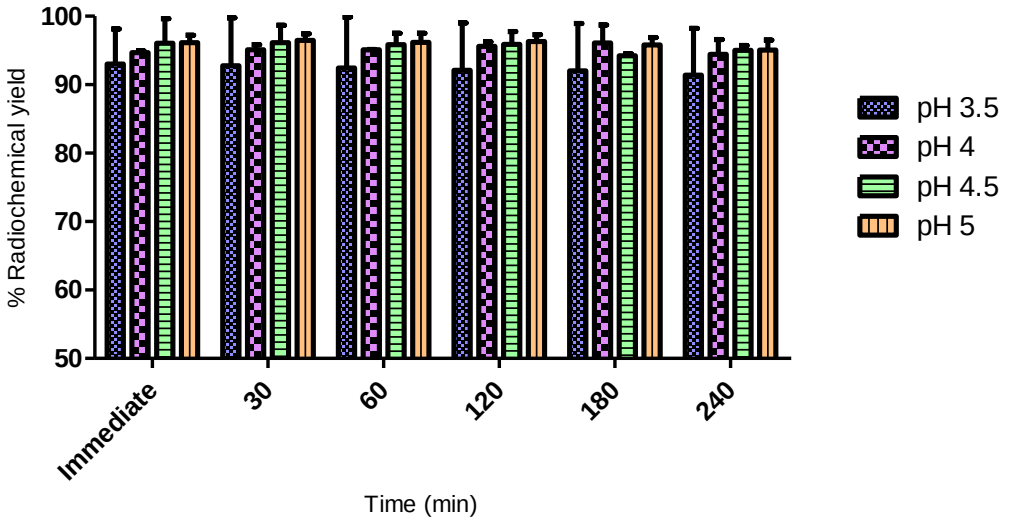

Figure 4: Relationship between pH variation and yield of labeling (n = 3).

The variation of pH demonstrated significant influence on the radiochemical yield only at pH 3.5. Thus, only the other pH values were analyzed for the formation of diasteroisomers (FIG. 5).

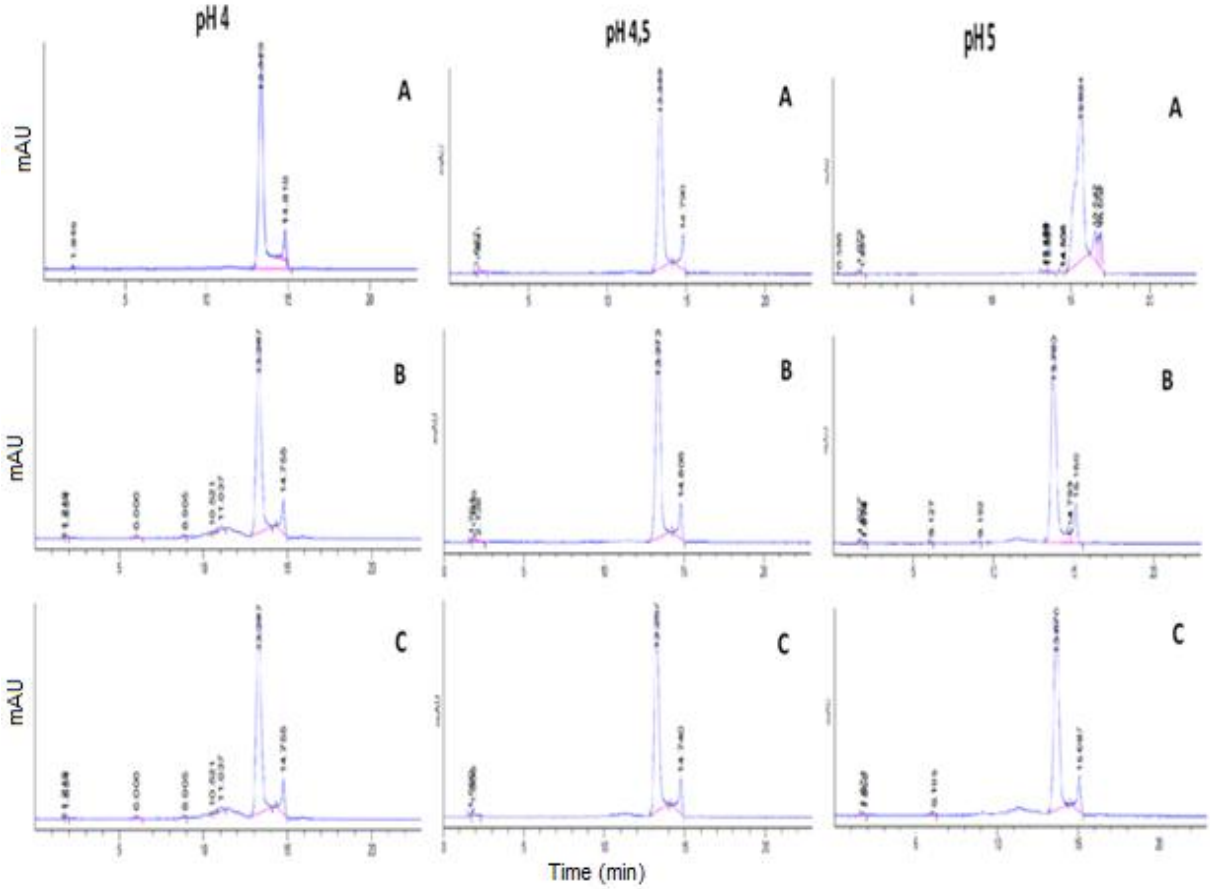

Figure 5: HPLC profile of of ^{68}Ga-PSMA-HBED-CC preparation: the influence of pH in the production of diasteroisomers.

In the labeling conditions using pH 5, high instability of ^{68}Ga-PSMA-HBED-CC was observed immediately after labelling. The thermodynamically most stable diastereomer (RR) was produced in minor amount, beyond the chromatogram shows many co-eluting peaks. At the following times the production of the most stable diasteroisomers was observed in HPLC profile. The labeling with pH 4 and 4.5 did not show considerable variations in the formation of diasteroisomers.

The specific activity of radiopharmaceuticals corresponds to the nuclide radioactivity per unit of mass (GBq/µmol) [19]. From the point of view of clinical application, this parameter is important in the development of new drugs, since the optimal specific activity can be found because when it is very high can cause radiolysis and thus denaturation of the peptide, and when specific activity is low, the unlabelled molecules compete with the labeled through the same receptors *in vivo* and may lead frames toxicity in patients [20].

The study of specific activity was based on activities used in clinical studies (80.5 GBq/µmol) [16]. When analyzing the results in TAB. 3, there is a direct relationship between the increase in specific activity and the decrease of radiochemical purity.

Table 3: Specific activity of ^{68}Ga-PSMA-HBED-CC

Mass of PSMA-HBED-CC (µg)	**Activity of $^{68}GaCl_3$ (GBq)**	**Specific activity of ^{68}Ga-PSMA-HBED-CC (GBq/µmol)**	**Radiochemical yield of ^{68}Ga-PSMA-HBED-CC (%) (TLC-SG)**	**Radiochemical yield of ^{68}Ga-PSMA-HBED-CC (%) (HPLC)**
5	0.37	46.25	96.97 ± 0.03 (n = 2)	100
5	0.55	68.75	95.61 ± 0.00 (n = 2)	98.24
5	0.74	92.5	95.05 ± 0.01 (n = 3)	98.27
5	1.11	138.75	62.16 ± 0.18 (n = 2)	63.01

Specific activities between 46.25 GBq/µmol - 68.75 GBq/µmol showed high radiochemical yield (> 95%) without purification, remained stable throughout the study period (4 hours) (Fig. 6).

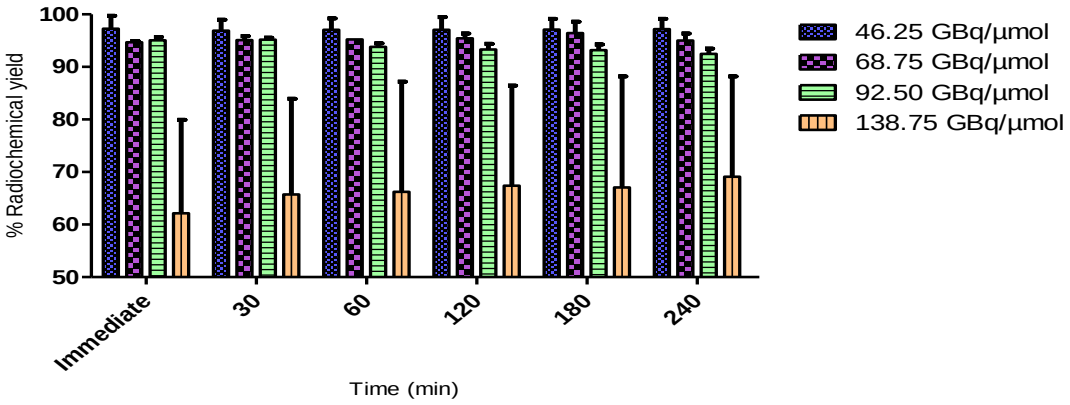

Figure 6: Relationship between specific activity variation and yield of labeling.

The specific activity of 92.5 GBq/µmol remained above 95% in only 30 minutes, decreased to 93.08 ± 0.01 and 138.75 GBq/µmol there was a marked decrease of radiochemical purity (62.12 ± 0.18) demonstrating probable breakdown of ^{68}Ga-PSMA-HBED-CC by radiolysis.

The labeled peptide supported a higher specific activity (92.50 GBq/µmol) to that used in clinical trials with stability for up to 4 hours, which makes possible its use.

The preparation showed good stability when stored under refrigeration (2 - 8 ° C) and at room temperature (15-35 ° C) (FIG. 7). The results suggest there is no need to use stabilizers.

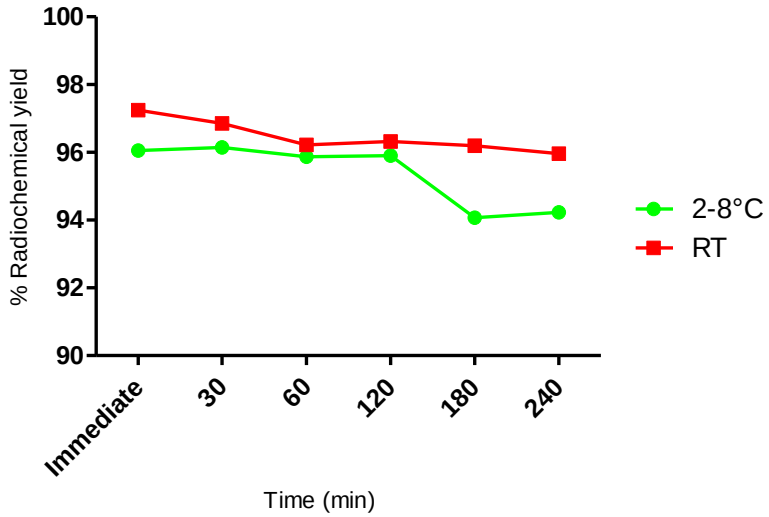

Figure 7: Stability of ^{68}Ga-PSMA-HBED-CC stored under refrigeration (2-8 ° C) and at room temperature (15-35 ° C). Conditions of radiolabeling: 5 µg of PSMA-HBED-CC peptide, 740 MBq of [^{68}Ga]$GaCl_3$ for 7 minutes at 95 ° C without stirring (TLC-SG).

4. CONCLUSIONS

The ^{68}Ga-PSMA-HBED-CC was produced in high specific activity and showed good stability at room temperature. These results stimulate future pre-clinical studies to determine the potential of this radiopharmaceutical for diagnostic application.

ACKNOWLEDGMENTS

The authors are grateful to CNEN and Radiopharmacy Center/IPEN for providing the financial resources.

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