Optimized production, quality control, biological evaluation and PET/CT imaging of $^{68}$Ga-PSMA-617 in breast adenocarcinoma model

**Abstract:** Optimized production, quality control and pre-clinical evaluation of $^{68}$Ga-PSMA-617 as a PET radiotracer for PSMA-positive malignancies as well as successful application in imaging of breast adenocarcinomas are reported. $^{68}$Ga-PSMA-617 radiolabeling and QC optimization, stability, log $P$, biodistribution in breast adenocarcinomas-bearing mice (direct and blockade studies) and also PET/CT imaging was performed. $^{68}$Ga-PSMA-617 complex was prepared in high radiochemical purity (>96%, ITLC, HPLC) and specific activity of 300–310 GBq/mM at 95 °C using 2–4 micrograms of the peptide in 10 min followed by solid phase purification. The tracer was stable in serum and final formulation for at least 120 min. The log $P$ was -1.98. Western blot test on the tumor cell homogenates demonstrated distinct existence of the PSMA on the surface. The biodistribution of the tracer demonstrated specific kidney and tumor significant uptake using blocking study. Significant tumor : blood and tumor : muscle ratio uptake observed at 30 min post-injection (2.69 and 19.1, respectively). A reduction of 40–80% off tumor uptake in the study time period observed using blocking $^{68}$Ga-PSMA-617. $^{68}$Ga-PSMA-617 can be proposing a possible tracer for PET imaging of breast adenocarcinomas and other breast malignancies.

**Keywords:** Breast adenocarcinoma, Western blot, $^{68}$Ga-PSMA-617, PET/CT, block test, biodistribution.

1 Introduction

Prostate cancer (PC) is the second most common cancer worldwide in male and the fourth most common cancer overall [1]. Prostate-specific membrane antigen (PSMA) is a catalyzing peptidase specific for N-acetyl-L-aspartyl-L-glutamate moiety [2] and its expression in nearly all the PCs is 10–80 folds higher than healthy prostate tissue leading to high clinical importance for effective theranostic agents via PSMA targeting [1]. With respect to peptidic nature of binding moiety of PSMA natural substrate and interesting pharmacokinetics of peptides radioligands such as facile synthesis, rapid wash-out and target visualization, and possible development of theranostic agents, many peptidic PSMA radioligands have been developed and used successfully in the diagnosis and therapy of human malignancies especially PC [3, 4]. $^{68}$Ga has been known as an excellent positron emitting radioisotope suitable for clinical utilizations. The physical characteristics of this radioisotope (positron emission (89%), low abundance of 1077 keV photon emission (3.22%) and the relatively short half-life ($t_{1/2} = 67.71$ min) [5] permit PET applications of the $^{68}$Ga-radiopharmaceuticals especially available in form of $^{68}$Ge/$^{68}$Ga generator as a useful hospital radiopharmaceutical system while maintaining an acceptable radiation dose to the patient [6]. Recently, a new modified chemical structure of DOTA-based synthesized PSMA, 2-[3-(1-Carboxy-5-[3-naphthalen-2-yl]-2-[[4-[[2-(4,7,10-tris-carboxymethyl-1,4,7,10-tetraaza-cyclocdec-1-yl)-acetylaminio]-methyl]-cylohexanecarbonyl]-amino]-propionylamino]-pentyl)-ureido]-pentanedioic acid (PSMA-617) (Figure 1), has been introduced showing high potential in the clinical management of advanced PCs in form of labeled with $^{177}$Lu [4] and $^{68}$Ga [7]. More studies have shown the effectiveness of the diagnostic PSMA complexes for detection of other tumors. For example, PSMA-targeted radionuclide therapy has been utilized for treatment of differentiated thyroid cancer [8]. PSMA is also
expressed on the other organs, and therefore radionuclide-labeled PSMA ligands can be applied as good candidates for imaging and/or treatment of other malignancies such as melanoma, renal cell cancer, and colon carcinoma and breast cancer.

Considering the importance of breast cancer early detection in order to reduce the morbidity in patients [9], as the most common cancer among women, and availability of PSMA on many breast cancers, we studied the possibility of \(^{68}\)Ga-PSMA-617 application in the detection of breast adenocarcinomas.

In the present work, the \(^{68}\)Ga-PSMA-617 complex has been prepared at the optimal conditions. The biodistribution of the complex was studied in breast adenocarcinoma bearing BALB/c mice using biodistribution method in normal and PSMA blocked animals as well as PET/CT studies. The data obtained in this study might be valuable to the application of \(^{68}\)Ga-PSMA-617 in breast cancer and as far as the authors knowledge has not been reported elsewhere.

2 Materials and methods

A prototype 40-mCi \(^{68}\)Ge/\(^{68}\)Ga generator, developed at Pars Isotope Co. (Tehran, Iran), was used in this study. PSMA-617 was provided from ABX (Radeberg, Germany). All other chemical reagents were purchased from Sigma-Aldrich Chemical Co. (UK). Whatman No. 2 paper was obtained from Whatman (UK). Radio-chromatography was performed by Whatman paper using a thin layer chromatography scanner, Bioscan AR2000, Paris, France. Reverse phase high performance liquid chromatography (RP-HPLC) was performed for radiolabeling and specific activity analysis of the final product using a KNAUER-D14163 system, Berlin, Germany using Mobile phase of A: Ultrapure water-TFA 1% (V/V); B: Acetonitrile HPLC Grade using gradient-elution: 0–3 min, A: 100%, B: 0%; 3–10 min, A: 50%, B: 50%; 10–15 min, A: 0%, B: 100%; Flow rate: 1.5 mL/min, Injection volume: 20 μL. The column used was MZ-Analysentechnik, ODS-H 5 μm (100 × 4.0 mm) and gamma detector was from Ray test, GABI gamma ray detector. The activity of the samples was measured by a p-type coaxial high-purity germanium (HPGe) detector (model: EGPC 80–200 R) coupled with a multichannel analyzer system. Calculations were based on the 511 keV peak for \(^{68}\)Ga. All values were expressed as mean ± standard deviation (Mean ± SD) and the data were compared using Student’s t-test. Statistical significance was defined as \(P < 0.05\). Animal studies were performed in accordance with the United Kingdom Biological Council’s Guidelines on the Use of Living Animals in Scientific Investigations, second edition.

2.1 Radiolabeling of PSMA-617 with \(^{68}\)GaCl\(_3\)

The QC/QA protocols for the \(^{68}\)Ge/\(^{68}\)Ga generator used in this study has been reported elsewhere and the eluted activity was analyzed carefully for radiolabeling according to a recent report [10]. The preparation of \(^{68}\)Ga-peptide radiopharmaceutical using the generator performed according to the recently reports [11]. Briefly, a stock solution of PSMA-617 with the concentration of 1 μg/μL in the distilled water was prepared. The first fraction of the eluted \(^{68}\)Ga was put away and the next three fractions including 1.5 mL of \(^{68}\)GaCl\(_3\) (20–30 mCi) were used for radiolabeling. Certain amount of PSMA-617 was added to the vial containing \(^{68}\)GaCl\(_3\) and the pH of the reaction mixture was adjusted utilizing HEPES. In order to obtain the optimized conditions, several experiments were performed by changing the ligand concentration, pH, and temperature and incubation time. Eight micro liter of water was then added to the final solution and the mixture was passed through a C\(_18\) Sep-Pak column preconditioned with 5 mL ethanol, 10 mL water and 10 mL air, respectively. The column was then washed with 0.5 mL ethanol and 1 mL.
of 0.9% NaCl. Radiochemical purity of the radiolabeled complex was checked using both HPLC and ITLC methods. Paper chromatography was carried out using Whatman No. 2 paper and 0.9% NaCl and 0.1 M sodium citrate as the mobile phase. HPLC was performed on the final preparation using a reverse phase column as described above.

2.2 Stability tests

The stability of $^{68}$Ga-PSMA-617 complex in room temperature was studied according to the conventional ITLC method. The radiolabeled complex was kept at room temperature for 120 min while being checked by ITLC at the specified time intervals (10, 20, 30, 45 and 120 min).

2.3 In vitro stability of $^{68}$Ga-PSMA-617 in presence of human serum

For stability of the complex incubated in human serum, the release of the $^{68}$Ga from the $^{68}$Ga-PSMA-617 complex was measured by ITLC. Final solution (200 μCi, 50 μL) was incubated in the presence of freshly prepared human serum (300 μL) (Purchased from Iranian Blood Transfusion Organization, Tehran) and kept at 37 °C for 2 h. Every 30 min to a portion of the mixture (50 μL), trichloroacetic acid (10%, 100 μL) was added and the mixture was centrifuged at 3000 rpm for 5 min followed by decanting the supernatant from the debris. The stability was determined by performing frequent ITLC analysis of supernatant using above mentioned ITLC system.

2.4 Determination of partition coefficient

Partition coefficient ($\log P$) of $^{68}$Ga-PSMA-617 was calculated followed by the determination of $P$ ($P = \text{the ratio of specific activities of the organic and aqueous phases}$). A mixture of 1 mL of 1-octanol and 1 mL of isotonic acetate-buffered saline (pH = 7) containing approximately 3.7 MBq of the radiolabeled gallium complex at 37 °C was vortexed 1 min and left 5 min. Following centrifugation at > 1200 g for 5 min, the octanol and aqueous phases were sampled and counted in an automatic well-type counter. A 500 μL sample of the octanol phase from this experiment was shaken again two to three times with fresh buffer samples. The reported $\log P$ values are the average of the second and third extractions from three to four independent measurements.

2.5 Developing breast adenocarcinoma-bearing BALB/c mice

The tumor xenograft development was performed according to the reported procedure [12]. The tumor was created via percutaneous implantation of adenocarcinoma breast tumor (by 2–3 mm$^3$ segments) in the right side of the flank of inbred female BALB/c mice (16–25 g, 6–8 weeks old, Pasteur Institute, Tehran, Iran). The biodistribution and imaging studies were performed when the tumor volume reached 70–80 mm$^3$.

2.6 Determination of PSMA expression in murine breast adenocarcinoma using western blot

In order to demonstrate the existence of PSMA expression in the available tumor cell surface a western blot analysis was performed using 0.75 mm thick, 12% T, 2.67% C polyacrylamide gels run at non-reducing conditions followed by transferring the protein bands onto PVDF membranes (0.45 μm pore size Invitrolon™ PVDF, Invitrogen, Carlsbad, CA, USA) using a tank blotting (Bio-Rad, Hercules, CA, USA) at constant current of 350 mA for 1.5 h. The membrane proteins were blocked with 1% of BSA in 50 mM Tris–HCl/150 mM NaCl/0.1% Tween-20, pH 7.4. The 2E9 antibody was diluted to 1:5000 as described previously [13], whereas ECL anti-mouse IgG (as secondary antibody) was used in dilution 1:10000. The horseradish peroxidase reagent system (ECL) were from GE Healthcare (Uppsala, Sweden). Staining was performed with Coomassie blue R350 for 1 h and de-stained according to the manufacturer’s recommendations.

2.7 Biodistribution of the radiolabeled complex in breast adenocarcinoma-bearing BALB/c mice at normal and target blocked conditions

The final $^{68}$Ga-PSMA-617 solution (5.55 MBq, 100 μL) was injected intravenously into the BALB/c mice bearing adenocarcinoma breast cancer through their tail vein. The total amount of radioactivity injected into each animal was measured by counting the 1-mL syringe before and after injection in a dose calibrator with fixed geometry. For blocking studies the animals pre-treated with 0.1 mg PSMA-617, 15 min prior to tracer administration via i.p. route. The biodistribution of the solutions
among tissues were determined by sacrificing of the mice at each selected intervals (15, 30, 60 and 120 min) after injection using the animal care protocols. Blood samples were rapidly taken after scarification. The tissues were weighed and rinsed with normal saline and their activities were determined with a p-type coaxial HPGe detector coupled with a multichannel analyzer. The percentage of injected dose per gram (% ID/g) for different organs was calculated by dividing the activity amount of each tissue (A) to the decay-corrected injected activity and the mass of each organ. Five mice were sacrificed for each interval. All values were expressed as mean ± standard deviation and the data were compared using Student’s t-test.

2.8 Imaging studies

PET/CT imaging was performed with a PET/CT scanner (Biograph 6 TrueX; Siemens Medical Solutions). Static PET images were acquired for 5 min with three sets of emission images immediately after injection of $^{68}$Ga-PSMA-617 and besides at 60 and 120 min post-injection in the BALB/c mice bearing tumor. In addition, PET emission scans were preceded by CT scans performed for anatomical reference and attenuation correction (spatial resolution 1.25 mm, 80 kV, 150 mAs) with a total CT scanning time of 20 s. Reconstruction was performed using the iterative algorithm with attenuation correction. The reconstruction settings were four iterations and 21 subsets to a $256 \times 256$ matrix, with a post filtering of 2 mm. Transmission data were reconstructed into a matrix of equal size by means of filtered back-projection, yielding a co-registered image set.

3 Results and discussion

The elution of $^{68}$Ge/$^{68}$Ga generator and Quality control of the eluted $^{68}$Ga as well as determination of chemical ionic impurities has been reported [14]. The results were as follows; Fe < 0.230 ppm, Sn < 0.220 ppm, Zn < 0.135 ppm and Ga < 0.1. Considerations were taken into account for $^{68}$Ga-radiolabeling using a generator to reach the desired radiochemical purity of the complex and the quality of the formulation for human applications [15]. The breakthrough of the generator was also determined using spectroscopic techniques in 48 h.

In order to reach the best radiolabeling yield with minimum unlabeled peptide as well as highest radiochemical purity, several experiments were carried out to obtain the maximum complexation yields, by varying different reaction parameters including the ligand concentration, pH, temperature and reaction time. The radiolabeling of the peptide at the optimized conditions (2.5 μg; 2.4 nmol) led to the formation of final tracer in 85–92% of radiochemical purity at 90–95 °C at the pH 3.5–4 using HEPES and 10 min seemed a sufficient time, however using a solid phase extraction applying C$_{18}$ cartridges a high radiochemical purity complex was obtained (>96%).

The optimizations were controlled using ITLC method using Whatman paper No.2. In both 0.1 M sodium citrate and 0.9% NaCl as the mobile phases, the radiolabeled compound remains at the origin, while free gallium cation migrates to higher $R_f$ (Figure 2).

HPLC experiments using a gradient of water : acetoniitrile (0–100 : 244 100–0% added: 1% trifluoroacetic acid) led to the fast removal of any free cation from the column with retention time (1.2 min), while

![Figure 2: ITLC chromatogram of $^{68}$GaCl$_3$ (right) and $^{68}$Ga-PSMA-617 (left) in 0.9% NaCl using Whatman No.2.](image)
\[ {^{68}\text{Ga}}\text{-PSMA-617} \text{ with high molecular weight was eluted after 4.5 min (Figure 3).} \]

### 3.1 Stability studies

The stability of \(^{68}\text{Ga}\)-PSMA-617 was investigated at room temperature and in human serum at 37 °C. The radiochemical purity of the complex remained > 96% at room temperature and in freshly prepared human serum at 37 °C even after 120 min of preparation.

### 3.2 Partition coefficient

As an hydrophil Ga-68 complex the partition coefficient (\(\log P\)) of \(^{68}\text{Ga}\)-PSMA-617 was calculated based on 1-octanol: isotonic acetate-buffered saline dispersion method and as expected showed a negative amount of −1.98 at pH = 7 consistent with many other Ga-68 peptides used in clinical trials.

### 3.3 Detection of PSMA at the tumor cell surface

The existence of PSMA in breast carcinomas in different species is well documented in reviews [16], however in this study, the Western blot was employed as an analysis for verification of PSA in native polyacrylamide gels. The 2E9 monoclonal antibody, used for detection, is known to recognize PSMA as both free and as complexed with other proteins. The single band observed within the range of 23–28 kDa of sample (Figure 4) agreed well with the previous reports showing the existence of PSMA in free form in seminal plasma [17].

### 3.4 Biodistribution of the radiolabeled complex in BALB/c mice b adenocarcinoma breast cancer using normal and blocked conditions

The tissue uptake of the radiolabeled complex was calculated as the percentage of the area under the curve of the related photo peak per gram of tissue (% ID/g) (Figure 5). \(^{68}\text{Ga}\)PSMA-617 demonstrated significant uptake in kidney as the major route of excretion. The maximum uptake in the kidney was occurred at 15 min post injection while decrease with time. As expected, small accumulation of activity was also observed in the tumor. Results showed rapid tracer clearance from the blood, while after 30 min of no significant activity was found in blood samples. No considerable accumulation was detected in the other organs. Prostate-specific membrane antigen is a type II membrane protein with folate...
Figure 5: Percentage of injected dose per gram (%ID/g) at 15, 30, 60 and 120 min after intravenously injection of \(^{68}\text{Ga}\)-PSMA-617 (5.55 MBq) into breast adenocarcinoma-bearing BALB/c mice and also receptor-blocked tumoral mice (%ID/g: percentage of injected dose per gram of tissue calculated based on the area under curve of 511 keV peak in gamma spectrum) (n = 5).

Table 1: Tumor : blood and tumor : muscle ratios of ID/g%

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>120</th>
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<tr>
<td>Tumor: blood</td>
<td>2.38</td>
<td>2.69</td>
<td>2.04</td>
<td>1.98</td>
</tr>
<tr>
<td>Tumor: muscle</td>
<td>17.07</td>
<td>19.10</td>
<td>14.63</td>
<td>10.63</td>
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Hydrolase activity produced by prostatic epithelium. The expression of this molecule has also been documented in extraprostatic tissues, including small bowel and brain. Previous studies using immunohistochemical analysis showed detectable PSMA levels in prostatic epithelium as well as proximal renal tubules. A subpopulation of neuroendocrine cells in the colonic crypts also exhibited PSMA immunoreactivity [18].

In 2011, Liu and colleagues demonstrated the higher expression of PSMA in human breast cancer cells (MDA-MB-231) detected by imaging with fluorescent PSMA inhibitors assuming that the results can be used to determine the role of PSMA in angiogenesis and factors [19].

In 2014, specific results showed the expression of PSMA in blood vessels of gliomas and breast cancer brain metastases providing a rationale for more comprehensive studies to explore PSMA targeted agents for treating secondary brain tumors with PSMA expressing vasculature [20].

Given that PSMA participates in angiogenesis, cell signaling, tumor survival, and invasion, characterizing its expression may help guide later investigations of the poorly understood process of low grade glioma progression to glioblastoma. Recently solid results have been published on the possibility of PSMA imaging of metastatic breast cancer [21], however no more details have been disclosed.

Interestingly many primary prostate adenocarcinomas, lymphomas, renal cell carcinomas, transitional cell carcinomas, colon carcinomas also overexpress PSMA protein on the surface [22]. These findings may offer the possibility of PSMA protein targeting of the mentioned tissues for theranostic procedures.

It is now well understood that PSMA otherwise known as glutamate carboxypeptidase II (GCPII) is a membrane bound protein that has been found to be highly expressed in prostate cancer as well as the neovascularity of a wide variety of tumours including glioblastomas, breast and bladder cancers as well as being implicated in a variety of neurological diseases including schizophrenia and Alzheimer's [23].

Considering the target: non target ratios for the tracer in tumor bearing model at all time intervals, it is suggested that 30 min post injection is a suitable imaging time. The tumor : blood and tumor : muscle uptake ratios are 2.69 and 19.1, respectively at 30 min (Table 1).

PSMA has been detected in the epithelium of kidney, liver and also breast [19]. Recently the first detailed assessment of PSMA expression in the tumor-associated vasculature of primary and metastatic
breast carcinomas has been reported and the researchers claimed that further studies are needed to evaluate whether PSMA has diagnostic and/or potential therapeutic value [24], this work findings can really elaborate on their claim and shows that at least in case of 68Ga-PSMA-617, it can be also used for PET imaging of some breast carcinomas.

As it is obviously shown in the blocking study the most important receptor rich organ i.e. kidney and the tumor uptake have significantly reduced while a pharmacological dose of cold PSMA-617 was applied to the animals prior to tracer administration. In this experiment, the kidney uptake is almost 2 times less at 15 min, and 30–40% less in 120 min. The chosen dose was based on the proposed human injectable dose extrapolation to rats based on the body weight. It is not obvious that longer distribution studies of the tracer would lead to higher uptake in target organs or not, however imaging studies in a tumor-bearing model has been presented in this work. Tumor uptake has also been reduced at all time intervals in blocking studies changing from 1.55% to 0.6% in 120 min. all these data demonstrated the receptor mediate/specific uptake of the tracer among PSMA.

3.5 PET/CT imaging studies

PET/CT images were acquired immediately after 68Ga-PSMA-617 injection and besides after 5–120 min in BALB/c mice bearing tumor (Figure 6). As can be seen, the only visible organs were the kidney and the breast cancer lesion. Other organs show slightly higher than the background activity. No lungs and liver uptake as well as spleen uptake can be observed. The removal of activity from the tumor and kidneys are similar and this suggests that the kidney uptake is a result of receptor binding and not only as an excreting organ. Possibly the best imaging time could be considered about 30 min post injection.

4 Conclusion

In this study, 68Ga was obtained from the SnO2 based 68Ge/68Ga generator. The results of quality control including radionuclide analysis, chemical and radiochemical purities indicated the high purity of the eluted 68Ga. Preparation of 68Ga-PSMA-617 radiolabeled compound
was performed at optimized conditions which showed the increase of the radiolabeling yield with the increment of temperature and ligand concentration. Radiochemical purity of the complex was reached higher than 96%. Also, specific activity of final product was 300–310 MBq/mmol in less than 10 min at the optimized conditions (2.5 μg of PSMA, pH of 3.5–4, temperature of 90–95 °C). The biodistribution of the tracer demonstrated specific kidney and tumor significant uptake using blocking study. Significant tumor: blood and tumor: muscle ratio uptake observed at 30 min post-injection (2.69 and 19.1, respectively). A reduction of 40–80% off tumor uptake in the study time period observed using blocking test. This work supports the application of ⁶⁸Ga-PSMA-617 as a suitable agent for breast adenocarcinoma PET imaging.

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References
