Development and Validation of a High-Pressure Liquid Chromatography Method for the Determination of Chemical Purity and Radiochemical Purity of a \([^{68}\text{Ga}]\)-Labeled Glu-Urea-Lys(Ahx)-HBED-CC (Positron Emission Tomography) Tracer

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ABSTRACT: Background: Prostate-specific membrane antigen (PSMA) has gained high attention as a useful biomarker in the imaging evaluation of prostate cancer with positron emission tomography (PET) during recent years. \([^{68}\text{Ga}]\)-labeled Glu-urea-Lys(Ahx)-HBED-CC (\([^{68}\text{Ga}]\)-PSMA-HBED-CC) is a novel PSMA inhibitor radiotracer which has demonstrated its suitability in detecting prostate cancer. Preparation conditions may influence the quality and in vivo behavior of this tracer, and no standard procedure for the quality control (QC) is available. The aim of this study was to develop a new rapid and simple high-pressure liquid chromatography method of analysis for the routine QC of \([^{68}\text{Ga}]\)-PSMA-HBED-CC to guarantee the high quality of the radiopharmaceutical product before release. Methods: A stepwise approach was used based on the quality by design concept of the International Conference of Harmonisation Q2 (R1) and Q8 (Pharmaceutical Development) guidelines in accordance with the regulations and requirements of European Association of Nuclear Medicine, Society of Nuclear Medicine, International Atomic Energy Agency, World Health Organization, and Italian Association of Nuclear Medicine and Molecular Imaging. The developed analytical test method was validated because a specific monograph in the pharmacopoeia is not available for \([^{68}\text{Ga}]\)-PSMA-HBED-CC. Results: The purity and quality of the radiopharmaceutical obtained according to the proposed method resulted high enough to safely administrate it to patients. An excellent linearity was found between 0.8 and 5 \(\mu\text{g/mL}\), with a detection limit of 0.2 \(\mu\text{g/mL}\). Assay imprecision (% CV) was <2%. Conclusions: The developed method to assess the radiochemical and chemical purity of \([^{68}\text{Ga}]\)-PSMA-HBED-CC is rapid, accurate, and reproducible, allowing routinely the use of this PET tracer as a diagnostic tool for imaging prostate cancer and also assuring patient safety.

1. INTRODUCTION

Prostate cancer (PCa) is now recognized as one of the most important medical problems facing the male population, and it is currently the second most common cause of cancer death in men. Until recently, choline-based positron emission tomography/computed tomography (PET/CT) represented the state-of-the-art radionuclide imaging technique for treatment selection. However, there have been numerous studies reporting a low sensitivity and specificity, especially in patients with prostate-specific antigen values below 3 ng/mL, with a detection rate reported to be only 40–60%. Recent studies have shown that the PCa has a high expression of the prostate-specific membrane antigen (PSMA), which makes it a promising target for developing small-molecule radiometal chelators. The PSMA is a type II transmembrane protein with high expression in prostate carcinoma cells, and a promising new target for specific imaging of PCa. Recently, an innovative \([^{68}\text{Ga}]\)-labeled ligand has been designed to target membrane PSMA in vivo using PET/CT.

There are several biological characteristics making the prostate-urea-based inhibitors of the PSMA an outstanding target for nuclear medicine. PSMA is a type II transmembrane protein with glutamate–carboxypeptidase activity and a known substrate, an ideal target for developing small-molecule radiopharmaceuticals which typically shows fast blood clearance and low background activity.

The acyclic Ga(III) chelator \(\text{N},\text{N}’,\text{N}’’\)-bis[2-hydroxy-5-(carboxethyl)benzyl] ethylenediamine-\(\text{N},\text{N}’,\text{N}’’\)-diacetic acid (HBED-CC) is introduced into the radiotracer as a lipophilic moiety, allowing a favorable interaction with the hydrophobic pocket of the PSMA receptor. The HBED-CC chelator enables efficient radiolabeling of Ga-68 even at ambient temperature.

However, in contrast to other clinically well-established radiometal chelators, HBED-CC forms three NMR-distinguishable diastereomers (RR, RS, and SS configurations), which are thermodynamically favored. Besides the influence of the temperature, the formation of the diaster-
eomers was reported to be pH- and concentration-dependent as well.\textsuperscript{13,14}

The two diastereomers have the same biological activity (evaluation of cell binding and internalization in human prostatic adenocarcinoma cell line) and the same radiochemical stability after the labeling procedure.

In a standard labeling protocol, $[^{68}\text{Ga}]-\text{PSMA-HBED-CC}$ is incubated at a pH of $\sim 4$ and heated at 95 °C. The thermodynamically favored diastereomer is formed; however, a small fraction of one of the other two diastereomers is still present in the labeling reaction without influencing the cell-binding properties of the final formulation of the PSMA-targeted radioligand $[^{68}\text{Ga}]-\text{PSMA-HBED-CC}$ prepared for the patient.\textsuperscript{13,14}

Therefore, there is a need for a quality control (QC) test capable of separating each impurity from $[^{68}\text{Ga}]-\text{PSMA-HBED-CC}$ and to accomplish it over a short duration.

Taking into account the above pieces of information and the complexity of radioactive gallium chemistry, the QC process is vital to ensure the safety and effectiveness of the radiolabeling procedure.

Finally, the Italian and European Pharmacopoeia do not provide any guidelines on the QC of $[^{68}\text{Ga}]-\text{PSMA-HBED-CC}$, and the literature reports only about the synthesis of the tracer but not about the QC method.\textsuperscript{13}

In recent years, radiopharmaceuticals with such complex characteristics gain clinical interest according to the increasing need of personalized management of the diseases requiring detection of the specific biological targets.

Figure 1. HPLC traces of the blank sample (a), PSMA (b), and Ga-PSMA (c). The peaks (b,c) correspond to (1), the thermodynamically more stable, and (2), the thermodynamically less stable diastereomer, respectively.
The continuing development of new tools for molecular imaging is not accompanied by a coherent effort in development, standardization, and validation of QC methods to guarantee high-quality radiopharmaceutical production, especially in the routine clinical setting.

More strong work is necessary when labeling procedures are complex and form different stereoisomers of the same molecule during isotope complexation to assess the diagnostic efficacy and safety of the final product.

The aim of this study was to develop and validate a rapid and simple high-pressure liquid chromatography (HPLC) method of analysis for the routine QCs of a $^{68}$Ga]-PSMA-HBED-CC PET tracer. More specifically, our aim was to prepare and characterize reference materials, as well as to determine a practical QC test for the routine use of $^{68}$Ga]-PSMA-HBED-CC samples.

2. RESULTS AND DISCUSSION

Under the chromatographic conditions described, Glu-urea-Lys(Ahx)-HBED-CC (PSMA-11), natGa-labeled reference Glu-urea-Lys(Ahx)-[Ga(HBED-CC)] (DKFZ-GaPSMA-11), and the internal standard peaks were well-resolved.

In Figure 1, typical chromatograms of the blank eluent are illustrated in comparison with spiked samples analyzed for the analytical HPLC method validation. The average retention times of the first and second diastereoisomers of PSMA-11 were 6.9 and 7.1 min, respectively, similar to those for the two diastereoisomers of DKFZ-GaPSMA-11.

The calibration curve for the determination of PSMA-11 and DKFZ-GaPSMA-11 was linear over the range 5−0.8 μg/mL. The linearity of this method was statistically confirmed for each calibration curve (Figure 2).

The correlation coefficient ($r^2$) for calibration curves was equal to 0.999, which is in accordance with the acceptance criteria (Table 2).

The relative standard deviation (RSD) value of the slope was equal to 0.21%. For each point of the calibration standard, the concentrations of PSMA-11 and GaPSMA-11 were recalculated from the equation of the linear regression curve and the average bias % values were found to be 97.90 and 97.98%, respectively.

The limit of quantitation (LOQ) for PSMA-11 and DKFZ-GaPSMA-11 was 0.8 μg/mL. As shown in Table 1, the coefficient of variation (CV %) was less than 2%.

Under the same gradient chromatographic conditions described above, $^{68}$Ga]-PSMA-HBED-CC and the internal standard peaks were well-resolved.

Figure 3 shows a typical chromatogram of $^{68}$Ga]-PSMA-HBED-CC analyzed for the analytical HPLC method validation.

The average retention times of the first diastereoisomer of $^{68}$Ga]-PSMA-HBED-CC was 7.1 min and the second one was 7.2 min. The calibration curve for the determination of $^{68}$Ga]-PSMA-HBED-CC was linear, and the linearity of this method was therefore statistically confirmed (Figure 4).

The correlation coefficient ($r^2$) for the calibration curve was equal to 0.997, and the average CV % was less than 2% (1.97%) which is in accordance with the acceptance criteria (Table 3).

3. CLINICAL USE OF $^{68}$Ga]-PSMA-HBED-CC

At the University Hospital of Parma, a Prostate Cancer Unit was established in October 2014. The multidisciplinary team of the Prostate Cancer Unit attends meetings every 2 weeks for case management to provide care for patients with prostate cancer at all its stages. The high-technology diagnostic procedures (choline-PET/CT, PSMA-PET/CT, mpMR, and ultrasound/magnetic resonance imaging US/MRI fusion biopsy) are decided by the team to guarantee appropriateness and optimization of resource utilization.

We started the PET/CT diagnostic activity of $^{68}$Ga]-PSMA-HBED-CC in April 2016 producing 123 batches for 162 patients with prostate cancer for staging, restaging, and radiotherapy planning.

A synthesis session was planned once per week at the beginning of the new diagnostic activity, and then it was increased until now with a daily production of $^{68}$Ga]-PSMA-HBED-CC.

Therefore, it has become evident about how the process has to be rapid and efficient in all phases and particularly during the QC steps.

4. CONCLUSIONS

The lack of specific literature about the QCs of $^{68}$Ga]-PSMA-HBED-CC has led us to develop and validate a simple and reproducible analytical HPLC method to analyze the produced radiopharmaceutical to implement it in the clinical setting.

Our validation protocol for QC includes not only typical analytical characteristics (% radiochemical purity, % radiochemical impurities, chemical purity, pH, integrity filter,
but also precision, accuracy, specificity, limit of detection and quantitation, linearity, and a range of HPLC methods to guarantee simple and safe implementation in the diagnostic activity.

The developed chromatographic method complied with all International Conference of Harmonisation (ICH) requirements during the course of time.

These validation results demonstrate the suitability of the method in determining the radiochemical and chemical purity of \(^{68}\text{Ga}\)-PSMA-HBED-CC.

More than 123 consecutive batches were analyzed using this method with the same results, thus proving its accuracy, robustness, and repeatability for the routine clinical application.

| reference value of concentration of PSMA [\(\mu g/mL\)] | calculated concentration [\(\mu g/mL\)] | calculated average concentration [\(\mu g/mL\)] | DS | CV % | average bias % (deviation of positive or negative from 100%) |
|----------------------------------------------------------|----------------------------------------|-----------------------------------------------|----|------|---------------------------------------------------------|
| 5                                                        | 5.00                                   | 5.00                                          | 0.01 | 0.11 | 0.08                                                     |
| 4                                                        | 3.94                                   | 3.94                                          | 0.02 | 0.53 | -1.40                                                   |
| 3.125                                                    | 3.00                                   | 3.00                                          | 0.01 | 0.18 | -3.87                                                   |
| 1.25                                                     | 1.24                                   | 1.24                                          | 0.02 | 1.80 | -0.80                                                   |
| 0.8                                                      | 0.76                                   | 0.76                                          | 0.01 | 1.49 | -4.50                                                   |

| reference value of concentration of Ga-PSMA [\(\mu g/mL\)] | calculated concentration [\(\mu g/mL\)] | calculated average concentration [\(\mu g/mL\)] | DS | CV % | average bias % (deviation of positive or negative from 100%) |
|------------------------------------------------------------|------------------------------------------|-----------------------------------------------|----|------|---------------------------------------------------------|
| 5                                                          | 5.00                                     | 5.00                                          | 0.04 | 0.73 | 0.08                                                     |
| 4                                                          | 3.95                                     | 3.94                                          | 0.08 | 1.93 | -1.45                                                   |
| 3.125                                                      | 3.01                                     | 3.01                                          | 0.03 | 1.01 | -3.74                                                   |
| 1.25                                                       | 1.24                                     | 1.24                                          | 0.02 | 1.85 | -0.48                                                   |
| 0.8                                                        | 0.76                                     | 0.76                                          | 0.01 | 1.49 | -4.50                                                   |
5. MATERIALS AND METHODS

5.1. Reagents and Chemical Syntheses. Synthesis of [68Ga]-PSMA-HBED-CC was performed using 10 μg of Glu-urea-Lys(Ahx)-HBED-CC (ABX, Advanced Biochemical Compounds, Radeberg, Germany).

The ligand was labeled with median 550 (300−800) MBq 68GaCl3 (half-life, 67.6 min) eluted from an IGG10068Ge/68Ga generator (Eckert & Ziegler, E&Z, Berlin, Germany) in a fully automated module (Scintomics GRP, Fuerstenfeldbruck, Germany) in combination with good manufacturing practice−grade disposable cassettes and reagent kit (ABX).

5.2. Synthesis Development. 68GaCl3 was eluted from a 68Ge/68Ga generator with 10 mL of sterile HCl (0.1 M) and collected into a prepurification cation exchange column, which was then eluted with a solution of NaCl (5 M). The eluate was added into the reaction vial, previously loaded with PSMA-HBED-CC [10 μg in 1.5 M 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES buffer solution) at pH = 4]. The mixture was incubated at 95 °C for 10 min. The reaction mixture after incubation was trapped onto a Sep-Pak C18 light cartridge, washed with water for injection Ph. Eur., and eluted with 2 mL of ethanol/water in the ratio of 1/1.

The final product was diluted with 10 mL of phosphate-buffered saline and sterilized by filtration through an inline 0.22 μm sterile filter with a poly(vinylidene difluoride) membrane.

5.3. QC Analysis of [68Ga]-PSMA-HBED-CC Formula- tion. The standard QC tests for [68Ga]-PSMA-HBED-CC were % radiochemical purity (HPLC), % radiochemical impurities of 68Ga3+ [HPLC and thin-layer chromatography (TLC)], chemical purity (HPLC and gas chromatography), pH (pH strips), integrity filter (pressure test), radionuclidic purity principal γ-photon, 68Ge content, 68Ga half-life (γ-ray spectrometry/counting), and sterility/endotoxin assay (sterility test and Limulus amebocyte lysate test).

Chromatograms and instant TLC (ITLC) strips (Varian iTLC-SG plates) were each analyzed with a scanner (Cyclone Plus storage phosphor system, PerkinElmer), Rf values were identified, and then peaks were integrated to determine the % area of a 68Ga species as a proportion of the total area. Additionally, HPLC was performed on a Dionex UltiMate 3000 HPLC system (Thermo Fisher Scientific) equipped with an Acclaim 120 C18 column (3 μm 120 Å (3.0 mm × 150 mm)) and a UV and a γ-detector (Berthold Technologies, Milan, Italy). The used solvents were (A) water +0.1% trifluoroacetic acid (TFA) and (B) acetonitrile +0.1% TFA. HPLC eluents, water, acetonitrile, and TFA were of high-grade purity.

The flow rate of the mobile phase was set at 0.6 mL/min, with a total run of 12 min.

The following gradient was used upon HPLC analysis: 0−2 min 3% B, 2−8 min 3% B to 60% B, 8−8.01 min 60% B to 90% B, 8.01−9 min 90% B, 9.01−12 min from 90% B to 3% B. Flow rate: 0.6 mL/min. The column temperature was maintained at 30 °C. For the detection of chemical impurities, the samples were also monitored with a UV detector at 220 nm. The software system Chromleon 7 was used to assemble the information.

The natGa-labeled reference Glu-urea-Lys(Ahx)-[Ga-(HBED-CC)] (DKFZ-GaPSMA-11) and the reference Glu-

Figure 3. Radio-HPLC trace of [68Ga]-PSMA-HBED-CC. The peak corresponds to (3), the thermodynamically more stable, and (4), the thermodynamically less stable diastereomer, respectively.

Figure 4. Calibration curve obtained with the average values of peak areas of 68Ga-PSMA.
urea-Lys(Ahx)-HBED-CC (PSMA-11) were purchased from ABX Radeberg (Germany).

The γ-ray spectrometry tests included the identification of principal γ-photon (499–521 keV peak) and 68Ge content (decay of 499–521 keV peak ≥ 48 h) using a large volume counter linked to a multichannel analyzer system (HPGe detector ORTEC GEM 30P-76).

The half-life of 68Ga was calculated after measuring the radioactivity of a sample in the dose calibrator at four consecutive intervals (5, 10, 15, and 20 min) and then using the equation: 

\[ t_{1/2} = \frac{\ln(1/2)}{\lambda} \]

where \( \lambda \) is decay constant.

5.4. Standard Solution. Stock solutions (5 μg/mL) and appropriate dilutions of Glu-urea-Lys(Ahx)-HBED-CC (PSMA-11) and natGa-labeled reference Glu-urea-Lys(Ahx)-[Ga(HBED-CC)] (DKFZ-GaPSMA-11) were prepared in CH3CN/H2O (1:1) and stored at −20 °C.

5.5. Validation of the HPLC Method To Determine Chemical Purity. Validation of the analytical method for the determination of chemical purity of [68Ga]-PSMA-HBED-CC was carried out according to ICH Q2 (R1) guidelines. The preparation method of the development of [68Ga]-PSMA-HBED-CC was not considered for chemical impurities, except for free gallium-68. Thus, analyses were performed using a series of standards containing [68Ga]-PSMA-HBED-CC and Ga-68.

5.5.1. Specificity. Specificity determination is performed by analyzing the mixture containing critical components that might be present in the finished product [68Ga]-PSMA-HBED-CC solution and by demonstrating that the method is capable to distinguish the various components present at the limited concentration for the considered standards. The preparation method of the development of [68Ga]-PSMA-HBED-CC was not considered for chemical impurities, except for free gallium-68. Thus, analyses were performed using a series of standards containing [68Ga]-PSMA-HBED-CC and Ga-68.

5.5.2. Linearity. Determination of linearity was done on sets of standard solutions with different concentrations for each of the analytes of interest (Ga-PSMA-HBED-CC and PSMA-11). Such solutions are usually prepared by serial dilution starting from a “mother” solution with the highest concentration (5, 4, 3.125, 1.25, and 0.8 μg/mL). The statistical function used is linear regression with least squares. The curve equation, the correlation coefficient, and the determination coefficient (R2) are calculated through the equation:

\[ y = ax + b \]

where \( y \) is the peak area, \( a \) is the slope, \( x \) is the analyte concentration, and \( b \) is the intercept.

5.5.3. Precision. Precision may be considered at different levels as a measure of repeatability or intermediate precision.

5.5.3.1. Repeatability. Repeatability may be calculated based on the content of standard Ga-PSMA-HBED-CC and PSMA-11. The statistical parameter of concern is the CV % or RSD, which is determined using the equation:

\[ CV\% = s/m \times 100 \]

where \( s \) is the standard deviation of the peak areas and \( m \) is the average of the peak areas.

The data are obtained by injecting five times the sample of the analytes with the concentration range used during the linearity test.

5.5.4. Accuracy. Accuracy is a measure of the degree of conformity of a value generated by a specific procedure to the assumed or accepted true value that is performed through bias % value.

Bias % value measures the difference between the expectation of test results about the different concentration for each of the analytes of interest (Ga-PSMA-HBED-CC and PSMA-11) and an accepted reference value.

Acceptance criterion is bias % > 95%.

5.5.5. Limit of Quantitation. Experimental LOQ has been determined by analyzing a series of diluted solutions of Ga-PSMA-HBED-CC and standard PSMA-11, until a concentration level quantified with a precision >95% is reached. The experimental value determined as above described the need to be confirmed through a precision analysis, using a sample at a concentration corresponding to the found LOQ. Acceptance criterion is CV % < 5%.

5.6. Validation of the HPLC Method To Determine Radiochemical Purity. Validation of the analytical method for the determination of radiochemical purity is presented here.

In Table 3, the validation parameters and their acceptance criteria are summarized:

| Test          | Acceptance Criteria |
|---------------|---------------------|
| specificity   | ≥2.5                |
| linearity     | R2 ≥ 0.99           |
| repeatability | CV % < 2%           |
| LOQ          | CV % < 5%           |
| accuracy     | bias % > 95%        |

In the validation of the methods for radioactive compounds, some of the ICH guidelines of validation parameters may not be of concern and do not apply.

5.6.1. Linearity. Considering the radioactive nature and the short half-life of 68Ga, the typical experimental approach based on the preparation of a series of solutions with different concentrations does not apply. On the contrary, in this case, one sample solution only, with a suitable radioactive concentration, is analyzed five times, at defined time intervals (15 min). Indeed, the radioactivity being the physical parameter of concern for radiochemical detectors, the radionuclide decay itself provides the necessary linear series of values. R2 may be extrapolated from the calibration curve by analyzing five different radioactive concentrations of [68Ga]-PSMA-HBED-CC.

5.6.2. Precision. Precision may be considered at different levels as a measure of repeatability or intermediate precision.

5.6.2.1. Repeatability. Here, also the same considerations apply as that described for linearity, that is, the decay of the radionuclide 68Ga inevitably leads to a decrease over time of the radioactivity. However, repeatability may be evaluated by analyzing a series of HPLC runs obtained with repetitive injections of a single [68Ga]-PSMA-HBED-CC sample and by recalculating the obtained peak area values with the decay equation: 

\[ A = A_0 - \lambda t \]

where \( A \) is the measured peak area, \( A_0 \) = corrected peak area, \( \lambda \) = decay constant of the radionuclide, and \( t \) = time interval between...
the considered injection and the first one, $t_{1/2} = \text{half-life (}^{68}\text{Ga} = 67.63 \text{ min)}$.

The peak area values normalized for decay may then be compared and yield a consistent statistical analysis. Average, standard deviation, and CV % are then calculated. Repeatability has to be determined in three different days, to verify the instrument outcome during the course of time.

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S.M., A.S., and L.R. have contributed to the organization of the content for this manuscript. S.M. and A.S. collected relevant information and prepared the draft. L.R. drafted and revised the manuscript. All authors read and approved the final manuscript. All authors have read and approved the paper for publication.

**Notes**

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### Abbreviations

CT, computed tomography; QC, quality control; CV %, coefficient of variation; GMP, good manufacturing practice; GRP, good radiopharmaceutical practices; GC, gas chromatography; LAL, *Limulus* amebocyte lysate; HPGe, high-purity germanium; HPLC, high-pressure liquid chromatography; ICH, International Conference of Harmonisation; MR, magnetic resonance; NBP-MN, Norme di Buona Preparazione in Nuclear Medicine; SPECT, single photon emission computed tomography; TLC, thin layer chromatography; TFA, trifluoroacetic acid; RSD, relative standard deviation; PCA, prostate cancer; PSA, prostate-specific antigen; PSMA, prostate-specific membrane antigen; PET, positron emission tomography; US, ultrasound

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