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Original Article

Good manufacturing practice production of [^{68}Ga]Ga-ABY-025 for HER2 specific breast cancer imaging

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Abstract: Therapies targeting human epidermal growth factor receptor type 2 (HER2) have revolutionized breast cancer treatment, but require invasive biopsies and rigorous histopathology for optimal patient stratification. A non-invasive and quantitative diagnostic method such as positron emission tomography (PET) for the pre-therapeutic determination of the presence and density of the HER2 would significantly improve patient management efficacy and treatment cost. The essential part of the PET methodology is the production of the radiopharmaceutical in compliance with good manufacturing practice (GMP). The use of generator produced positron emitting ^{68}Ga radionuclide would provide worldwide accessibility of the agent. GMP compliant, reliable and highly reproducible production of [^{68}Ga]Ga-ABY-025 with control over the product peptide concentration and amount of radioactivity was accomplished within one hour. Two radiopharmaceuticals were developed differing in the total peptide content and were validated independently. The specific radioactivity could be kept similar throughout the study, and it was 6-fold higher for the low peptide content radiopharmaceutical. Intrapatient comparison of the two peptide doses allowed imaging optimization. The high peptide content decreased the uptake in healthy tissue, in particular liver, improving image contrast. The later imaging time points enhanced the contrast. The combination of high peptide content radiopharmaceutical and whole-body imaging at 2 hours post injection appeared to be optimal for routine clinical use.

Keywords: Affibody, breast cancer, clinical study, HER2, GMP, Gallium-68

Introduction

Epidermal growth factor receptor type two (HER2) belongs to the HER family composed of four transmembrane receptor tyrosine kinases, and it is overexpressed in malignant tumors such as breast, ovary, lung, colorectal and urothelial cancers and is also present in normal epithelial cells [1-3]. Signaling of HER2 receptors results in cell division, suppression of apoptosis and increase of cell motility. HER2 is overexpressed in up to 25% cases of breast cancer and is associated with poor survival [4-6].

Anti-HER2 therapy with antibodies such as trastuzumab, pertuzumab, and trastuzumab emtansine or small molecule tyrosine kinase inhibitors such as lapatinib improves survival of patients. HER2 expression confirmation is required for patient selection and prediction of response [4, 6-10]. It should be mentioned that only 1 out of 3 patients respond to this expensive treatment with inherent side effects. Currently HercepTest® is used to select patients that might benefit from trastuzumab (Herceptin®) treatment and it is the best-known example of a commercialized theranostics. However, it requires invasive tissue sampling with pitfalls such as sampling error or highly invasive sampling from bone and brain lesions, heterogeneity of receptor expression within a lesion and between the primary tumor and metastasis as well as patient discomfort and side effects such as infection and hemorrhage. Moreover, multiple biopsies for the determination of HER2 expression heterogeneity and receptor expression change over time, and

treatment response monitoring are rarely possible in clinical practice [10-13].

Hence, there is an unmet need for a safe and non-invasive whole body accurate quantification of HER2 expression in a routine clinical setting. This could be accomplished by molecular imaging techniques, in particular positron emission tomography (PET). It would allow staging, prognosis, patient stratification, quantification of the receptor expression and drug dose estimation, early monitoring of the treatment response and resistance, residual disease, follow-up and relapse. In addition, receptor heterogeneity can be profiled. Such a diagnostic method would result in personalized medicine providing possibility to optimize therapeutic response and avoid futile treatments, minimize risks and toxicity as well as reduce cost and patient distress [14]. For example, a clinical intrapatient study with variable amount of administered ^{68}Ga-labeled somatostatin analogue has previously demonstrated significance of individualized patient management [15]. An important prerequisite for such application is development of imaging agents with high sensitivity and specificity for HER2 receptor.

Various radiolabeled agents based on antibodies, antibody fragments, EGFR natural ligand, Affibody® molecules as well as tyrosine kinase inhibitors targeting human epidermal growth factor receptor family have been tested pre-clinically and clinically [16, 17]. The number of antibody based radioimmunotherapeutic agents is considerable, however, the slow antibody pharmacokinetics and clearance from blood circulation result in high radiation dose to the normal tissue, suboptimal image contrast, and prolonged patient examination duration [18, 19]. In addition, radiolabeled trastuzumab may compete for the same target with the therapeutic antibody thus reducing possibility of receptor expression monitoring during the therapy. Reduction of the size to a F(ab')$_2$ fragment improves the pharmacokinetics and dosimetry, however lesion detection rate deteriorates [20]. Anti-HER2 Affibody® molecules (**Figure 1**) are peptide ligands with high affinity for HER2 receptors and favorable pharmacokinetics and clearance from non-target tissue [21-25]. They have a high melting point and rapidly re-fold upon (partial) denaturation which, e.g. may happen during the harsh radiolabeling conditions. The second generation Affibody® molecule, ABY-025, binds selectively to HER2 receptors with picomolar affinity. Importantly, the binding site differs from that of trastuzumab and pertuzumab thus allowing imaging during the respective treatment [26].

A clinical study using [^{111}In]In-ABY-025/SPECT/CT (single photon emission tomography with computer tomography) enabled non-invasive differentiation of HER2-positive and negative lesions and confirmed the feasibility of examination despite of ongoing antibody treatment [27]. The inherent advantages of PET over SPECT technique such as higher resolution, sensitivity, accurate quantification and dynamic scanning argues for an exchange of ^{111}In with ^{68}Ga. It might result in higher detection rate especially with regard to smaller lesions due to the higher resolution and superior scatter correction of PET versus SPECT. Because of the higher image contrast the examination time might be reduced from 24 hours, which was found to be optimal using [^{111}In]In-ABY-025, to 1-2 hours decreasing the logistical challenge, cost and patient discomfort. The study using [^{111}In]In-ABY-025 with a peptide dose of approximately 100 μg indicated some uptake in the normal liver that may compromise image contrast and accuracy of small liver metastasis detection [27]. The reduction of normal liver uptake might be achieved by optimising the amount of the administered peptide as it was demonstrated in a clinical study of patients affected by neuroendocrine tumors using [^{68}Ga]Ga-DOTA-TOC/PET-CT and three different levels of injected peptide amount [15]. In this study [^{68}Ga]Ga-ABY-025 was therefore prepared in two peptide doses and the radiochemistry was developed to ascertain stable levels of specific radioactivity for each peptide dose.

Other advantages of ^{68}Ga are its availability from a simple ^{68}Ge/^{68}Ga generator system on demand independent on external distributors or a cyclotron; high quality PET images; low radiation dose to the patient and personnel; short scanning time; possibility of repetitive examinations; highly reproducible and straightforward labeling chemistry amenable to automation and kit preparation [14, 17, 28, 29]. The generator is simple in use, has long shelf-life ($t_{½}$(^{68}Ge)=270.95 d), and meets criteria of an ideal generator in terms of efficient separation of the daughter and parent elements; physical half-life of parent allowing rapid daughter

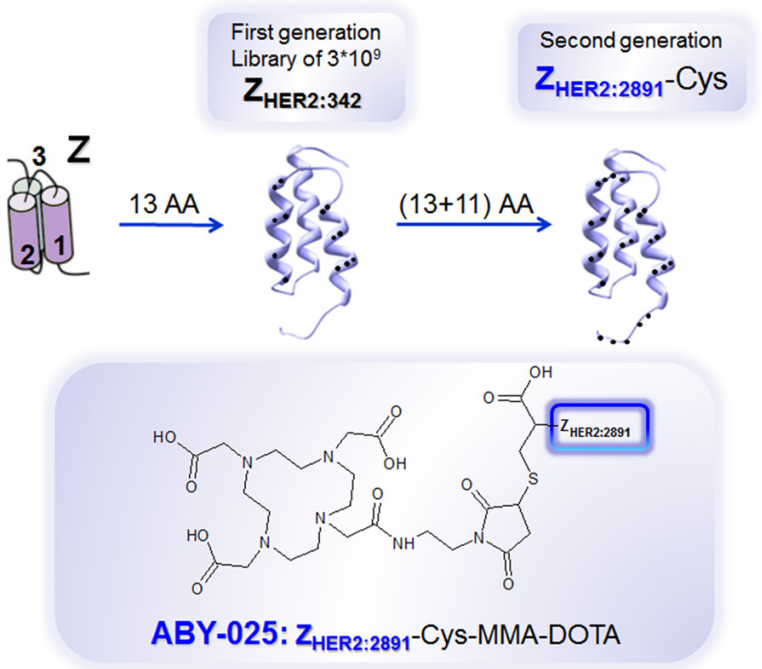

Figure 1. Development of anti-HER2 Affibody® molecules. The variation of 13 amino acids (AA) on the binding surface of a 3 helix peptide structure (Z) resulted in a combinatorial library containing billions of variants from which the 1st generation HER2-binding Affibody molecule, $Z_{HER2:342}$, was selected. Further modification of the non-binding surface resulted in the second generation Affibody molecule, $Z_{HER2:2891}$, with higher thermal stability and hydrophilicity, diminished background interactions with immunoglobulins and production flexibility by peptide synthesis or recombinant expression as well as fully retained *in vitro* and *in vivo* functionality. $Z_{HER2:2891}$ was modified by addition of a unique terminal cysteine for site-specific conjugation to the bifunctional chelator, 1,4,7,10-tetraaza cyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide (MMA-DOTA). $Z_{HER2:2891}$-Cys binds selectively to HER2 with high affinity (K_D: 60 pM).

regrowth after generator elution; stable granddaughter with no radiation dose to the patient; long shelf-life; effective shielding of the generator, minimizing radiation dose to the user and expenses of hot cells; sterile and pyrogen-free output of the generator.

The absence of European regulatory framework such as guidance documents, guidelines, decisions, and directives specific to PET radiopharmaceuticals made it difficult to conduct clinical trials and introduce new imaging agents into clinical routine. However, the situation has been constantly improving during recent years. There are currently a number of guidelines, non-binding and binding regulatory documents available and in preparation indicating that regulatory bodies and authorities recognize the need for regulations specific to PET radiopharmaceuticals. Solutions that may allow the clinical use of small scale preparation radiopharmaceuticals without obligation to apply for manufacturing authorization or clinical trial might become possible in the future [30-33]. European regulation which is currently in force (Directive 2001/83) [34] applies good manufacturing practice (GMP) to medicinal products intended for the market. It is not fully relevant to in-house produced and consumed PET radiopharmaceuticals, but it is the only way to ensure quality and safety. Traditional GMP may easily hinder any clinical development of PET radiopharmaceuticals and their availability for patients and thus it should be applied with common sense and practicality as well as rational and proportionate risk assessment. Another restricting legislative document is Directive 2001/20/EC which makes the PET radiopharmaceutical clinical trial application preparation challenging [35]. This document will be replaced with new Clinical Trial Regulation in the nearest future putting forward more rational requirements to radiopharmaceuticals [36], however currently Directive 2001/20/EC is in force.

Part B of the EANM "Guidelines on good radiopharmacy practice (GRPP)" presents small-scale "in house" production of radiopharmaceuticals that are not intended for industrial production, sale or distribution and provides general and specific requirements to radiopharmaceutical production in research and clinical trials as well as clinical routine care [37]. Most importantly for the present work, ^{68}Ga-related European Pharmacopoeia monographs [38, 39] were released reflecting the acceptance of obvious benefits of ^{68}Ga/PET-CT to the patient management [14]. Despite the appearance of some specific documentation and regulation

for PET radiopharmaceuticals there are still remaining challenges, e.g. the absence of Investigational Medicinal Product Dossier (IMPD) specific to PET radiopharmaceuticals. Currently the facilitation of the entry of novel radiopharmaceuticals into clinical practice still relies mostly on magisterial and officinal preparation/prescription and compassionate use under responsibility of the prescribing physician.

The aim of the present work was to develop, qualify, validate, document, and establish the production of a ^{68}Ga-labeled Affibody molecule ([^{68}Ga]Ga-ABY-025, **Figure 1**) in compliance with GMP and GRPP for clinical trials of patients affected by breast cancer. The development considered two independent productions for [^{68}Ga]Ga-ABY-025 imaging agents of variable peptide amount in order to investigate the impact of the peptide administered amount on the biodistribution, image contrast and lesion detection rate.

Materials and methods

Facilities, equipment, and materials

The aseptic production was conducted in a GMP grade A workstation (unidirectional laminar airflow workbench (LAFW)) situated in a cleanroom with GMP grade B air quality. The ^{68}Ge/^{68}Ga generator and block heater were placed in the LAFW. The temperature in the cavity of the block heater was controlled by a thermometer.

A high performance liquid chromatography system (LaChrom, Hitachi, VWR) consisting of an L-2130 pump, UV detector (L-2400), and a radiation flow detector (Bioscan) coupled in series was used for product quality control. Separation of the analytes was accomplished using an endcapped analytical column with stationary reversed phase (C-4; Vaydac-C4; 50×4.6 mm; particle size: 3 μm). The conditions were as follows: A=10 mM TFA; B=70% acetonitrile (MeCN), 30% H_2O, 10 mM TFA with UV-detection at 220 nm; linear gradient elution: 0-2 min at 25% B, 2-7 min at 25 to 100% B, 7-10 min at 100% B; flow rate was 1.0 ml/min. Data acquisition and handling were performed using the EZChrom Elite Software Package.

The GMP grade drug product, ABY-025, was provided by Affibody AB. The peptide ligand was produced recombinantly in *Escherichia coli* and subsequently conjugated chemically with the bifunctional chelator 1,4,7,10-tetraaza cyclododecane-1,4,7-tris-acetic acid-10-maleimidoethylacetamide (MMA-DOTA) at the C-terminal cysteine. The manufacturer followed the relevant GMP guidelines, i. e. the EU directive 2003/94/EC and ICH Q7 guideline for APIs. Safety pharmacology and toxicology studies in animals revealed no ABY-025 related toxicity. Certificates of compliance and certificates of analysis were obtained from the manufacturer. ABY-025 (Molecular weight: 7.6 kDa) was supplied in a sterile 10 ml capped glass vial containing a solution of 100 μg ABY-025 in 0.5 ml of 0.2 M sodium acetate (pH 5.3). The product was sterile, non-pyrogenic, and without bacteriostatic preservatives.

The purchased chemicals were used without further purification: HCl (ultrapure, Merck), Acetate buffer (pH 4.6, Sigma-Aldrich), Sterile water (Fresenius Kabi), Phosphate buffer (Apoteket AB), NaOH (10 M, Sigma-Aldrich), Ethanol (APL), Water (Fluka, TraceSelect), Trifluoroacetic acid (Merk, Darmstadt, Germany).

^{68}Ge/^{68}Ga generator qualification and validation

The starting material, ^{68}Ga ($t_{1/2}$=68 min, β^+=89%, and EC=11%) used for the manufacture of the radioactive agent was obtained from a ^{68}Ge/^{68}Ga-generator (1850 MBq, IGG100, Eckert & Ziegler Isotope products), manufactured under ISO certificates ISO 13485:2003 and ISO 9001:2000. The generator is a closed system employing a long-lived parent radionuclide ^{68}Ge ($t_{½}$=270.8 days) immobilized on inorganic stationary phase (titanium dioxide). The generator components are of medical grade, or made of materials approved for medical use. The core of the generator consists of a borosilicate glass tube with polyether ether ketone end plugs connected to inlet and outlet lines. A secondary inlet and outlet pass the outer casing providing possibility for the elution of the generator. The difference in chemistry of ^{68}Ge and ^{68}Ga and their interaction with the inorganic stationary phase allows their separation and extraction of the liberated ^{68}Ga by simple elution with 0.1 M hydrochloric acid. The hydrochloric acid molarity of 0.1 provides acidic environment of pH 1 preventing the growth of microbiological entities. Potential non-radioac-

Table 1. Summary of the validation parameters and the respective specifications defined in the Ph. Eur. monograph for ^{68}Ge/^{68}Ga generator eluate

Parameter	Acceptance criteria	Result*
Appearance	clear, colorless	clear, colorless
Radionuclidic identity, Half-life determination	62 min to 74 min	67.96 ± 0.01 min
Radionuclidic identity, gamma spectrum	511 + 1077 keV	511 + 1077 keV
Radionuclidic purity	> 99.9%	> 99.999%
^{68}Ge breakthrough	< 0.001%	0.000095%
Sterility test	sterile	sterile
Endotoxins test	< 175/V EU/ml	< 0.25 EU/ml
pH	< 2	1
Fe	< 10 μg/GBq	ND**
Zn	< 10 μg/GBq	0.035 μg/GBq**

*Mean ± SD of three measurements; **Specified by the manufacturer.

tive impurities are titanium from the column, zinc as decay product of ^{68}Ga, sodium and boron originating from glass housing. The metal ion content of the eluate was investigated using Inductive Coupled Plasma-Mass Spectrometry and was provided by the manufacturer (Eckert & Ziegler). The amount of detected metal impurities was less than defined limit in the European Pharmacopeia monograph [39]. The appearance of the ^{68}Ga eluate was clear and colorless.

For the preparation of the 0.1 M HCl eluent ultrapure hydrochloric acid (30%, 9.45 M, MERCK) was diluted with sterile water to yield 0.1 M HCl in a sterile flask. The procedure was performed in LAFW (class A). Sterile syringes were used to flush the generator with the eluent. The generator was eluted regularly and the ^{68}Ga radioactivity and volume readings were recorded in the register. The elution profile was determined by fractionating and measuring the ^{68}Ga radioactivity in each successive fraction of the eluate. The ^{68}Ga elution yield was expressed as the ratio between the obtained ^{68}Ga and the ^{68}Ge radioactivity (in equilibrium with the ^{68}Ga daughter nuclide) on the generator column at the time of elution. Aliquots (0.5 ml) of the generator eluate were counted immediately using an ionization chamber with NaI(Tl) scintillation detector.

According to the Ph Eur monographs the ^{68}Ge breakthrough should be calculated as a percentage of the eluted ^{68}Ga radioactivity. Aliquots of the generator eluates were counted using an ionization chamber with NaI(Tl) scintillation detector immediately after elution and in the well-type NaI(Tl) scintillation counter 48 h post elution in order to determine the ^{68}Ge breakthrough in the eluate. The ^{68}Ge contamination was expressed as % ^{68}Ge in the ^{68}Ga eluate (**Table 1**). During the production of the radiopharmaceutical the final product was purified in order to additionally assure radionuclidic purity. The ^{68}Ge breakthrough level was controlled periodically (once per month).

The identity and radionuclidic purity of ^{68}Ga was confirmed by: A) the determination of the half-life; B) the measurement of the radiation emitted (annihilation photons of 511 keV and characteristic gammas of 1077 keV); C) the measurement of gamma radiation emitted 48 h post elution when ^{68}Ga decayed to a level that permits the detection of possible impurities. For the determination of ^{68}Ga half-life ($t_{½}$) the generator was eluted with 0.1 M HCl (5 ml) and the eluate radioactivity was measured in an ionization chamber every 15 min for 6 hours. Radioactivity was plotted as a function of time with subsequent fit of an exponential function. The half-life was calculated using the decay constant (λ) value from the fit function (**Equation 1**) and (**Equation 2**). Alternatively, radioactivity readings were converted to their logarithms and plotted as a function of time.

$$A(t)=A(0)*\exp(-\lambda t) \quad (1)$$

$$t_{½} = \frac{\ln(2)}{\lambda} \qquad t_{½} = \frac{\ln(2)}{\lambda} = \frac{0.693}{0.0102} = 67.956 \quad (2)$$

Radioactivity measurements were carried out with an ultra-pure germanium detector (ORTEC, Oak Ridge, Tennessee) connected to an 8192 channel PC-based multichannel analyzer (The Nucleus, Oak Ridge, Tennessee). The detector was calibrated for energy and efficiency with a standard ^{152}Eu source (Amersham Pharmacia

Table 2. Summary of the product specifications and validation results for three consecutive validation productions of low peptide content [^{68}Ga]Ga-ABY-025 (ABYA)

Test	Acceptance criteria	ABYA12001	ABYA12002	ABYA12003
Radiochemical purity	> 91%; no unknown impurity corresponds to > 5%	96.8	97.3	95.3
pH	4-8.5	7.4	7.4	7.4
Radioactivity concentration	5-100 MBq/ml	40.0	55.4	54.0
Radioactivity	50-500 MBq	340	388	432
Volume	2-10 ml	8.5	7.940	7.520
Color	colorless	colorless	colorless	colorless
Specific radioactivity	1-100 MBq/nmol	54.8	33.7	37.2
Radionuclidic purity	> 99.9%	99.9999993	99.999999	99.9999975
^{68}Ge breakthrough	< 0.001%	0.0000007	0.000001	0.0000025
Amount of peptide	< 100 μg	46.8	86.6	82.1
Sterility test	sterile	sterile	sterile	sterile
Endotoxins test	< 3 EU/ml	< 0.25	< 0.25	< 0.25
Stability	RCP > 91% within 60 min	96.6	96.2	95.9

Biotech, Uppsala, Sweden). Accuracy of the calibration was within 1 keV. The first measurement was performed approximately 10 min post elution. The eluate was placed at 2 m distance from the detector to keep dead time below 10 %. The measurement of gamma radiation was repeated 24 hours post elution when ^{68}Ga decayed to a level that permitted the detection of possible long-lived impurities. Due to very low intensity of the radiation, the sample was placed directly to the casing of the detector, and 12 hour acquisition time was used.

The kinetics of ^{68}Ga accumulation was investigated by collecting generator eluate at predetermined time points and measuring the radioactivity in the ionization chamber. Generator elution efficiency was determined at the time of secular equilibrium as percentage of the eluted radioactivity of the radioactivity expected theoretically with decay correction for ^{68}Ge. Three consecutive eluates were collected on different days and sent to the microbiology laboratory for determination of the sterility and endotoxin amount.

Production of low peptide dose [^{68}Ga]Ga-ABY-025 (ABYA)

Production and quality control of the tracer was accomplished within one hour in the LAFW. A fractionation method was used for the labeling [40]. The first fraction of 1.5 ml was sent to the waste and the next 1.5 ml containing over 85% of the total radioactivity was collected into a sterile Eppendorf vial with pre-dispensed acetate buffer containing sodium hydroxide (250 μl). The pH was controlled with indicator paper to provide 4.6 ± 0.4. The required amount of the radioactivity was transferred into a vial with ABY-025, followed by addition of ethanol (200 μl) and heating for 15 min at 75°C. After the reaction completion the crude product was purified on an Oasis HLB SPE cartridge eluting the final product with 1 ml of 50% ethanol. The resulting product was diluted to 8 ml with sterile phosphate buffer for the formulation and the solution was passed through two sequentially connected 0.22 μm sterile filters into a sterile 10 ml capped glass bottle. The sterile filter integrity was controlled. A sample was taken for the determination of the identity, radiochemical purity, peptide concentration and pH. The total radioactivity of the product was then measured in an ionization chamber. The product vial was labeled with batch number, preparation date and time, calibration time, radioactivity at calibration time, radioactivity concentration at calibration time.

Three consecutive productions for the validation of the manufacturing process were performed. The quality control tests were validated using HPLC technique. The chemical purity, radiochemical purity and amount of the peptide were determined by HPLC. A sample of the product was kept for subsequent determination of ^{68}Ge content. The stability of the product at room temperature was monitored by UV-Radio-HPLC for 3 hours. The products were sent for the test of sterility and endotoxins. Tests performed on the finished product and specifications are summarized in **Table 2**.

Table 3. Summary of the product specifications and validation results for three consecutive validation productions of high peptide content [^{68}Ga]Ga-ABY-025 (ABYB)

Test	Acceptance criteria	ABYB12001	ABYB12002	ABYB12003
Radiochemical purity	> 91%; no unknown impurity corresponds to > 5%	96.5	96.2	97.3
pH	4-8.5	7.4	7.4	7.4
Radioactivity concentration	5-100 MBq/ml	54	66.6	75.8
Radioactivity	50-500 MBq	431.8	433.0	474.0
Volume	2-10 ml	8.500	7.070	7.570
Color	colorless	colorless	colorless	colorless
Specific radioactivity	0.1-100 MBq/nmol	8.2	10.4	10.8
Radionuclidic purity	> 99.9%	99.999998	99.9999975	99.9999974
^{68}Ge breakthrough	< 0.001%	0.000002	0.0000025	0.0000026
Amount of peptide	< 500 µg	398.6	385.4	398.7
Sterility test	sterile	sterile	sterile	sterile
Endotoxins test	< 3 EU/ml	< 0.25	< 0.25	< 0.25
Stability	RCP > 91% within 60 min	96.8	96.4	97.3

Production of high peptide dose [^{68}Ga]Ga-ABY-025 (ABYB)

After the ^{68}Ga-labeling reaction completion as described for ABYA, the resulting product was mixed with 400 µg of ABY-025 (in 2 ml of acetate buffer) and the total volume was adjusted to 8 ml with sterile phosphate buffer (5 ml) for the formulation. The final solution was passed through two sequentially connected 0.22 µm sterile filter into a sterile glass vial. The sterile filter integrity was controlled. A sample was taken for determination of the identity, radiochemical purity, peptide concentration and pH. The total radioactivity of the product was then measured in an ionization chamber.

Three consecutive productions for the validation of the manufacturing process were performed. The quality control tests were validated using HPLC technique. The chemical purity, radiochemical purity and amount of the peptide were determined by HPLC. A sample of the product was kept for subsequent determination of ^{68}Ge content. The stability of the product at room temperature was monitored by UV-Radio-HPLC for 3 hours. The products were sent for the test of sterility and endotoxins. Tests performed on the finished product and specifications are summarized in **Table 3**.

Quality control of [^{68}Ga]Ga-ABY-025

It is not feasible to determine the radionuclide identity and purity, endotoxin level and sterility as well as ^{68}Ge content in the final product prior to the patient administration due to the ^{68}Ga physical decay. These parameters were controlled as a part of production validation and were periodically tested according to the extemporaneous preparation approach.

As mentioned above, the chemical purity, radiochemical purity and amount of the peptide were determined by UV-Radio-HPLC. The recovery of radioactivity from the analytical column was investigated in order to confirm that no radioactive product or impurities were left on the column. Three replicate runs were performed collecting the HPLC effluent from the whole run. The HPLC column was replaced with a piece of tubing and the injection of the sample was repeated collecting the same volume as with the column attached. The radioactivity of all effluents (similar volume) was measured in a well-type NaI(Tl) scintillation counter corrected for dead-time and for radioactive decay. The radioactivity readings were decay and volume corrected. The tests were performed both for the product ([^{68}Ga]Ga-ABY-025) and free ^{68}Ga(III). Specificity, linearity, and precision as repeatability were validated for both UV- and Radio-detectors.

Regulation and permission

The study was approved by the Swedish Medical Products Agency (EudraCT 2012-005228-14; NCT01858116), the Regional Board of Medical Ethics, and the local radiation protection ethics committee.

Clinical studies

The patient demographic data, clinical study outline and protocols, PET examination proto-

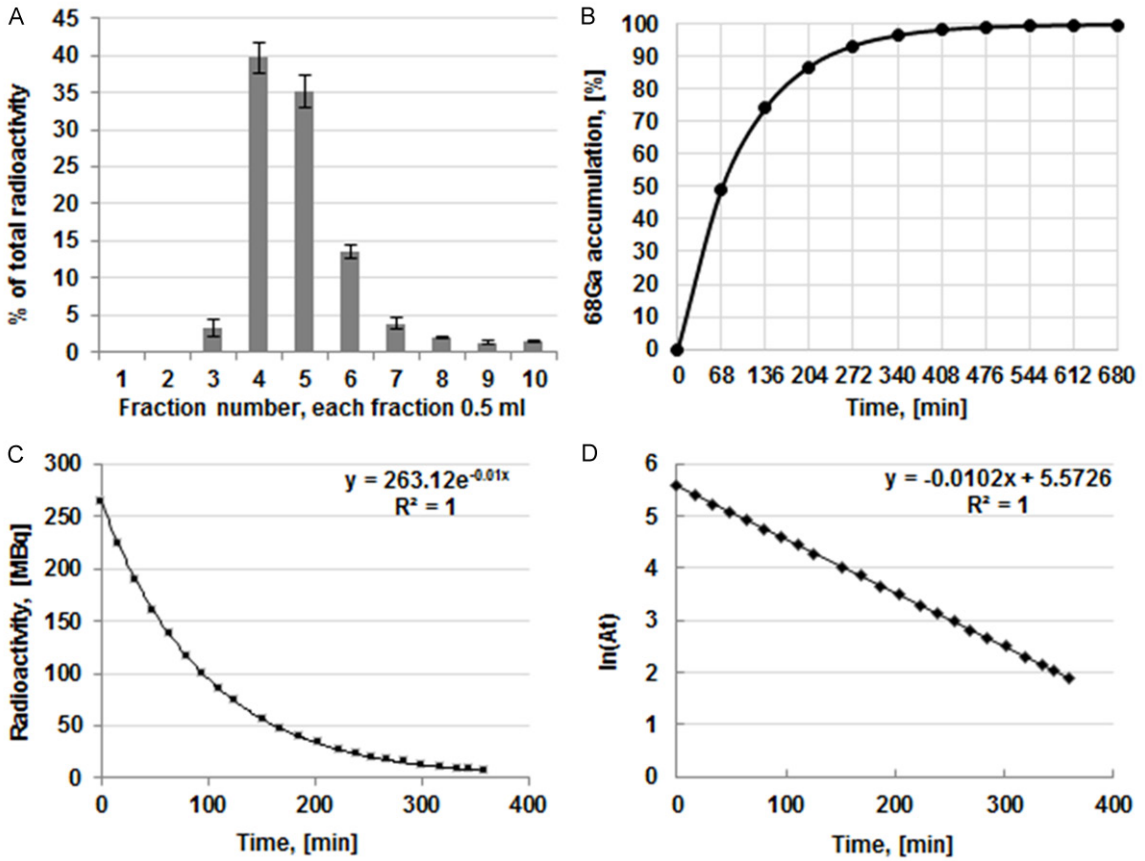

Figure 2. A. Elution profile of the ^{68}Ge/^{68}Ga generator where each fraction is 0.5 ml. Ten fractions were collected giving a total eluted volume of 5 ml; B. ^{68}Ga accumulation presented as percentage of the maximal possible radioactivity at secular equilibrium; C. Radioactivity of the ^{68}Ga eluate measured in the ionization chamber and plotted as a function of the time point of the measurement; D. Radioactivity readings converted to their logarithms and plotted as a function of time.

cols and data evaluation methods have been published elsewhere [41]. The patients received bolus intravenous administration. PET/CT study was performed twice with low and high peptide doses, respectively. In each occasion dynamic acquisition during 0-45 minutes was followed by static images after 1, 2 and 4 hours.

Results

Generator qualification and validation

The ^{68}Ge/^{68}Ga generator was validated according to the parameters defined in the Ph. Eur. monograph [38]. The respective results are summarized in **Table 1**. The kinetics of ^{68}Ga accumulation was investigated by collecting generator eluate at pre-determined time points and measuring the radioactivity in the ionization chamber. The top fraction of 1.5 ml contained 90 ± 5% of total eluate radioactivity (**Figure 2A**). The accumulation kinetics of ^{68}Ga corresponded to the theoretically expected results (**Figure 2B**). Generator elution efficiency was 75 ± 5% as determined at the time of secular equilibrium as percentage of the eluted radioactivity of the total radioactivity expected theoretically with decay correction for ^{68}Ge. The identity of ^{68}Ga was confirmed by the determination of the half-life from the periodical radioactivity measurements, function fitting (**Figure 2C**, **2D**), and calculation using λ value from the fit function (**Equation 1**) and (**Equation 2**). Alternatively, radioactivity readings were converted to their logarithms and plotted as a function of time. The λ value of 0.0102 corresponds to a half-life of 67.96 ± 0.05 min. Furthermore, the identity of ^{68}Ga and radionuclidic purity were confirmed by gamma ray spectrometry using an ultra-pure germanium detector connected to an 8192 channel PC-based multi-

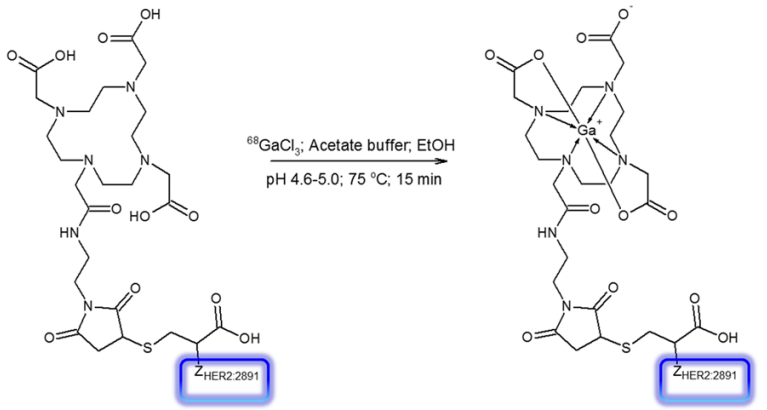

Figure 3. Schematic representation of [^{68}Ga]Ga-ABY-025 production. Success rate: 100% (N=6 + 34); non-decay corrected RCY: 61.3 ± 6.7; RCP: 99.1 ± 0.4; SRA (ABYA): 25.1 ± 4.6; SRA (ABYB): 3.8 ± 0.8.

channel analyzer. The analysis was performed directly after generator elution and 24 hours later. The only gamma photons detected from the decay of ^{68}Ge had energies of 511 keV and 1077 keV characteristic to ^{68}Ga being in secular equilibrium with ^{68}Ge and corresponding to annihilation photons and characteristic gammas of 1077 keV. No other gamma radiation signals were detected. The radionuclidic purity determined from the measurements of ^{68}Ga eluate radioactivity directly after and 48 hours post elution, when ^{68}Ga decayed to a level that permits the detection of possible impurities, resulted in > 99.999%. Sterility and bacterial endotoxin tests of the generator eluate showed no growths of aerobic or anaerobic bacteria and as low endotoxin level as ≤ 0.25 EU/ml.

Production of [^{68}Ga]Ga-ABY-025 with low and high peptide content

GMP-compliant, reliable and highly reproducible production (success rate: 100% (N=40); **Figure 3**) with control over the product peptide concentration and radioactivity was accomplished within one hour in the LAFW. The eluate fractionation method [40] was modified and used for the production. The parameters: time, temperature, radical scavenger, buffer concentration, pH, product purification step, product formulation, and sterile filtration were investigated and optimised. Although the Affibody molecules are known for their high thermal stability up to 90°C, in this particular case the highest radiochemical yield was obtained at 75°C. The concentration of acetate buffer was investigated in the range of 0.05-1 M with the highest radiochemical yield and robust production obtained at 0.1 M.

The crude product was purified on a solid phase extraction cartridge, formulated in phosphate buffer and sterile filtered into a sterile 10 ml capped glass bottle. Quality control was conducted using UV-Radio-HPLC. The manufacturing was a continuous process without isolation of the active substance. Final product purification excluded contamination with ^{68}Ge and provided radiochemical purity (RCP) of 99.1 ± 0.4%. Decay-corrected radiochemical yield was of 61.3 ± 6.7% with duration of the production of 45-60 min. The amount of the starting radioactivity used for the radiopharmaceutical manufacturing intended for the clinical examinations was 640 ± 130 MBq. Addition of EtOH as a radical scavenger improved RCP.

The radiopharmaceuticals were supplied in a solution of sterile phosphate buffer and ethanol (< 10%) in a total volume of 6-10 ml with pH of 4-8. The acceptance criterion for the radiochemical purity was higher than 91% and no unknown radioactive impurities with each contribution of more than 5%. The formulated product was passed through two 0.22 μm sterile filter disks coupled in series. The product was delivered in a sterile 10 ml capped glass bottle (Apoteket AB, Sweden) with a label providing the unique batch code and product information.

The final volume of the radiopharmaceutical solution intended for the bolus injection was kept similar (7.85 ± 0.57 ml; RSD=7.3%). The accurate determination of the injected radioactivity and peptide amount was accomplished by the administration of the whole preparation volume and measurement of the radioactivity in all syringes and containers before and after administration as well as peptide concentration determination by UV-HPLC.

Two production procedures were developed for the manufacture of the ^{68}Ga-labeled investigational medicinal product (IMP) with low and

Table 4. Tests and methods used for the quality control of each batch of [^{68}Ga]Ga-ABY-025

Test	Acceptance criteria	Method
Identity	The retention time corresponds to ABY-025	UV-HPLC
Radiochemical purity	Total labeled side-products < 9% and no unknown impurity corresponds to > 5%	Radio-RP-HPLC
Chemical purity and peptide concentration	Absence of new unknown peaks in the UV-trace. Determination of the peptide concentration.	UV-RP-HPLC
Radioactivity	50-500 MBq	Ionization chamber
Specific radioactivity	5-50 MBq/nmol	Calculated
pH	4-8	pH indicator strip
Volume, [ml]	2-10	Syringe
Color	Colorless	Visual inspection
Sterile filter integrity	Intact integrity	Manual

Table 5. Summary of the UV-Radio-HPLC quality control method validation tests and results

Description	Acceptance criteria	Result
Specificity-UV detector (Relative standard deviation)	≤ 5%	0.64 (N=8)
Linearity-UV detector Pearson correlation coefficient (R^2)	> 0.99%	0.9998
Precision-UV detector (Relative standard deviation)	≤ 5%	2.04 (N=6)
Specificity-Radio detector (Relative standard deviation)	≤ 5%	0.64 (N=8)
Column recovery (free ^{68}Ga)	≥ 95%	> 99
Column recovery ([^{68}Ga]Ga-ABY-025)	≥ 95%	> 98
Stability (during 3 h)	> 91%	> 94

high content of the peptide respectively consuming 1 and 5 vials of the drug product. They were considered and validated as two separate production processes. Three consecutive productions for the validation of the manufacturing process for both low and high dose agents were performed. The key parameters were determined and conformed to the predetermined specifications (**Tables 2** and **3**). As mentioned above, the process was robust and reproducible with 100% success rate with 6 validation and 34 delivery productions. The chemical purity, radiochemical purity and amount of the peptide were determined by UV-Radio-HPLC. The radionuclidic purity was determined by gamma spectroscopy as part of ^{68}Ge/^{68}Ga generator validation. Stability tests on the finished product were conducted measuring radiochemical purity by UV-Radio-HPLC during 3 hour storage at room temperature for three consecutive productions during the production validation. The radiochemical purity after 60 min storage at room temperature was over 95% for both ABYA and ABYB, i.e. higher than the acceptance criterion of 91%. A sample of the product was kept for subsequent determination of ^{68}Ge content and resulted in less than 10^{-5}%. Sterility and endotoxin level of the finished product were controlled in 3 production validation batches for each of the low and high dose tracers with approved results. Specific radioactivity determined by the ratio of the radioactivity measured in an ionization chamber and the total amount of ABY-025 determined by UV-HPLC was 25.1 ± 4.6 MBq/nmol and 3.8 ± 0.8 MBq/nmol for ABYA and ABYB, respectively. Tests performed on the finished product, specifications and results are summarized in **Tables 2** and **3**. Tests and methods used for the quality control are summarized in **Table 4**.

Quality control

The HPLC method for the determination of [^{68}Ga]Ga-ABY-025 radiochemical purity and concentration by UV and radio detection was validated with respect to specificity, linearity, repeatability, radioactivity recovery from the HPLC column as well as stability of the product at room temperature (**Table 5**). The calibration of the UV-signal was conducted in order to enable accurate determination of the peptide content in the final product (**Figure 4A**). The specificity was examined to ensure that it was the right molecule that was analyzed and that the tracer molecule was separated from major impurities in the batch. The analyte needs to be sufficiently retained on the column providing

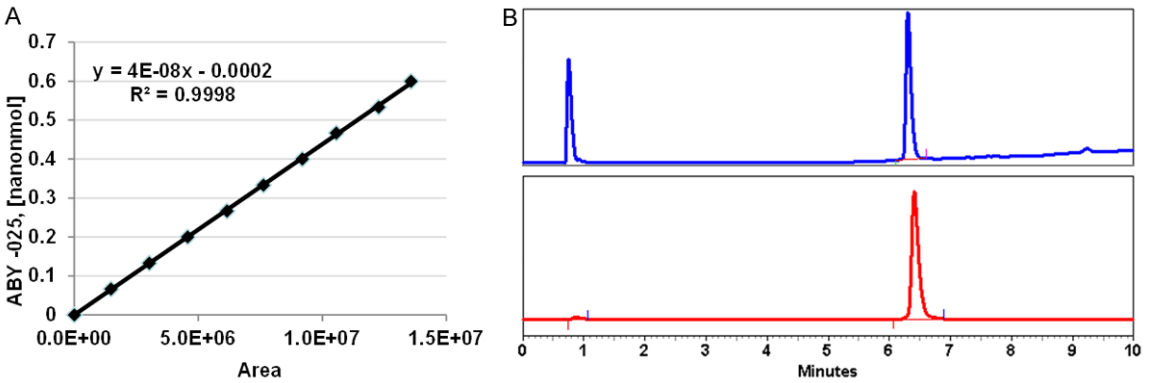

Figure 4. A. Validation of UV-HPLC quality method: UV calibration of ABY-025; B. Typical HPLC-chromatograms with UV absorption profile (upper panel) and radioactivity signal profile (lower panel) of [^{68}Ga]Ga-ABY-025.

distinct retention time. The ABY-025 drug product in acetate buffer solution was used for the validation and verification of its separation from impurities, buffers, and excipients. The detection limit was investigated in order to assure sufficient amount and absorption of the peptide for adequate UV signal intensity and respective identification of the radiosignal (**Figure 4B**). The repeatability was determined by injecting a standard solution six times. The concentration range of the solution was at the same concentration level as it would be expected during the product analysis. The recovery of radioactivity from the column was investigated in order to demonstrate that no radioactive product or impurities were left on the column and thus the output results reflected the actual radiochemical purity. Specificity, linearity, and precision as repeatability were validated for both UV- and Radio-detectors and met the acceptance criteria. A number of solid phase extraction cartridges (SPE) and HPLC columns were tested in order to assure high recovery. In particular, the recovery from the HPLC column was over 98% allowing adequate quality control. The recovery from SPE was above 80% providing acceptable and reproducible product yield.

Clinical studies

The results on the clinical examination protocol compliance and safety, uptake of [^{68}Ga]Ga-ABY-025 in normal tissues and lesions, test-retest examinations as well as correlation with biopsy tests have been reported elsewhere [41]. In particular, fraction of the administered radioactivity present in the normal liver was higher for ABYA (15%) as compared to ABYB (9%) one hour after injection. The similar washout rate resulted in higher liver ABYA SUVs even at later time points. The uptake difference was statistically significant.

Discussion

Regulatory aspects

The prime aims of GMP are the assurance of the product quality, subject safety, traceability as well as reliability and robustness of the manufacturing process. The quality and safety of the radiopharmaceutical must be adequate for the intended use. It should be mentioned that existing regulations allow some flexibility in the amount of data that needs to be submitted accompanying an Investigational New Drug (IND, USA) application or application for clinical trial authorization (CTA, EU), depending on the goals of an investigation, the specific human testing being proposed, and the expected risks. The core question is the relation between the rational and proportionate risk assessment and the patient benefit. For radiopharmaceuticals sub-pharmacological doses are administered and the potential risks to human subjects are less than that for a traditional phase I study with a therapeutic drug. The recognition of the microdosing concept (≤ 100 μg or ≤ 30 nanomoles for peptides/proteins) by EMEA and FDA [42-45] and introduction of the Exploratory Investigational New Drug (eIND) guidelines reduce the demand on toxicity studies and respective cost burden allowing validation requirements relevant to PET radiopharmaceuticals [46, 47]. This is possible because of the

Table 6. Binding and non-binding regulatory documents

European Pharmacopoeia monograph
Gallium (^{68}Ga) chloride solution for radiolabeling (2464)
Gallium (^{68}Ga) edotreotide injection (2482)
Radiopharmaceutical Preparations (0125)
Pharmaceutical Preparations (2619)
Extemporaneous preparation of radiopharmaceutical preparation (5.19.)
Parenteral preparations (0520)
Bacterial endotoxins (20614)
EudraLex Volume 4 (GMP)
Annex 1, Manufacture of sterile med products
Annex 3, Manufacture of radiopharmaceuticals
Annex 13, Investigational medicinal products
Guideline
Guidelines on good radiopharmacy practice, EANM
Guidelines on current good radiopharmacy practice (cGRPP) for the small scale preparation of radiopharmaceuticals, EANM
General International Conference on Harmonization (ICH) guidelines on validation of analytical procedures, EMA/CPMP/ICH/381/95
General International Conference on Harmonization (ICH) guidelines on quality risk management, EMA/CHMP/ICH/24235/2006
International Conference on Harmonization (ICH) guidance on nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals, EMA/CPMP/ICH/286/95
Regulation
European Medicines Agency Committee for Medicinal Products for Human Use. Guideline on the requirements for quality documentation concerning investigational medicinal products in clinical trials. EMA/CHMP/BWP/534898/2008
Directive
Directive 2001/20/EC
Directive 2001/83/EC
Other
Medical internal radiation dose format (MIRD)
International commission on radiological protection (ICRP)

high sensitivity of PET and consequently use of nonpharmacological radiopharmaceutical doses. It should also be mentioned that adverse reactions to PET radiopharmaceuticals are extremely rare and with no serious or life-threatening events [48]. In addition, Annex 3 of Eudralex on Manufacture of radiopharmaceuticals recognizes that there are acceptable methods, other than those described in this annex, which are capable of achieving the principles of quality assurance.

Still it is a challenging task to prepare an Investigational Medicinal Product Dossier (IMPD) since the existing dossier is considered for conventional pharmaceuticals and does not take into account peculiarities of a radiopharmaceutical, especially aimed for PET [49]. Thus the standard format of the dossier should be critically analysed and rationally adjusted to the needs and limitations of in-house small scale PET radiopharmaceutical production under radiochemistry environment within a frame of academic clinical research. The IMPD covers two main sections: the drug substance (the active pharmaceutical ingredient, API) and the drug product or finished product. The present work concerns the latter with emphasis on the chemical and pharmaceutical part. Recently Radiopharmacy and Drug development Committees of the EANM proposed a guideline for the preparation of an Investigational Medicinal Product Dossier (IMPD) for a radiopharmaceutical to be used in a clinical trial [50]. The guideline advises where the standard IMPD format is not suitable and allows easier understanding of the requirements. The approach used in this study resonates very strongly with the EANM suggestions. In particular, the “Drug substance” or API subsection of a standard IMPD is not applicable since the drug substance is not isolated and characterized during PET radiopharmaceutical manufacturing/production. Thus, only the final product can be defined, and “Investigational medicinal product under test” subsection is completed. The information regarding manufacturing of ABY-025 drug substance and drug product, which was the active ingredient of the final investigational medicinal product was presented separately from the radiopharmaceutical part and is not discussed here.

Our work on the radiopharmaceutical production development and validation as well as writing of the investigational medicinal product dossier was guided by a number of binding and non-binding documents listed in **Table 6**. In particular, the manufacturing and quality controls were set up in compliance with applicable regulations from current European Union-Good Manufacturing Practices (EU-GMP), current Good Radiopharmacy Practice (cGRPP) and European Pharmacopoeia (Ph. Eur.). The focus of the present work was on the quality section of the IMPD covering the aspects of Chemistry/Radiochemistry, Manufacturing and Control (CMC) which assures subject safety through the appropriate and tested production as well as defined quality, e.g. identity and purity of the investigational medicinal product. Potential risks of in-house manufacturing due to deviation from GMP requirements may be acceptable on a case-by-case basis on an individual and documented quality risk management (QRM) according to ICH Q9 guidelines [51]. Risk acceptance with regard to radiopharmaceuticals is influenced by small volume, immediate application, and short shelf-life.

Generator and labeling chemistry

The generator is involved in the radiopharmaceutical production process and thus should comply with the requirements that assure: product quality; safety of patients; traceability of the process; reliability and robustness of the performance. The qualification and validation of the performance of a chromatographic generator includes the investigation of its elution profile, elution efficiency, the extent of radionuclidic contamination of the eluate, contamination of the eluate with other metal cations and column material matrix. The primary documents to adhere are the European Pharmacopoeia monographs on Gallium (^{68}Ga) chloride solution and Gallium (^{68}Ga) edotreotide injection [38, 39]. Other helpful documents are listed in **Table 6**. The parameters of a ^{68}Ge/^{68}Ga generators that should be validated according to the specifications given in the Ph. Eur. monographs are summarized in **Table 1**. In addition, ^{68}Ga accumulation kinetics (**Figure 2B**) allows choice of the generator elution and tracer production frequency. Daily elution or elution 3-4 h prior to synthesis is recommended in order to keep the metal cation impurities at lower level [40].

One of the critical generator quality parameters is the breakthrough of the long-lived parent radionuclide, ^{68}Ge ($t_{½}$=270.8 d). Accumulation of ^{68}Ge on the column may occur if the generator is not eluted regularly. It is recommended to pre-elute the generator 4-24 hours prior to the intended production [40]. The limit of < 0.001% defined in the European Pharmacopeia monographs was based on a hypothetical assumption of total accumulation of ^{68}Ge(IV) radioactivity in the bone marrow with an infinite retention. However, ^{68}Ge(IV) biodistribution studies conducted in rats with extrapolation to the human organ and whole-body radiation dosimetry demonstrated that the limit defined in the monograph might be increased at least 100 times without compromising patient safety [52]. ^{68}Ge has no biological function or pharmacological activity and it demonstrated fast elimination without organ accumulation. It was also shown that ^{68}Ge(IV) was not chelated by DOTA-TOC and thus deposition of ^{68}Ge in the sites of DOTA-based imaging agents accumulation was also excluded [52]. This observation is valid in the case of [^{68}Ga]Ga-ABY-025 as well. However currently the monograph is in power and thus the product specification should comply with the defined limit and the ^{68}Ge breakthrough was monitored continuously once a month. ^{68}Ge breakthrough was monitored for both generator eluate and the product, and it was 3 to 5 orders lower than defined in the Ph. Eur. Sterility and apyrogenicity are crucial quality parameters that were controlled also for the generator eluate even though it is known that the acidic environment is not favorable to the microbiological growth and it was confirmed by loading a ^{68}Ge/^{68}Ga generator column intentionally with various bacteria and fungi in exhaustive amounts and following their survival during two weeks [53]. The risk of incidental microbial contamination was found to be very low. The acceptance, qualification and validation of the generator was summarized in a separate Standard Operating Procedure (SOP) and Master Batch Document (MBD) documents that were then used as appendixes in the IMPD.

Quality and characteristics of the generator eluate including eluate volume, ^{68}Ga radioactivity concentration, HCl eluent molarity, content of metal cationic impurities influence the efficiency of ^{68}Ga-labeling chemistry. Aspects such as pH, prevention of Ga(III) precipitation and colloid formation, radiolysis of vector molecules,

competition of metal cations in the labeling chemistry should be taken into consideration in the radiopharmaceutical production development. The generator eluate inevitably contains a number of metal cations that may compete and interfere with the ^{68}Ga-labeling reaction. Stable Zn(II) which is the product of ^{68}Ga decay accumulates continuously in the generator column [54] and is a strong competitor in complexation with DOTA-comprising agents. Metal cation and ^{68}Ge content can be reduced by regular elution and elution prior to the synthesis as well as by eluate and product purification [40]. The collection of the eluate top fraction decreases the eluate volume and increases ^{68}Ga concentration [40]. Consequently, it improves the radioactivity incorporation, decreases the reaction time and required ligand amount. However, it does not remove metal cation impurities and parent ^{68}Ge requiring, respectively high ligand amount to compensate for the metal cation content, and product purification from ^{68}Ge. The required relatively low SRA of ABYA and ABYB allowed sufficiently high amount of ABY-025 to be used for the labeling synthesis and thus a simpler fractionation method was employed.

Since the generator elution is conducted with hydrochloric acid eluent the labeling requires buffers for the correct pH adjustment necessary for the complexation. Moreover, weak buffer complexation capability is also essential in order to act as a stabilizing agent and prevent ^{68}Ga(III) precipitation and colloid formation. A number of buffering systems such as HEPES, acetate, succinate, formate, tris, glutamate were studied with HEPES, acetate and succinate buffers demonstrating better characteristics [40, 55]. In particular, HEPES and acetate buffers are both biocompatible, with no toxicity issue, providing relevant pH, and functioning as stabilizing agents. However, at lower ligand concentration, HEPES is more preferable. Nevertheless, from the regulatory point of view acetate has an advantage since HEPES is not approved for human use and thus purification and additional quality control (QC) analyses are required resulting in further time and resource consumption. Thus in the present study the acetate buffer was used. The concentration of the acetate buffer was optimized in order to avoid excess buffer that might compete with DOTA if used in excess [40] on one hand and on the other hand to assure right and stable pH as well as utilize the stabilizing function of the acetate and exclude ^{68}Ga precipitation and colloid formation [54].

The use of large radioactivity amount with increased ^{68}Ga concentration may elevate the risk of radiolysis caused by the formation of free radicals such as hydroxyl and superoxide radicals in aqueous solutions. Thus presence of radical scavengers such as ascorbic acid, gentisic acid, thiols, human serum albumin, or ethanol might be necessary. In the case of [^{68}Ga]Ga-ABY-025, additional radio-signals in front of the product signal were observed indicating possible radiolysis, especially considering presence of sensitive amino acid residues such as methionine and tryptophan. Addition of EtOH as a radical scavenger improved RCP. The advantages of using ethanol are its biocompatibility without toxicity or immunoreactivity issues and GMP compatibility. Most often it does not interfere with labeling reaction, and has no biological target binding capability. In addition, it can also aid the solubility of lipophilic precursors.

Two production procedures were developed for the manufacture of the radiopharmaceuticals with low and high content of the peptide respectively consuming 1 and 5 vials of the drug product. They were considered and validated as two separate production processes. In the production of ABYB 400 μg of the peptide was added during the product formulation in order to avoid peptide losses on the product purification step and assure reproducibility of the total peptide content in the final product. The choice was also justified by the high thermal stability, fast re-fording, and maintenance of biological activity of the peptide as well as sterility of the ABY-025 drug product solution. Non-clinical safety studies were conducted previously for ABY-025 encompassing tissue cross reactivity studies, standard safety pharmacology tests for central nervous system in rats, respiratory and cardiovascular effect in cynomolgus monkeys, repeated dose toxicity studies in rats and cynomolgus monkeys of two week duration as well as toxicokinetics. In the clinical trial using ^{111}In-labeled ABY-025 no adverse effects were observed [27]. The low peptide content ABYA falls under the microdosing category [56] and thus pharmacological and toxicological safety might be of no concern. The high peptide content ABYB (56 ± 2 nmol) exceeded the microdosing limit

of 30 nmol per injection however repeated dose toxicity study in rats and cynomolgus monkeys demonstrated safety upon administration of 800-fold higher dose.

The product identification was performed by UV-Radio-HPLC using as a reference standard the metal-free precursor, ABY-025, which demonstrated the same UV-HPLC retention time as for [^{68}Ga]Ga-ABY-025 with correction for the delay between UV and Radio detectors. It was used prior to the finished product quality control in order to: control the HPLC system performance; use it for a single point calibration; and avoid interference with the UV signal of the finished product and allow for the determination of the peptide content. The accurate determination of the injected peptide amount was enabled by UV-HPLC analysis according to a validated method and calibration plots as well as monitoring the radioactivity distribution among vials and syringes.

Two productions a day could be conducted four hours apart. The pre-buffering of the eluate prior to the addition to the reaction mixture allowed a possibility to use the radioactivity amount that would provide desired product SRA independent on the age of the generator. Despite the variable volume of the eluate used in the labeling procedure the production was highly reproducible and robust. The required amount of the injected peptide was assured by the use of the same quantity of the peptide in the production and the administration of the total product volume. The radiopharmaceuticals demonstrated high stability with RCP of > 95% during 3 hours at room temperature. However, in order to provide injected radioactivity amount as similar as possible for all patients the radiopharmaceutical was intended for immediate use. The variation in SRA depended also on the delay between the product delivery and patient administration (range: 15-45 min). However, it should be stressed that the biodistribution variation depends more on the amount of the injected peptide than SRA per se.

GMP-compliant, reliable and highly reproducible production with control over the product peptide concentration and radioactivity was accomplished within one hour. The amount of injected radioactivity (212 ± 46 MBq) was similar throughout the clinical study. This was achieved due to the moderation of the radioactivity amount used in the labeling synthesis by the generator eluate pre-buffering that allowed preparation of required specific radioactivity. Consequently, the specific radioactivity could be kept similar and it was approximately 6-fold higher for the low peptide content ABYA preparation. The final product purification excluded contamination with ^{68}Ge and provided radiochemical purity (RCP) of over 95%. The manual production procedure applied in this study corresponds to the basic steps of manufacturing under GMP environment where generator eluate was added to a reaction vessel, followed by the product purification, formulation, sterile filtration and release after the quality control. Thus the production was conducted with aseptic technique and terminal sterile filtration, and the final radiopharmaceutical did not contain original generator eluate solution. The qualification of the production method was followed by validation in order to establish documented evidence providing high degree of assurance that the process would consistently produce a product meeting its predetermined specifications and quality attributes.

The impact of administered peptide amount

One of the study aims was the investigation of the impact of the injected peptide amount on the biodistribution, detection rate and image contrast. Our earlier clinical experience demonstrated influence of the total amount of administered peptide agent ([^{68}Ga]Ga-DOTA-TOC) on the organ distribution [15]. Three sequential examinations with gradually increasing total amount of injected peptide were performed in the same patient affected by neuroendocrine tumor on the same day. As the peptide amount increased to 50 μg the uptake in the metastases improved while it was decreased in liver and spleen. Further elevation of the peptide amount up to 500 μg resulted in considerable uptake decrease in both lesions and healthy organs. This clinical example [15] demonstrates necessity of the optimization of the radiopharmaceutical SRA.

Three major methods of radiolabeling, namely using full generator eluate volume, eluate fractionation or eluate preconcentration could be employed [54]. Since the lower specific radioactivity tracer was expected to yield better imaging quality when using [^{68}Ga]Ga-ABY-025, the simpler fractionation method was selected. In the present study the patients received bolus intravenous administration of the low (ABYA, 78

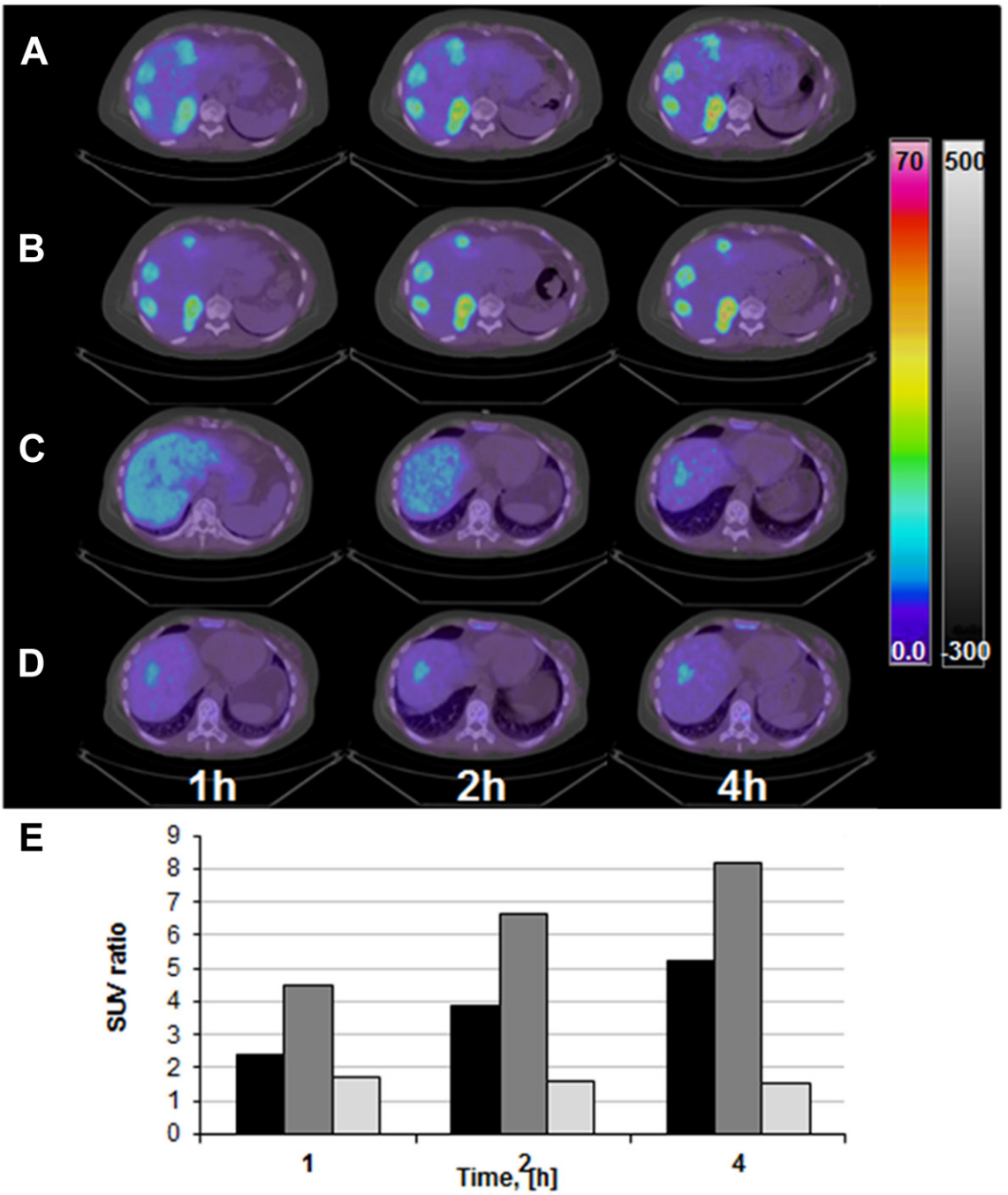

Figure 5. Transaxial fused PET-CT images of the liver with metastases after administration of low peptide dose (ABYA, A, C) and high peptide dose (ABYB, B, D) at 1, 2 and 4 hours after injection of the radiopharmaceuticals. (E) The bar graph demonstrates that the metastasis image contrast was lower for ABYA (■ tumor-to-background ratio) as compared to ABYB (■ tumor-to-background ratio) and gradually increased with time. The background uptake was constantly higher for the ABYA (■ background ratio of ABYA-to-ABYB). The images are normalized to the same color scale of SUV. Grey and colored scale bars correspond, respectively to CT and PET images.

± 8 μg) and high (ABYB; 427 ± 19 μg) peptide content radiopharmaceuticals on two occasions one week apart [41]. In each occasion dynamic acquisition during 0-45 minutes was followed by static images after 1, 2 and 4 hours (**Figure 5**). The influence of the specific radioactivity of the radiopharmaceutical on its tissue distribution, image contrast, and consequently lesion detection capability is illustrated in **Figure 5**. The examples given here clearly dem-

onstrate higher normal liver uptake in the case of ABYA of higher specific radioactivity and thus lower total amount of the administered peptide (**Figure 5A** and **5C**). In one case the detection rate and the lesion uptake were found similar during the examination with ABYA and ABYB (**Figure 5A** and **5B**). While in the other case the lesion could not be localized during the examination using ABYA at 1 and 2 hours after injection due to most probably rather high liver physiological uptake (**Figure 5C**). The lesion visualization became possible 4 hours later (**Figure 5C**). While the lower specific radioactivity of ABYB lead to the lower liver uptake with the lesion evident already at 1 hour after injection (**Figure 5D**). The image contrast increased with the time and radioactivity washout from the normal liver tissue for both ABYA and ABYB (**Figure 5A-D**). The ratio of the lesion-to-liver uptake and thus contrast was higher for the high peptide content ABYB. As mentioned, the image contrast increased with the time for both ABYA and ABYB, however the background uptake was constantly higher for ABYA (**Figure 5E**). The combination of high peptide content and whole-body imaging at 2 hours after injection appeared to be optimal for routine clinical use.

Conclusion

Two peptide dose radiopharmaceuticals were developed based on [^{68}Ga]Ga-ABY-025 in order to establish an optimal protocol for HER2-imaging in patients with metastatic breast cancer. GMP/GRPP-compliant, reliable and highly reproducible production with control over the product peptide concentration and injected peptide and radioactivity amount was accomplished within one hour. The specific radioactivity could be kept similar throughout the study due to the moderation of the radioactivity amount by the generator eluate pre-buffering. Final product purification excluded contamination with ^{68}Ge and provided radiochemical purity of over 95%. The study was approved by Swedish medical products agency. The intrapatient comparison of the two peptide doses allowed imaging optimization, and the combination of high peptide content radiopharmaceutical and whole-body imaging at 2 hours after injection appeared to be optimal for routine clinical use.

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Disclosure of conflict of interest

Anders Wennborg and Joachim Feldwisch are employees of Affibody AB.

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