Erik de Blois

Radiochemical Aspects of Receptor Scintigraphy:

labeling with radiometals, optimisation and radiochemical purity

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labeling with radiometals, optimisation and radiochemical purity

Radiochemische aspecten van receptor scintigrafie:
Labeling met radiometalen, optimalisatie
en radiochemische zuiverheid

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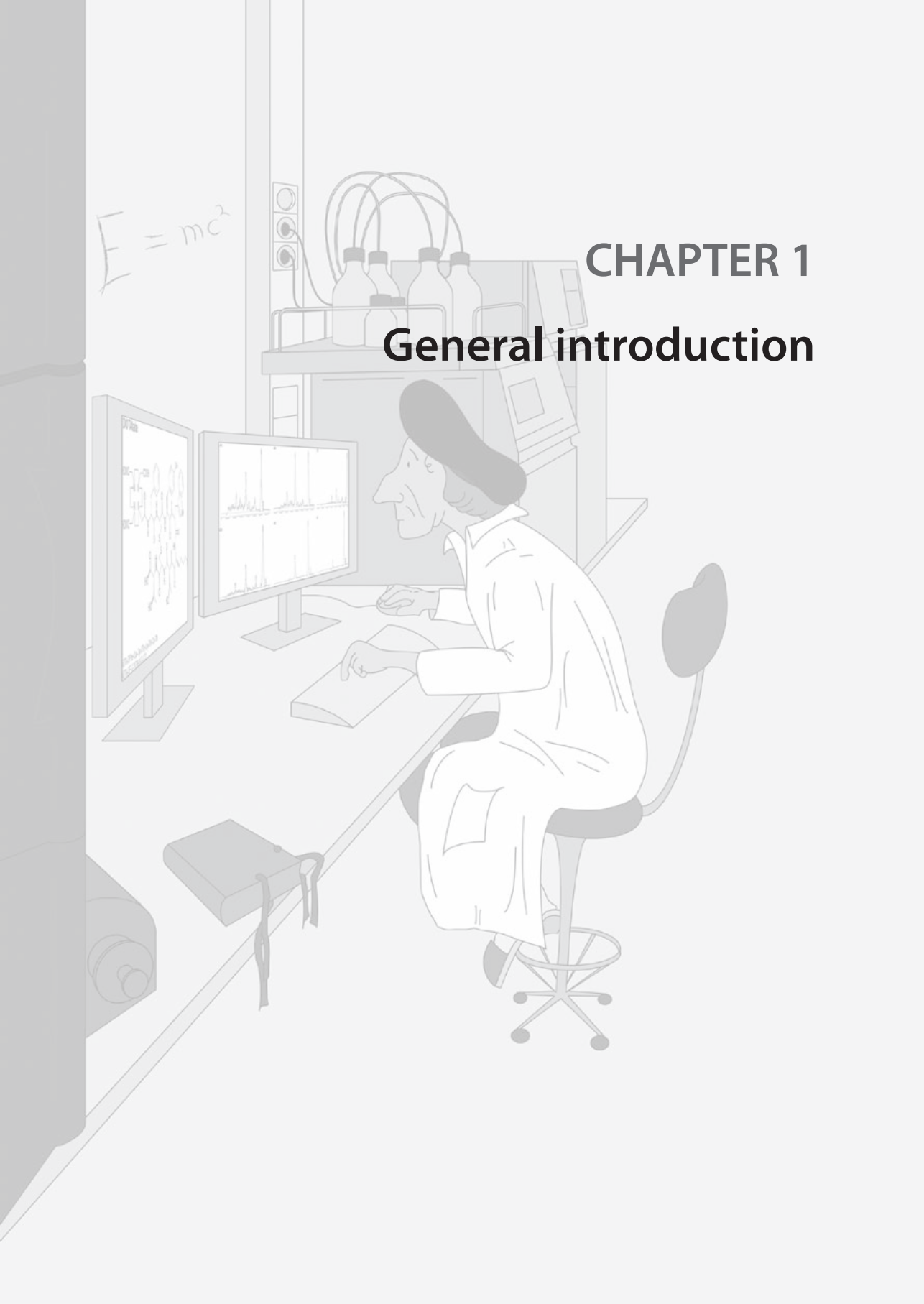

CHAPTER 1

General introduction

General aspects of peptide receptor targeting

Radiolabeled peptides have shown to be an important class of radiopharmaceuticals for imaging and therapy of malignancies over-expressing receptors of regulatory peptides. These peptides have high affinity and specificity for their receptors. The majority of these receptors are present at different levels in different tissues and tumors. This thesis focuses on the application of regulatory peptides radiolabeled with ^{68}Ga , ^{111}In or ^{177}Lu. Due attention is given to the current status of research, limitations and future perspectives of the application of these radiolabeled peptides for imaging and therapy. Limitations and potential future directions are also considered **[1].**

It has been reported that Peptide Receptor Scintigraphy (PRS) with radiolabeled peptides has shown to be a sensitive and specific technique, for instance to demonstrate the presence of somatostatin (SS) receptors on various tumors *in vivo* **[2, 3]**. With this technique, SS receptor-positive primary tumors and metastases are visualised. As soon as the success of peptide receptor imaging for tumor visualisation became clear, the next logical step was to label these peptides with radionuclides emitting α- or β-particles, including Auger or conversion electrons, and to perform Peptide Receptor Radionuclide Therapy (PRRT) **[1]**.

Receptor-mediated binding and uptake of peptides

Due to their relatively small size, peptides have favourable pharmacokinetics and are cleared rapidly from the circulation, with rapid excretion of the intact peptides or metabolites in faeces and/or urine **[4, 5]**. This frequently results in a high level of accumulation of these products in their target and in high target to non-target ratios. After binding of the ligand to its receptor, the cell reacts with a physiological response, for instance the secretion of bioactive peptides (see Table **1**).

The receptors of regulatory peptides are normally present at low levels of 10^{-9} to 10^{-13} mol/g tissue **[6-8]**. Such a range of receptor densities was also determined in SS receptor-positive tumors in patients **[8]**.

Table 1. Regulatory peptides with concordant pharmacological responses.

Regulatory peptide	Pharmacological response
Bombesin (BN)	smooth muscle contraction, gut hormone release
Somatostatin (SS)	stimulation of inhibition of secretion

Note that all peptides have stimulatory characteristics, however, SS stimulates the inhibition of secretion, thus, eventually SS has inhibitory effects

This implies that the sensitivity of detection of SS receptor-positive tumors by PRS might be improved by administration of an optimised dose of radioligand (nmol). Improvement here means, high tumor uptake and low tissue background to have an optimal tumor to background ratio. However, optimized dose encounter all kind of radiochemical limitations such as high specific activity, metal ion impurities and radiolysis. The next paragraph of the introduction are focussing on these radiochemical items **[1]**.

Maximum achievable specific activity

To optimize the dose of a radioligand (nmol) a high specific activity (SA) is often needed for PRS or PRRT with peptides that can only be administered in small quantities. In that case high SA is the only way to image or give a therapeutic dose to the tumor. High SA as stated here is expressed as amount of activity (GBq) coupled to a minimal amount of peptide mass (nmoles). Maximum SA is related to the half-life of radionuclide and possible influence of competition of other ions (see Table **2**).
Thus, given the fact that a certain amount of activity is necessary for detection, even low amounts (in mass) of ^{111}In-DOTA/DTPA-peptides can be administered. DOTA-conjugated peptides, such as the stabilised SS-analogs DOTA-tate and DOTATOC were developed for PRRT, labeled with ^{177}Lu. In order to be successful for PRRT, high SA's are required **[1]**.

Table 2. Physical characteristics of the radionuclides mentioned in this study **[1]**

	^{68}Ga	^{111}In	^{177}Lu
Production method	Generator	Cyclotron	Reactor
		(p,2n)	(n, gamma)
Target	^{68}Ge	^{112}Cd	^{176}Lu
Decay product	^{68}Zn	^{111}Cd	^{177}Hf
t½ [days]	4.7x10^{-2}	2.83	6.71
nmoles per GBq	0.01	0.58	1.39
Maximal specific activity [GBq.nmol^{-1}]			
Theory[a]	102	1.72	0.72
Practice	>1.0	0.82	0.12[b],0.42[c]

a: Since 1 nmol DTPA or DOTA can incorporate 1 nmol nuclide in theory, this number indicates the maximal theoretical specific activity of the radiolabeled DTPA- or DOTA-peptides
b: data from (n,gamma) reactor produced ^{177}Lu from enriched ^{176}Lu **[9]**,
c: ^{177}Lu reactor produced via (n,gamma) from enriched ^{176}Yb**[62]**

There are also a number of biological factors dictating the need for a high specific activity, some examples are:

- The affinity of the ligand for its receptor, but also the amount of available receptors limit the amount of ligand or radioligand that can be administered for *in vivo* use. **[4]**.
- Endocytotic mechanisms that affect the cellular internalisation of peptides may become desensitised at high peptide concentrations **[10]**, which results in lower uptake of activity into target tissue.

Unfortunately, the need for high specific activity is often compromised by conflicting radiochemical parameters that determine reaction rates and yields, i.e. the rate of formation of the metal-DOTA complexes increases with pH, but on the other hand the solubility of In^{3+}, Y^{3+} and Lu^{3+} decreases when pH is increased **[11]**. Moreover, reaction kinetics differ for each radionuclide and reactions can be hampered by contaminants of ions, including ions from target material and decay products **[9]**. This subject will be further described below in **Influence of metal ions on DOTA-labeling.**

Radiolabeling and radiochemical purity

Radiolabeling, handling and quality control of radiolabeled regulatory peptides are performed differently throughout the world, e.g. the addition of quenchers to prevent radiolysis are variable.

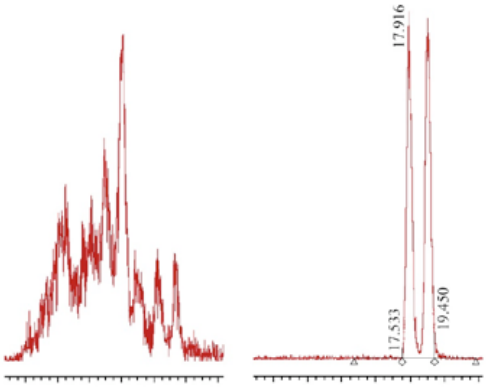

Figure 1. HPLC-chromatograms of OctreoScan with and without quenchers 24h after radiolabeling. The two major peaks (17.9-19.2 min) in the HPLC-chromatograms were caused by stereo isomers of the ^{111}In-labeled DTPA **[12]**.

There is a lack of fundamental knowledge regarding the destruction of radiopeptides in aqueous solution due to radiolysis. Radiolysis is here defined as the degradation of radiopeptides in aqueous solution caused by radiation and free radicals generated by radiolysis of water molecules **[13-17]**.
Comparison of radiolabeling and quantification of radiochemical purity (RCP) between different High-performance Liquid Chromatography systems (HPLC) and between laboratories is not standardized. Moreover, since there are no criteria or guidelines to qualify HPLC-quality controls, we present reproducible parameters for comparison of RCP between different HPLC-systems and laboratories in this thesis.
We also present an overview from literature and data from our studies how to reduce radiolysis by the addition of different quenchers and thus how to maintain RCP of radiolabeled peptides, especially focused on regulatory peptides **[18]**.

Dosimetry can provide an indication of the amount and nature of radiolysis to radiopeptides. Micro- and macrodosimetry models have been used for the direct and indirect damage pathway in water **[19]**. Damage to the peptides by low LET (low energy transfer) radiation is well described by the average energy absorption in the reaction vial, i.e. by macrodosimetry **[19]**. Radiolysis of radiopeptides depends on several parameters such as concentration of activity and radionuclide, type of radionuclide/radiation, temperature and the concentration of the peptide analog **[16]**. All these factors influence radiolysis and thus %RCP. Choice of quenchers is therefore important to protect radiolabeled peptides during labeling and maintain RCP after labeling procedure. The RCP was measured by HPLC, in an attempt to find a relation between radiation dose and radiolysis of RCP. A spherical geometry dosimetry model **[20]** was used to derive the absorbed dose rates obtained in vials being used in the measurements.

The complete emission spectrum of the specific nuclides (^{111}In and ^{177}Lu) were taken into account. The absorbed fractions of energy and the total absorbed dose factors S were calculated for spheres with various volumes of tissue (with a density 1 g/cm^3). A macroscopic dosimetry model was developed to calculate the dose rates in vials being used for radiolabeling and to predict RCP of radiopeptides in the absence of quenchers.

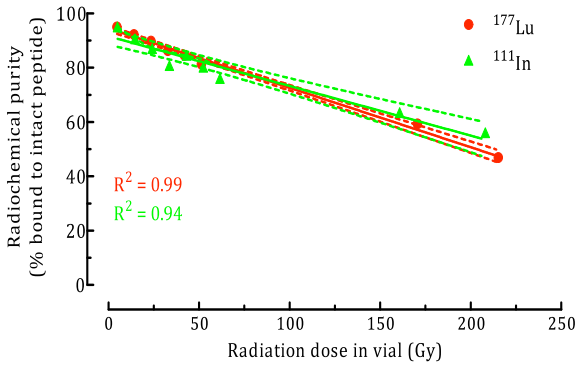

Figure 2. Correlation of the Monte Carlo calculation model in Gy vs RCP in practice. Two nmoles DOTA-minigastin (MG11) were labeled with ^{111}In (110 MBq) or ^{177}Lu (23 MBq), respectively. 24h corresponding to 200 Gy in the reaction vial. Moreover, if radiolabeling performed in the absence of quenchers radiolytic effects can be anticipated **[18]**.

A linear correlation between RCP and dose (Gy) was found (see Fig. **2**). This provides the ability to predict RCP of radiopeptides, according to calculations from the Monte Carlo model. This model proves the influence of radiolysis of radiopeptides and high activity in small volumes and thus the necessity of the addition of quenchers.

Quality control of radiolabeled peptides

Incorporation of the radiometal can be determined by ITLC-SG (Instant Thin Layer Chromatography Silica Gel), while RCP is frequently used to express the portion of radioligand that is present in the desired chemical form **[21]**. RCP, as mentioned above cannot be determined with ITLC-SG, but requires techniques such as HPLC. RCP measurement of desired (=intact) and non-intact forms of radiolabeled DTPA- and DOTA-peptides requires chromatographic separation of these forms. Typically RCP of radiolabeled peptide analogs measured by HPLC is expressed as % of radiodetected peak area (e.g. μV.sec^{-1}) of the intact radiolabeled peptide *vs.* all other radio peaks measured during the same HPLC-analyses.

Since tumor uptake of these peptides is largely dependent on the receptor binding radiolytic decomposition of the radiopepide may lead to the decreased therapeutic and diagnostic efficacy. Therefore, quenchers are frequently added. Several quenchers are described in literature for protection against radiolysis **[22-32]**. Since radiolysed radiopeptides often differ in charge and shape from the intact peptide, radiolysis can be quantified by HPLC **[18]**.

There are many reports on the determination of RCP by HPLC, including accuracy, linearity, precision, repeatability, detection limit **[21, 33, 34]**. With differences in eluens, gradient flow, column type and length there might be an increase variation in finding impurities, and thus variation in RCP. For comparison of RCP between different HPLC-systems and laboratories we therefore suggest standardisation of these HPLC-settings. Standardisation of separation method includes HPLC-eluens, gradient or isocratic, flow, column type and length, detection and interpretation of chromatograms. Therefore, in our opinion, RCP are actually expressed in percentages of arbitrary units. In this thesis we would like to propose minimal requirement for RCP by HPLC and radiodetection for radiolabeled DTPA or DOTA-peptides. These requirements are important with the aim to standardize RCP measurements for reliable comparisons of RCP quantifications between different systems/laboratories **[18]**.

Influence of metal ions on DOTA-labeling

The presence of trace metals could have severe influence on the incorporation since these metal ions compete with the radionuclide for the DOTA chelator. The effect of the addition of unlabeled (cold) Y, In and Lu to the ^{177}Lu was investigated as described by Breeman et al 2003 **[9]**. As expected, the dilution of the SA of the radionuclide decreased its incorporation in the DOTA-chelator in a concentration-dependent manner. The addition of nuclides such as Hf, Zr and Sr had no effect on the % incorporation of the radionuclides, indicating that these nuclides are not competitors under these reaction conditions. In contrast, addition of nuclides such as Fe, Cd, Cu, Zn clearly (Fig. **4**) showed that they are strong competitors for the incorporation of the radionuclide (^{177}Lu and ^{111}In) in the DOTA-chelator.
The radiolabeling of DOTA-peptides with ^{111}In is presented by Breeman et al 2007 (also part of this introduction **[1]**). These results indicate that In^{3+} and Cd^{2+} have similar reaction kinetics under these reaction conditions. And, although the stability constants of DOTA for In^{3+} and Cd^{2+} differ considerably, 23.9 and 20.9, respectively, at pH 7, the described incorporations and competitions are determined by kinetics (Fig. **3**).

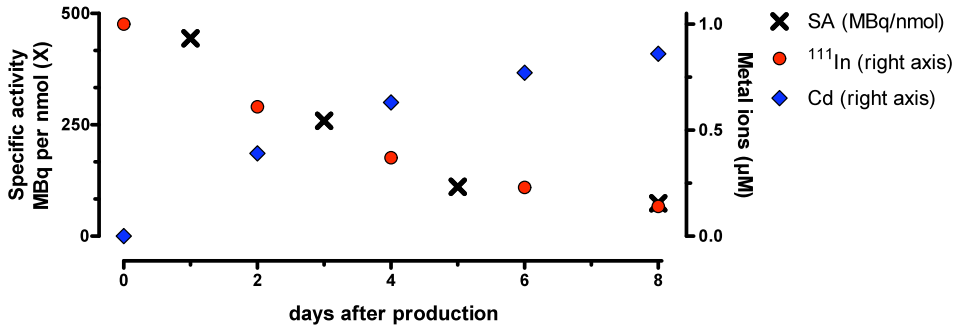

Figure 3. Maximal achievable SA of ^{111}In-DOTA-peptide in daily practice, expressed as function of time post production of ^{111}In. Decrease of SA is mainly influenced by the presence and ingrowth of Cd.

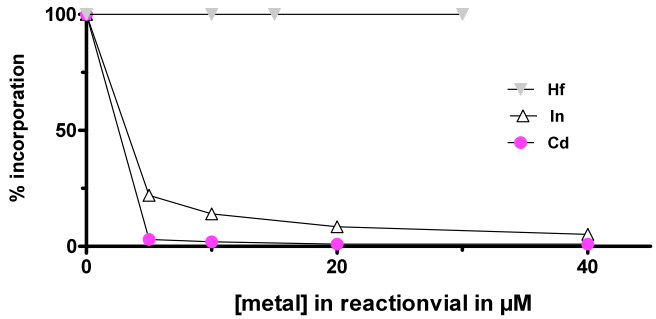

Figure 4. Effects of contaminants on the incorporation of ^{111}In in a DOTA-peptide by the controlled addition of non-radioactive ions of In^{3+} and Cd^{2+}, measured as described earlier **[9]**. This illustrates that the ever present Cd^{2+}-ions compete with In^{3+}-ions for the incorporation in DOTA, while the addition of Hf^{4+} ions (hafnium, the decay product of ^{177}Lu) has no effect on the % incorporation of ^{111}In and indicates that Hf^{4+} is no competitor for $^{111}In^{3+}$**[1].**

In this thesis optimization of ^{68}Ga chemistry is described as well. As described above for ^{111}In and ^{177}Lu, metal ions influence radiolabeling. Metal ions introduced by i.e. eluting the ^{68}Ge, ^{68}Ga generator are also influencing radiolabeling.

^{68}Ge/^{68}Ga generator's

^{68}Ga represents one of the very early radionuclides applied to Positron Emission Tomography (PET) imaging at a time when even the wording PET itself was not established, long time before the usage of e.g. fluorine-18. This because of the availability of ^{68}Ga via the first ^{68}Ge/^{68}Ga generators **[35-37]**.
Interest and research in ^{68}Ge and ^{68}Ga (Fig. **5**) started in the early 1950s, initially as a spin off from the discovery of ^{68}Ge in fission products **[38, 39].**

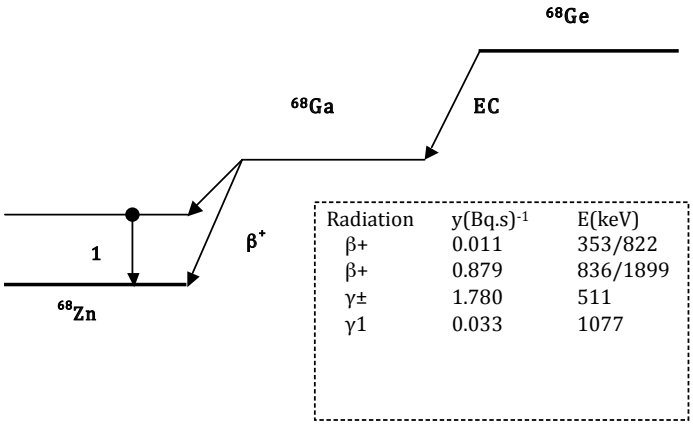

Figure 5. Decay scheme of ^{68}Ge (Half-life 270 days) to ^{68}Ga (68 minutes) to stable ^{68}Zn, releasing different types of radiation.

Most of ^{68}Ge for ^{68}Ge/^{68}Ga generators is now a cyclotron-produced radio-metal **[36, 40, 41]**. The ^{68}Ge/^{68}Ga generator has been under study since the 1970s, and gallium chemistry has been extensively reviewed **[11, 42-46]**. The ^{68}Ga-eluate requires time-consuming (i.e. solvent extraction, ion exchange, pyrolysis and/or evaporation) and/or tedious handling (^{68}Ga is also a 1.8-MeV β^+-emitter, 88% abundance) prior to radiolabeling **[46-49]**. A major advantage of the ^{68}Ge/^{68}Ga generator is its continuous source of ^{68}Ga, independent from an on-site cyclotron. A revival started in the 1970s, when several other ^{68}Ge/^{68}Ga generator systems were developed that resulted in reliable sources of the positron emitter ^{68}Ga. Thanks to the pioneering achievement of radiochemists in Obninsk, Russia, a new type of ^{68}Ge/^{68}Ga generators became commercially available in the first years of the 21st century **[50]**. Already 68 min after the former elution 50% of the equilibrium value is reached, while after 4h this is >90%. Generator eluates based on hydrochloric acid provided cationic ^{68}Ga instead of inert ^{68}Ga-complexes, opening new pathways of Me^{3+} based radiopharmaceutical chemistry. Besides the better quality of the ^{68}Ga-scans also patient logistics are improved as well as and a lower patient dose in compassion with ^{111}In-Octreoscan (0.023 mGy/MBq for ^{68}Ga **[51]** and 0.19 mGy/MBq for ^{111}In **[52]**).

Trace metals ions in ^{68}Ga chemistry

Since ^{68}Ga is generator based and eluted with hydrochloric acid, the ^{68}Ga eluate also contains Zn^{2+} formed by ^{68}Ga decay. There are also other metal cations present that originate from the matrix material, or are present in the eluent hydrochloric acid in the first place. For example, the total amount of metal contaminants (Ga, Ge, Zn, Ti, Sn, Fe, Al and Cu) in the eluate of a SnO_2-based generator was reported to be <10 ppm (<3 ppm Zn^{2+}; <1 ppm for each of the other ions)**[53]**. In fractionated eluate from a generator with a TiO_2 matrix, concentrations of $Fe^{2+/3+}$ and Zn^{2+} up to 50 nM were found **[50]**. The influence of metal ions in ^{68}Ge/^{68}Ga generator eluates on ^{68}Ga labeling has always been considered, and efforts have been made to remove these contaminants, together with the long-lived radiochemical impurity ^{68}Ge ($t_{1/2}$=270 days). Multiple purification methods and labeling of ^{68}Ga are described **[46, 50, 53, 54]**. Muller *et al* reported a NaCl based purification method removing impurities like metal ions including long-lived radiochemical impurity ^{68}Ge **[55, 56]**.

Influence of metal ion contaminants on ^{68}Ga labeling has been assessed systematically. A study by Velikyan et al investigated how $^{68}Ga^{3+}$ incorporation of DOTATOC is affected by the presence of Fe^{3+}, Al^{3+} and In^{3+}**[57]**. Simecek *et al* and Oehlke *et al* **[58, 59]** studied the influence of the most relevant metal ions contained in generator (Al, Cu and Zn) eluates on the $^{68}Ga^{3+}$ incorporation in to important macrocyclic chelators including DOTA. The presence of Al^{3+} in the ^{68}Ga eluate had no influence on labeling of all investigated chelators at concentrations below 30 mM final concentration. Since such high Al^{3+} concentrations are very unlikely to ever occur in generator eluates, Al^{3+} contamination is considered irrelevant. Fe^{3+} can be regarded as a particularly problematic contaminant because its physical properties. The presence of 10 mM of Fe^{3+} had little effect on the ^{68}Ga-labeling yield for phosphinates (>90%), whereas that of the carboxylates was decreased almost to a negligible level. The influence of Cu^{2+} on ^{68}Ga labeling of individual chelators appears to be quite similar to that of Zn^{2+}. Cu^{2+} exerts its effect on $^{68}Ga^{3+}$ incorporation at concentrations approximately ten times lower than that of Zn^{2+}, which is why Cu^{2+} deserves more attention than Zn^{2+} when considering the chemical purity of generator eluates.

Oehlke *et al* **[58]** mentioned also that ^{68}Ga-DOTA-TATE transmetalates with Cu^{2+} at 95°C.
Overall it seems that Cu^{2+} is the most critical contaminant for ^{68}Ga, affecting labeling to a greater extent than Zn^{2+}. This is important as Cu^{2+} has been occasionally detected in generator eluates **[53, 60, 61]**. Furthermore, although its influence on labeling is less pronounced, Zn^{2+} is of significance. In the generator, it is accumulated in the course of ^{68}Ga decay, and a critical Zn^{2+} level will eventually be reached when no elution is performed. However, this effect should not be overestimated; after elution of any generator, it takes approximately one week to reach a Zn/Ga molar ratio of 100, and even after a week, not more than a total amount of 1 nmol of Zn^{2+} is formed in a 1 GBq generator.

AIM AND OUTLINE OF THIS THESIS

In the introduction of this thesis an overview is given of several options how optimize radiolabeling including quality control of radiolabeled peptides. Furthermore, related (labeling)limitations, all performed to improve receptor scintigraphy (PRS) and receptor radionuclide therapy (PRRT) are described. One of the options is to improve labeling efficacy. In this thesis somatostatin and bombesin analogs used as peptide models. To increase specific activity, radiochemical condition must be improved and protection of radiolabeled peptide analogs for targeting tumor specific receptors as well.

Therefore the aims of thesis are:

I: Optimize and compare purification methods used for ^{68}Ga peptide chemistry and increased labeling yields and specific activity.

II: To accurately measure and monitor radiochemical purity of radiolabeled peptides by UPLC/HPLC. Furthermore compare and determine effectiveness of quenchers to maintain high radiochemical purity.

In short, make ^{68}Ga-, ^{177}Lu- and ^{111}In-labeled peptide more applicable for (pre-)clinical application.

Part I of this thesis: Investigation of ^{68}Ga labeling chemistry

PET imaging is of growing interest for PRS. Therefore the field of Nuclear Medicine was more focussed on improvement of ^{68}Ga chemistry and comparison of ^{68}Ga labeling procedures to increase their (pre-)clinical applicability. A major advantage of a ^{68}Ge/^{68}Ga generator is its continuous source of ^{68}Ga, independent from an on-site cyclotron. In this thesis a comparison was made of different labeling and purification techniques to increase i.e. ^{68}Ga yields. Comparison and improvement of purification techniques was made to decrease total synthesis time to a minimum (<30 min) including quality control **(chapter 2 and 3)**. The possibility to decrease waste stream of ^{68}Ga chemistry was also investigated. Liquid waste after ^{68}Ga-peptide synthesis contain small amounts of ^{68}Ge and must be collected and stored as long lived radioactive liquid waste, therefore we developed a purification method to decrease the waste stream to a minimum by solidification **(chapter 4)**.

Part II of this thesis: Investigation of radiolysis and measurement of radiochemical purity

In this part of the thesis attention is given to current status research, limitations and future perspectives of the application of radiolabeled peptides for PRS and PRRT **(chapter 5)**. Elements of basic science and preclinical and clinical aspects in general were investigated. Since only intact radiolabeled peptide has high affinity to its receptor, it is important to protect radiolabeled peptide for radiolysis and to inject it only with high RCP. Therefore investigation was performed how to protect radiolabeled peptides by using different quenchers and combinations of quenchers to maintain RCP **(chapter 6 and 7)**.
The focus for improvement of bombesin-based (BN) imaging has predominantly relied on development of new BN-based analogs with enhanced affinity for the gastrin releasing peptide receptor(GRPR). In this thesis we investigated the impact of various experimental factors in order to further improve PRS. Therefore we compared pharmacokinetics by adjusting experimental conditions as injected peptide mass, co-injection of peptide and way of purification of radiolabeled peptide **(chapter 8)**.

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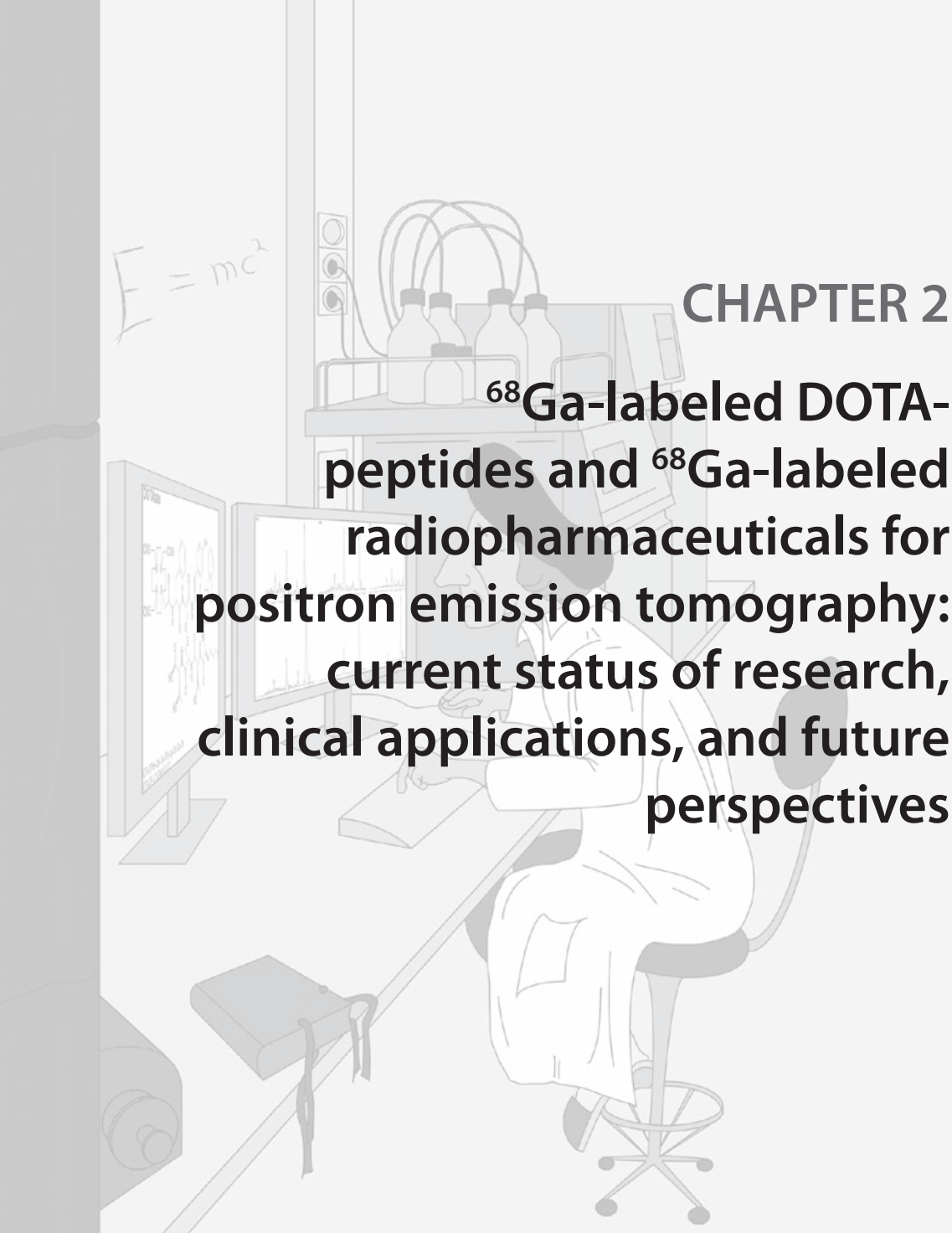
PART I

Investigation of ^{68}Ga labeling chemistry



CHAPTER 2

^{68}Ga-labeled DOTA-peptides and ^{68}Ga-labeled radiopharmaceuticals for positron emission tomography: current status of research, clinical applications, and future perspectives

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Abstract

In this review we give an overview of current knowledge of ^{68}Ga-labeled pharmaceuticals, with focus on imaging receptor-mediated processes. A major advantage of a ^{68}Ge/^{68}Ga generator is its continuous source of ^{68}Ga, independently from an on-site cyclotron. The increase in knowledge of purification and concentration of the eluate and the complex ligand chemistry has led to ^{68}Ga-labeled pharmaceuticals with major clinical impact. ^{68}Ga-labeled pharmaceuticals have the potential to cover all today's clinical options with ^{99m}Tc, with the concordant higher resolution of positron emission tomography (PET) in comparison with single photon emission computed tomography. ^{68}Ga-labeled analogs of octreotide, such as DOTATOC, DOTANOC, and DOTA-TATE, are in clinical application in nuclear medicine, and these analogs are now the most frequently applied of all ^{68}Ga-labeled pharmaceuticals. All the above-mentioned items in favor of successful application of ^{68}Ga-labeled radiopharmaceuticals for imaging in patients are strong arguments for the development of a ^{68}Ge/^{68}Ga generator with Marketing Authorization and thus to provide pharmaceutical grade eluate. Moreover, now not one United States Food and Drug Administration–approved or European Medicines Agency–approved ^{68}Ga-radiopharmaceutical is available. As soon as these are achieved, a whole new radiopharmacy providing PET radiopharmaceuticals might develop.

Introduction

Interest and research in ^{68}Ge and ^{68}Ga (half-life of 9 months and 68 min, respectively.) started in the early 50's, initially as a spinoff from the discovery of ^{68}Ge in fission products **[1, 2]**. Most of ^{68}Ge for ^{68}Ge/^{68}Ga generators is now a cyclotron-produced radio-metal, formed from ^{69}Ga by a (p,2n) reaction (Table **1**) **[3-5]**. A major advantage of a ^{68}Ge/^{68}Ga generator is its continuous source of ^{68}Ga, see (Fig. **1**), independently from an on-site cyclotron.

Table 1.

^{68}Ge is a cyclotron-produced radio-metal, formed from ^{69}Ga by a (p,2n) reaction:
$^{69}Ga \xrightarrow{p,2n} {}^{68}Ge \xrightarrow{EC} {}^{68}Ga \xrightarrow{\beta^+} {}^{68}Zn$

^{68}Ga half-life = 68 min, specific activity ≡ 3.63*10^{-13} gram-atoms per 37 MBq ^{68}Ga. In theory, 1 nmol DOTA-peptides can complex 10^{-9} gram-atoms Ga, corresponding to 102 GBq ^{68}Ga. After ~2.5 h equal amounts of ^{68}Zn and ^{68}Ga are present in the generator **[6]**.

The ^{68}Ge/^{68}Ga generator was first described in 1960 **[7]**, for a historical overview see these sources **[3, 8-10]**. A revival started in the 1970s, when several other ^{68}Ge/^{68}Ga generator systems were developed that resulted in reliable sources of the positron emitter ^{68}Ga. Renewed interest in ^{68}Ga has recently arisen for several reasons. First, positron emission tomography (PET) has developed during the last decade from a research tool into a powerful diagnostic and imaging technique for routine clinical application.

Second, ^{68}Ge/^{68}Ga generators have been developed which produce suitable eluates for labeling that can be converted into a ^{68}Ga-labeled pharmaceutical for PET studies **[9, 11-18]**. Third, there are many DOTA-peptides that can be labeled with ^{68}Ga. Fourth, a variety of monofunctional and bifunctional chelators have been developed which allow the formation of stable $^{68}Ga^{3+}$complexes and convenient coupling to biomolecules. Fifth, the availability of PET radiolabeled pharmaceuticals by the introduction of ^{68}Ga in radiopharmacy, independent of an on-site cyclotron opened new applications and possibilities. Coupling of ^{68}Ga to small peptides and biomolecules was recently reviewed **[3, 8, 10, 19, 20]**, and ^{68}Ga is potentially an alternative to ^{18}F- and ^{11}C-based radiopharmacy **[20]**.

Last but not least, equipment, including generators, purification and concentration of eluate, techniques of radiolabeling, robotics and PET cameras has improved over the last decade. Preclinical application of many ^{68}Ga-labeled pharmaceuticals has been developed and all today's options with ^{99m}Tc are potentially covered, for example myocardial perfusion and function, blood flow, renal function, liver function, and clinical application (Mäcke and Andre **[20]**, Fani *et al.* **[8]**, Roesch and Riss **[3]**, Wadas *et al.* **[10]**).

The term *radiopharmaceutical* has several meanings: any radiolabeled molecule intended for human use, and from a regulatory point of view, a radiopharmaceutical must be sterile, pyrogen free, safe for human use, and efficacious for a specific indication **[21]**. Therefore, we will not use that term here. None of the developed ^{68}Ga-labeled pharmaceuticals has Food and Drug Administration (FDA) or European Medicines Agency (EMA) approval. Also, no FDA- or EMA-approved ^{68}Ge/^{68}Ga generator is available. Therefore, all current clinically applied ^{68}Ga–labeled pharmaceuticals are prepared on site through a so-called magisterial preparation **[22-24]**. Here we give an overview of the state of current knowledge and research of many ^{68}Ga-labeled pharmaceuticals of the last decade, with focus on the application of ^{68}Ga-labeled pharmaceuticals for imaging receptor-mediated processes.

Physical characteristics of ^{68}Ge, ^{68}Ga and the ^{68}Ge/^{68}Ga generator

The theoretical ingrowth of ^{68}Ga from the parent nuclide ^{68}Ge in a ^{68}Ge/^{68}Ga generator is shown in Fig. **1** and Table **1**. Already 68 min after the previous elution of the generator, 50 % of the maximal activity of ^{68}Ga is reached, and after 4 h this is >90 % (Fig. **1**). ^{68}Ga decays to 89% by positron emission and 11% via electron capture. The average positron energy per disintegration is 740 keV (E_{max} = 1.9 MeV), which is higher, for example, than that of ^{18}F and potentially leads to lower resolution during scanning **[8]**.

^{68}Ge/^{68}Ga generators have been developed with many different inorganic carriers such as Al_2O_3, CeO_2, SnO_2, TiO_2, ZrO_2 **[8, 25-32]** and organic carriers like pyrogallol **[33]** and eluted with different eluents, including ethylenediaminetetraacetic acid, 8-hydroxyquinoline and HCl. All have their pros and cons, and most have recently been reviewed **[3, 8, 10, 19]**.

Purification and eluate concentration

The major limitations for direct use of ^{68}Ga for radiolabeling of DOTA-peptides for clinical PET applications are the large volume of generator eluate, high [H^+], ^{68}Ge breakthrough, and potential metal ion impurities **[6, 8, 34, 35]**. However, although the concentration of these metals (eg, Zn, Sn, Ti and Fe) are low (i.e., at sub parts per million level), their concentration can still be much higher than the concentration of ^{68}Ga. If

one supposes a concentration of Zn^{2+}, Sn^{4+}, Ti^{4+} or $Fe^{2+/3+}$ of 1 ppm *versus* 370 MBq ^{68}Ga in 6 mL, then the ratios, expressed in gram-atoms are approximately 1000, 1250, 2500 and 2500, respectively.
The presence of 4+ metals, such as Ti^{4+} is irrelevant, **[34-38]** because Ti^{4+} does not compete with Ga^{3+} in the incorporation in DOTA. By contrast, the presence of Fe^{3+} is very relevant, because Fe^{3+} competes with Ga^{3+} for the incorporation in DOTA as a result of a very limited difference in physical chemistry between Ga^{3+} and Fe^{3+}. However, as Pourbaix reported, Fe^{3+} is hardly present, because Fe^{2+} is the stable oxidation state of iron instead of Fe^{3+} in acid systems, and Fe^{2+} is much less a competitor for Ga^{3+} for the incorporation in DOTA **[39]** at labeling pH 3-5 **[34, 35]**. However, the situation is quite different in neutral or basic solutions **[39]**. More detailed data regarding competitions, impurities, and kinetics of labeling DOTA-peptides with ^{68}Ga were described previously **[19, 35].** Chemistry of eluate concentration and purification of TiO_2-based generators was recently summarized by Ocak *et al* **[14]**. Briefly, there are 3 different methods of purification and/or concentration of ^{68}Ga: (1) fractionated elution, (2) anion chromatography, and (3) cation chromatography. Fractionated elution results in a ready-to-use eluate containing approximately 80 % of the elutable ^{68}Ga activity **[6, 35, 37, 40]**. Anion and cation exchange resins are used to reduce ionic impurities, reduce acidity, and to concentrate the generator eluate **[6, 13, 35, 37, 40, 41]**. These techniques for purification and concentration of the eluate can also be applied for the Good Manufacturing practice-produced SnO_2-based $^{68}Ge/^{68}Ga$ generator (iThemba Labs, Cap Town, South Africa) and eventually labeling DOTA-peptides successfully **[6]**.

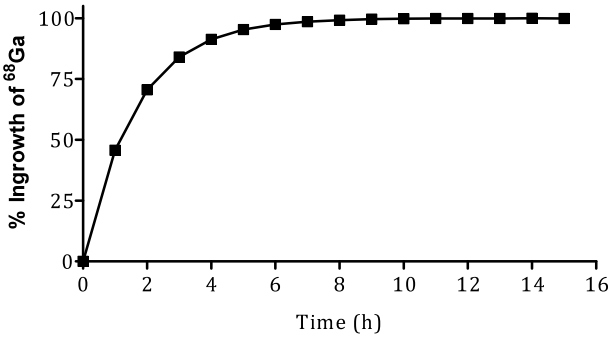


Figure 1. Theoretical ingrowth of ^{68}Ga from a $^{68}Ge/^{68}Ga$ generator. Eight hours after the previous elution there is an equilibrium (>99%) of formation of ^{68}Ga and decay to ^{68}Zn (Table **1**). Already 68 minutes after the previous elution, 50% of the equilibrium value is reached, whereas after 4 hours this is >90%.

^{68}Ge breakthrough

Since the discovery of ^{68}Ge in fission products **[1, 2]** and the possibility of medical application of ^{68}Ga, there has been renewed interest in its biochemical and pharmacological properties. The presence of ^{68}Ge activity in the eluate of $^{68}Ge/^{68}Ga$ generator, although low, has frequently been an item of concern for clinical application of ^{68}Ga-labeled pharmaceuticals. Rosenfeld **[2]** reported studies on the metabolism of germanium in the 1950s. Metabolic studies on the absorption, transport, distribution, storage, and

excretion of inorganic Ge in rats have been conducted. The data demonstrate that Ge is rapidly absorbed after oral, subcutaneous, intramuscular, or intraperitoneal administration. When injected directly into the circulation or absorbed following oral or parenteral administration, Ge is transported unbound by plasma proteins. Germanium is rapidly eliminated from the blood, excreted via the urine and feces; the kidney being the main excretory organ (ratio kidney vs liver in rats 6).
Rosenfeld **[2]** concluded Ge is not deposited selectively but is widely distributed among all the organs. No accumulation was observed by any tissue even after many weekly doses. The biochemical data support the view that Ge is relatively inert metabolically and are consonant with its remarkably low (chemical) toxicity. With an excretion of 50% during in the first hour after administration and a half-life of 30 minutes in the kidneys, the current conclusion is that Ge does not accumulate in bone.
With the techniques as described above**,** ^{68}Ge activity in eluate can be reduced by anion or cation exchange. Another additional "safety net" (to reduce ^{68}Ge activity administration) is frequently incorporated in the preparation of the final solution before administration by reversed rhase C_{18} or solid phase extraction columns **[6, 22, 42]**. These columns retain ^{68}Ga-DOTA-complexes, whereas ^{68}Ge and uncomplexed ^{68}Ga pass through the column **[22]**.
A typical example of a final ^{68}Ga- and ^{68}Ge-containing solution that is ready for clinical administration has a radionuclide purity (RNP), expressed as the ratio in activities of ^{68}Ge over ^{68}Ga, in the range of 10^{-6} and 10^{-8} **[6, 13, 41, 42]**. If one supposes a clinical administration of 100 MBq ^{68}Ga-DOTATOC, this will result in an effective dose of 2.3 mSv (0.023 mSv/MBq **[43]**), whereas the effective dose of 100 Bq ^{68}Ge (supposing RNP of 10^{-6}) will be $0.6*10^{-5}$ mSv (0.06 mSv/MBq ^{68}Ge **[44]**).

DOTA-peptides labeled with ^{68}Ga

G protein-coupled receptors, like somatostatin receptors are frequently overexpressed on human tumor cells **[23, 45, 46]**. Somatostatin receptor-targeted imaging, initially with [^{123}I-Tyr^3]octreotide and later with [^{111}In-DTPA^0]octreotide (OctreoScan; Covedien, Hazelwood, MO), was important for imaging and diagnostics of neuroendocrine tumors in nuclear medicine **[3, 8, 20, 47]**. Radiolabeled peptides targeting G protein-coupled receptors with DOTA as bifunctional chelator were developed and have shown in vivo stability, favorable pharmacokinetics (PK) and high and specific receptor-mediated tumor uptake **[12]**. Another boost for nuclear medicine came with the somatostatin receptor-targeting radiopeptide [^{68}Ga-DOTA^0, Tyr^3]octreotide (^{68}Ga-DOTATOC, Fig. **2**), in the diagnostic imaging of somatostatin receptor-positive tumors. In preclinical studies it has shown superiority over its ^{111}In-labeled congener; however, this is not surprising because amongst others, the resolution of PET is higher in comparison with single photon emission Computed tomography (SPECT) **[11, 12, 15-17]**. Because DOTATOC and other DOTA analogs have proven high in vitro and in vivo stability, most of the investigated radiolabeled peptides have the bifunctional DOTA as chelator. However, alternative chelators are still being developed, such as NOTAGA and acyclic chelates, all with their specific advantages and disadvantages **[3, 8, 19, 20, 48-50]**. An additional beneficial effect of the presence of Ga^{3+} in the DOTA cavity in DOTA-pharmaceuticals is the increased receptor affinity, as compared to other trivalent metals in the DOTA cavity, such as In^{3+} or Y^{3+}, **[9, 35, 45]** probably due to differences in coordination chemistry **[3, 10, 51, 52]**.

PK of ^{68}Ga-labeled pharmaceuticals

The uptake kinetics of ^{68}Ga-DOTA-peptides, such as DOTATOC, DOTANOC and DOTA-TATE (for structural formula see legends Fig. **2**) are rapid **[12, 19]** and in good agreement with the half-life of ^{68}Ga. The small size of these molecules display desirable PK properties, because the process of binding with their G protein-coupled receptor is rapid, as well as their clearance, which are both required for successful imaging **[53]**.

Other ligands that bind to G protein-coupled receptors, such as analogs of octreotide, cholecystokinin and gastrin-releasing peptide also display rapid PK and clearance and favorable for the half-life of ^{68}Ga. In addition, successful modification of PK with spacers between the chelator and the biomolecule is reported **[52]**.

Because of the mismatch in half-life of ^{68}Ga and the PK of most intact monoclonal antibodies (MoAb) imaging (radioimmunoimaging) with ^{68}Ga-labeled MoAb is not considered as a potential application **[54]**. The promise of radioimmunoimaging by *γ*-scintigraphy has not fully lived up to expectations, mostly because differences in biodistribution and PK between animals and humans **[55]**. PK of ^{68}Ga-labeled F(ab')$_2$ fragments of a mouse MoAb are more rapid and favorable for ^{68}Ga **[56]**, showed good target-to-background ratios at approximately 2 hours after administration, and were successfully applied in animal models **[57]**. HER2-expressing tumors in breast cancer patients were successfully imaged with ^{68}Ga-labeled affibodies ABY-002 **[11]**.

Specific radioactivity

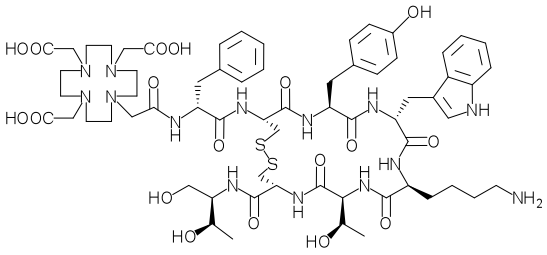
There are many factors that influence the interaction of a radioligand with its receptor. In saturable regulatory peptide binding processes (i.e., in vitro radioimmunoassay and receptor binding), the signal-to-background ratio is often improved by increasing the specific radioactivity (expressed of activity units per mass units of ligand, eg, MBq per nmol) of the ligand. In *in vivo* experiments it was shown that, contrary to what was expected, the percentage uptake of radiolabeled octreotide analogs in octreotide receptor-positive tissues is not optimal at the lowest dose of maximum specific radioactivity; rather, the uptake is a bell-shaped function of the injected mass, initially increasing followed by a decreased uptake. These findings might be the result of 2 opposing effects, i.e., a positive effect of increasing ligand concentrations on the rate of internalization by ligand-induced receptor clustering and a negative effect because saturation of the receptor at increasing ligand concentrations. **[58]** This implies that the sensitivity of detection of somatostatin receptor-positive tumors by peptide receptor scintigraphy might be improved by administration at optimized dose of radioligand, as was found for other radioligands. **[59-61]** These findings have been confirmed in patients for [^{111}In-DTPA^0]octreotide **[58, 62]** and led to increased quality of imaging and with a significantly increased uptake in tumors.

From the above-mentioned data it can be concluded that the amount of administered radioactivity and the amount of ligand can be concluded; thus specific radioactivity is a potent tool and can be applied for optimizing personal patient peptide dose in Peptide Receptor Radionuclide Therapy (PRRT) **[17, 19, 63-65]**.

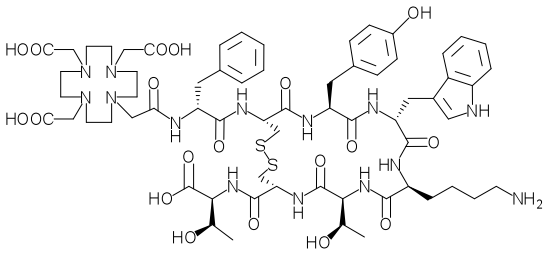
In a PRRT study, with [^{90}Y-DOTA^0,Tyr^3]octreotide (^{90}Y-DOTATOC), DOTATOC was radiolabeled with ^{86}Y (^{86}Y

is a positron emitter, with a half-life of 15 h) for dosimetric measurements to replace ^{90}Y **[66]**. Jonard *et al* **[67]** presented data on tissue distribution after the administration of ^{86}Y-DOTATOC, labeled with various amounts of DOTATOC (range of 50 to 500 µg); the kidney dose was not affected; however, tumor dose decreased (in absolute uptake) with higher peptide amounts. In analogy herewith, Velikyan *et al* **[18]** also investigated the impact of peptide mass on binding to neuroendocrine tumor somatostatin receptors in vivo using ^{68}Ga-DOTATOC as tracer at a constant high specific activity, preceded by injection of 0, 50 and 250 or 500 µg of octreotide (Sandostatin®, Novartis, Stein, Switzerland), administered 10 min before the tracer. Nine patients with gastroenteropancreatic neuroendocrine tumors were included. Six of them underwent 3 sequential PET/computed tomography (CT) examinations with intravenous injections of ^{68}Ga-DOTATOC (after administration of octreotide) and carried out in 1 day.

DOTA-TOC



DOTA-TATE



DOTA-NOC

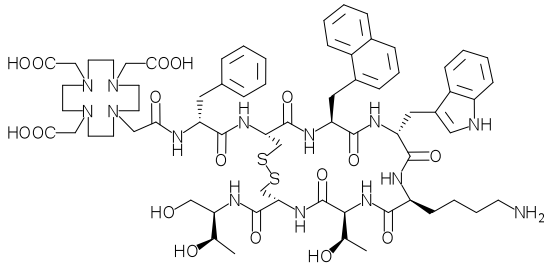


Figure 2. Structural formula of DOTATOC ([DOTA^0, Tyr^3]octreotide), DOTANOC [DOTA^0,1-Nal^3]octreotide and DOTA-TATE ([DOTA^0,Tyr^3,Thr^8]octreotide), Labeled with ^{68}Ga, these analogs are most clnically applied of all ^{68}Ga-Labeled pharmaceuticals in nuclear medicine

Three patients were examined by dynamic and static PET/CT for PK and dosimetric calculations. The tracer accumulation in the tumors varied and depended on the total amount of the preadministered octreotide. In 5 of 6 patients, the highest tumor-to-normal tissue ratio was found when 50 µg of octreotide was preadministered. Thus again, optimizing mass improved image contrast. However, 1 patient showed a continuously increasing tumor uptake even with higher octreotide preadministered. The application of ^{68}Ga-labeled ligand for optimizing therapeutic applications of concordant radiotherapeutic labeled ligand needs further dosimetric studies. A relation (such as in PK and clearance) between the ligands, labeled with ^{68}Ga versus the therapeutic radionuclide (eg, ^{90}Y or ^{177}Lu) at early time points also needs to be established.

Robotics for automated labeling

Initially all labeling procedures were done manually. Finger dosimetry measurements during elution of the generator and radiolabeling revealed doses of 60 µSv per 555 MBq and 1.5 mSv per administration of 10 MBq in rats **[35]**. Introduction of robotics increased reproducibility and it also opened the routinely application of ^{68}Ga-labeled analogs for radiopharmacies. The development of automated modules included reduction of ^{68}Ge activity, impurities and volume in the ^{68}Ga activity containing eluate of the ^{68}Ge/^{68}Ga generator, and eventually minimizing the reaction volume for DOTA-peptides **[6, 13, 14]**. Different strategies for radiolabeling with robotics were applied, including ion exchange chromatography techniques, variation in labeling time **[35-38]**, methods of heating **[36, 37, 68]**, labeling temperature **[35-37, 68]**, and application of different materials to reduce "sticking" or other uncontrolled loss of ligand **[35, 41, 69]**. Eventually all reported robotics were successful, with labeling yields >95% and specific radioactivity >50 MBq/nmol **[14, 37, 38, 68, 70]**.
To our knowledge, no finger dosimetry data while applying robotics have been reported.

Regulatory affairs

Efforts to obtain approval for Marketing Authorization for radionuclide generators (such as the ^{68}Ge/^{68}Ga generator), labeling kits, etc have recently been reported **[23, 24, 71, 72]**. Because the eluate of a ^{68}Ge/^{68}Ga generator is considered to be a medicinal product and all ingredients for clinical administration should be approved for pharmaceutical use, aspects such as the choice of a buffer for pharmaceutical use are also investigated and reported **[22]**. Moreover, not only should the ingredients be of pharmaceutical grade, the ingredients themselves should also be of high chemical purity, because they should not interfere with the incorporation of Ga^{3+} as was investigated for the choice of the buffer for clinical application, with ^{68}Ga DOTATOC as model **[22]**. Sterility of the eluates from TiO_2- and SnO_2-based ^{68}Ge/^{68}Ga generator has been reported **[6, 35]**. For overviews to the regulations for radiopharmaceuticals in early-phase clinical trials in the European Union see the "Guideline to Regulations for Radiopharmaceuticals in Early-Phase Clinical Trials in the EU" by Verbruggen *et al* **[24]** and for the U.S. in "Operation of a Radiopharmacy for a Clinical Trial" by Norenberg *et al* **[71]**.

Considering, the increase in knowledge on the chemistry of Ga^{3+}, the developments in robotics, and eventually successful application of ^{68}Ga-labeled radiopharmaceuticals for imaging in patients, all are strong arguments for the development of a ^{68}Ge/^{68}Ga generator with Marketing Authorization, and to provide pharmaceutical grade eluate [23]. As soon this is achieved, a whole new radiopharmacy providing PET radiopharmaceuticals might develop.

Applications

Most neuroendocrine tumors express somatostatin receptor subtype 2 **[45, 46, 73]**. Somatostatin receptor-targeted imaging is important in nuclear medicine.

In general, analogs of octreotide have high affinities for somatostatin receptor subtype 2 ,3 and 5, however there are differences in affinity per receptor subtype per analog. DOTANOC has very high affinity for receptor subtype 2 ,3 and 5 **[11, 15, 20, 74-76]**. In an intraindividual clinical study comparing the diagnostic efficacy of ^{68}Ga-DOTA-NOC and ^{68}Ga-DOTA–TATE, it was demonstrated that ^{68}Ga-DOTA-NOC is superior to ^{68}Ga-DOTA–TATE, **[52]** thus Prasad and Baum**[16]** use ^{68}Ga-DOTANOC for routine receptor PET/CT of neuroendocrine tumors. Prasad *et al* **[15]** also state that for proper staging of neuroendocrine tumors and also for the follow-up, it is more appropriate to use ^{68}Ga-DOTA-NOC receptor PET/CT rather than CT or ^{68}Ga-DOTA-NOC PET alone. The addition of morphological information from CT was found to be absolutely essential in pinpointing the exact site of the primary tumor, particularly in the abdominal region. ^{68}Ga-DOTA-NOC PET/CT caused a therapy modification in more than half of the patients **[77]**.

Another possible clinical application of ^{68}Ga-labeled pharmaceuticals include the techniques and set-up as described by Velikyan *et al* **[18]** with ^{68}Ga-DOTATOC to optimize the interval in time between withdrawal of octreotide and PRRT. In short, the use of variable amounts unlabeled octreotide in combination with, eg, ^{68}Ga-DOTATOC at high specific radioactivities might also be a tool for optimizing personal patient peptide dose in PRRT.

This might also be true for other radiodiagnostics, such as DOTABOC (another octreotide analog, or the -ATE versions like DOTANOC-ATE **[17, 19, 20, 78]**) and can be investigated, also according to the procedure guidelines for PET/CT tumor imaging with ^{68}Ga-DOTA-conjugated peptides **[79]**, preferably in an intraindividual clinical study.

Development of other DOTA-peptides with high affinity to other G protein-coupled receptors with overexpression on human tumors (eg, receptors of gastrin-releasing peptide, glucagon-like peptide) and integrins like arginine-glycine-aspartic acid (RGD), are still ongoing and successfully applied in patients **[74]** (Dr R. Baum, personal communication, 2010). There are many other ^{68}Ga-labeled pharmaceuticals developed, such as melatonin-stimulating hormone, **[80]** amino acids **[81]** and siderophores for aspergillosis **[82]**, and analogs with potentials to replace nowadays options in nuclear medicine and labeled with ^{99m}Tc. In addition, there are more applications such as coupling of ^{68}Ga to small peptides and biomolecules, as was recently reviewed **[3, 8, 20]**. ^{68}Ga is therefore potentially an alternative to ^{18}F- and ^{11}C-based radiophamacy **[19]**. However, up to now, not one ^{68}Ga-labeled pharmaceutical has FDA or EMA approval. For a recent overview consult these sources **[3, 8, 20]**.

Future perspectives

In a review on the continuing role of radionuclide generator systems for nuclear medicine, Knapp and Mirzadeh **[83]** state that "The continuing role of radionuclide generator systems for nuclear medicine, that "despite the availability of the ^{68}Ge/^{68}Ga generator application of ^{68}Ga radiopharmaceuticals may suffer from the complex ligand chemistry required for Ga^{3+} complexation to useful tissue-specific radiopharmaceuticals." Indeed, at that time (1994) no ^{68}Ga-labeled pharmaceuticals were in clinical studies and currently still not one EMA- or FDA-approved ^{68}Ga-labeled pharmaceuticals is available **[21, 72]**.

However, as Nunn **[84]** states "Molecular imaging must be accepted as not just good science but also as central to routine patient management in the personalized medicine of the future. There is an urgent need to reduce the cost (i.e., time and money) of developing imaging agents for routine clinical use. The mismatch between the current regulations and personalized medicine includes molecular imaging and requires the engagement of the regulatory authorities to correct. This is a new venture in both molecular imaging and targeted drugs. However, there are various regulatory, financial, and practical barriers that must be overcome to achieve this aim". We would like to express our vision that with current knowledge and techniques, applications of ^{68}Ga-labeled pharmaceuticals have a new future; however, there are indeed various barriers that must be overcome.

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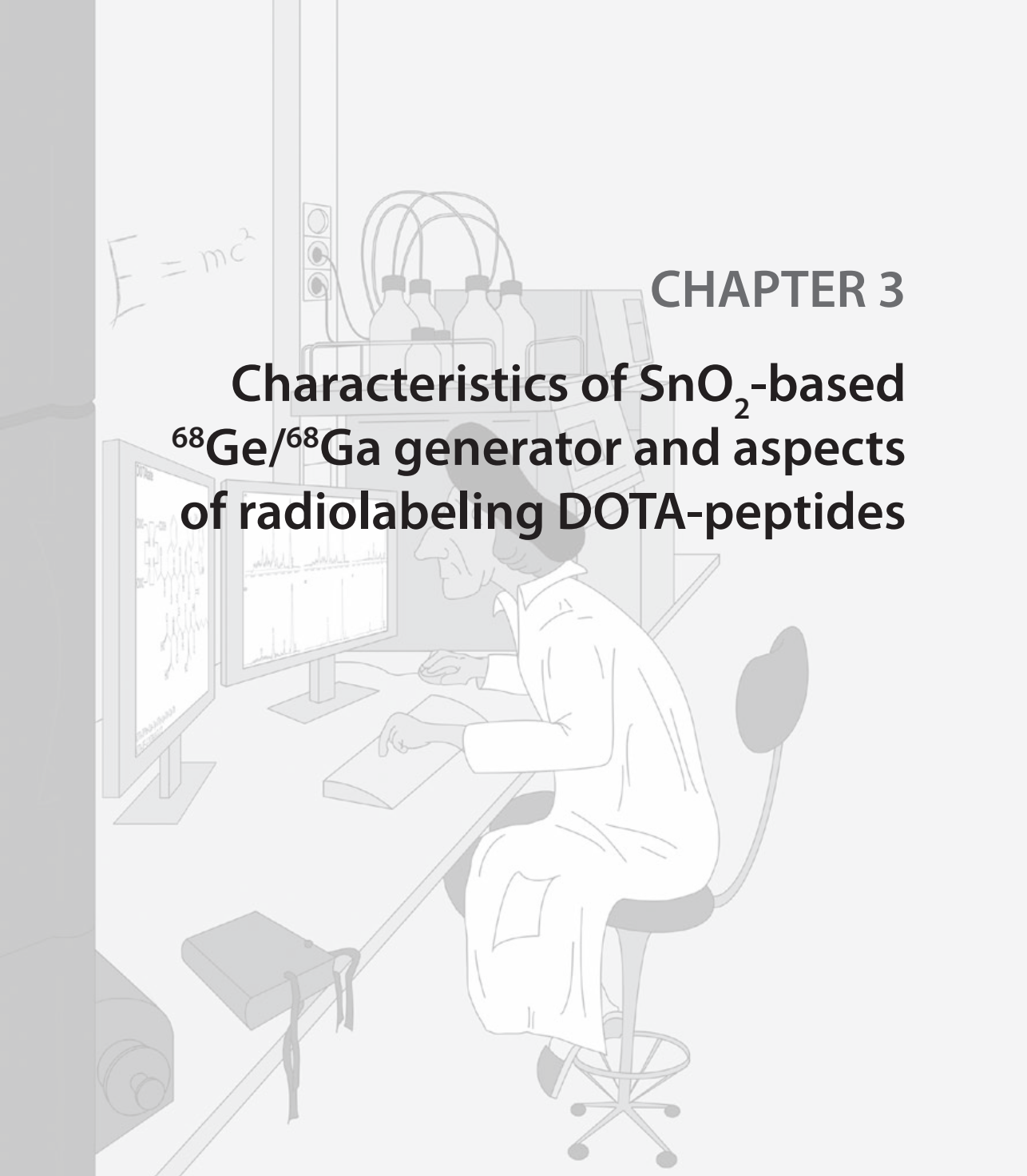

CHAPTER 3

Characteristics of SnO_2-based $^{68}Ge/^{68}Ga$ generator and aspects of radiolabeling DOTA-peptides

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Abstract

Objectives: PET scintigraphy with ^{68}Ga-labeled analogs is of increasing interest in Nuclear Medicine and performed all over the world. Here we report the characteristics of the eluate of SnO_2-based ^{68}Ge/^{68}Ga generators prepared by iThemba LABS (Somerset West, South Africa). Three purification and concentration techniques of the eluate for labeling DOTA-TATE and concordant SPE purifications were investigated.

Methods: Characteristics of 4 SnO_2-based generators (range 0.4-1 GBq ^{68}Ga in the eluate) and several concentration techniques of the eluate (HCl) were evaluated. The elution profiles of SnO_2-based ^{68}Ge/^{68}Ga generators were monitored, while [HCl] of the eluens was varied from 0.3-1.0 M. Metal ions and sterility of the eluate were determined by ICP. Fractionated elution and concentration of the ^{68}Ga eluate were performed using anion and cation exchange. Concentrated ^{68}Ga eluate, using all three concentration techniques, was used for labeling of DOTA-TATE. ^{68}Ga-DOTA-TATE-containing solution was purified and RNP increased by SPE, therefore also 11 commercial available SPE columns were investigated.

Results: The amount of elutable ^{68}Ga activity varies when the concentration of the eluens, HCl, was varied, while ^{68}Ge activity remains virtually constant. SnO_2-based ^{68}Ge/^{68}Ga generator elutes at 0.6 M HCl > 100% of the ^{68}Ga activity at calibration time and ± 75 % after 300 days. Eluate at discharge was sterile and Endotoxins were < 0.5 EU/mL, RNP was always < 0.01 % . Metal ions in the eluate were < 10 ppm (in total). Highest desorption for anion purification was obtained with the 30 mg Oasis WAX column (>80%). Highest desorption for cation purification was obtained using a solution containing 90% acetone at increasing molarity of HCl, resulted in a ^{68}Ga desorption of 68 ± 8 % . With all ^{68}Ge/^{68}Ga generators and for all 3 purification methods a SA up to 50 MBq/nmol with >95% incorporation (ITLC) and RCP (radiochemical purity) by HPLC ±90% could be achieved. Purification and concentration of the eluate with anion exchange has the benefit of more elutable ^{68}Ga with 1 M HCl as eluens. The additional washing step of the anion column with NaCl and ethanol, resulted in a lower and less variable [H^+] in the eluate, and, as a result the pH in the reaction vial is better controlled, more constant, and less addition of buffer is required and concordant smaller reaction volumes. Desorption of ^{68}Ga-DOTA-TATE of SPE columns varied, highest desorption was obtained with Baker C_{18} 100 mg (84 %). Purification of ^{68}Ga-DOTA-TATE by SPE resulted in an RNP of < 10^{-4}%.

Conclusions: Eluate of SnO_2-based ^{68}Ge/^{68}Ga generator, either by fractionated elution as by ion exchange can be used for labeling DOTA-peptides with ^{68}Ga at a SA of 50 MBq/nmol at >95% incorporation and a RCP of ± 90% . SPE columns are very effective to increase RNP.

Introduction

Since the 1970s, several ^{68}Ge/^{68}Ga generator systems have been developed to provide a reliable source of the positron-emitter ^{68}Ga (half-life 68 min) that can readily be converted into radiopharmaceuticals for Positron Emission Tomography (PET) studies. There has been renewed interest in ^{68}Ga for several reasons. Firstly, PET has developed from a research tool to routine clinical application over the last decade. Secondly, the production of ^{68}Ge/^{68}Ga generators from which the eluate is suitable for labeling in a clinical environment. Thirdly, the availability of compounds, such as small peptides that can be labeled with ^{68}Ga **[1-5]**.

The theoretical ingrowth of ^{68}Ga from the parent nuclide ^{68}Ge in a ^{68}Ge/^{68}Ga generator is shown in Fig. **1**. Eight hours after the former elution there is an equilibrium (>99%) of formation of ^{68}Ga and decay to ^{68}Zn (see Table **1**). Already 68 min after the former elution 50% of the equilibrium value is reached, while after 4 h this is > 90% (Fig. **1**).

Recently Ocak *et al.* **[6]** summarized the 3 different methods of purification and/or concentration of the ^{68}Ga from eluate of TiO_2-based generators by fractionated elution, and by anion- and cation chromatography. In short, fractionated elution (a) results in a ready to use eluate containing approximately 80 % of the elutable ^{68}Ga activity **[1, 2, 4]**. Whereas anion (b) and cation (c) exchange resins are used to reduce ionic impurities and, to concentrate the generator eluate and to reduce acidity **[3-5]**. The major limitations for direct use of ^{68}Ga for radiolabeling of peptides for clinical PET applications are the large volume of generator eluate, high [H^+], ^{68}Ge breakthrough and potential metal ion impurities. More detailed data regarding competitions impurities and kinetics was described previously **[1, 4, 7]**.

Loc'h *et al*. **[8]** reported on the elution characteristics of a SnO_2-based ^{68}Ge/^{68}Ga generator, while [HCl] of the eluens was varied from 0.5-10 M and showed decreased activities of ^{68}Ga and ^{68}Ge in the eluate at lower [HCl]. McElvany *et al.* also confirmed the elution profile, low breakthrough and chemical impurities of the SnO_2-based generator **[9]**.

Recently iThemba LABS (Somerset West, South Africa) launched a under Good Manufacturing Practice (GMP) conditions produced SnO_2-based ^{68}Ge/^{68}Ga generator **[10]**.

Here we report the characteristics of the eluate of this SnO_2-based generator, the results of the 3 purification and concentration techniques of the eluate, and the results of labeling a DOTA-peptide.

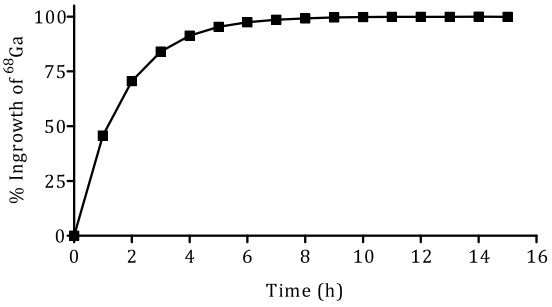

Figure 1. Theoretical ingrowth of ^{68}Ga from a ^{68}Ge/^{68}Ga generator. 8 h after the former elution there is an equilibrium (> 99 %) of formation of ^{68}Ga and decay to ^{68}Zn (see Table **1**). Already 68 min after the former elution 50 % of the equilibrium value is reached, while after 4 h this is > 90 %.

Table 1.

^{68}Ge is a cyclotron-produced radio-metal, formed from ^{69}Ga by a (p,2n) reaction:
$^{69}Ga \xrightarrow{p,2n} {}^{68}Ge \xrightarrow{EC} {}^{68}Ga \xrightarrow{\beta^+} {}^{68}Zn$

^{68}Ga t½ = 68 min, specific activity ≡3.63*10^{-13} mol per 37 MBq ^{68}Ga. In theory 1 nmol DOTA-peptides can complex 10^{-9} gram atoms Ga, corresponding to 102 GBq ^{68}Ga. After ~2.5 h equal amounts of ^{68}Zn and ^{68}Ga are present in the generator, see Fig. **8**.

Methods and materials

$^{68}Ge/^{68}Ga$ generator eluate purification procedures

$^{68}Ge/^{68}Ga$ generators were prepared by iThemba LABS (Somerset West, South Africa) by loading the parent ^{68}Ge (half-life 271 days) onto a modified SnO_2 column. $^{68}Ge/^{68}Ga$ generators of 370, 555, 740 and 1110 MBq $^{68}GaCl_3$ (10, 15, 20 and 30 mCi, respectively) were investigated. The generators were eluted 3-24 h after prior elution at a flow of 3-6 $mL.min^{-1}$, total elution volume 6 mL, with Suprapur HCl (Merck, Modderfontein, South Africa) or Ultrapure HCl (J.T. Baker, Deventer, The Netherlands) in fractions of 0.5 mL at different [HCl], range 0.3-1.0 M, increments of 0.1 M. Eluted ^{68}Ga and ^{68}Ge activities were monitored and quantified per fraction as described earlier **[1]**. All ^{68}Ga activity data are expressed decay corrected. Used chemicals were reagent grade (Sigma-Aldrich, Zwijndrecht, The Netherlands), unless specified otherwise.

Elutions of the generators were optimized so that the eluate could be used for labeling DOTA-peptides, as described earlier **[1]**. The following parameters were investigated: elution profiles, volume peak activity of ^{68}Ga (a), and ^{68}Ge (b), the concentration in the eluate of Ga, Ge, Zn, Ti, Sn, Fe, Al and Cu by ICP (c) and sterility tests of the eluate (d).

Breakthrough and RadioNuclidic Purity (RNP)

In literature breakthrough is used for different expressions, first to express the ratio of the activity of a parent radionuclide (e.g. ^{68}Ge or ^{99}Mo) in the eluate vs. activity of the parent on the column. However, the actual activity of the parent ^{68}Ge on the column is frequently not known or uncertain. Secondly, breakthrough is nowadays also in use to express the ratio of activities in the eluate of the parent radionuclide (here ^{68}Ge) vs. daughter radionuclide (here ^{68}Ga). Another expression is RadioNuclidic Purity (RNP), which is defined as the ratio of activity of a radionuclide vs. the activity of another radionuclide. We define RNP here as the ratio of activities of ^{68}Ge vs. ^{68}Ga in the eluate, expressed in % .

Analysis metal ions in eluate by ICP

Metal ions in the eluate were determined using a Jobin-Yvon Ultima ICP Spectrometer (Wirsam Scientific & Precision Equipment, Auckland Park, South Africa). Concentrations of the following metals were determined: Ga, Ge, Zn, Ti, Sn, Fe, Al and Cu 1000 ppm. Ga, Ge, Zn, Ti, Sn, Fe, Al and Cu standard solutions were obtained from Industrial Analytical (Halfway House, South Africa) and Alfa Aesar (Kyalami, South Africa). The standard solutions were used to prepare one standard solution containing 100 ppm of each of these metals. The 100 ppm standard solution was further diluted to prepare a 1, 5 and 10 ppm standard solution. The standard solutions, made up in 0.6 M HCl, were run to obtain a calibration curve. The undiluted eluate was analyzed.

Sterility tests of eluate

Dehydrated Tryptic soy broth and Thioglycolate broth were used to determine the sterility of the generator eluate. Tryptic soy broth was added aseptically (through a filter) to the sample provided and incubated at 25 ± 2.5ºC for 14 days. Thioglycolate broth was added aseptically (through a filter) to another sample and was incubated at 32 ± 2.5ºC for 14 days. Samples were checked daily for microbiological

growth. The media remains clear if no microbiological growth occurs. Endotoxins by the chromogenic method were measured by LAL test (Lonza, Walkersville, MD, USA).

Anion chromatography

At [HCl] >5.0 M, Ga^{3+} ions form the negatively charged gallium tetrachloro complex $[GaCl_4]^-$. This property can be used advantageously to concentrate the eluted gallium, since the $[GaCl_4]^-$ complex can be adsorbed quantitatively on a strong anion exchange resin, as described by Meyer *et al*. **[3]**. We investigated anion resin AG 1-X8, 200-400 Mesh (BioRad, Veenendaal, the Netherlands) in an in-house made cartridge. In addition, the amount of resin was varied between 50-500 mg. The following commercially available anion columns were also investigated: Oasis® WAX (2 forms: 30 and 225 mg, Waters, Etten-Leur, the Netherlands) and Chromabond PS-HCO_3 column (45 mg), Macherey-Nagel, Düren, Germany.

Briefly, columns containing anion resin (further referred as anion column) were pre-conditioned before use with 2 mL ethanol and 2 mL 5 M HCl. Two mL of the eluate (e.g. 1 M HCl), including >80 % of ^{68}Ga activity, was added to 4 mL 7 M HCl, total volume is 6 mL, final [HCl] is 5 M. This (6 mL) solution and 2 mL of 5 M HCl were eluted through the anion column. Subsequently, ^{68}Ga was desorbed with ≥0.4 mL of Milli-Q **[3]**. Desorption is expressed as % eluted ^{68}Ga of total ^{68}Ga activity applied to the column.

In order to reduce $[H^+]$ in the main containing ^{68}Ga activity fraction, an extra rinse with 2 mL 5M NaCl was introduced and investigated, followed by 2 mL ethanol to reduce $[Cl^-]$. Finally, ^{68}Ga was desorbed with ≥0.4 mL of Milli-Q. After each washing step the anion column was flushed with air or nitrogen.

Cation chromatography

Literature data indicate that, with the increase of the content of acetone in the HCl solutions of the same acid concentration, the affinity of $^{68}Ga^{3+}$ for the organic cation resin decreases **[11]**. Cation purification was investigated as *f*[acetone] and as *f*[HCl] while monitoring ^{68}Ga desorption and RNP of the eluate as also described by Fritz *et al.* **[12]** and Zhernosekov *et al.* **[5, 12]**. Cation purification was performed with cation resin: AG 50W-X8, 200-400 Mesh (BioRad, Veenendaal, The Netherlands), in an in-house made cartridge. In addition, the amount of resin was varied, between 50-500 mg.

Briefly, columns containing cation resin (further referred as cation column), were pre-conditioned before use with 2 mL Milli-Q and 1 mL 4 M HCl. Total eluate (6 mL), was eluted through the cation column, 0.6 mL of 0.15 M HCl/80 % acetone was used to wash the cation column. ^{68}Ga activity was desorbed with 0.4 mL of different mixtures of acetone and HCl. After each washing step the cation column was flushed with air or nitrogen.

Radiolabeling and quality control

DOTA-TATE, (DOTA-D-Phe^1,Tyr^3-octreotate, DOTA=1,4,7,10-tetraazacyclo-dodecane-1,4,7,10-tetraacetic acid) MW: 1436 g/mol, (BioSynthema, St Louis, MO, USA) was used as peptide model to investigate radiolabeling with ^{68}Ga, as described earlier **[1]**. DOTA-TATE was dissolved in Milli-Q water, final peptide concentration 10^{-3} M. A solution containing DOTA-TATE, radioactivity (typically 200 µL in Milli-Q, post ion chromatography) and 1 M HEPES (200 µL) or a solution containing DOTA-TATE, radioactivity (typically 100 µL of the fractionated eluted ^{68}Ga in 0.6 M HCl,) and 2.5 M sodium acetate (37 µL) was heated for

10 min at 80°C in a temperature-controlled heating block as described previously **[7]**. Radiolabeling was at pH between 3,5-4 and were performed in small volumes, typically 50–150 µL, using double-sealed polypropylene reaction tubes (PCR thermocycler tubes, maximal volume 150 µL, MoBiTec, ITK Diagnostics, Uithoorn, The Netherlands). Radiolabelings in volumes ≥ 0.15 mL and ≤ 1.5 mL were performed in polypropylene vials (IKS International, Rosmalen, The Netherlands). Ethanol was used as quencher during radiolabeling, after labeling ascorbic acid and methionine were added. All 3 described methods for handling the eluate (fractionated elution, and both ion chromatography techniques) were used for labeling DOTA-TATE, and incorporation was investigated **[7]**, minimal peptide mass was 1 nmol **[7],** while specific activity (SA) was varied up to 50 MBq/nmol. Quality control, including the addition of 5 µL 4 mM EDTA post radiolabeling was performed as described earlier **[1, 7]**. In short: incorporation of ^{68}Ga was measured by Instant Thin Layer Chromatography Silica Gel (ITLC-SG), eluens: 0.1 M sodium citrate (pH 5) and/or with 1 M ammonium acetate/ methanol (1:1 v/v) **[1, 2]**. Radioactivity on the ITLC strips was measured with a dose calibrator (e.g. Veenstra VDC-405, Joure, The Netherlands) or Packard Cyclone Phosphor Imaging system with OptiQuant software (PerkinElmer, Groningen, The Netherlands). RadioChemical Purity (RCP) was measured by HPLC, e.g. a Waters Breeze HPLC system, 1525 binair pump. A Symmetry C_{18} column (5 µm, 4.6 mm x 250 mm, Waters, Etten-Leur, The Netherlands), mobile phase 0.1 % TFA (A) methanol (B). Gradient profile was: 0-2 min 100 % A (flow 1 mL/min), 2-3 min 55 % B (flow 0.5 mL/min), 3-20 min 60 % B (flow 0.5 mL/min), 20-20.01 min 100 % B (flow 1 mL/min), 20.01- 25 min 100 % A (flow 1mL/min), 25.01- 30 min 100 % A (flow 1mL/min). HPLC injections were performed via a Waters 717 autosampler (injection volume 200 µL). Radioactivity was monitored with a Unispec MCA γ- detector (Canberra, Zelik, Belgium).

Solid Phase Extraction (SPE) by C_{18} reversed phase extraction

^{68}Ge activity will be present in the eluate, whereas ^{68}Ge activity can not be quantified in the presence of abundant ^{68}Ga activity, therefore a "Safety Net" to eliminate ^{68}Ge activity is preferred. Although ^{68}Ge activity is already reduced by above-mentioned anion and cation column chromatography **[2, 3, 5, 6]**, ^{68}Ga-labeled peptides can also be purified by SPE on a C_{18} reversed phase column **[2]**. ^{68}Ga-labeled peptides are retained on this type of column, while colloids, non-incorporated $^{68}Ga^{3+}$ and $^{68}Ge^{4+}$ are not retained, thus SPE could therefore indeed be a "Safety Net" prior to clinical administration of ^{68}Ga-labeled peptides.

The following commercially available 11 SPE cartridges were tested: Strata-X C_{18} 30 mg (Phenomenex, Utrecht, the Netherlands), Chromabond Shorty (10 and 30 mg) and Chromabond C_{18} 100 mg (Macherey-Nagel, Düren, Germany), Sep-Pak C_{18}, Sep-Pak light C_{18}, Sep-Pak plus C_{18}, Sep-Pak C_{18} Vac 3cc, Sep-Pak C_{18} Vac 6cc and Oasis HLB plus 250 mg (Waters, Etten-Leur, The Netherlands), J.T. Baker C_{18} 100 mg (Mallinckrodt Baker, Deventer, The Netherlands).

Columns were pre-conditioned with 2 mL of methanol followed by 2 mL of Milli-Q.

For adsorption experiments of ^{68}Ga-DOTA-TATE on SPE columns, DOTA-TATE was labeled at a fixed SA of 35 MBq ^{68}Ga per nmol, and transferred to the pre-conditioned SPE columns. To quantify adsorption eluates were collected in separate vials. For desorption study SPE columns were flushed with 2 fractions of 1 mL Milli-Q, 2 fractions of 1 mL ethanol and eluates were collected in separate vials, ^{68}Ga and ^{68}Ge activity in these vials were quantified as described previously **[1].**

Statistic analysis

Statistical analysis was performed using Student's *t* test (Graph-Pad, Prism4, San Diego, CA, USA). Statistical significance was defined at $p < 0.05$.

Results

Elution profiles

Fig. **2** shows cumulated elutable ^{68}Ga activity in fractions of 0.5 mL at various [HCl]. The amount of elutable ^{68}Ga activity decreases dramatically at lower [HCl] as eluens, while ^{68}Ge activity in the eluate remains constant. After ± 4 mL » 95 % of elutable ^{68}Ga was collected, and > 99 % at 6 mL (Fig. **2**). One h after the former elution, the eluted ^{68}Ga activity was 40–45 % , and this increased to 90–95 % after 4 h. Although elutions with 1 M HCl resulted in more ^{68}Ga activity, we preferred to continue with less acidity, according the following compromise: less acidity (0.6 M HCl vs. 1 M), however at less activity (-25 % , Fig. **2**). Thus, unless otherwise indicated, the ^{68}Ge/^{68}Ga generators were eluted with 0.6 M HCl. Eluted ^{68}Ga activity of the generators at 0.6 M HCl ranged from > 100 % (at calibration time) to ± 75 % after 300 days, and concordant with iThemba LABS specifications. Figure **3** shows the elution profile of ^{68}Ga activity: void volume was ± 2.0 mL, ± 80 % of total activity was collected in 1.5 mL, while ^{68}Ge activity is constant per fraction.

Metal ions in eluate

Metal ions (Ga, Ge, Zn, Ti, Sn, Fe, Al and Cu) in the eluate were always < 10 ppm (in total, and for Zn < 3 ppm and for all other metals < 1 ppm) and up to 300 days after calibration time.

RNP

RNP was always < 0.01 % . However, incidentally spikes in RNP (< 0.025 %) were evident, generally after long weekends when daily elutions of the generator was not possible or when the generator was not eluted for extended period of time. Always, after a few elutions, returning to required specification of < 0.01 % RNP. Therefore, daily elutions are recommended to maintain generator integrity beyond the 9 months.

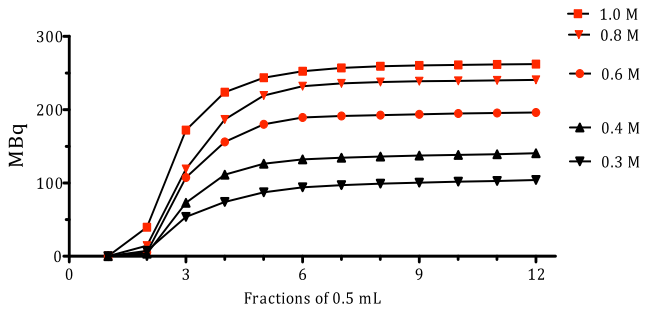

Figure 2. Cumulative ^{68}Ga activity after fractionated elution of the ^{68}Ge/^{68}Ga generator ≥ 24 h post prior elution in fractions of 0.5 mL as *f*[HCl] (range 0.3-1.0 M). Total volume eluted was 6 mL. More than 90 % of the elutable ^{68}Ga activity was eluted after 6 fractions of 0.5 mL. 75 % of the ^{68}Ga activity can be eluted with 0.6 M HCl (mean ± SD, n≥ 2). Experiments were performed at intervals of 1–2 days.

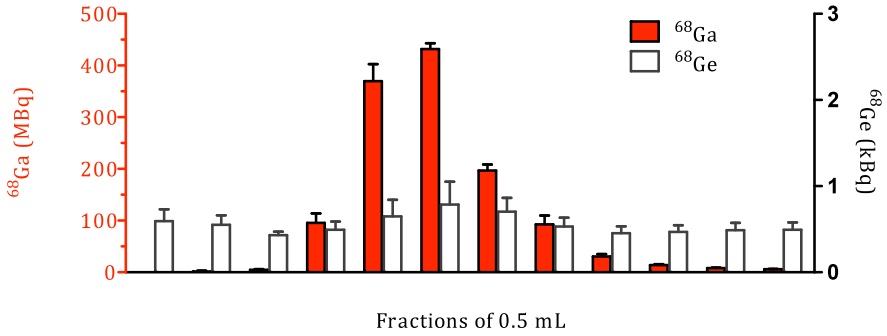

Figure 3. ^{68}Ge and ^{68}Ga after fractionated elution of the ^{68}Ge/^{68}Ga generator with 0.6 M HCl ≥ 24 h post prior elution. ^{68}Ga is expressed in MBq and ^{68}Ge is expressed in kBq (mean ± SD, n=5). Experiments were performed at intervals of 1–2 days.

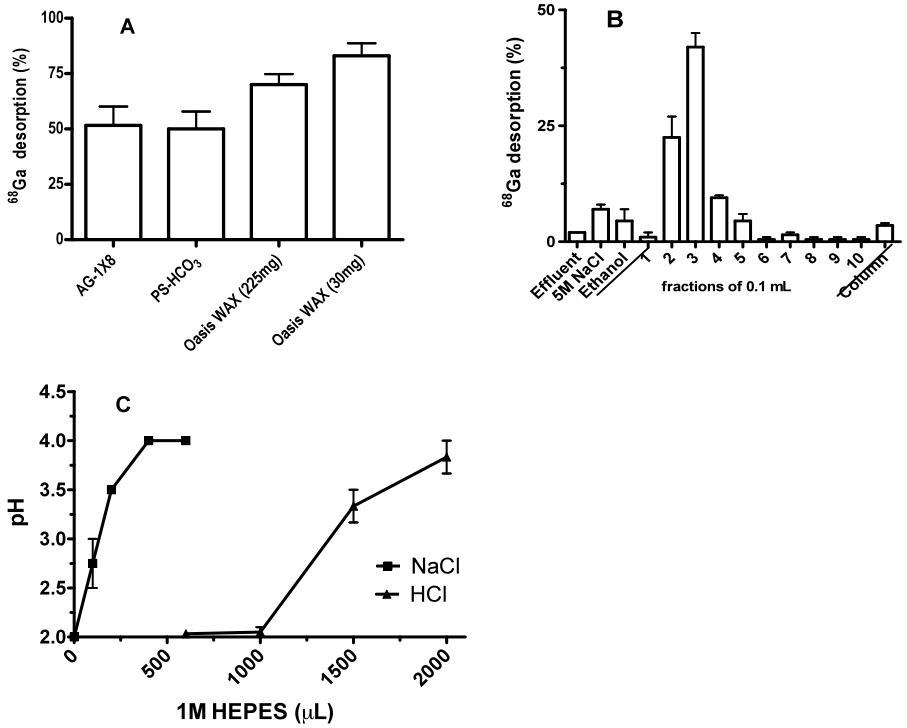

Figure 4A. Anion purification using 3 different types of resins. Desorption of ^{68}Ga of total eluate ranged between 53 and 83 % (mean ± SD, n ≥ 2). Best result were obtained with the 30 mg Oasis WAX (83 %) **(B).** Anion purification procedure using 30 mg Oasis WAX. Desorption of ^{68}Ga is 79 % in 0.4 mL and 83 % in 1 mL of Milli-Q (mean ± SD, n ≥ 2) **(C).** pH of 0.4 mL ^{68}Ga eluate, desorbed from Oasis WAX anion column after purification as described above (Fig. **4B**), after addition of HEPES (1 M). Anion column was rinsed with HCl (▲) or NaCl (■). To adjust to pH 4, 5-times less HEPES (1 M) is required after the extra rinse with 5 M NaCl (mean ± SD, n ≥ 2).

Sterility of eluate

Sterility at discharge always met specification requirements: Endotoxins were < 0.5 EU/mL.

Anion chromatography

^{68}Ga activity in 6 mL 5 M HCl was adsorbed (> 98 %) on all anion columns. Desorption of ^{68}Ga activity from total ^{68}Ga activity on anion columns ranged between 50 and 83 % (Fig. **4A**). AG 1-X8 200-400 mesh resin loaded on to in-house made reload cartridge showed best ^{68}Ga desorption (± 55 %) using 250 mg (data not shown) with no further increase in ^{68}Ga desorption with more mass of resin.
As Figure **4A** shows, highest desorption was obtained with the 30 mg weak anion Oasis WAX column. Of total ^{68}Ga column activity ± 40 % was desorbed in the first 0.1 mL Milli-Q (Fig. **4B**, fraction 3), and in 0.4 mL Milli-Q (fractions 2-5, Fig. **4B**) 79 % was desorbed, and 83 % was desorbed in 1 mL Milli-Q.
In order to decrease acidity of the eluate of the anion column, H^+ was replaced by Na^+ while keeping [Cl^-] constant at 5 M. No statistically significant changes in ^{68}Ga desorption were observed while decreasing at constant [Cl^-]. As Figure **4C** clearly shows less 1 M HEPES is required to adjust pH of 0.4 mL eluate after the additional 5 M NaCl rinsing step: 2 mL 1M HEPES → 0.4 mL 1M HEPES. Fractions 2-5 (0.4 mL, Fig. **4B**) were directly used for radiolabeling by adding peptide and HEPES.

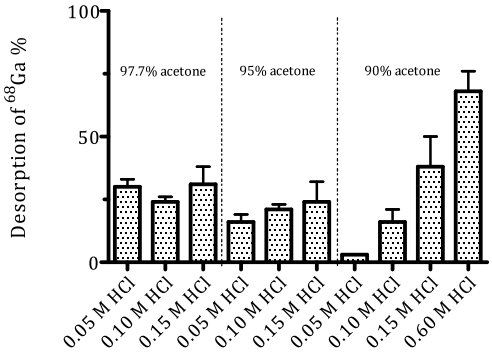

Figure 5. Cation purification using 3 different [acetone], while varying [HCl] (range 0.05-0.6 M). The lower the [HCl] the less desorption of ^{68}Ga. Best ^{68}Ga desorption (68 ± 8 %) was obtained using 90 % acetone/0.6 M HCl (mean ± SD, n ≥ 2).

Cation chromatography

^{68}Ga in 6 mL eluate was adsorbed on the cation column (> 98 % adsorption of ^{68}Ga). The cation column was rinsed with 0.15 M HCl/80 % acetone. Solution containing 90 % acetone at increasing molarity of HCl, resulted in a better ^{68}Ga desorption (Fig. **5**). Therefore ± 0.4 mL of 0.6 M HCl/90 % acetone was used and resulted in a 68 ± 8 % desorption of ^{68}Ga. This fraction was directly used for radiolabeling by adding peptide and HEPES to acquire pH 3-4. AG 50W-X8 200-400 mesh resin loaded on to an in-house made reload cartridge showed best desorption at 250 mg, with no further increase in adsorption or desorption of ^{68}Ga with increasing mass of resin.

Radiolabeling

Main ^{68}Ga activity peak could directly be used for radiolabeling. With all 4 ^{68}Ge/^{68}Ga generators and for all 3 methods a SA up to 50 MBq/nmol with > 95 % incorporation (ITLC) and RCP ± 90 % could be achieved (Fig**. 6**).

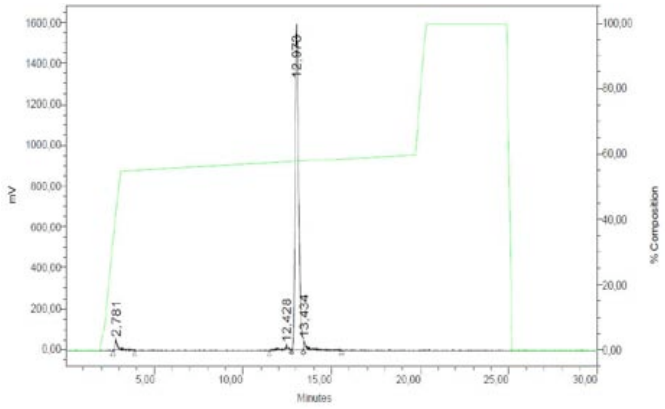

Figure 6. HPLC chromatogram of non-peptide bound ^{68}Ga (Rt: 2.78), ^{68}Ga-DOTA-TATE (Rt: 12.97). Right y-axis: ^{68}Ga activity (mV), x-axis presenting Rt (min). Green line (left y-axis) presents gradient of 0.1 % TFA/methanol.

Solid Phase Extraction (SPE) by C_{18} reversed phase extraction

Desorption of ^{68}Ga-DOTA-TATE from the 11 SPE cartridges varied, range 15 – 84 % (Fig. **7**). Highest desorption of ^{68}Ga-DOTA-TATE (84 %) was obtained with Baker C_{18} 100 mg.

Adsorption of ^{68}Ge activity was always < 5 % , purification of ^{68}Ga-DOTA-TATE by SPE resulted in an RNP of < 10^{-4} % .

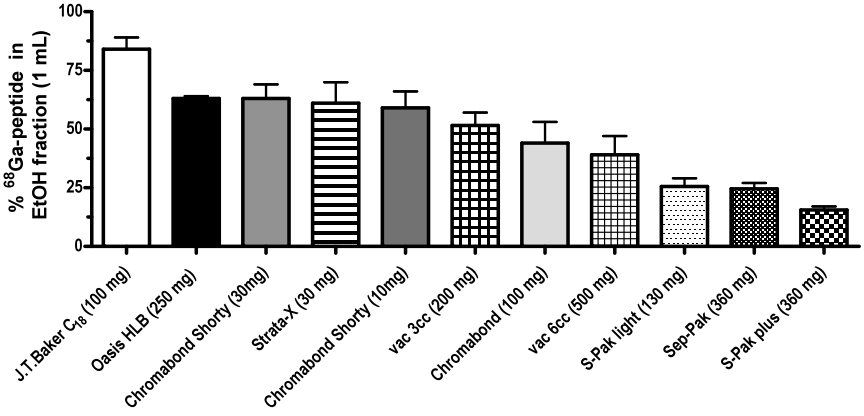

Figure 7. Solid Phase Extraction (SPE) C_{18} reversed phase extraction using 11 different C_{18} columns. Best results are obtained with a Baker C_{18} 100 mg (mean ± SD, n ≥ 2).

Discussion

Characteristics of $^{68}Ge/^{68}Ga$ generators and eluate

$^{68}Ge/^{68}Ga$ generators are frequently delivered with a given activity of ^{68}Ge on the column, however, the elutable amount of ^{68}Ga activity is not stated and depends, among others on the carrier and eluate. For most commercially available TiO_2-based $^{68}Ge/^{68}Ga$ generators holds, e.g. 1000 MBq $^{68}GeCl_4$ on the column, and ± 900 MBq ^{68}Ga can be eluted shortly after production, and after 1 half-life of ^{68}Ge the elutable ^{68}Ga activity is reduced to ± 350 MBq, thus ± 70 % **[1, 2].** iThemba Labs delivers their SnO_2-based generator with a given elutable ^{68}Ga activity, which is > 100 % in the beginning and ± 75 % after 1 half-life of ^{68}Ge.

Purification and concentration by ion chromatography

Less ^{68}Ga activity is eluted from the generator with 0.6 M HCl vs. 1 M HCl (at ± 25 % less ^{68}Ga activity, Fig. **2**), at concordant less acidity in the eluens (0.6 vs. 1 M HCl). However, if the eluate will be purified and concentrated by ion chromatography there is no need to lower the molarity of the eluens to 0.6 M HCl. For anion chromatography 1 M HCl is even preferred, since this results in higher eluted ^{68}Ga activity (Fig. **2**), and less addition of concentrated HCl is required to form $[GaCl_4]^-$ complex at 5 M HCl and to adsorb ^{68}Ga on an anion column **[3]**.

Eluate purification and concentration by cation chromatography with BioRad's AG 50W-X8 200-400 Mesh had high adsorption (> 98 %) and desorption (68±8 %) of ^{68}Ga activity. Zhernosekov *et al.* **[5]** reported higher rates of adsorption and desorption (> 99 %) of ^{68}Ga activity with BioRad's AG 50W-X8 resin 400 Mesh.

Radiolabeling DOTA-peptides

Reaction kinetics will improve at higher concentrations of the reactants, thus small volumes are preferred. With high ^{68}Ga volume activity (in MBq per mL) better incorporations and higher SA could be achieved **[1]**). Therefore, in theory, higher SA will be achievable with high activity $^{68}Ge/^{68}Ga$ generators, while 9 month later ^{68}Ga volume activity will decrease to ± 35 % of initial ^{68}Ga volume activity. This decrease in ^{68}Ga volume activity is less dramatic after purification and concentration of the eluate by ion exchange chromatography.

We demonstrate here the main ^{68}Ga activity peak could directly be used for radiolabeling of DOTA-TATE, with all $^{68}Ge/^{68}Ga$ generators by fractionated elution and for both ion chromatography techniques. A SA up to 50 MBq/nmol with > 95 % incorporation (ITLC) and RCP ± 90 % could be achieved (Fig. **6**).

RCP of radiopeptides by ITLC is performed world-wide, including in radiopharmacies. In our opinion the value of RCP needs to be reconsidered. RCP can also be measured by HPLC, a far more sensitive technique and can be used to make a relative comparison between 1 or several radiolabeled compounds, e.g. to show the difference in ways of storage or maintaining RCP. We would like to underline that RCP by HPLC is a "soft" parameter. RCP is frequently expressed as the % between the activity in the main peak, vs. total activity. However, there is no standard for HPLC separations, nor for the software to calculate. In short, RCP data can be confusing for comparisons between HPLC systems. Therefore, interdepartmental or interlaboratory comparisons are currently of arguable value.

Finger dosimetry during radiolabeling can be high **[1]**. Luckily robotics have been developed and have proven to be very efficient, for all 3 techniques **[2, 3, 6, 13, 14]**. Although not reported, to our knowledge, reduction in finger dose by robotics can be anticipated. Fingerdosimetry during preclinical administration in small animals is still a concern, e.g. 1.5 mSv per administration of 10 MBq are reported **[1]**.
Current robotics for radiolabeling are applying micro columns with concordant small void and elution volumes. And, although, the columns are flushed with air or nitrogen after each washing step. Very small droplets of HCl in columns and adherent tubing may still remain and will be included in the following elution, including the wash to desorb ^{68}Ga activity. This resulted often in a too low pH in the reaction vial for radiolabeling, and, as a consequence prevented full incorporation of ^{68}Ga activity in DOTA-peptides, such as DOTA-TATE. Therefore, we investigated an additional washing step of the anion column with NaCl and ethanol, and this indeed resulted in a lower and less variable [H^+] in the eluate. As a result the pH in the reaction vial was better controlled, more constant, and less addition of buffer was required (Fig. **4C**), which also resulted in smaller reaction volumes.

Clinical application

With high SA bioactive peptides with potential pharmacological side-effects, such as agonistic analogs of bombesin, CCK, substance P can now be injected in patients. Another advantage is the selection on a personal base of these radiolabeled ^{68}Ga-DOTA-peptides (such as analogs of octreotide) available for PRRT. Thus factors that optimize target uptake, such as the choice of the best radioligand and concordant timing and dosage (in MBq and nanomoles), can now be studied in each patient as suggested **[1, 15-17]** and confirmed in patients **[15]**, and in patients with ^{68}Ga-DOTA-TOC by Velikyan *et al*.**[18]**.
The sterility of the eluate and the Endotoxin level (< 0.5 EU per mL) make the eluate suitable for clinical application. However, there are many other requirements, such as those relating to establishment of chemical, radiochemical and RNP of the eluate, the granting of a marketing authorisation for a ^{68}Ge/^{68}Ga generator is dependent on the condition that it is manufactured under GMP conditions. Whereas, although the ^{68}Ge/^{68}Ga generator produced by iThemba Labs is GMP-produced, the eluate of such a generator is to be considered as an active substance used as a starting material for a medicinal product for human use. Article 46 (f) of European Directive 2001/83/EC and Article 50 (f) of Directive 2001/82/EC, as amended by Directives 2004/27/EC and 2004/28/EC respectively, place new obligations on manufacturing authorisation holders to use only active substances that have been manufactured in accordance with GMP for starting materials **[19]**.
For clinic applications the ^{68}Ge/^{68}Ga generator has high potential, however, currently ^{68}Ga from these generators cannot be used for the production of ^{68}Ga-labeled tracers in clinical routine**[17, 20]**.
Apart from the need for an authorised ^{68}Ge/^{68}Ga generator of "medicinal quality", the use of ^{68}Ga-labeled agents as radiopharmaceuticals is dependent on many conditions, rules and laws. The simplest and most straightforward way to permit the use of such tracers in an authorised way would be for a manufacturer of medicinal products to obtain a marketing authorisation for one or more labeling kits for the preparation of ^{68}Ga-labeled radiopharmaceuticals and to make these kits available on the market **[17]**.

Metal ions in eluate

The presence of metal ions, like Zn in the eluate is frequently a concern for labeling DOTA-peptides with radiometals as ^{68}Ga, since the incorporation of radiometals in DOTA-peptides is negatively influenced **[1, 7]**. Zn ions will always be present in the eluate of ^{68}Ge/^{68}Ga generators, due to formation of ^{68}Zn as decay product of ^{68}Ga (Table **1**).

The amount of formed ^{68}Zn is dependent on the time between prior elution, see Fig. **1** and **8A** and **B**. Therefore, the generator should preferably be eluted 3-24 h prior to the elution for radiolabeling when high SA are required. In theory after ≈ 3 days post prior elution the ratio in gram atoms of Zn vs. Ga = 50, which, still results in an achievable theoretical SA of 2 GBq ^{68}Ga per nmol DOTA-peptide (Fig. **8A** and **B**). All other metals were low (< 1 ppm), including Sn, as also reported by Loc'h**[8]**. However, although the concentration of these metals (Zn, Sn, Ti and Fe) are low, their concentration can be much higher than the concentration of ^{68}Ga.

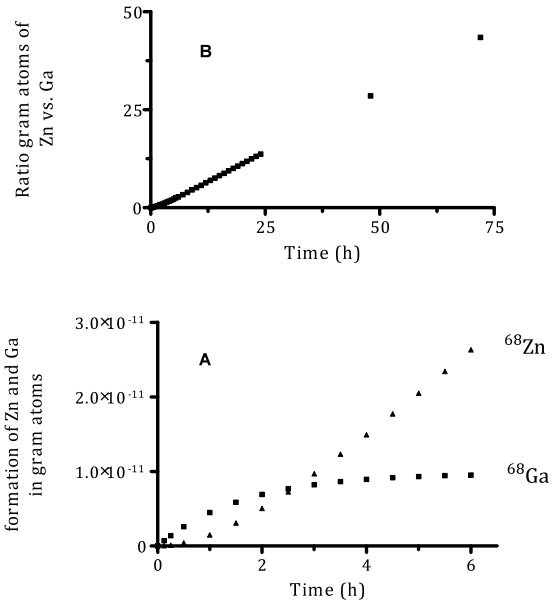

Figure 8 A: Ingrowth of [^{68}Ga] and [^{68}Zn] as *f*(time) in hours post prior elution of 1 GBq ^{68}Ge **B:** Ratio of [^{68}Ga] and [^{68}Zn] in eluate as *f*(t) post prior elution: As an example: 1 GBq ^{68}Ga corresponds with 10^{-11} gram atoms ^{68}Ga. Maximal theoretical SA of ^{68}Ga-labeled DOTA-peptides at t=0 is therefore 102 GBq per nmol **[5]**. In theory, even after ≈ 3 days post prior elution the ratio in gram atoms of [Zn]/[Ga] = 50, which still results in a theoretical maximal achievable SA of 2 GBq ^{68}Ga per nmol.

As an example, suppose a concentration Zn, Sn, Ti or Fe of 1 ppm vs 370 MBq ^{68}Ga in 6 mL; the ratios, expressed in gram atoms are 1000, 1250, 2500 and 2500, respectively.

Fani *et al*. discusses the use of several generator systems giving ^{68}Ga in a directly usable form for radiolabeling DOTA-peptides **[20]**. However, as Fani states, the presence of oxides from the carrier in the

eluate is not neglible, ruling out the possibility for clinical use **[20]**. As we have demonstrated and report here, the eluate from the iThemba Labs SnO_2-based has low metal impurities. Direct use of the eluate for radiolabeling DOTA-peptides resulted in radiopeptides at full incorporation, and with high SA.

Solid Phase Extraction (SPE) by C_{18} Reversed Phase Extraction

SPE provided an extra safety net for clinical applications since it reduced ^{68}Ge activity and ^{68}Ga (non-incorporated and colloids), e.g. it reduced ^{68}Ge activity by a factor of ± 100 **[2, 21]**, RNP increased to a level of 10^{-4} % . Moreover, ^{68}Ge has a short biological half-life, and preliminary data resulted in an effective dose of 0.034 Sv/MBq **[22]**. Further studies hereon are ongoing.

Desorption of ^{68}Ga-labeled peptides varied, and was peptide and SPE column type dependent. Investigations with other ^{68}Ga-labeled DOTA-peptides revealed different elution patrons, therefore further studies for each radiopeptide and SPE column type will be required.

Preclinical Application

Application of animal SPECT and PET for receptor-mediated processes, such as regulatory peptides can only be performed at high SA of the radiolabeled peptides. Moreover, high ^{68}Ga volume activity, and in small volumes (injections per 25 g mouse are limited to maximal 0.2 mL) are required, while radiolysis of radiopeptides under these conditions are reported **[23]**. Studies to maintain RCP of these radiopeptides, thus on preventing radiolysis, e.g. by the addition quenchers are ongoing. Another limitations for direct use in animals is the presence of ethanol post SPE, necessitating the removal of ethanol, e.g. by evaporation, however during this process (volume reduction and time) radiolysis continues.

Conclusion

SnO_2-based ^{68}Ge/^{68}Ga generator elutes at 0.6 M HCl > 100 % of the ^{68}Ga activity
at calibration time and ± 75 % after 300 days. The eluate is sterile at discharge, contains low Endotoxins (< 0.5 EU/mL), low amounts of metal ions (< 10 ppm in total), and RNP of < 0.01 % . Eluate by fractionated elution, and after purification and concentration by anion or cation exchange can all be used for labeling DOTA-peptides with ^{68}Ga at a SA of 50 MBq/nmol. Purification and concentration of the eluate with anion exchange has the benefit of more elutable ^{68}Ga with 1 M HCl as eluens. The additional washing step of the anion column with NaCl and ethanol, resulted in a lower and less variable [H^+] in the eluate, and, as a result the pH in the reaction vial is better controlled, more constant, and less addition of buffer is required (Fig. **4C**), and concordant smaller reaction volumes.

SPE columns are very effective to increase RNP of ^{68}Ga-DOTA-TATE. Desorption of ^{68}Ga-DOTA-TATE within tested C_{18} columns varies. Highest desorption (84 %). Is obtained with Baker C_{18} 100 mg.

Acknowledgements

We would like to thank Dr N Rossouw (iThemba Labs) and the working groups of the COST D38 and COST BM0607 of the European Community for stimulating discussions. We are grateful to Grant Sedres and Claudia Davids, Martijn Visser, Halil Okzal, Kevin Jonkers, and Rory de Zanger for expert technical assistance.

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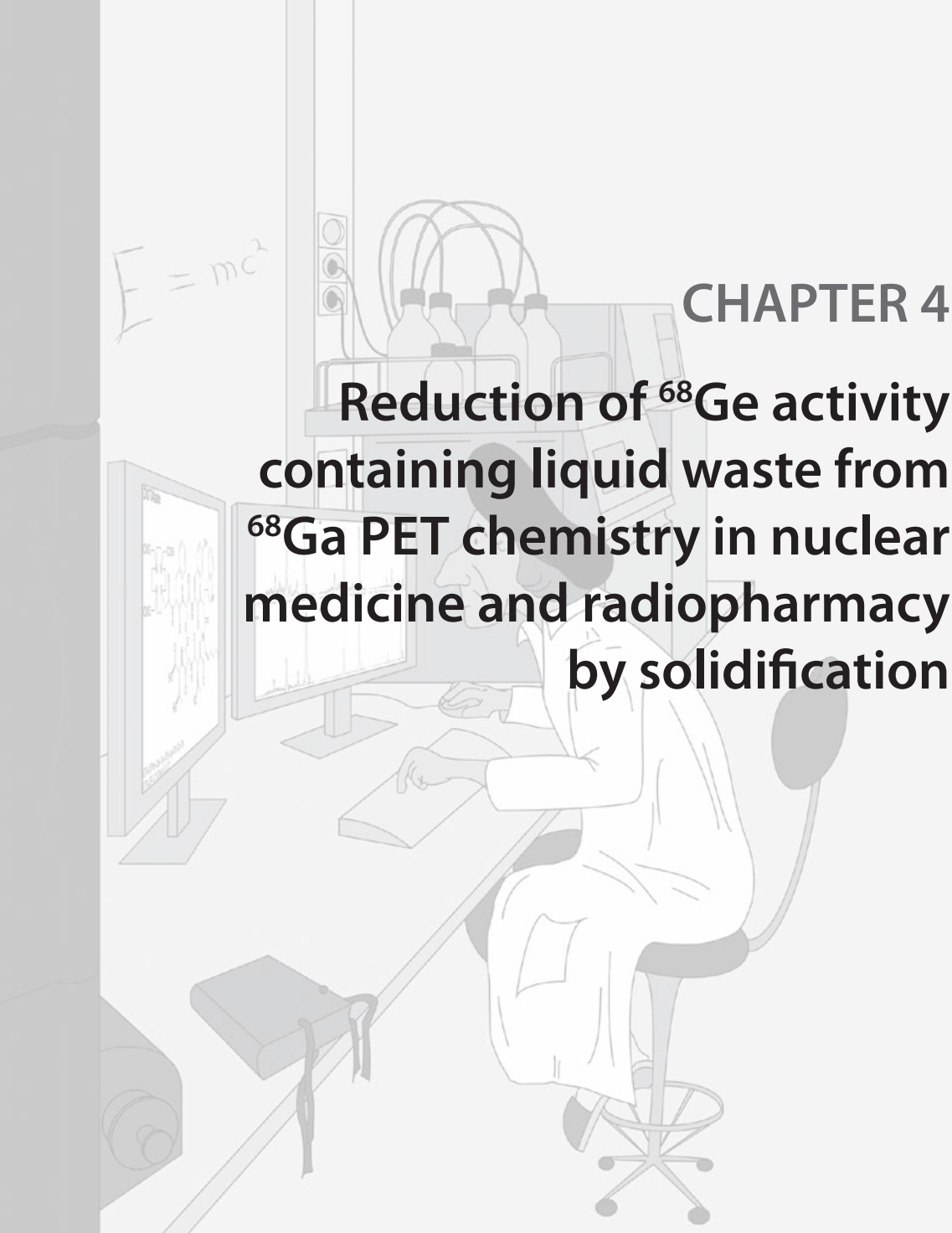

CHAPTER 4

Reduction of ^{68}Ge activity containing liquid waste from ^{68}Ga PET chemistry in nuclear medicine and radiopharmacy by solidification

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Abstract

PET with ^{68}Ga from the TiO_2- or SnO_2- based ^{68}Ge/^{68}Ga generators is of increasing interest for PET imaging in nuclear medicine. In general, radionuclidic purity (^{68}Ge vs. ^{68}Ga activity) of the eluate of these generators varies between 0.01 and 0.001%. Liquid waste containing low amounts of ^{68}Ge activity is produced by eluting the ^{68}Ge/^{68}Ga generators and residues from PET chemistry. Since clearance level of ^{68}Ge activity in waste may not exceed 10 Bq/g, as stated by European Directive 96/29/EURATOM, our purpose was to reduce ^{68}Ge activity in solution from >10 kBq/g to <10 Bq/g; which implies the solution can be discarded as regular waste. Most efficient method to reduce the ^{68}Ge activity is by sorption of TiO_2 or Fe_2O_3 and subsequent centrifugation. The required 10 Bq per mL level of ^{68}Ge activity in waste was reached by Fe_2O_3 logarithmically, whereas with TiO_2 asymptotically. The procedure with Fe_2O_3 eliminates ≥ 90% of the ^{68}Ge activity per treatment. Eventually, to simplify the processing a recirculation system was used to investigate ^{68}Ge activity sorption on TiO_2, Fe_2O_3 or Zeolite. Zeolite was introduced for its high sorption at low pH, therefore ^{68}Ge activity containing waste could directly be used without further interventions. ^{68}Ge activity containing liquid waste at different HCl concentrations (0.05–1.0 M HCl), was recirculated at 1 mL/min. With Zeolite in the recirculation system, ^{68}Ge activity showed highest sorption.

Introduction

PET scintigraphy with ^{68}Ga-labeled peptides is of increasing interest in PET imaging in nuclear medicine **[1-8]** and performed at [40 centres in Europe (current situation, 2010). In general, radionuclidic purity (RNP) of the eluate of these generators varies between 0.01 and 0.001% (^{68}Ge vs. ^{68}Ga activity). Liquid waste containing low amounts of ^{68}Ge activity (further referred as ^{68}Ge waste) is produced by eluting the ^{68}Ge/^{68}Ga generators and residues from PET chemistry. For several reasons in our facility, solid ^{68}Ge containing waste (t½ = 9 months) is preferred over liquid waste. Since clearance level of ^{68}Ge activity in waste may not exceed 10 Bq/g, as stated by European Directive 96/29/EURATOM, our aim was to reduce ^{68}Ge activity in solution from >10 kBq/g to <10 Bq/g; which implies the solution can be discarded as regular waste. As an example: when a 1,110 MBq ^{68}Ge/^{68}Ga generator with a RNP of 0.005%, is eluted with 6 mL 1 M HCl, the eluate contains 55.5 kBq ^{68}Ge activity. Eluting a 1,110 MBq ^{68}Ge/^{68}Ga generator three times a day, 200 days per year, and for 1 year will result in 33.3 MBq in 3.6 L (9.25 kBq/g). Although national legislation within the EU may vary per country, the rules may only be more strict. For example in our country, The Netherlands, legislation does not allow to store radioactive waste with half-lives of more than 100 days at local institutes for more than 2 years. After 2 years it is obligatory to store the here described waste in a special external waste facility, and at additional costs. In this facility waste is preferably compressed, therefore, liquid content is limited to<1% (v/w). The purpose of this study was to quantify ^{68}Ge activity in our ^{68}Ge liquid waste, and to concentrate and to transform ^{68}Ge liquid waste to solid waste. In short: $^{68}Ge_{liquid} \rightarrow {}^{68}Ge_{solid}$, initially on a small scale. Liquid ^{68}Ge waste was transformed to ^{68}Ge solid waste by sorption of TiO_2, Fe_2O_3 (325 and 500 mesh, respectively, Sigma-Aldrich, Zwijndrecht, The Netherlands) and Zeolite ($Na_2Al_2Si_3O_{10}{\cdot}2H_2O$, Zeolyst international, Conshohocken, PA, USA). Elution of ^{68}Ge/Ga generator is performed with HCl, therefore ^{68}Ge waste is acidic. Zeolite was investigated because high sorption at low pH could be achieved (manuscript Kamalika Roy submitted).

Experimental

^{68}Ge-containing liquid waste

Fractionated elution of the generator revealed that the activity of ^{68}Ge (e.g. Bq per mL) was constant during elution, whereas the main ^{68}Ga activity could be collected in a small volume (1 mL)**[1]**. Eluate from generators and left-over after ion-exchange prior radiolabeling is acidic and contain a certain amount of ^{68}Ge activity **[4, 8]**. Quantification of ^{68}Ge activity was performed in a welltype gamma counter as described earlier **[8]**. During 5 years of daily practice with 4TiO_2- and 4SnO_2-based ^{68}Ge/^{68}Ga generators (0.4–2 GBq per generator) the total amount of ^{68}Ge activity was 160 MBq in 48 L.

TiO_2 and Fe_2O_3 as sorbent

Sorption of ^{68}Ge activity on variable amounts of TiO_2 and Fe_2O_3 was investigated with 1 mL samples of ^{68}Ge waste as f[pH] (pH range 1–10) and time (range 1–24 h). These results were used for further up scaling. Typical example: 150 mL of ^{68}Ge waste was mixed with ±1.5 g of TiO_2 or Fe_2O_3 and centrifuged at 200xg. One mL of supernatant was taken as a sample and ^{68}Ge activity was quantified 24 h after sampling as described earlier **[1].**

Recirculation system

Eventually, to simplify the processing of $^{68}Ge_{liquid} \rightarrow {}^{68}Ge_{solid}$ a recirculation system was used to investigate ^{68}Ge activity sorption (see Fig. **1**) on TiO_2, Fe_2O_3 and Zeolite. Euate of ^{68}Ge/Ga generator is acidic, therefore Zeolite was also investigated because high sorption at low pH could be achieved (manuscript Kamalika Roy submitted). ^{68}Ge activity on TiO_2, Fe_2O_3 or Zeolite was monitored time dependently. Recirculation system contained ±1 g of sorption material enclosed with cotton, glass fiber and filters of 35 and 0.45 µm.

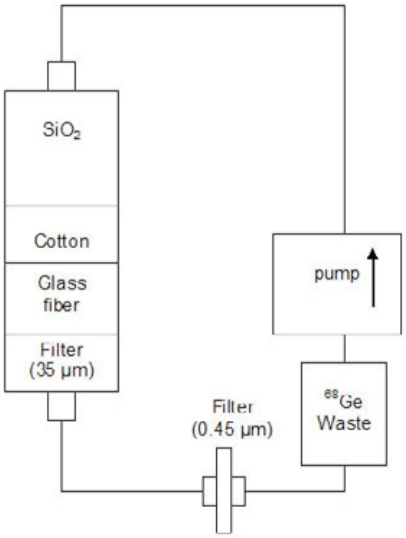

Figure 1. Recirculation system containing ± 1g of TiO_2, Fe_2O_3 or Zeolite as sorption material enclosed with cotton, glass fiber and filters of 35 and 0.45 µm. ^{68}Ge activity containing waste was recirculated at a flow rate of ± 1 mL/min. Samples of the circulating liquid were taken at indicated time points (see also Figs. **5** and **6**)

Results and discussion

Firstly, sorption on TiO_2, Fe_2O_3 was investigated time dependently. Sorption of ^{68}Ge activity on TiO_2 (0.5 g/50 mL ^{68}Ge waste) was low (<20%) at pH<2 and high (>99%) at pH >6 (Fig. **2**). Sorption of ^{68}Ge activity on Fe2O3 (0.5 g/50 mL ^{68}Ge waste), showed better result at pH 1–3 (>75% Fe_2O_3 and<25% TiO_2) and increased also as f[pH] up to >99% at pH 8. Optimal sorption was obtained at pH ≥5 (Fig. **2**). Secondly sorption on TiO_2, Fe_2O_3 was investigated as ¦[mass] and additions of sorption material.

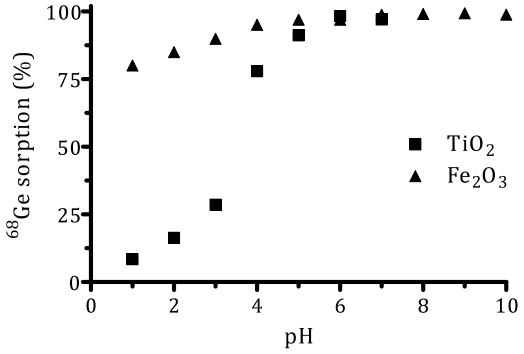

Figure 2. Sorption of ^{68}Ge (10 kBq/gram) on Fe_2O_3 or TiO_2 was investigated as f(pH). After 24 h the samples were centrifuged. Supernatant was decanted and ^{68}Ge activity was quantified. Sorption of ^{68}Ge activity using TiO_2 or Fe_2O_3 was optimal at pH >8.

Sorption of ^{68}Ge activity increased after each addition of sorption material. Moreover, Fe_2O_3 surprisingly continues to lower the ^{68}Ge activity logarithmically after each addition of Fe_2O_3, whereas with TiO_2 the value of 10 Bq ^{68}Ge per mL level was reached asymptotically. The procedure with Fe_2O_3 as sorption material reduces the ^{68}Ge activity by approximately 90% after each addition of sorption material (Fig. **3**), eg. From >10 kBq/g to <10 Bq/g in six procedures.

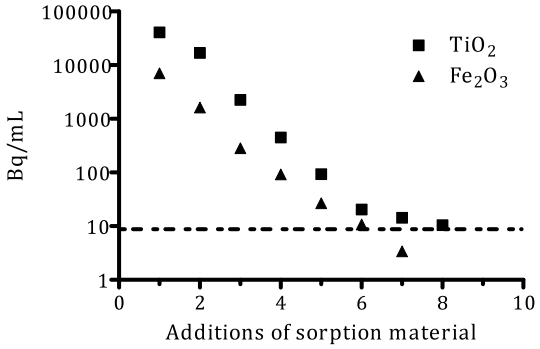

Figure 3. Samples of ^{68}Ge/^{68}Ga generator eluate (50 mL) were used to investigate ^{68}Ge activity sorption on 0.5 g TiO_2 or 0.5 g Fe_2O_3. pH of the eluate was controlled by addition of phosphate buffer, final pH was ~8, and gently vortexed. Twenty-four hours after each addition, the 50 mL was centrifuged and a sample of the supernatant was collected to quantify ^{68}Ge activity.

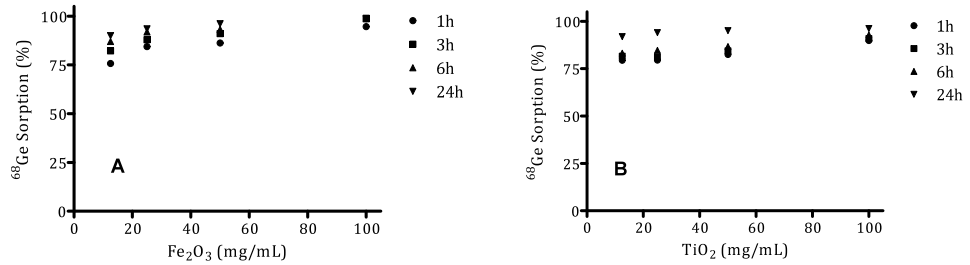

Figure 4. Complexation of ^{68}Ge activity of ^{68}Ge containing liquid waste (pH 8) with Fe_2O_3 (**A**) or TiO_2 (**B**) as sorbent as function of time (1-24h) and mass of sorbent (10-100 mg/mL).

Low amounts (10 mg/mL) of sorbent showed similar ^{68}Ge activity sorption at later time points in comparison with an amount of 100 mg/mL (Fig. **4A-B**). Zeolite was introduced since this has high sorption at low pH, therefore it was tested as (pH) (Fig. **5**). With Zeolite in the recirculation system (Fig. **1**), ^{68}Ge waste (0.05–1.0 M HCl) showed highest sorption (Figs. **6** and **7**). In our facility, eluate purification and concentration with anion chromatography with 5 M HCl is also applied **[4].** The waste stream is thus very acidic, but Zeolite as sorption material showed similar results as with 1 M HCl(Fig. **7**). Summarized, recirculation system, using Zeolite as sorbent, is more efficient, less time consuming and reduces ^{68}Ge waste mass in comparison with TiO_2 and Fe_2O_3 procedure. The overall ^{68}Ge containing liquid waste was 160 MBq in 48 L and could be concentrated to 160 MBq in<1 kg solid waste

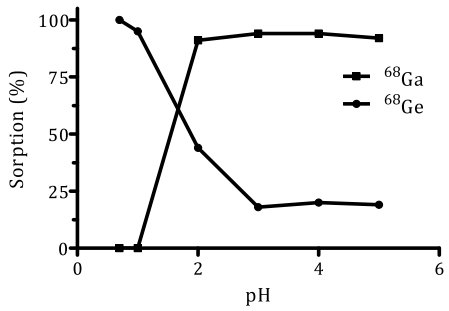

Figure 5. Sorption of ^{68}Ge on Zeolite was investigated as *f* (pH). After 24 h the samples were centrifuged. Supernatant was decanted and ^{68}Ge activity was quantified. Sorption of ^{68}Ge activity using Zeolite was optimal at pH <1.

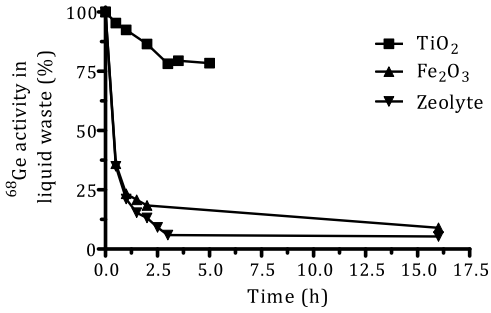

Figure 6. Recirculation system with ^{68}Ge waste in 1 M HCl. One gram of sorption material was enclosed, see Fig. **1**. ^{68}Ge activity was quantified at indicated time points. Zeolite showed optimal sorption of ^{68}Ge activity within 4 h (> 95%).

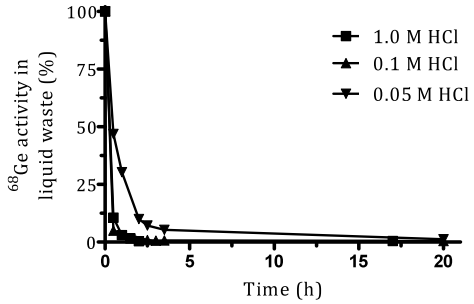

Figure 7. Since Zeolite showed an optimal sorption of ^{68}Ge activity (Fig. **5**), a column containing Zeolite (± 1g, Fig. **1**) was tested with ^{68}Ge waste in 0.05, 0.1 or 1.0 M HCl. ^{68}Ge activity was quantified at indicated time points. Recirculation system using higher [HCl] showed high sorption of ^{68}Ge activity at earlier time points.

Conclusion

TiO_2, Fe_2O_3 and Zeolite as sorbent, lower ^{68}Ge-containing liquid waste down to 10 Bq/g. Recirculation procedure using Zeolite as sorbent is preferred since less intervention with ^{68}Ge containing liquid waste has to be performed and solid waste mass is reduced.

Acknowledgement

The authors wish to thank Timo Kleijn for technical assistance.

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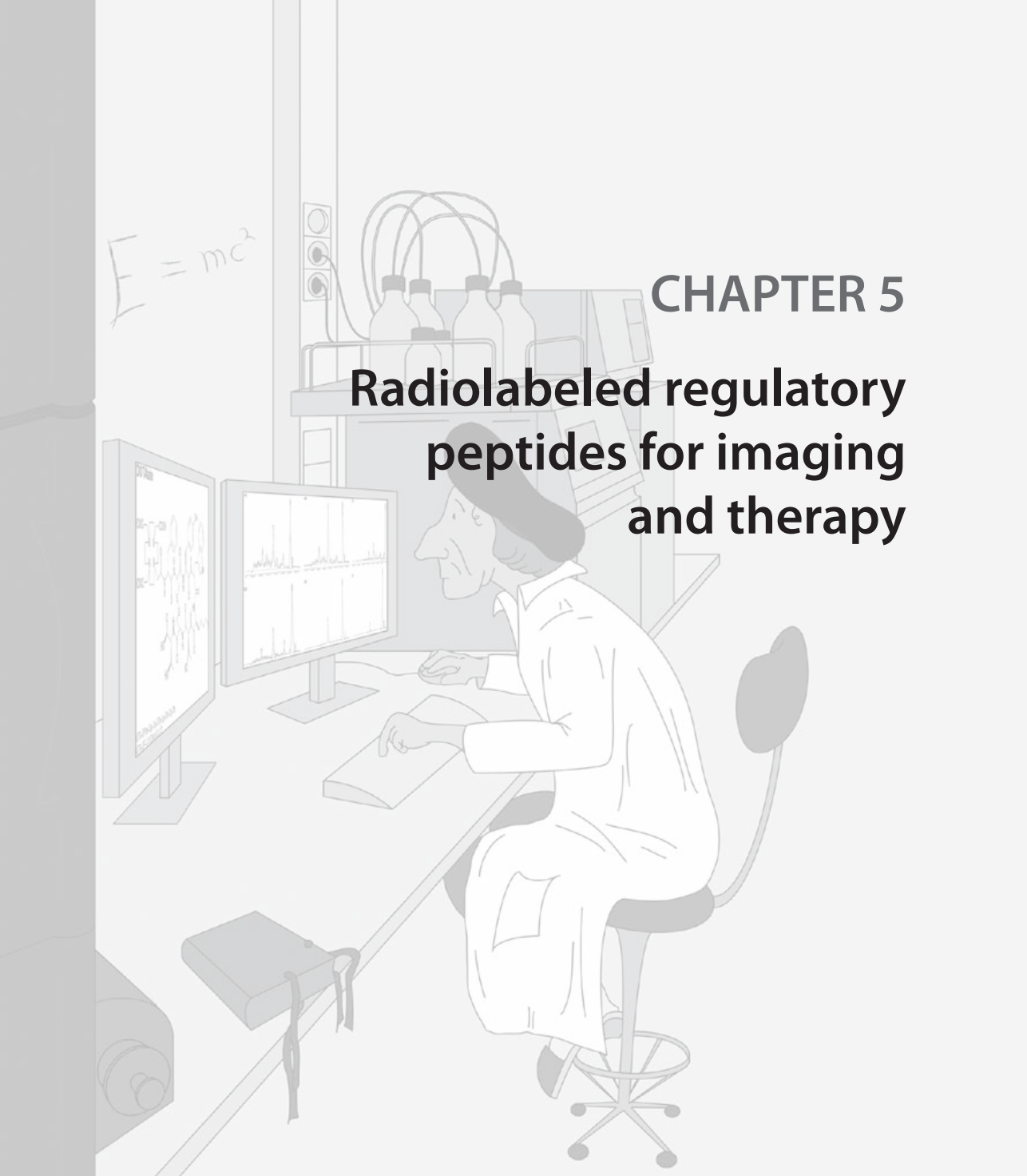
PART II

Investigation of radiolysis and measurement of radiochemical purity



CHAPTER 5

Radiolabeled regulatory peptides for imaging and therapy

Wouter A. P. Breeman, Dik J. Kwekkeboom, Erik de Blois,
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Abstract

Radiolabeled peptides have shown to be an important class of radiopharmaceuticals for imaging and therapy of malignancies expressing receptors of regulatory peptides. These peptides have high affinity and specificity for their receptors. The majority of these receptors are present at different levels in different tissues and tumors. This review focuses on the application of regulatory peptides radiolabeled with $^{67/68}$Ga , ^{90}Y, ^{111}In or ^{177}Lu. Due attention is given to the current status of research, limitations and future perspectives of the application of these radiolabeled peptides for imaging and radiotherapy. It also covers elements of the basic science and preclinical and clinical aspects in general, however, mostly based on somatostatin receptor-mediated imaging and therapy. New analogs, chelators, radionuclides and combinations thereof are discussed.

General aspects of peptide receptor targeting

Introduction

Peptide receptor imaging with radiolabeled peptides has been shown to be a sensitive and specific technique, for instance to demonstrate the presence of somatostatin receptors on various tumors *in vivo* **[1, 2]**. With this technique, somatostatin receptor-positive primary tumors and metastases can be visualised. As soon as the success of peptide receptor imaging for tumor visualisation became clear, the next logical step was to label these peptides with radionuclides emitting α- or β-particles, including Auger or conversion electrons, and to perform peptide receptor radionuclide therapy. Since somatostatin-14 (somatostatin) and its analogs are the most frequently used of all peptides for imaging and peptide receptor radionuclide therapy, their application is the main focus of this review. Extensive general reviews on this subject have been published recently **[3-12]** as well as reviews focused on the somatostatin receptor **[13-16]**.

Somatostatin

Somatostatin is a cyclic disulphide-containing peptide hormone of 14 amino acids. It is present throughout the central nervous system (CNS), including the hypothalamus, the cerebral cortex and the brain stem, and in the gastrointestinal tract, such as the pancreas. Somatostatin receptors have been identified in the CNS, the gastrointestinal tract, and on many cells of neuroendocrine origin. However, non-neuroendocrine cells, such as lymphocytes, also possess these receptors. In the CNS, somatostatin acts as a neurotransmitter. The general inhibitory effect of somatostatin on hormone secretion of various glands led to the concept of possible beneficial effects of somatostatin in the treatment of diseases based on gland hyper function or overproduction of hormones by endocrine-active tumors. However, somatostatin itself turned out to be unsuitable for such treatment. After intravenous administration in man, somatostatin has a plasma half-life of ≈ 3 min, owing to rapid enzymatic degradation. As long ago as the early 1980s somatostatin analogs were developed that were more resistant to enzymatic degradation than somatostatin itself. The molecule was modified in various ways while preserving most of the biological activity of the original molecule. Introduction of D-amino acids and shortening

of the molecule to the bioactive core sequence resulted in eight amino acid-containing somatostatin analogs such as octreotide. Octreotide and analogs are widely used for the symptomatic treatment of neuroendocrine-active tumors, such as growth hormone-producing pituitary adenomas and gastroenteropancreatic (GEP) tumors. The biology and clinical application of somatostatin and analogs have been described extensively **[17, 18]** and **[19, 20]** for reviews. In addition to their use as carriers of radionuclides, the specificity and high affinity of these peptides for their receptors are also exploited under preclinical conditions for the targeting of other cargo such as cytotoxics **[21]**, liposomes **[22, 23]**, radionuclides and contrast agents to specific tissues and tumors **[24, 25]**. The affinity of these peptides for their G-protein coupled receptors are in the nanomolar range.

Somatostatin receptor subtypes

Somatostatin receptors have been described in a large number of human primary tumors and cell lines. With the use of different radioiodinated somatostatin analogs it was possible to differentiate somatostatin receptors pharmacologically. Initially, two somatostatin receptor classes were described: class 1 was defined as displaying a relatively high affinity for the biologically stable ligands, such as octreotide, while class 2 had no affinity for the latter. Five different human somatostatin receptor subtypes have been cloned and splice variants have been reported. These have been named somatostatin receptor subtypes 1–5, according to chronological order of discovery. Each somatostatin receptor subtype is the product of a single gene located on a different chromosome. This allows a tissue-specific regulation of their expression and suggests diverse functions of the somatostatin receptors in different organs. Structurally, the somatostatin receptor subtypes belong to the family of G protein-coupled receptors characterised by seven transmembrane domains. The extracellular part is responsible for the ligand binding, while the intracellular domains transduce the signal into the cell **[18, 26]**. Four of the genes are intronless, the exception being somatostatin receptor subtypes 2 **[27]**. The somatostatin receptor subtype 2 comprises two isoforms generated from the same gene: subtype 2a, the unspliced form, and subtype 2b, a 23 amino-acid shorter splice variant **[28]**. The function of the subtype 2b is still unknown. All subtypes bind somatostatin-14 and somatostatin-28 (a polypeptide of 28 amino acids with somatostatin-14 at its C-terminus) with high affinity, while the affinities of numerous somatostatin analogs for the five somatostatin receptor subtypes differ considerably. Pharmacological studies in cells transfected with complementary DNA encoding the various somatostatin receptor subtypes have demonstrated that somatostatin receptor subtypes 2, 3 and 5 correspond to the formerly named class 1 receptors. The somatostatin receptor subtypes 1 and 4 correspond to class 2. Recently affinity profiles (IC_{50}) for human somatostatin receptor subtypes 1-5 of a series of somatostatin analogs have been reported, see Table **1** **[29-31]**. In Fig. **1-3** structural formulae of 3 somatostatin analogs are presented.

Studies were performed on cells stably transfected with the five different human somatostatin receptor subtypes (sst1-5), and ^{125}I-[Leu^{8}, D-Trp^{22}, Tyr^{25}]-somatostatin-28 as universal radioligand, and the peptides as competitors at increasing concentrations. IC_{50} is the concentration of the ligand that inhibits the binding of the radioligand for 50%, and is an indication for the affinity of the ligand for its receptor, data are expressed in nM, mean ± SEM. Data of these DOTA-conjugated somatostatin analogs complexed with Lu are not yet available in literature.

Table 1. Affinity profiles (IC_{50}) for human somatostatin receptor subtypes 1-5 (sst1-sst5) of a serie of somatostatin analogs, adapted from [29-31], *: [30], **: [31]

Peptides	sst 1	sst 2	sst 3	sst 4	sst 5
SS-28	5.2 ± 0.3	2.7 ± 0.3	7.7 ± 0.9	5.6 ± 0.4	4.0 ± 0.3
Octreotide	>10,000	2.0 ± 0.7	187 ± 55	>1,000	22 ± 6
[DTPA^0]octreotide	>10,000	12 ± 2	376 ± 84	>1,000	299 ± 50
[In-DTPA^0]octreotide	>10,000	22 ± 4	182 ± 13	>1,000	237 ± 52
[DOTA^0]octreotide	>10,000	14 ± 3	27 ± 9	>1,000	57 ± 22
[DTPA^0,Tyr^3]octreotate	>10,000	3.9 ± 1	>10,000	>1,000	>1,000
[DOTA^0,Tyr^3]octreotate	>10,000	1.5 ± 0.4	>1,000	453 ± 176	547 ± 160
[Y-DOTA^0,Tyr^3]octreotate	>10,000	1.6 ± 0.4	>1,000	523 ± 239	187 ± 50
[DOTA^0,Tyr^3]octreotide	>10,000	14 ± 3	880 ± 324	>1,000	393 ± 84
[Y-DOTA^0,Tyr^3]octreotide	>10,000	11 ± 2	389 ± 135	>10,000	114 ± 29
[Ga-DOTA^0,Tyr^3]octreotide	>10,000	2.5 ± 0.5	613 ± 140	>1,000	73 ± 21
[In-DOTA^0,Tyr^3]octreotide *	>10,000	4.6 ± 0.2	120 ± 26	230 ± 82	130 ± 17
In-DOTA-NOC **	>10,000	2.9 ± 0.1	8 ± 2	227 ± 18	11.2 ± 3.5
Y-DOTA-NOC **	>1,000	3.3 ± 0.2	26 ± 1.9	>1,000	10.4 ± 1.6

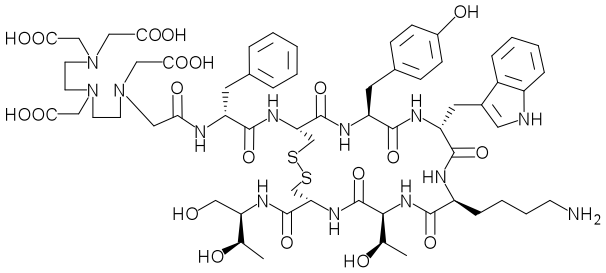
As will also be discussed later, the presence of metal in the chelator also effects the IC_{50}-values and the biodistribution of these radioligands. Receptor scintigraphy with radioactive somatostatin analogs is based on the visualisation of octreotide-binding somatostatin receptors: somatostatin receptor subtypes 2, 3 and 5 (for reviews: **[1, 17, 19, 20, 32]**). Patel postulated that in most instances somatostatin receptor subtypes operate in concert, rather than as individual members **[27]**. Nonetheless, there is evidence for somatostatin receptor subtype-selective actions, such as arrest of cell growth through somatostatin receptor subtypes 1, 2, 4 and 5, and unique cytotoxic actions by somatostatin receptor subtype 3 **[27]**. It was also reported that somatostatin receptor subtypes have different rates of internalisation, somatostatin receptor subtype 3 has the highest rate and subtype 1 even fails to internalise **[19, 33]**. For small and rapidly degradable (radio)ligands that do not (or hardly) internalise, the result will be a short residence time of the radioactivity at their target, which might hamper their application in nuclear medicine **[15]**. However, most of these models are in *in vitro* models and still have to be proven *in vivo* **[33]**.

Due to their relatively small size, peptides have favourable pharmacokinetics and are cleared rapidly from the circulation, with rapid excretion of the intact peptides or metabolites in faeces and/or urine **[3, 15]**. The pharmacokinetics of the process of binding and internalisation is illustrated since the intravenous administration of excess of ligand 30 min post radioligand failed to displace the radioligand from its receptor, thus the radioligand is already internalised **[34]**. This frequently results in a high level of accumulation of radioactivity in their target and in high target to non-target ratios.

Receptors and acceptors

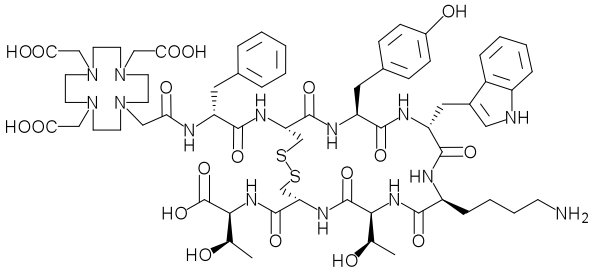
Receptors are frequently defined as proteins that bind their ligands with high affinity and selectivity, and, depending on occupancy, produce signals that ultimately influence cell function and/or growth **[35]**. Examples of regulatory peptides are presented in Table **2**, the majority of the concordant receptors

Figure 1. DTPA-Octreotide



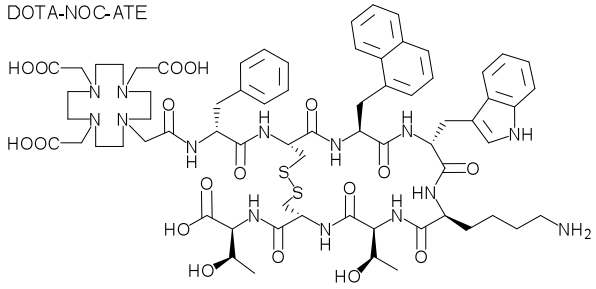
DTPA-DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr(ol)
DTPA-DF-C-F-DW-K-T-C-T(ol)

Figure 2. DOTA-TATE



DOTA-DPhe-Cys-Phe-DTrp-Lys-Thr-Cys-Thr
DOTA-DF-C-F-DW-K-T-C-T

Figure 3. DOTA-NOC-ATE



DTPA-DPhe-Cys-(1-Nal)-DTrp-Lys-Thr-Cys-Thr
DTPA-DF-C-(1-Nal)-DW-K-T-C-T

Figures 1-3. Structural formulae [DTPA^0]octreotide (=OctreoScan), [DOTA^0, Tyr^3]octreotide (=DOTATOC) and DOTA-NOC-ate. [DTPA^0]octreotide is an octreotide analog conjugated with the chelator DTPA at octreotides N-terminal side (Fig. **1**). It was designed for scintigraphy with ^{111}In, however, DTPA is not suitable for therapeutic β-emitting radionuclides such as ^{90}Y and ^{177}Lu, the macrocyclic DOTA is more suitable here for, and forms kinetically and thermodynamically stable complexes. In addition, the amino acid phenylalanine at the third position (Phe^3) in octreotide is replaced by another amino acid tyrosine (Tyr^3), which resulted in a peptide with higher affinity for the somatostatin receptor subtypes 2, 3 and 5, see Table **1**. DOTATOC is thus the abbreviation of **DOTA**-**T**yr^3-**Oc**treotide . In DOTA-tate (Fig. **2**) the alcohol group at the COOH-terminus of octreotide is replaced by a carboxylic acid group, and led to increased affinity and selectivity for somatostatin receptor subtype 2, see Table **1**. As can also be seen in Table **1** the affinity of the analogs varies for the different somatostatin receptor subtypes, but are also influenced by the different metals. The explanation hereof is the coordination of the metal in the chelator, and effects the charge of the molecule, and effects not only the IC_{50} (Table **1**), but also its biodistribution, (e.g. see also Fig. **9**). DOTA-NOC-ate is an analog of DOTA-NOC **[31]**, with a C-terminal carboxy group (Fig. **3**).
DOTA=[1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid]; NOC=Nal^3-octreotide;
DTPA= Diethylenetriaminepentaacetic acid.

belong to the family of G protein-coupled receptors **[3, 10]**. After binding of the ligand to its receptor, the cell reacts with a physiological response, for instance the secretion of bioactive peptides (see Table **2**). The physiological response to the binding of somatostatin to its receptor(s) includes the inhibition of secretion of bioactive substances such as growth hormone, insulin and glucagon. This is employed for instance in the medical treatment of acromegaly with octreotide. In contrast, binding of peptides such as bombesin, substance P and vasoactive intestinal peptide often results in the stimulated secretion of bioactive substances and concordant pharmacological effects. For the applications in nuclear medicine, however, we are interested in binding and internalisation of radiolabeled targeting peptides, preferably with a minimum physiological response of the tissues. The receptors of regulatory peptides are normally present at low levels of 10^{-9} to 10^{-13} mol/g tissue **[24, 35, 36]**. Such a range of receptor densities was also determined in somatostatin receptor-positive tumors in patients **[35]**. Other peptides bind to members of other receptor families for targeting anticancer drugs, such as the tyrosine kinase receptors, but the utilization of these receptors for scintigraphy or receptor-targeted radiotherapy has been limited so far. Other membrane proteins that can be employed for these purposes are another type of receptors. These receptors are also involved in so-called receptor-mediated endocytosis **[37]**. Their major physiological function, however, resides in the cellular uptake of essential factors such as vitamins, lipids, minerals and polypeptides rather than eliciting a biological response **[37]**. Lipoprotein receptors and related proteins such as megalin are also typical examples of such receptors. These receptors are also known as acceptors **[37]**. In contrast, these acceptors are involved in transport of ligands (such as transferrin, LDL, apoferritin, asialoglycoprotein and folate) and suffer less from the above-mentioned biochemistry in that they are present at much higher effective numbers and are less restricted by saturation. Therefore, these are better suited to be used in MRI-based visualization techniques **[38, 39]**. The effects of saturation of receptors will also be discussed in **Effect of dose and specific activity.**

Table 2. A selection of regulatory peptides with concordant pharmacological responses.

Regulatory peptide	Pharmacological response
Bombesin	smooth muscle contraction, gut hormone release
Cholecystokinin	gall bladder contraction
Somatostatin	stimulation of inhibition of secretions
Substance P	contraction and relaxation of vascular smooth muscle
Vasoactive Intestinal Peptide	vasodilatation, water and electrolyte secretion in gut

Note: that all peptides have stimulatory characteristics, however, somatostatin stimulates the inhibition of secretion, thus, eventually somatostatin has inhibitory effects

Magnetic Resonance: contrast agents and sensitivity

Development of targeted magnetic resonance imaging (MRI) contrast agents will dramatically expand the range of MRI applications by combining the non-invasiveness of and high spatial resolution of MRI with specific localisation of molecular targets. However, due to the intrinsically low sensitivity (in comparison to nuclear medicine, *see below and in* **[24, 35, 40, 41]**), high local concentrations of the contrast agent at the target are required to generate detectable MRI contrast. In case of the injection

of 370 MBq of a ^{11}C-labeled PET (Positron Emission Tomography) tracer with a specific activity of 370 MBq · nmol^{-1} (thus representing a dose of 1 nmol ligand) uniform distribution throughout a 70 kg body would result in a concentration of $1.4 \cdot 10^{-11}$ M. This concentration can be easily detected by modern gamma cameras. Further concentration of the injected material in the tissues expressing the receptors, will, of course, improve detection **[35]**. For MRI, the threshold for achieving a 50% increase in image enhancement is attained at a tissue concentration of approximately $5 \cdot 10^{-7}$ moles per gram ($5 \cdot 10^{-4}$ M) at 2 Tesla using a standard T1 weighted spin echo pulse sequence **[35]**. Thus, contrast agent concentrations of 10^{-5} to 10^{-4} M are required **[24, 40, 42]** which exceed the concentration of regulatory peptide receptors. Although the physics is favourable the biology is less so, even before saturation of these receptors unwanted physiological responses can be expected, like hypotension and flushes as reported with DTPA-substance P **[43]**.
Moreover, receptors are frequently heterogeneously distributed **[20, 44]** and low in concentration **[35]**. With internalisation and retention comes the threat of increased toxicity because of the large amounts of contrast agent involved **[24, 41]**. Encouraging results *in vitro* for the folated-gadolinium-dendrimers targeted to folate-receptors expressing leukemic cells are reported, which induce receptor-specific significant changes in relaxation times **[45]**. For the future minimum concentrations for the visibility of MRI contrast agent of a next generation dendrimer with maximal relaxivity agent are predicted at $5.2 \cdot 10^{-12}$ M and for super paramagnetic iron particles at $1.6\ 10^{-11}$ M **[35]**.

There are many aspects that influence the interaction of a radioligand with its receptor, including the **Effect of dose and specific activity** In saturable regulatory peptide binding processes (i.e. *in vitro* techniques, such as radioimmunoassay and receptor binding), the signal to background ratio is often improved by increasing the specific radioactivity of the ligand. However, *in vivo* results showed that, contrary to what was expected, the percentage uptake of radiolabeled [DTPA^0]octreotide in octreotide receptor-positive tissues is not optimal at the lowest possible dose of maximum specific radioactivity. Rather, the uptake expressed as percentage of the administered dose is a bell-shaped function of the injected mass. These findings might be the result of two opposing effects, i.e. a negative effect due to saturation of the receptor at increasing ligand concentrations and a positive effect of increasing ligand concentrations on, for instance, the rate of internalisation by ligand-induced receptor clustering **[15]**. This implies that the sensitivity of detection of somatostatin receptor-positive tumors by peptide receptor scintigraphy might be improved by administration of an optimised dose of radioligand, as was found for other radioligands **[46-49]**. These findings have also been confirmed in patients, in whom it was shown that coupling a standard dose of 220 MBq ^{111}In to less than 5 µg [DTPA^0]octreotide led to decreased quality of imaging with a significantly reduced uptake in tumors **[15, 50]**. Therefore, [DTPA^0] octreotide scans should always be performed using at least 10 µg peptide. Anderson *et al*. reported reduced uptake in somatostatin receptor-positive tumors in a patient after the single administration of 40 µg peptide *versus* multiple injections of 10-20 µg peptide **[51, 52]**. These data indicate that the receptor can rapidly be saturated, this also implies that the range for optimal uptake versus injected amount of lgand is rather small. Moreover, Jonard *et al*. presented data on tissue distribution after the administration of [^{86}Y-DOTA^0,Tyr^3]octreotide (^{86}Y-DOTATOC) in the range of 50 to 500 µg: the kidney dose was not affected, however, tumor dose here also decreased relatively with higher peptide amounts **[53]**.

From the above-mentioned data it can be concluded that high specific activity is necessary. Further studies are needed to investigate and optimize the amount of ligand to be administered. This subject, together with dose fractionation, will also be discussed in the following sections.

Maximal achievable specific activity

^{111}In-DTPA-peptides have proven to be very useful in peptide receptor scintigraphy. ^{111}In-DTPA-peptides can be labeled up to a level of 1.7 GBq.nmol^{-1} and that is near the theoretical maximum, see Table **3**. Thus, given a certain amount of radioactivity is necessary for detection (by for instance a gamma camera), for optimal target uptake of radioactivity even low amounts (in mass) of ^{111}In-DTPA-peptides can be administered.

Table 3. Physical characteristics of a selection of radionuclides

Production method	^{67}Ga	^{68}Ga	^{90}Y	^{111}In	^{177}Lu
	Cyclotron (p,2n)	Generator	Generator	Cyclotron (p,2n)	Reactor (n, gamma)
Target	^{68}Zn	^{68}Ge	^{90}Sr	^{112}Cd	^{176}Lu
Decay product	^{67}Zn	^{68}Zn	^{90}Zr	^{111}Cd	^{177}Hf
t½ [days]	3.26	4.7E-2	2.67	2.83	6.71
nmoles per GBq	0.68	0.01	0.55	0.58	1.39
Maximal specific activity [GBq·nmol^{-1}]					
Theory[a]	1.48	102	1.81	1.72	0.72
Practice	0.37	>1.0	0.41	0.82	0.12[b],0.42[c]

[a]: One nmol of a DTPA- or DOTA-peptide can incorporate 1 nmol nuclide in theory, this number indicates the maximal theoretical specific activity of the radiolabeled DTPA- or DOTA-peptides.
[b]: data from (n,gamma) reactor produced ^{177}Lu from enriched ^{176}Lu **[54]**.
[c] ^{177}Lu reactor produced via (n,gamma) from enriched ^{176}Yb **[55]**. As an example, 1 nmol of DOTA-peptide can incorporate 0.42 GBq ^{177}Lu.

Since ^{90}Y- and ^{177}Lu-DTPA-peptides lack stability they are not suitable for peptide receptor radionuclide therapy. Therefore, DOTA-conjugated peptides, such as the stabilised somatostatin analogs [DOTA^0, Tyr^3]octreotide (DOTATOC) and [DOTA^0, Tyr^3]octreotate (DOTA-tate, see Fig. **2**) were developed, labeled with radionuclides such as ^{90}Y and ^{177}Lu **[56]**. As mentioned above, the optimal range of the amount of injected (radio)ligand *versus* target uptake is small.
Thus, in order for these radiolabeled peptides to be successfully used in peptide receptor radionuclide scintigraphy and therapy, high specific activities of these radiolgands are required. There are a number of biological factors dictating the need for a high specific activity (SA):
1. For *in vivo* use the affinity of the ligand for its receptor, but also the amount of available receptors limit the amount of ligand or radioligand that can be administered. Increasing the amount of ligand or radioligand will result in a saturation of the receptor, and as a consequence a progressive decrease in fraction of administered label bound and internalised by receptor-expressing tissues and tumors **[15]**.
2. For peptides that display pharmacological side effects, such as DOTA-substance P or DOTA-bombesin (Table **2**), only very small quantities of peptides are tolerated and intravenous administration is limited

to less than 1 nmol per minute **[43, 57-61]**, whereas a high specific activity will reduce the total peptide amount to be administered. However, treatment of cancer without side effects are scarce. This will be discussed in detail in **Dose of radiolabeled DTPA- and DOTA-peptides**.

3. Endocytotic mechanisms that affect the cellular internalisation of peptides may become desensitised at high peptide concentrations **[62]**, which results in lower uptake of radioactivity into target tissue.

4. In addition, for *in vitro* use, the investigations aimed at measuring receptor-binding affinities require low concentrations of these radioligands (e.g. 10^{-10} M) in order to measure receptor-ligand interactions accurately.

Several physical factors influence the highest achievable specific activity of radioligands, e.g. ^{177}Lu-DOTA-tate can be radiolabeled in theory at a level of 0.72 GBq ^{177}Lu per nmol ligand (Table **3**). However, in ^{177}Lu is (n, gamma) reactor-produced from enriched ^{176}Lu, the presence of ^{176}Lu reduces the maximally achievable specific activity in theory at best to 0.12 GBq ^{177}Lu per nmol ligand (Table **3**), In daily practice in our facilities we produce ^{177}Lu-DOTA-tate at a specific activity of 40 MBq per nmol. Recently, (n, gamma) reactor-produced from enriched ^{176}Yb revealed a higher achievable specific activity: 0.42 GBq ^{177}Lu per nmol DOTA-tate (Breeman, unpublished results)(Table **3**).

Unfortunately, the need for high specific activity is often compromised by conflicting parameters, such as the pH and solubility of the radionuclide during radiolabeling. The pH determines reaction rates and yields, i.e. the rate of formation of the metal-DOTA complexes increases with pH, but on the other hand the solubility of In^{3+}, Y^{3+} and Lu^{3+} decreases when pH is increased **[63]**. Moreover, reaction kinetics differ for each radionuclide and reactions can be hampered by contaminants, including contaminants from target material and decay products, see Table **3 [54]**.

A typical reaction mixture for radiolabeling is 37 GBq (1 Ci) $^{177}LuCl_3$ in 1 mL 0.01 M HCl with 1 mg DOTA-tate in 2.5 mL 50 mM sodium-ascorbate and gentisic acid and a final pH (after reconstitution) of 4. Reaction kinetics for labeling DOTA-peptides differ per radionuclide, e.g. with ^{90}Y and ^{177}Lu, reactions at pH 4–4.5 were completed after 20 min at 80°C, while labeling with ^{111}In took 30 min at 100°C **[54]**. After the Radiolabeling and cooling the reaction mixture to room temperature a chelator, such as DTPA or EDTA is added. There are several reasons for this addition. First. since it is hard to take a representative sample from a solution containing DOTA-conjugated analogs labeled with radionuclides that are known to form colloids. For example, in the accurate determination of unchelated ^{90}Y during the standard quality control by ITLC or HPLC, the unchelated ^{90}Y will be rapidly bound to the origin of the ITLC or to HPLC column. This will result in a false identification of the incorporation or radiochemical purity, respectively **[64]**. The addition of a chelator can solve this problem, and the addition is therefore necessary. Second, the free ionic fraction of radionuclide in radiolabeled DOTA-peptides can effectively be complexed by the addition of chelator *in vitro*, and this results in an efficient complexation of the free ionic fraction of radionuclide and excretion as such **[56, 65]**. Since the free ionic fraction of radionuclide in radiolabeled DOTA-peptides can be complexed and rerouted *in vivo* effectively the specification for the % of incorporation is now lowered to 97 %. An example of the effect of contaminants, including contaminants from target material and decay products, is the presence of Cd in ^{111}In, see Table **3 [54]**.

In Fig. **4** the concentration of ^{111}In (measured by Inductively Coupled Plasma Emission Spectrophotometer (ICP) and the intrinsically present ^{111}Cd (calculated) in the ^{111}In-solution are presented as a function of time post the production of ^{111}In. The concentration of Cd, which is a competitor for ^{111}In for the

incorporation in DOTA, was also determined by ICP-measurements at t=0 (i.e. directly after production) the concentration of Cd was already 0.5 µM (range 0.4-0.6 µM, n=5 batches), indicating the presence of target material (=^{112}Cd, see Table **4**). The concentration of ^{111}In at t=0 is 1 µM, see Fig. **4**.

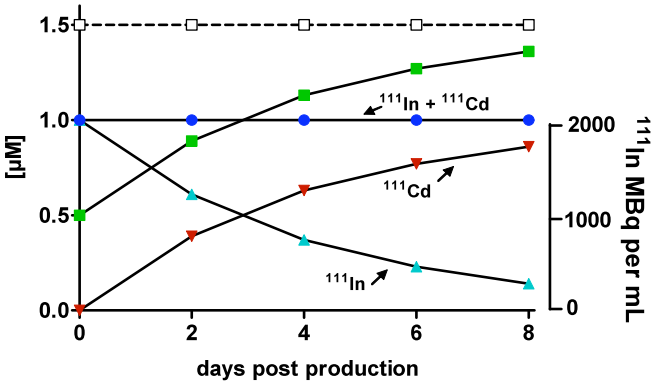


Figure 4. The concentration of ^{111}In (▲, measured), ^{111}Cd (▼, calculated) and Σ(^{111}In+ ^{111}Cd)(●) are presented as a function of time. The concentration of Cd determined by ICP at t=0 (i.e. directly after production) was already 0.5 µM, indicating the presence of target material (=^{112}Cd, ■). The cumulated concentration of ^{111}In, ^{111}Cd, and ^{112}Cd is presented (□). Cd competes with ^{111}In for the incorporation in the DOTA-chelator (see Fig. **6**), therefore, the maximal achievable specific activity of ^{111}In-DOTA-peptides will depend on the concentration of competing contaminants such as Cd. In practice, the theoretical maximal achievable specific activity of 1.72 GBq per nmol DOTA-peptide (see Table **3**), will never be reached. If, for instance, only ingrowing ^{111}Cd is present: the maximal achievable specific activity after one half-life (t½= 2.83 days) will, as a consequence, also be half of the theoretical value of 1.72 GBq ^{111}In per nmol DOTA-peptide.

The effects of the ever-present contaminants such as Cd^{2+} ions on the incorporation of ^{111}In in DOTA-peptides are illustrated in Fig. (**5**). First the effect of the controlled addition of ^{115}In ("non-radioactive indium"): adding ^{115}In, thus diluting the specific activity of ^{111}In will increase the competition of ^{111}In for incorporation in the DOTA-chelator. As a negative control: the addition of Hf^{4+} ions (hafnium, the decay product of ^{177}Lu) has no effect on the % incorporation of ^{111}In (Fig. **5**) and indicates that Hf^{4+} is no competitor for $^{111}In^{3+}$. In contrast, the addition of ions of Cd^{2+} or In^{3+} clearly shows that these ions are strong competitors for $^{111}In^{3+}$ for the incorporation in the DOTA-chelator (Fig. **5**).

Table 4. ^{111}In: Method of production, physical constants, specific activity (SA) of ^{111}In and the maximal theoretical SA of ^{111}In-labeled DOTA-peptides

^{111}In is a cyclotron-produced radiometal, formed from ^{112}Cd by a (p,2n) reaction: **^{112}Cd →^{111}In →^{111}Cd**
t½= 2.83 days, specific activity of ^{111}In ≡ $2.15 \cdot 10^{-11}$ mol per 37 MBq ^{111}In. In theory 1 nmol DOTA-peptide can complex 1 nmol In, corresponding to 1.72 GBq ^{111}In (Table **3**).

The ^{111}In-containing stock solution is diluted by the manufacturer (Mallinckrodt Medical, Petten, the Netherlands) at the day of production to a concentration of approximately 1700 MBq ^{111}In per mL,

corresponding to 1 nmol ^{111}In per mL, or 1 μM ^{111}In. ^{111}In decays to ^{111}Cd, implying that the amount of ^{111}In remaining and that of ^{111}Cd formed can be calculated (see Table **3** and Fig. **4**) as a function of time, using the t½= 2.83 days.

To illustrate these effects of target material and decay products of ^{111}In in the $^{111}InCl_3$ stock solution: the radiolabeling of DOTA-peptides with ^{111}In is presented, while measuring the highest achievable specific activity as a function of time over a week of use of $^{111}InCl_3$ (Fig. (**6**))**[54]**. Thus, as shown in Fig. **6**, the maximal achievable specific activity decreases as a function of time after the production of ^{111}In, as the concentration of ingrowing ^{111}Cd increases (for details hereon see also legend Fig. **4**). These results indicate that In^{3+} and Cd^{2+} have similar reaction kinetics under these reaction conditions. And, although the stability constants of DOTA for In^{3+} and Cd^{2+} differ considerably, 23.9 and 20.9, respectively, at pH 7, the here described incorporations and competitions are determined by kinetics. Stability constants of DOTA with Hf^{4+} and of DOTA-peptides with In^{3+}, Cd^{2+} or Hf^{4+} are currently not available in literature. Analogously with Cd in ^{111}In, achieving high specific activities with ^{67}Ga will also be very difficult owing to the [$^{67/68}$Zn], present from target (^{68}Zn) and formed during decay (Table **3**). Even if the [^{68}Zn] is low or zero, at the end of production and purification of ^{67}Ga, after one half-life of ^{67}Ga the [^{67}Zn]=[^{67}Ga]. There are, however, radionuclides with have much higher specific activities, and not only in theory. For the positron-emitter ^{68}Ga.even specific activities of > 1GBq ^{68}Ga per nmol DOTATOC and DOTA-tate are reported **[66, 67]**(Table **3**).The uptake of ^{68}Ga-DOTATOC in somatostatin receptor-positive tissues, such as pancreas and adrenals was the highest encountered in a rat model, while uptake in kidney was lowest **[67]**. These results confirm the data in mice **[68]** and in patients **[69, 70]**. Besides gammas,^{67}Ga also emits Auger (0.1-8 KeV) and internal conversion electrons (80-90 KeV), and might therefore be suitable for peptide receptor radionuclide therapy **[11]**. Moreover, the current peptide receptor radionuclide scintigraphy clinical trials with ^{90}Y-DOTATOC and ^{177}Lu-DOTA-tate have bone marrow and kidney as the dose-limiting organs. The reported reduction in kidney uptake and the concordant higher tumor uptake of $^{67/68}$Ga-DOTATOC enhances the potential role of ^{67}Ga-DOTATOC and ^{67}Ga-DOTA-tate in peptide receptor radionuclide scintigraphy.

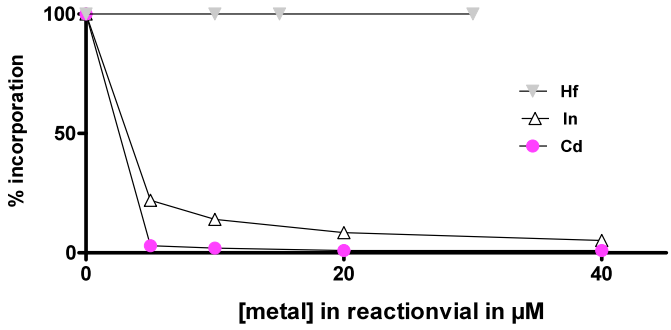


Figure 5. Effects of contaminants on the incorporation of ^{111}In in a DOTA-peptide by the controlled addition of non-radioactive ions of In^{3+} and Cd^{2+} **[54]**. This illustrates that the ever present Cd^{2+}-ions compete with In^{3+}-ions for the incorporation in DOTA, while the addition of Hf^{4+} ions (hafnium, the decay product of ^{177}Lu) has no effect on the % incorporation of ^{111}In and indicates that Hf^{4+} is no competitor for $^{111}In^{3+}$.

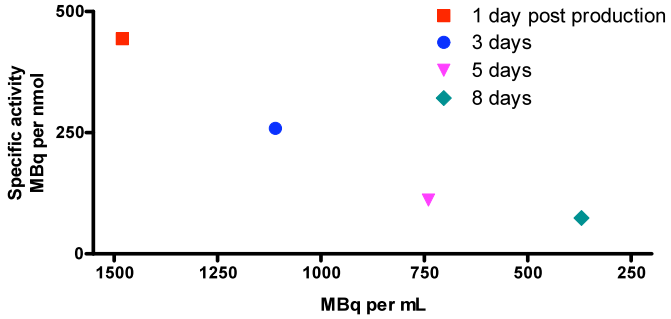


Figure 6. Maximal achievable specific activity of ^{111}In-DOTA-peptide, expressed as a function of time *post* the production of ^{111}In.

Dose of radiolabeled DTPA- and DOTA-peptides

As mentioned above, the optimal dose in mass of a radioligand for peptide receptor radionuclide scintigraphy and therapy needs to be determined. In an attempt to quantify the optimise the dose in order of amounts of mass of ligands and doses of radioactivity the following assumptions and calculations are made. Suppose the optimal and tolerated mass per min of a ligand is **A** nmol·min^{-1}. Thus here presented as administrated dose ligand per min. And suppose this radioligand can be administrated for **B** hours. And suppose with a ligand that can be labeled with a radionuclide at a specific activity of **C** MBq·nmol^{-1}. Taken these factors together, this will result in a radioactive dose of **A**·**C** MBq·min^{-1} and to be administered for **B** min. These values for **A** and **B** has to be determined. For instance, the maximal tolerated value for **A** in patients is frequently subjective and thus arguable. In addition, we are here discussing treatment of cancer, and in case of lack of alternatives, whereas, there are not many options to treat cancer without side-effects. The values for **C** for Ga^{3+}, In^{3+}, Y^{3+} and Lu^{3+} in theory and practice can be found in Table **3**. An example is the administration of bombesin to patients, less than 1 nmol of ligand per min is tolerated **[57, 58]**. Suppose there is no difference in side-effects in patients between bombesin and DOTA-bombesin, then the value of **A** will be maximal 1 nmol per min. Based on our experience of routinely radiolabeling of ^{177}Lu-DOTA-tate, a specific activity of 40 MBq ^{177}Lu per nmol DOTA-tate is achievable (*vide supra*), and suppose this value also holds for other DOTA-peptides, including DOTA-bombesin. Thus **C** will have a value of 40 MBq per nmol. Eventually the term **A**·**C** will have a maximal level of 40 MBq·min^{-1}, and can be administered for **B** min. In summary, the administration of 400 MBq ^{177}Lu-DOTA-bombesin will take 10 min or 4000 MBq ^{177}Lu-DOTA-bombesin will take 100 min.

Moreover, the potentials or success rate for peptide receptor radionuclide scintigraphy and therapy depend on many items, such as the amount of accumulation and retention of radioactivity in target and non-target tissue (thus target to background ratio), and minimal level of detection of the gamma camera. For peptide receptor radionuclide therapy, the radiosensitivity and radiobiology of these tissues need also to be included. Based on these data dosimetric calculation can be made.

Peptide receptor radionuclide therapy in patients with radiolabeled somatostatin analogs

As mentioned earlier, the majority of endocrine GEP tumors possess somatostatin receptors and can therefore be visualized using the radiolabeled somatostatin analog [^{111}In-DTPA^{0}]octreotide. A logical sequence to this tumor visualization *in vivo* was to treat with inoperable or metastasised endocrine GEP tumors with radiolabeled somatostatin analogs.

Clinical studies with [^{111}In-DTPA^{0}]octreotide

Because at that time no other chelated somatostatin analogs labeled with beta-emitting radionuclides were available, early studies in the mid- to late 1990s used [^{111}In-DTPA^{0}]octreotide for peptide receptor radionuclide therapy. Initial studies with high dosages of [^{111}In-DTPA^{0}]octreotide in patients with metastasised neuroendocrine tumors were encouraging with regard to symptom relief, but partial remissions (PRs) were exceptional. Two out of 26 patients with GEP tumors who were treated with high dosages of [^{111}In-DTPA^{0}]octreotide, and who received a total cumulative dose of more than 20 GBq (540 mCi), had a decrease in tumor size of in between 25 and 50%, as measured on CT scans (Fig. **7**). None, however, had PR (Table **5**). In another study in 27 patients with GEP tumors, PR was reported in 2/26 patients with measurable disease (Table **5**). Both series had relatively high numbers of patients who were in a poor clinical condition upon study entry. Also, many had progressive disease when entering the study. The most common toxicity in both series was due to bone marrow suppression. serious side-effects consisted of leukaemia and myelodysplastic syndrome (MDS) in 3 patients who had been

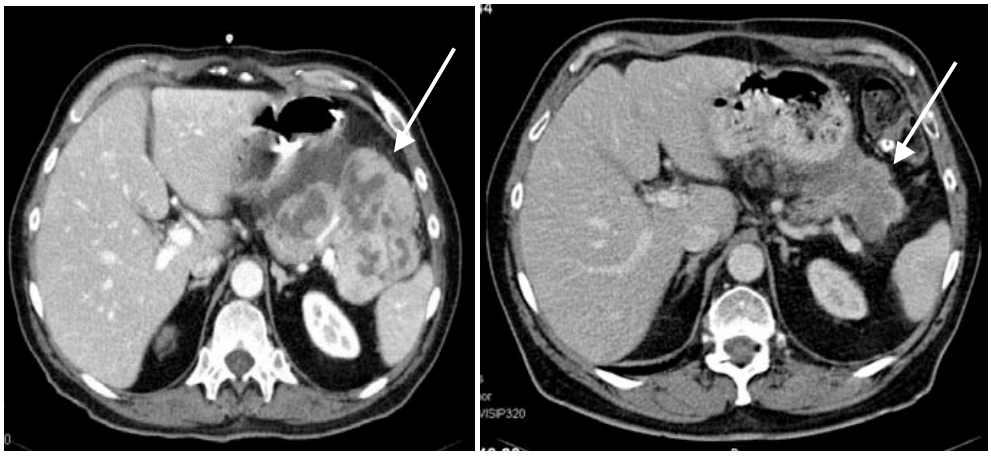


Figure 7. CT scan images in a patient with a non-functioning neuroendocrine tumor of the tail of the pancreas (arrows) before (left) and 6 weeks after the last treatment with ^{177}Lu-DOTA-tate. Before the treatment, the patient had lost more than 15 kg of body weight. At the time of scanning 8 months later (right), he had regained 14 kg. Scaling is identical. Notice the decrease in tumor size and the increase in body circumference.

treated with total cumulative doses of > 100 GBq (2.7 Ci) (and estimated bone marrow radiation doses of more than 3 Gy) [71]. One of these patients had also been treated with chemotherapy, which may have contributed to or caused this complication. Anthony *et al*. reported renal insufficiency in one patient which was probably not treatment-related, but due to pre-existent retroperitoneal fibrosis **[72]**. Transient liver toxicity was observed in 3 patients with widespread liver metastases. Although in both series favourable effects on symptomatology were reported, CT-assessed tumor regression was observed only in rare cases. This is not surprising, since ^{111}In-coupled peptides are not ideal for peptide receptor radionuclide therapy because of the small particle range and therefore short tissue penetration.

Table 5. Tumor response in patients with gastroenteropancreatic tumours, treated with different radiolabeled somatosatin analogs

Center	Ligand	Patient number	CR	PR	MR	SD	PD	CR+PR
Rotterdam **[71]**	OctreoScan	26	0	0	5 (19%)	11 (42%)	10 (38%)	**0%**
New Orleans **[72]**	OctreoScan	26	0	2 (8%)	NA	21 (81%)	3 (12%)	**8%**
Milan **[77]**	^{90}Y-DOTA-TOC	21	0	6 (29%)	NA	11 (52%)	4 (19%)	**29%**
Basel **[74,75]**	^{90}Y-DOTA-TOC	74	3 (4%)	15 (20%)	NA	48 (65%)	8 (11%)	**24%**
Basel **[76]**	^{90}Y-DOTA-TOC	33	2 (6%)	9 (27%)	NA	19 (57%)	3 (9%)	**33%**
Rotterdam **[8,79]**	^{90}Y-DOTA-TOC	54	0	4 (7%)	7 (13%)	33 (61%)	10 (19%)	**7%**
Rotterdam **[85]**	^{177}Lu-DOTA-TOC	131	3 (2%)	32 (26%)	24 (19%)	44 (35%)	22 (18%)	**28%**

CR: complete remission, PR: patrial remission, MD: minor response, SD: stable disease, PD: progressive disease, NA: not available Clinical studies with ^{90}Y-DOTATOC

The next generation of somatostatin receptor mediated radionuclide therapy used a modified somatostatin analog, [Tyr^3]octreotide, with a higher affinity for the somatostatin receptor subtype 2 (Table **1**), and a different chelator, DOTA instead of DTPA, in order to ensure a more stable binding of the intended beta-emitting radionuclide ^{90}Y. Using ^{90}Y-DOTATOC different phase-1 and phase-2 peptide receptor radionuclide therapy trials have been performed. Otte *et al*. **[73]** and Waldherr *et al*. **[74, 75]** (Basel, Switzerland) reported different phase-1 and phase-2 studies in patients with neuroendocrine GEP tumors. In their first reports, using a dose escalating scheme of 4 treatment sessions up to a cumulative dose of 6 GBq (160 mCi)/m^2, and at which time renal protection with amino acid infusion was not performed in half of the patients, renal insufficiency developed in 4/29 patients. The overall response rate in GEP tumor patients who were either treated with 6 GBq (160 mCi)/m^2 **[74]**, or, in a later study, with 7.4 GBq (200 mCi)/m^2 in 4 doses **[75]**, was 24% (Table **5**). In a subsequent study, with the same dose of 7.4 GBq (200 mCi)/m^2 administered in two sessions, complete and partial remissions were found in one third of 36 patients **[75]** (Table **6**). It should be emphasized, however, that this was not a randomised trial comparing 2 dosing schemes. Chinol *et al.*, described dosimetric and dose-finding studies with ^{90}Y-DOTATOC with and without the administration of kidney protecting agents **[76-78]**. No major acute reactions were observed up to an administered dose of 5.6 GBq (150 mCi) per cycle. Reversible grade 3 haematological toxicity was found in 43% of patients injected with 5.2 GBq (140 mCi), which was

defined as the maximum tolerated dose per cycle. None of the patients developed acute or delayed kidney failure, although follow-up was short. Partial and complete remissions were reported by the same group in 28% of 87 patients with neuroendocrine tumors **[76]**. In a more detailed publication from the same group, Bodei *et al*. report the results of a phase-1 study in 40 patients with somatostatin receptor positive tumors, of whom 21 had GEP tumors **[77]**. Cumulative total treatment doses ranged from 5.9-11.1 GBq (160 to 300 mCi), given in 2 treatment cycles. Six of 21 (29%) patients had tumor regression (Table **5**). Median duration of the response was 9 months.

Another study with [^{90}Y-DOTA^{0},Tyr^{3}]octreotide is a multicentre phase-1 study which was performed in Rotterdam (the Netherlands), Brussels (Belgium) and Tampa (USA), in which 60 patients received escalating doses up to 14.8 GBq (400 mCi)/m^2 in 4 cycles or up to 9.3 GBq (250 mCi)/m^2 single dose, without reaching the maximum tolerated single dose **[8, 79]**. The cumulative radiation dose to kidneys was limited to 27 Gy. All received amino acids concomitant with ^{90}Y-DOTATOC for kidney protection. Three patients had dose-limiting toxicity: one liver toxicity, one thrombocytopenia grade 4 (<25·10^9/L), and one MDS. Four out of 54 (8%) patients who had received their maximum allowed dose had PR, and 7 (13%) had a minor response (MR) (25-50% tumor volume reduction) (Table **5**). The median time to progression in the 44 patients who had either stable disease (SD), MR, or PR was 30 months.

Despite differences in protocols used, complete plus partial remissions in most of the different studies with ^{90}Y-DOTATOC are in the same range, in between 10 and 30%, and therefore better than those obtained with [^{111}In-DTPA^{0}]octreotide.

Clinical studies with ^{177}Lu-DOTA-tate

The somatostatin analog [DTPA^{0},Tyr^{3}]octreotate, differs from [DTPA^{0},Tyr^{3}]octreotide only in that the C-terminal threoninol is replaced with threonine (Table **1**, and Fig. **1-3**). Compared with [DTPA^{0},Tyr^{3}]octreotide, it shows an improved binding to somatostatin receptor-positive tissues in animal experiments **[80]**. Also, its DOTA-coupled counterpart, DOTA-tate (=[DOTA^{0},Tyr^{3}]octreotate, (Fig. **2, 3** and Table **1**), labeled with the ß- and gamma-emitting radionuclide ^{177}lutetium (^{177}Lu, see also Table **3**), was reported very successful in terms of tumor regression and animal survival in a rat model **[81, 82]**. As mentioned above a nine-fold increase in affinity was reported for the somatostatin receptor subtype 2 for DOTA-tate if compared with DOTATOC, and a six to seven-fold increase in affinity for their yttrium-loaded counterparts (Table **1**)**[29]**.

In a comparison in patients, it was found that the uptake of radioactivity, expressed as percentage of the injected dose of ^{177}Lu-DOTA-tate, was comparable to that after [^{111}In-DTPA^{0}]octreotide for kidneys, spleen and liver in patients **[83]**, but was 3 to 4-fold higher for 4 of 5 tumors **[81]**. Therefore, ^{177}Lu-DOTA-tate potentially represents an important improvement because of the higher absorbed doses that can be achieved to most tumors with about equal doses to potentially dose-limiting organs and because of the lower tissue penetration range of ^{177}Lu if compared with ^{90}Y, which may be especially important for small tumors.

The first treatment effects of ^{177}Lu-DOTA-tate therapy were described in 35 patients with neuroendocrine GEP tumors, who had a follow-up of 3 to 6 months after receiving their final dose **[84]**. Patients were treated with dosages of 3.7, 5.6, or 7.4 GBq (100, 150, or 200 mCi) ^{177}Lu-DOTA-tate, up to a final cumulative dose of 22.2-29.6 GBq (600-800 mCi), with treatment intervals of 6-9 weeks.

The effects of the therapy on tumor size were evaluable in 34 patients. Three months after the final administration a complete remission (CR) was found in one patient (3%), PR in 12 (35%), SD in 14 (41%), and progressive disease (PD) in 7 (21%), including 3 patients who died during the treatment period. The side-effects of treatment with ^{177}Lu-DOTA-tate were few and mostly transient, with mild bone marrow depression as the most common finding.

More recently, an analysis of this treatment in 131 patients with neuroendocrine GEP tumors was reported **[85]**. Fifty-five of the 131 (42%) patients had documented progressive disease within 1 year before the start of the therapy, 37 (28%) had stable disease, and in 39 (30%) information on disease progression was absent. Treatment intervals were 6-10 weeks, except in 4 patients who had persistent thrombocytopenia and in 13 others because of reasons unrelated to the treatment. In 116 patients, the final intended cumulative dose of 22.2-29.6 GBq (600-800 mCi) was administered. Ten of the 15 remaining patients died of progressive disease before completing their treatment.

Serious side-effects occurred in 2 patients. One patient in whom in the year preceding the therapy serum creatinine concentrations had risen from 60-70 µmol/L to 90-100 µmol/l, and who had a urinary creatinine clearance of 41 mL/min when entering the study, eventually developed renal insufficiency 1.5 years after receiving her last treatment. In another patient who had diffuse liver metastases from an endocrine pancreatic tumor which had grown rapidly in the months preceding the therapy, an increase in upper abdominal pain and a deterioration of liver functions occurred in the days and weeks following the first administration. The patient developed hepatorenal syndrome and died after 5 weeks.

WHO toxicity grade 3 or 4 anaemia (Hb 4.0-4.9 or <4.0 mmol/L, respectively), leucocytopenia (WBC 1.0-1.9 or $<1.0 \cdot 10^9$/L, respectively), or thrombocytopenia (platelets 25.0-49.9 or $<25 \cdot 10^9$/L, respectively) occurred after 0.4% and 0.0%, 1.3% and 0.0%, and 1.5% and 0.2% of the administrations, respectively.

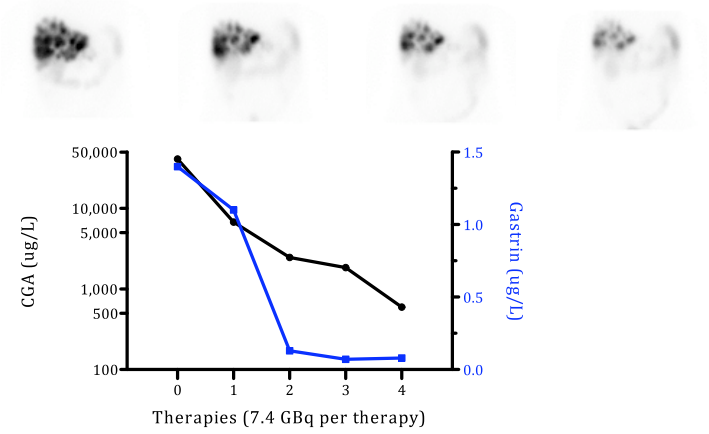


Figure 8. Upper part: post therapy gamma camera scans in a patient with metastatic gastrinoma after the first to fourth therapy cycle (left to right) with 7.4 GBq (200 mCi) ^{177}Lu-DOTA-tate per therapy. Note the intense uptake in the multiple liver metastases and the decrease of tumor uptake after each therapy cycle. This phenomenon usually predicts a favourable treatment outcome. Lower part: also notice the significant decrease of the serum tumor markers chromogranin-A (CGA) and gastrin as the administered cumulative dose increases. The patient had a partial remission (PR; more than 50 % tumor volume reduction measured bidimensionally on CT scans) after completing the therapy.

Tumor size could be evaluated in 125 patients. CR was found in 3 (2%) patients, PR in 32 (26%), MR in 24 (19%), SD in 44 (35%), and PD in 22 (18%) patients, including the 10 patients who died before the intended cumulative dose was reached (Table **5**, Fig. **8**).

As an illustration a series of gamma camera scans is presented in a patient with metastatic gastrinoma after the first to fourth therapy cycle with 7.4 GBq (200 mCi) ^{177}Lu-DOTA-tate per therapy, with concordant decrease of the serum tumor markers chromogranin-A (CGA) and gastrin as the administered cumulative dose increases (Fig. **8**). Higher remission rates were positively correlated with high uptake during pretherapy [^{111}In-DTPA^{0}]octreotide scintigraphy and a limited number of liver metastases, whereas PD was significantly more frequent in patients with a low Karnofsky Performance Score (KPS) and extensive disease.

Median follow-up in the 103 patients who either had SD or tumor regression was 16 months and median time to progression was more than 36 months.

Comparison of the different treatments

Treatment with radiolabeled somatostatin analogs is a promising new tool in the management of patients with inoperable or metastasised neuroendocrine tumors. The results that were obtained with ^{90}Y-DOTATOC and ^{177}Lu-DOTA-tate are very encouraging, although a direct, randomised comparison between the various treatments is lacking. Also, the reported percentages of tumor remission after ^{90}Y-DOTATOC treatment vary. This may have several causes: The administered doses and dosing schemes differ: some studies use dose-escalating schemes, whereas others use fixed doses; also, there are several patient and tumor characteristics that determine treatment outcome, such as amount of uptake on the [^{111}In-DTPA^{0}]octreotide scintigraphy, the estimated total tumor burden, and the extent of liver involvement. Therefore, differences in patient selection may play an important role in determining treatment outcome. Other factors that can have contributed to the different results that were found in the different centres performing trials with the same compounds, may be differences in tumor response criteria, and centralized vs. decentralized follow-up CT scoring. Therefore, in order to establish which treatment scheme and which radiolabeled somatostatin analogs or combination of analogs is optimal, randomised trials are needed.

Outlook for future concepts and research

It can be concluded from literature and the above-mentioned arguments that peptide receptor radionuclide scintigraphy can be performed with ^{111}In-DTPA-and DOTA-peptides. However, although DOTA-peptides can also be labeled with therapeutic radionuclides at high specific activity, this specific activity might be not high enough for peptide receptor radionuclide therapy with ligands like DOTA-substance P and DOTA-bombesin, particularly when administered as a bolus and, as a consequence. The delivery of sufficient amounts of radioactivity to these targets may be too low for scintigraphy and therapy. However, there may be several other ways to circumvent these limitations, such as different ways of administration, long-lasting infusions, fractionating the dose or combinations hereof **[34, 51, 86]**. Intra-arterial **[87]** or intratumoral administration **[88]**, and further successful increase in specific

activity of the radionuclides are reported, see Table **3 [55]**. The use of other chelators, like acyclic chelators might improve the specific activity of the peptides **[89]**. Peptides with more than one chelator are currently under investigation **[40]**, enabling to incorporate more radioactivity per ligand. Preliminary reports state however that kidney uptake are also increasing, thus eventually the ratio of tumor versus kidney is not improved.

Another approach is the use of an antagonist of the ligand. However, only with intact binding of the antagonist to the receptor and without the dose-dependent physiological response of the agonist, the use of ligands labeled with short-lived radionuclides might be possible, especially α-emitters **[90, 91]**. α-emitting radionuclides have a high Linear Energy Transfer (high energy deposition within a short range), consequently the cell kill probability is high, but only if the target (e.g. DNA) is within range. Nevertheless, the applications of targeting peptides without internalisation might be hampered, as we reported for DTPA- and DOTA-bombesin antagonists **[92]**. Improvements of the characteristics of the radioligand might also be achieved by investigating the role of co-ordination chemistry in the development of these peptides, linkers and chelators **[3, 64, 93, 94]**, and by studies of the pharmacokinetics of the peptide derivatives **[95]**.

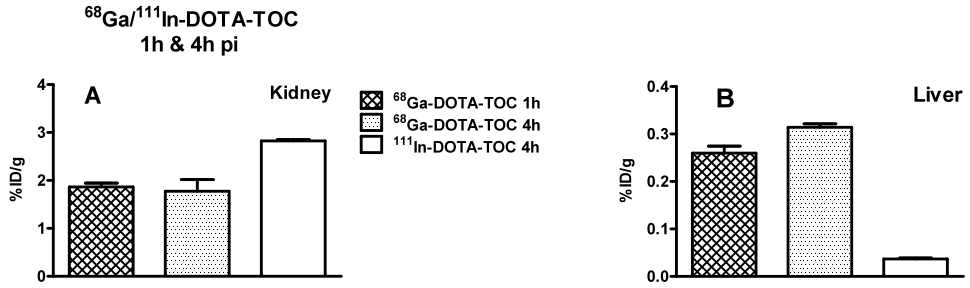


Figure 9 A-B. Uptake of radioactivity in liver and kidney after the administration of ^{68}Ga-DOTATOC at 1 and 4 h pi and ^{111}In-DOTATOC at 4 h pi in rats (n=3), expressed as mean ± SD **[67]**. Note the difference in uptakes, and this can be explained by the difference in charge between the radioligands, see text.

Molecular weight, lipophilicity and charge of the radioligand are important properties that determine the clearance via liver or kidney. An example hereof is the effect of the radionuclides ^{68}Ga and ^{111}In in DOTATOC on the tissue distribution in rats, see Fig. **9 A-B [67, 68]**. The explanation for this finding lies in the difference in co-ordination of Ga^{3+} compared with In^{3+} **[68]**. However, a too rapid clearance might prevent sufficient tumor accumulation for peptide receptor radionuclide scintigraphy and therapy, whereas a more stable ligand with side effects also has its drawback. The balance between clearance and stability must be determined and is most likely to be product- and application-dependent **[95]**. An important item for the success of peptide receptor radionuclide therapy is the application of the knowledge from radiobiology, like the research on the radiosensitivity of tumor (and within types of tumor) and normal tissue, the effects of sensitisers **[21, 96-100]**, and in combinations, such as peptide receptor radionuclide scintigraphy **[12]** and cytotoxics. However, *in vitro* the down regulation of receptors as an effect of cytotoxics **[101]**, or glucocorticoids **[102]**, or up regulation of receptors by estrogens are reported **[103, 104]**.

With all the above-mentioned possibilities in mind and although radiolabeled regulatory peptides have been investigated and successfully applied for peptide receptor radionuclide scintigraphy and therapy for nearly 2 decades, there are still many possibilities to improve and fully exploit peptide receptor radionuclide scintigraphy and therapy.

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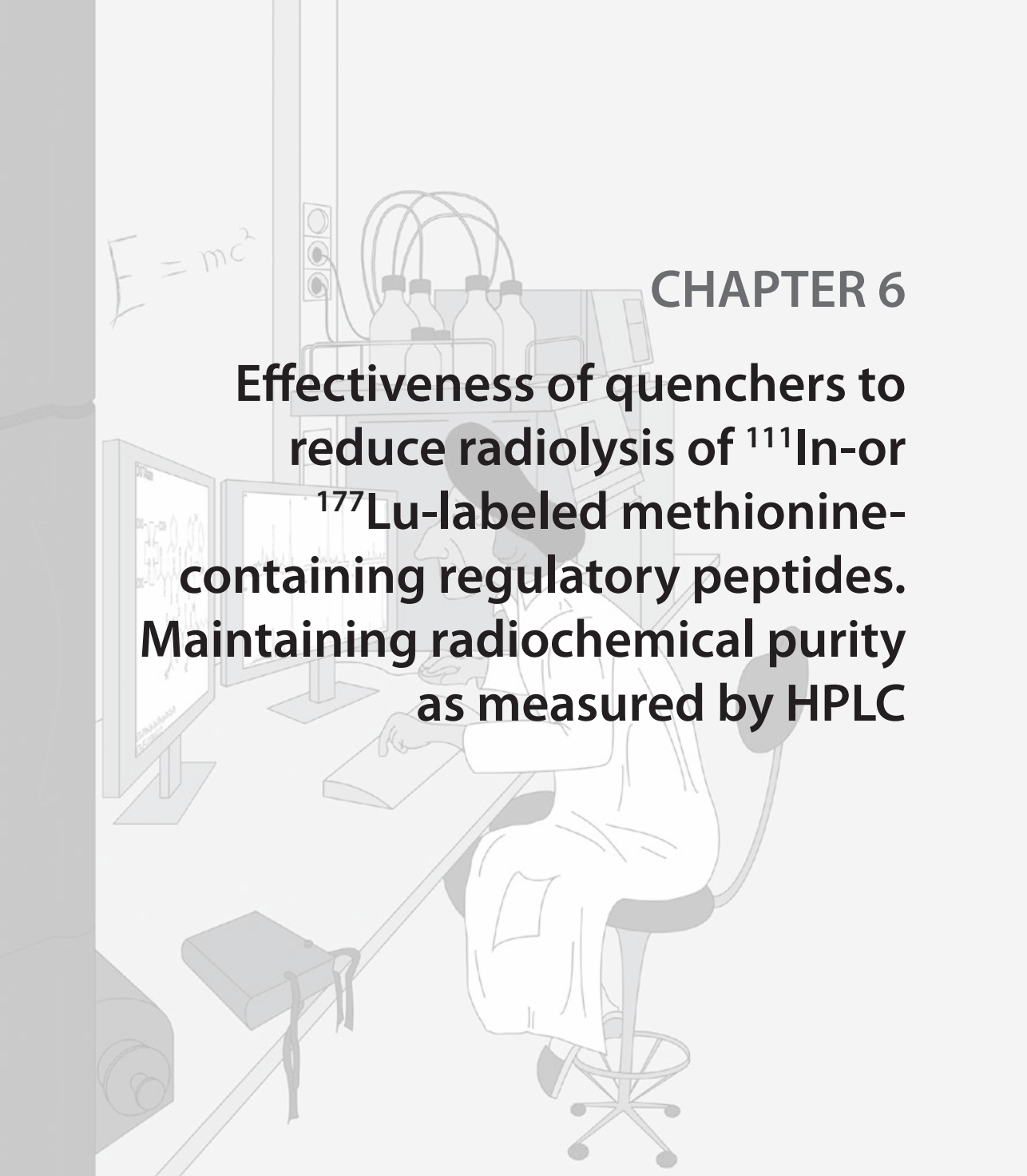

CHAPTER 6

Effectiveness of quenchers to reduce radiolysis of ^{111}In-or ^{177}Lu-labeled methionine-containing regulatory peptides. Maintaining radiochemical purity as measured by HPLC

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Abstract

An overview how to measure and to quantify radiolysis by the addition of quenchers and to maintain RadioChemical Purity (RCP) of vulnerable methionin-containing regulatory peptides is presented. High RCP was only achieved with a combination of quenchers. However, quantification of RCP is not standardized, and therefore comparison of radiolabeling and RCP of regulatory peptides between different HPLC-systems and between laboratories is cumbersome. Therefore we suggest a set of standardized requirements to quantify RCP by HPLC for radiolabeled DTPA- or DOTA-peptides.
Moreover, a dosimetry model was developed to calculate the doses in the reaction vials during radiolabeling and storage of the radiopeptides, and to predict RCP in the presence and absence of quenchers.
RCP was measured by HPLC, and a relation between radiation dose and radiolysis of RCP was established. The here described quenchers are tested individually as f(concentration) to investigate efficacy to reduce radiolysis of radiolabeled methionin-containing regulatory peptides.

Introduction

General aspects of peptide receptor targeting

Radiolabeled regulatory peptides such as somatostatin, cholechocystokinin (CCK), minigastrin (MG) or Gastrin-Releasing Peptides (GRP) analogs are applied in Nuclear Medicine for Peptide Receptor Scintigraphy (PRS) and Peptide Receptor Radionuclide Therapy (PRRT). Peptides for PRS are labeled with β^+ or γ-emitters, like ^{68}Ga and ^{111}In **[1-3]**, and for PRRT labeled with α or β^--emitting nuclides like ^{213}Bi or ^{177}Lu **[4-6].** These regulatory peptides are predominantly peptides conjugated with a chelator (such as DTPA or DOTA) and complexed with a radiometal. For various reasons, including preventing receptor saturation the injected amount of these regulatory peptides are low and in mice and rats in the range of nanomoles (10^{-9} moles) or even down to picomoles (10^{-12} moles) **[7-10]**.

Radiolabeled methionin-containing regulatory peptides

Here we focus on regulatory methionin (Met)-containing GRP and MG peptide analogs which are used for imaging of receptor mediated processes **[1, 3, 11-13]**. Radiolabeling, handling and quality control of radiolabeled regulatory peptides are worldwide performed differently. E.g. the addition of quenchers to prevent radiolysis are variable. Incorporation of the radiometal can be determined by ITLC-SG (Instant Thin Layer Chromatography Silica Gel), while RCP is frequently used to express the portion of radioligand that is present in the desired chemical form **[14]**. RCP, as mentioned above can not be determined with ITLC-SG, but requires techniques such as High Pressure Liquid Chromatography (HPLC, see paragraph RCP). RCP measurement of the different forms of desired (=intact) and non-intact forms of radiolabeled DTPA- and DOTA-peptides requires chromatographic separation of these forms.

Radiolysis of radiopeptides

There is a lack of fundamental knowledge regarding the destruction of radiopeptides in aqueous solution due to radiolysis. Radiolysis is here defined as the degradation of radiopeptides in aqueous solution caused by radiation and free radicals generated by radiolysis of water molecules **[15-19]**.

Comparison of radiolabeling and quantification of RCP between different HPLC-systems and between laboratories is not standardized. Moreover, since there are no criteria or guidelines to qualify HPLC-quality controls, we would like to suggest to create reproducible parameters for comparison of RCP between different HPLC-systems and laboratories.
Here we also present an overview from literature and data from our study how to reduce radiolysis by the addition of different quenchers and thus how to maintain RCP of radiolabeled peptides, especially focused on vulnerable Met-containing regulatory peptides. A macroscopic dosimetry model was developed to calculate the dose rates in vials being used for radiolabeling and to predict RCP of radiopeptides in the absence of quenchers.

Vulnerability of met-containing regulatory peptides

Currently, there is a growing interest in Nuclear Medicine on development of regulatory peptides, like CCK, MG and GRP receptor-mediated PRS and PRRT. These small native regulatory peptides contain Met in their pharmacophore, while Met is rapidly oxidized in to Met-sulfoxide, as described for DOTA-MG11 and DOTA-AMBA *(Fig. **1**)* **[13, 20]**, which results in loss of receptor affinity **[13, 21].** Whereas MG has affinity to the CCK subtype 2 receptor (CCK-2) **[22]** and bombesin has affinity to GRP receptor.
Radiolysed radiopeptides and radiometabolites have reduced receptor affinity and therefore will circulate in the body with increased radiation toxicity to organs such as liver and bone marrow **[23]**.
^{111}In- or ^{177}Lu-labeled Met-containing regulatory peptides are known to be vulnerable to radiolysis and oxidation during and after radiolabeling **[13, 21]**, and will be discussed in paragraph **Radiolysis of Radiolabeled Met-Containing Regulatory Peptides**.

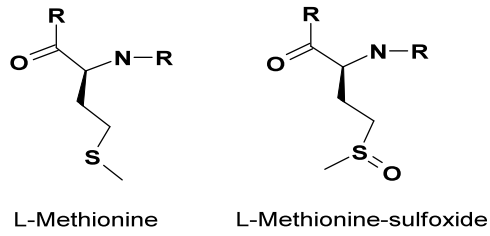

Figure 1. Met in the pharmacophore of peptides, as in MG or bombesin is rapidly oxidized in to Met-sulfoxide, and results in loss of receptor affinity

Radicals in aqueous solution

Free radicals, first described by Moses Gomberg **[24, 25]**, are charged molecules or atoms with a non-paired electron in the outer shell. Non-paired electrons show an extreme degree of chemical reactivity. Radicals have a lifetime of fractions of a second (e.g. the •OH radical has a lifetime of 10^{-9} s). **[26]**. Free radicals are very reactive towards organic molecules, including peptides. To reduce radiolysis and stabilize the radiopeptides, a quencher is often used during or/and after the radiolabeling. The radiolysis of aqueous solutions produces reactive species (•OH, H•, e^-_{aq}, H_2O_2) that may react with the peptide in the reaction solution **[15-19]**. It is well established that the hydroxylation of an aromatic ring appears to be

the principal reaction initiated by hydroxyl radicals in a aqueous solution of aromatic amino acids **[15, 27]**. Here described Met-containing regulatory peptides contain also aromatic amino acids like tyrosine (Tyr) and tryptophan (Trp) which are thus sensitive to radiolysis as well **[15, 27]**.
Moreover, in analogy with the intrinsic quenching effect of Met and seleno-Met (Se-Met) on Met-containing regulatory peptides **[13, 20]**, here we report the addition of Tyr and Trp as intrinsic quenchers to maintain RCP.

Radiolysis of radiolabeled met-containing regulatory peptides

In this overview we also present our study on the protection of the radiolabeled MG and bombesin analogs against radiolysis. Since tumor uptake of these peptides is largely dependent on the receptor binding radiolytic decomposition of the radiopepide *(see Fig. **2**)* may lead to the decreased therapeutic and diagnostic efficacy. Therefore, addition of quenchers (also known as radiolytic stabilizers, scavengers, stabilizers or anti-oxidants) are frequently used. Several quenchers are described in literature *(see Fig. **3**)* for protection against radiolysis **[20, 23, 28-33]**.

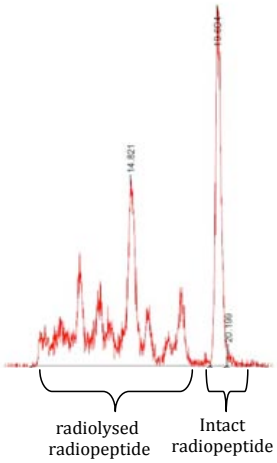

Figure 2. Typical HPLC-chromatogram ^{111}In-DOTA-MG11 in the absence of quenchers. By altering polarity of the HPLC-gradient, damaged radiopeptides are separated from intact radiolabeled peptides.

For the here presented study of the protection of the radiolabeled MG and bombesin analogs we used DOTA- and [DTPA-Pro^{1} ,Tyr^{4}] bombesin (further referred as DTPA-Bombesin), 14-amino acid peptides including Met in their pharmacophore *(see Fig. **4A**)* and DOTA-MG11, a 8-amino acid Met-containing peptide *(see Fig. **4B**)*. Maintaining pharmacophore intact in radiolabeled peptide is crucial for its receptor binding and the biological activity of the peptide. For comparison of radiolabeling and influence of individual quenchers and mixtures of quenchers, DOTA- and DTPA-conjugated bombesin analogs and MG are labeled. A typical reaction mixture for radiolabeling is 60 MBq of ^{177}Lu- or $^{111}InCl_3$ in 0.01-0.05 M HCl, 2 nmoles peptide dissolved in Milli-Q water, and sodium acetate as buffer (≤2µL of 2.5M) and 10µL of (0.05-0.1M) described quenchers *(Fig. **3**)*, in a final volume of 140 µL (final pH 4-4.5).

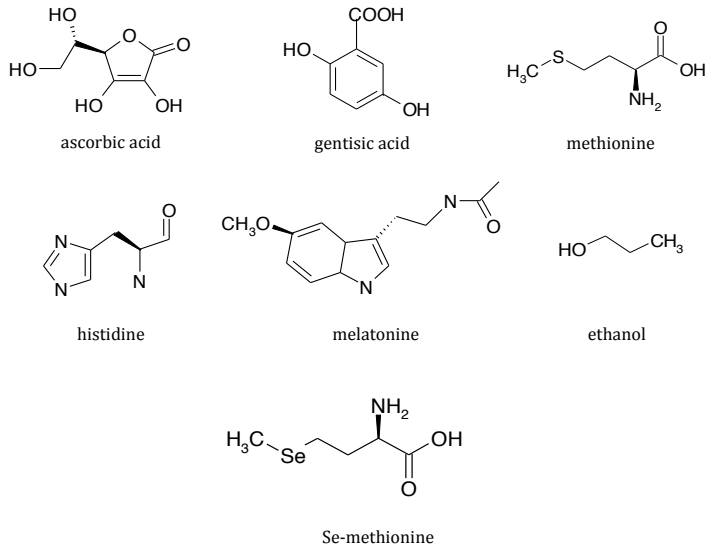

Figure 3. Chemical structure of investigated quenchers

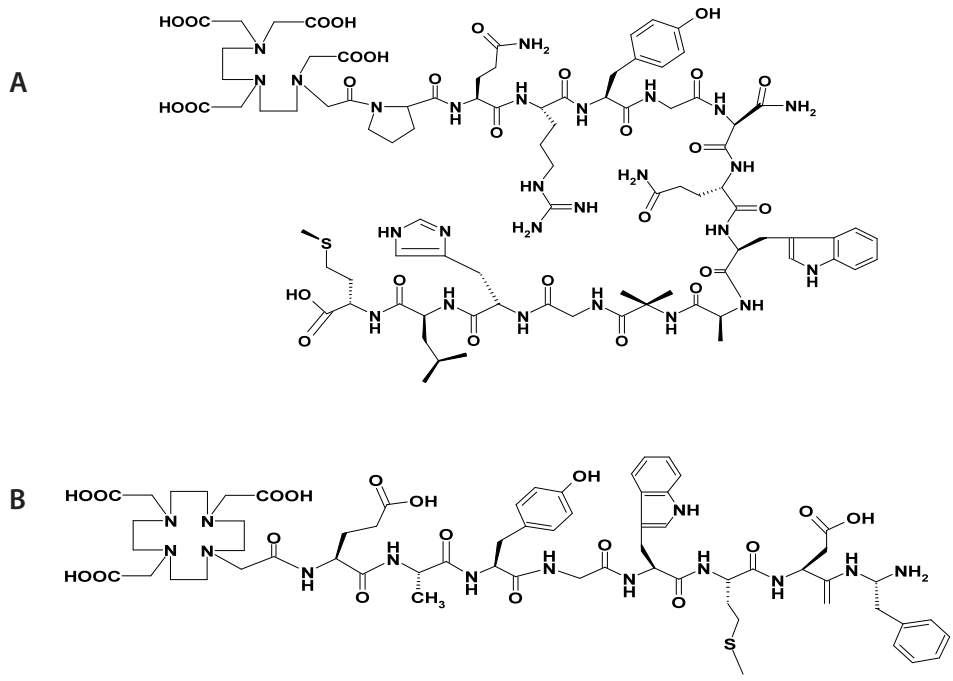

Figure 4. Chemical structure of DTPA- bombesin **(A)** and DOTA-MG11 **(B)**

DOTA-peptides were heated for 10 min 80°C in a heating block as described earlier **[34]**. DTPA-peptides were incubated for 10 min at room temperature **[35]**. Quality control of DOTA-peptides was performed after the addition of 5 µL 4 mM DTPA post radiolabeling **[36, 37]**. Quality control includes incorporation of ^{111}In or ^{177}Lu as measured by ITLC-SG **[38]** and RCP of radiolabeled DTPA- and DOTA-peptides as measured by HPLC, which is further defined in paragraph **RCP**. RCP of radiopeptides was studied as *f*(time post radiolabeling) at room temperature. The addition of quenchers to reduce radiolysis, and thus maintain RCP, is frequently described, but how accurate can RCP actually be quantified/measured? **[13, 20, 23, 30]**.

Comparison of RCP of radiopeptides between different HPLC-systems (including columns, conditions, gradient, detector, software etc), and between laboratories are not standardized. Therefore description of requirements and criteria's are essential for an accurate comparison between different HPLC-systems and laboratories.

RCP

Since radiolysed radiopeptides often differ in charge and shape *vs.* structure of intact radiolabeled peptide, radiolysis of radiolabeled peptide can be quantified by HPLC. Typically RCP of radiolabeled MG or bombesin analogs measured by HPLC is expressed as % of radiodetected peak area (e.g. µV.sec^{-1}) of the intact radiolabeled peptide *vs.* all other radio peaks measured during the same HPLC-analyses. *(Fig. **2**).* All HPLC-measurements are corrected for background. Even at low activity measurement influence of background was <3% on RCP

There are many reports on the determination of RCP by HPLC, including accuracy, linearity, precision, repeatability, detection limit **[14, 39, 40]**. To our knowledge, there are no criteria to qualify a HPLC separation method as perfect, good or good enough. With differences in eluens, gradient, flow, column type and length and might increase variation in finding impurities, and thus variation in RCP.

For comparison of RCP between different HPLC-systems and laboratories we therefore suggest standardisation of these HPLC-settings. Standardisation of separation method includes HPLC-eluens, gradient or isocratic, flow, column type and length, detection and interpretation of chromatograms.

Therefore, in our opinion, RCP are actually expressed in percentages of arbitrary units.

Here we would like to suggest minimal requirement for RCP by HPLC and radiodetection for radiolabeled DTPA- or DOTA-peptides, which should include base-to-base separation of:

A. At least 2 peaks of the DTPA conformations of a radiolabeled DTPA-peptide **[41]**,
B. Metal-peptide *vs.* peptide for DTPA and DOTA-peptide **[42, 43]**,
B. For Met-containing regulatory peptides: Met-sulfoxide radiopeptide vs intact radiolabeled peptide **[13]**.

Base-to-base separation is described at page 26 of **Reviewer Guidance Validation of Chromatographic Methods [39]**.

These requirements are important to standardize RCP measurements for reliable comparisons of RCP quantifications between different systems/laboratories. The tools in analytical chemistry are constantly improving, e.g. as recently reported by Asti et al**[42]** for a base-to-base chromatographic separation of a DOTA-peptide labeled with different not radioactive metal-ions. How this new technique will effect the radiodetection, including sensitivity of radiopeptides are currently under investigation.

Quenchers

Most quenchers are anti-oxidants, which readily reacts with hydroxyl and super oxide radicals as described above in paragraph **Radicals in aqueous solution [15-17, 19]**. In general, a quencher for therapeutic and diagnostic radiopharmaceutical should have the following characteristics:

A. The ability to quench the radiopharmaceutical for a reasonable period of time for preparation, release, storage and shelf life of the radiopeptide **[23]**,
B. Low or no toxicity of the quencher itself when it is used for animal and human administration.

Quenchers can be added to the reaction vial of the radiopharmaceutical for PRS or PRRT before or after the radiolabeling. Unfortunately, quenchers could have negative effects on labeling kinetics and thus on efficacy of labeling time and final incorporation. Radiolabeling DOTA-peptides with ^{111}In and ^{177}Lu requires a heating procedure (e.g. 10 min 80°C)**[36]** and thus peptides and quencher(s) are subjected to a certain degree of thermolysis. In case of post-labeling addition of quenchers, the quencher is not exposed to the thermolysis process; thus quenchers may have longer quenching effects and higher quenching efficiencies. The post-radiolabeling approach is particularly useful when addition of a large amount of quencher in the radiopharmaceutical formulation interferes with the radiolabeling .
Almost all living mechanisms, proteins and peptides are build-up from amino acids. These amino acids have all there own vulnerability related to radiolysis. In analogy with Met as quencher **[13, 20]** to prevent Met-containing radiopeptides against radiolysis, we also investigated other amino acids, such as Trp, Tyr, histidin (His) as quenchers and achieve more information of the vulnerability of the amino acids independently. Therefore, we performed radiolabeling in the presence of an excess of several amino acids *(see Fig. **5**)* Added amino acids were selected on their vulnerability as described in paragraph **Radicals in aqueous solution**. Experiments resulted in 2 amino acids with potential quenching effects. These amino acids, Met and His, could maintain RCP at 50%, which is higher in comparison to other selected amino acids *(Fig. **5**)*.

In literature, quenchers such as gentisic acid **[23]**, ascorbic acid **[21, 23, 30]**, Se-Met **[20, 21]**, melatonin **[29, 31, 32]**, and ethanol **[28, 33]** has been described and added to the reaction mixtures to prevent radiolysis. Also RCP obtained with addition of mixtures of quenchers like gentisic acid, ascorbic acid and ascorbate in combination with Se-Met are described **[20, 23]**. To our knowledge, structural comparison and combination of described quenchers *(see Fig. **3**)*, within one peptide model, to optimize protection of radiolabeled peptide against radiolysis are not described earlier.

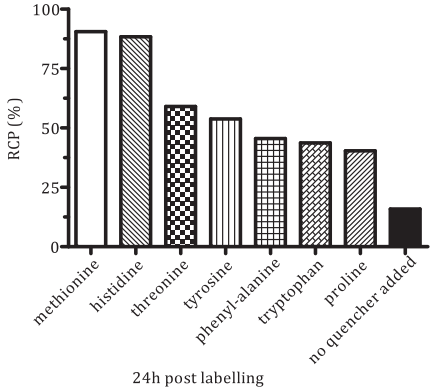

Figure 5. In order to investigate the intrinsic effect of amino acids as quenchers, several potential radiolysis sensitive amino acids were selected and tested on vulnerability and thus effect of quenching. Met-containing regulatory radiopeptides like ^{111}In-DTPA-bombesin were monitored in the presence of selected L-amino acids, after optimizing quencher concentration (See paragraph **Quenchers**). Applied quencher concentration in reaction vial was 35 mM. X-as presents different amino acids and Y-axis is expressed in RCP (%) 24h after labeling. With ^{111}In-DTPA-bombesin as a model, the addition of Met and His maintained RCP best (>50%) in comparison with no quencher added.

Unfortunately, results obtained in those studies could not be compared with here described RCP because final reaction volume, amount of activity and peptide amount and thus specific activities are different from our models. All above described quenchers were tested individually as *f*(concentration) to investigate efficacy of preventing radiolysis for here described radiolabeled Met-containing regulatory peptides, e.g. for Met final concentrations of 10, 35 and 50 mM were investigated. HPLC-analyses of labeling mixtures with the addition of different quenchers concentrations were performed at T=0 (directly after the radiolabeling) and up to 7 days after radiolabeling *(Fig. **6**)*. Obtained RCP's resulted in a minimum concentration of quencher which have the maximum effect on maintaining RCP of radiopeptide. Moreover, HPLC-chromatograms of radiopeptides with the addition of different quenchers showed quencher-specific HPLC-chromatogram profiles *(Fig. **7A, B**)*. Combination of the HPLC-chromatogram profiles resulted in combination of quenchers which maintained RCP of radiolabeled Met-containing regulatory peptides for longer periods of time, up to 7 days. Finally, mixtures of quencher were tested with radiolabeling of other Met-containing GRP analogs like DOTA-AMBA and DOTA-pesin and resulted in a maintained RCP (>90%) up to 7 days after radiolabeling *(Fig. **8A, B**)* **[4, 44, 45]**.

Dosimetry

Radiolysis of peptides is caused by several mechanisms including:
1) direct damage by ionizing electrons and
2) indirect damage by radiation-induced reactive radicals like oxygen.
Dosimetry can provide an indication of the amount and nature of radiolysis to radiopeptides. Micro- and macrodosimetry models have been used for the direct and indirect damage pathway in water **[46]**. Damage to the peptides by low LET (low energy transfer) radiation is well described by the average energy absorption in the reaction vial, i.e. by macrodosimetry **[46]**. Radiolysis of radiopeptides depends

on several parameters such as concentration of activity and radionuclide, type of radionuclide/radiation, temperature and the concentration of the peptide analog **[19]**. All these factors influence radiolysis and thus %RCP (defined as described in paragraph **RCP**). Choice of quenchers is therefore important to protect radiolabeled peptides during labeling and maintain RCP after labeling procedure. The RCP was measured by HPLC, in an attempt to find a relation between radiation dose and radiolysis of RCP.
A spherical geometry dosimetry model **[47]** was used to derive the absorbed dose rates obtained in vials being used in the measurements. The complete emission spectrum of the specific nuclides (^{111}In and ^{177}Lu) were taken into account. The absorbed fractions of energy and the total absorbed dose factors S were calculated for spheres with various volumes of tissue (with a density 1 g/cm^3). Absorbed dose S-values were used for calculating the dose rate and dose in the production vial by the product of the amount of radioactivity A (in MBq) and S (in mGy/MBq.s):

$$D(t)=A_0 e^{-\lambda t} \times S$$

The dose is then calculated by integration over time of the dose rate, which can be approximated by multiplication with the incubation time T, when T<<T½ (decay half time of radionuclide):

$$\dot{D}(T)=A_0 \times S \times T$$

Parameters of input for dose calculations were decay half-time ($T_{1/2}$) and decay constant λ (= ln(2)/$T_{1/2}$), amount of radioactivity A_0 and time of incubation T. The dose factors S are indicated in table 1. With this model the relation of the absorbed dose (Gy) and obtained RCP of the radiopeptides were investigated. Typically, one CCK-analog, DOTA-MG11 and GRP-analog DOTA-bombesin were labeled with ^{111}In or ^{177}Lu. RCP was quantified .

According to the calculations, 110 MBq ^{111}In vs 23 MBq ^{177}Lu in a final volume of 200 µL have similar dose rates (4.3 mGy/s vs. 4.2 mGy/s) *(see table 1)*. Under these condition, DOTA-MG11 and DOTA-bombesin were labeled with 110 MBq ^{111}In vs. 23 MBq ^{177}Lu. In an attempt to obtain a measurable decrease of RCP of maximum 50 % within 24h different amounts of activity and volume were used in the calculations to compare labeling procedure described in paragraph of **Radiolysis of radiolabeled met-containing regulatory peptides**. RCP was determined at indicated time points *(see Fig. **9A-B**)*.

Radiolysis of ^{111}In- and ^{177}Lu-labeled peptide was monitored by HPLC and separation between radiolysed and non-radiolysed radiopeptide was optimized as described in paragraph of **RCP**.
We found a linear correlation between RCP and dose (Gy) *(see Fig. **9A-B**)*. This provides the possibility to predict RCP of radiopeptides, in accordance with the Monte Carlo calculations. Quenchers have the ability to reduce radiolysis. Therefore the absence of quenchers during these experiments as shown in *Fig. **9A-B*** are a necessity, otherwise the effect of the dose (Gy) will be underestimated. Volume for intravenous administration for small animals is limited (e.g 0.2 mL for 25 g mouse) and the amount of activity required for preclinical scanning of small animals is relatively high.

Therefore relatively high volume activity of the Met-containing regulatory radiopeptides is unavoidable and thus make the addition of quencher even more essential. This calculation model proves the influence of radiolysis of radiopeptides and high activity in small volumes and thus the necessity of the addition of quenchers.

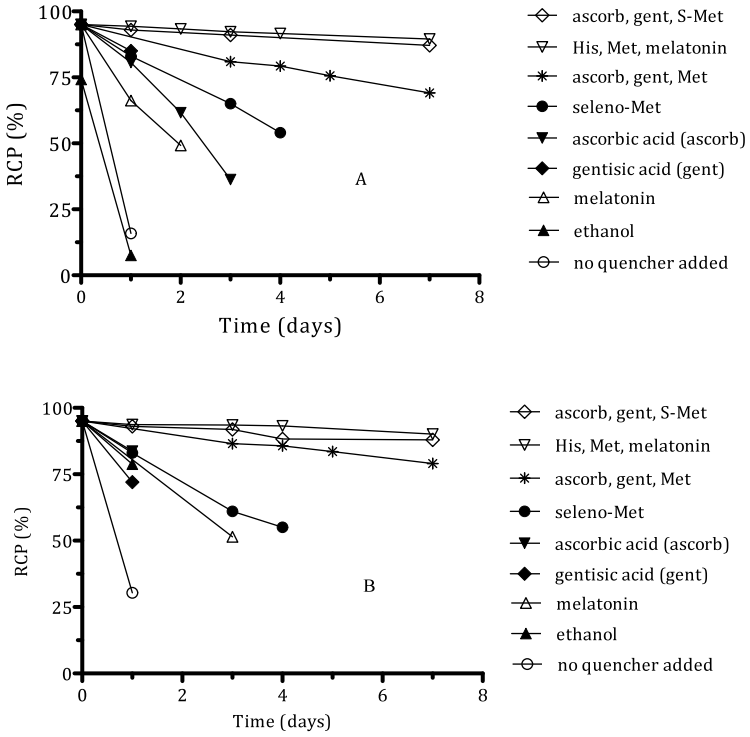

Figure 6. ^{111}In-DTPA-bombesin **(A)** and ^{111}In-DOTA-MG11 **(B)** in the presence of quenchers or combinations of quenchers. X-axis presenting time (days) and Y-axis expressed in % RCP. These figures show the effect of quenchers and combination separately. The maximum error of inter and intra-observer was <3% and were performed n=≥2

Table 1: Dose factors S for ^{111}In an ^{177}Lu for different volumes

Vial volume (μL)	^{111}In (mGy/MBq.s)	^{177}Lu (mGy/MBq.s)
10	0.464	2.18
50	0.280	1.31
100	0.0492	0.227
200	0.039	0.182
500	0.01	0.0461

Outlook for future concept and research

Optimization and maintaining RCP with quenchers for ^{111}In- or ^{177}Lu-labeled DOTA-MG11 and DTPA- or DOTA-bombesin can be used as a model for ^{111}In- or ^{177}Lu-labeled Met-containing regulatory peptides and other radiopeptides or proteins containing amino acids like Met, Trp and Tyr. However, maintaining RCP with here described mixtures of quenchers is not a guarantee for protection of other peptides or proteins. Radiolabeling with addition of quencher mixtures to maintain RCP must be investigated nuclide and peptide dependently.

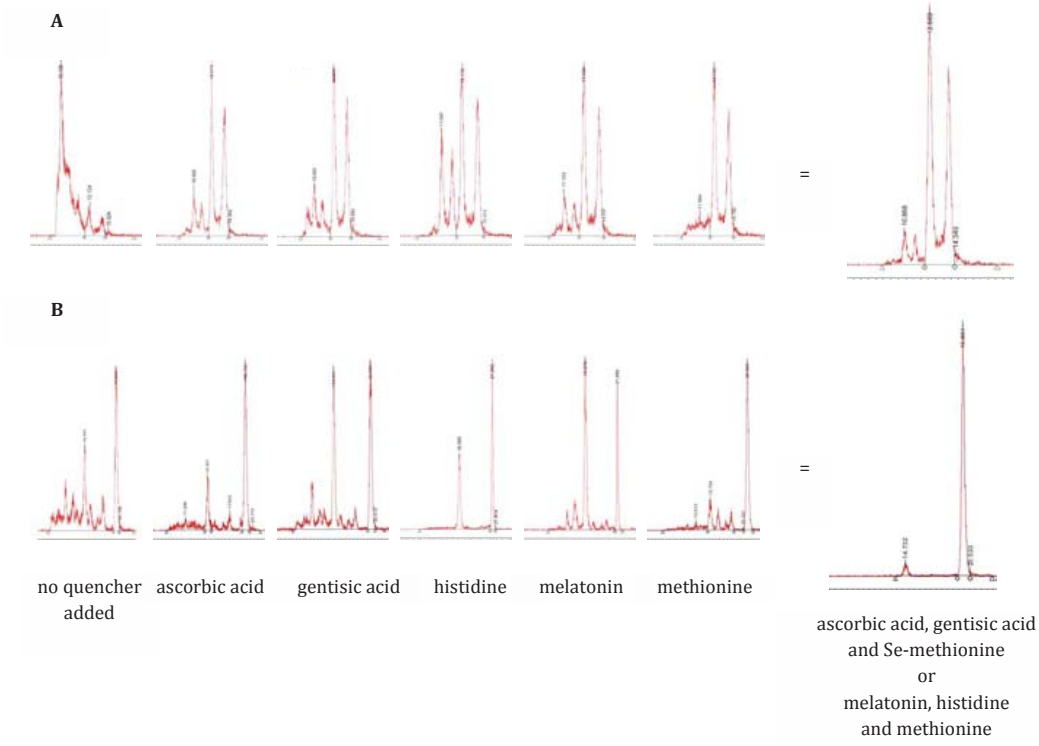

Figure 7. ^{111}In-DTPA-bombesin **(A)** and ^{111}In-DOTA-MG11**(B)** and effect of the difference quenchers and mixtures of quenchers 24h after radiolabeling. Applied quencher concentration after optimizing quencher concentration (See paragraph **Quenchers**) in reaction vial were: 3.5 mM for gentisic acid and ascorbic acid, 10 mM for Se-Met, 0.35 mM for melatonin and 7% (v/v) for ethanol. **(A)** HPLC-chromatograms of ^{111}In-DTPA- bombesin 24h after labeling. Chromatograms show specific patterns of radiolysed peptide in the presence of different quenchers. As shown in the HPLC-chromatograms, the 2 major peaks (13.5-14.5 min) are caused by stereo isomeres of the ^{111}In-labeled DTPA **[41]**, also seen with ^{111}In-DTPA-octreotide (OctreoScan®) Two major pre-peaks can be seen after radiolysis of Met in DTPA-bombesin (see HPLC-chromatogram Fig. **7A**) and are caused by stereo isomers of ^{111}In-DTPA **(B)** HPLC-chromatograms of ^{111}In-DOTA-MG11 at 24h. As shown in HPLC-chromatograms several peaks were formed in absence and presence of quenchers. The oxidation of Met and can only be prevented by addition of an excess of Met or Se-Met. The best RCP was achieved with a combination of quenchers.

Five to 10 % of the non-intact ^{111}In- or ^{177}Lu-labeled DOTA-MG11 and DTPA- or DOTA- bombesin was already formed during the short (heating)labeling procedure as described above, moreover non-intact peptide could already be present from impurities, e,g, originated from peptide synthesis. PRS or PRRT with DOTA-conjugated Met-containing CCK or bombesin analogs at high RCP is a challenge, therefore further research for chelators are required. Moreover, chelators should include the following characteristics: high kinetic and thermodynamic stability, preferably also with a therapeutic isotope like ^{177}Lu, and without the need of a heating procedure, this might help to maintain RCP **[48].**

The receptor affinity of Met-sulfoxide ^{111}In-DOTA-MG11 decreased dramatically **[13]** as also described for other bombesin analogs like ^{177}Lu-AMBA **[20]**. There is however a lack of knowledge about affinity of other radiolysed radiopeptides, i.e. for damaged Trp or Tyr. Unfortunately these specific radiolysed radiopeptides were not identified and characterised in this study and the influence on receptor affinity is currently unknown.
Overall, as shown in the overview of HPLC-chromatograms *(Fig. **8A-B**)* mixtures of to 7 quenchers were made that could maintain the RCP of Met-containing radiopeptide ≥ 90% days after labeling *(see Fig. **9A-B**)*.

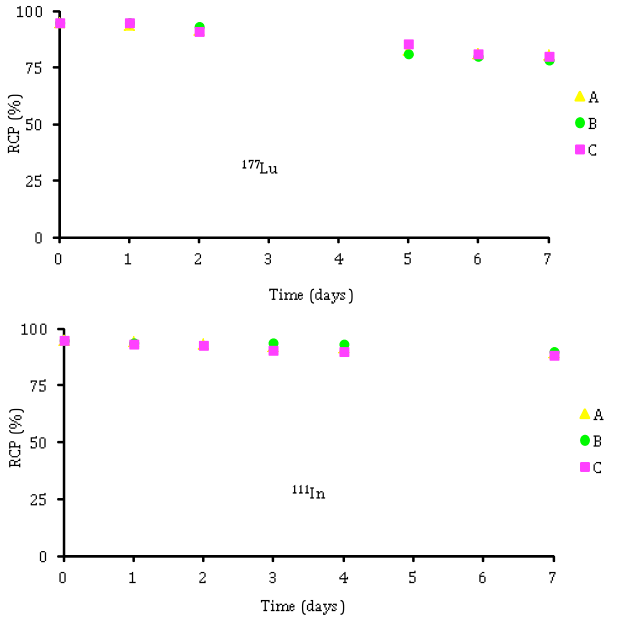

Figure 8. ^{111}In- and ^{177}Lu-DOTA-bombesin **(A)** ^{111}In- and ^{177}Lu-DOTA-MG11 **(B)** ^{111}In and ^{177}Lu-DOTA-pesin **(C)** in the presence of optimal combinations of quenchers are described. Pesin was tested to monitored the applicability of quencher mixture on other GRP analogs. X-axis is expressed in days and Y-axis expressed in % RCP. All 3 peptides could be stabilized at a RCP of ≥90% up to 7 days after radiolabeling.

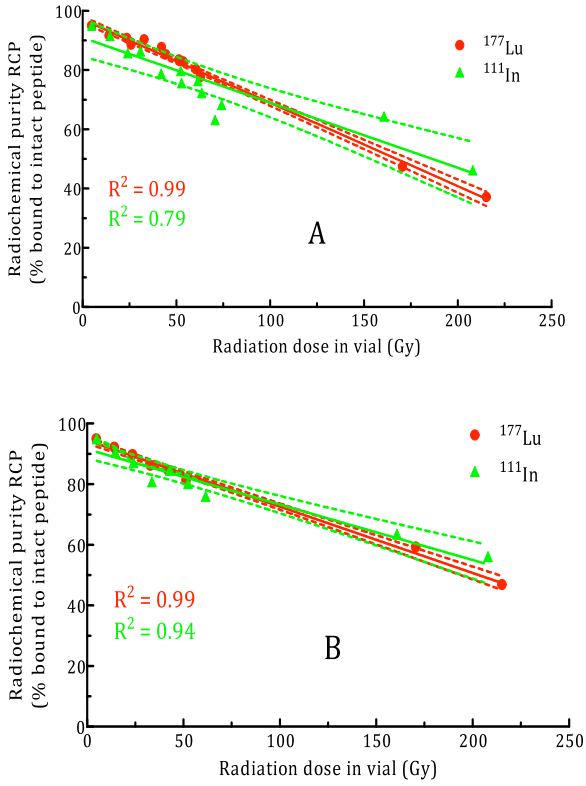

Figure 9. Correlation of the Monte Carlo calculation model in Gy vs RCP in practice. Two nmoles DOTA- bombesin **(A)** or DOTA-MG11 **(B)** were labeled with ^{111}In (110 MBq) or ^{177}Lu (23 MBq), respectively. The X-axis is expressed in dose (Gy) and Y-axis in RCP (%). 24h corresponding to 200 Gy in the reaction vial. Labeling DOTA-peptides requires a heating procedure **[36]**. For comparison of RCP caused by radiolysis we corrected the RCP for thermolysis which was induced during the heating procedure. Correction was performed based on the comparison of HPLC chromatograms of a radiolabeling with the addition of quenchers, as performed in figure 8, vs. no quencher added at T=0 after radiolabeling. **[23].** Moreover, radiolabeling in the absence of quenchers radiolytic effects can be anticipated. Labeling of the compounds were performed in small vial and in a total volume of 200 µL. Sodium acetate 2.5 M (< 2µL) was used to control the pH, final pH 4-4.5. For activity measurement NaI-cristal (Scionix Holland BV, Bunnik, The Netherlands) and by Canberra software (Canberra, Zellik, Belgium) connected to a HPLC-system (Waters, Etten-Leur, The Netherlands). Peaks of the radiopeptide and unlabeled radionuclide were separated by HPLC **[13]** and the amount of radiolysis quantified by integration of the peaks by Breeze HPLC software (Waters, Etten-Leur, Waters), as shown in figure 2. Dotted lines express the 95% accuracy of the lines which are also corresponding to the correlation factor (R).

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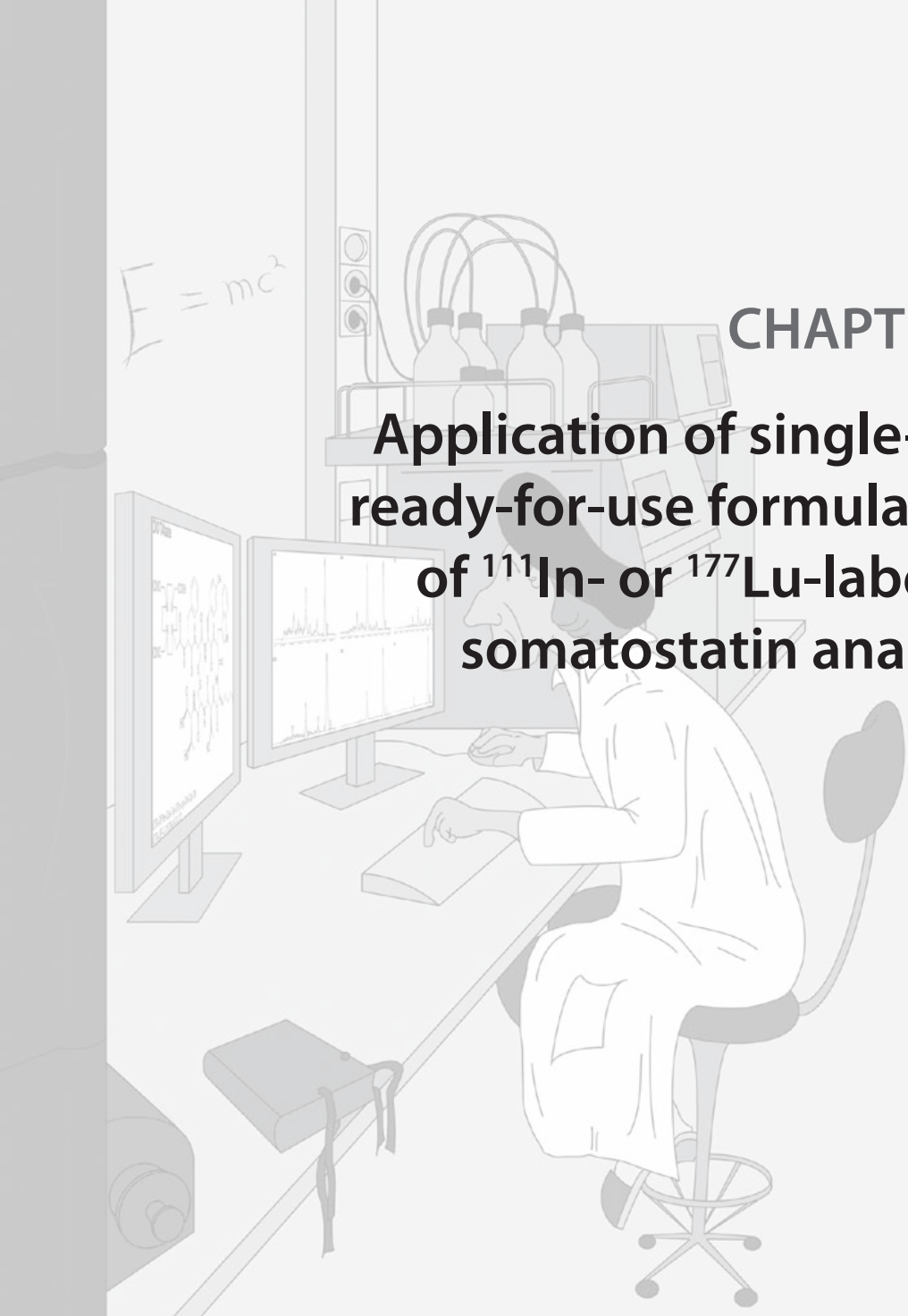

CHAPTER 7

Application of single-vial ready-for-use formulation of ^{111}In- or ^{177}Lu-labeled somatostatin analogs

Erik de Blois, Ho Sze Chan, Rory de Zanger, Mark Konijnenberg, Wouter A.P. Breeman

Applied Radiation and Isotopes 85 (2014) 28–33

Abstract

For the sake of safety it would be desirable to store and transport the ready-for-use liquid formulation (diagnostics and therapeutics) of radiolabeled peptides. The use of ethanol, in combination with a mixture of gentisic- and ascorbic acid, has superior effects on stabilizing radiolabeled somatostatin analogs. As a consequence, ^{111}In- and ^{177}Lu-labeled somatostatin analogs can be stored and transported in a single-vial ready-for-use liquid formulation up to 7 days after radiolabeling.

Introduction

Here we describe the presence of quenchers **[1]** in a single-vial liquid pharmaceutical formulation of a radiolabeled peptide, in a quantity sufficient to prevent radiolysis of the formulation. Radiolabeled peptides are generally stored and transported in the form of multi-vial kit formulations **[2]**. Usually the contents of these vials are lyophilized or frozen and should be brought into solution subsequently in a mutual reaction to produce the intended radiolabeled peptide. For the sake of safety it would be desirable to be able to store and transport the ready-for-use liquid formulation of the radiolabeled peptide. Then, a physician could administer the labeled peptide without a radiochemical reaction, simply by diluting the contents of the vial in a radiopharmaceutical liquid that can be administered by injection or by infusion **[3]**.

During labeling, storage and transport of the somatostatin analogs (further referred as SS-analogs) the peptide is exposed to radicals by produced by i.e. ^{177}Lu or ^{111}In as radionuclide. Radiolysis of aqueous solutions produces reactive species (•OH, H•, e^-_{aq}, H_2O_2) that may react with the peptide in the reaction **[2, 4-6]**. Here we describe how to preserve high radiochemical purity – and thus to increase the storage and transport time of radiolabeled SS-analogs – with the use of quenchers. The literature reports successful addition of quenchers such as gentisic acid **[7]**, ascorbic acid **[8-10]**, methionine **[11]** and ethanol **[3, 10, 12, 13]** to the reaction mixtures in various combinations and concentrations prior to radiolabeling to prevent radiolysis.
In this study, stability and radiolysis of radiolabeled SS-analogs were monitored by HPLC. HPLC methods were optimized to distinguish between non-labeled and radiolabeled peptides *vs.* the radiolysed peptides. The effect of quenchers on the stability of radiolabeled SS-analogs, even under therapeutic conditions was optimized and monitored up to 7 days after radiolabeling.

Methods and materials

^{111}In/^{177}Lu labeling of SS-analogs

DOTA-TATE ([DOTA^0,Tyr^3]octreotate), DOTA-NOC ([DOTA^0-Nal^3]octreotide) and DTPA-Octreotide ([DTPA^0]-octreotide) were purchased from BioSynthema, (St Louis, MO, USA). $^{111}InCl_3$ was purchased from Covedien (Petten, The Netherlands) and $^{177}LuCl_3$ from IDB Holland (Baarle Nassau, The Netherlands). During optimisation, the typical reaction mixture for radiolabeling consisted of 60 MBq of ^{177}Lu- or $^{111}InCl_3$ in 0.01-0.05 M HCl with 2 nmoles peptide dissolved in Milli-Q water, sodium acetate as buffer (≤2 µL of 2.5

M) and 10 µL of quenchers in a final volume of 0.14 mL (final pH 4-4.5). To inhibit oxidation and radiolysis, quenchers were added in various combinations and concentrations prior to radiolabeling as described previously (***Radiolabeled SS-analogs in presence of quenchers mixtures***). Quenchers included ascorbate (Bufa BV, Uitgeest, The Netherlands), gentisic acid (Tyco Health Care, Petten, The Netherlands), ethanol (Sigma-Aldrich Zwijndrecht, The Netherlands) and methionine (Fluka Biochemika, Switzerland). Radiolabeling of DOTA-TATE and other DOTA-conjugated SS-analogs with ^{111}In or ^{177}Lu requires heating for 15 min at 80°C **[14]**. DTPA-peptides were incubated for 10 min at room temperature (20-22°C) **[15]**. After cooling to room temperature, quality control of DTPA- and DOTA-peptides was performed. Quality control includes incorporation of ^{111}In or ^{177}Lu as measured by ITLC-SG **[16]** and RCP of radiolabeled DTPA- and DOTA-peptides as measured by HPLC **[1]**. RCP of radiopeptides was determined as function of time post radiolabeling at room temperature at regular time intervals. In order to avoid false positive quality control results due to colloid formation **[7, 17]**, quality control of DOTA-peptides was assessed after the addition of 5 µL 4 mM DTPA post radiolabeling **[18, 19].** Any non-incorporated ^{111}In or ^{177}Lu will be rapidly captured by the addition of DTPA. ^{111}In-DTPA, and ^{177}Lu-DTPA after i.v. administration, will be rapidly excreted via the kidneys **[19, 20]**.

DOTA-TATE labeling at therapeutical level (patient's dose) was performed under the kit formulation as previously reported **[21]** in a concentrated form (60 GBq in 3 mL) as described above. After QC Lu-DOTA-TATE was diluted with saline (100 mL) for patient infusion. We investigated the storing conditions of a ^{177}Lu-labeled DOTA-TATE and DOTA-NOC patient dose (3.7-7.4 GBq) after dilution with saline (5-100 mL) to maintain high RCP.

HPLC

HPLC grade methanol and trifluoroacetic acid (TFA) were purchased from Mallinckrodt Baker (Deventer, The Netherlands). All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). SS-analogs were analysed with a HPLC system (Breeze, Waters, Etten-Leur, The Netherlands), containing a 1525 binary pump and a UV detector (W2487 Waters Dual λ Absorbance Detector). UV absorption was measured at 278nm. A Symmetry C_{18} column (5mm x 4.6mm x 250mm, Waters, Etten-Leur, The Netherlands) was used with a gradient profile as described earlier **[22],** mobile phase 0.1% TFA (A) and methanol (B). Sample injections on the HPLC were performed via a Waters 717 autosampler (injection volume <200 µL). Radioactivity was monitored with a system including a NaI detector, digital multichannel analyzer and dedicated software (MetorX B.V, Goedereede, The Netherlands), connected to the HPLC system.

Dosimetry within the reaction vial

In order to investigate the influence of dose (Gy) on the radiolysis of SS-analogs, dose in the reaction mixture during the labeling procedure and storage was calculated according to the spherical geometry dosimetry model **[23]**. This model derives the absorbed dose rates in the vials used. The complete emission spectrum of the specific nuclides (^{111}In and ^{177}Lu) was taken into account used in the calculations. Dose was also calculated during storage up to 7 days using different volumes (5, 50 and 100 mL) containing a therapeutical amount (3.7 GBq) of ^{177}Lu-DOTA-TATE.

Optimizing quencher concentration

To obtain maximum protection of radiolabeled SS-analogs and minor effect in pharmacokinetics, quencher concentration in reaction mixture was investigated time dependently. DOTA-TATE was radiolabeled with 60 MBq ^{111}In or ^{177}Lu in the presence of different concentrations of quenchers and 2 nmol of DOTA-TATE in a final volume of 0.14 mL. Ascorbic acid and gentisic acid were investigated with final concentrations of 1-20 mM, 1-50 mM for methionine and 2-20 % (v/v) for ethanol.

Radiolabeled SS-analogs in the presence of quenchers mixtures

Applied quencher concentrations after optimization in reaction vial were: 3.5 mM for ascorbic acid and gentisic acid, 10 mM for methionine and 7-10% (v/v) for ethanol. Single quenchers and combinations of those quenchers were applied and RCP's were measured by HPLC up to 7 days after radiolabeling. RCP measurements were stopped when RCP decreased below 50 %.

Purging labeling mixture with nitrogen (N_2) or oxygen (O_2)

To investigate any influence of O_2 on the formation of radicals, which would lower the RCP, the reaction mixture after radiolabeling was purged for 1h with N_2 or O_2 (100 mL/min). We hypothesized that N_2 would decrease the O_2 concentration and thereby positively influence RCP. In contrast, purging with O_2 would increase the oxygen concentration and thereby influence RCP negatively. RCP was measured by HPLC up to 7 days after radiolabeling.

Maintaining RCP and biological activity (receptor affinity)

Autoradiography with stabilized ^{111}In-DOTA-TATE (upper line, figure **4A**) was performed as previously reported **[24-26]** on rat brain sections up to 7 days after radiolabeling. Internalisation with stabilized ^{111}In-DOTA-TATE was performed using CA20948 somatostatin receptor expressing cells as described previously **[27],** also up to 7 days after radiolabeling.

Results

Dosimetry within the reaction vial

Dose (Gy) within reaction mixture during labeling procedure and storage was calculated for ^{111}In- and ^{177}Lu-containing vials.

Fig. **1A** shows the differences is dose with a factor of 5. Fig. **1B** shows the calculated dose during storage using different volumes (5, 50 and 100 mL) of saline containing a therapeutical amount (3.7 GBq) of ^{177}Lu. Maximum dose was obtained (7.5 kGy) in a volume of 5 mL saline.

Optimizing quencher concentration

Quencher concentrations in reaction mixture were investigated time dependently by measuring RCP during storage time. Each quencher was investigated separately for its minimal concentration with maximum quenching effect *(Fig. **2**)*. Quencher mixtures were optimized to achieve maximum protection using these minimal concentrations. Under the experimental conditions the optimal quencher concentrations were 3.5 mM for ascorbic and gentisic acid, 10 mM for methionine and 10 % (v/v) for

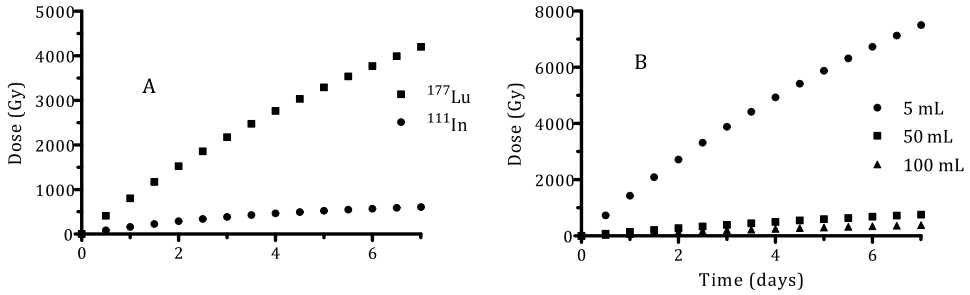

Figure 1. Dose calculations spherical geometry dosimetry model **[23]**. X-axis shows time (days); Y-axis dose (Gy). **(A)** Doses for ^{111}In- and ^{177}Lu-containing vials within standardised reaction mixture up to 7 days of storage. Reaction mixture containing 60 MBq of ^{177}Lu- or $^{111}InCl_3$ labeled SS-analog in a final volume of 0.14 mL. In comparison to ^{111}In, Therapeutic nuclide ^{177}Lu resulted in a 5 times higher dose (Gy). **(B)** Dose calculations using different volumes (5, 50 and 100 mL) were performed up to 7 days of storage for vials containing 3.7 GBq of ^{177}Lu.

ethanol. These concentrations had no effect on pharmacokinetics, so complete incorporations (>99%) within described incubation time.

Radiolabeled SS-analogs in the presence of quencher mixtures

Reaction mixture (60 MBq ^{111}In or ^{177}Lu, 2 nmol DOTA-TATE in 0.14 mL) containing ascorbic and gentisic acid (3.5 mM) and extra addition of ethanol clearly stabilized ^{111}In-DOTA-TATE. Addition of ethanol 10 % (v/v) stabilize ^{111}In-DOTA-TATE during 7 days; higher concentrations (>10 % (v/v)) have no additional effect *(Fig. **3A-B**)*.

We also investigated the influence of ethanol on the RCP of solutions of OctreoScan ([^{111}In-DPTA^0] octreotide) during storage. The stability of these solutions was compared with a commercially available kit formulation (OctreoScan) after reaction of their ingredients; this commercial kit contains a mixture of gentisic- and ascorbic acid as quenchers. Addition of ethanol to the gentisic- and ascorbic acid containing OctreoScan kit (see instructions for use) even improve stability of radiolabeled peptide *(Fig. **4A-B**)*.

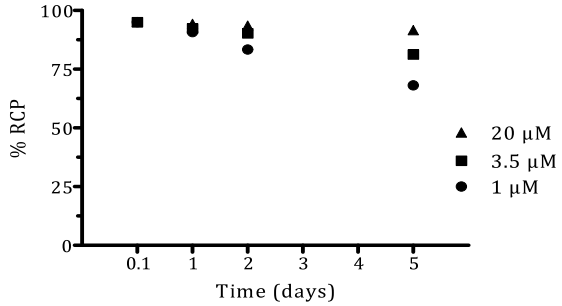

Figure 2. ^{111}In-DOTA-TATE in the presence of three different concentrations of ascorbic acid. X-axis shows time (days); Y-axis RCP (%). Labeling was performed with 60 MBq ^{111}In and 2 nmol of DOTA-TATE in a final volume of 0.14 mL. This figure shows the effect of quencher concentration over time. Under same conditions, ^{177}Lu-DOTA-TATE showed similar results.

The optimal quencher mixtures as described above was tested within a broad range of specific activity of ^{111}In-DOTA-TATE (50-190 MBq/nmol, *Fig.* ***5A***) and activity concentration (0.14-1.4 GBq/mL, *Fig.* ***5B***). The results were similar to those for RCP *(Fig.* ***3-4****)*.
Fig. **6** shows the optimized storing conditions of ^{177}Lu-DOTA-TATE and ^{177}Lu-DOTA-NOC. Comparable results were obtained with ^{177}Lu-DOTA-TATE at a therapeutical level (3.6-7.4 GBq, see *Fig.* ***7***) and maintained high RCP after dilution in saline (5-100 mL).

Purging labeling mixture with N_2 and O_2
Purging the reaction mixture with N_2 after radiolabeling resulted in ±10% higher RCP after 5 days of storage. Purging with O_2 resulted in RCP values obtained with traditional storage (data not shown).

Maintaining RCP and biological activity (receptor affinity)
In order to prove the biological activity of the radiolabeled SS-analogs, we studied internalisation and autoradiography during the 7 days of RCP monitoring (*Fig.* ***4****)*. Results showed a constant and specific binding and internalisation of SS-analogs (data not shown) **[28]**.

Discussion

RCP quantification
Since radiolysed radiopeptides often differ in charge and shape *vs.* structure of intact radiolabeled peptides, radiolysis of radiolabelled peptide can be separated by HPLC and quantified by radiodetection. Typically, RCP of radiolabeled SS-analogs measured by HPLC is expressed as % of radiodetected peak area ($\mu V.sec^{-1}$) of the intact radiolabeled peptide *vs.* all other radio peaks measured during the same HPLC-analyses.

There are no quality criteria within the field of Nuclear Medicine to qualify HPLC-separation methods **[29]**. Nevertheless, differences in eluents, gradient, flow, column type and length might result in the detection of different degrees of impurity, and thus variation in RCP.
Therefore, in our opinion, RCP are actually expressed in percentage of arbitrary units **[1]**. Moreover, to enable comparison, HPLC runs were performed under standardized conditions. All HPLC-measurements were corrected for background. Even at low activity, the influence of background on calculated RCP was <3 %.

Addition of ethanol as a quencher
Adding ethanol during or after labeling procedure could have severe side effects in patients with a neuroendocrine carcinoid tumor. Intravenous administration of a ^{177}Lu-labeled SS-analogs mixture containing ethanol could lead to carcinoid syndrome **[30]**. Long et al showed that development of a carcinoid syndrome could be blocked by administration of somatostatin **[31]**. Unfortunately there is no standard maximum tolerated amount of ethanol which can be administered to these patients, since this might depend on tumor mass. A fully automated system for labeling of different SS-analogs, including a C_{18} separation using 2.5 mL of 50% of ethanol, has been applied in many patients world–wide, without

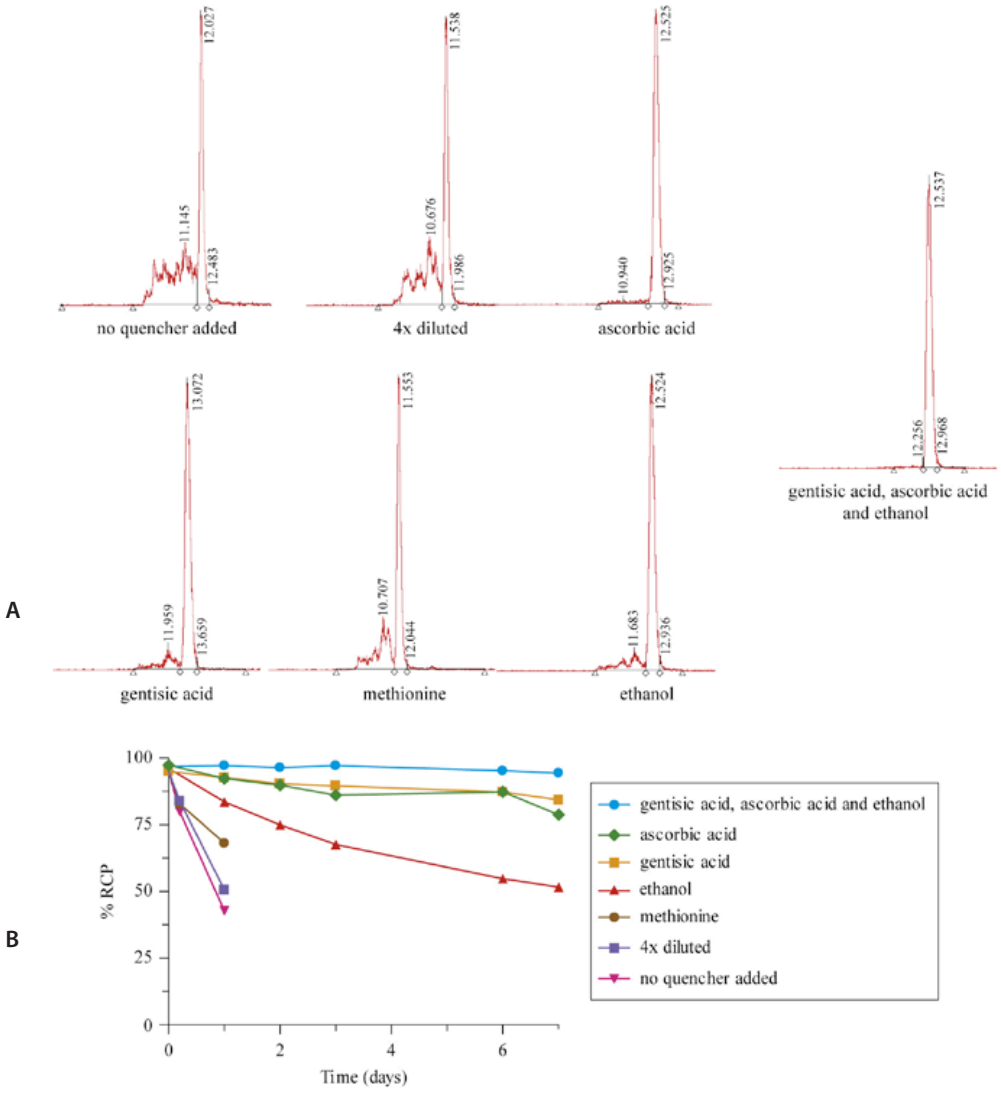

Figure 3. A: HPLC-chromatograms of ^{111}In-DOTA-TATE and effect of the difference quenchers and mixtures of quenchers 24h after radiolabeling. Applied quencher concentrations after optimization (See paragraph ***optimizing quencher concentration***) in reaction vial were: 3.5 mM for gentisic acid and ascorbic acid, 10 mM for methionine, and 7-10% (v/v) for ethanol. HPLC-chromatograms 24 h after radiolabeling can be compared in a relative way and show specific patterns of radiolysed peptide in the presence of different quenchers, which indicates that quenchers specifically scavenge different chemical groups and/or formed radicals **B:** ^{111}In-DOTA-TATE in the presence of quenchers or combinations of quenchers up to 7 days after radiolabeling. This figure shows the effect of quenchers and combination separately. The maximum error of inter and intra-observer was <3 % and were performed n≥2.

reports of carcinoid crisis **[32]**. According Serdons et al. **[33]** radiolabeled peptides can safely be used without removal of the ethanol after appropriate dilution with normal saline to a concentration of ethanol not exceeding 10% and injection volume below 20 mL.

The ready-for-use liquid formulation of the radiolabeled peptide allows physicians to administer labeled peptide without a radiochemical reaction – simply by diluting if necessary.

Users still should be aware that while dilution indeed results in lower dose radiation, however this also decreases quencher concentration and thus negatively influences RCP during further storage. Therefore after dilution radiolabeled peptide should be administered as rapidly as possible to decrease the influence of radiolysis on radiolabeled peptide. To overcome this problem we recommend diluting the liquid formulation with quencher added to maintain a constant quencher concentration.

Moreover sterility is still a matter of concern, there are microbiological implications for 7-day shelf-life. When the ready-for-use liquid formulation of the radiolabeled peptide will be used over a longer time period sterility should be determined before administration, this could be performed by measuring endotoxins in final liquid formulation.

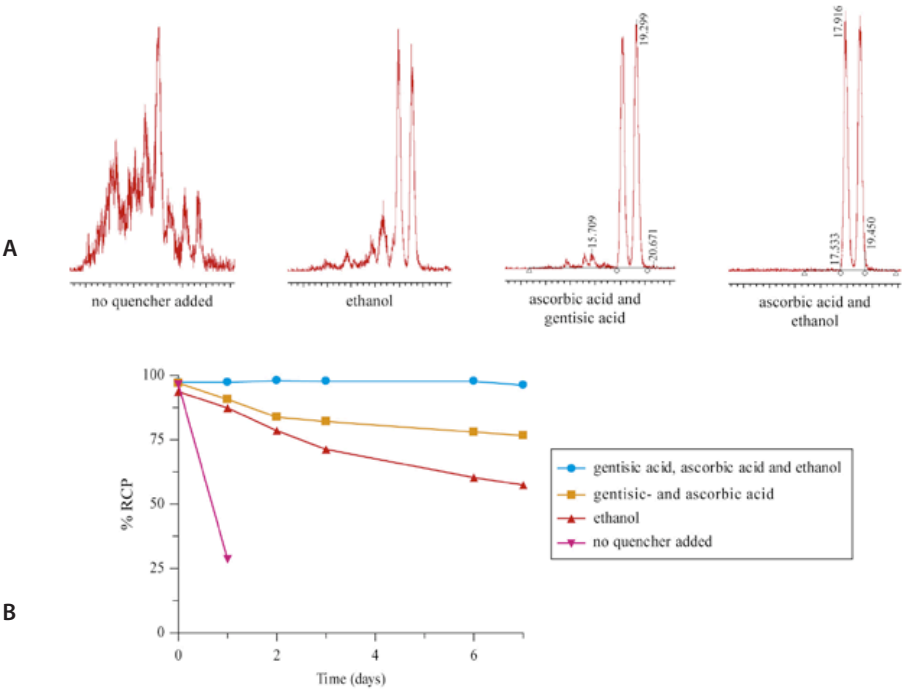

Figure 4 A. HPLC-chromatograms of OctreoScan and effect of the difference quenchers and mixtures of quenchers 24h after radiolabeling. Applied quencher concentrations after optimization as described in paragraph ***optimizing quencher concentration***. HPLC-chromatograms 24 h after radiolabeling show specific patterns as well, as also described in figure 3A. The two major peaks (17.9-19.2 min) in the HPLC-chromatograms were caused by stereo isomers of the ^{111}In-labeled DTPA **[9]**. The two major pre-peaks seen after radiolysis were caused by stereo isomers of ^{111}In-DTPA (see HPLC-chromatogram of gentisic and ascorbic acid). **B:** OctreoScan in the presence of quenchers or combinations of quenchers. X-axis shows time (days); Y-axis % RCP. These figures show the effect of quenchers and combination up to 7 days after radiolabeling.

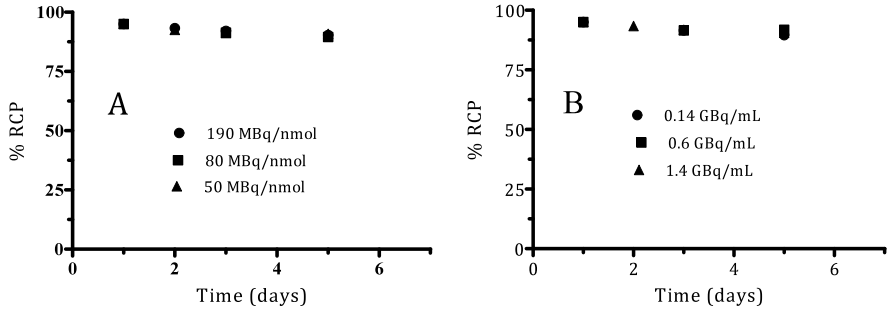

Figure 5. ^{111}In labeled DOTA-TATE as function of time in the presence of gentisic acid, ascorbic acid and 7% of ethanol (v/v) and the influence of specific activity (50-190 MBq/nmol) **(A)** or by decrease of the concentration of activity (0.14-1.4 GBq/mL) **(B)**. Labeling was performed in a final volume of 0.14 mL and only the peptide amount (1-4 nmoles) or activity (50-190 MBq) was adjusted. X-axis shows time (days); Y-axis RCP (%). Both figures show the applicability of the quencher mixture within a broad ranch of specific activities, thus independent of the peptide mass or activity concentration (MBq/mL).

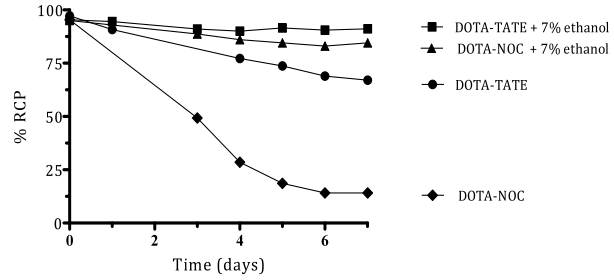

Figure 6. Therapeutic labeling of DOTA-TATE and DOTA-NOC containing 3.7 GBq of ^{177}Lu . X-axis shows time (days); Y-axis in RCP (%). For patient administration, labeling mixtures were diluted in saline, final volume 100 mL. Under these conditions RCP was monitored up to 7 days after radiolabeling in the presence or absence of 7% of ethanol (v/v). Addition of ethanol showed a substantial beneficial effect on maintaining the RCP.

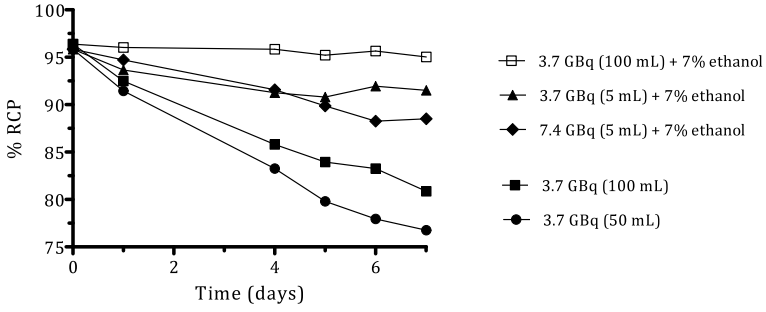

Figure 7. Dilution in saline (5, 50 or 100 mL) of a therapeutic dose (3.7or 7.4 GBq) of ^{177}Lu-DOTA-TATE in the presence or absence of 7% of ethanol (v/v) up to 7 days after radiolabeling. X-axis shows time after radiolabeling (days); Y-axis in RCP (%).

Conclusion

Our experiments showed that ethanol, in combination with a mixture of gentisic- and ascorbic acid has a superior effect in stabilizing radiolabeled peptides. This property enables to store and transport ^{111}In- and ^{177}Lu-labeled SS-analogs in a single-vial, ready-for-use liquid formulation up to 7 days after radiolabeling.

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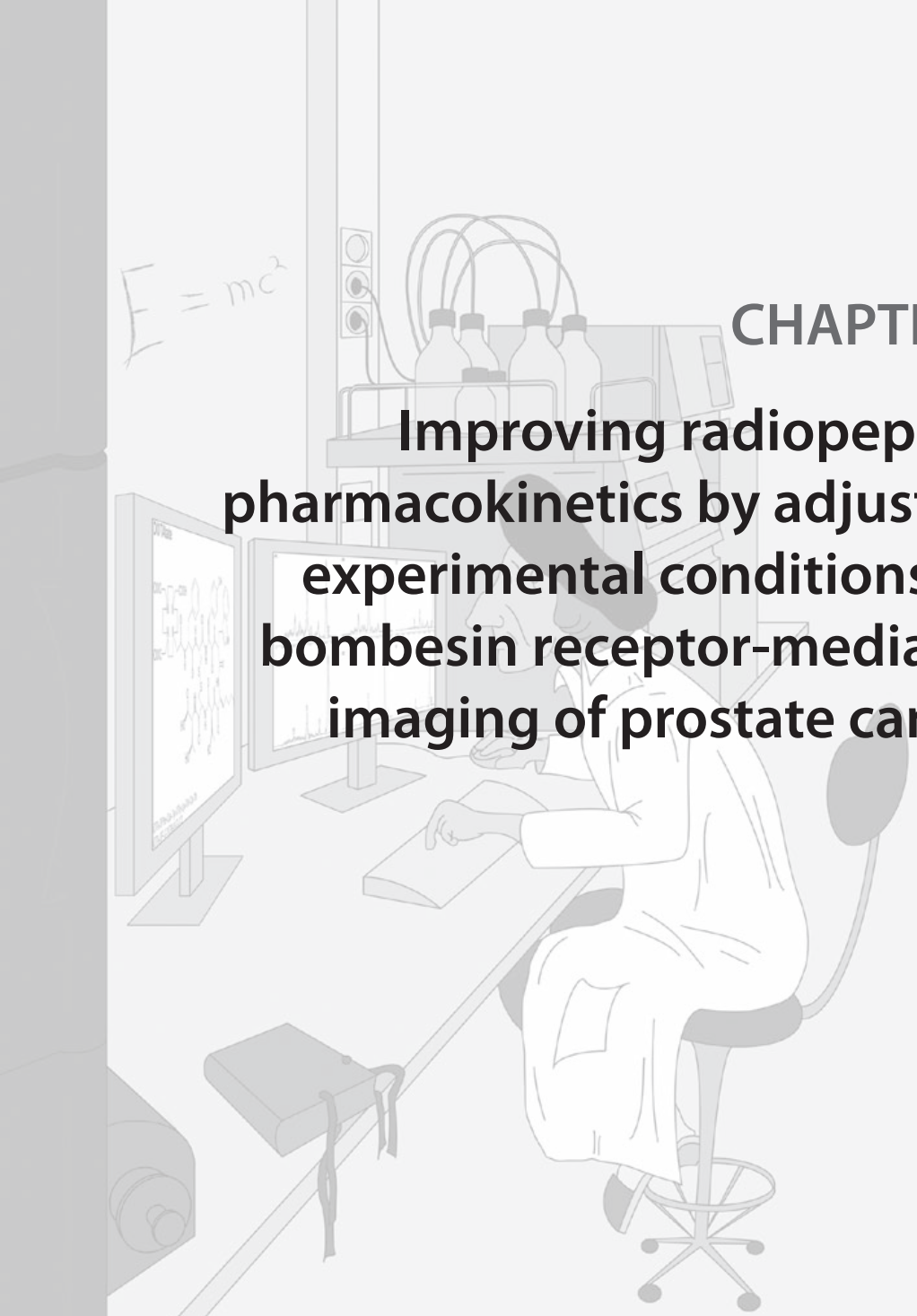

CHAPTER 8

Improving radiopeptide pharmacokinetics by adjusting experimental conditions for bombesin receptor-mediated imaging of prostate cancer

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Abstract

Aim: Prostate cancer (PC) is a major health problem. The Gastrin Releasing Peptide Receptor (GRPR) offers a promising target for staging and monitoring of PC since it is overexpressed in PC and not in normal prostatic tissue. To improve GRPR-mediated imaging we investigated the impact of various experimental conditions on pharmacokinetics using the ^{111}In-labelled bombesin (BN) analogue DOTA-AMBA that binds to the GRPR with high affinity. Besides the frequently used PC3 cell line, the androgen sensitive VCaP celline was used as human PC xenografts in nude mice.
Methods: Non-purified [^{111}In]DOTA-AMBA was compared with HPLC-purified [^{111}In]DOTA-AMBA. Effect of specific activity was studied administrating a constant amount of activity (0.1 MBq) [^{111}In]DOTA-AMBA supplemented with different amounts of DOTA-AMBA (1-3000 pmol). GRPR was saturated with Tyr^4-Bombesin, 1 and 4h prior to injection of [^{111}In]DOTA-AMBA.
Results: GRPR-positive tumour tissue showed a significant 2 to 3-fold increase in absolute uptake after HPLC-purification while a stable tumour-to-pancreas ratio remained. Low peptide amounts (10 pmoles) resulted in a decline in uptake of 43 % in tumour, 49 % in kidney and 92 % in pancreas. Tumour-to-pancreas ratio improved six-fold from 0.1 ± 0.0 at 10 pmol up to 0.6 ± 0.2 at 3000 pmol ($p<0.01$). GRPR saturation 4h prior to injection of [^{111}In]DOTA-AMBA resulted in improvement of the tumour-to-pancreas ratio from 0.10 ± 0.3 to 0.22 ± 0.2 ($p<0.01$) and tumour-to-kidney ratio increased from 0.92 ± 0.16 up to 3.45 ± 0.5 ($p<0.01$).
Conclusions: Besides specific peptide characteristics also the experimental conditions, such as HPLC-purification, variations in peptide mass and GRPR saturation prior to [^{111}In]DOTA-AMBA administration affect radiopeptide pharmacokinetics. Experimental conditions therefore need to be carefully selected in order to compose standardised protocols for optimal targeting.

Introduction

Prostate cancer (PC) is the most frequently diagnosed cancer among men in the Western world and the second cause of cancer related death **[1]**. PSA-based screening of PC has dramatically increased early diagnosis **[2]**, but for optimal management of disease it is essential to discriminate between organ-confined disease and PC beyond the borders of the gland. Current imaging techniques are not sufficiently accurate to determine extent of PC at diagnosis and to visualise micrometastases **[3-5]**. Therefore more sensitive imaging techniques are required.

Nuclear imaging - using single photon emission computed tomography (SPECT) and positron emission tomography (PET) - might offer applications for such desired accurate PC imaging. The GRPR constitutes an interesting target for peptide-receptor radionuclide imaging and therapy. GRPR is a G-protein coupled receptor overexpressed on membranes of PC while not or less on normal prostate **[6]**. BN and its mammalian counterpart GRP, are natural ligands for the GRPR and bind to it with high affinity. The 14 amino acids at C-terminus of the 27 amino acid GRP are responsible for the biological activity and correspond to those of BN **[6]**. Several radiolabelled BN-based analogues have been developed and tested in preclinical and clinical settings as diagnostic and therapeutic tools for management of PC in

patients. Our group has performed a standardised study on PC targeting efficacy of five radiolabelled BN-based analogues **[7]**. In this study, among others, the agonist DOTA-CH_2CO-Gly-[4-aminobenzoyl]-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH_2 (further referred as AMBA) was demonstrated to be one of the best BN-based analogues with relatively high in vivo stability, high tumour uptake and retention **[8]**. Also, AMBA is one of the few BN-based analogues that have been used for PET imaging in patients **[9, 10]**.

The focus for improvement of BN-based imaging (by increasing tumour-to-background ratio) has predominantly relied on development of new BN-based analogues with enhanced affinity for the GRPR **[8, 10-12]**. Instead, in the present study we investigated the impact of various experimental factors in order to further improve peptide receptor-mediated imaging.
To ensure purity of the labelled peptide many study groups perform purification of BN-based analogues by high performance liquid chromatography (HPLC) between labelling and administration. In order to determine the impact of this purification step, we studied the effect of HPLC-purification on BN biodistribution.
An essential parameter to evaluate the performance of targeted imaging probes is the ratio between radioactivity in tumour and that in background organs. Contrast between the two is dependent on the amount of administered peptide. BN-based analogues have already pharmacological effects after administration in nanomole quantities. The high affinity GRPR have al low capacity in tumour and other tissues and also the expression varies between tissues **[8, 13, 14]**. Therefore, in the present study we investigated GRPR saturation of target versus non-target organs using different peptide amounts **[15]**. We studied the impact of specific activity on tumour-to-background ratio by varying injected peptide amount while injecting a constant radioactivity level. Specific activity in this study is expressed as amount of activity (MBq) vs. pmoles of peptide. Specific activity was investigated using a fixed amount of radioactivity (0.1 MBq) while varying the amount peptide (1-3000 pmoles)

GRPR-binding of BN-based agonists like AMBA causes internalisation of the receptor **[12, 16]** and over time gradually re-expression of receptors on the cell membrane occurs indicating receptor turn-over. Tumour cells are reported to have a higher metabolic rate than normal tissue **[17, 18]**. We therefore hypothesized that GRPR turn-over is faster in PC than in normal tissue resulting in a higher tumour-to-background ratio. We also performed biodistribution studies to evaluate the impact of GRPR saturation with Tyr^4-Bombesin (further referred to as Tyr^4-BN) 1 and 4 h prior to the injection of ^{111}In-labelled DOTA-AMBA (further referred to as [^{111}In]AMBA).

The aim of this study was to investigate the impact of various experimental factors, like HPLC-purification of [^{111}In]AMBA, the effects of specific activity by increasing peptide amount and GRPR saturation prior to the injection of [^{111}In]AMBA on tumour uptake. Biodistribution studies were performed administrating BN-based agonist ([^{111}In]AMBA) intravenously to athymic nude mice bearing GRPR-expressing PC-3 or VCaP **[19, 20]** xenografts. The data show that all factors affect GRPR-mediated biodistribution and thereby influence prostate cancer imaging.

Materials and methods

Cell culture

Human PC cells were cultured in RPMI (LONZA, Verviers, Belgium) supplemented with fetal calf serum (GIBCO Invitrogen Co, Grand Island, NY, USA), 5 % for PC-3 and 10 % for VCaP cells. An additional penicillin/streptomycin antibiotic solution (LONZA, Verviers, Belgium; 10.000 Units/mL penicillin, 10.000 Units/mL streptomycin) was added to medium of both cell lines (5 mL/500 mL). Cells were grown in T175 Cellstar tissue culture flasks (Greiner Bio-one GmBH, Frickenhausen, Germany) at 37°C in a humidified atmosphere containing 5 % CO_2. Cells were passaged using a Trypsin/EDTA solution (LONZA, Verviers, Belgium), Trypsine-Versene 170000 U/L and EDTA 200 mg/L. Cells were grown to near confluence after which they were harvested and counted. Suspensions of approximately 5 x 10^6cells were yielded and dissolved in 100 µL PBS for subcutaneous injection in xenografts.

Prostate cancer xenografts

Six to seven-weeks old male Swiss nu/nu mice (Charles River, Kißlegg, Germany) were inoculated subcutaneously with PC-3 or VCaP cells (5 x 10^6cells/100µL PBS) on the right shoulder. Maximum 4 mice per cage were kept in 14 x 13 x 33cm^3 individually ventilated cages (Techniplast BV, Wognum, The Netherlands), on Woody-Clean sawdust type BK8/15 (Technilab-BMI, Someren, The Netherlands) under a 12 h light-dark cycle, at 50 ± 5 % relative humidity and controlled temperature (~22°C). Mice had access to irradiated chow and acidified drinking water *ad libitum*. Experiments were initiated when tumours were established and clearly visible ranging from 200-500 mm^3 (3-5 weeks after inoculation).

The study was approved by the Animal Experimental Committee (DEC) of the Erasmus MC and performed in agreement with the Dutch Animal Experimentation Act (1977) and the European Convention for Protection of Vertebrate Animals Used for Experimental Purposes (Strasbourg, 18 March 1986).

Radiolabelling of AMBA and quality control

AMBA (MW: 1503 g/mol) was kindly provided by Prof. Dr. H.R. Mäcke (University Hospital Basel, Switzerland) and was dissolved in Milli-Q water, final peptide concentration 10^{-3} M. $^{111}InCl_3$ (DRN 4901, 370 MBq/mL in HCl, pH 1.5–1.9) was obtained from Covedien (Petten, The Netherlands). All chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands) at the highest available grade. Radioactivity was measured in a VDC-405 dose calibrator (Veenstra Instruments BV, Joure, The Netherlands).
Small volume reactions were performed in conical vials as described **[21]**. Sodium acetate was used to control pH (3.5-4) and quenchers were used for stabilization **[22]**. Vials were placed in a temperature-controlled heating block. AMBA, $^{111}InCl_3$ and quenchers (methionin, ascorbic acid and gentisic acid) were heated for 20 min at 80°C. After cooling down DTPA was added to bind potential non-incorporated ^{111}In. Instant thin-layer chromatography Silica Gel (ITLC-SG) was performed mobile phase both sodium citrate 0.1 M and ammonium acetate 1 M / methanol (1:1 v/v) as mobile phase**[23, 24]**. Radioactivity on the ITLC strips was subsequently detected using a Phosphor Imaging system, Packard Cyclone, with OptiQuant software (PerkinElmer, Groningen, The Netherlands). HPLC quality control and purification were performed using a Breeze HPLC system with a 1525 binary pump (Waters, Etten-Leur, The Netherlands).

Radioactivity was monitored with a radioactivity monitoring system, including a NaI detector, digital multichannel analyzer and dedicated software (MetorX B.V, Goedereede, The Netherlands), was connected to the HPLC-system. For separation a Symmetry 5 µm, 4.6 mm x 250 mm C_{18} column (Waters, Etten-Leur, The Netherlands) was used. HPLC gradient was applied as described **[21]**. Injections of 200 µL were performed with a 717plus autosampler (Waters, Etten-Leur, The Netherlands).

HPLC purification of [^{111}In]AMBA

To study the biodistribution effects of specific activity on imaging we needed to obtain a fixed and well-characterized amount of [^{111}In]AMBA. Hereto we optimized HPLC-separation between non-labelled AMBA and [^{111}In]AMBA (see Fig. **1**). HPLC-purified [^{111}In]AMBA was quantified by dose calibrator (e.g. Veenstra VDC-405, Joure, The Netherlands), supplemented with know amounts non-labelled AMBA (range 1 to 3000 pmol), and diluted with saline for injection (0.1 MBq per animal). The peptide mass of HPLC-purified [^{111}In]AMBA from the main peak was considered negligible, since 37 MBq ^{111}In ≈ 2.15x10^{-11} gram atoms. For stabilisation and reduction of radiolysis methionin, ascorbic acid and gentisic acid were added as quenchers directly after HPLC-purification.

Biodistribution studies

Mice (either non- tumour-bearing, PC-3 or VCaP tumour-bearing) were injected with [^{111}In]AMBA solution via the lateral tail vein. Animals were euthanized by cervical dislocation at fixed time points post injection (p.i.). Study groups consisted of 3-5 mice. Tumour and other selected tissues were excised, weighed, and radioactivity was determined in a LKB-1282-compugamma γ-counter (Perkin Elmer, Groningen, The Netherlands). Data were expressed as percentage of the injected dose per gram of tissue (%ID/g).

Effect of HPLC-purified [^{111}In]AMBA on biodistribution

A biodistribution study comparing tissue distribution of [^{111}In]AMBA and HPLC-purified [^{111}In]AMBA was performed. An equal peptide amount (10 pmol) and radioactivity (0.1 MBq) of both was injected in PC-3 and VCaP bearing mice (n=4/group). Mice were euthanized 1 h p.i..

Effect of specific activity of [^{111}In]AMBA on biodistribution

A biodistribution study in PC-3 bearing mice was performed comparing tissue distribution after injections of constant radiodose (0.1 MBq) with increasing amount of AMBA, range 1-3000 pmoles. Purification was performed as described in paragraph ***HPLC purification of [^{111}In]AMBA,*** mice (n=4 per condition) were euthanized 1 h p.i..

Effect of GRPR saturation on biodistribution

The effect of pre-dosing (further referred as GRPR saturation) prior to the administration of 0.1 MBq/10 pmoles of HPLC-purified [^{111}In]AMBA was studied in PC-3 bearing mice with or without injection of Tyr^4-BN (60 nmol) injected via the lateral tail vein.

Three groups were compared: (I) no saturation, (II) 1 h and (III) 4 h after GRPR saturation by 60 nmol Tyr^4-BN of (n=4/time point). Mice were euthanized 1h after injection of HPLC-purified [^{111}In]AMBA.

Effect of HPLC-purified AMBA prior to labelling and organic solvents

We performed a biodistribution study in order to investigate whether the HPLC procedure itself caused differences in uptake. In this study we used [^{111}In]AMBA in which the non-labelled AMBA peptide was purified by HPLC prior to labelling and compared that to HPLC-purified [^{111}In]AMBA. 0.1 MBq/10 pmoles of this [^{111}In]AMBA was administered to PC-3 bearing via the lateral tail vein (n=4).

Since HPLC-purification requires aqueous solutions containing various organic eluents such as methanol, ethanol or acetonitrile to separate labelled and non-labelled peptides, small amounts of these organic solvents will be injected. To investigate the influence on GRPR uptake non HPLC-purified [^{111}In]AMBA was supplemented with these commonly used organic solvents before administration. 0.1 MBq/10 pmoles of [^{111}In]AMBA supplemented with these solvents was administered to PC-3 bearing via the lateral tail vein (n=4/solvent).

Effect of Sep-Pak C_{18} purification of [^{111}In]AMBA on biodistribution

In contrast to HPLC-purification, separation procedures using Sep-Pak C_{18} do not separate [^{111}In]AMBA from non-labelled AMBA. Consequently, after purification of labelled peptide, to injection solution no additional of non-purified AMBA mass was needed. 0.1 MBq/10 pmoles of this Sep-Pak C_{18} purified [^{111}In]AMBA was administered to PC-3 bearing mice via the lateral tail vein (n=4).

Effect supplementing [^{115}In]AMBA on biodistribution

In order to study the differences in affinity and uptake between labelled and non-labelled AMBA, biodistribution was performed in which we added HPLC-purified [^{115}In]AMBA to HPLC-purified [^{111}In]AMBA. 0.1 MBq/10 pmoles of this [$^{111/115}$In]AMBA was administered to PC-3 bearing via the lateral tail vein (n=4).

Statistics

Statistical analyses were performed using the unpaired 2-tailed t-test in SPSS 11.01 (SPSS Inc., Chicago, United States). Statistical significance was assumed if p-values were <0.05.

Results

HPLC purification of [^{111}In]AMBA

Purification of [^{111}In]AMBA solution resulted in carrier free product, as shown in Fig. **1**. Main peak on HPLC-chromatogram containing carrier free [^{111}In]AMBA, retention time (RT) 16.1 min, while RT for non-labelled AMBA was 14.6 min. Radiochemical purity measured directly after labelling, was always **>**90 %. Typically radiochemical purity of radiolabelled bombesin analogues measured by HPLC is expressed as % of radiodetected peak area (e.g. $\mu V.sec^{-1}$) of the intact radiolabelled peptide *vs.* all other radio peaks measured during the same HPLC-analyses.

Effect of HPLC-purified [^{111}In]AMBA on biodistribution

When administrating non-purified [^{111}In]AMBA in PC-3 bearing mice tumour uptake was 3.3 ± 0.7 %ID/g and pancreas uptake was 28.6 ± 2.2 %ID/g 1 h p.i (Fig. **2**). After injection of HPLC-purified [^{111}In]AMBA, higher uptakes ($p<0.01$) for both tissues were obtained: tumour uptake was 6.8 ± 1.1 %ID/g and pancreas uptake was 60.5 ± 14.0 %ID/g 1h p.i. (Fig. **2**). However, uptake was significantly higher in all gastro-intestinal organs.

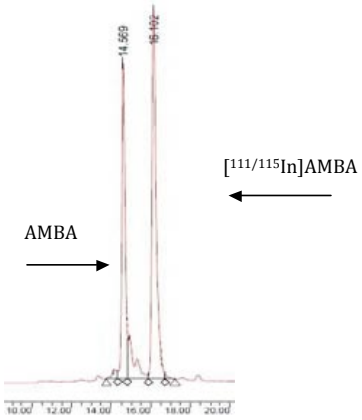

Figure 1. HPLC chromatogram at 278 nm showed base-to-base separation between AMBA and $^{111/115}$In-labelled AMBA. In this case, UV absorbance could be visualized since we added ^{115}In with a final mol/mol ratio of 2 : 1 (AMBA vs ^{115}In) before (radio)labelling. Difference in retention time between AMBA and [$^{111/115}$In]AMBA is 1.5 min.

In VCaP-tumour bearing mice the same tendency was seen. Uptake in tumour increased significantly from 1.6 ± 0.2 %ID/g for non-purified [^{111}In]AMBA up to 4.3 ± 1.3 %ID/g after injection of HPLC-purified [^{111}In]AMBA at 1 h pi.

Since pancreatic tissue is GRPR-postive, there is a relatively high uptake. Uptake was also increased when HPLC-purified [^{111}In]AMBA : 23.4 ± 2.9 %ID/g and 70.0 ± 20.3 %ID/g (Fig. **2**), respectively. Tumour-to-pancreas ratio remained unchanged in both experiments.

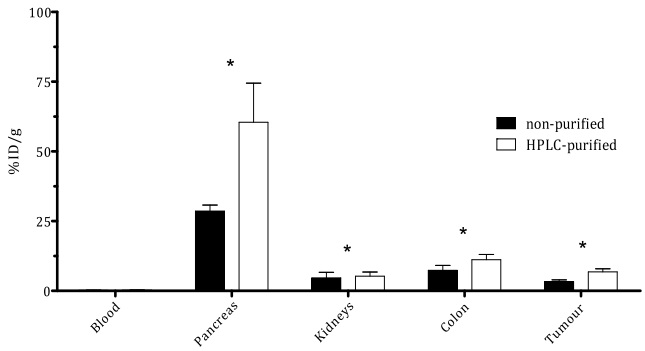

Figure 2. Biodistribution results of non-purified versus HPLC-purified [^{111}In]AMBA in PC-3 bearing mice, 1h post injection.

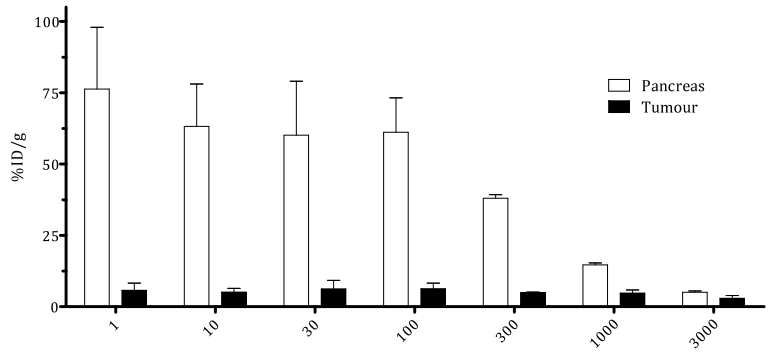

Figure 3. Uptake in pancreas and tumour after administration of HPLC-purified [^{111}In]AMBA with variable specific activities. A constant amount of radioactivity (0.1 MBq) was supplemented with 1-3000 pmoles. X-axis depicts peptide amount in pmol.

Effect of specific activity of [^{111}In]AMBA on biodistribution

Injecting [^{111}In]AMBA with a fixed amount of radioactivity (0.1 MBq) while varying the peptide amount significantly influenced PC3 tumour uptake. Lower peptide mass resulted in a 57 % decline (p=0.09) in tumour uptake between 10 pmol (5.7 ± 2.5 %ID/g) and 3000 pmol (2.9 ± 1.0 %ID/g, see Fig. **3**).

Significant higher pancreas uptake was observed at lower peptide amounts (<100 pmol). Increasing peptide amount resulted in a 80 % reduction in pancreas uptake. Injection of 10 pmol [^{111}In]AMBA resulted in a pancreas uptake of 63.2 ± 14.9 %ID/g vs 5.1 ± 0.4 %ID/g after 3000 pmol. Due to a rather stable tumour uptake and decreased pancreas uptake, tumour-to-pancreas ratios improved six-fold from 0.1 ± 0.0 at 10 pmol up to 0.6 ± 0.2 at 3000 pmol.

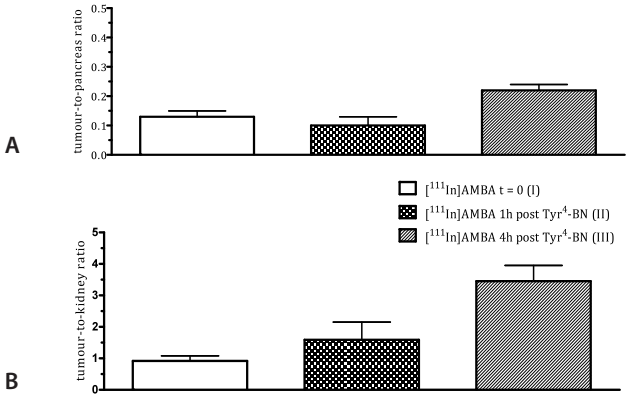

Figure 4. Tumour-to-pancreas (**A**) and tumour-to-kidney (**B**) ratio after administerion of purified [^{111}In]AMBA at different time points after injection of 60 nmoles Tyr^4-BN in PC-3 bearing mice.

Uptake in kidneys showed a gradual decrease at higher peptide amount. Uptake decreased 51 % from 4.3 ± 1.0 %ID/g (10 pmol) down to 2.0 ± 0.3 %ID/g (3000 pmol). Tumour-to-kidney ratio remained stable with an average ratio of 2.1 ± 0.6.

Effect of GRPR saturation on biodistribution

Tumour uptake of [^{111}In]AMBA at 1 h p.i. without GRPR saturation was 3.9 ± 0.6 %ID/g (l).

[^{111}In]AMBA tumour uptake 1h after GRPR saturation, showed a decreased uptake (1.3 ± 0.5 %ID/g ($p<0.01$)), while 4h after GRPR saturation uptake was 2.4 ± 0.7 %ID/g. Pancreas uptake of [^{111}In]AMBA without GRPR saturation was 29.8 ± 1.7 %ID/g 1h p.i.. Uptake was lowered ($p<0.001$) when GRPR saturation was applied (12.2 ± 0.6 %ID/g 1h and 10.8 ± 3.0 %ID/g 4h, respectively). As a result tumour-to-pancreas ratio improved ($p<0.01$) from 0.10 ± 0.3 up to 0.22 ± 0.2 (Fig. **4A**).

Tumour-to-kidney ratios significantly increased after GRPR saturation. Without GRPR saturation, kidney uptake was 4.4 ± 1.5 %ID/g 1 h p.i., while [^{111}In]AMBA uptake after GRPR saturation at 1 h and 4 h was 0.8 ± 0.1 %ID/g and 0.7 ± 0.1 %ID/g. As a result also tumour-to-kidney ratio increased ($p <0.01$) from 0.92 ± 0.16 up to 3.45 ± 0.5 (Fig. **4B**).

Effect of HPLC-purified AMBA Prior to labelling and organic solvents

No increase in uptake of GRPR-positive tissues was obtained after administering [^{111}In]AMBA which was HPLC-purified prior to labelling (Table **1**). A tendency towards higher uptake in GRPR-expressing organs was seen after addition of organic solvents (Table **1**).

Effect of Sep-Pak C_{18} purification of [^{111}In]AMBA on biodistribution

Again, biodistribution data of Sep-Pak C_{18} purified [^{111}In]AMBA did not differ from non HPLC-purified [^{111}In]AMBA (Table **1**).

Effect supplementing [^{115}In]AMBA on biodistribution

As compared to HPLC purified [^{111}In]AMBA, the solution of [$^{111/115}$In]AMBA showed no significant differences in GRPR uptake (data not shown). Clearly, substitution of [^{111}In]AMBA by [^{115}In]AMBA did not affect GRPR affinity so biodistribution data were similar.

Discussion

Nuclear imaging offers new applications for the desired accurate imaging of PC. Targeting of GRPR with radiolabelled BN has shown promising results in preclinical studies **[7, 8, 11, 12]**. A variety of BN-based analogues have been developed and tested for *in vivo* stability, GRPR affinity and specificity. Since, not only specific tracer characteristics, but also experimental conditions like HPLC-purification of labelled peptide, peptide mass and GRPR saturation may affect GRPR-targeting performance of BN-based analogues, we have focused on these aspects is this study, experimental conditions during purification are summarized in Table **1**.

Table 1. Uptake in %ID/g in pancreas (and tumour) after administration of variable [^{111}In]AMBA solutions in (VCaP tumour-bearing) mice.

	Tumour (%ID/g)			Pancreas (%ID/g)		
	mean	**SD**	**P-value**	**mean**	**SD**	**P-value**
HPLC-purification						
Non-purified (control)	-	-	-	19.4	4.1	
Purified before labelling	-	-	-	29.3	17.4	0.39
Purified after labelling	-	-	-	41.8	6.0	**0.01*
Addition of organic solvent						
Non-purified (control)	5.4	0.9		42.9	5.1	
Methanol 14 % (v/v)	5.5	1.4	0.73	48.0	6.9	0.49
Ethanol 14 % (v/v)	7.5	2.5	0.25	55.0	1.8	**0.02*
Acetonitril 14 % (v/v)	7.4	0.6	**0.03*	62.0	5.9	**0.01*
Sep-Pak C_{18}						
Non-purified (control)	-	-	-	22.5	1.5	
Purified	-	-	-	27.1	7.5	0.30

- indicates that this values have not been obtained.
* indicates significance compared to control

We aimed at optimising GRPR-targeting. Many factors showed to have an effect on the GRPR-mediated uptake, while GRPR-expressing PC3 and VCaP xenografts were used as model. The androgen-resistant PC3 cell line was chosen because of its use in the vast majority of studies. In addition, the less applied VCaP cell line was added since it has GRPR-expression comparable with PC3 and is more clinically relevant because of its expression of the androgen receptor and ability to secrete PSA **[19, 20]**. Therefore, being a more realistic model for future peptide-based imaging as well as therapeutic studies.

Effect of HPLC-purified AMBA and organic solvents

A tendency towards higher uptake in GRPR-expressing organs was seen after addition of organic solvents like ethanol, methanol or acetonitrile. These differences, however, could not fully explain the significant differences seen with HPLC purified [^{111}In]AMBA. Further studies are ongoing.

Effect of GRPR saturation and injection of different specific activity on biodistribution

There is no consensus in literature about the optimal BN peptide amount for optimal targeting in mice. We previously reported a peptide mass for GRPR-targeting between 10 pmol and 50 pmol for analogue [[^{111}In]DTPA-Pro^{1},Tyr^{4}]BN in non-tumour-bearing Lewis rats to obtain the highest tumour background ratio **[15]**. In experimental studies using mice often 10 pmol of radiolabelled BN is injected **[10, 11]**. Our current study clearly showed that differences in peptide amount have a significant influence on BN biodistribution. The best tumour-to-background ratios were reached when injecting 3000 pmol of purified [^{111}In]AMBA (Fig. **3**). This was caused by decrease in tracer uptake in most background organs, especially pancreas, while tumour uptake remained fairly stable. This implies that the GRPR saturation is faster in background- than in tumour tissue, probably caused by a higher metabolic rate **[17, 18]**. Similar results - although GRPR saturation of the tumour seemed not to be reached - were seen in a study by

Lantry *et al*. In this study [^{177}Lu]AMBA with various peptide amounts (between 1.7 pmol and 426 pmol) was administered to PC-3 bearing mice **[8]**.

In our study we assumed that the main peak of [^{111}In]AMBA, shown at HPLC-chromatogram (Fig. **1**) only contains carrier free [^{111}In]AMBA. It needs to be mentioned that the [^{111}In]AMBA peak might also contain AMBA labelled with other metals like Fe and Zn. Total peptide mass collected from HPLC might therefore be higher than calculated. Since we neglected the amount of [^{111}In]AMBA from HPLC we have added 10 pmol AMBA to achieve final injection solution of 0.1 MBq (10 pmol). In theory, if the total amount of peptide was injected into the HPLC and it would be completely incorporated with ^{111}In and trace metals, this would result in an extra peptide mass of 10 pmol. Therefore, after addition of 10 pmol AMBA, the maximum peptide mass of [^{111}In]AMBA solution is 20 pmol. As concluded earlier, this would not affect biodistribution since GRPR-mediated tumour uptake remains stable up to 300 pmol.

Biodistribution of BN was also affected when GRPR was saturated prior to the injection of radiolabelled [^{111}In]AMBA. It resulted in significantly lower uptakes in tumour, pancreas and kidney. At 4h after GRPR saturation, a recovery in tumour uptake of HPLC-purified [^{111}In]AMBA was seen while restoration of uptake in pancreas and kidney was not observed.
Basso *et al*. reported that in man a BN infusion of 15 ng/kg per minute resulted in the maximal gastrin response **[25]**. These side-effects might influence biodistribution explaining the increased uptake in GRPR-negative kidneys after administration large amounts of AMBA **[25]**.

Noticeably, GRPR saturation goes with a reduction in tumour uptake (%ID/g) and therefore higher amounts of peptide are required to equal uptake when GRPR saturation is performed. The need for higher peptide amounts may result in more pharmacological side-effects, especially when GRPR agonists are being used. The use of BN-based antagonists instead might will however likely solve this problem **[26]**.

Conclusion

In the present study we investigated the experimental factors that interfere with GRPR-targeted biodistribution using the BN-based analogue AMBA as a tracer. We demonstrated that HPLC-purification of [^{111}In]AMBA, variations in increased peptide amount and GRPR saturation prior to the administration of [^{111}In]AMBA all affect radiopeptide biodistribution in mice. All described experimental variations influence tumour uptake and often tumour-to-background ratio was affected as well. Since details in study design strongly affect GRPR-based biodistribution, experimental conditions need to be carefully selected to compose standardised protocols for optimal targeting.

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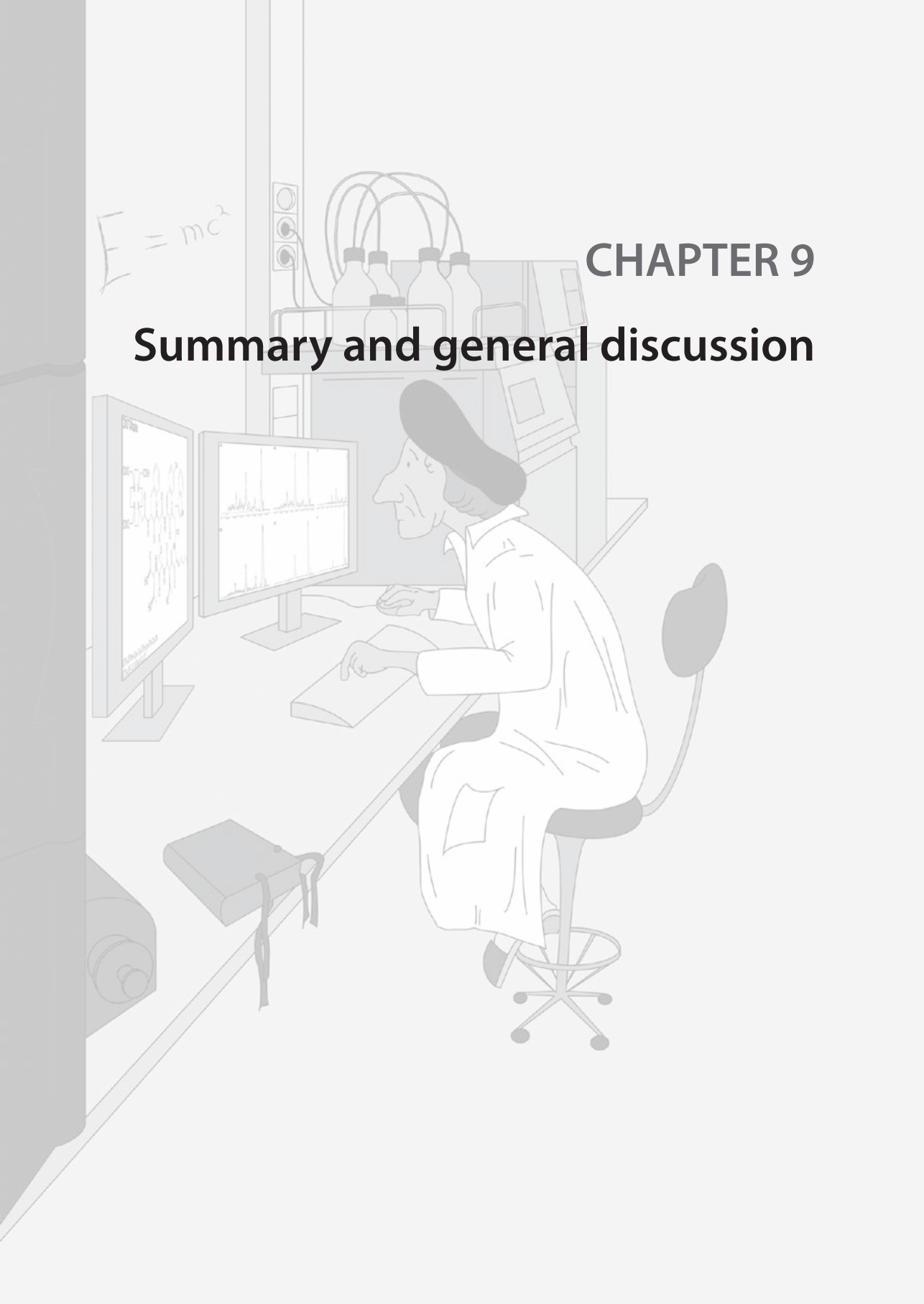

CHAPTER 9

Summary and general discussion

PART I: INVESTIGATION OF ^{68}GA LABELING CHEMISTRY

^{68}Ga PET imaging technology

PRS and PRRT of receptor-positive tumors are currently being performed using DOTA or DTPA-conjugated analogs such as somatostatin (SS) like octreotide (DOTATOC or OctreoScan) **[1, 2]** or octreotate (DOTA-tate) **[1, 3-7]** Also bombesin-analogs (BN) like DOTA-Tyr^4-bombesin **[8, 9]** and DOTA-AMBA **[10-14]** are used. The application of ^{68}Ga-labeled DOTA-conjugated peptides for positron emission tomography (PET), with concordant superior imaging technology, has already been demonstrated in preliminary studies in patients **[15-20]**.

Besides their use as PET imaging agents, these peptides might be a tool for research on individual receptor status and for optimal fine tuning of PRS and PRRT (in terms of timing and dosage).

Renewed interest in ^{68}Ga has recently arisen for several reasons. First, positron emission tomography (PET) has developed during the last decade from a research tool into a powerful diagnostic and imaging technique for routine clinical application.

Second, ^{68}Ge/^{68}Ga generators have been developed that produce suitable eluates for labeling that can be converted into a ^{68}Ga labeled pharmaceutical for PET studies **[17, 21-27].** Third, there are many DOTA-peptides that can be labeled with ^{68}Ga.

Fourth, a variety of monofunctional and bifunctional chelators have been developed that allow the formation of stable $^{68}Ga^{3+}$complexes and convenient coupling to biomolecules. Fifth, the availability of PET radiolabeled pharmaceuticals by the introduction of ^{68}Ga in radiopharmacy, independent of an on-site cyclotron, opened new applications and possibilities. Coupling of ^{68}Ga to small peptides and biomolecules was recently reviewed and described **[28-32]** and ^{68}Ga is potentially an alternative to ^{18}F- and ^{11}C-based radiopharmacy **[29]**. Last but not least, equipment, including generators, purification and concentration of eluate, techniques of radiolabeling, robotics, and PET cameras, has improved during the last decade.

Preclinical application of many ^{68}Ga-labeled pharmaceuticals has been developed, and all of today's options with ^{99m}Tc are potentially covered, for example, myocardial perfusion and function, blood flow, renal function and liver function **[28, 29, 31, 32]**.

None of the developed ^{68}Ga-labeled pharmaceuticals has acquired Food and Drug Administration (FDA) or European Medicines Agency (EMA) approval. Also, no FDA- or EMA-approved ^{68}Ge/^{68}Ga generator is available. Therefore, all current clinically applied ^{68}Ga-labeled pharmaceuticals are prepared on site through a so-called magisterial preparation **[33-35]**. **Chapter 1** presents an overview of the status of current knowledge and investigations of many ^{68}Ga-labeled pharmaceuticals, with a focus on the application of these radiopharmaceuticals for imaging of receptor-mediated processes.

Optimization of ^{68}Ga peptides labeling

Increasing applications of generator based ^{68}Ga radio-pharmaceuticals for diagnostic procedure, but also for treatment planning, ask for optimisation of ^{68}Ge/^{68}Ga generators both from chemical and regulatory points of view.

Gallium is element number 31 in the periodic table of the elements. Belonging to group 13. Elements of this group have three valence electrons , loose three valence electrons to become ions and form mostly 3+ ions. Apart from the low melting metallic form, ionic gallium can exist in two oxidation states, Ga^{+} and Ga^{3+}, Only the Ga^{3+} is stable under aqueous conditions and thus, only the Ga^{3+} is of relevance for in vivo application **[31, 36]**.

Another main characteristic of the Ga^{3+} is its high susceptibility to aqueous hydrolysis at moderately acidic to basic pH-values. At pH-values higher than 3, the cation rapidly forms oxide and hydroxide species. A variety of different species of low solubility can be found between pH 3.5 and 7.5 (see Fig. **1**.) The above-mentioned species however, tend to form colloidal or pseudo-colliodal precipitates which cannot be subjected to complexation anymore.

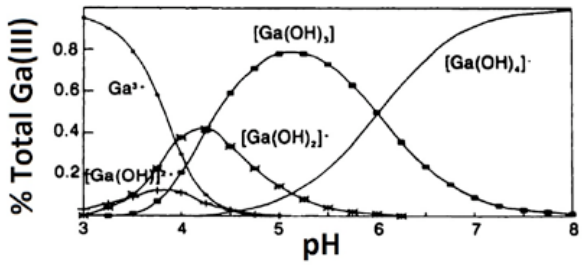

Figure 1: Specification of a 10^{-9} M aqueous solution Ga(III) **[37]**.

These chemical properties and other limitations like the large volume of generator eluate, high concentration of H^{+}, ^{68}Ge breakthrough and potential metal ion impurities could cause difficulties for direct use of ^{68}Ga for radiolabeling of peptides effectively **(chapter 2)**. In the past, it was investigated how to reduce this large volumes, acidity and impurities for a (pre)-clinical applications using a TiO_2 based generator **[38]**. More detailed data regarding competitions impurities and kinetics was described previously **[38-41]**.

Quantification and purification of ^{68}Ge

To implement different ^{68}Ga-labeled DOTA-conjugated peptides analogs PET scintigraphy with ^{68}Ga-labeled peptides is performed at >40 centres in Europe. In general, radionuclidic purity (RNP) of the eluate of these generators varies between 0.01 and 0.001% (^{68}Ge vs. ^{68}Ga activity). Liquid waste containing low amounts of ^{68}Ge activity is produced by eluting the $^{68}Ge/^{68}Ga$ generators and residues from PET chemistry and exceeds waste disposable limits (10 Bq/g). The purpose of **chapter 3** was to quantify ^{68}Ge activity in our ^{68}Ge liquid waste, and to concentrate and to transform ^{68}Ge liquid waste to solid waste. Since ^{68}Ge can be trapped on a small column both liquid and solid waste is reduced to a minimum. Liquid ^{68}Ge waste was transformed to ^{68}Ge solid waste by sorption of TiO_2, Fe_2O_3 and Zeolite **[42]** and we are able to reduce liquid ^{68}Ge waste to neglectable quantities. All activity is concentrated on a small column containing Zeolite (<10g).

PART II: INVESTIGATION OF RADIOLYSIS AND RADIOCHEMICAL PURITY

Radiopharmaceuticals

Radiolabeled peptides have shown to be an important class of radiopharmaceuticals for imaging and therapy of malignancies expressing receptors of regulatory peptides. These peptides have high affinity and specificity for their receptors. The majority of these receptors are present at different levels in different tissues and tumors. This part of the thesis focuses on the application of regulatory peptides radiolabeled with ^{111}In or ^{177}Lu. Due attention is given to current status research, limitations and future perspectives of the application of these radiolabeled peptides for imaging and radiotherapy are discussed in the introduction. It also covers elements of basic science and preclinical and clinical aspects in general, however mostly based on SS and BN receptor-mediated imaging and therapy. Since only intact radiolabeled peptide have high affinity to this receptor, it is important protect radiolabeled peptide for radiolysis and to inject it only with high RCP [43].

Radiolysis and RCP

In this thesis we present an overview how to measure and to quantify radiolysis by the addition of quenchers and to maintain RCP of regulatory peptides is presented in **chapter 4 and 5**. The literature report successful addition of quenchers such as gentisic acid **[44]**, ascorbic acid **[14, 44, 45]**, methionine **[46],** melatonin and ethanol **[14, 47-55]** to the reaction mixtures in various combinations and concentrations prior to radiolabeling to prevent radiolysis. The quenchers were tested individually as function of concentration to investigate their efficacy to protect against radiolysis of radiolabeled methionine-containing regulatory peptides. High RCP was only achieved with a combination of quenchers.

However, quantification of RCP is not standardized, and therefore comparison of radiolabeling and RCP of regulatory peptides between different HPLC-systems and between laboratories is cumbersome. Therefore we suggested a set of standardized requirements to quantify RCP by HPLC for radiolabeled DTPA- or DOTA-peptides. To predict influence of a certain concentration of activity (MBq) on RCP a macroscopic dosimetry model was developed. With this model we could calculate the dose rates in vials being used for radiolabeling and predict RCP of radiopeptides in the absence of quenchers. RCP was measured by HPLC, and a relation between radiation dose and radiolysis of RCP was established.

Requirements for RCP

There is a lack of fundamental knowledge regarding the destruction of radiopeptides in aqueous solution due to radiolysis. Radiolysis is defined as the degradation of radiopeptides in aqueous solution caused by radiation and free radicals generated by radiolysis of water molecules **[56-60]**. Comparison of radiolabeling and quantification of RCP between different HPLC-systems and between laboratories is not standardized. Moreover, since there are no criteria or guidelines to qualify HPLC-quality controls of radiotracers, we suggest to create reproducible parameters for comparison of RCP between different HPLC-systems and laboratories.

There are many reports on the determination of RCP by HPLC, including accuracy, linearity, precision, repeatability, detection limits **[61-63]**. To our knowledge, there are no criteria to qualify a radiodetection based HPLC separation method. With differences in eluens, gradient, flow, column type and length there might be an increase variation in finding impurities, and thus variation in RCP. Standardisation of separation method includes HPLC-eluens, gradient or isocratic, flow, column type and length, detection and interpretation of chromatograms. Therefore, in our opinion, RCP is actually expressed in percentages of arbitrary units. Therefore we suggested minimal requirement for RCP by HPLC and radiodetection for radiolabeled DTPA or DOTA-peptides which should include base-to-base separation of:

a. At least 2 peaks of the DTPA conformations of a radiolabeled DTPA-peptide **[64]**
b. Metal-peptide *vs.* peptide for DTPA and DOTA-peptide **[65, 66]**
c. For Met-containing regulatory peptides: Met-sulfoxide radiopeptide vs intact radiolabeled peptide **[46]**

These requirements are important to standardize RCP measurements for reliable comparisons of RCP quantifications between different systems/laboratories.

Ready-for-use liquid formulation

For safety reasons it would be desirable to store and transport the ready-for-use liquid formulation (diagnostics and therapeutics) of radiolabeled peptides. In **chapter 5** we describe the presence of quenchers in a single-vial liquid pharmaceutical formulation of a radiolabeled peptide, in a quantity sufficient to prevent radiolysis of the formulation. Radiolabeled peptides are generally stored and transported in the form of multi-vial kit formulations. Usually the contents of these vials are lyophilized or frozen and should be brought into solution subsequently in a mutual reaction to produce the intended radiolabeled peptide. For health physics reasons it would be desirable to be able to store and transport the ready- for-use liquid formulation of the radiolabeled peptide. This could avoid similar labeling of radiopharmaceuticals in different hospitals.

So, a physician could administer the labeled peptide without performing extra handling like a radiochemical reaction by local radiochemist, but simply by diluting the contents of the vial in a radiopharmaceutical liquid that can be administered by injection or by infusion **[48]**. Here we describe how to maintain RCP and thus prolong storage time. As a result this increases the availability of the ready-for-use radiolabeled SS-analogs to worldwide. In this thesis, stability and radiolysis of radiolabeled SS-analogs were monitored by HPLC. HPLC methods were optimized to distinguish between non-labeled and radiolabeled peptides vs. the radiolysed peptides. The effect of quenchers on the stability of radiolabeled SS-analogs, even under therapeutic conditions was optimized and monitored up to 7 days after radiolabeling.

Pharmacokinetics

The focus for improvement of BN-based imaging (by increasing tumor-to-background ratio) has predominantly relied on development of new BN-based analogs with enhanced affinity for the GRPR **[10, 67-69]**. Instead, in **chapter 6** we investigated the impact of various experimental factors in order to

further improve uptake in target and target background ratio of peptide receptor-mediated imaging. To ensure purity of the labeled peptide many study groups perform purification of BN-based analogs by HPLC between labeling and administration. In order to determine the impact of this purification step, we studied the effect of HPLC-purification mass dependently by performing biodistribution studies.
An essential parameter to evaluate the performance of targeted imaging probes is the ratio between activity in tumor vs. background organs. Contrast between the two is dependent on the amount of administered peptide. BN-based analogs have already pharmacological effects after administration in nanomole quantities. The high affinity GRPR have a low capacity in tumor and other tissues and also the expression varies between tissues **[10, 70, 71]**. Therefore, in the present study we investigated GRPR saturation by injecting an increasing amount of peptide (bellshape) and measuring target vs. non-target organs. From this bellshape an optimal peptide dose was calculated for further applications **[72]**. Furthermore we studied the impact of specific activity (MBq/nmol) on tumor-to-background ratio by varying injected peptide amount while injecting a constant amount of activity (MBq).
GRPR-positive tumor tissue showed a significant 2 to 3-fold increase in absolute uptake after HPLC-purification while a stable tumor-to-pancreas ratio remained. Low peptide amounts resulted in a decline in uptake in tumor, kidney and pancreas. Tumor-to-pancreas ratio improved six-fold. GRPR saturation 4h prior to injection of ^{111}In-DOTA-AMBA resulted in improvement of the tumor-to-pancreas ratio. All these finding increased knowledge of how to improve tumor uptake and to perform a proper BN-based image. Nevertheless it also shows the increase importance of labeling of BN analogs with high SA.

Conclusion

With all the above-mentioned options in mind and although radiolabeled regulatory peptides have been investigated and successfully applied for peptide receptor radionuclide scintigraphy and therapy for nearly 2 decades, there are still many options to improve and fully exploit peptide receptor radionuclide scintigraphy and therapy.

Outlook for future concepts and research

It can be concluded from literature and the above-mentioned arguments that peptide receptor radionuclide scintigraphy can be performed with either ^{111}In- or ^{177}Lu -labeled DTPA- or DOTA-peptides. However, although DOTA-peptides can also be radiolabeled at high specific activity, this specific activity might be not high enough for PRS and PRRT with ligands which have pharmalogical side effects using small amounts (nmoles), the delivery of sufficient amounts of activity (MBq) to these regions may be too low for scintigraphy and therapy.
However, there may be several other ways to circumvent these limitations. Since Maximum SA is directly related to half-life of isotope i.e. for imaging the use of PET radionuclides (^{68}Ga) and for therapy α-emitters could be good alternative. α-emitting radionuclides have a high Linear Energy Transfer (high energy deposition within a short range), consequently the cell kill probability is high, but only if the target (e.g. DNA) is within range.
Also improvements of the characteristics of the radioligand might be achieved by investigating the role

of linkers and chelators **[73-76]**, and by studies of the pharmacokinetics of the peptide derivatives **[77]**. The tools in analytical chemistry are constantly improving, e.g. as recently reported by Asti *et al.* **[65]** for a base-to-base chromatographic separation by UPLC of a DOTA-peptide labeled with different not radioactive metal-ions. How this new technique will affect the radiodetection, including sensitivity of radiopeptides is currently under investigation and is already proven to be very promising.
To increase SA, purity and content of the used peptides is very important. Therefore we
developed an alternative and indirect method to quantify DOTA-peptide, which include titration of the DOTA-moiety with non-radioactive metal ions This titration is a indirect and alternative method to quantify purity and content of DOTA-peptides, even in the presence of UV-absorbing additions and/or impurities

Optimization and maintaining RCP with quenchers for ^{111}In or ^{177}Lu-labeled SS-, MG- or BN-peptides can be used as a model bombesin or ^{111}In- or ^{177}Lu-labeled regulatory peptides and other radiopeptides or proteins containing amino acids like Met, Trp and Tyr. However, maintaining RCP with described mixtures of quenchers is not a guarantee for protection of other peptides or proteins. Addition of quencher mixtures to maintain RCP must be investigated nuclide and peptide dependently. PRS or PRRT with DOTA-conjugated Met-containing CCK or bombesin analogs at high RCP is a challenge, therefore further research for chelators are required. Moreover, chelators should include the following characteristics: high kinetic and thermodynamic stability, preferably also with a therapeutic isotope like ^{177}Lu, and without the need of a heating procedure, this might help to maintain RCP **[78]**. The receptor affinity of radiolysed peptide decrease dramatically **[10]**. There is a lack of knowledge about affinity of other radiolysed radiopeptides, i.e. for damaged Trp or Tyr. Unfortunately these specific radiolysed radiopeptides were not identified and characterised in this study and the influence on receptor affinity is currently unknown.

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Nederlandse samenvatting

Kanker is een steeds meer voorkomende ziekte in onze samenleving. Onderzoek en ontwikkeling van nieuwe diagnostiek en behandelmethoden voor kankerpatiënten worden daarom steeds belangrijker. De Nucleaire Geneeskunde maakt gebruik van radioactieve stoffen om zo tumoren in beeld te kunnen brengen met behulp van gamma- of PET camera (Positron Emission Tomografie, zie ^{68}Ga PET beeldvormende technologie). In dit proefschrift wordt een overzicht gegeven van verschillende bestaande en nieuwe radiochemische technieken voor visualisatie van verschillende soorten kanker **(hoofdstuk 1)**.

Radioactieve peptiden zijn stoffen die gebruikt worden voor het scannen en het in beeld brengen van kanker. Peptiden zijn kleine eiwitten die specifiek binden aan receptoren. Receptoren zijn ook eiwitten die voorkomen in en op cellen in het lichaam. Deze receptoren-eiwitten hebben een dusdanige vorm dat de bijhorende peptiden daar precies in passen, zoals iedere sleutel op zijn eigen slot past. Elk type peptide bindt specifiek aan zijn eigen soort receptor. Door deze specifieke binding tussen peptiden en receptoren worden signalen afgeven binnen de cel. Deze receptor-peptide bindingen zorgen dus voor communicatie tussen cellen binnen het lichaam. Tumorcellen brengen vaak receptoren in overvloed tot expressie in en op de tumorcellen. Wanneer deze receptoren, aanwezig op de tumorcellen, binden met de radiopeptiden kunnen met behulp van een PET of gammacamera deze tumoren worden gevisualiseerd. PET is een beeldvormende techniek die zijn toepassing vindt voor diagnostiek. Wereldwijd zijn er op dit moment meer dan 40 centra begonnen met het toepassen van ^{68}Ga gelabelde peptiden.
Bovengenoemde methode van tumorvisualisatie wordt Peptide Receptor Scintigrafie (PRS) genoemd. Er zijn verschillende radionucliden die specifieke eigenschappen hebben: bijvoorbeeld gammastraling bij verval (voor diagnostiek) en β-straling (voor therapie). Deze radionucliden kunnen worden gekoppeld aan het peptide die er voor zorgen dat er schade optreedt aan de tumorcel. Dit betekent dat op deze manier de tumorcellen kunnen worden behandeld. Deze behandelingsmethode wordt Peptide Receptor Radionuclide Therapie (PRRT) genoemd. In dit proefschrift zijn onder andere de (labelings) beperkingen beschreven, om de PRS en de PRRT te verbeteren.
Om bijwerkingen en eventuele verzadiging van de receptoren bij het injecteren van peptiden te voorkomen kan er slechts een beperkte hoeveelheid van het peptide worden toegediend aan patiënten. Om toch genoeg aan radioactiviteit toe te kunnen dienen voor het uitvoeren van een optimale scan, is een van de opties het verbeteren van de labelingsmethode. Wanneer er meer peptide gekoppeld zijn aan een radionuclide, betekent het dat er in totaal meer radioactiviteit gebonden is.
Om met een hoge resolutie te kunnen scannen gaat dit ten koste van de gevoeligheid van een gammacamera. Dit betekent dat er dan meer radioactiviteit nodig is om een scan te maken maar wel met een betere beeldkwaliteit. Een nadeel uit bovenstaande voorbeelden is dat dit in beide gevallen leidt tot een hogere dosis aan straling in oplossing. Er is daarom onderzoek gedaan naar een mogelijkheid voor betere bescherming tegen radiolyse van het radiopeptide. Radiolyse is het beschadigen van peptide door hoge dosis aan straling. Door de hogere dosis straling is er een verhoogde kans dat het peptide wordt beschadigd, zelfs voordat het wordt geïnjecteerd. Het beschadigde peptide verandert in structuur en vorm zodat deze niet meer past op zijn receptor. Dit resulteert vervolgens in minder binding van het geïnjecteerde radioactief gelabelde peptide aan receptoren. De hoeveelheid schade aan radioactief gelabelde peptide door radiolyse worden uitgedrukt in percentage radiochemische zuiverheid (RCP).

RCP is gedefinieerd als het percentage van de intacte hoeveelheid radiopeptide in de desbetreffende oplossing ten opzichte van alle aanwezige radioactiviteit.
Er is momenteel een gebrek aan fundamentele kennis voor wat betreft de radiolyse van radiopeptiden in waterig milieu. Radiolyse is gedefinieerd als de degradatie van radiopeptiden in waterige oplossingen, veroorzaakt door straling en vrije radicalen gegenereerd bij bestraling van watermoleculen. Om de invloed van radiolyse te kunnen onderzoeken zijn er in dit proefschrift twee type peptiden als model gebruikt: DOTA- of DTPA-geconjugeerde analogen van somatostatine en bombesine (voor structuren zie de figuren 1-3 hoofdstuk 5). Deze peptiden worden radioactief gelabeld en worden specifiek gebruikt voor visualisatie van neuro-endocrine, prostaat-, borst-tumoren op een gamma- of PET camera.
In dit proefschrift is ook onderzoek gedaan naar de implementatie van ^{68}Ga als PET isotoop. Om verschillende redenen is er tegenwoordig een verhoogde interesse voor het ^{68}Ga. Ten eerste zijn er verschillende ^{68}Ge/^{68}Ga generatoren op de markt die ^{68}Ga produceren; dat vervolgens kan worden omgezet in ^{68}Ga-gelabelde peptiden. Ten tweede zijn er veel verschillende DOTA-peptiden (voor structuren zie de figuren 1-3 hoofdstuk 5) welke gelabeld kunnen worden met ^{68}Ga. Ten derde, de beschikbaarheid van een PET radionuclide, door de introductie van ^{68}Ge/^{68}Ga generators, onafhankelijk van een cyclotron, genereert nieuwe toepassingen en mogelijkheden. Als laatste punt zijn er de sterk verbeterde apparaten zoals apparatuur voor radiolabeling met behulp van robotics.

Doelstellingen van dit proefschift

Bovengenoemde punten resulteren in de volgende doelstellingen van dit proefschrift:

I: Optimaliseren en vergelijken van zuiveringstechnieken gebruikt voor ^{68}Ga labelingen en verhogen van de labeling opbrengsten en hoeveelheid gelabelde radiopeptide.

II: Het accuraat meten en monitoren van de radiochemische zuiverheid van het radiopeptide met daarvoor bestemde chromatografische apparatuur zoals HPLC en UPLC. Vaststellen van de effectiviteit van toevoeging van verschillende quenchers (beschermen tegen radiolyse) en de onderlinge vergelijking, om zo een goede radiochemische zuiverheid van het radiopeptide te kunnen behouden na labeling.

In het kort samengevat: het meer toepasbaar maken van ^{68}Ga-, ^{177}Lu- of ^{111}In gelabelde peptiden.

^{68}Ga PET beeldvormende technologie

^{68}Ga-gelabelde DOTA-geconjugeerde peptiden voor PET scans zijn een middel om onderzoek te doen aan de individuele receptor-status van tumoren van patiënten. Hiermee kan men dus de PRS en PRRT optimaliseren.
Hoofdstuk 2 van dit proefschrift geeft een overzicht van de huidige kennis en onderzoeken naar verschillende ^{68}Ga gelabelde moleculen, maar dan vooral gefocust op de toepassing van peptiden voor de beeldvorming van receptor peptide interacties (ook wel receptor-gemedieerde processen genoemd).

Optimalisatie van ^{68}Ga gelabelde peptiden

De chemische eigenschappen van het ^{68}Ga isotoop en andere beperkingen zoals grote volumes van ^{68}Ga eluaat, hoge concentraties aan zuren en andere verontreinigingen, kunnen ervoor zorgen dat

de labeling van ^{68}Ga met DOTA-peptiden niet volledig tot stand komen. De toenemende toepassing van de ^{68}Ga gelabelde peptide binnen de kliniek vraagt daarom naar een optimalisatie van vooral de reactiekinetiek.
Al deze punten worden behandeld en oplossingen voor deze problemen worden beschreven in **hoofdstuk 3**.

Kwantificatie en opzuiveren van ^{68}Ge

In het eluaat van de ^{68}Ge/^{68}Ga generator is altijd ^{68}Ge aanwezig. Ook al is dit een lage concentratie, valt deze concentratie aan ^{68}Ge wellicht niet binnen de regels voor regulier afval die vastgesteld zijn door de overheid. In **hoofdstuk 4** is onderzoek gedaan naar

a. hoe nauwkeurig ^{68}Ge in kleine hoeveelheden kan worden gekwantificeerd en
b. hoe het volume van het ^{68}Ge bevattende vloeibaar afval kan worden gereduceerd. Hiervoor is een speciale kolom ontwikkeld om zo het ^{68}Ge te concentreren, zelfs in vaste vorm.

Onderzoek naar radiolyse en radiochemische zuiverheid

Het tweede deel van het proefschrift (zie tweede doelstelling) richt zich vooral op peptiden gelabeld met ^{111}In (indium) en ^{177}Lu (lutetium). Ook is hier een overzicht gegeven van de status van dit onderzoek tot nu toe, de beperkingen en de toekomstige mogelijkheden van de receptor-gemedieerde radiopeptiden **(hoofdstuk 5)**. Omdat alleen intact peptide kunnen binden aan hun receptor (in het lichaam), is het erg belangrijk om deze peptiden te beschermen tegen radiolyse. Er mag idealiter dus alleen intact radiopeptide worden geïnjecteerd met een hoge RCP.

Eisen voor meten van RCP

Een belangrijk punt beschreven in **hoofdstuk 6** is het kwantificeren van RCP. Kwantificatie van RCP is niet gestandaardiseerd. Dat maakt een inter en intra vergelijk tussen verschillende HPLC systemen en laboratoria gecompliceerd. Ondanks dat RCP te beïnvloeden is door onder andere verschillende loopvloeistoffen, kolommen en gradiënten, zijn er tot op heden nog geen criteria voor scheidingen op HPLC van radiopeptiden. Er is in ieder geval geen consensus binnen de specialisten en overheden. Daarom zijn in dit hoofdstuk een aantal standaard criteria voorgesteld waaraan een goede scheiding van radiopeptiden moet voldoen om een onderling vergelijk tussen laboratoria mogelijk maken. Ook is er een verband gelegd tussen de stralingsdosis en het kwantificeren van RCP van de radiopeptiden.

Radiolyse en RCP

In de **hoofdstukken 6 en 7** wordt een overzicht gegeven hoe radiolyse kan worden gekwantificeerd. In de literatuur zijn verschillende quenchers onderzocht en beschreven om radiolyse van radiopeptiden te voorkomen. In deze hoofdstukken is een overzicht gegeven van de effecten, de concentratie afhankelijkheid en de kracht van het combineren van deze quenchers.

Voor toediening gereed maken van radiopeptide

Vanwege ondermeer veiligheidsreden is het wenselijk om een samenstelling van oplossingen te hebben om radiopeptiden na de radiolabeling (zowel voor diagnostiek als therapie) te kunnen bewaren

en zo op ieder moment voor toediening gereed te hebben gedurende meerdere dagen per week. In **hoofdstuk 7** worden deze mogelijkheden beschreven. Er worden verschillende quenchers toegevoegd in verhoudingen die radiolyse van radiopeptiden beperken.

Normaal gesproken worden de verschillende onderdelen voor de uitvoering van radiolabeling van een peptiden in aparte oplossingen vervoerd. De inhoud van deze verschillende flesjes, zoals peptide en buffers, is vaak gevriesdroogd en moeten voor radiolabeling worden opgelost en worden gemengd met de oplossing die het radionuclide bevat. Om ongewenste (be)straling te voorkomen is het wenselijk om de voor toediening gereed gemaakte radiopeptiden te kunnen bewaren en te vervoeren. Dit voorkomt dezelfde soort handelingen in de ziekenhuizen. De bedoeling is dat de voor toediening gereed gemaakte oplossing alleen in een injectiespuit behoeft te worden opgetrokken en kan worden toegediend zonder dat extra handelingen moeten worden uitgevoerd.

Optimalisatie van de *in vivo* verdeling van radiopeptide

De kwaliteit van de beeldvorming op de afdeling Nucleaire Geneeskunde is onder andere afhankelijk van de verdeling van de radioactiviteit na injectie *in vivo*. In **hoofdstuk 8** is onderzoek gedaan naar het verbeteren van de beeldvorming van het bombesine peptide. Bombesine receptoren komen in hoge mate voor op prostaat-tumoren. Een belangrijk punt is daarom om meer radiopeptide aan de receptoren in en aan de tumor te binden. Er is in dit hoofdstuk vooral onderzoek gedaan naar de invloeden van bereiding en opwerken van radiopeptiden met behulp van zuiveringstechnieken. Om de kwaliteit en de zuiverheid van radiopeptiden te kunnen waarborgen, besluiten verschillende onderzoeksgroepen ertoe voor om het radiopeptide op te zuiveren. Dat wil zeggen dat het radiopeptide van het peptide wordt gescheiden. In dit hoofdstuk is onderzoek gedaan naar het effect van deze verschillende manieren van zuiveringen op de verdeling *in vivo*.

Het is belangrijk om te weten wat het massa effect is van het toe te dienen radiopeptide en dus welke hoeveelheid radiopeptide de beste *in vivo* verdeling geeft. De variaties en resultaten uit dit soort experimenten geven ons meer kennis op welke wijze de tumoropname voor deze peptiden verbeterd kan worden en laten vooral zien hoe belangrijk het is om een gelabeld bombesine analoog te hebben met een hoge RCP.

Conclusie

Zoals beschreven in bovenstaande onderzoeken zijn PRS en PRRT al meer dan twee decennia succesvol. Toch zijn er nog steeds mogelijkheden om deze techniek en de toepassing ervan te verbeteren. De resultaten van de hier beschreven onderzoeken geven meer kennis en inzicht in de verschillende processen van bijvoorbeeld de radiochemische technieken en zorgen uiteindelijk voor een verbetering van de toepassingen. Dit resulteert in een betere beeldvorming en dus in het eerder in beeld brengen van kanker en vooral in een effectieve therapie.

Abbreviations

%ID/g	percentage injected dos per gram
µmol	micromolar
µSv	microSievert
µV.Sec	microvolt per second
AMBA	DOTA-CH_2CO-Gly-[4-aminobenzoyl]-Gln-Trp-Ala-Val-Gly -His-Leu-Met-NH_2
BN	bombesin
CCK	cholecystokinin
CGA	chromogranin-A
Ci	Curie
CNS	central nervous system
COST	European Cooperation in Science and Technology
CR	complete remission
CT	computed tomography
DEC	animal experimental committee
DNA	deoxyribonucleic acid
DOTA	1,4,7,10-tetraazacylcodecane-N,N',N'',N'''-tetra-acetic acid
DOTA-BOC	DOTA°, benzyl-3-thienylalanine^3]octreotate
DOTA-NOC	[DOTA°,Naphthyl^3]octreotate
DOTANOC-ATE	[DOTA°,Tyr^3]octreotate
DOTA-TATE	[DOTA°, Naphthyl^3,Thr^8]octreotate
DOTA-TOC	[DOTA°,Tyr^3]octreotide
DTPA	diethylene triamine penta-acetic acid
EDTA	ethylenediaminetetraacetic acid
EMEA	European Medicines Agency
EU	European Union
EU/mL	endotoxin units per millilitre
FDA	Food and Drug Administration
GBq	Gigabecquerel (10^9 Bq)
GEP	gastroenteropancreatic
GMP	Good Manufacturing Practice
GRP	gastrin-releasing peptides
GRPR	gastrin releasing peptide receptor
Gy	Gray
Hb	haemoglobin
HER2	human epidermal growth factor receptor 2
His	histidine
HPLC	high-performance liquid chromatography
IC_{50}	inhibiting concentration 50%
ICP	inductively coupled plasma
ITLC-SG	instant thin layer chromatography silica gel
KBq	Kilobecquerel (10^3 Bq)
KeV	Kilo electron volt
KPS	karnofsky performance score
LET	low energy transfer
MBq	Megabecquerel (10^6 Bq)
mCi	milliCurie

MDS	myelodysplastic syndrome
Met	methionine
MeV	Mega electron volt
MG11	minigastin analog 11
mmol	millimolar
MoAb	monoclonal antibody
MR	minor response
MRI	magnetic resonance imaging
mSv	milliSievert
n, gamma	neuton, gamma (γ) reaction
nmol	nanomolar
OctreoScan	[DTPA^0]octreotide
p2n	proton, 2 neutron reaction
PC	prostate cancer
PCR	poly chain reaction
PET	positron emission tomography
PK	pharmacokinetics
ppm	part per million
PR	partial response
PRRT	peptide receptor radionuclide therapy
PRS	peptides receptor scintigraphy
PSA	prostate specific androgen
RCP	radiochemical purity
RGD	arginine-glycine-aspartic acid
RNP	radionuclidic purity
RT	retention time
S Factor	total absorbed dose
SA	specific activity
SD	stable disease
SD	standard deviation
SEM	standard deviation of the mean
Se-Met	seleno-methionine
SPE	solid phase extraction
SPECT	single-photon emission computed tomography
SS	somatostatin
TFA	trifluoroacetic acid
Trp	thryptophan
UPLC	ultra-performance liquid chromatography
UV	ultra violet
WBC	white blood cells
WHO	World Health Organisation

Dankwoord

Hier ligt 'het boekje', mijn proefschrift dan voor mijn neus. Na jaren van onderzoek, opleidingen en cursussen is het zover. Dit had ik uiteraard alleen nooit voor elkaar gekregen. Daarom wil ik van deze gelegenheid gebruik maken om een woord van dank uit te spreken.

Mijn promotoren: Prof dr. J.F. Verzijlbergen: Beste Fred, ook al bent u nog niet zo lang hoofd van de afdeling Nucleaire Geneeskunde en ken ik u nog niet zo lang, toch kunnen we het goed vinden! Hartelijk dank voor al uw hulp en begeleiding tijdens het traject! Uw taalvaardigheid en verbeteringen van teksten heb ik zeer op prijs gesteld!

Mijn promotor, Prof dr. H.Th. Wolterbeek van het Reactor instituut Delft: Beste Bert, ik heb altijd veel bewondering voor u gehad, de ongelofelijke hoeveelheid kennis die u heeft over dit vakgebied is voor mij bewonderingswaardig. Maar aan de andere kant de nuchterheid en de dosis humor maakt u een bijzondere persoonlijkheid. Daarom vind ik het ook een eer dat u één van mijn promotoren bent.

Mijn Co-promotor W.A.P. Breeman: Beste Wout, toen ik bij u stage kwam lopen op het radiochemie lab, inmiddels zo'n 14 jaar geleden, had ik nooit verwacht dat dit ooit zou leiden tot dit proefschrift. U heeft mij weten te motiveren tot verder studeren en hebt mij ook altijd gesteund. Ook het vele geduld voor wat betreft het nakijken van de manuscripten en verslagen heb ik zeer op prijs gesteld. U heeft mij geleerd kritisch naar mij zelf en naar anderen te kijken, maar toch altijd met flinke dosis humor. Dit maakt onze samenwerking binnen het radiochemie team zeer plezierig! Met andere woorden: zonder uw hulp en bijdrage had ik hier nooit gestaan. Hartelijk dank voor alles!!

Graag wil ik ook de overige leden van de commissie bedanken:
Prof. dr. P.M. van Hagen, Prof. dr. T.J. Visser en Prof. dr. A.G. Vulto: ik wil u hartelijk bedanken voor het snelle beoordelen van het proefschrift. Dear Prof. dr. H.J. Wester, it is an honour for me that you are in my board of my commission! Thank you very much for the very positive comments on my thesis, I appreciate this very much! I hope to have a fruitfull collaboration in the near future. Ook wil ik graag Prof. dr. P.H. Elsinga bedanken voor het zitting nemen in de promotie-commissie op 25 november.

Daarnaast wil ik graag mijn dank uitspreken tot iedereen van de Afdeling Nucleaire Geneeskunde: voor de gezelligheid en de prettige sfeer in de afgelopen jaren! Jammer dat door de verbouwingen en de verhuizing het contact op een laag pitje staat, maar daar komt op korte termijn verandering in, dan verhuist de radiochemie in zijn geheel naar de Cyclotron Rotterdam, onder de afdeling!

In het bijzonder wil ik noemen, Jacqueline Renirie voor hulp bij de planning en invullen en versturen van brieven en formulieren. De MNAA's wil ik bedanken voor o.a. het altijd inboeken en uitpakken van de binnengekomen radioactiviteit. Erica, Jopie, Linda, Heleen, Pieter, Arthur, Carlijn, Emar, Lies en Sharia dank daarvoor! En Arthur veel succes met jouw nieuwe uitdagingen! Van mij ben je niet af, ik zit straks alleen maar dichterbij! Verder alle collega's van de prekliniek (Jan, Marleen, Stuart, Monique, Sandra en Gaby) onder leiding van Marion de Jong, dank voor alle bijdragen wat betreft cel experimenten, autoradiografieën en in vivo experimenten. Harald, Linda en Saskia, al werken jullie inmiddels ergens

anders, bedankt voor de prettige samenwerking! Ook alle PhD'ers (Simone, Kristell, Constanza, Joost, Stefan, Sander en Ingrid), bedankt voor de goede samenwerking. Labelingen en kwaliteitscontroles van 'van alles en nog wat' doe ik altijd met veel plezier voor jullie! Ik hoop ook op een plezierige voortzetting daarvan. Marleen, een dappere beslissing van je om over te stappen naar de MNAA groep! Heel veel succes daar en ook met je nieuwe studie! In het bijzonder wil ik Stuart bedanken voor het tekenen van de voor- en achterkant van mijn boekje. Bijzonder hoe je dat zo op papier krijgt!

Mark Konijnenberg wil ik bedanken voor de uitvoering van ingewikkelde berekeningen en maken van de gelikte figuren. Ongelofelijk hoe je dat doet! Beste Rogier, hartelijk dank voor de plezierige samenwerking. O.a. het AMBA stuk had wel wat voeten in de aarde.., maar het is gelukt! Dear Boxue, Thank you for very much for the nice cooperation, hope you will find a new challenge soon.

Veel van de experimenten beschreven in de artikelen van dit proefschrift heb ik samen uitgevoerd met stagiaires. Daarom wil ik hier in het bijzonder noemen Ho Sze, Rory, Gabriëlle, Kevin, Halil, Martijn, Jeffrey, Timo, Madi, Esther, Jolanda, Martin en Louann. Bedankt voor jullie inzet; ik heb met heel veel plezier met jullie gewerkt. Leuk om te zien dat de stage bij ons op de afdeling de meesten van jullie heeft gestimuleerd om verder te leren!

De paranimfen wil ik in het bijzonder bedanken. Ho Sze, we kennen elkaar al een behoorlijke poos! Begonnen als mijn stagiaire, maar nu bijna een race tegen de klok wie het eerst gaat promoveren. We hebben inmiddels heel wat meegemaakt op de afdeling. Ik waardeer je behulpzaamheid enorm; je staat altijd voor mij klaar. Het maakt echt niet uit voor wat, in het weekend of wat dan ook, jij bent er gewoon! Ik hoop dat je jezelf kunt blijven motiveren zodat je spoedig je boekje kan afronden! Ik wil nog niet denken aan je vertrek want ik ga je enorm missen.

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Lieve vrienden. Bedankt voor jullie begrip en luisterend oor als ik weer eens begon te vertellen over mijn werk aan de bar in de "kippenschuur"☺. Jullie hebben mij er doorheen gesleept!

Uiteraard wil ik mijn familie en schoonfamilie niet vergeten! Lieve pa en ma. Daar is 'ie dan, het proefschrift van jullie (schoon)zoon! Het heeft behoorlijk wat voeten in de aarde gehad en zonder jullie begrip en steun was het niet gelukt! Het is soms moeilijk om precies uit te leggen wat de onderzoeken allemaal inhouden, maar toch doen jullie altijd je best om mij te begrijpen. Super bedankt mam dat u menig keer wilde oppassen op Rick en Steff zodat ik in alle rust op 'de Hoogeveen' kon schrijven!

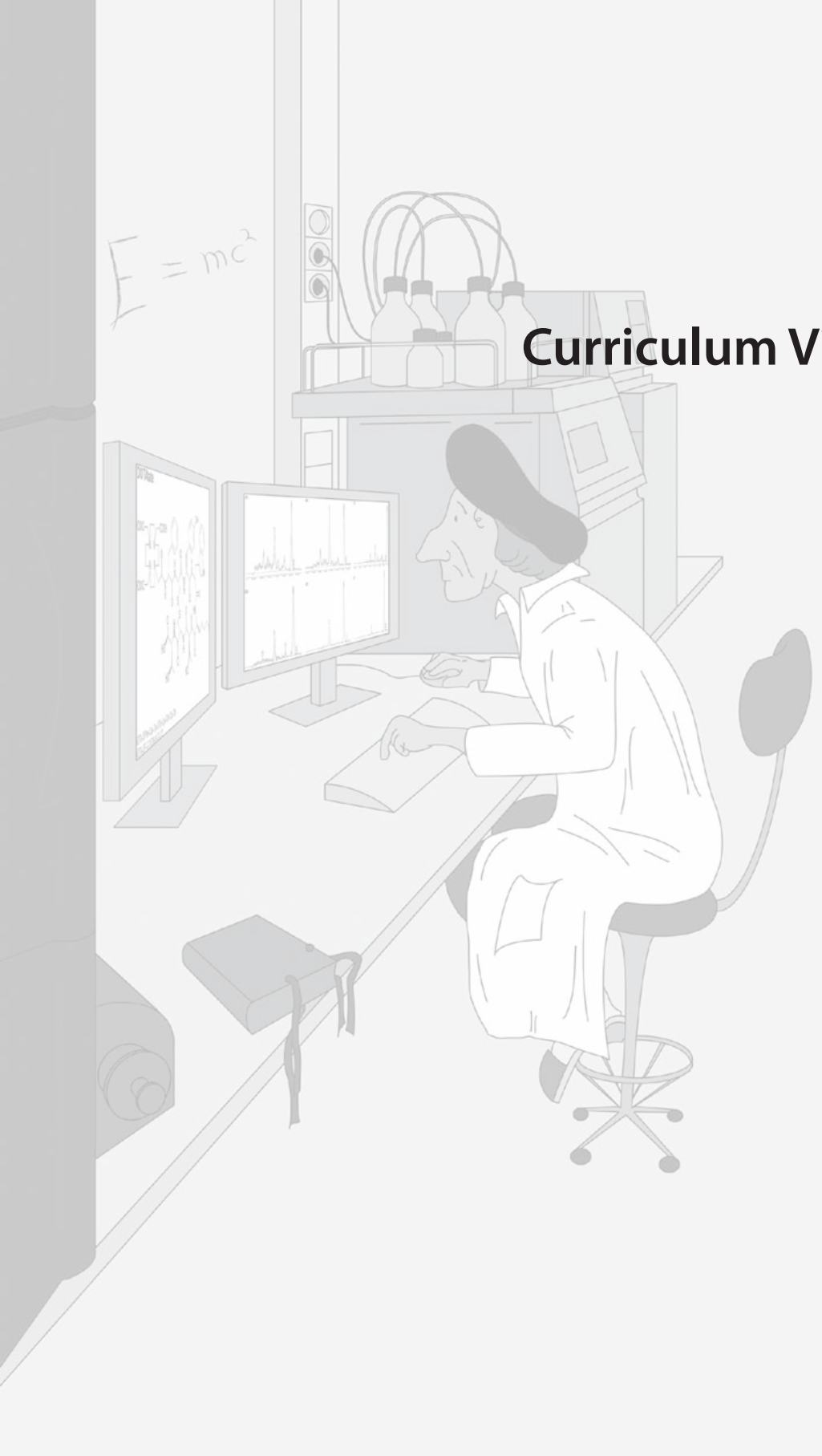
Hans, Marian, Bart, Margo, Gerdine, Anton en natuurlijk alle kinderen, Patrick, Jessica en Ingrid. Ik hoop dat het "leken praatje" ook voor jullie wat duidelijkheid geeft over wat ik nu precies allemaal doe. Ik hoop nu meer tijd te krijgen voor andere leukere dingen.

En als laatste mijn gezin:
Lieve Rick en Steff, ook al beseffen jullie nog niet wat papa allemaal aan het doen is, ik wil jullie toch bedanken. Jullie hebben zonder dat je dat beseft mij gestimuleerd! Rick zijn ondeugendheid en zijn lach, Steff je altijd en altijd vrolijke gezicht, je grote glimlach op je gezicht! Ik hoop nu meer tijd voor jullie te hebben! Jullie zijn mijn aller kostbaarste bezit!

Lieve Marieke, vele uren heb ik achter de computer versleten, vele avonden afwezig in een hoekje turend naar een computerscherm, zelfs in de weekenden! Zeker het afgelopen jaar drukte dit behoorlijk op onze relatie. Je was en je bent er **altijd** voor mij!! Zonder jou had ik het nooit gered! Nu het voorbij is kunnen we er samen zijn voor ons gezin. Schat ik kan niet zonder je!!

Erik

Curriculum Vitae



Erik de Blois werd geboren op 22 juli 1981 te Zeist. In juni 1997 behaalde hij zijn MAVO diploma aan het Driestar college te Gouda, waarna hij begon aan de middelbare laboratoriumopleiding (MLO) aan het Zadkine college te Rotterdam, afstudeerrichting Biotechnology. Na een stage op de afdeling Nucleaire Geneeskunde (begeleider: Dr. W.A.P. Breeman) studeerde hij af in maart 2002. In 2003 is hij de deeltijd studie Bio medisch labonderzoek gaan doen aan de hogere laboratoriumopleiding (HLO) te Utrecht, afstudeerrichting moleculaire biologie. Hij studeerde af in juni 2005. Vervolgen is hij in 2006 een Engelstalige deeltijd master opleiding "Drug Innovation" gaan doen aan de Universiteit van Utrecht, afstudeerrichting pharmaceutical sciences. Hij studeerde af in juni 2009.

Al vanaf het behalen van zijn MLO diploma (februari 2002) is hij werkzaam op de afdeling Nucleaire Geneeskunde als Research analist in de radiochemie groep van Dr. W.A.P. Breeman (toenmalig hoofd Prof. Dr. E.P. Krenning; heden Prof Dr. J.F. Verzijlbergen) tot heden. Tijdens zijn werkzaamheden binnen dezelfde groep heeft hij gewerkt aan de ontwikkeling van nieuwe peptiden voor visualisatie en behandeling van tumoren. De resultaten van de onderzoeken zijn samengevat en beschreven in dit proefschrift.

List of publications

1) Accurate assessment of whole-body retention for PRRT with ^{177}Lu using paired measurements with external detectors
Liu B, **de Blois E**, Breeman WAP, Konijnenberg MW, Wolterbeek HT and Bode P.
Curr Top Med Chem., in press

2) Alternative method to determine Specific Activity of ^{177}Lu by HPLC
Breeman WAP, de Zanger R, Chan HS and **de Blois E.**
Curr Top Med Chem., in press

3) Therapeutic application of CCK2R-targeting PP-F11: influence of particle range, activity and peptide amount.
Konijnenberg MW, Breeman WAP, **de Blois E**, Chan HS, Boerman OC, Laverman P, Kolenc-Peitl P, Melis M and de Jong M.
EJNMMI Research 2014, 4:47

4) Determination of peptide content and purity of DOTA-peptides by metal ion titration and UPLC: an alternative method to monitor quality of DOTA-peptides.
Breeman WAP, Chan HS, **de Blois E**.
J Radioanal Nucl Chem. 2014 May 13

5) Inhibited osteoclastic bone resorption through alendronate treatment in rats reduces severe osteoarthritis progression.
Siebelt M, Waarsing JH, Groen HC, Müller C, Koelewijn SJ, **de Blois E**, Verhaar JA, de Jong M, Weinans H.
Bone. 2014 Jun 13

6) Aspects on radiolabeling of ^{177}Lu-DOTA-TATE: After C_{18} purification re-addition of ascorbic acid is required to maintain radiochemical purity.
Maus S, **de Blois E**, Ament SJ, Schreckenberger M, Breeman WAP.
Intern J of Diagn Imag 2014 March; 1(1) 5-12

7) Increased physical activity severely induces osteoarthritic changes in knee joints with papain induced sulphate-glycosaminoglycan depleted cartilage.
Siebelt M, Groen HC, Koelewijn SJ, **de Blois E**, Sandker M, Waarsing JH, Müller C, van Osch GJ, de Jong M, Weinans H.
Arthritis Res Ther. 2014 Jan 29;16(1):R32

8) Application of single-vial ready-for-use formulation of ^{111}In- or ^{177}Lu-labelled somatostatin analogs.
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9) Improving radiopeptide pharmacokinetics by adjusting experimental conditions for bombesin receptor-mediated imaging of prostate cancer.
de Blois E, Schroeder RPJ, de Ridder CMA, Van Weerden WM, Breeman WAP, De Jong M.
Q J Nucl Med Mol Imaging. 2013 Jun 19.

10) Effectiveness of quenchers to reduce radiolysis of ^{111}In- or ^{177}Lu-labelled methionine-containing regulatory peptides. Maintaining radiochemical purity as measured by HPLC.
de Blois E, Chan HS, Konijnenberg M, de Zanger R, Breeman WA.
Curr Top Med Chem. 2012;12(23):2677-85.

11) Iodination and stability of somatostatin analogues: comparison of iodination techniques. A practical overview.
de Blois E, Chan HS, Breeman WAP.
Curr Top Med Chem. 2012;12(23):2668-76.

12) ^{68}Ga-labeled DOTA-peptides and ^{68}Ga-labeled radiopharmaceuticals for positron emission tomography: current status of research, clinical applications, and future perspectives.
Breeman WA, **de Blois E**, Chan HS, Konijnenberg M, Kwekkeboom DJ, Krenning EP. Semin Nucl Med. 2011 Jul;41(4):314-21. doi: 10.1053/j.semnuclmed.2011.02.001. Review.

13) Comparison of the binding and internalization properties of 12 DOTA-coupled and ^{111}In-labelled CCK2/gastrin receptor binding peptides: a collaborative project under COST Action BM0607.
Aloj L, Aurilio M, Rinaldi V, D'ambrosio L, Tesauro D, Peitl PK, Maina T, Mansi R, von Guggenberg E, Joosten L, Sosabowski J.K., Breeman W.A., **De Blois E**, Koelewijn S, Melis M, Waser B, Beetschen K, Reubi JC, de Jong M. Eur J Nucl Med Mol Imaging. 2011 Aug;38(8):1417-25. Epub 2011 Apr 20.

14) Gastrin-releasing peptide receptor-based targeting using bombesin analogues is superior to metabolism-based targeting using choline for in vivo imaging of human prostate cancer xenografts.
Schroeder RP, van Weerden WM, Krenning EP, Bangma CH, Berndsen S, Grievink-de Ligt CH, Groen HC, Reneman S, **de Blois E**, Breeman WA, de Jong M.
Eur J Nucl Med Mol Imaging. 2011 Jul;38(7):1257-66. Epub 2011 Mar 23.

15) Reduction of ^{68}Ge activity containing liquid waste from ^{68}Ga PET chemistry in nuclear medicine and radiopharmacy by solidification
de Blois E, Chan HS, Roy K, Krenning EP and Breeman WAP.
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16) Characteristics of SnO_2-based ^{68}Ge/^{68}Ga generator and aspects of radiolabelling DOTA-peptides.
de Blois E, Chan HS, Naidoo C, Prince D, Krenning EP, Breeman WAP.
Appl Radiat Isot. 2011 Feb;69(2):308-15. Epub 2010 Nov 23.

17) A standardised study to compare prostate cancer targeting efficacy of five radiolabelled bombesin analogues.
Schroeder RP, Müller C, Reneman S, Melis ML, Breeman WA, **de Blois E,** Bangma CH, Krenning EP, van Weerden WM, de Jong M.
Eur J Nucl Med Mol Imaging. 2010 Jul;37(7):1386-96. Epub 2010 Feb 25.

18) Optimised labeling, preclinical and initial clinical aspects of CCK-2 receptor-targeting with 3 radiolabeled peptides.
Breeman WA, Fröberg AC, **de Blois E**, van Gameren A, Melis M, de Jong M, Maina T, Nock BA, Erion JL, Mäcke HR, Krenning EP.
Nucl Med Biol. 2008 Nov;35(8):839-49.

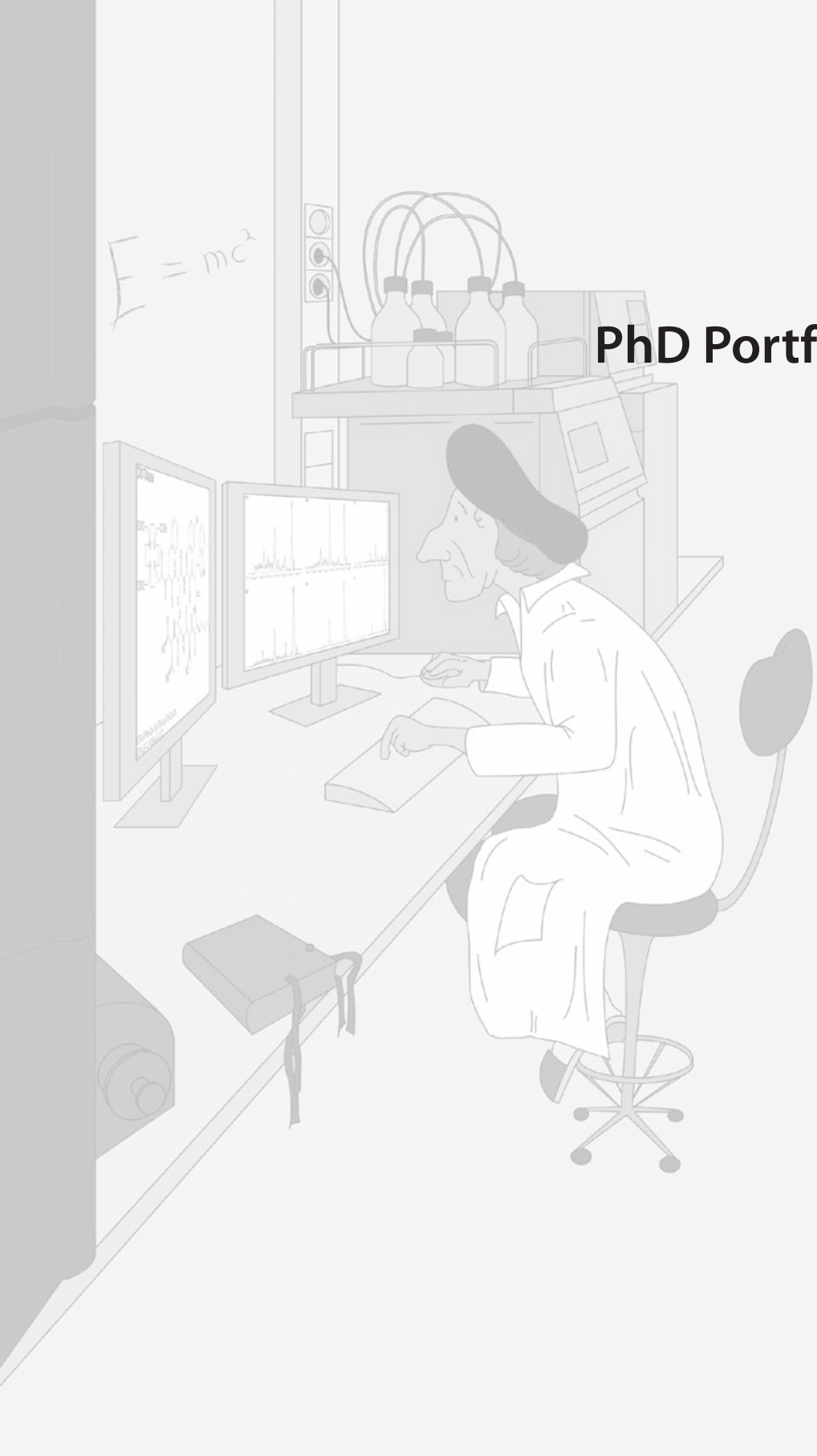
19) Molecular imaging of reduced renal uptake of radiolabelled [DOTA^0,Tyr^3]octreotate by the combination of lysine and Gelofusine in rats.
Rolleman EJ, Bernard BF, Breeman WAP, Forrer F, **de Blois E**, Hoppin J, Gotthardt M, Boerman OC, Krenning EP, de Jong M.
Nuklearmedizin. 2008;47(3):110-5.

20) Radiolabelled regulatory peptides for imaging and therapy.
Breeman WA., Kwekkeboom DJ, **de Blois E**, de Jong M, Visser TJ, Krenning EP
Anticancer Agents Med Chem. 2007 May;7(3):345-57. Review.

21) Radiolabelling DOTA-peptides with ^{68}Ga.
Breeman WA, de Jong M, **de Blois E**, Bernard BF, Konijnenberg M, Krenning EP
Eur J Nucl Med Mol Imaging. 2005 Apr;32(4):478-85. Epub 2005 Jan 18.

22) Reduction of skeletal accumulation of radioactivity by co-injection of DTPA in [^{90}Y-DOTA^{0},Tyr^{3}] octreotide solutions containing free $^{90}Y^{3+}$.
Breeman WA, De Jong MT, **De Blois E**, Bernard BF, De Jong M, Krenning EP
Nucl Med Biol. 2004 Aug;31(6):821-4.

PhD Portfolio



Summary of PhD training and teaching

PhD training

General Courses

Health physics course level 3 — 2006
Technical University, Delft, The Netherlands

Rapporteren in het engels — 2006
Erasmus MC, Rotterdam, The Netherlands

Laboratory Animal Science Art 9 — 2007
University Utrecht, The Netherlands

Empower 2 HPLC/UPLC software — 2011
Waters, Etten-Leur, The Netherlands

Biomedical English Writing Course — 2013
Erasmus MC, Rotterdam, The Netherlands

Visualising the invisible: from single molecule super-resolution to patient — 2013
Erasmus MC, Rotterdam, The Netherlands

^{89}Zr labeling course — 2014
(VU, Amsterdam, The Netherlands)

Presentations / poster at conferences

European Cooperation in Science and Technology (COST) — 2008
Krakow, Poland **(1 poster)**

European Cooperation in Science and Technology (COST) — *2009*
Waschau, Poland **(1 oral)**

International Symposium on Radiopharmaceutical Sciences (ISRS) — *2009*
Edmonton, Canada **(5 posters)**

Radiochemie.nl/ NKRV — 2006-2014
Rotterdam, Delft, Groningen, Amsterdam, Nijmegen, The Netherlands **(7 orals)**

World Federation of Nuclear Medicine and Biology (WFNB) — 2010
Cape Town, South Africa **(1 oral, 2 posters)**

International Symposium on Radiopharmaceutical Sciences (ISRS) — 2011
Amsterdam, The Netherlands **(2 poster)**

European Cooperation in Science and Technology (COST) — 2011
Sorrento, Italy **(1 oral)**

Nederlandse Vereniging voor Nucleaire Geneeskunde (NVNG) 2012
Nijmegen, The Netherlands **(1 oral)**

Rotterdamsche Chemische Kring 2012
Rotterdam, The Netherlands **(1 oral)**

International Symposium on Radiopharmaceutical Sciences (ISRS) 2013
Jeju, South Korea **(5 poster)**

European Symposium on Radiopharmacy and Radiopharmaceuticals (ESRR) 2014
Pamplona, Spain **(1 oral, 1 poster)**

Society of Nuclear Medicine and Molecular imaging, Annual Meeting (SNM) 2014
St Louis, USA **(3 posters)**

Attendance to national /international congresses whithout oral or poster presentation

International Symposium on Radiopharmaceutical Sciences (ISRS) 2007
Aachen, Germany

MINI-SYMPOSIUM 25 years of Radiopeptide Research 2011
Department of Nuclear Medicine, Erasmus MC, Rotterdam, The Netherlands

Waters UPLC user meeting 2012
Oostende, Belgium

Teaching activities:

Training School on PET-Radionuclide generators for trivalent metals: 2009
^{68}Ge/^{68}Ga and ^{44}Ti/^{44}Sc (COST)
Mainz, Germany

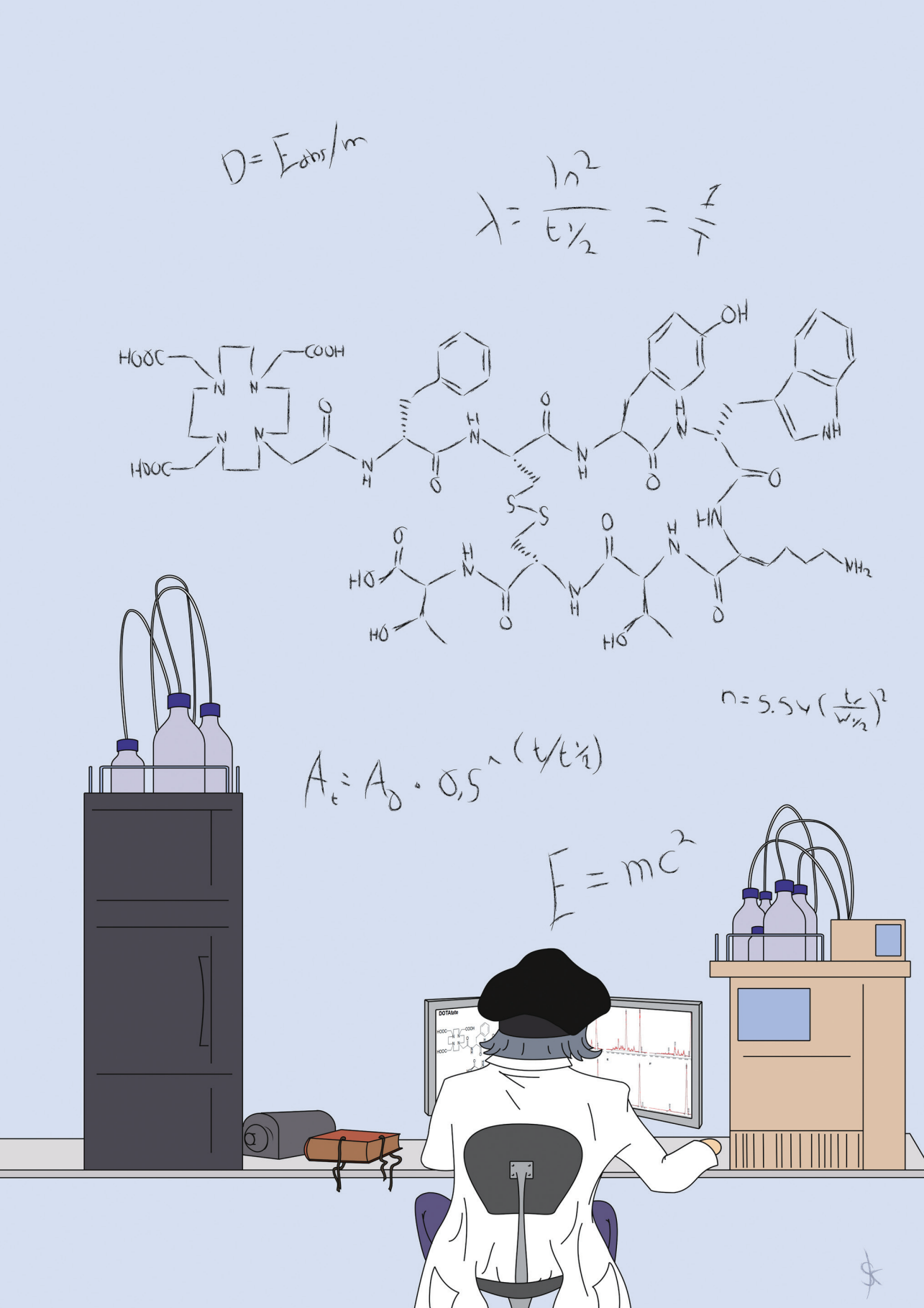
ACQUITY UPLC H-Class school for the Lab Practioner 2011
Waters, Etten-Leur, The Netherlands

Short term scientific meeting (STSM) 2013
Optimization of Studies with ^{177}Lu-SG5 in Small Animals
NCSR "Demokritos", Athens, Greece

Upgrade and repair of UPLC and HPLC 2014
implementation of separation techniques of ^{213}Bi and ^{68}Ga-peptides
New Mexico Center for Isotopes in medicine (NMCIM), Albuquerque, USA

Supervising students:

10 MLO-students	(6 - 18 months)	2005- 2014
7 HLO-students	(4.5 – 7 months)	2007 - 2013
Junior science students		2009 - 2012


D = E_abs / m
λ = ln 2 / t½ = 1 / τ
HOOC
COOH
HOOC
OH
NH
HO
HO
HO
HN
NH2
n = 5.54 (t_r / W½)²
A_t = A_0 · 0,5^(t/t½)
E = mc²
DOTAtate