Development and evaluation of a $^{44}Ti/^{44}Sc$ radionuclide generator and labeling of biomolecules with ^{44}Sc and ^{68}Ga for PET imaging.

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Abstract

Non-invasive molecular-imaging technologies are playing a key role in drug discovery, development and delivery. Positron Emission Tomography (PET) is such a molecular imaging technology and a powerful tool for the observation of various deceases *in vivo*. However, it is limited by the availability of vectors with high selectivity to the target and radionuclides with a physical half-life which matches the biological half-life of the observed process. The $^{68}Ge/^{68}Ga$ radionuclide generator makes the PET-nuclide anywhere available without an on-site cyclotron. Besides the perfect availability ^{68}Ga shows well suited nuclide properties for PET, but it has to be co-ordinated by a chelator to introduce it in a radiopharmaceuticals.

However, the physical half-life of ^{68}Ga of (67.7 min) might limit the spectrum of clinical applications of ^{68}Ga-labelled radiodiagnostics. Furthermore, ^{68}Ga-labelled analogues of endoradiotherapeuticals of longer biological half-live such as ^{90}Y- or ^{177}Lu-labeled peptides and proteins cannot be used to determine individual radiation dosimetry directly.

Thus, radionuclide generator systems providing positron emitting daughters of extended physical half-life are of renewed interest. In this context, generator-derived positron emitters with longer physical half-life are needed, such as ^{72}As ($T_{½}$ = 26 h) from the $^{72}Se/^{72}As$ generator, or ^{44}Sc ($T_{½}$ = 3.97 h) from the $^{44}Ti/^{44}Sc$ generator.

In this thesis the implementation of radioactive gallium-68 and scandium-44 for molecular imaging and nuclear medical diagnosis, beginning with chemical separation and purification of ^{44}Ti as a radionuclide mother, envestigation of pilot generators with different elution mode, building a prototype generator, development and ivestigation of post-processing of the generator eluate, its concentration and further purification, the labeling chemistry under different conditions, *in vitro* and *in vivo* studies of labeled compounds and, finally, *in vivo* imaging experiments are described.

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1. Introduction

1.1. Gallium

Positron emission tomography (PET) is a powerful tool in non-invasive and quantitative medical imaging, augmenting – in several interesting aspects - other imaging methods such as computer tomography (CT) or magnetic resonance imaging (MRI). PET is one of the rapidly developing molecular imaging technologies, both in the context of a more sensitive imaging technology and more sensitive radiotracers. For this technique, emission of positrons by β^+-emitters or, more precisely, the γ-radiation originating from their annihilation with electrons, is detected. At present, PET facilities are still dependent on an on-site cyclotron, since the predominantly used radioisotopes (^{18}F, ^{11}C, ^{13}N, and ^{15}O) are cyclotron-produced and have very short half-lives. The clinically most commonly used positron emitting radionuclide is ^{18}F ($T_{½}$ = 110 min), and ^{18}F-labeled 2-fluoro-2-deoxyglucose is the dominating PET tracer. This renders PET an expensive imaging technique. Today the majority of PET studies are performed with ^{18}F radiopharmaceuticals requiring an on-site cyclotron or shipment from a site in close proximity to the place where the investigation is performed.

Another option to support hospitals with relevant PET radio-nuclides is using radionuclide generators. Generator based radionuclides would allow easier availability and more flexibility in use. The decay of a long-lived parent nuclide to a short-lived PET daughter provides an inexpensive and convenient alternative (Rösch and Knapp, 2003).

Recently, the positron emitter ^{68}Ga has gained more attention as it is now available from commercially distributed radionuclide generators. The high positron branching of 89% and the kit-type of radiopharmaceutical syntheses offer excellent parameters for the routine use of ^{68}Ga labelled tracers in nuclear medicine using state-of-the-art positron emission tomography (PET) and PET/CT. The generator provides ^{68}Ga ($T_{½}$ = 67.7 min) from the long-lived ^{68}Ge ($T_{½}$ = 270.8 d), which is absorbed on a TiO_2 or SnO_2 matrix. ^{68}Ga-based radiopharmaceuticals are thus available independently of an on-site cyclotron. In addition, they may enable medical PET scans at a fraction of the usual cost. The physical half-life of 67.7 min and is thus compatible with most bio-targeting applications. The chemical aspects of utilization ^{68}Ga have recently been reviewed (Fani et al., 2008, Roesch and Riss, 2010).

Consequently, the generator-based radionuclide ^{68}Ga is getting into the focus of researchers and clinicians especially for radiolabeling of biomolecules. The advent of small peptides has boosted the interest in this radionuclide as its short half-life of 67.7 min is well suited to their rapid pharmacokinetics (Hofmann et al., 2001) and results in reduced radiation burden for the patient. Additionally, radiolabelling with trivalent metals such as ^{68}Ga is straight forward using direct reaction with DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) derivatized peptide conjugates. This is exemplified by the use of [^{68}Ga]-DOTATOC (DOTA-D-Phe^{1}-Tyr^{3}-octreotide) for diagnosis of somatostatin receptor positive tumors (Hofmann et al., 2001, Gabriel et al., 2007).

Until now, different types of ^{68}Ge/^{68}Ga generators were described by absorption of the accelerator produced parent radionuclide ^{68}Ge onto different solid column materials, such as metal oxides (Al_2O_3, TiO_2, or SnO_2), organic pyrogallol–formaldehyde resins (Schuhmacher and Maier, 1981), a styrene-divinyl-benzene copolymer containing N-methylglucamine (Nakayama et al., 2003) or alpha-ferric oxide (Ambe, 1988). Among them, TiO_2-based generators have become commercially available that are eluted with 0.1 M HCl, and are currently most widely used. The major limitations for direct use of ^{68}Ga for radiolabeling of peptides for clinical PET applications are large volumes of generator eluate, high H^+ concentration, ^{68}Ge breakthrough and potential metallic impurities. Several approaches have been described to overcome these limitations. Meyer et al. (2004) and Velikyan et al. (2004) used a micro-chromatography column containing anion-exchange resins, where ^{68}Ga is retained by forming an anionic tetrachloro-complex $[GaCl_4]^-$ in concentrated HCl to get rid of cationic impurities and to concentrate the generator eluate. Additionally Velikyan et al. (2004) used microwave heating during the radiolabeling process to eliminate an additional purification step for the ^{68}Ga labeled peptide and to improve specific radioactivity of radiolabeled peptide.

Breeman et al. (2005) used the fractionated generator eluate directly without any further purification step achieving specific activities of the radiolabeled peptide of up to 1 GBq nmol^{-1}. A fully automated system was developed by using direct fractionated generator eluate for the preparation ^{68}Ga-DOTA derivatized peptides with high pharmaceutical quality and high yields (Decristoforo et al., 2007, Ocak et al., 2010).

Zhernosekov et al. (2007) reported an efficient and simplified method for preparation of ^{68}Ga-labeled radiopharmaceuticals using a microchromatography column containing cation-

exchange resins. This method combines volume reduction with purification from metals including ^{68}Ge to provide ^{68}Ga in a useful form for direct radiolabeling.

In aqueous solutions, gallium is stable only as a trivalent cation. It cannot be incorporated into the structure of targeting vectors by covalent bonding, but must be complexed by a ligand that is conjugated to the biological vector. The Ga^{3+} ion possesses a d^{10} electron configuration and accepts different coordination numbers (usually 4–6), while not displaying preference for any particular coordination polyhedron. At pH>4, formation of colloidal hydroxide $[Ga(OH)_3]_n$ commences. Although this does not generally inhibit complex formation, radiolabeling is nevertheless substantially hampered due to formation of insoluble colloids (particularly at high activities) and their adhesion to the surface of the reaction vessel. At pH values above 8, a watersoluble hydroxo complex, $[Ga(OH)_4]$, is formed. As ligand exchange with the tetrahydroxo complex is a much slower process than complexation of free Ga^{3+}, complexation is achieved best at pH<4.

Ligands for ^{68}Ga-based PET radiopharmaceuticals should ideally combine the following set of properties.

1) Stability: Ga^{III} complexes should be as stable as possible; a kinetic inertness of the complex is more important than high thermodynamic stability.

2) Quick complexation under radiochemical conditions: Formation of Ga^{III} complexes should be fast at low temperatures, low concentration, and minimal excess of the ligand. A desirable ligand will chelate Ga^{3+} in solutions of nanomolar concentration at room temperature within minutes.

3) Selectivity: The ligand should ideally be selective for Ga^{3+} ion. Particularly, complexation of serum metals like Ca^{2+}, Mg^{2+}, and Zn^{2+} ions (the last being produced by decay of ^{68}Ga) should be disfavored in order to avoid transmetallation in vivo or diminishing of radiochemical yield.

4) Conjugation ability: The chelating unit has to possess a functional group which allows covalent binding to the targeting vector (biomolecule) without a significant derogation of complexation performance.

5) Long shelf-life: In medical applications, excellent chemical stability is necessary.

6) Accessibility: Preparation of the compound in practical amounts should be quick, facile, and inexpensive.

DOTA derivatives are currently the working horses of bioconjugational chemistry related to medical imaging, including ^{68}Ga applications (Breeman et al., 2005, Breeman and Verbruggen, 2007). This is mainly due to the commercial availability of ready-to-use mono-unprotected precursors like DOTA (tBu)$_3$ (Heppeler et al., 1999), which, in turn, is rooted in the fact that lanthanide (III) complexes of DOTA derivatives and its conjugates have been extensively used as MRI contrast agents (Caravan et al., 1999, Hermann et al., 2008, Aime et al., 2005) and radiotherapeutics (Van Essen et al., 2007). However, NOTA like ligands show a much better selectivity towards Ga^{3+} ion and their complexes are more stable, as the size of the NOTA cavity is almost ideal for this ion (Clarke and Martell, 1991, Broan et al., 1991, Craig et al., 1989).

1.2. Scandium

Recently, the $^{68}Ge/^{68}Ga$ radionuclide generator has shown significant potential for molecular imaging (Rösch et al., 2003, Rösch et al., 2004, Zhernosekov et al., 2007, Roesch and Riss, 2010). The high positron branching of 89% and the kit-type of radiopharmaceutical syntheses offer excellent parameters for the routine use of ^{68}Ga labelled tracers in nuclear medicine using state-of-the-art positron emission tomography (PET) and PET/CT. A clinical breakthrough was achieved demonstrating the superior possibilities of ^{68}Ga-DOTA-octreotide derivatives for localising neuroendocrine tumours, in particular if PET/CT is used. However, the physical half-life of ^{68}Ga of (67.7 min) might limit the spectrum of clinical applications of ^{68}Ga-labelled radiodiagnostics. Furthermore, ^{68}Ga-labeled analogues of endoradiotherapeuticals of longer biological half-live such as ^{90}Y- or ^{177}Lu-labeled peptides and proteins cannot be used to determine individual radiation dosimetry directly.

Thus, radionuclide generator systems providing positron emitting daughters of extended physical half-life are of renewed interest (Zhernosekov et al., 2007). In this context, generator-derived positron emitters with longer physical half-life are needed, such as ^{72}As ($T_{½}$ = 26 h) from the $^{72}Se/^{72}As$ generator, or ^{44}Sc ($T_{½}$ = 3.97 h) from the $^{44}Ti/^{44}Sc$ generator (Rösch and Knapp, 2003).

^{44}Sc is its cyclotron-independent availability *via* the $^{44}Ti/^{44}Sc$ radionuclide generator system. The long-lived ^{44}Ti produces a short-lived ^{44}Sc, which subsequently decays to stable ^{44}Ca. The physical half-life of ^{44}Sc is $T_{½}$ = 3.97 h, its positron branching is 94.27 % (Cameron and Singh, 1999). ^{44}Sc despite its high β^+-branching (94.3%) shows additional 99.9% photon emission of 1157.0 keV, generated by ^{44}Ti. The ^{44}Ti half-life varies in different studies from 39 to 66.6 years (Moreland and Heymann, 1965, Alburger and Harbottle, 1990, Norman et al., 1998, Ahmad et al., 1999, Hashimoto et al., 2001). Most recent studies revealed a half-life of 60.6±1.3 years (Norman et al., 1998, Ahmad et al., 1999, Hashimoto et al., 2001).

A crucial issue in the development of $^{44}Ti/^{44}Sc$ radionuclide generator systems consists in the production of ^{44}Ti. The $^{45}Sc(p,2n)^{44}Ti$ process seems to be an effective nuclear reaction, however, due to the long physical half-life, cyclotrons of high proton flux are mandatory.

For preparation of $^{44}Ti/^{44}Sc$ radionuclide generators, several radiochemical criteria are relevant, such as effective separation strategies providing high ^{44}Sc elution yields and low ^{44}Ti

breakthrough, high long-term stability, and type of Sc eluates useful for subsequent labelling reactions (i.e. low volume, low pH, high purity etc.), cf. (Rösch and Knapp, 2003).

Sc(III) is strongly adsorbed from oxalic acid solution, and its oxalate complex is selectively destroyed by the addition of hydrochloric acid. These data can be used as the basis of a method for the anion exchange separation of Sc(III) and Ti(IV) in oxalic acid / hydrochloric mixtures (Walter, 1958). In fact, there are only a few reports on studies to develop ^{44}Ti/^{44}Sc radionuclide generators. Using 0.2 M HCl / 0.1 M $H_2C_2O_4$ mixture on Dowex-1 resin, 60-70 % elution yield of ^{44}Sc in 30-50 ml was reported (Green and Hillman, 1967). A solvent extraction technique with an organic phase of 1 % 1-phenyl-3-methyl-4-capryl-pyrazolone-5 in methyl isobutyl led to >90 % recovery of Sc in less than 10 ml with a Ti contamination of < 10^{-6} (Mirza and Aziz, 1969). Elution yields of 42-46 % and decontamination factor of $5 \cdot 10^4$ were reported in studies with 0.01 M HCl as an eluent and ^{44}Ti being adsorbed on inorganic ZrO_2 as an analogue of Ti (IV) (Seidl and Lieser, 1973), cf. Table 1. However, clinically relevant generators have not yet been described. As those generators have not existed in the past, there was – consequently – no radiopharmaceutically and / or clinically development of ^{44}Sc-labelled radiopharmaceuticals. Even such basic informations whether DOTA could be an adequate ligand for coordinating ^{44}Sc were not available.

Year of study	Activity of ^{44}Ti (MBq)	Yield of ^{44}Sc (%)	Eluate volume (ml)	Separation factor
Green and Hillman, 1967	not given	60-70	30-50	$2 \cdot 10^4$
	not given	60-70	50	10^3 (after 40 elution)
Seidl and Lieser, 1973	0.037	42-46	30	$5 \cdot 10^4$

TABLE 1. Comparison of different ^{44}Ti/^{44}Sc radionuclide generators

In general, the trivalent metal Sc(III) appears particularly relevant, as it may be used for labelling of radiopharmaceuticals based on bifunctional chelators, established for coordinating lanthanides such as stable Gd(III) or ^{177}Lu, as well as for radioactive rare earth metals such as ^{90}Y, or radio nuclides like ^{111}In and ^{68}Ga. Due to the increasing medical applications of trivalent radiometals in diagnosis and therapy, the ^{44}Ti/^{44}Sc generator could possibly provide an interesting route for PET-imaging using ^{44}Sc labelled tracers. As a β^+-emitter, it could be

applied for planning and dosimetric calculations in endoradiotherapy based on the therapeutic radio-nuclides previously mentioned, but also for matching β^--emitting ^{47}Sc radiopharmaceuticals (Mausner et al., 1995).

The generator-derived ^{44}Sc appears to be appropriate in various directions. Hosain et al. (1977) already proposed ^{44}Sc as a PET radionuclide for studying bone disease. As a trivalent radiometal it may be used to synthesize radiopharmaceuticals based on bifunctional chelators, established to coordinate currently used other trivalent radionuclides in diagnosis and therapy, such as ^{68}Ga and ^{111}In or ^{90}Y and ^{177}Lu, as well as non-radioactive Gd(III). Due to the increasing medical applications of trivalent radiometals, the $^{44}Ti/^{44}Sc$ generator could possibly provide a new option for PET/CT imaging.

Macrocyclic chelators that form very stable complexes with metal cations are of paramount interest for radiopharmaceutical design. DOTA-conjugated peptides, such as the octreotide and octreotate somatostatin analogues are readily labeled with radionuclides such as ^{68}Ga, ^{90}Y, ^{111}In, and ^{177}Lu (Win et al., 2007, Breeman et al., 2003) being widely used for the detection and staging of neuroendocrine tumours. However, PET/CT imaging of these or other (DOTA) conjugated peptides or proteins is restricted to the rather short physical half-life of ^{68}Ga.

In recent years the Peptide Receptor Radionuclide Therapy (PRRT) is utilizing synthetic peptides as vectors for radionuclides such as ^{90}Y and ^{177}Lu. This has been accomplished with the aid of Positron Emission Tomography (PET) which involves the same vector molecules labeled with positron emitters, which was first demonstrated with ^{68}Ga labelled somatostatin analogues for diagnostics imaging of neuroendocrine tumors. This is turn resulted in the growing interest in other positron emitters obtained in generator systems described in the overview by Welch and McCarthy (2000) as well as by Rösch and Knapp (2003). So far the $^{68}Ge/^{68}Ga$ generator remains the most popular PET generator, it is also commercially offered by several manufacturers. The use of ^{44}Sc with half-life more than 3 times longer than that of ^{68}Ga may be an useful alternative not only for diagnostic purposes but also for dosimetry studies and further therapy planning with the use peptides labeled with the β^-emitting ^{47}Sc as a radiotherapeutic agents (Mausner et al., 1995). The chemistry of Sc^{+3} is similar to that of the Lanthanides, or the "Lanthanide like" elements. Due to its small ionic radius it is also chemically similar to Aluminium and Gallium. Hence chelators developed for the complexation of Gallium and the Lanthanides could be used as well for the complexation of

Scandium, the thermodynamic stability constants of the above M^{+3}-DOTA complexes are similar (Voila-Villegas and Doyle, 2009).

Generally small neuropeptides, such as Somatostatin (SST) and Gastrin Releasing Peptide (GRP)/BN analogs, labeled with γ- and/or β^--emitting radionuclides have been, and are still being investigated for their ability to bind to receptors which are overexpressed in a variety of malignant tissues (Prevost et al., 1993, Hofland et al., 1995, Dasgupta, 2004)**.** The affinity to these receptors of the designed chelatorpeptide construct may vary depending on the metal incorporated into the complex (Reubi et al., 2000). Consequently, it will be a challenge to characterize the binding affinities of ^{44}Sc-DOTA (or other ligands) –conjugated tumor membrane peptides.

2. Aims and Scopes

Non-invasive molecular-imaging technologies are playing a key role in drug discovery, development and delivery. Positron Emission Tomography (PET) is such a molecular imaging technology and a powerful tool for the observation of various deceases in vivo. However, it is limited by the availability of vectors with high selectivity to the target and radionuclides with a physical half-life which matches the biological half-life of the observed process.

Among diagnostic approaches in nuclear medicine oncology, PET provides the most accurate and biologically sensitive technique to localize and to quantify tumour specific biochemical interactions. However, the commonly used isotops ^{11}C ($T_{½}$ = 20 min), ^{13}N ($T_{½}$ = 10 min), ^{15}O ($T_{½}$ = 2 min) and ^{18}F ($T_{½}$ = 110 min) have short half-lifes and are cyclotron dependent.

In this context, using radionuclide generators seems to be adequate. The decay of a long-lived parent nuclide to a short-lived PET daughter provides an inexpensive and convenient alternative. Radionuclide generator systems as means of separating a desired radioisotope from its parent and other contaminants have proved to be extremely useful. The radiochemical separation concept, however, should meet certain requirements in order to be of real value:

1. It must rapidly yield the generator daughter in high purity in a chemical form suitable for further application;

2. The parent material must remain, or be reconverted readily to a form with which the process can be repeated and

3. It should be simple to handle, operate and shield. Potential radionuclide generators have been considered since decades.

The $^{68}Ge/^{68}Ga$ (^{68}Ge: $T_{½}$ = 270.8 d; ^{68}Ga: $T_{½}$ = 67.7 min, 89 % β^+) and $^{44}Ti/^{44}Sc$ (^{44}Ti: $T_{½}$ = 60 a; ^{44}Sc: $T_{½}$ = 3.97 h, 94.3 % β^+) radionucled generators satisfy all three requirements, that makes them desireable for PET using. Both of these radionuclides can be used for labeling various biomolecules for PET diagnosis. However, in contrast to the routenly used $^{68}Ge/^{68}Ga$ radionuclide generator, a $^{44}Ti/^{44}Sc$ radionucled generator was not developed till nowadays. Only few studies of ^{44}Sc “milking” systems with low activity were done before. ^{44}Sc has a special interest for PET because of it longer half-life compared to ^{68}Ga.

To introduce these new isotopes and to label ligands for radiopharmacy and PET, several criteria have to be studied:

1. Optimization of generator elution to produce daughter nuclide suitable for further use

2. Development and evaluation of radiochemical separation and purification of generator eluate suitable for a possible future routine application in radiopharmacy and nuclear medicine

3. Development and evaluation of a labeling chemistry of biomolecules

4. *in vivo* and *in vitro* studies of labeled compounds

5. *in vivo* imaging in an appropriate animal model

This thesis describes all five aspects in detail, gives an in-depth review of the current status of literature and implements new technical and chemical solutions to the various problems arising when introducing new isotopes to nuclear medicine and PET.

However, the aspects are different for the two generator systems. Consequently, the main focus of the present work is

a) in the case of the ^{68}Ge/^{68}Ga generator to

1. Improvement of post-processing to get eluate free from acetone

2. Labeling studies of various biomolecules at different conditions

3. Preclinical studies of ^{68}Ga-labeled biomolecules

and b) in the case of the ^{44}Ti/^{44}Sc generator to

4. Development and evaluation of an adequate generator model

5. Development and evaluation of post-processing of the generator eluate, its concentration and purification

6. Development and evatuation of labeling procedure

7. Preclinical studies of ^{44}Sc labeled biomolecules

This thesis describes the implementation of radioactive gallium-68 and scandium-44 for molecular imaging and nuclear medical diagnosis, beginning with chemical separation and purification of ^{44}Ti as a radionuclide mother, investigation of pilot generators with different elution mode, building a prototype generator, development and evaluation of post-processing of the generator eluate and the labeling chemistry, *in vitro* and *in vivo* studies of labeled compounds and, finally, *in vivo* imaging experiments using μPET.

Most of these individual problems have been addressed in separate research projects, which have been already published within the last 3 years (III, IV, V) or were recently submitted to peer-review journals (I, VII).

The present thesis will thus in the following chapter collect those scientific papers. Only a few of the topics mentioned (II, VI) are still not published and will be presented here as manuscripts.

The papers presented are:

1. N.S. Loktionova, A.N. Belozub, D.V. Filosofov, K.P. Zhernosekov, T. Wagner, A. Türler, F. Rösch, *A ^{44}Ti/^{44}Sc radionuclide generator for potential application of ^{44}Sc-based PET-radiopharmaceuticals*, submitted to Applied Radiation and Isotopes

2. M. Fellner, N. Loktionova, B. Bisalski, N. Bausbacher, V. Kubícek, P. Hermann, F. Rösch, O. Thews, *^{68}Ga-BPAMD: PET-imaging of bone metastases with a generator based positron emitter*, prepared for Nuclear Medicine and Biology

3. Johannes Notni, Petr Hermann, Jana Havlíčková, Jan Kotek, Vojtěch Kubíček, Jan Plutnar, Natalia Loktionova, Patrick Johannes Riss, Frank Rösch, and Ivan Lukeš, *A Triazacyclononane-Based Bifunctional Phosphinate Ligand for the Preparation of Multimeric ^{68}Ga Tracers for Positron Emission Tomography*, Chem. Eur. J. 16, 7174 – 7185 (2010)

4. Filosofov D.V., Loktionova N.S. and Roesch F., *A ^{44}Ti/^{44}Sc radionuclide generator for potential application of ^{44}Sc-based PET-radiopharmaceuticals*,

Radiochim. Acta 98, 149-156 (2010)

5. Pruszynski M., Loktionova N.S., Filosofov D.V., Roesch F., *Post-elution processing of $^{44}Ti/^{44}Sc$ generator-derived ^{44}Sc for clinical application*, Applied Radiation and Isotopes 68, 1636–1641(2010)

6. Marek Pruszyński, Agnieszka Majkowska, Natalia S. Loktionova, Frank Rösch, *Radiolabeling of DOTATOC and DOTATATE with the longer-lived, generator-derived trivalent metallic positron emitter ^{44}Sc*. In preparation for Applied Radiation and Isotopes

7. E. Koumarianou, N.S. Loktionova, M. Fellner, F. Roesch, O.Thews, D. Pawlak, S.C. Archimandritis and R. Mikolajczak, *DOTA-BN[2-14]NH_2 labeled with ^{68}Ga & ^{44}Sc PET tracers. In vitro and animal studies evaluation.* Submitted to Journal of Nuclear Medicine and Biology.

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3. Manuscripts

3.1. Improved column-based radiochemical processing of the generator produced ^{68}Ga

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Keywords: ^{68}Ga, radionuclide generator, post-processing of eluates, radiolabelling, PET

Abstract

An improved chemical strategy for processing of the generator produced 68Ga was developed based on processing of the original ^{68}Ge/^{68}Ga generator eluate on a micro-column. Direct pre-concentration and purification of the eluted ^{68}Ga is performed on a cation-exchang resin in hydrochloric acid / acetone media. A supplementary step based on a second micro-column filled with a second resin allows direct re-adsorption of ^{68}Ga eluted from the cation-exchanger. ^{68}Ga is finally striped from the second resin with a small volume of pure water. For this purpose a strong anion-exchanger and a novel extraction chromatographic resin based on tetraalkyldiglycolamides are characterized. The strategy allows on line pre-concentration and purification of ^{68}Ga from the original generator eluate. The supplementary column allows transferring ^{68}Ga with high radionuclide and chemical quality in the aqueous solution with small volume and low acidity useful for direct radiolabelling reactions.

1. Introduction

The $^{68}Ge/^{68}Ga$ radionuclide generator provides an excellent source of positron emitting ^{68}Ga for the application of ^{68}Ga-labeled compounds using PET. However, currently available "ionic" $^{68}Ge/^{68}Ga$ radionuclide generators are not necessarily optimized for the routine synthesis of ^{68}Ga-labelled radiopharmaceuticals in a clinical environment. The eluates have rather large volumes (up to 5 to 10 ml for a complete generator elution), a high acidity (0.1 to 1.0 N, depending on the generator type), an initial breakthrough of ^{68}Ge in the range of 10^{-3} to 10^{-2} %, increasing with time or usage frequency, and metallic impurities such as stable Zn^{II} (generated by the on-generator-decay of ^{68}Ga), Ti^{IV}, Sn^{IV} or other metals as a consequence of use of the metal oxide based generator matrixes.

Recently, we have introduced a generator associated post-processing approach to adsorb ^{68}Ga on line from generator eluates on a micro cation-exchange column, to purify it using a HCl / acetone mixture of 0.15 M HCl / 80% acetone. Subsequently the radionuclide is desorbed from the resin quantitatively with 0.4 ml of a 0.05 M HCl / 98% acetone solution. The process takes 3 minutes only and allows obtaining 96% of ^{68}Ga with the highest radionuclide and chemical purity in the form useful for the radiolabelling reaction (Zhernosekov et al., 2007). Thus the technique is successfully applied for the preparation of ^{68}Ga-labelled peptides for clinical studies (Asti et al., 2008). Contents of the acetone in the reaction mixture are low and non-toxic. They might be, however, avoided for the direct *in vivo* applications or for the radiolabelling reactions performed under high temperature.

Alternatively, ^{68}Ga can be pre-concentrated on an anion exchanger because of high distribution coefficients of Ga(III) (Schumacher, Kraus, Meyer). This technique does not allow, however, direct pre-concentration from the original generator eluate and adjustment of the hydrochloric acid concentration must be done. Finally ^{68}Ga is eluted with small volume of pure water.

The aim of the present study is to develop an improved column-based chemical strategy combining aspects of the both methods. Direct pre-concentration of ^{68}Ga from the original eluate and its purification is supposed to be performed on the cation-exchanger (Zhernosekov et al., 2007). The ^{68}Ga can be eluted with hydrochloric acid solutions at high concentration > 2 - 3 M. For an effective transfer of the purified ^{68}Ga into the aqueous phase of low acidity and small volume a secondary micro-column is introduced into the process, which allows

direct re-adsorption of gallium eluted form the cation-exchanger and can be finally striped with a small volume of pure water. For this propose anion-exchanger and a novel extraction chromatographic resin based on tetraalkyldiglycolamides (DGA) is characterized in details.

2. Materials and Methods

2.1. Chemicals and reagents

Only analytic reagent grade chemicals and Milli-Q water (18.2 MΩ·cm; Millipore) were used. The ion exchange resins AG 50W-X4 (-400 mesh, H^+-form) and AG 1-X8 (200-400 mesh, Cl^--form) were purchased at Bio-Rad Laboratories (Richmond, CA, USA). The extraction chromatographic resins based on N,N,N',N'-tetra-n-octyldiglycolamide (TODGA) and N,N,N',N'-tetrakis-2-ethylhexyldiglycolamide (TEHDGA) 50–100 μm particle size were provided by Eichrom Technologies. 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) conjugated with D-Phe^1-Tyr^3-octreotide (DOTATOC), GMP-grade, was obtained from piChem R&D (Graz, Austria).

2.2. ^{68}Ge/^{68}Ga radionuclide generator

A generator based on a modified TiO_2 phase adsorbing $^{68}Ge^{IV}$ was obtained from Cyclotron Co. Ltd.. In the present study, a 250 MBq device was used. The ^{68}Ge content in the eluate was 10±2 kBq.

2.3. Determination of Ga^{III} and Ge^{IV} distribution on DGA

5 ml 0.1 M HCl generator eluates were mixed with 12 M HCl solution to obtain $^{68}Ga^{III}$, $^{68}Ge^{IV}$ solutions with hydrochloric acid concentrations in the range of 0.1 – 8 M. The uptake of Ga^{III} and Ge^{IV} ions by the DGA resins was measured by combining 2.5 ml of the solution with about 50 mg of the resin in a glass vial. The samples were shaken well for 1 h. After equilibration with the resin, the aqueous phase was filtered through 20 μm PTFE syringe filter. All experiments were performed at room temperature between 20 and 23 °C. Weight distribution ratios D_w were calculated using the following equation:

$$D_w = (A_0 - A_s)\cdot V/A_s\cdot m, \quad (1)$$

where A_o and A_s are the aqueous phase activity before and after equilibration, V is the volume of the aqueous phase in milliliters and w is the weight of the resin in grams. D_w values were then converted to k' (the resin capacity factor) as described elsewhere (Horwitz et. al., 2005a):

$$k' = 0.57 \cdot D_w. \quad (2)$$

2.4. Micro-columns

For the preparation of micro-columns, simillar as described previously (Zhernosekov et al., 2007), 50 mg of wet cation exchangers AG 50W-X8 (minus 400 mesh in H^+-form), 50 mg of wet anion exchanger AG 1-X8 (200-400 mesh, Cl^--form) and 100 mg DGA resin (Eichrom Technologies, 50 – 100 μm particle size) were used.

2.5. Pre-concentration and purification of ^{68}Ga on cation exchanger

The first step of concentration and purification of the initial ^{68}Ge/^{68}Ga generator eluate was performed on a micro cation exchange column. The generator was eluted with 7 ml of 0.1 M HCl on line through the chromatographic column. Next, the column was eluted with a 1 ml solution of 80% acetone / 0.15 M HCl to remove the main parts of the chemical and radiochemical impurities of the generator eluate (Zhernosekov et al., 2007).

To desorb the purified ^{68}Ga from the cation exchanger, the column was striped with hydrochloric acid solutions. HCl concentrations of 1 to 8 M and of 0.5, 1.0 and 2.0 ml volumes have been tested.

2.6. Processing of ^{68}Ga on anion exchanger and DGA resin

Adsorption of ^{68}Ga on the anion exchanger and DGA resin was investigated by dynamic loading of the activity on the columns with hydrochloric acid solutions of 1 to 8 M concentrations and of 0.5, 1.0 and 2.0 ml volume.

2.7. Combining cation exchange with anion exchange

A closed system was assembled by connecting the micro-column and the generator using standart capillary and 3-way valves (Figure 3a). The system could be operated using single-use syringes. The generator was eluted directly trough the cation-exchange column (direction

1 on Fig. 3a). 3 ml of the 5 or 4 M HCl solution was used to strip the cation-exhange column online trough the anion-exchange or DGA column (direction 3 on Fig. 3a), respectively. Finally, 68Ga was desorbed from anion exchanger or DGA resin with pure water (direction 4 on Fig. 3a).

2.8. Measurement of radioactivities

Measurement of ^{68}Ga was accomplished in a dose calibrator M2316 (Messelektronik, Dresden GmbH). The absolute activity of ^{68}Ge was analysed by γ-spectrometry using an HPGe-detector at least 2 days after the corresponding radionuclide generator elution.

2.9. Radiolabeling of DOTATOC

For labeling reactions, 2 mL tubes (Eppendorf) were used. Heating was performed in ThermoMixer MHR13 from HLC BioTech. For labeling of octreotide derivatives, DOTA-D-Phe^{1}-Tyr^{3}-conjugated octreotide (DOTATOC) was used.

2.10. Analyses of contents of acetone

The acetone content in the processed fractions was analysed by gas chromatography (HP 6890 series GC system).

3. Results and Discussion

3.1. Direct pre-concentration of the ^{68}Ga

Direct pre-concentration of the generator produced ^{68}Ga can be effectively performed on a miniaturized cation-exchange column. Application of HCl /acetone media allows purification of the activity from trace of ^{68}Ge, Zn(II), Ti(IV) and even Fe(III) (Zhernosekov et al., 2007). In contrast to our previous work, elution of 68Ga from cation exchanger is performed in concentrated HCl solutions. As these are not applicable, however, for direct radiolabelling reaction, a transfer of 68Ga into an aqueous phase of low acidity and small volume is introduced based on a second micro-column. For this propose strong anion exchanger and extraction chromatographic resin DGA were characterized.

3.2. ^{68}Ga(III) behavior on DGA resin

Commercially available DGA resins have recently been proposed as very promising for processing of radionuclides such as ^{90}Y(III) or ^{177}Lu(III) for medical use (Horwitz et. al., 2005a; Horwitz et. al., 2005b). Figure 1 shows the dependency of Ga(III) and Ge(IV) uptake on hydrochloric acid concentration on DGA. The DGA resins show negligible gallium adsorption at low acid concentration up to 1 M. The retention of the cation exchange resin, however increases rapidly with increasing hydrochloric acid concentration. Thus, in contrast to strong anion exchanger such as AG 1-X8 (Nelson, 1963), the DGA resins show increased adsorption to trivalent gallium at lower HCl concentration, but TODGA resin has a little bit higher coefficients than TEHDGA. Ge(IV) has only negligible retention on the TODGA resin in a wide range of the HCl concentration. For further investigation TODGA resin was chosen.

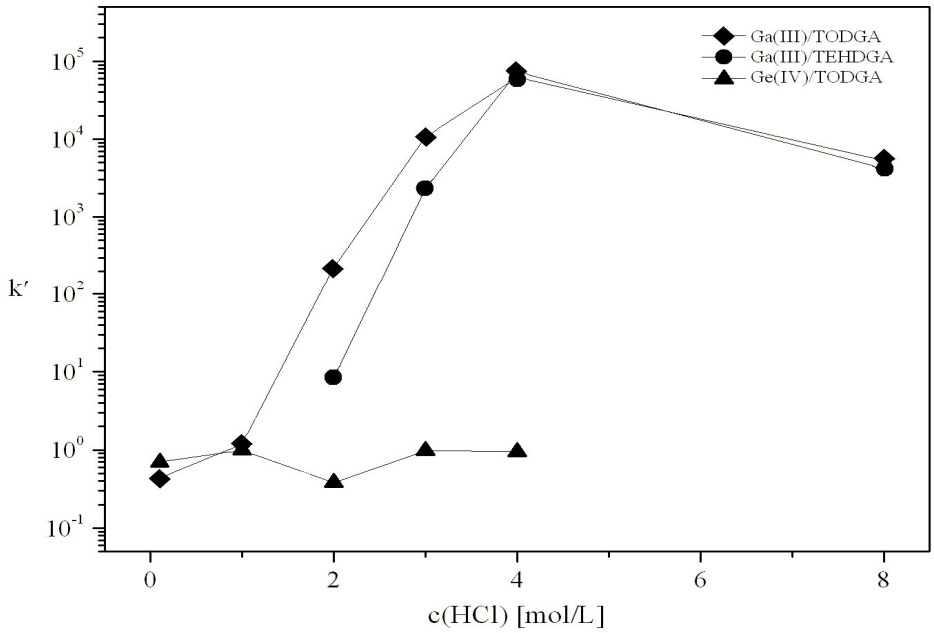

Figure 1. k' for Ga(III) and Ge(IV) on DGA resin (50 – 100 μm) versus HCl concentration, 1 h equilibration time, room temperature.

3.3. Pre-concentration and purification of ^{68}Ga on cation exchanger

^{68}Ga is quantitatively adsorbed from the original generator eluate on the cation-exchange column and effectively purified using 80% acetone / 0.15 M HCl solution (Zhernosekov et al., 2007). Optimum desorption of ^{68}Ga from the cation-exchange column was achieved with 2 ml of 2 to 4 M HCl solutions (Figure 2a), which corresponds to known characteristics of distribution coefficients of GaIII on strong cation exchangers (Nelson et al., 1963).

3.4. Characterisation of anion exchanger and TODGA resin columns

Adsorption efficiency of ^{68}Ga on the anion-exchange and TODGA column from HCl solutions at different acid concentration is given in Figs 2b and 2c. An effective retention of ^{68}Ga on the TODGA resin could be achieved at > 3-4 M HCl, whereas not less than 5-6 M HCl concentration was needed for nearly complete adsorption of ^{68}Ga on AG1-X8. Elution of the activity could be quantitatively performed in pure water for both resins. However, 300 µl of H_2O was used to strip ^{68}Ga from the anion-exchange column and up to 1000 µl was needed for its elution from the TODGA column.

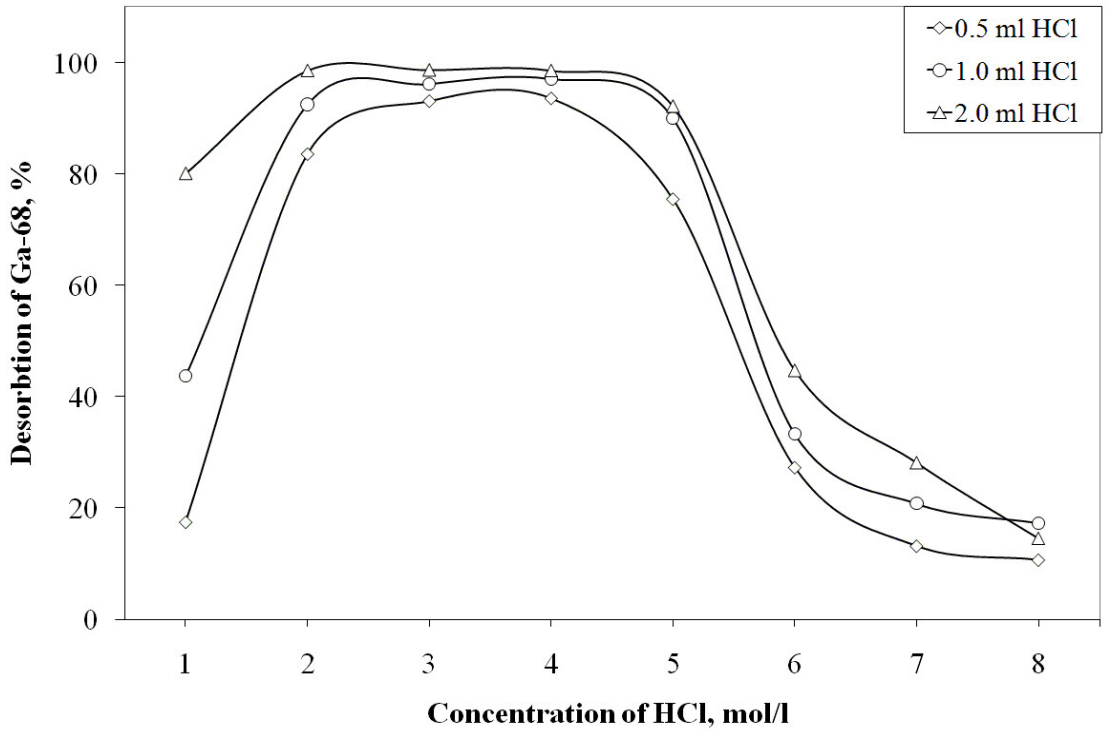

Figure 2a. Yields of ^{68}Ga, eluted from the cation exchange resin with 0.5, 1.0, 2.0 ml of various concentrations of HCl.

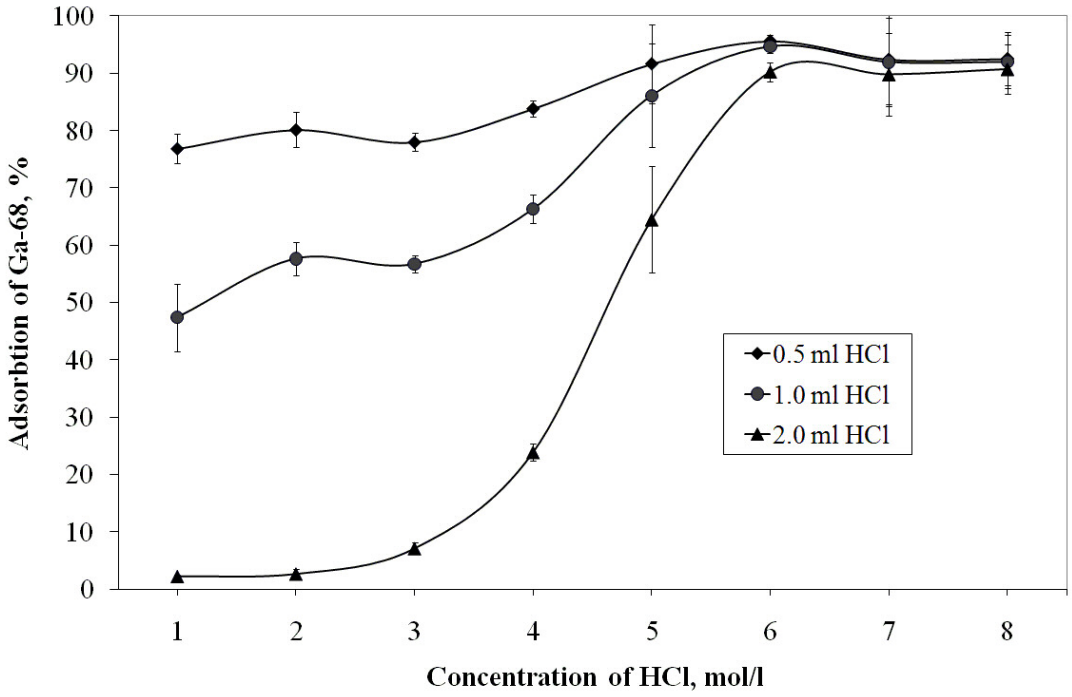

Figure 2b. Yields of ^{68}Ga, adsorbed at the AG 1-X8 resin (50 mg) with 0.5, 1.0, 2.0 ml of various concentrations of HCl used to desorb ^{68}Ga from the AG 50W-X8.

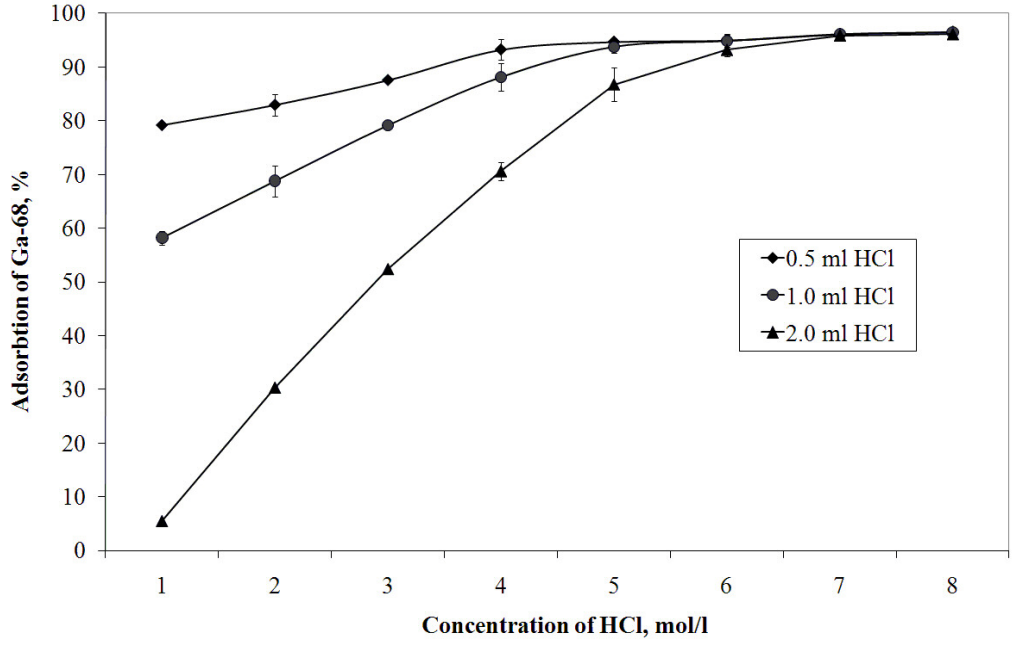

Figure 2c. Yields of ^{68}Ga, adsorbed at the TODGA resin (100 mg) with 0.5, 1.0, 2.0 ml of various concentrations of HCl used to desorb ^{68}Ga from the AG 50W-X8.

3.5. Improved column-based ^{68}Ga processing using column combinations

Quantitative desorption of ^{68}Ga from cation-exchange column is achieved at 2 to 4 M HCl, which corresponds to known distribution characteristics of GaIII on strong cation exchangers (Nelson et al., 1963). However, 2 to 4 M HCl solutions of GaIII are not adequate to adsorb GaIII on the strong anion exchangers such as AG 1-X8. Thus HCl concentrations of 6 to 8 M are needed. The extraction chromatographic resin TODGA shows, in contrast, a higher adsorption profile to trivalent gallium at lower acids concentration. Thus, an effective 68Ga adsorption can be performed at 4 M HCl concentration. In this context, 5 M and 4 M HCl solutions were applied for desorption of ^{68}Ga from cation-exchanger and readsorption of the activity on anion-exchange and DGA columns, respectively.

A conceptual flowsheet of the system including combination of cation-exchange and anion-exchange or TODGA columns is presented in Fig. 3b. The columns are connected consecutively via 3-way valves. In the first step (1) 7 ml 0.1 M HCl pass through the generator and the cation exchange resin into the waste A. Step (2) consists in eluting the cation exchanger with 1 ml of hydrochloric acid/ acetone solution into waste B. The third step (3) is the nearly quantitative transfer of ^{68}Ga from the cation exchange column onto the anion exchanger or TODGA resin with 3 ml of 5 M or 4 M HCl, respectively. While ^{68}Ga is quantitatively adsorbed on both resins, the 3 ml of HCl continues to the waste vial B. From the supplementary anion-exchange or TODGA columns, ^{68}Ga is eluted using 0.3 ml or 1.0 ml of water, respectively, (4) into the product vial. With 98% effectivity for the cation exchange part, the 92% and 98 % yield obtained for desorbing ^{68}Ga from the anion exchange and chromatographic TODGA columns, respectively, the overall yield in the final 0.3-1.0 ml water fraction is 87±5 % (for AG 1-X8) and 96 % (for TODGA resin) related to the initial generator eluate (Table 1).

Table 1. The distribution of ^{68}Ga and ^{68}Ge for every eluted fraction. I – AG 50W-X8, II – AG 1-X8 or TODGA.

N	Solution	Via	Volume, ml	Yield, %	
				^{68}Ga	^{68}Ge
AG 50W-X8 (50 mg) only [a]					
1	0.1 M HCl	I	7	0.16±0.05	97.08±1.99
2	N1	I	1	1.43±0.18	2.92±0.46
3	N2	I	0.4	**97.82**±2.01	**$3\cdot10^{-2}\pm2\cdot10^{-3}$**
4	4 M HCl	I	1	0.41±0.09	$5\cdot10^{-3}\pm1\cdot10^{-4}$
5	Water	I	1	0.18±0.02	$3\cdot10^{-3}\pm2\cdot10^{-4}$
AG 50W-X8 (50 mg) + AG 1-X8 (50 mg) [b]					
1	0.1 M HCl	I	7	0.41±0.57	94.36±2.44
2	N1	I	1	4.50±1.87	3.87±1.52
3	5 M HCl	I+II	3	5.54±6.98	1.23±0.98
4	Water	II	0.3	**86.55±4.82**	**$1\cdot10^{-2}\pm1\cdot10^{-2}$**
5	4 M HCl	I	1	2.67±2.08	0.42±0.15
6	Water	I	1	0.33±0.35	0.11±0.05
AG 50W-X8 (50 mg) + TODGA (100 mg) [b]					
1	0.1 M HCl	I	7	0.58±0.06	91.53±1.2
2	N1	I	1	2.20±0.09	6.75±1.19
3	4 M HCl	I+II	3	1.35±0.16	1.47±0.30
4	Water	II	1.0	**95.63±0.21**	**$1\cdot10^{-2}\pm2\cdot10^{-2}$**
5	Water	I	1	0.24±0.05	0.25±0.06

[a] data from work of Zhernosekov et al., 2007, total breakthrough of ^{68}Ge is 170 kBq,

[b] experimental data, total breakthrough of ^{68}Ge is 10 kBq.

For recondition of the columns the cation exchange resin should be washed with 1 ml 4 M HCl (5) and 1 ml of water (6), consecutively, into the waste A. The anion exchange resin should be washed with 1 ml of 8 M HCl (7) into the waste B.

Figure 3. Sketch of $^{68}Ge/^{68}Ga$ generator elution with cation and anion exchange columns in tandem (a) and indication of detailed flows (b).

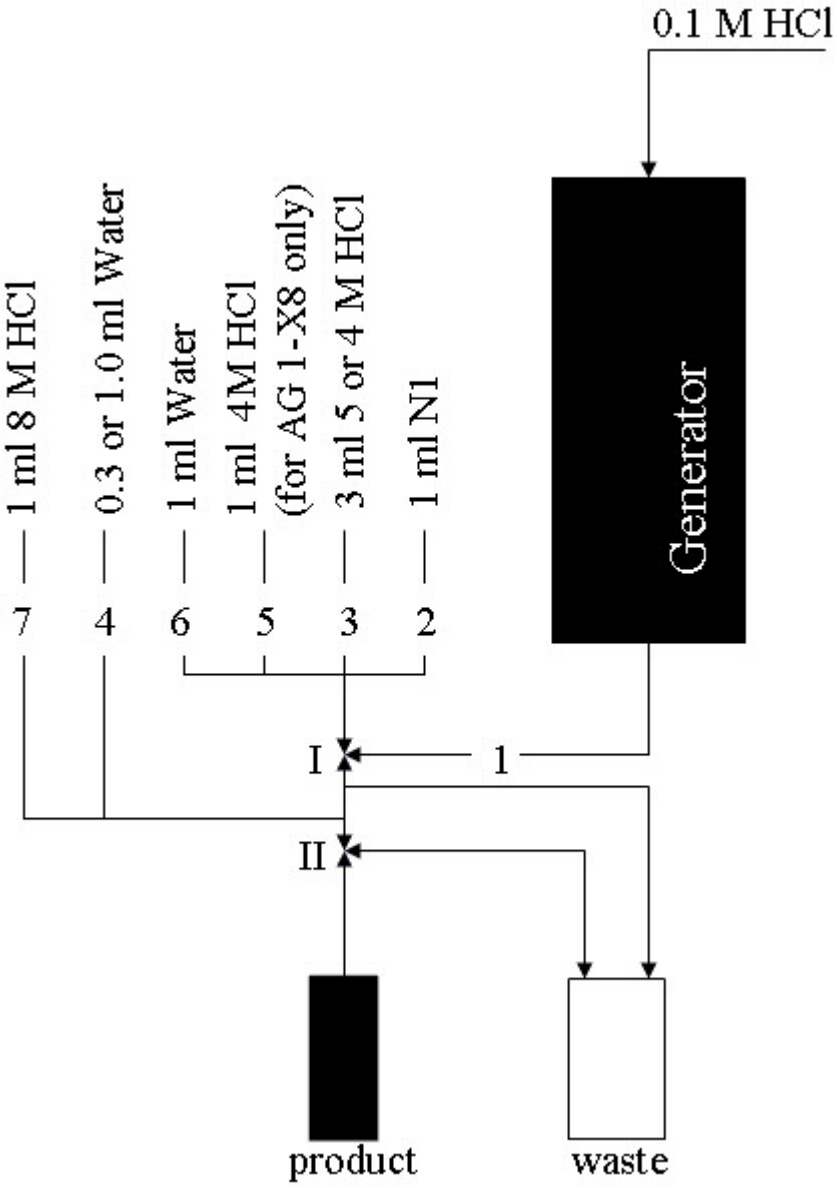

Figure 3a. I – 50 mg AG 50W-X8, II – 50 mg AG 1-X8 or 100 mg TODGA.

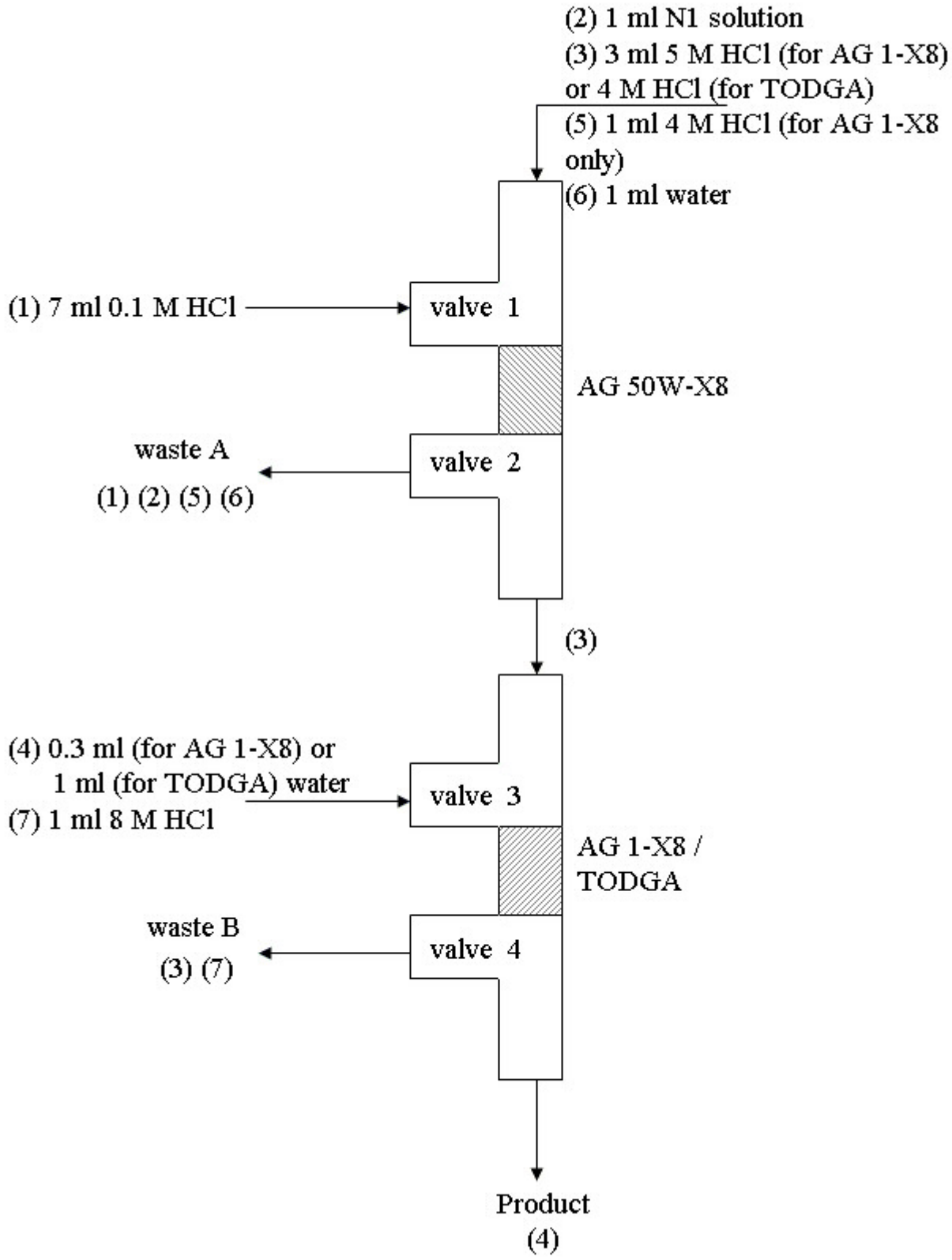
(2) 1 ml N1 solution
(3) 3 ml 5 M HCl (for AG 1-X8)
or 4 M HCl (for TODGA)
(5) 1 ml 4 M HCl (for AG 1-X8
only)
(6) 1 ml water
(1) 7 ml 0.1 M HCl
valve 1
AG 50W-X8
waste A
(1) (2) (5) (6)
valve 2
(3)
(4) 0.3 ml (for AG 1-X8) or
1 ml (for TODGA) water
(7) 1 ml 8 M HCl
valve 3
AG 1-X8 /
TODGA
waste B
(3) (7)
valve 4
Product
(4)

Figure 3b.

The complete processing takes about 5 min only. Final 68Ge contents were below 10^{-2} %, indicating additionally purification of ^{68}Ga generator eluates from the initial breakthrough of ^{68}Ge by a factor 10^5. The pH of the final ^{68}Ga fraction was 0.3±0.1 according to the hydrochloric acid residues on the columns. This pH can be shifted towards pH 2 by using diluted sodium hydroxide solution instead of pure water.

The final ^{68}Ga fraction was used for preparation of ^{68}Ga-DOTATOC. For this propose 0.5 M NaAc buffer solution was used to adjust the pH of the reaction mixture to 2.5-3. The radiolabelling was performed utilizing 14 nmol of the peptide providing 98±2 % labelling yield.

4. Conclusions

A combined protocol of processing ^{68}Ge/^{68}Ga generator eluate has been developed. It utilises the significant advantages of cation-exchenger based processing of ^{68}Ga. To avoid any presence of acetone in radiolabelling mixtures processing, ^{68}Ga was transferred from the cation-exchanger to the supplementary micro-column filled with strong anion exchanger or a novel extraction chromatographic TODGA resin. An effective processing of generator produced $^{68}Ga^{III}$ could be performed within 5 min only. The $^{68}Ga^{III}$ preparation could be obtained in a reduced volume with high chemical and radiochemical purity in a form useful for radiolabelling reactions. Overall yields of ^{68}Ga for anion exchanger and TODGA resins are 87% and 96%, respectively.

Acknowledgements

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3.2. ^{68}Ga-BPAMD: PET-imaging of bone metastases with a generator based positron emitter

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Keywords: Ga-68, macrocylic ligand, bisphosphonate, bone metastases

Abstract

Purpose: Bone metastases are a serious aggravation for patients suffering from cancer. Therefore, early recognition of bone metastases is of great interest for further treatment of patients. Bisphosphonates are widely used for imaging bone lesions with ^{99m}Tc. Using the ^{68}Ge/^{68}Ga generator together with a macroyclic bisphosphonate a comparable PET-tracer comes into focus.

Procedures: The bisphosphonate DOTA-like ligand BPAMD was labelled with ^{68}Ga. [^{68}Ga]BPAMD was investigated concerning binding to hydroxyapatite and stability. The tracer's *in vivo* accumulation was determined on healthy rats and bone metastases bearing animals by μ-PET.

Results: BPAMD was labelled efficiently with ^{68}Ga after 10 min at 100 °C. [^{68}Ga]BPAMD showed high stability within 3h and high binding to hydroxyapatite. Consequently μ-PET experiments revealed high accumulation of [^{68}Ga]BPAMD in regions of pronounced remodelling activity like bone metastases.

Conclusions: [^{68}Ga]BPAMD is of great interest for diagnosis of bone metastases. The straight forward ^{68}Ga-labelling could be transferred to a kit-preparation of a cyclotron-independent PET tracer, that could be used in many clinical sites using the ^{68}Ge/^{68}Ga generator.

1. Introduction

Besides lung and liver, the bones are most frequently affected by metastases. 60-80 % of these metastases are caused by breast or prostate carcinoma. Bone metastases occur in many cases at an early stage of the tumour disease, however their symptoms are recognized rather late [1,2]. Hence, a diagnosis of bone metastases in an early state together with a subsequent therapy is of great importance for patients.

^{99m}Tc-phosphonates are well-established tracers for the diagnosis of bone metastases in nuclear medicine using planar imaging or single photon emission tomography (SPECT) [3]. However, due to the higher spatial resolution of positron emission tomography (PET), adequate pharmaceuticals with positron emitters would be of great potential. The superior imaging quality in the case of PET/CT imaging is clearly demonstrated by using [^{18}F]fluoride [4]. Nevertheless PET features also quantification of the tracer accumulation in the tissues investigated, compared to SPECT, which cannot afford this. Additionally, non-cyclotron dependent PET-tracers, i.e. radionuclide generator based derivatives, would provide the required availability for instant tracer synthesis and PET/CT diagnosis. For this attempt the Germanium-68/Gallium-68 generator with the positron emitter ^{68}Ga ($T_{1/2}$ = 67.7 min; high positron branching = 89%) represents a promising system. Having had recent supply difficulties of the ^{99}Mo/^{99m}Tc generators [5,6], this Gallium generator comes into focus of nuclear medicine diagnostics.

Using the cationic post-processing of the generator eluate, it is an excellent source for synthesizing and evaluating new tracers [7,8]. Previous studies on first generation phosphonates [9] provided following requirements and restrictions: 1. open chained ligands like EDTMP (Fig. 1) exhibit low stability, this means high amounts of free ligand has to be injected (> 1.5 mg / kg body weight) in order to keep the complex stable; 2. phosphonate groups in ligands which are needed for complexing Ga^{3+} are not available for binding to apatite (e.g. NOTP; Figure 1), thus free phosphonates are necessary [9].

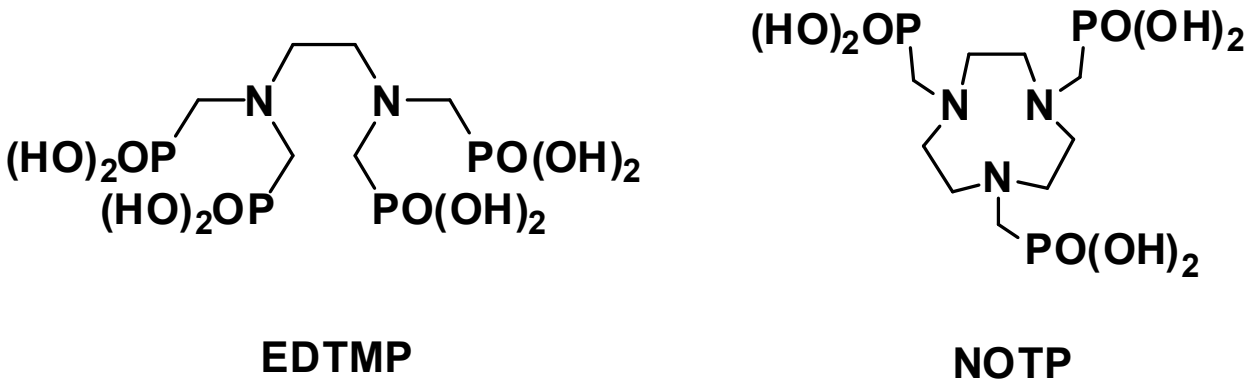

Figure 1. Ligands EDTMP and NOTP used in former study investigating ^{68}Ga-complex formation and binding to hydroxyapatite

Bisphosphonates are known to have high and fast binding to apatite structures especially those of high biological activity [10] and are well-established ligands for ^{99m}Tc-labelled SPECT tracers. Most used phosphonates for ^{99m}Tc are bisphosphonates like methylene diphosphonate (MDP), dicarboxypropane diphosphonate (DPD) and hydroxymethylene diphosphonate (HDP). Chemically combining bisphosphonates with a positron emitter, however, would be of great potential for the *in vivo* imaging of bone metastases using PET/CT, particularly in times of global ^{99}Mo shortage as seen in the last 2 years.

Combining these results led to a new generation of DOTA bisphosphonates. One out of these is (4-{[bis-(phosphonomethyl))carbamoyl]methyl}-7,10-bis(carboxymethyl)-1,4,7,10-tetraazacyclo-dodec-1-yl)acetic acid (BPAMD; Figure 2) [11,12], which was labelled with ^{68}Ga in nanomolar scale in water after 10 min heating at 100 °C and has been used for a first human application [13].

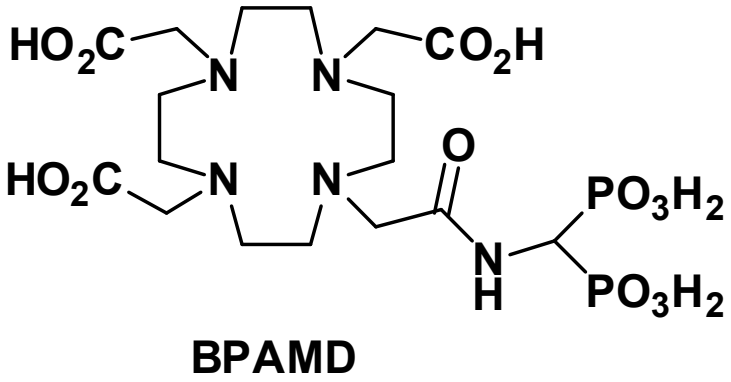

Figure 2. Macrocyclic bisphosphonate BPAMD investigated in this study

Bisphosphonates in general are of another benefit. Compounds like alendronate, risedronate or zoledronate do not only bind to the hydroxyapatite structure of the bone, they are also interacting in the HMG CoA reductase pathway and are inhibiting the farnesyl diphosphate synthase (FPPS) [10,14]. The bisphosphonates mimic pyrophosphates and thus inhibit the FPPS meaning that bisphosphonates have a biochemical target and not only a physical one by binding to hydroxyapatite.

2. Materials and Methods

2.1. Generator

Germanium-68 ($T_{1/2}$ = 270.8 d) provides the positron emitter Gallium-68 as an easily available and relatively inexpensive source of a PET nuclide. A Cyclotron Obninsk Ltd. Co. (Russian Federation) generator was used, with Germanium-68 fixed on a solid phase of modified titanium dioxide. Gallium-68 is eluted from the generator with 10 mL 0.1 M HCl and is on line-immobilized on a strong acidic cation exchanger. A mixture of hydrochloric acid and acetone further removes impurities such as zinc, iron and titanium as well as ^{68}Ge generator breakthrough (0.15 M HCl / 80 % acetone). Subsequently, ^{68}Ga is eluted quantitatively in 400 µL of a second mixture of HCl and acetone (0.05 M HCl / 97.6 % acetone) from the cation exchange resin [7, 8]. This fraction serves as an ideal low-volume, low acidic and chemically highly pure source of ^{68}Ga for subsequent labelling.

2.2. BPAMD Synthesis and Labelling

BPAMD was prepared by published method [11,12]. In brief, Triethyl orthoformate (1.2 eq.), diethyl phosphite (3 eq.), and dibenzylamine (1 eq.) were reacted at 150 °C for 5 h with condenser and additional 24h without. Reaction mixture was partitioned between $CHCl_3$ and 5 % aqueous NaOH and washed with brine. The crude product was purified by gradient column chromatography over ethanol/ hexane yielding tetraethyl (*N*,*N*-dibenzyl)aminomethyl-bis(phosphonate) (43 %). This compound was hydrogenated using 10 % Pd/C in ethanol, yielding tetraethyl aminomethyl-bis(phosphonate) in 96 % yield as colorless oil. This product was dropped in a solution of cloracetyl chloride in acetonitrile, cooled to -40 °C. Treated with charcoal and removing excess of chloracetyl chloride by co-distillation with toluene gave the chloroacetamide as colourless oil (96 %). Finally the chloroacetamid was dissolved in acetonitrile together with K_2CO_3 and added to a solution of t-Bu_3DO3A•HBr in acetonitrile with K_2CO_3. Reaction overnight, subsequently evaporation of the solvent and deprotection of the tertbutyl ester yielded the product BPAMD after purification over a strong cation exchanger (39 %).

A stock solution of BPAMD (1 mg/mL water) was prepared and aliquots were used for labelling studies. ^{68}Ga labelling itself was performed in 5 mL millipore water in 11 mL Technevials by adding the 400 µl of purified and concentrated ^{68}Ga fraction. Through variation of reaction time (1-10 min), temperature (60 to 100 °C), different amounts of the complex ligands (5-50 nmol) as well as reaction pH (pH = 1-5), optimum reaction parameters for ^{68}Ga complex formation were analysed. All experiments studying labelling kinetics were conducted in a thermomixer system (HLC Biotech HeatingThermoMixer MHR 13). For the human application, the thermomixer can be replaced by a simple heating block available at most clinical sites with a ^{68}Ge/^{68}Ga generator.

2.3. Radioactive Analysis

Determination of radiochemical labelling yield and complex formation kinetics were carried out by two TLC systems: A: Merck SG-60 silica plates developed with 0.25 M citrate buffer pH 4;
B: Macherey Nagel cellulose TLC plates (Polygram Cel 300, 0.1 mm) developed with 2:1 mixture of B1:B2 (B1: 9 mL millipore water; 0.6 mL HCl (conc. 37 %); 88 mL acetone / B2:

2,4-pentadione). After drying and developing the TLCs, they were measured on a Canberra Packard Instant Imager.

Additionally a HPLC system was developed for quality control using a strong anion exchange column (Partisil 10 SAX 250x4 mm) on a Dionex HPLC system (P680 HPLC, UVD 170U) connected with a Raytest Gabi 2x2" radioactivity detector. The solvent system was a gradient of 1 M phosphate buffer (pH = 3, A) and 1 M sodium citrate (B): flow 1.5 mL/min;0-5 min 100 % A; 5-7 min 100 % A to 70 % A/ 30 % B; 7-12 min 70 % A/ 30 % B; 12-14 min 70 % A/ 30 % B to 100 % A; 14-20 min 100 % A.

2.4. Purification of [^{68}Ga]BPAMD

Non-complexed ^{68}Ga had to be removed prior to further evaluations. This was performed by passing the reaction mixture over strong cation exchanger (Strata-X-C 60 mg, conditioned with 1 mL 4 M HCl and 1 mL water). The non-complexed gallium was immobilized on the exchanger while the [^{68}Ga]BPAMD complex passed to the product vial. In order to adjust the pH to 7.4, aliquots of a 100 mg/mL sodium HEPES solution were added.

2.5. Binding studies on Hydroxyapatite

Binding studies on synthetic hydroxyapatite (Hap) were applied to simulate the binding of the different ^{68}Ga ligand complexes to bone structures. For this purpose, 20 mg Hap were incubated in 1 mL isotonic saline for 24 h. The test itself was performed by the addition of 50 µL of the ^{68}Ga-complexes to the Hap fraction. After vortexing for 10 seconds, the probes were incubated for 10 min at ambient temperature. The samples were centrifuged and the supernatant was removed. The Hap fraction was washed with 0.5 mL saline. This solution contained less than 2% of the overall ^{68}Ga radioactivity.

The ^{68}Ga radioactivity in combined liquids and the Hap fraction was measured in a curiemeter (Aktivimeter Isomed 2010, MED Nuklear-Medizintechnik Dresden GmbH). ^{68}Ga complex binding to Hap was determined as percent of ^{68}Ga absorbed to Hap.

2.6. Stability study

For stability study, 3 mg of apo-transferrin was suspended in 1 mL PBS buffer. 400 µL of purified [^{68}Ga]BPAMD was added. The mixture was shaken in the thermomixer at 37 °C for up to 180 min. The solution was analyzed by TLC using 0.25 M citrate buffer pH 4. For

control, additional vials were prepared by the same method only missing of one compartment (chelator or transferrin) and therefore serving as complete binding of ^{68}Ga to transferrin (without chelator) and only [^{68}Ga]BPAMD (without transferrin).

2.7. Animals and tumours

Animal imaging experiments were performed either in healthy rats (Copenhagen rat, body weight 190 g) and in animals bearing induced bone metastases (Sprague-Dawley rats). Animals were allowed access to food and acidified water ad libitum before the investigation. All experiments had previously been approved by the regional animal ethics committee and were conducted in accordance with the German Law for Animal Protection and the UKCCCR Guidelines [15].

For metastases experiments cells of the Walker 256 carcinoma has been used, a cell line which is known to form bone metastases [16]. Walker 256 cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) at 37°C under a humidified 5% CO_2 atmosphere and sub cultivated once per week. For tumour implantation male Sprague-Dawley rats (Charles River Wiga, Sulzfeld, Germany; body weight 320 to 480 g) housed in the animal care facility of the University of Mainz were used in this study. For metastasis induction Walker 256 cells were injected into the superficial caudal epigastric artery. In brief, after an inguinal incision the subcutaneous preparation of the epigastric artery was performed. After distal ligation of the artery a thread was looped loosely around the proximal end. Following a small incision of the artery wall a catheter (o.d. 0.5 mm, i.d. 0.25 mm) was inserted and fixed with the thread. $2x10^5$ cells were suspended in 200 µL isotonic saline and injected into the catheter. After tightening the proximal ligature the catheter was removed, the operating field was cleaned with ethanol and the cutaneous incision was closed. Tumour cells were always implanted into the right leg whereas the contralateral side served as a control. PET imaging was performed two weeks after tumour cell injection an interval during which bone metastases in the distal femur or the tibia were formed in approximately 80% of the animals (data not shown).

2.8. µ-PET experiments

PET imaging was performed in spontaneously breathing rats under isoflurane anaesthesia (2% isolflurane, 98% oxygen). [^{68}Ga]BPAMD was prepared with 30 nmol ligand using the

described conditions. After purification and pH adjustment, a solution was obtained ready for injection.

The PET imaging was performed on a microPET Focus 120 small animal PET (Siemens/Concorde, Knoxville, USA). During PET measurements the animals were placed in supine position. For imaging of the healthy animals the scanning region was placed over the chest whereas in the tumour experiments the region was located over the lower limb from the pelvis down to the feet. After a 15 min transmission scan with an external ^{57}Co source, dynamic PET studies were acquired in 2D mode. The radiotracer was administered as a bolus injection via the tail vein. For the measurement of the tracer distribution in a healthy animal the injected dose was 15 MBq. In the metastases experiments the dose was 20.5±0.5 MBq. Afterwards, PET images were obtained for a total measuring interval of 60 min. Finally, a whole body scan (60-75 min post injection) was performed.

For analysis the PET listmode data were histogrammed into 14 to 20 frames with varying time frames (1-5 min) and reconstructed using OSEM algorithm. μ-PET image quantification was applied using PMOD software (PMOD Technologies Ltd.). Volumes-of-interests (VOIs) were defined for tumour and reference tissue (contralateral bone). Ratios of tumour to reference tissue were calculated from integral image between 10' and 60' after tracer injection.

3. Results

3.1. Complex formation, Analysis and Purification

The elution of $^{68}Ga^{3+}$ from the generator and the on line-processing of the eluate are performed within less than five minutes. Figures. 3 to 5 compare typical labelling reactions for BPAMD depending on temperature (Figure 3, 60 to 100 °C), amount of ligand (Figure 4, 5-50 nmol) and reaction pH (Figure 5, pH = 1-5).

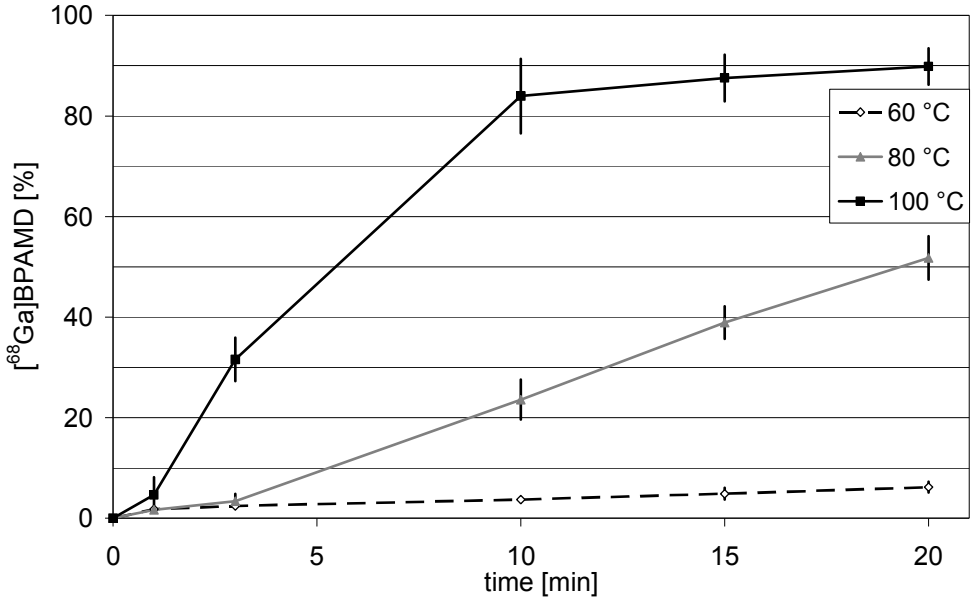

Figure 3. Radiochemical yield of [^{68}Ga]BPAMD at different temperatures with 20 nmol BPAMD at pH = 3

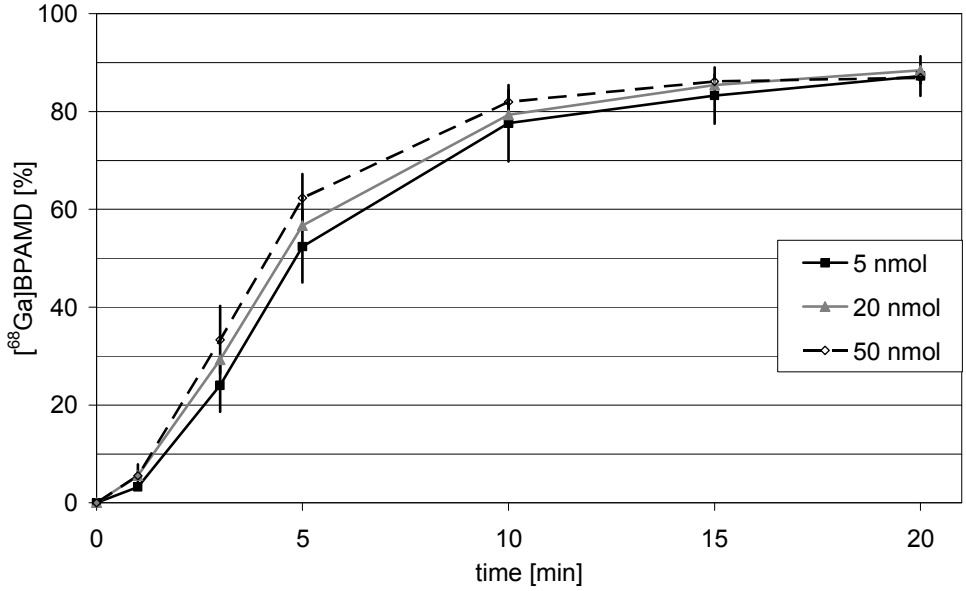

Figure 4. Radiochemical yield of [^{68}Ga]BPAMD at 100 °C and pH = 3 with different amounts of ligand (5-50 nmol)

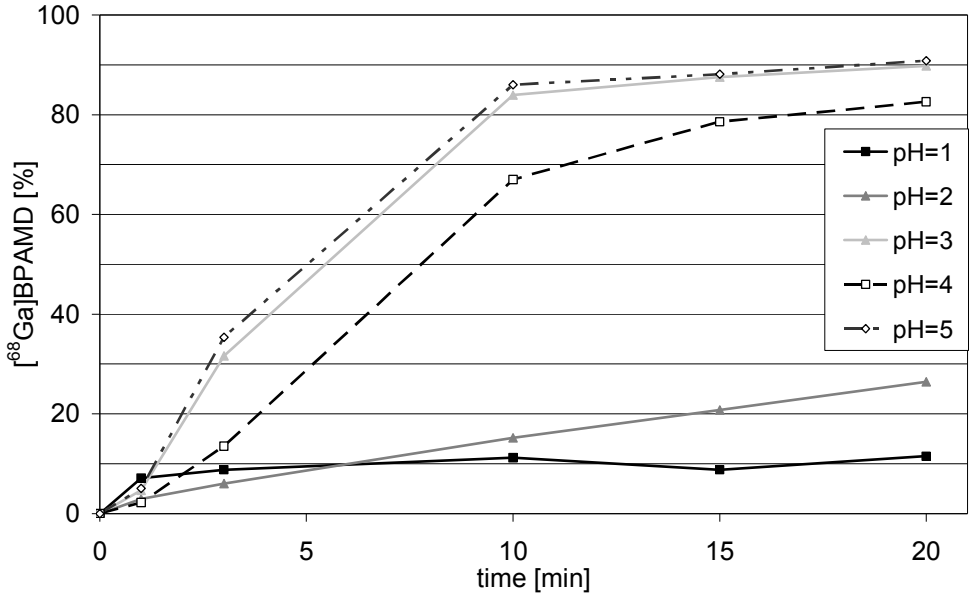

Figure 5 . Radiochemical yield of [^{68}Ga]BPAMD using 20 nmol ligand at 100 °C with pH varied from 1 to 5

Labelling finally proceeds at temperatures of 100 °C within 10 min in a total volume of 5 mL and at optimal pH of 3 to yield 90 % of [^{68}Ga]BPAMD. This was checked by TLC and HPLC: TLC with sodium citrate showed the complex with an $R_f = 0.5$ and uncomplexed ^{68}Ga with $R_f = 0.0$ and 0.9 (due to formation of ^{68}Ga-citrate with solvent front and possibly colloidal ^{68}Ga at the origin) whereas the second solvent system showed 2 spots only and takes only 5 min for developing, compared to about 15 min for the citrate TLC. The complex [^{68}Ga]BPAMD with $R_f = 0.0$ and the uncomplexed ^{68}Ga with $R_f = 0.8$ (Figure 6).

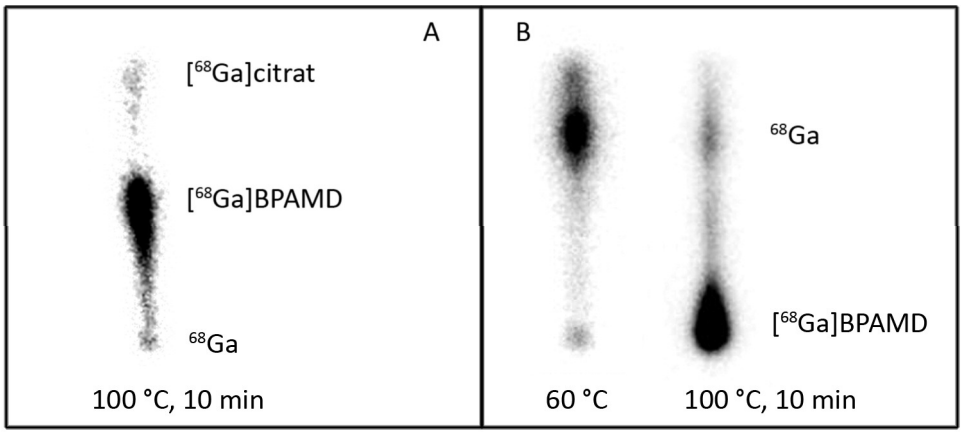

Figure 1. A) Citrate TLC of the reaction mixture of [^{68}Ga]BPAMD, R_f = 0.0: ^{68}Ga, R_f = 0.5: [^{68}Ga]BPAMD, R_f = 0.9: ^{68}Ga-citrate; B) cellulose TLC with solvent system B1:B2 (2:1) with R_f = 0.0: [^{68}Ga]BPAMD, R_f = 0.8: ^{68}Ga

HPLC chromatogram revealed two peaks, the complex [^{68}Ga]BPAMD at 3.2 min and the uncomplexed ^{68}Ga at 8.5 min (Figure 7). Both analytical methods were found to be consistent among the analysis methods (90 % by TLC compared to 91 % by HPLC). The reaction mixture was purified from uncomplexed ^{68}Ga using 60 mg Strata-X-C columns yielding pure [^{68}Ga]BPAMD (> 98 % radiochemical purity by HPLC, Figure 8). Prior to using the purified ^{68}Ga-species for binding and μ-PET studies, pH was adjusted to 7.4 by adding aliquots of a sodium HEPES solution.

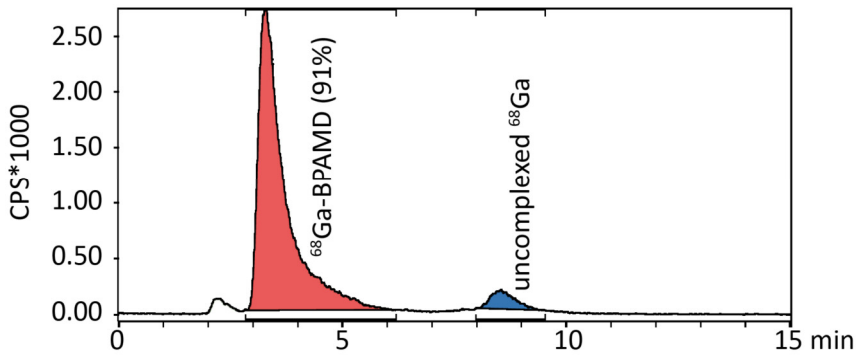

Figure 7. HPLC radioactivity chromatogram of the reaction mixture of [^{68}Ga]BPAMD (20 nmol BPAMD, 100 °C, 10 min); retention times: [^{68}Ga]BPAMD at 3.2 min, uncomplexed ^{68}Ga at 8.5 min

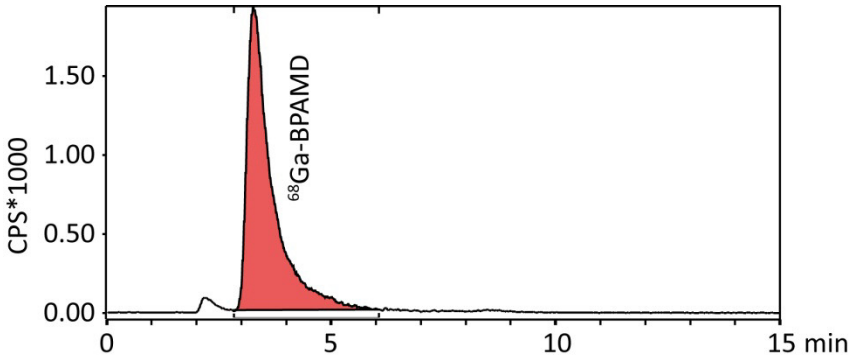

Figure 8. HPLC radioactivity chromatogram of purified [^{68}Ga]BPAMD, radiochemical purity > 98 %

3.2. Hydroxyapatite Binding

Hydroxyapatite experiments showed binding of 81.5 ±0.5 % for [^{68}Ga]BPAMD. Compared to former studies under the same conditions with the NOTA tri-phosphonate (NOTP) and a open chained phosphonate (EDTMP) the binding is lower than for [^{68}Ga]EDTMP (Figure 9). However, due to very low *in vivo* stability of EDTMP [9], [^{68}Ga]BPAMD is more favourable.

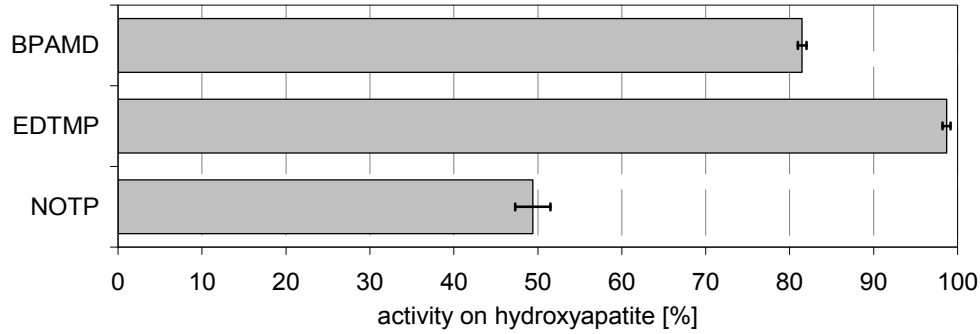

Figure 9. binding of ^{68}Ga complexes (only ligands are given) to hydroxyapatite; comparison of BPAMD to ligands used in previous study (EDTMP, NOTP) [9]

3.3. Stability Study

Stability study of [^{68}Ga]BPAMD against 3 mg apo-transferrin revealed a decomposition of 9.1 ± 0.6 % within 3 h at 37 °C. However, this decomposition was also present in PBS buffer under the same experimental conditions but with a lower value of 4.2 ± 0.8 %.

3.4. µ-PET Small Animal Study

Imaging the [^{68}Ga]BPAMD uptake in a healthy animal showed that the tracer is accumulated in bones most prominent in sections with a relatively high turnover of the bone matrix. Under physiological conditions rats show high remodelling activity in the joints. For this reason the [^{68}Ga]BPAMD accumulation was highest in the shoulder and along the backbone (Figure 10). Compared to solid bones the activity in the joints of the vertebral column was higher by a factor of 2.49 (humerus), 2.88 (sternum) or 2.08 (scapula). These data indicate that the new tracer preferentially accumulates in metabolically active bone regions.

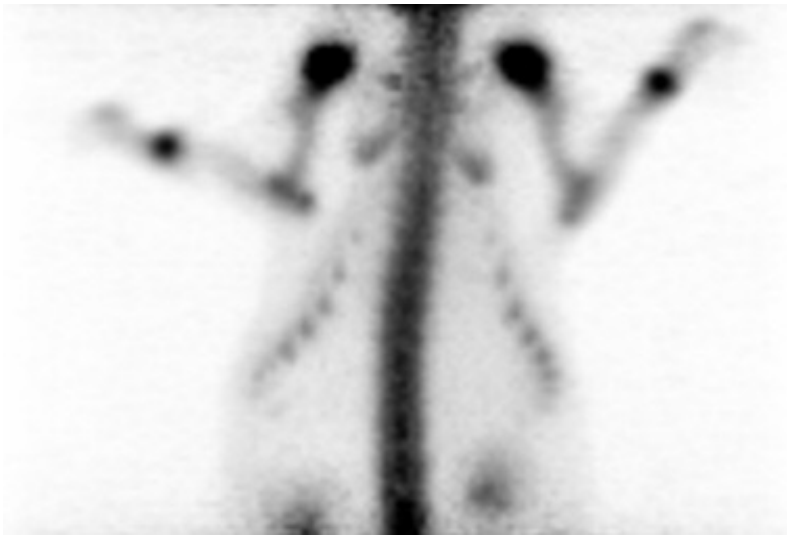

Figure 10. *Example of the tracer accumulation in bones of a healthy rat.*

For the metastasis experiments Walker 256 cells were injected into the superficial caudal epigastric artery of the right leg of 7 animals. Within 2 weeks five of them developed a bone metastasis in the tibia (confirmed by histology after PET imaging) whereas 2 animals did not show any signs of tumour growth. All metastases were located in the proximal end or the shaft of the tibia. Using [^{68}Ga]BPAMD for PET imaging the tracer was accumulated within the metastases (Figure 11). Compared to the contralateral tibia (which served as an intraindividual control) the accumulation in the metastatic lesion was 3.96 times higher.

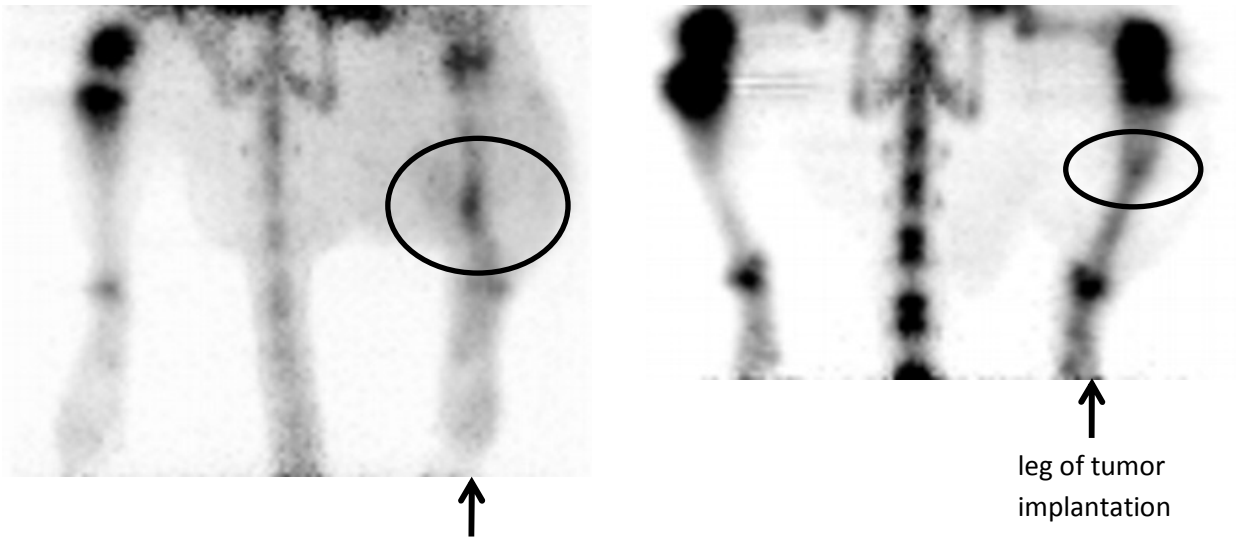

Figure 11: Two examples of tracer accumulation in bone metastases (circles). Tumour cells were injected into the superficial caudal epigastric artery of the right leg. PET imaging was performed two weeks after tumour implantation.

4. Discussion

4.1. ^{68}Ga-Complex formation, analysis and purification

Complex formation of [^{68}Ga]BPAMD is fast and results in high yields, however, it was not possible to gain the tracer in quantitative yield. Around 90% seems to be the maximum for 10 min reaction time with heating to 100°C. Nevertheless the synthesis of [^{68}Ga]BPAMD is easy and followed by the purification method, the tracer can be obtained in 20-25 min starting from elution of the generator in 62% decay corrected yield, ready for further application. As important the fast and easy labelling procedure for a clinical adoption is, also the fast analysis and quality control of the final product is of great significance, not to have the product decaying while waiting for the analytics. Thus, the presented TLC and HPLC system, having the results ready in less than 10 min, is of great advantage.

4.2. Hydroxyapatite binding

Binding experiments with hydroxyapatite show a trend for the phosphonates and are an easy possibility to distinguish between compounds applicable for further studies for example in animals and those that will not be evaluated further. However, in literature one can find many

different experimental setups [17-19]. Therefore this is only a hint for further experiments with a tracer like [^{68}Ga]BPAMD, it does not show any biological activity of the tracer, as it is proposed for the bisphosphonates, inhibiting the FPP synthase [10,14]. Under the same experimental conditions, this basic information indicates the purpose of additional experiments for a compound like [^{68}Ga]BPAMD.

4.3. Stability study

Investigation of the complex stability at 37 °C showed a small decrease over a time of 3 h in PBS buffer and against apo-transferrin. Nevertheless, this little decomposition of the [^{68}Ga]BPAMD is only of minor value (4.2 % in PBS; 9.1 % against apo-transferrin), if free ^{68}Ga will bind to transferrin *in vivo* which shows a fast blood clearance in general [20,21]. Therefore this slight decomposition is no problem for animal or human application.

4.4 µ-PET Small Animal Study

In the animal experiments it was shown that the new tracer basing on the macrocyclic bisphosphonate BPAMD labelled with ^{68}Ga is taken up into the bone especially in regions with a high remodelling activity. For this reason, in particular in the epiphysis of the bones close to joints and in the backbone high activity of the tracer was found. These regions could be visualized with a high spatial resolution (Fig. 10).The animal model used for induction of bone metastases by injecting tumour cells into the superficial caudal epigastric artery (a branch of the femoral artery) is known to lead to orthotopic metastases [22]. Using the cell line Walker 256 metastases were induced in 70% of the animals, a rate, which is comparable to previous results with the same model. In these experiments approximately 80% of the animals had osteolytic lesions, which were located mostly in the proximal shaft or the head of the tibia. For this reason, the animal model used is a realistic representation of bone metastases of breast or prostate cancers. It is important to point out that at the site of the bone tumour no additional injury was made leading to a forced remodelling in the bone. Activity changes in the bone were solely by the incorporation of the tumour cells.

In these experimental metastases the new tracer showed a significant accumulation (Fig. 11). In all animals exhibiting tumours in the leg, the lesions were clearly identifiable in the PET images. The relative activity of the tracer was approximately 4-times higher than in the cotralateral leg without a tumour. The imaging allowed to identify metastases with a diameter

of 1-3 mm. These results clearly indicate that [^{68}Ga]BPAMD is suitable as a tracer for bone metastases in PET imaging.

5. Conclusion

Investigation of labelling kinetics of BPAMD with ^{68}Ga showed best efficency at pH = 3-5 and 100 °C for 10 min with a radiochemical yield of 90 %. However, quantitative yield could not be achieved, therefore purification from uncomplexed ^{68}Ga was appled using strong cation exchanger columns. For quality control two systems by TLC and HPLC were developed, showing the high purity (>98 %) of the product [^{68}Ga]BPAMD in less than 10 min. Studies to hydroxyapatite revealed 81.5% binding of the tracer to the synthetic bone material. Decomposition of [^{68}Ga]BPAMD when challenged with transferrin was very small (9.1 % after 3h), leading to first *in vivo* experiments on a healty rat with μ-PET. Images showed high accumulation on joints, implying high remodelling activity of these bone structures in rats. Consequently in further experiments Walker 256 carcinoma cells were injected on the right leg of rats, developing osteolytic lesions, located mostly in the proximal shaft or the head of the tibia. Injection of [^{68}Ga]BPAMD and μ-PET imaging focused on the legs of the animals indicated high contrast between the bone lesion and healthy bone in the same animal (contrast factor = 3.96).

Finally [^{68}Ga]BPAMD is of great interest for nuclear medicine. By usingthe ^{68}Ge/^{68}Ga generator the tracer can be obtained in a fast and easy process. Having the higher spatial resolution as well as quantification PET-technique is superior to SPECT with ^{99m}Tc-phosphonates and it is cyclotron independent.

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3.3. A Triazacyclononane-Based Bifunctional Phosphinate Ligand for the Preparation of Multimeric ^{68}Ga Tracers for Positron Emission Tomography

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Keywords: gallium, macrocyclic ligands, potentiometry, radiopharmaceuticals. stability constants

Abstract:

For application in positron emission tomography (PET), **PrP9**, a N,N',N''-trisubstituted triazacyclono-nane with methyl(2-carboxyethyl)phosphinic acid pendant arms, was developed as $^{68}Ga^{3+}$ complexing agent. The synthesis is short and inexpensive. Ga^{III} and Fe^{III} complexes of **PrP9** were characterized by single-crystal X-ray difraction. Stepwise protonation constants and thermodynamic stabilities of metal complexes were determined by potentiometry. The Ga^{III} complex possesses a highthermodynamic stability (log $K_{[GaL]} = 26.24$) and a high degree of kinetic inertness. ^{68}Ga labeling of **PrP9** is possible at ambient temperature and in a wide pH range, also at pH values as low as 1. This means that for the first time, the neat eluate of a TiO_2-based $^{68}Ge/^{68}Ga$ generator (typically consisting of 0.1 m HCl) can be directly used for labeling purposes. The rate of ^{68}Ga activity incorporation at pH 3.3 and 20°C is higher than for the established chelators DOTA and NOTA. Tris-amides of **PrP9** with amino acid esters were synthesized to act as models for multimeric peptide conjugates. These conjugates exhibit radio-labeling properties similar to those of unsubstituted **PrP9**.

1. Introduction

Positron emission tomography (PET) is a powerful tool in non-invasive and quantitative medical imaging, augmenting other imaging methods such as computer tomography (CT) or magnetic resonance imaging (MRI). For this technique, emission of positrons by β^+-emitters or, more precisely, the γ-radiation originating from their annihilation with electrons, is detected. At present, PET facilities are still dependent on an on-site cyclotron, since the predominantly used radioisotopes (^{18}F, ^{11}C, ^{13}N, and ^{15}O) are cyclotron-produced and have very short half-lives. This renders PET an expensive imaging technique. Recently, the positron emitter ^{68}Ga has gained more attention as it is now available from commercially distributed radionuclide generators. In such devices, ^{68}Ga is produced by decay of the parent nuclide ^{68}Ge ($T_{½}$ = 271 d), which is absorbed on a TiO_2 or SnO_2 matrix. ^{68}Ga-based radiopharmaceuticals are thus available independently of an on-site cyclotron and enable medical PET scans at a fraction of the usual cost. It possesses a half-life of ≈ 68 min and is thus compatible with most bio-targeting applications. The topic of utilization of ^{68}Ga in PET has recently been reviewed[1]. In addition, there are two more cyclotron-produced gallium isotopes suitable for nuclear imaging: 1) ^{67}Ga is a γ-emitter ($T_{½}$ = 78.3 h) used in single-photon emission computed tomography (SPECT) and therapy; 2) β^+-emitting ^{66}Ga with an intermediate half-life of 9.4 h did not receive much attention in the past[2], but the in- terest in this radioisotope has revived recently[3].

In aqueous solutions, gallium is stable only as a trivalent cation. It cannot be incorporated into the structure of targeting vectors by covalent bonding, but must be complexed by a ligand that is conjugated to the biological vector. The Ga^{3+} ion possesses a d^{10} electron configuration and accepts different coordination numbers (usually 4–6), while not displaying preference for any particular coordination polyhedron. At pH > 4, formation of colloidal hydroxide $[Ga(OH)_3]_n$ commences. Although this does not generally inhibit complex formation, radiolabeling is nevertheless substantially hampered due to formation of insoluble colloids (particularly at high activities) and their adhesion to the surface of the reaction vessel. At pH values above 8, a water-soluble hydroxo complex, $[Ga(OH)_4]^-$, is formed. As ligand exchange with the tetrahydroxo complex is a much slower process than

complexation of free Ga^{3+}, complexation is achieved best at pH < 4.

Ligands for ^{68}Ga-based PET radiopharmaceuticals should ideally combine the following set of properties.

1) Stability: Ga^{III} complexes should be as stable as possible; a kinetic inertness of the complex is more important than high thermodynamic stability.

2) Quick complexation under radiochemical conditions: Formation of Ga^{III} complexes should be fast at low temperatures, low concentration, and minimal excess of the ligand. A desirable ligand will chelate Ga^{3+} in solutions of nanomolar concentration at room temperature within minutes.

3) Selectivity: The ligand should ideally be selective for Ga^{3+} ion. Particularly, complexation of serum metals like Ca^{2+}, Mg^{2+}, and Zn^{2+} ions (the last being produced by decay of ^{68}Ga) should be disfavored in order to avoid transmetallation in vivo or diminishing of radiochemical yield.

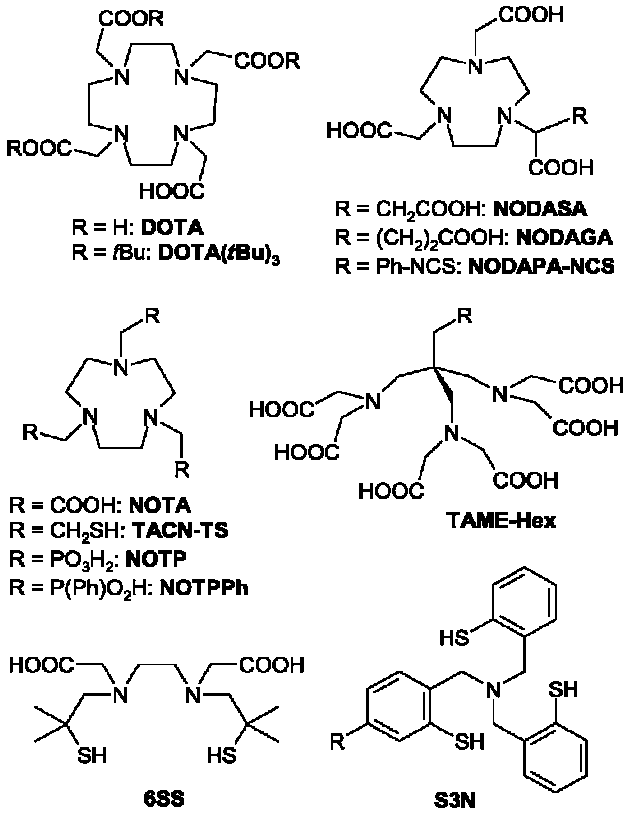
4) Conjugation ability: The chelating unit has to possess a functional group which allows covalent binding to the targeting vector (biomolecule) without a significant derogation of complexation performance.

5) Long shelf life: In medical applications, excellent chemical stability is necessary.

6) Accessibility: Preparation of the compound in practical amounts should be quick, facile, and inexpensive.

A choice of ligands (some of them representing a family of compounds) which have been proposed or have already been used for application in ^{68}Ga radiopharmaceuticals is depicted here (see references [8, 12, 18, 19, 21, 23, 27, 58–64]). All of them have advantages and drawbacks. Aza-macrocycle based ligands generally form complexes with increased thermodynamic and, more importantly, kinetic stability compared to open-chain or tripod ligands[4, 5]. However, in case of the first group, a considerable barrier has to be overcome in order to place the metal ion into the ligand cavity, thus causing slow complexation kinetics. The open-chain ligands are less rigid and do not have a cavity of a particular size, which is why they are less selective for particular ions, but show faster complex formation[6, 7]. Despite of the amazing

thermodynamic stabilities of the resulting complexes[8], ligands containing thiol groups are disfavored because of their tendency to degrade, albeit slowly, by oxidation. Also, the comparably low acidity of sulfhydryl groups hampers complex formation in acidic media, and derivatives suitable for bioconjugation are difficult to synthesize[9].



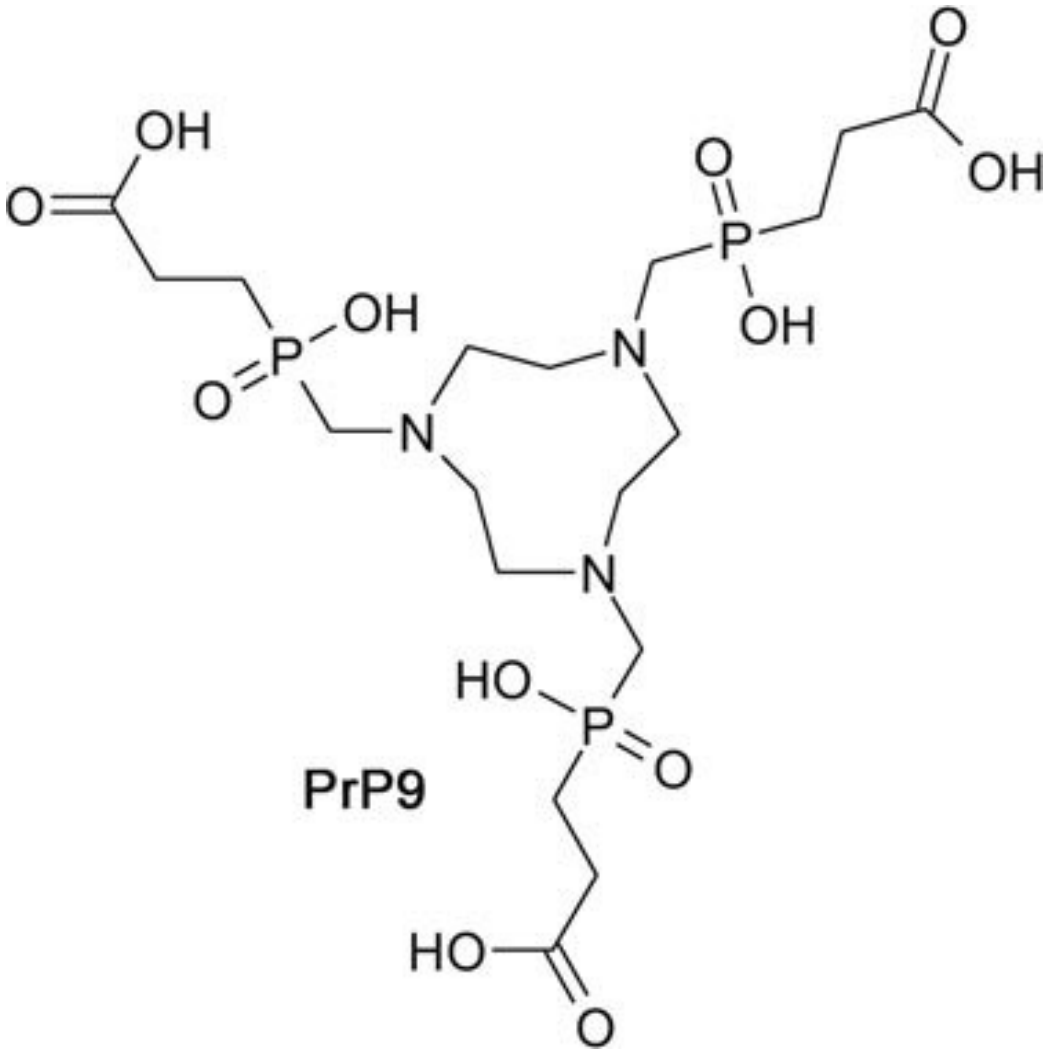
DOTA derivatives are currently the working horses of bioconjugational chemistry related to medical imaging, including ^{68}Ga applications[10, 11]. This is mainly due to the commercial availability of ready-to-use mono-unprotected precursors like DOTA(tBu)$_3$[12], which, in turn, is rooted in the fact that lanthanide(III) complexes of DOTA derivatives and its conjugates have been extensively used as MRI contrast agents[13-16] and radiotherapeutics[17]. However, NOTA-like ligands show a much better selectivity towards Ga^{3+} ion and their complexes are more stable, as the size of the NOTA cavity is almost ideal for this ion[118-20]. Conjugable NOTA derivatives like NODASA[21], NODAGA[22], NODAPA-NCS[23] as well as other thiocyanato-equipped NOTAs[24, 25]

are thus much better suited for the synthesis of ^{68}Ga radiopharmaceuticals.

With all these considerations in mind, we devised a ligand structure that combines the advantages of the different structural motifs. The novel chelator **PrP9** (shown here) is structurally related to some known [9]aneN_3 derivatives[26-29]. Unlike the above-mentioned ligands, it contains phosphinates instead of carboxylates as primary coordination sites. Due to the lower pK_a value of phosphinic acids (mostly 1) compared to aliphatic carboxylic acids (typically 6 4) [30], metal-ion complexation should be possible at much lower pH values than in case of NOTA and DOTA, thereby expanding the pH range of application. Furthermore, the distant carboxylates are supposed to act as "pre-coordination" sites: at very low concentrations typical for radiochemistry, fast, open-chain-like interactions with metal ions are supposed to help to increase the effective metal concentration close to the ligand cavity and therefore increase complexation rate. The structure is thus expected to combine advantages of both macrocycle-based chelators (stability and selectivity) and open-chain ligands (fast complex formation). In addition, phosphinic acid and carboxylic groups exhibit different reactivity, which can be utilized to functionalize the carboxylates without having to protect the phosphinate moieties. The availability of three carboxylic acid moieties for conjugation furthermore paves the way towards multimeric tracers.

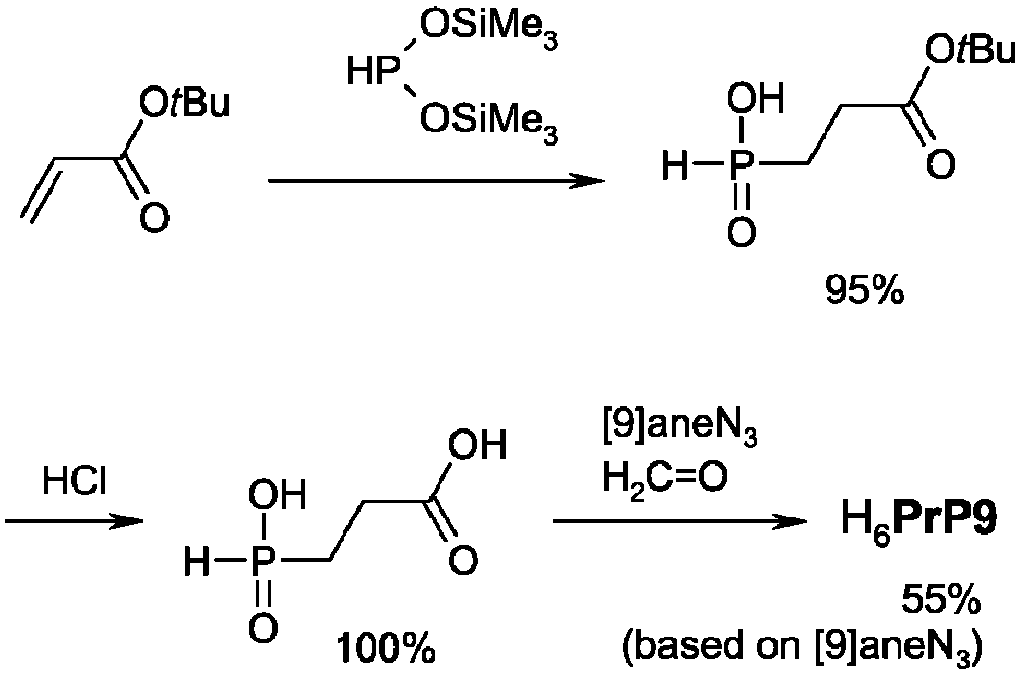
2. Results and Discussion

Throughout this paper the abbreviation **PrP9** (systematic name of **PrP9** is 1,4,7-triazacyclononane-1,4,7-tris[methyl(2-carboxyethyl)phosphinic acid]) is used regardless of proto- nation state, except in cases in which the distinction is necessary for comprehension.



2.1. Ligand synthesis

The preparation protocol for **PrP9** is extremely short ; there are just three steps starting from stock chemicals and only the last reaction involves the azamacrocycle. All steps have satisfying to excellent yields. The complete synthesis is outlined in Scheme 1 and can be carried out in less than three days. All required materials are commercially available and, apart from the amine [9]aneN_3, they are also inexpensive. In addition, the synthesis requires very little workup effort. One extraction after the first step and just a simple ion exchange chromatography after the third step, followed by recrystallization, are necessary in order to obtain a very pure product. The procedure can be scaled up without problems, as has been proven by preparing 15 g of **PrP9** in a single batch. For these reasons, we hold the view that **PrP9** has good prospects in providing a basis for the synthesis of bioconjugated chelators.



Scheme 1. Synthesis of the hexaprotonated (zwitterionic) form of the ligand **PrP9**.

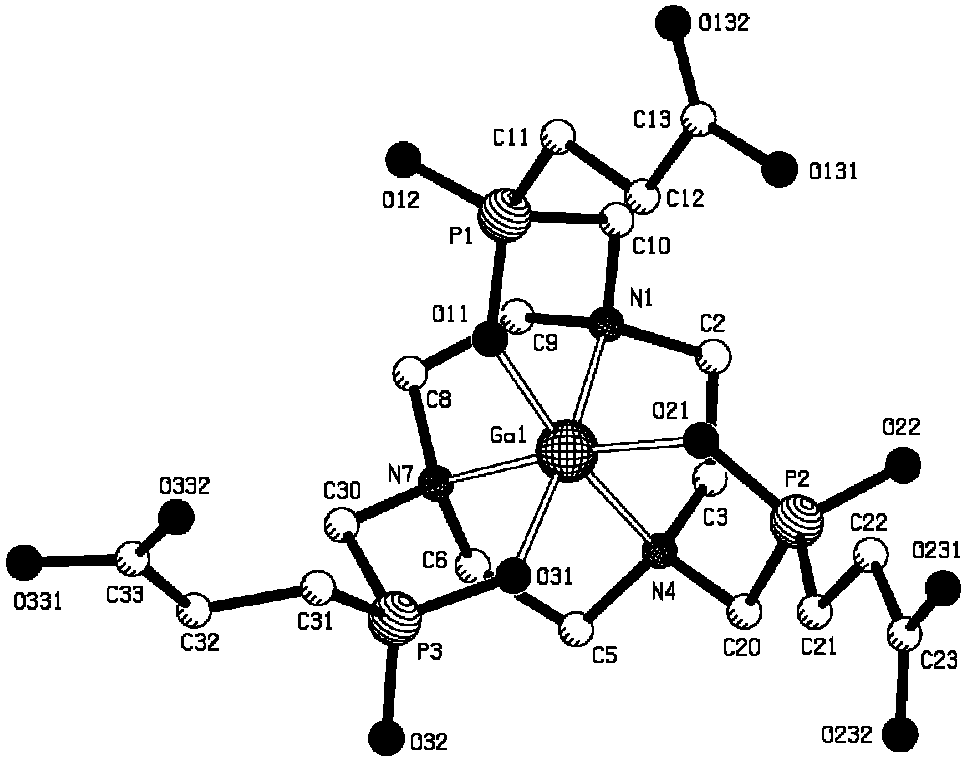


Figure 1. Molecular structure of the [Ga(H_3**PrP9**)] complex in [Ga-(H_3**PrP9**)]·6H_2O (hydrogen atoms are omitted for clarity).

2.1. Complex synthesis and solid-statestructures

Although **PrP9** forms complexes with a variety of metal ions, precipi- tates suitable for X-ray diffraction and analysis could only be obtained for [Ga(H_3**PrP9**)] and [Fe(H_3**PrP9**)]. Two phases were btained for each, differing only in the amount of water of crystallization. From acetone/water mixtures, the complexes crystallized as dihydrate and monohydrate, respectively, whereas a simple evaporation of an aqueous solu- tion afforded isostructural hexahydrates. In both compounds the chelate cages in the two phases are quite similar and just one example is depicted (Figure 1; for the Fe^{III} complex see Supporting Information, Figure S1). Coordination bond lengths and angles of the two Ga^{III} structures are almost identical, whereas the two Fe^{III} phases exhibit small structural differences (see Table 1). The parameters of the [Ga-(H_3**PrP9**)] structures are similar to other hexacoordinated Ga^{III} complexes[31]. The lattices contain enantiomeric *Λδδδ-RRR/Δλλλ-SSS* [Ga(H_3**PrP9**)] complex units with the metal in a trigonal-antiprismatic coordination sphere and the propionic acid moieties being located above the complex cage. An identical structural arrangement has been found in the [Ga(NOTPPh)] complex[32]. A similar combination of chelate-ring conformation and pendant-arm helicity has been found in the structurally related compounds [Ga(NOTA)][19] and [Ga(NODASA)][21] (Table 2), as well as in other Ga^{III} complexes with six-membered pendant arm chelate rings[33, 34]. Interestingly, a different situation is encountered for Fe^{III} complexes: for phosphorus-containing ligands, pendant arm helicity is opposite to macrocycle chelate rings (*Λδδδ/Δλλλ*, similarly as found in the Ga structures); for ligands with carboxylate pendant arms it is the same (*Λδδδ/Δλλλ*; for more information see the Supporting Information).

Table 1. Selected parameters of the complex moieties in [Fe-(H_3**PrP9**)]·nH_2O and [Ga(H_3**PrP9**)]·nH_2O in the solid state [distances given in Å, angles in °].

	[Fe-(H_3**PrP9**)]·nH_2O		[Ga(H_3**PrP9**)]·nH_2O	
	n = 1	n = 6	n = 2	n = 6
M-N1	2.210(2)	2.178(2)	2.120(2)	2.108(1)
M-N4	2.232(2)	2.198(2)	2.124(2)	2.128(1)
M-N7	2.189(2)	2.178(2)	2.125(2)	2.106(1)
M-O11	1.927(1)	1.948(1)	1.926(1)	1.937(1)
M-O21	1.932(1)	1.947(1)	1.924(1)	1.931(1)
M-O31	1.934(1)	1.946(1)	1.929(1)	1.932(1)
N1-M-O31	162.27(5)	164.54(6)	169.64(6)	170.07(5)
N4-M-O11	162.19(5)	164.73(6)	169.63(6)	169.88(5)
N7-M-O21	164.31(5)	165.41(6)	169.95(6)	170.21(4)
N1-NQ-OQ-O11[a,b]	48.41	49.91	52.08	51.82
N4-NQ-OQ-O21[a,b]	47.79	49.44	51.90	51.55
N7-NQ-OQ-O31[a,b]	51.01	51.37	52.78	53.16
NQ-OQ[a]	2.3929	2.3966	2.3722	2.3788
M-NQ[a]	1.487(2)	1.4540(2)	1.3594(2)	1.3506(2)
M-OQ[a]	0.9063(2)	0.9428(2)	1.0128(2)	1.0284(2)
N_3/O_3 planes ∠	3.57(8)	1.63(6)	0.51(3)	1.22(5)

[a] NQ and OQ are the barycenters of the N and O planes, respectively.

[b] Torsion angle.

Table 2. Comparison of some structural parameters of [Ga(H_3**PrP9**)] with analogous complexes [distances given in A, angles in 8].

	N-Ga-O[a]	Torsion ∠[b]	OQ-NQ[c]	Ga-OQ[c]	Ga-NQ[c]
[Ga(H3**PrP9**)][d]	169.9	52.2	2.376	1.355	1.021
[Ga(NOTPPh)][32]	168.3	52.1	2.370	1.391	0.979
[Ga(NOTA)][20]	167.4	47.6	2.329	1.318	1.012
[Ga(H-NODASA)][21]	165.5	44.4	2.363	1.333	1.030

[a] Average of values for opposing N and O atoms. [b] For definition of torsion angle see Table 1. [c] NQ and OQ are the barycenters of the N_3 and O_3 planes, respectively. [d] Average values of two structures.

Comparison of average "diagonal" N-Ga-O and torsion angles of structurally related Ga^{III} complexes (Table 2) reveals that the values of those derived from phosphinate ligands, and particularly of [Ga (H_3**PrP9**)], are more close to those of an ideal octahedron (1808 and 608, respectively). Also, the N_3 and O_3 planes are slightly more rotated. In all structures regarded, gallium is located closer to the N_3 than to the O_3 plane; the difference is most pronounced in the structure of the phenylphosphinate complex [Ga-(NOTPPh)]. Comparing all the parameters characterizing the ligand cavity, it can be stated that, among these ligands, **PrP9** fits best for Ga^{III}. The Fe-O and Ga-O distances in [Ga (H_3**PrP9**)] and (Fe-(H_3**PrP9**)] (see Table 1) are almost identical, but Fe-N bonds are significantly longer than Ga-N linkages. Also, the coordination environment of Fe^{III} is more distorted, the average N-M-O and the torsion angles of the Ga^{III} complexes being closer to the ideal values. This means that the cavity size of **PrP9** is slightly less suitable for Fe^{III} (ionic radius 0.69 Å) than for Ga^{III} (ionic radius 0.76 Å).

2.3. Solution structure of [Ga(PrP9)]

In both ^{31}P and ^{71}Ga NMR spectra, single resonances were found (42.4 and ≈135 ppm, respectively). The ^{31}P NMR thus proves that solid-state and solution structures are identical, that is, only one non-fluxional pair of enantiomers *Λδδδ-RRR/Δλλλ-SSS* is present. The half-width of the Ga resonance (n1/2 ≈400 Hz) is larger than in case of [Ga (NOTA)] (210 Hz)[19] and more close to the values found for complexes of related phosphorus containing ligands, the methylenephosphonate analogue $[Ga(NOTP)]^{3-}$ (430 Hz)[35] or methylene (phenGyl)-phosphinate analogue [Ga(NOTPPh)] (560 Hz) [32].

In the ^1H NMR spectra, only two sets of overlapping triplets at 2.16 and 2.76 ppm, originating from protons of the propionate side arms, can be unambiguously assigned. The resonances of the remaining protons form a set of overlapping multiplets, indicating a rigid structure of the whole complex, which appears to be ^1H NMR spectra measured at temperatures of 20–80℃ indicate a high rigidity of the ligand skeleton. Up to 60℃, the splitting pattern of the ring protons and the nitrogen-bound side- arm protons (N-CH_2-P) remains unchanged; some signal coalescence starts at 80℃. This is in accordance with the respective observations made for [Ga-(NOTA)] , [Ga(NOTPPh)] , and $[Ga(NOTP)]^{3-}$ complexes[19, 32, 35].

2.4. Thermodynamic and mechanistic studies

Potentiometry and NMR techniques were employed for determination of protonation constants and complex stabilities as well as for in- vestigation of the mechanism of complex formation.

The stepwise protonation constants of the ligand (Figure 2) are log K_a = 11.48, 5.44, 4.84, 4.23, 3.45, and 1.66. The protonation sequence can be estimated by a comparison of these data with those for similar ligands (see also Sup- porting Information, Table S2) [27, 28, 36]. The first two protonations should occur on the ring nitrogen atoms, followed by three protonations on the side-arm carboxylates. The other protons are most likely bound on the remaining ring nitrogen atom and/or phosphinate groups. Thus, not all phosphinate groups are protonated, even in solutions with a very low pH (< 1) and therefore are still able to complex Ga^{3+} ion. Basicity of the ring nitrogen atoms is somewhat lower than in case of NOTA[37], but higher than for triazacyclono-nane-derived methylphosphinic acid ligands[27, 28, 35], presumably due to higher overall negative charges of the **PrP9** anions. Basicity of the distant carboxylate groups is in the usual range for carboxylic acids[38, 39].

The stability constants were determined for complexes of **PrP9** with several biologically important metal ions as well as some lanthanides. For Y^{3+} and Lu^{3+} ions, the measure- ments were not possible, as insoluble precipitates were obtained, most likely due to formation of complex polymers containing protonated ligand molecules. The data given in Table 3 clearly show the preference of **PrP9** for the Ga^{3+} ion. Complexes with Cu^{2+} and Zn^{2+} are about ten, and those with Mg^{2+} and Ca^{2+} approximately 20 orders of magnitude less stable. Furthermore, stability constant values for the related metal-ion pairs Mg^{2+}/Ca^{2+} and La^{2+}/Gd^{3+} illustrate the ligand's selectivity for small ions, as for both couples the stability constant of the larger ion is about two orders of magnitude lower. Similar selectivities have been observed for NOTA[37, 40] and its phosphinate analogues[27, 28, 35]. (For a comparison of stability constants and more distribution diagrams see Supporting Information; Table S4, Figures S2 and S3). Also, **PrP9** fits into the correlation of overall basicity of these ligands with the stability constants of their Cu^{II} and Zn^{II} complexes[30].

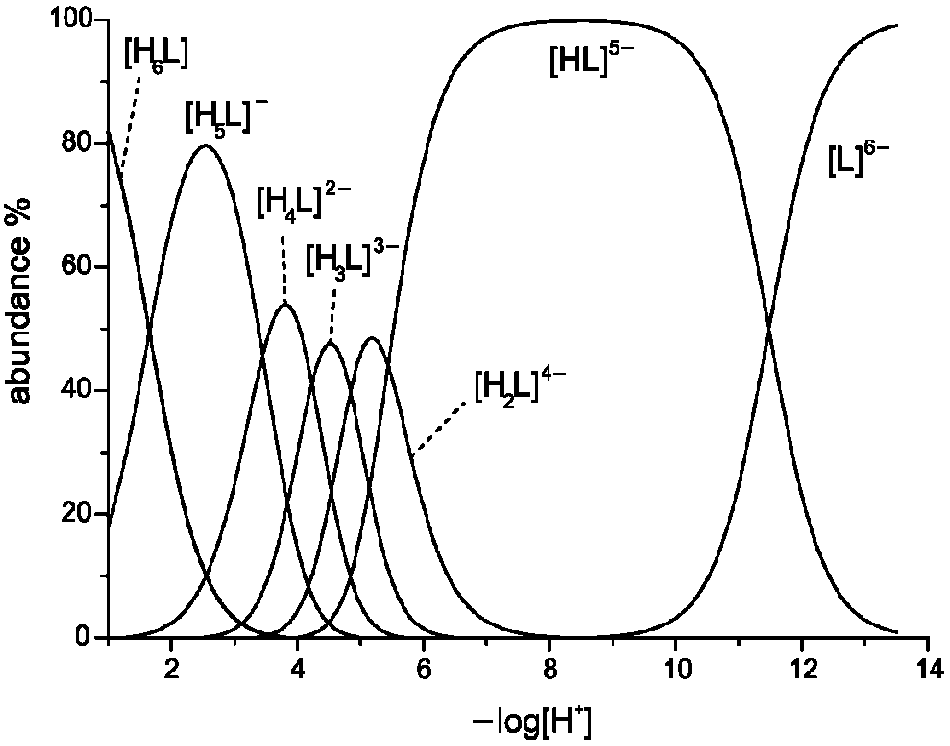


Figure 2. Distribution diagram for the protonation of **PrP9**.

The distribution diagram (Figure 3) shows that the 1:1 complex is triply negatively charged under physiological pH. In solutions containing two equivalents of Ga^{3+}, the primary complex appears to be able to bind an additional Ga^{3+} ion, most likely by weak interaction with the side-arm carboxylates and/or the phosphoryl oxygen atoms (that is, in the form of an "out-of-cage" complex). This shows the ability of the carboxylates to direct Ga^{3+} ions towards the ligand cavity, which we believe is the main cause for the unusually fast complex formation.

From Figure 3 it is furthermore apparent that the Ga^{III} complex is fully formed, even at the beginning of titration, in the form of a fourfold protonated species, $[Ga(H_4PrP9)]^+$, the corresponding fourth protonation constant being log K_a 6 0.7 (Table 3). Hence, the $K_{[GaL]}$ value obtained from potentiometry is determined by the competitive ligand exchange reaction with an hydroxide anion, which, in order to represent the complexes' actual thermodynamic stability, must be in thermodynamic equilibrium. This reaction, however, turned out to proceed

surprisingly slowly and this had to be taken into account in order to obtain correct results. During the normal "in-cell" titration, we observed no precipitate of $Ga(OH)_3$ and pH readout quickly stabilized over the whole pH range of measurement (1.5–12).

Table 3. Stability constants [log K] and stepwise protonation constants [log K_A] of complexes of **PrP9** with selected metal ions.

	Ga^{3+}	Cu^{2+}	Zn^{2+}	Mg^{2+}	Ca^{2+}	La^{3+}	Gd^{3+}
$L + M \leftrightarrow LM$	26.24	16.85	16.88	7.84	6.04	11.26	13.46
$LM + H \leftrightarrow HLM$	5.2	5.14	5.17	6.49	7.94	6.22	4.80
$HLM + H \leftrightarrow H_2LM$	4.5	4.66	4.68	5.00	4.98	5.00	4.80
$H_2LM + H \leftrightarrow H_3LM$	3.8	3.95	3.96	4.74	4.50	3.95	3.78
$H_3LM + H \leftrightarrow H_4LM$	0.7	1.33		4.29		3.32	3.38
$LM(OH) + H \leftrightarrow LM(H_2O)$	9.9	12.24	12.63		13.04	11.32	10.42
$LM(OH)_2 + H \leftrightarrow LM(OH)(H_2O)$						12.30	11.23
$LM + M \leftrightarrow LM_2$	7.3	3.32	2.43	2.95	2.80		
$LM_2 + H \leftrightarrow HLM_2$		4.79	4.71	5.9			
$LM_2(OH) + H \leftrightarrow LM_2(H_2O)$	2.5			8.5	12.8		

From the data thus collected, a stability constant of $\log K_{[GaL]} = 35.65$ was calculated. However, this value appeared unrealistically high compared to the one of the [Ga(NOTA)] complex ($\log K_{[GaL]} = 31.0$)[18], since **PrP9** exhibits a lower overall basicity than NOTA. When the reaction was given a timespan of about four weeks in order to reach equilibrium ("out-of-cell" titration), we obtained a more realistic value of $\log K_{[GaL]} = 26.24$, which is, as expected, lower than that of [Ga(NOTA)]. Even at pH 11, the reaction half-life was ≈60 h (see Supporting Information, Figure S7); finally, at pH 13 the reaction was completed within minutes. A possible explanation is the high negative charge of the $[Ga(\mathbf{PrP9})]^{3-}$ ion, which could be hampering the approach of OH^- to the central ion[18]. This shows that neglecting the kinetics of the ligand-hydroxide competition for the gallium(III) ion can easily lead to erroneous values for thermodynamic stabilities; such kinetic inertness may give a false impression of a higher stability constant. For polydentate/rigid ligands, equilibration time generally should be checked carefully, even in the alkaline pH

region at which, until now, equilibriums have been supposed to establish quickly. The carboxylate protonation constants for the Ga^{III} complex obtained by both "non-equilibrium" (log K_a = 5.14, 4.54, and 3.65) and "out-of-cell" titrations were identical (Table 3). This confirms a fast and quantitative complexation of Ga^{3+} ions even at pH 6 1.5, which was also observed during NMR measurements and under radiochemical conditions (see below).

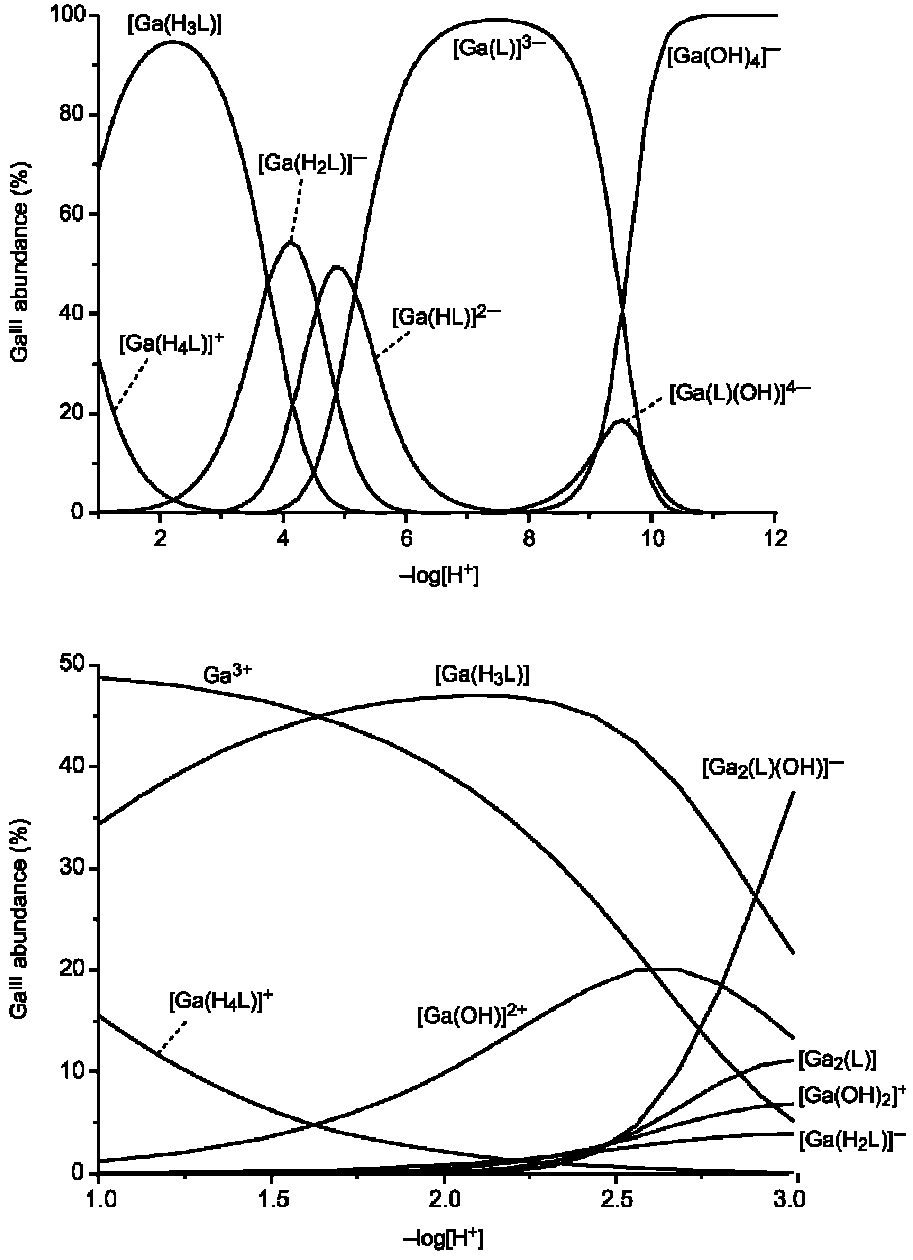


Figure 3. Distribution diagrams for the Ga^{III}/**PrP9** systems (ligand-to- metal-ratio : upper : L/M = 1:1, lower : L/M = 1:1; c_L = 4 mm).

In addition, we like to draw attention to another aspect of the observed kinetics. Although at physiological pH hydroxide anions will compete with the ligand in the [Ga(**PrP9**)] complex, this process is very slow compared to the ^{68}Ga half-life of 68 min. In practice, decay limits the lifetime of any ^{68}Ga-labeled compound to a couple of hours. For applications in nuclear medicine, the high "non-equilibrium" stability constant value of $\log K_{[GaL]} \approx 35$ that has been measured in a comparable timeframe is therefore more decisive than the true long-term thermodynamic stability, since equilibration requires weeks which renders it irrelevant it terms of ^{68}Ga radiochemistry.

The complexation mechanism was also investigated by ^{31}P and ^{71}Ga NMR sectroscopy. Between pH 1.5 and 8, quantitative complexation occurs immediately after mixing equimolar amounts of Ga^{3+} ions and **PrP9** without any inter- mediates being detectable. However, at initial pH values of 1.3, 1.0, and 0.8, quantitative complexation requires 8, 65, and 90 min, respectively (Figure 4 and Figures S6 and S7 in the Supporting Information). Thus, additional NMR signals could be observed, presumably corresponding to "out-of-cage" complexes. The chemical shift of the ^{71}Ga NMR signal at $\approx$ -30 ppm belongs to some species possessing a rather symmetric O_6 coordination environment that is very probably formed from phosphinate and carboxylate oxygen atoms (Figure 4). The transient ^{31}P and ^{71}Ga NMR peaks should correspond to the "out-of-cage" complexes as interconversion of isomeric "in-cage" octahedral complexes is energetically demanding and thus does not occur at room temperature. The ^{31}P NMR peak at $\approx$42.5 ppm was observed in all final reaction mixtures and can be assigned to a minor diastereomer formed during the process of complex formation.

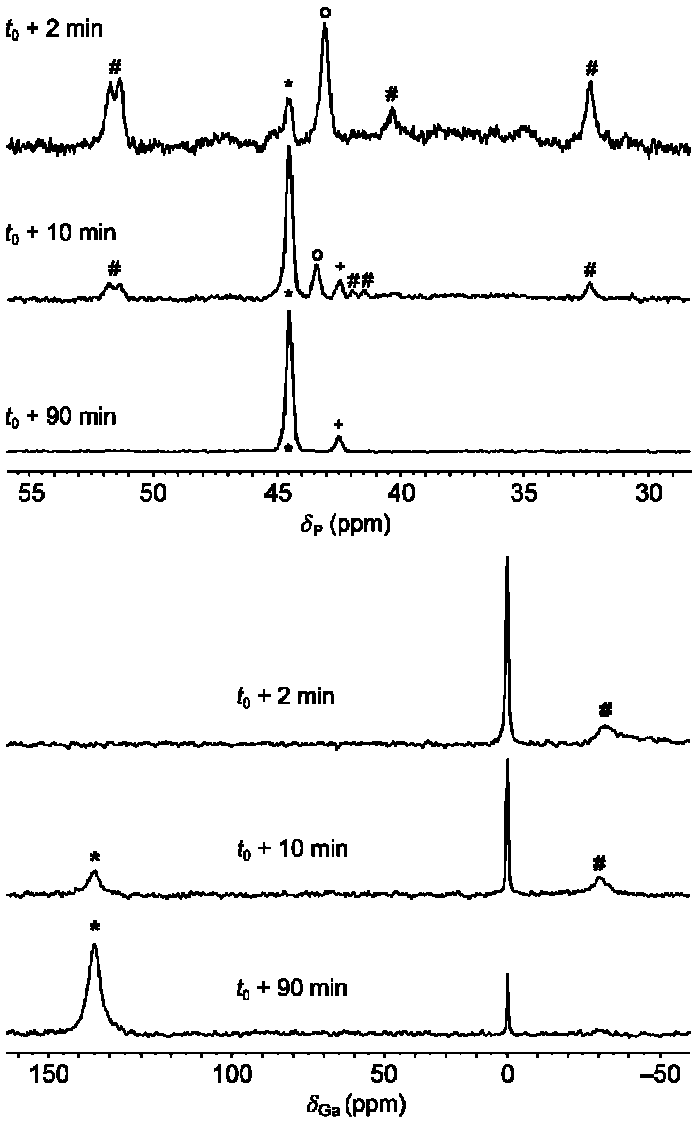


Figure 4. Complexation of Ga^{3+} with **PrP9** as followed by ^{31}P (top) and ^{71}Ga NMR (bottom) (M/L = 1, pH 0.8, 25°C). Peak labeling: *: final ("in cage") complex, #: intermediates, ○: free ligand. In the ^{31}P NMR spectra, the small peak at 6 42.5 ppm (labeled +) probably corresponds to a dia-stereometric complex.

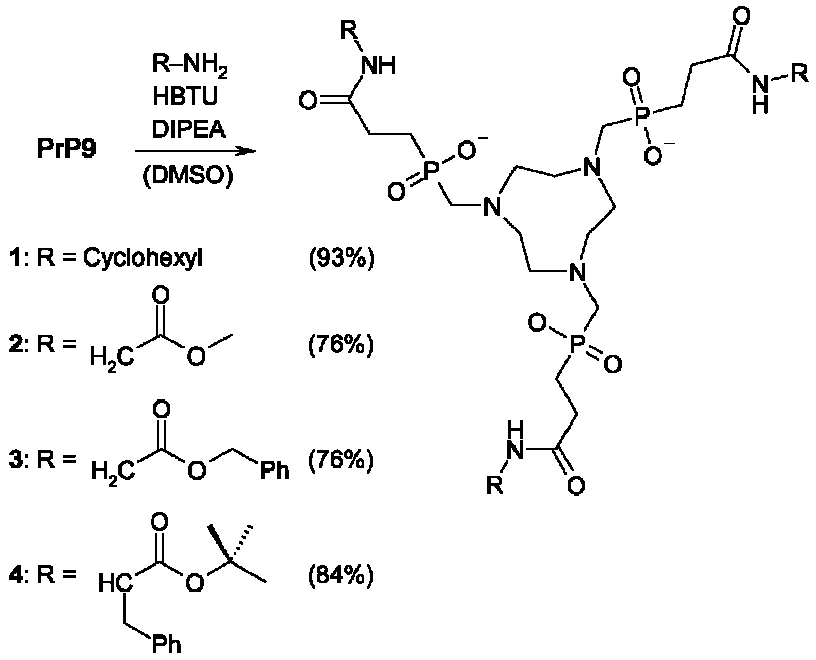
Moreover, complexation could be observed even under extremely acidic conditions, that is, in 1, 2, or 5 m hydrochloric acid. In 1 M HCl, complete extinction of the $[Ga\text{-}(H_2O)_6]^{3+}$ signal in the ^{71}Ga NMR spectrum requires about 12 d, whereas in 2 M HCl solution an equilibrium was reached within 30 d (in which approximately 30 % of "free" gallium is present). Finally, in 5 M HCl the ^{71}Ga

resonance of the "in-cage" complex could not be detected after a period of 30 d. However, a ^{31}P NMR resonance of $\delta \approx 40$ ppm and some other signals appeared, indicating that some Ga^{3+} – ligand interactions (probably formation of the "out-of-cage" complex) occurs even in extremely acidic media.

When a sample of [Ga(**PrP9**)] was dissolved in 6 M $HClO_4$ at 25°C, no decomplexation was observed over a period of seven months. A comparable degree of stability has been observed for [Ga(NOTA)] in aqueous HNO_3 (pH —0.7)[19]. Hence we conclude that even under harsh conditions, protons are not able to effectively compete with Ga^{3+} ion for the ligand's basic sites (the ring nitrogen atoms), which renders the complex extremely inert. As observed by potentiometry, the "in-cage" complex, once formed, is protonated on the phosphinate phosphoryl oxygen atom(s) instead, re- sulting in $[Ga(H_4L)]^+$ (Figure 3) or even multiply protonated species.

2.5. Conjugation

Clearly, the carboxylate moieties of **PrP9** are predestined to act as conjugation sites for biomolecules. However, the scope of this study is mainly to deliver proof of principle, rather than the development of actual PET tracers. At this stage, we only intended to assess the general feasibility of such derivatization as well as the radiolabeling properties of such conjugates. Therefore, triamides of **PrP9** with cyclohexylamine and amino acid esters were prepared as model compounds for conjugates with for example, (oligo)peptides (Scheme 2). From some common peptide coupling agents investigated, uronium reagents like HBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate) and TBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) gave the best results. Five equivalents of amine and about eight equivalents of coupling agent were found to be sufficient for complete derivatization. Subsequently the triamides 1–4 could be purified very conveniently by diafiltration through a membrane with 0.5 kDa cutoff. The compounds, obtained as the Na^+ salts, were easily soluble in water.



Scheme 2. Functionalization of **PrP9** with amines, leading to conjugates 1–4.

Concerning derivatization, the most significant advantage of **PrP9** is that protection of the phosphinate moieties during the coupling reaction was found to be unnecessary. Hence, no final deprotection step following conjugation has to be taken into account, which simplifies conjugation protocols. On the one hand, this could render further protection of certain targeting vectors unnecessary ; on the other hand, utilization of a vector that does not require any protection at all offers the possibility of a single-step synthesis of a de- sired bioconjugate. Moreover, due to the availability of three equal conjugation sites, the access to multimeric trac- ers is provided in a very convenient way.

2.6. ^{68}Ga labeling

Labeling of the unsubstituted ligand **PrP9** with ^{68}Ga was performed by employing various temperatures and pH values, according to an established protocol reported earlier[41, 42]. It allows for a very precise adjustment of pH and temperature and is therefore well suited for the investigation of labeling properties. Figure 5 shows that at pH 3, nearly complete (> 95 %) incorporation of activity is achieved almost

instantaneously at 60°C and above. Even at 40°C and room temperature, decrease in labeling performance is just marginal, requiring three instead of one minute to reach a > 95 % plateau. Variation of pH at a constant temperature of 60°C revealed that instantaneous labeling occurs for pH values from 3–5, whereas for complete radioactivity incorporation at lower pH, slightly longer reaction times are required. Moreover, for the derivatives 1–4 an almost complete incorporation of activity occurs nearly as rapidly, as in the case of the unsubstituted compound (see Supporting Information, Figure S8). Hence we conclude that functionalization of the side arms does not substantially affect complex formation; this result is somewhat surprising but nevertheless quite satisfying.

The most striking feature of **PrP9** and its derivatives is their ability to incorporate $^{68}Ga^{3+}$ at pH values as low as 1, which has two major advantages. Firstly, at a pH below 3 there is no perturbing formation of the colloidal hydroxide and all activity is available for radiolabeling in form of free $[^{68}Ga(H_2O)_6]^{3+}$. The second aspect is more a practical one: The very popular commercially available TiO_2-based $^{68}Ge/^{68}Ga$ generator systems are commonly eluted with 0.1 m HCl, the eluate thus having pH of ≈1. None of the common chelators used for ^{68}Ga radiopharmaceuticals can be sufficiently labeled directly with this eluate. Hence, processing of the eluate[41] or addition of buffers has been mandatory, whereby the latter do not play an innocent role in terms of chemical interactions during the labeling process, as well as with respect to legal and regulatory issues in radiopharmaceutical production. We therefore exemplarily performed labeling of the benzylglycine conjugate 3 using the neat eluate from a TiO_2-based ^{68}Ga generator (Cyclotron Co., Obninsk, Russia). Figure 6 illustrates that although radioactivity incor- poration occurs significantly slower than at pH 3, a radio-chemical incorporation of > 95 % after 10 min is achieved by using 14 nmol of the ligand, an amount which is in the usual range for clinical production of ^{68}Ga tracers[42, 43]. This shows the feasibility of such labeling under conditions common in clinical practice.

We also performed a direct comparison of **PrP9** with NOTA and DOTA, the most common chelating units in ^{68}Ga chemistry. Here, we employed another commonly used labeling method[10]. HEPES buffer was used to adjust the pH of the eluate from a SnO_2-based generator (from iThemba LABS, South Africa, eluted

with 1 m HCl) to a value of 3.3. We chose this pH in order to ensure a fair and realistic comparison of the ligands, since it has been found to be optimal for both DOTA-[43] and NOTA-conjugated peptides[44]. The results of the labeling at ambient temperature (≈20°C), depicted in Figure 7, show that **PrP9** exhibits superior performance than DOTA and is also ahead of NOTA, although the velocity of radioactivity incorporation of the latter is in a comparable range. It is evident that at lower pH, the advance of **PrP9** will increase because both competitors exhibit drastically reduced labeling yields in more acidic media[43, 44].

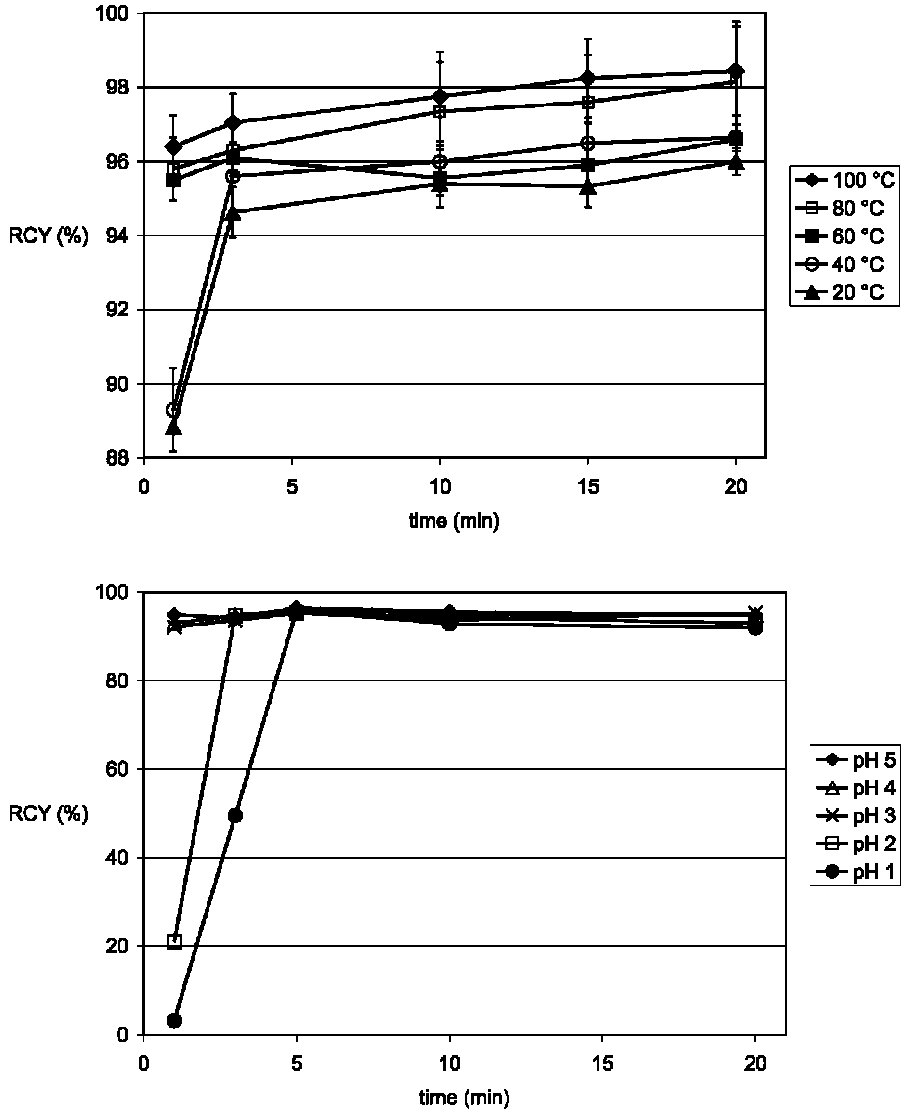


Figure 5. Radiochemical yield (RCY; ^{68}Ga incorporation) for labeling of **PrP9** (14 nmol in 5 mL buffered water, 120–160 MBq) upper: pH 3, 20–100°C; lower: pH 1–5, 60°C.

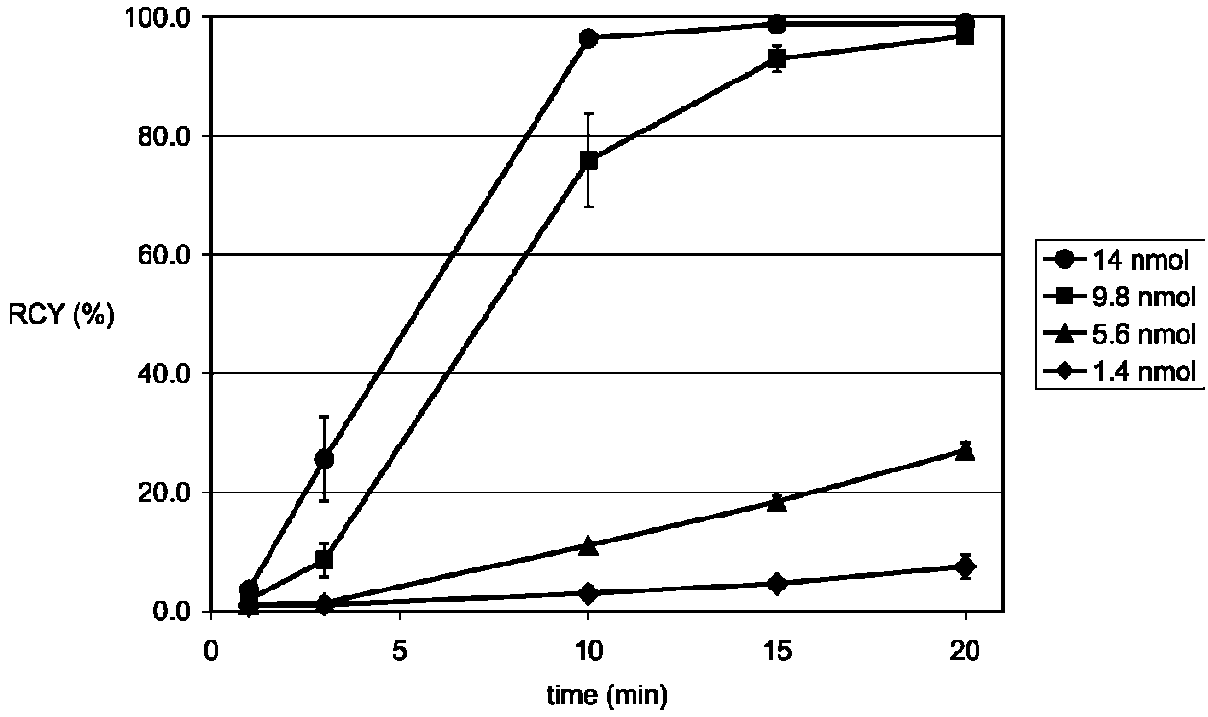


Figure 6. Radiochemical yield (^{68}Ga incorporation) for labeling of 3 (60 MBq in 400 mL, 60°C, pH 1, different molar amounts of 3).

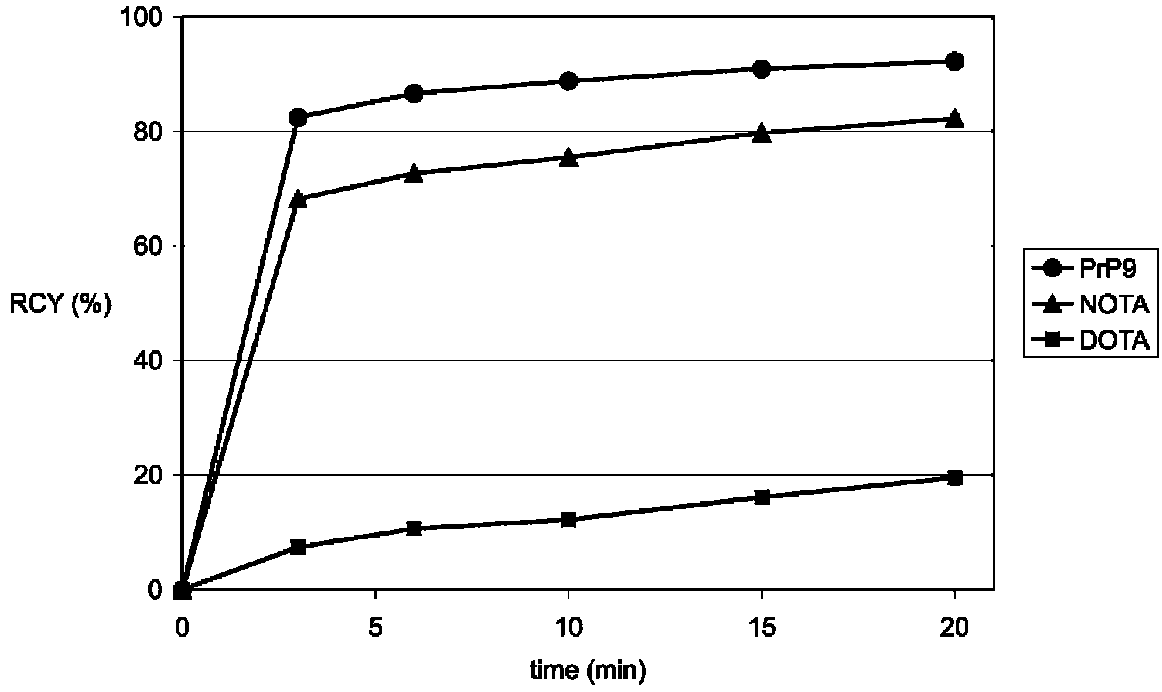


Figure 7. Radiochemical yield (^{68}Ga incorporation) for labeling of **PrP9**, NOTA, and DOTA (10 nmol of ligand, 60 MBq in 110 mL HEPES-buffered solution, pH 3.3, 20°C). Errors were too small to be displayed at this scale.

3. Conclusion

Desirable properties of Ga^{III} chelators for application in ^{68}Ga-based nuclear imaging include high complex stability, fast and selective complex formation, ability to be conjugated, long shelf life, and accessibility. Our data show that the novel ligand system **PrP9** introduced herein does fulfill all of these requirements.

Complex formation kinetics under common chemical as well as under radiochemical conditions is exceptionally fast, being superior to the established ligand systems DOTA and NOTA. This is owed to the assumed complexation mechanism: initially, the Ga^{3+} ion is coordinated very quickly by the pendant carboxylates (open-chain-like interaction, leading to "out-of-cage" complexes). The ion is thereby brought into direct vicinity of the main chelation site, thus accelerating transfer to the cage by increasing the effective concentration close to the macrocyclic cavity. Moreover, decomplexation under very acidic as well as under neutral and moderately alkaline conditions is extremely slow. This is of utmost importance as problems caused by dissociation/trans- metallation in biosystems can therefore be ruled out, and in ^{68}Ga radioimaging, the risk of uncontrolled distribution of $^{68}Ga^{3+}$ activity is eliminated. The fast complex formation allows for milder labeling conditions (e.g. , at ambient temperature), thereby expanding the scope of ^{68}Ga radiolabeling towards targeting vectors with low thermal stability (as for example, antibodies or their fragments).

Another useful effect of the complexation mechanism is that formation of colloidal hydroxide is prevented; no formation of precipitates was observed during complexation reactions over the entire pH range. Hence, the ligand can be considered suitable for ^{68}Ga radiolabeling even at pH≈5 and above. This expansion of applicable labeling pH range, together with straightforward labeling at room temperature, enables its use in radiotracers featuring more sensitive targeting vectors (e.g., antibodies or their fragments). Furthermore, the Ga^{III} complex is quantitatively formed even below pH 1. This entails the most significant improvement concerning ^{68}Ga radiochemistry, as the pH range for optimal labeling is expanded towards quite acidic solutions. For the first time, ^{68}Ga labeling can be performed using the neat eluate from TiO_2-based $^{68}Ge/^{68}Ga$ generators (typically 0.1m HCl, pH 1). Due to possible degradation of sensitive biomolecules at such pH, this might finally turn out to be of lesser practical relevance. However, it means that for **PrP9** there is no strict necessity to keep the pH in a very narrow range from 2.5–4 during the labeling procedures (as required,

for example, for DOTA peptides). This might render the fully automated syntheses of 68Ga radiotracers more robust, in which adjustment of labeling pH is often a crucial issue.

In addition, the synthesis of **PrP9** itself is fast, simple, and scalable; particularly in comparison with other ligand systems bearing additional carboxylic groups suitable for conjugation like DOTA(tBu)$_3$ or NODAGA(tBu)$_3$. Another novel and important characteristic is that conjugation by amide formation needs no protection (and therefore no final deprotection) as metal coordination sites and functional groups for conjugation are different in nature. However, the most striking advantage of **PrP9** is to provide easy access to multimers. We are not aware of any other system of such simple structure that could be multiply conjugated to amine substrates in a single synthetic step. We therefore hold the view that although monofunctionalization can be easily achieved as well by performing the coupling reaction by using a large excess of the ligand, the true designation of the compound is the preparation of multimeric tracers.

All things considered, we believe that **PrP9** has truly the potential to boost the development of ^{68}Ga-based nuclear medicine and molecular imaging by providing facile and cost-efficient access to ^{68}Ga radiopharmaceuticals with superior properties. Further investigations concerning the behavior in biosystems as well as the preparation of conjugates with targeting vectors are currently under way.

4. Experimental Section

4.1. Materials and methods

All reagents used were of analytical grade. Dry solvents were used only where indicated and dried according to established procedures. The preparation of [9]aneN$_3$ followed essentially a published procedure[45], with little alterations as described earlier[46]. NMR spectra were recorded using a UNITY Inova (400 MHz) or a VNMRS (300 MHz) spectrometer from Varian. ^{1}H and ^{13}C NMR shifts are referenced to TMS. ^{31}P NMR shifts are given relative to 85% aq. H_3PO_4. ^{71}Ga NMR shifts are referenced to a 1.0 M aqueous solution of Ga(NO$_3$)$_3$. Elemental analysis was performed using a Heraeus Vario EL III system. Ultrafiltration/diafiltration was performed using a Millipore setup (consisting of a 50 mL stirred cell model 8050, CDS10 selector valve, and RC800 mini-reservoir), in

combination with Ultracel cellulose acetate membranes, filter code YC05, NWML 500Da. Diluted HCl for elution of $^{68}Ge/^{68}Ga$ generators was prepared from HCl (suprapure) and water (ultrapure; both from Merck).

4.2. Syntheses

The full synthetic route to of H_6**PrP9** includes two steps to obtain the phosphorus precursor; its synthesis has been developed in our laboratory and published by some of us before[47]. However, as efficacy and yields were significantly improved, we report the complete protocol starting from commercially available chemicals, including the mentioned

steps.

4.3. Synthesis of H_6PrP9:

Dry ammonium hypophosphite (0.3 mol, 25 g) and hexamethyldisilazane (0.48 mol, 77 g, 100 mL) were heated to 105°C under argon atmosphere with stirring, whereupon gaseous ammonia was evolved. *Caution! The intermediate $HPG(OSiMe_3)_2$ is pyrophoric!* After 4 h the mixture was cooled to room temperature, dry dichloromethane (200 mL) was added and the solution was cooled in an ice bath. Then tert-butyl acrylate (213 mmol, 27.3 g, 31 mL) was added through a syringe and the mixture was stirred for additional 12 h at room temperature. The mixture was hydrolyzed by transferring it into ethanol (500 mL) by means of a capillary and evaporated to dryness. The crude product was dissolved in chloroform (300 mL) and extracted with two portions (60 mL each) of 3% aq. HCl. The aqueous phases were combined and re-extracted with chloroform (3×50 mL). The combined organic extracts were dried over anhydrous sodium sulfate and the solvent removed to afford [2-(tert-butyloxycarbonyl)ethyl]phosphinic acid as a colorless, viscous oil (39.5 g, ca. 203 mmol, 95%), with a purity of 96% according to ^{31}P NMR. The remaining 4% impurity is the P-disubstituted product, which is inert in the following reactions and thus does not need to be removed. The crude phosphinic acid was dissolved in a mixture of ethanol (100 mL) and conc. aq. HCl (100 mL) and heated under reflux for 12 h. Then all volatiles were removed in vacuo to yield (2-carboxyethyl)phosphinic acid (34.2 g, ca. 203 mmol, 100%) as a colorless oil which solidifies upon standing. This compound and 1,4,7-triazacyclononane (45 mmol, 5.8 g) were dissolved in 6m aq. HCl (120 mL) and heated to 70°C. Para-formaldehyde (0.6 mol, 18 g) was added in small portions with stirring during a period of 24 h, while progress of the reaction was monitored with ^{31}P NMR spectroscopy. Then the solvents were distilled off in

vacuo and the remaining HCl was removed by repeatedly adding small portions of water and evaporating to dryness. The crude product was purified by chromatography on ion exchange resin (DOWEX 50×8, H^+-form, column size 25×6 cm, eluent: water). Impurities were removed with the first 700 mL of eluate. The next fraction of approx. 1.8 L containing the pure product was concentrated in vacuo to a volume of 40 mL and methanol (300 mL) was added, whereupon the product slowly crystallized. After 1 h, isopropanol (150 mL) was added and the suspension was cooled for several hours in order to ensure a complete precipitation. The product was filtered off, washed with isopropanol and diethyl ether, and dried in vacuo to yield H_6**PrP9**·$2H_2O$ (15.4 g, 55% based on triazacyclononane) as a colorless, fine-crystalline powder. M.p. 218–219°C; ^{1}H NMR (300 MHz, D_2O): δ=2.07 (dt, J_3^{HH}=7.8 Hz, J_3^{PH}=13.7 Hz, 6H; C(O)-CH_2), 2.68 (dt, J_3^{HH}=7.7 Hz, J_2^{PH}=13.2 Hz, 6H; P-CH_2), 3.46 (d, J_2^{PH}=5.7 Hz, 6H; N-CH_2-P), 3.53 ppm (s, 12H; ring-CH_2); ^{13}C{^{1}H} NMR (75.4 MHz, D_2O): δ=25.1 (d, J1 PC=94 Hz, P-C-C), 26.8 (C(O)-C), 51.5 (ring-C), 54.6 (d, J_1^{PC}=89 Hz, N-C-P), 177.3 ppm (d, J_3^{PC}=13 Hz, C=O); ^{31}P{^{1}H} NMR (121.4 MHz, D_2O): δ=40.0 ppm; MS (ESI positive): m/z (%): 602 (21) $[M+Na]^+$, 626 (100) $[M+2Na-H]^+$, 648 (22) $[M+3Na-2H]^+$; MS (FAB positive): *m/z*: 580 $[M+H]^+$; elemental analysis calcd (%) for $C_{18}H_{36}N_3O_{12}P_3$·$2H_2O$ (615.44): C 35.13, H 6.55, N 6.83; found C 35.04, H 6.60, N 6.67.

4.4. Synthesis of [Ga (H_3PrP9)]·nH_2O

H_6PrP9·$2H_2O$ (0.65 mmol, 400 mg) and $GaCl_3$ (0.65 mmol, 115 mg) were dissolved in water (1 mL). After brief heating, the pH value of the solution was found to be -0.4. An aqueous solution of 5% NH_3 was added until a pH of 2.3 was reached and the complex was allowed to precipitate for 24 h. The solid was filtered off and recrystallized from water (1 mL). The crystals were filtered off and dried in vacuo, whereupon the material apparently loses a fraction of the co-crystallized water, as the initially large transparent crystals disintegrate to give 320 mg of a colorless powder. The single crystals of [Ga(H_3**PrP9**)]·$6H_2O$ suitable for X-ray diffraction were obtained from water and those of [Ga(H_3**PrP9**)]·$2H_2O$ by diffusion of acetone vapor into an aqueous solution of the complex. ^{1}H NMR (300 MHz, D_2O): δ= 2.13–2.21 (m, 6H; C(O)-CH_2), 2.69–2.79 (m, 6H; P-CH_2), 3.10–3.28 (m, 6H; N-CH_2-P), 3.35–3.48 ppm (m, 12H; ring-CH_2); ^{13}C{^{1}H} NMR (101 MHz, D_2O): δ=21.8 (d, J_1^{PC}=100 Hz, P-C-C), 24.2 (C(O)-C), 49.7 (ring-C), 53.3 (d, J_3 PC=11 Hz, ring-C), 55.4 (d, J_1^{PC}=85 Hz, N-C-P), 174.0 ppm (d, J_3^{PC}=13 Hz, C=O); ^{31}P{^{1}H} NMR (121.4 MHz, D_2O): δ= 42.4 ppm; ^{71}Ga NMR (122.0 MHz, D_2O): δ=135.2 ppm; MS (ESI negative): *m/z*: 644/646 $[M-H]^-$; MS (ESI

positive): m/z: 668/670 $[M+Na]^+$, 690/692 $[M+2Na-H]^+$, 712/714 $[M+3Na-2H]^+$; elemental analysis calcd (%) for $C_{18}H_{33}N_3O_{12}P_3Ga \cdot 1.3H_2O$ (669.53): C 32.29, H 5.36, N 6.28; found C 32.27, H 5.40, N 6.12.

4.5. Synthesis of [Fe (H_3PrP9)]·nH_2O

H_6**PrP9**·$2H_2O$ (0.2 mmol, 123 mg) and $[Fe(acac)_3]$ (0.2 mmol, 72 mg) were dissolved in water (0.5 mL) and isopropanol (0.1 mL). When heated to reflux, a red solution initially obtained turned into a light yellow one after several minutes. After cooling to ambient temperature and standing for several hours, yellow crystals had formed, which were filtered off and recrystallized from water (0.5 mL). The precipitate was filtered off and dried in vacuo. During drying, it apparently loses a fraction of the co-crystallized water, as the initially transparent crystals disintegrate to result in a yellow powder (88 mg). The single crystals suitable for X-ray diffraction were obtained directly from water as $[Fe(H_3$**PrP9**$)]\cdot 6H_2O$ and by diffusion of acetone vapor into the aqueous solution as $[Fe(H_3$**PrP9**$)]\cdot H_2O$. MS (ESI negative): *m/z*: 631 [M-H]; MS (ESI positive): *m/z*: 655 $[M+Na]^+$, 677 $[M+2Na-H]^+$, 699 $[M+3Na-2H]^+$, 721 $[M+4Na-3H]^+$; elemental analysis calcd (%) for $C_{18}H_{37}N_3O_{14}P_3Fe$ (668.26): C 32.35, H 5.58, N 6.29; found: C 32.20, H 5.63, N 6.09.

4.6. Preparation of conjugates-general procedure

H_6**PrP9**·$2H_2O$ (0.2 mmol, 123 mg) was dissolved in dry DMSO (3 mL). Then diisopropylethylamine (DIPEA, 2 mmol, 260 mg, 0.35 mL) was added; in case the coupled amine was in the hydrochloride form, 3 mmol (390 mg, 0.5 mL) of DIPEA were used. The amine or amino acid ester hydrochloride (1 mmol) was added and the mixture was stirred for 5 min. Then TBTU (1.6 mmol, 0.5 g) was added in small portions within 10 min. The mixture was left to react for variable time (see individual compound data) and afterwards diluted with water (50 mL). For workup, solutions were concentrated to 15 mL by ultrafiltration through a membrane with 0.5 kDa MWCO. Diafiltration with aqueous NaCl (0.05m, 300 mL) removed all impurities. The solutions were further concentrated to 10 mL and desalted by diafiltration with pure water (100 mL). Subsequent lyophilization of the retentate afforded the sodium salts of the products as off-white to pale yellow, voluminous solids.

4.7. Tris(cyclohexylamide)-PrP9 (1)

Cyclohexylamine (1 mmol, 100 mg, 115 mL), reaction time: 10 min. Yield: 179 mg (93%); ^{1}H NMR (400 MHz, D_2O): δ=1.08–1.27 (m, 15 H), 1.52–1.55 (m, 3H), 1.64–1.67 (m, 6H), 1.72–1.80 (m, 12H), 2.30–2.37 (m, 6H; P-CH_2), 3.05 (d, J_2 ^{PH}= 6.0 Hz, 6H; N-CH_2-P), 3.19 (br s, 12H; ring-CH_2), 3.45–3.52 ppm (m, 3H); $^{13}C\{^{1}H\}$ NMR (75.4 MHz, D_2O): δ=24.3, 24.9, 26.9 (d, J1 ^{PC}=92 Hz, P-C-C), 28.7, 31.9, 48.9, 50.6, 53.6 (d, J_1^{PC}=92 Hz, N-C-P), 174.2 ppm (d, J_3^{PC}=17 Hz, C=O); $^{31}P\{^{1}H\}$ NMR (161.9 MHz, D2O): δ=38.5 ppm; MS (ESI negative): *m/z*: 843 $[M+Na-2H]^+$; elemental analysis calcd (%) for Na(H_2**1**)·6H_2O ($C_{36}H_{80}N_6O_{15}P_3Na$; 952.96): C 45.37, H 8.46, N 8.82; found: C 45.20, H 8.35, N 8.74.

4.8. Tris{(O-methyl)glycyl}PrP9 (2)

Glycine methyl ester hydrochloride (1 mmol, 125 mg), reaction time: 45 min. Yield: 138 mg (76%); ^{1}H NMR (400 MHz, D_2O): δ=1.70–1.81 (m, 6H; CH_2-C(O)N), 2.36–2.45 (m, 6H; P-CH_2), 3.08 (d, J_2^{PH}=6.0 Hz, 6H; N-CH_2-P), 3.16 (br s, 12H; ring-CH_2), 3.68 (s, 9H; CH_3), 3.94 ppm (s, 6H; CH_2-C(O)O); $^{13}C\{^{1}H\}$ NMR (75.4 MHz, D_2O): δ=24.7 (d, J_1^{PC}=90 Hz, P-C-C), 31.0, 43.9, 53.1, 55.4, 56.0 (d, J_1^{PC}=99 Hz, N-C-P), 174.8, 178.6 ppm (d, J_3^{PC}=16 Hz, C=O); $^{31}P\{^{1}H\}$ NMR (161.9 MHz, D_2O): d=38.5 ppm; MS (ESI negative): *m/z*: 813 $[M+Na-2H]^-$; elemental analysis calcd (%) for Na_2(H_2**2**)·4.8H_2O ($C_{27}H_{59.6}N_6O_{19.8}P_3Na$; 901.1): C 35.99, H 6.67, N 9.33; found C 35.98, H 6.35, N 9.19.

4.9. Tris{(O-benzyl)glycyl}PrP9 (3)

Glycine benzyl ester hydrochloride (1 mmol, 202 mg), reaction time: 50 min. Yield: 173 mg (76%); ^{1}H NMR (400 MHz, D_2O): δ=1.73–1.80 (m, 6H; CH_2-C(O)N), 2.38–2.43 (m, 6H; P-CH_2), 3.05 (d, J_2^{PH}=5.6 Hz, 6H; N-CH_2-P), 3.14 (br s, 12H; ring-CH_2), 3.75 (s, 6H; CH_2-C(O)O), 4.85 (s, 6H; CH_2-Ph), 7.05–7.12 ppm (m, 15H; C_6H_5); $^{13}C\{^{1}H\}$ NMR (100.6 MHz, D_2O): δ=24.1 (d, J_1^{PC}=90 Hz, P-C-C), 25.7, 38.6, 47.6, 50.6 (d, J_1^{PC}=93 Hz, N-C-P), 64.5, 125.5, 125.8, 126.0, 132.5, 168.4, 173.1 ppm (d, J_3 ^{PC}=16 Hz, C=O); $^{31}P\{^{1}H\}$ NMR (161.9 MHz, D_2O): δ=38.3 ppm; MS (ESI negative): m/z: 1042 $[M+Na-2H]^-$; elemental analysis calcd (%) for Na (H_2**3**)·5H_2O ($C_{45}H_{72}N_6O_20P_3Na$; 1132.99): C 47.70, H 6.41, N 7.42; found C 47.52, H 6.37, N 7.38.

4.10. Tris{(O-tert-butyl)-l-phenylalanyl}PrP9 (4)

L-Phenylalanine *tert*-butyl ester hydrochloride (1 mmol, 258 mg), reaction time: 50 min. Yield: 223 mg (84%); ^{1}H NMR (400 MHz, D_2O): δ=1.10 (s, 27H; CH3), 1.73 (br s, 6H; CH2-

C(O)N), 2.36 (br s, 6H; P-CH_2), 2.77–2.86 (br s, 6H; CH_2-Ph), 3.04 (br s, 6H; N-CH_2-P), 3.16 (br s, 12H; ring-CH_2), 4.33 (t, J= 4.8 Hz, 3H; CH), 6.97–7.07 ppm (m, 15H; C_6H_5); $^{13}C\{^1H\}$ NMR (100.6 MHz, D_2O): δ=24.2 (d, J1 ^{PC}=103 Hz, P-C-C), 24.7, 25.7, 34.6, 47.6, 50.6 (d, J_1^{PC}=92 Hz, N-C-P), 52.3, 79.7, 124.0, 125.7, 126.7, 133.9, 169.1, 172.1 ppm (d, J_3^{PC}=16 Hz, C=O); $^{31}P\{^1H\}$ NMR (161.9 MHz, D_2O): δ=38.2 ppm; MS (ESI negative): *m/z*: 1210 [M+Na-2H]$^-$; elemental analysis calcd (%) for Na (H_2**4**)·6H_2O ($C_{57}H_{98}N_6O_{21}P_3Na$): C 51.89, H 7.49, N 6.37; found C 51.70, H 7.61, N 6.24.

4.11. Crystal structure determination

The diffraction data were collected on a Nonius Kappa CCD diffractometer (Enraf–Nonius) at 150(1) K with MoKa radiation (λ=0.71073 Å) and analyzed by using the HKL program package[48, 49]. The structures were solved using direct methods and refined by full-matrix least-squares techniques (SIR92[50] and SHELXL97[51]). Scattering factors for neutral atoms were included in the SHELXL97 program. All non-hydrogen atoms were refined anisotropically. The hydrogen atoms were located in electron density map. Hydrogen atoms at carbon atoms were fixed in the theoretical positions. Those belonging to carboxylate and water oxygen atoms were fixed in original positions using the riding model with $U_{eq}(H)=1.2U_{eq}$. In the structure of [Ga(H_3**PrP9**)]·2H_2O, one of the oxygen atoms of one carboxylate moiety was best refined disordered in two positions with mutual occupancy of 79 and 21%. CCDC-749080 ([Fe (H_3**PrP9**)]·H_2O), -749083 ([Fe-(H_3**PrP9**)]·6H_2O), -749081 ([Ga (H_3**PrP9**)]·2H_2O), and -749082 ([Ga-(H_3**PrP9**)]·6H_2O) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif. For crystallographic parameters see also Supporting Information, Table S5.

4.12. Potentiometry

The stock/titration solutions used (aq. HCl, ≈0.03m; NMe4OH, ≈0.2m) and metal chlorides or nitrates were the same as in previous studies[52, 53]; the known amount of HCl was added to the stock solution of $GaCl_3$ to prevent hydrolysis. Titration conditions: 25.0±0.1°C; I=0.1 M (NMe_4Cl); -log[H^+] range of 1.7–11.9 or until precipitation of a metal hydroxide occurred; starting volume 5 mL; ligand concentration ≈0.004m; presaturated wet argon as an inert gas. The titration system consisted of a PHM 240 pH meter, a 2 mL ABU 900 automatic piston burette and a GK 2401B combined electrode (all Radiometer, Denmark). At least three

parallel titrations were carried out for each metal-to-ligand molar ratio (1:1 and 2:1); ≈40 points per each titration. In the case of Ga^{3+} ("equilibrium" titration) and Gd^{3+} ions, the complexation was too slow for a conventional titration. Thus the "out-of-cell" method was used: 30 (Ga^{3+}) or 25 (Gd^{3+}) points per titration; -log[H^+] range of 1.5–10.5 (Ga^{3+}/L=1:1), 1.5–3.1 (Ga^{3+}/L=2:1) or 1.8–6.0 (Gd^{3+}/L=1:1); starting volume 1 mL; prepared under argon; equilibrium time at room temperature four weeks (Ga^{3+} systems) or 1 d (Gd^{3+} system). Solutions were kept in tightly closed ground glass tubes below -log[H^+]<6. For -log[H^+]>6, solutions were flame sealed into ampoules in order to protect the solutions against atmospheric CO_2 during standing, as alkaline solutions in the closed ground tubes gave irreproducible results. The solution -log[H^+] in each tube/ampoule for out-of-cell titration was measured separately with a freshly calibrated electrode (as given below). The constants determined by this technique showed higher standard deviations due to less precise measurements and a smaller number of experimental points. At least two parallel titrations for each metal-to-ligand ratio (1:1 and 2:1) were performed. The constants (with standard deviations) were calculated using the OPIUM program[54–56]. The program minimizes the criterion of the generalized least-squares method using the following calibration function:

$$E = E_0 + S\log[H^+] + j_1[H^+] + j_2[OH^-]$$

in which the additive term E_0 contains the standard potentials of the electrodesused and contributions of inert ions to the liquid-junction potential, S corresponds to the Nernstian slope (the actual value of which should be close to the theoretical value) and the $j_1[H^+]$ and $j_1[OH^-]=j_2Kw/[H^+]$ terms are the contributions of the H^+ and OH^- ions to the liquid-junction potential. It is clear that j_1 and j_2 cause deviation from a linear dependence of E on pH only in strongly acidic and strongly alkaline solutions. The calibration parameters were determined from titration of the standard HCl with the standard NMe_4OH before each ligand or ligand–metal titration to give a pair of calibration/titration, which was used for calculations of the constants. All constants determined are concentration constants. The water ion product pKw (13.81) and stability constants of the $M^{2/3+}$-OH^- systems included into the calculations were taken from literature[38, 39, 57].

4.13. NMR measurements

The Ga^{III}–**PrP9** complex formation was followed by ^{31}P and ^{71}Ga NMR spectroscopy at room temperature (25°C). Typical conditions: $GaCl_3$ hydrate (15 mg) was dissolved in distilled

water (5 mL) and the pH was adjusted to 1.3, 1.0 or 0.8 with 0.5m aq. HCl. The ligand hydrate (10.3 mg) was pre-weighed into a 3 mL vial and quickly dissolved in this $GaCl_3$ solution (1 mL) to give a solution containing metal ions and ligand in equimolar amounts. This moment represented zero on the timeline (t=0). The reaction mixture was transferred as quickly as possible to a 5 mm NMR tube and placed in the NMR spectrometer. Typically, the first spectra were obtained at t=2 min. After the end of complexation reaction, the final pH of the solution was determined again. The ^{71}Ga NMR spectra were quantified against signal of $[Ga(OH)_4]^-$ (5 mm, pH 13) in the insert tube. Identity of the major isomer in the solution with that isolated in the solid state was confirmed by addition of the isolated complex into NMR sample after finishing the complexation reaction.

The dissociation of the Ga^{III}–**PrP9** complex in alkaline media was investigated by ^{71}Ga NMR spectroscopy ([GaL]=5 mM, 25°C). The complex was preformed by mixing of the equimolar amount of Ga^{3+} salt and the ligand dissolved in water. This solution was evaporated in vacuum and dissolved in water to give a stock solution of the complex. An increasing abundance of $[Ga(OH)_4]^-$ was quantified against 5 mm $[Ga\ (H2O)_6]^{3+}$ solution in 0.1m HNO_3 in an insert tube. The experiments were carried out at pH 11.0 (0.2 M CAPS buffer) and pH 13 (0.1 M NaOH). For decomplexation in acidic media, the complex was preformed in the same way and dissolved in 5 M $HClO_4$. The tube was stored at room temperature for seven months while ^{31}P and ^{71}Ga NMR spectra were recorded regularly.

4.14. ^{68}Ga labeling—standard labeling procedure[41]

A 10 mL Mallinckrodt standard glass vial was charged with Millipore water (5 mL) containing the ligand (14 nmol). Elution of the TiO_2-based ^{68}Ge/^{68}Ga generator (from Cyclotron Co, Obninsk, Russia) was performed with 0.1 M HCl (7 mL). ^{68}Ga activity was concentrated on a small cation-exchange resin (AG 50-W×8, 400 mesh, H^+-form) and purged with an acetone/HCl-mixture (1 mL). [^{68}Ga]$GaCl_3$ (120–160 MBq, ≅1.2–1.6 pmol) was eluted with a 2.44% solution of 0.05m HCl in acetone (400 mL) directly into the reaction vessel. The vial was heated to the specified temperature for 20 min. Samples were withdrawn from the reaction mixture at 1, 3, 10, 15 and 20 min and analyzed by radio-TLC (TLC sheets silica gel 60, mobile phase: 0.1 M sodium citrate in water).

The labeling of **3** with the neat generator eluate was performed by directly dissolving the specified amount of ligand **3** in the eluate (1.7 mL, typically containing ≈60 MBq of ^{68}Ga,

≅0.6 pmol) and subsequent heating of the solution to 60°C. Probing and analysis were performed as described above. The stability of **3** under these conditions was checked by heating a solution of **3** (2 mg) dissolved in HCl (1 mL, 0.1m) to 60°C for 20 min. HPLC analyses (using a 20× 4 mm C18 column, flow rate 1 $mLmin^{-1}$, gradient: in 20 min from 30 to 70% MeCN in H_2O, both eluents containing 0.1% trifluoroacetic acid), were performed immediately after dissolving and after 20 min of heating. The chromatograms showed no significant differences and were practically identical to those of the compound dissolved in pure water.

For labeling in HEPES-buffered solution (comparison of **PrP9**, NOTA, and DOTA), a generator with an SnO_2 matrix (from iThemba LABS, South Africa, total eluted ^{68}Ga activity ca. 1500 MBq) was eluted with 1 M HCl. A fraction of 1.25 mL containing the highest activity was mixed with a solution of HEPES (600 mg) in water (Merck ultrapure, 500 mL). From this solution aliquots of 100 mL (containing about 60 MBq (≅0.6 pmol) each) were transferred into eppendorf cups. 10 mL of solutions of the ligand (1 $\mu mol\ mL^{-1}$) were added and mixed well. The pH of the mixture was 3.3. Samples were withdrawn after 4, 7, 10, 15, and 20 min and analyzed by TLC as described above.

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3.4. A ^{44}Ti/^{44}Sc radionuclide generator for potential application of ^{44}Sc-based PET-radiopharmaceuticals

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Summary

^{44}Ti/^{44}Sc radionuclide generators are of interest for molecular imaging. The 3.97 h half-life of ^{44}Sc and ist high positron branching of 94% may stimulate the application of ^{44}Sc labelled PET radiopharmaceuticals. However, both ^{44}Ti production and ^{44}Ti/^{44}Sc generator design represent challenges for basic radiochemistry.

About 5 mCi (185 MBq) of ^{44}Ti were obtained *via* the Sc(*p,* 2*n*) nuclear reaction. ^{44}Ti was separated from 1.5 g of massive scandium targets in multi-step procedures, including exchange chromatography on cation exchange resin (AG 50W-X8, 200–400 mesh, H^+-form). In order to design a robust ^{44}Ti/^{44}Sc generator concept, distribution coefficients of Ti(IV) and Sc(III) on both AG 1-X8 (200–400 mesh, Cl^--form) and AG 50W-X8 (200–400 mesh, H+-form) resins eluted with HCl and HCl/$H_2C_2O_4$ solution of various concentrations were investigated systematically. Optimal conditions for efficient separations of both radionuclides have been determined for AG 1-X8 resin and mixtures of 0.07 M HCl and 0.005 M $H_2C_2O_4$.

A 5 mCi generator was prepared on an AG 1-X8 column (H = 150 mm, D = 3 mm, V_0 = 0.55 mL). The system achieved elution of 180 MBq ^{44}Sc in 20 mL of eluate solution. The breakthrough of ^{44}Ti is 90 Bq. This corresponds to an excellent separation factor of 2×10^6.

In the context of long-term stability of ^{44}Ti/^{44}Sc generators, a “reverse” type of washing steps after each elution using 0.07 M HCl/0.005 M $H_2C_2O_4$ mixtures appeared to be essential.

1. Introduction

Radionuclide generator systems as means of separating a desired radioisotope from its parent and other contaminants have proved to be extremely useful. The radiochemical separation concept, however, should meet certain requirements in order to be of real value: (1) it must rapidly yield the generator daughter in high purity in a chemical form suitable for further application; (2) the parent material must remain, or be reconverted readily to a form with which the process can be repeated and (3) it should be simple to handle, operate and shield. Potential radionuclide generators have been considered since decades, *e.g.* [1].

For PET imaging, the ^{82}Sr/^{82}Rb generator with its relatively long-lived mother radionuclide has already shown significant clinical value. Recently, the ^{68}Ge/^{68}Ga radionuclide generator has shown significant potential for molecular imaging [2-4]. The high positron branching of 89% and the kit-type of radiopharmaceutical syntheses offer excellent parameters for the routine use of ^{68}Ga labelled tracers in nuclear medicine using state-of-the-art positron emission tomography (PET) and PET/CT. A clinical breakthrough was achieved demonstrating the superior possibilities of ^{68}Ga-DOTA-octreotide derivatives for localising neuroendocrine tumours, in particular if PET/CT is used. However, the physical half-life of ^{68}Ga ($T_{½}$ = 67.71 min) might limit the spectrum of clinical applications of ^{68}Ga labelled radiodiagnostics. Furthermore, ^{68}Ga labelled analogues of endoradiotherapeuticals of longer biological half-live such as ^{90}Y- or ^{177}Lu-labelled peptides and proteins cannot be used to determine individual radiation dosimetry.

Thus, radionuclide generator systems providing positron emitting daughters of extended physical half-life are of renewed interest [4].

An important characteristic of positron-emitting ^{44}Sc is its cyclotron-independent availability *via* the ^{44}Ti/^{44}Sc radionuclide generator system. The long-lived ^{44}Ti produces a short-lived ^{44}Sc, which subsequently decays to stable ^{44}Ca. The physical half-life of ^{44}Sc is $T_{½}$ = 3.97 h, its positron branching is 94.27 % [5]. The ^{44}Ti half-life varies in different studies from 39 to 66.6 years [8-12]. Most recent studies revealed a half-life of 60.6±1.3 years [10-12].

A crucial issue in the development of ^{44}Ti/^{44}Sc radionuclide generator systems consists in the production of ^{44}Ti. The ^{45}Sc(p,2n)^{44}Ti process seems to be an effective nuclear reaction, however, due to the long physical half-life, cyclotrons of high proton flux are mandatory.

For preparation of $^{44}Ti/^{44}Sc$ radionuclide generators, several radiochemical criteria are relevant, such as effective separation strategies providing high ^{44}Sc elution yields and low ^{44}Ti breakthrough, high long-term stability, and type of Sc eluates useful for subsequent labelling reactions (i.e. low volume, low pH, high purity etc.), cf. [4].

Sc(III) is strongly adsorbed from oxalic acid solution, and its oxalate complex is selectively destroyed by the addition of hydrochloric acid. These data can be used as the basis of a method for the anion exchange separation of Sc(III) and Ti(IV) in oxalic acid / hydrochloric mixtures [13]. In fact, there are only a few reports on studies to develop $^{44}Ti/^{44}Sc$ radionuclide generators. Using 0.2 M HCl / 0.1 M $H_2C_2O_4$ mixture on Dowex-1 resin, 60-70 % elution yield of ^{44}Sc in 30-50 ml was reported [14]. A solvent extraction technique with an organic phase of 1 % 1-phenyl-3-methyl-4-capryl-pyrazolone-5 in methyl isobutyl led to >90 % recovery of Sc in less than 10 ml with a Ti contamination of $< 10^{-6}$ [15]. Elution yields of 42-46 % and decontamination factor of $5 \cdot 10^4$ were reported in studies with 0.01 M HCl as an eluent and ^{44}Ti being adsorbed on inorganic ZrO_2 as an analogue of Ti (IV) [16].

1. Materials and Methods

1.1. Chemicals and reagents

Oxalic acid of analytical reagent grade was obtained from Merck (Darmstadt, Germany). All other chemicals were pure reagent grade and used as received unless otherwise specified. The ion exchange resins AG 1-X8 (200-400 mesh, Cl^--form) and AG 50W-X8 (200-400 mesh, H^+-form) were purchased at Bio-Rad Laboratories (Richmond, CA, USA). Deionized Milli-Q water (18.2 MΩ·cm; Millipore) was used in all reactions.

1.2. Production of ^{44}Ti

Five mCi of ^{44}Ti were produced utilizing the $^{45}Sc(p,2n)^{44}Ti$ process at an internal proton beam of $E_p \approx 25$ MeV. The 1.5 g Sc targets were covered by intermediate layers of Ag in order to reduce ^{65}Zn contaminations. The target system was capable to withstand long-term irradiations at up to 200 μA as described in [17].

^{46}Sc (T½ = 83.79 d) was produced *via* neutron capture on ^{45}Sc at the TRIGA research reactor Mainz as a tracer both for the determination of Sc(III) distribution coefficients and to follow the separation of ^{44}Ti from the irradiated scandium target.

1.3. Measurement of radioactivities

The absolute radioactivity of ^{44}Sc, ^{46}Sc and ^{44}Ti was measured by γ-spectrometry using a high-purity germanium (HPGe) detector. For detection of ^{44}Ti(^{44}Sc), the 1157 and 1499 keV γ-emissions were used for decay equilibrium. The ^{44}Ti radioactivity was measured also at 67.9 (88 %) and 78.4 (94.5 %) keV. Measurements of ^{44}Ti radioactivity were performed 120 h after ^{44}Sc measurements (more than 30 half-lives of ^{44}Sc).

All radioactive materials were handled according to approved protocols at the Institute of Nuclear Chemistry at Johannes Gutenberg University.

Measurement of ^{44}Sc high radioactivities was accomplished in a dose calibrator M2316 (Messelektronik, Dresden GmbH). The Curie-meter ^{18}F setting was used with a multiplication factor of 0.7, to account for absolute ^{44}Sc activity.

A small animal PET scanner (Siemens MicroPET Focus 120) was used to analyse the local distribution of ^{44}Ti(^{44}Sc) on the generator columns.

1.4. Separation of ^{44}Ti and ^{44}Sc

The irradiated 1.5 g irradiated scandium target was dissolved in 18 mL of 2 M HCl and basic separations of silver as well as co-produced ^{65}Zn and ^{109}Cd have been performed as described earlier [17].

For further separations of macroscopic scandium two aliquots A) ^{44}Ti in 9 mL 2 M HCl (96.6 MBq) and B) ^{44}Ti in 9 mL 2 M HCl (103.5 MBq) were spiked with ^{46}Sc. For cation exchange chromatography, a large column (H = 350 mm, S = 2 cm^2, V_0 = 35 mL) of AG 50W-X8, 200-400 mesh (H^+-form) was washed with 1500 mL of 4 M HCl and 50 mL H_2O. Aliquot A was brought in the column followed by 7.5 mL H_2O and 8 mL 1 M HCl consecutive. After that, aliquot B was brought in the column, then 9 mL H_2O and 17.5 mL 1 M HCl. The column was washed with 45 mL 1 M HCl, 30 mL 2 M HCl, 160 mL 3 M HCl, 200 mL 0.5 M $H_2C_2O_4$ consecutively, *cf.* Table 1.

The second separation was performed using a similar column (H = 360 mm, S = 2 cm^2, V_0 = 36 mL) with AG 50W-X8, 200-400 mesh (H^+-form). The column was washed with 1000 mL of 4 M HCl and 50 mL H_2O. The fraction N 5 from the first separation was brought in the column, then 170 mL H_2O, 45 mL 1 M HCl, 180 mL 2 M HCl and 190 mL 4 M HCl, consecutively. Measurements have been performed similar to the protocol used for the first separation, *cf.* Table 2.

1.5. Final purification of ^{44}Ti

The first purification was performed on a medium-scale column (H = 170 mm, D = 3 mm, V_0 = 0.6 mL) with AG 1-X8 resin, 200-400 mesh (Cl^--form). The column was washed with 10 mL 12 M HCl, 10 mL 1 M HCl, 10 mL H_2O and 10 mL 12 M HCl, consecutively. The fractions N 23-24 (from second separation) were brought in the column, then 8.6 mL 12 M HCl, 4.5 mL 8 M HCl, 8 mL 1 M HCl, consecutively, *cf.* Table 2, Fig.1.

The second purification was made on a smaller column (H = 150 mm, D = 3 mm, V_0 = 0.55 mL) using cation exchange chromatography with the resin AG 50-X8, 200-400 mesh (H^+-form). The column was washed with 40 mL 4 M HCl and 5 mL H_2O. The ^{44}Ti fraction N 4 (from first separation) as isolated in the anion exchange chromatography in 2 M HCl, as well as the fractions N 22, 25 (from second separation), and N 13-18 (from first purification) as obtained in the initial separations in 2 M HCl, were brought in the column, then 10 mL 1 M HCl and 20 mL 0.3 M HCl were applied. The ^{44}Ti was eluted with 1 M HCl and ^{56}Co with 4 M HCl beginning with fraction N 122, *cf.* Fig.2.

1.6. Determination of K_d values of Ti and Sc in HCl/$H_2C_2O_4$ solution of various concentrations

K_d values for both Sc(III) and Ti(IV) were determined in batch experiments using different concentrations of HCl/$H_2C_2O_4$ mixtures. ^{44}Ti(^{44}Sc) and ^{46}Sc were used as isotopic tracers for Ti(IV) and Sc(III) distributions. A stock solution of ^{44}Ti (30 kBq) and ^{46}Sc (1 mg, 20 mCi) was dried and dissolved in 100 µL 0.1 M $C_2H_2O_4$ (solution X). Aliquots were prepared in Eppendorf 1.5 mL vials with 100 mg of AG 1-X8 (200-400 mesh, Cl^--form) or AG 50-X8 (200-400 mesh, H^+-form). To all solutions 1 mL of HCl/$H_2C_2O_4$ mixture was added, than 5 µL of solution X was added to the solutions 001-009.

A solution Y - an aliquot with ^{44}Ti (117 kBq) with the remaining solution of X and with ^{46}Sc - was dried and dissolved in 0.025 M $H_2C_2O_4$. 5 µL of solution Y was added to vials 010-016.

Another solution Z was prepared from a fraction of ^{44}Ti (27 kBq) with the remaining solution Y and with ^{46}Sc dried and dissolved in 0.005 M $H_2C_2O_4$. 5 µL of solution Z was added to vials 017-026. Each of the vials was shaken for 2 d. Then 400 µL of the liquid phase was taken from every vial, and radioactivity A was measured on the curie-meter.

K_d was calculated by the following equation:

$$K_d = (4\ A\text{-}10\ A')/A'$$

A – activity of a whole vial with resin, A' – activity of a 400 µL sample of the solution after ion-exchange reaction.

1.7. Preparation and evaluation of pilot ^{44}Ti/^{44}Sc radionuclide generators

Two columns made of PEEK (diameter 3 mm, length 40 mm) were prepared in the institute's workshop. Both columns were filled with AG 1-X8, 200-400 mesh, in Br^--form. The columns were washed with 5 mL 12 M HCl and 5 mL H_2O two times. Finally, they were washed with 5 mL 0.1 M $H_2C_2O_4$.

A sample of ^{44}Ti was evaporated to dryness and taken up with 420 µL of 0.1 M $H_2C_2O_4$. The solution obtained was divided into two parts. To each sample 2 mL 0.1 M $H_2C_2O_4$ were added. The two ^{44}Ti fractions of 300 kBq activity were transferred to the generators.

Both generators were eluted using 10 mL of 0.2 M HCl / 0.1 M $H_2C_2O_4$ solutions. While generator G1 was eluted in a standard procedure, *i.e.* in a single direction ("direct"), the generator G2 was additionally regenerated after each elution using 0.2 M HCl / 0.1 M $H_2C_2O_4$ in alternating direction ("reverse"). Elution of both generators was carried out 3 times a week.

1.8. Improvement of ^{44}Ti distribution profiles of a pilot Ti^{44}/Sc^{44} radionuclide generator

Elution of the generator G2 was carried out 3 times a week as described earlier. After 50 elutions, the ^{44}Ti distribution profile was analysed using µPET registration. Subsequently, the generator was washed using 4 mL of 0.1 M $H_2C_2O_4$ and 2 mL of 1 M HCl / 0.1 M $H_2C_2O_4$ mixture and again 4 mL of 0.1 M $H_2C_2O_4$ consecutively in "reverse" direction. This operation was repeated 5 times. After the "regeneration" protocol, the generator G2 was scanned again.

^{44}Ti distribution profiles on the column of the pilot generator G2 after 50 elutions (a) and after regeneration with using 0.1 M $H_2C_2O_4$ and 1 M HCl / 0.1 M $H_2C_2O_4$ mixture solutions (b) are compared graphically in Fig. 5.

1.9. Preparation of a 5 mCi ^{44}Ti/^{44}Sc radionuclide generator

For the final generator, a larger column (H = 150 mm, D = 3 mm, V_0 = 0.55 mL) was made of PEEK and filled with resin AG 1-X8 (200-400 mesh, Br^--form). The column was washed with 20 mL 12 M HCl and 10 mL H_2O. Finally, it was washed with 10 mL 0.1 M $H_2C_2O_4$. The fractions with purified ^{44}Ti (185 MBq) were dried and dissolved in 20 mL 0.1 M $H_2C_2O_4$. This solution was brought into the generator and the generator was washed with 0.07 M HCl / 0.005 M $H_2C_2O_4$ mixture in "reverse" direction. Two days later, the generator was eluted for the first time using 20 mL of 0.07 M HCl/0.005 M $H_2C_2O_4$. Eluate aliquots were collected for each 2 mL. One week later, the activity of ^{44}Ti in these samples were analysed by means of γ-spectrometry.

Within several months, the generator was eluted each week according to the same protocol.

3. Results and Discussion

3.1. Separation of ^{44}Ti and macroscopic Sc

About 99.9 % of the ^{44}Ti produced in the Sc(p, 2n) nuclear reaction have been isolated in a 48 mL fraction of 2 M HCl. As expected, the scandium separation was not complete with about 500 μg scandium still present in that ^{44}Ti fraction. A second chromatographic separation provided a more complete separation with a separation factor of about 10^5, *i.e.* less than 10^{-3}% (about 15 μg) of the initial scandium still remaining in the ^{44}Ti fraction. In total, about 99.6% of the ^{44}Ti activities have been recovered following cation exchange purification of the no-carrier-added radionuclide from about 1.5 g of a macroscopic scandium target.

Tables 1 and 2 show the results for the ion exchange chromatography for two basic separations, the activities of ^{44}Ti and ^{44}Sc measured in the different fractions using different detectors, *i.e.* Curie-meter and γ-ray spectroscopy. Further purification appeared to be useful. The fractions N 23 and N 24 (Table 2) thus were further purified using anion exchange chromatography, as described later.

Table 1. Initial ^{44}Ti / Sc separation parameter. AG 50W-X8, 200-400 mesh (H^+-form), H = 350 mm, S = 2 cm^2, V_0 = 35 mL. Sample N 5 is highlighted as this aliquot was used for subsequent separation.

N	Solution	V [ml]	Activity [MBq]			
			Curie-meter		γ-spectroscopy	
			1st day	2nd day	^{44}Ti	^{46}Sc
1	1 M HCl	40	0.197	0.153	0	0
2	1 M HCl	45	0.888	0.827	0	0
3	1 M HCl	45	0.386	0.314	0	0
4	2 M HCl	47	1.089	1.271	0.18	0
5	*3 M HCl*	48	*924.7*	*1040.0*	*204.35*	*2.56*
6	3 M HCl	49	56.96	14.91	0.100	2.36
7	3 M HCl	41	23.84	7.231	0.410	1.14
8	3 M HCl	18	8.260	2.752	0.007	0.280
9	0.5 M $H_2C_2O_4$	32	13.33	6.570	~0.020	~0.150
10	0.5 M $H_2C_2O_4$	47	2.367	0.667	0.025	0.109
11	0.5 M $H_2C_2O_4$	37	1.661	0.398	0.008	0.077
12	0.5 M $H_2C_2O_4$	39	-	0.420	0.008	0.850

3.2. Purification of ^{44}Ti

After second separation ^{44}Ti still contains about 15 µg ^{nat}Sc and ^{56}Co (7 MBq), therefore further purification was performed.

Results for the ion exchange chromatographic purifications are shown in Figs. 1-2 for the two purification procedures. Activities have been analysed using a Curie-meter at two different time points. The ^{44}Ti fraction N 1-12, *cf.* Fig. 1, contain > 99% of the overall ^{44}Ti activity. The amount of ^{nat}Sc and the contamination of ^{56}Co are below the detection limit. This highly-pure ^{44}Ti was finally used to prepare the first ^{44}Ti/^{44}Sc generator. The fractions 13-18 (Fig. 1), as well as sample N 4 from the 1st (Table 1) separation and 22, 25 from 2nd (Table 2) were transferred to another purification process, *cf.* Fig. 2.

Table 2. Second ^{44}Ti / ^{nat}Sc separation. AG 50W-X8, 200-400 mesh (H^+-form), H = 360 mm, S = 2 cm^2, V_0 = 36 mL. Samples *N 23* and *24* are highlighted as these aliquots were used for subsequent purification.

N	Solution	V [ml]	Activity [MBq]			
			Curie-meter		γ-spectroscopy	
			1st day	2nd day	^{44}Ti	^{46}Sc
13	1 M HCl	30	0.036	0.002		
14	1 M HCl	30	0.055	0.008		
15	1 M HCl	30	0.062	0.014		
16	1 M HCl	30	0.082	0.018		
17	1 M HCl	30	0.082	0.020		
18	1 M HCl	30	0.088	0.020		
19	1 M HCl	30	0.088	0.019		
20	1 M HCl	30	0.082	0.014		
21	2 M HCl	30	0.077	0.011		
22	2 M HCl	30	1.692	2.206	0.54	
23	*2 M HCl*	30	*561.4*	*771.4*	*188.42*	
24	*2 M HCl*	30	*154.8*	*209.0*	*51.04*	
25	2 M HCl	30	13.80	12.99	0.06	
26	2 M HCl	30	0.844	0.295	0.004	
27	4 M HCl	30	1.546	0.064	0.003	0.0004
28	4 M HCl	30	95.90	5.139		1.017
29	4 M HCl	30	46.52	3.105		0.614
30	4 M HCl	30	24.16	1.631		0.323
31	4 M HCl	30	12.85	0.704		0.140
32	4 M HCl	30	8.974	0.358		0.071
33	4 M HCl	30	5.440	0.181		0.036

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well as sample *N* 4 from the 1st (Table 1) separation and 22, 25 from 2nd (Table 2) were transferred to another purification process, *cf.* Fig. 2.

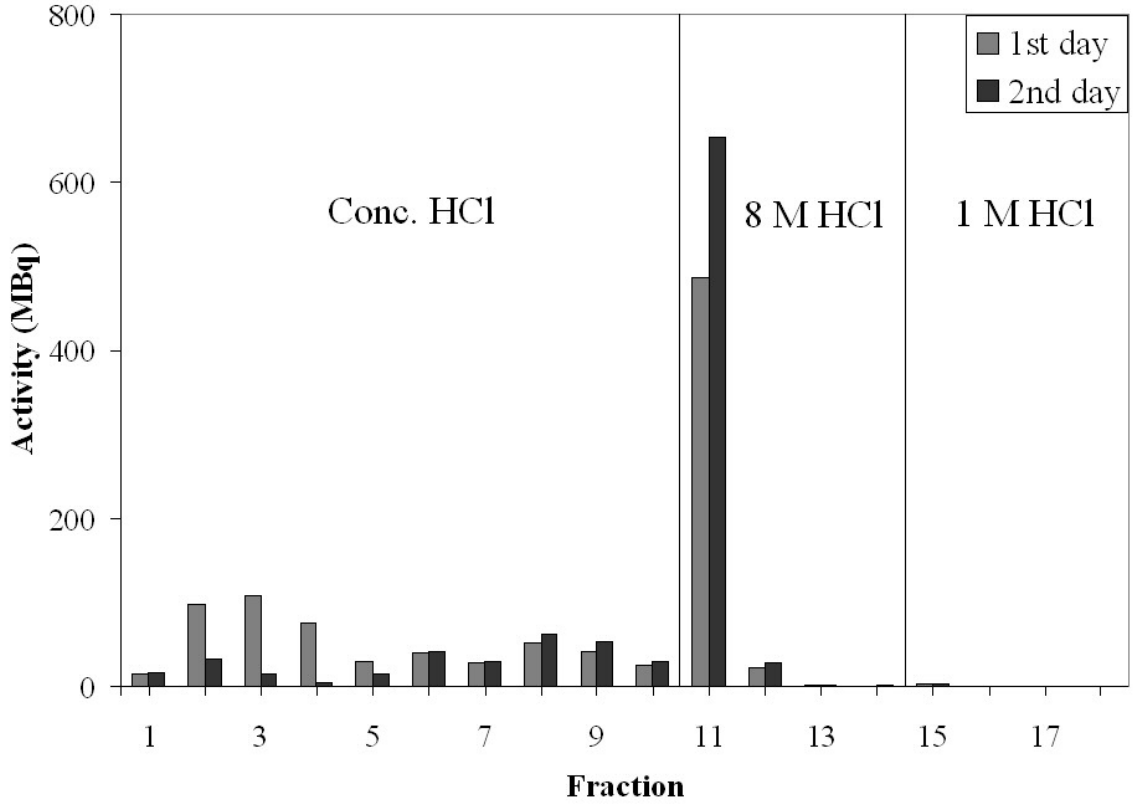

Figure 1. The distribution of ^{44}Ti after the 1st purification. AG 1-X8 (200-400 mesh, Cl^--form). Each fraction volume is 1.2 mL. Activity measured on Curie-meter.

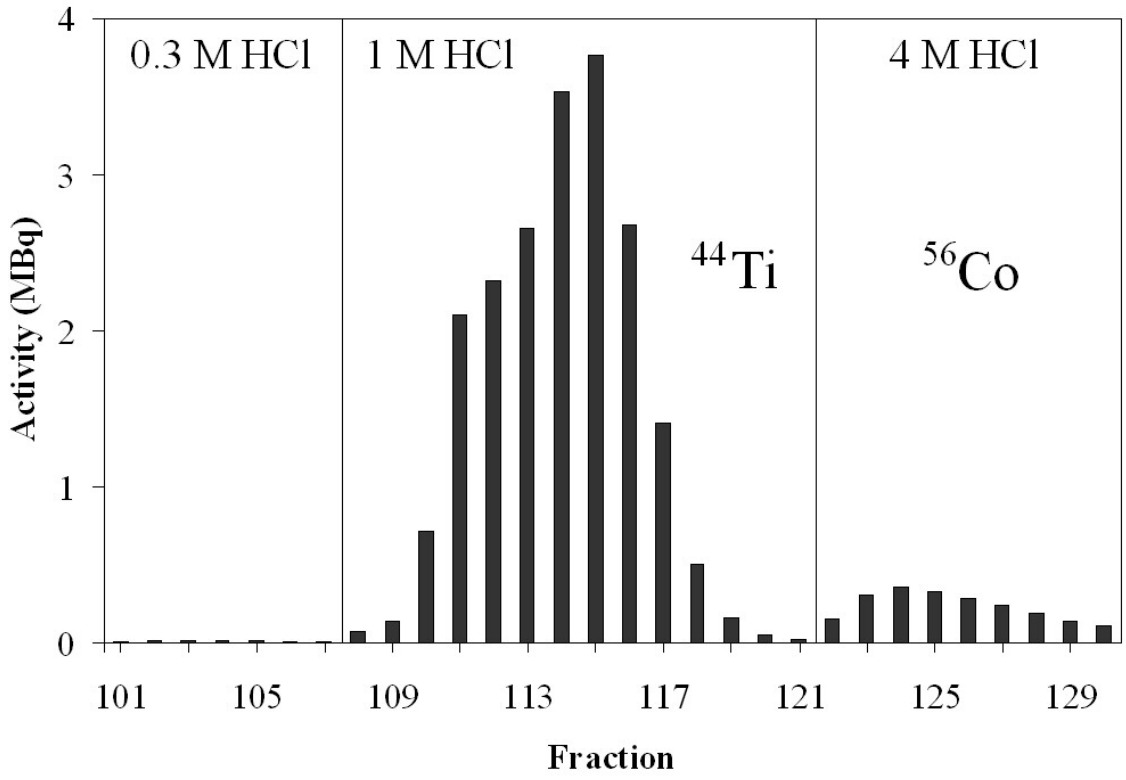

Figure 2. Distribution of ^{44}Sc and ^{44}Ti after the 2nd purification. AG 50-X8 (200-400 mesh, H^+-form). Each fraction volume is 2 mL. Activity measured on Curie-meter.

Fractions N 108-119 are very pure, with ^{nat}Sc and ^{56}Co separated quantitatively. The highly-pure fractions *N* 110-111 were used for the preparation of two low-activity pilot-generators G1 and G2, in order to investigate the radiochemical design of ^{44}Ti/^{44}Sc generators in terms of high ^{44}Sc elution yields and low ^{44}Ti breakthrough, but also long-term stability.

3.3. Determination of distribution coefficients of Ti(IV) and Sc(III) on ion exchange resins

Results of the K_d values obtained for the two different ion exchange resins and the various mixtures are shown in Table 3.

Table 3. Distribution coefficients of Ti(IV) and Sc(III) in various $HCl/H_2C_2O_4$ mixtures for cation and anion exchange resins. $HCl/H_2C_2O_4$ mixtures providing the most useful K_d values are indicated in italic numbers.

	N	Concentration of solution, mol/l		K_d			
				AG 50-X8		AG 1-X8	
		$H_2C_2O_4$	HCl	Ti(IV)	Sc(III)	Ti(IV)	Sc(III)
X	001	0.1	0	-	-	>1000	184
	002	0.1	0.05	-	-	>1000	41
	003	0.1	0.1	-	-	>1000	14
	004	0.1	0.15	<< 1	12.0	>1000	5.1
	005	*0.1*	*0.20*	<< 1	10.7	*>1000*	*1.7*
	006	0.1	0.30	<< 1	7.0	370	0.2
	007	0.1	0.50	<< 1	11.2	105	<< 1
	008	0.1	0.75	~0.5	14.0	-	-
	009	0.1	1.0	<< 1	8.1	17	<< 1
Y	010	0.025	0	1.0	201	>1000	954
	011	0.025	0.025	1.0	148	>1000	168
	012	0.025	0.050	0.6	129	>1000	40.9
	013	0.025	0.075	1.8	128	>1000	14.2
	014	*0.025*	*0.125*	3.3	124	*1050*	*2.68*
	015	0.025	0.175	3.1	120	410	0.3
	016	0.025	0.250	2.9	119	290	<< 1
Z	017	0.005	0	32	7619	>1000	2340
	018	0.005	0.025	30.4	2378	>1000	67.2
	019	0.005	0.0375	34.2	2242	>1000	24.0
	020	0.005	0.05	33.6	2665	>1000	10.9
	021	*0.005*	*0.065*	*28.2*	*1872*	*>1000*	*4.0*
	022	0.005	0.08	33	1715	844	1.27
	023	0.005	0.10	33	1646	688	0.71
	024	0.005	0.125	25.6	1398	457	<< 1
	025	0.005	0.25	-	-	46	<< 1
	026	0.005	0.5	-	-	3.8	<< 1

Accordingly, optimum conditions for efficient separations and for the design of generators could be to elute AG 1-X8 resins with 0.2 M HCl/0.1 M $H_2C_2O_4$, 0.125 M HCl/0.025 M $H_2C_2O_4$ or 0.06-0.08 M HCl/0.005 M $H_2C_2O_4$ mixtures.

In the context of the further use of ^{44}Sc, *e.g.* for radiopharmaceutical syntheses, it appeares favourable to use eluate mixtures of lower salt concentration, such as 0.06-0.08 M HCl / 0.005 M $H_2C_2O_4$. For other studies, such as, *e.g.* to investigate the breakthrough of ^{44}Ti even at increasing numbers of elutions, 0.2 M HCl/0.1 M $H_2C_2O_4$ mixtures can be used as well (*cf.*

"Evaluation of ^{44}Ti/^{44}Sc radionuclide generators"). In this case, the somewhat lower K_d values for Ti(IV) compared to the more diluted eluate mixtures, may allow following the eventual breakthrough of ^{44}Ti at a limited number of elutions already.

3.4. Evaluation of pilot ^{44}Ti/^{44}Sc radionuclide generator

Fig. 3 illustrates the yield of ^{44}Sc obtained for the increasing number of elutions for both generator types G1 and G2. After about 15 elutions, the activity of ^{44}Sc eluted started to drop, which is due to the increasing breakthrough of ^{44}Ti.

In contrast to G1, the "reverse" elution protocol applied to generator G2, showed a constant yield of ^{44}Sc elutions for the complete 50 elution runs applied. This ^{44}Sc elution profile corresponds with the breakthrough of ^{44}Ti as shown in Fig. 4.

The "direct" elution strategy of pilot generator G1 results in an increasing breakthrough of ^{44}Ti, which results in a 50 % desorption of ^{44}Ti after about 30 elutions, and an almost complete release of ^{44}Ti after 50 elution. In contrast, the breakthrough of ^{44}Ti in the case of the "reverse" type elution scheme is negligible for the first 10 elutions, and is increasing only slightly in the following 40 elutions. The maximum breakthrough of ^{44}Ti is about 0.2 %.

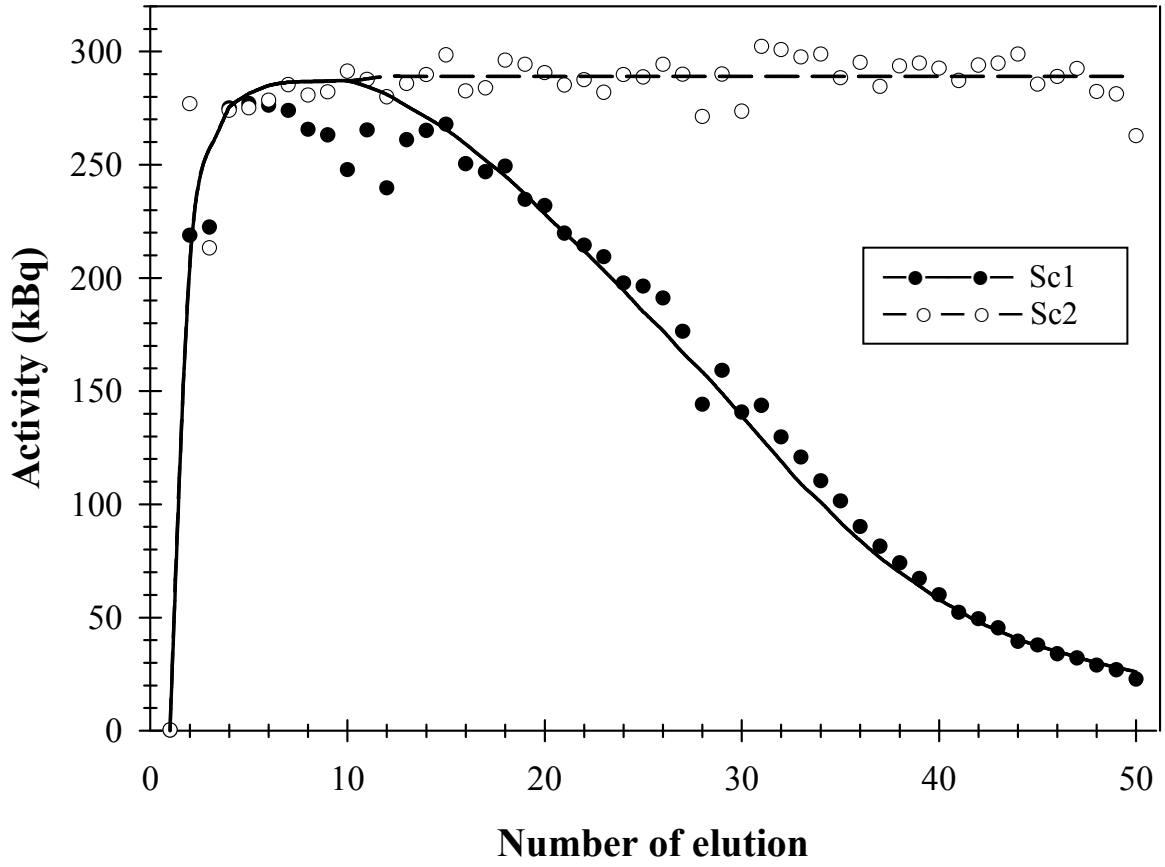

Figure 3. Yield of ^{44}Sc for increasing number of elutions for "direct" (Sc1) and "reverse" (Sc2) elution modes after 50 elutions

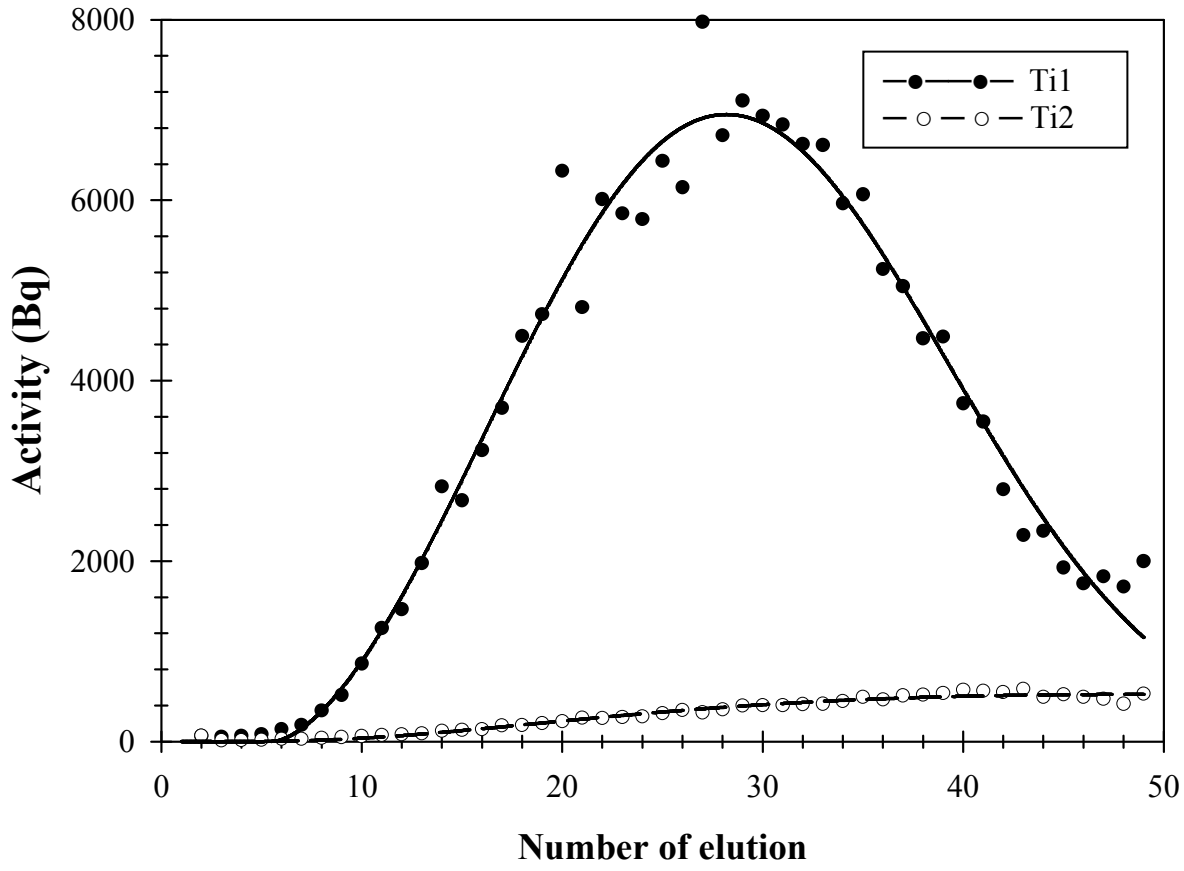

Figure 4. Breakthrough of ^{44}Ti for increasing number of elutions for "direct" (Ti1) and "reverse" (Ti2) elution modes after 50 elutions

3.5. Regeneration of ^{44}Ti distribution profiles on the column

"Reverse" elution modes obviously provide high retention of ^{44}Ti on the column. However, the distribution of ^{44}Ti is still changing with increasing elutions. We tested the possibility to improve the distribution profile by "reverse" elution with different composition of $H_2C_2O_4$/HCl solutions.

The coronar distribution of ^{44}Ti(^{44}Sc) on the pilot generator G2 after 50 elutions was analysed by µPET imaging. Quantitative data on the distribution of ^{44}Ti/^{44}Sc of the generator column are illustrated in Fig. 5. Each slice unit is about 0.5 mm. Obviously, the zone of ^{44}Ti became narrower after a "regeneration" procedure.

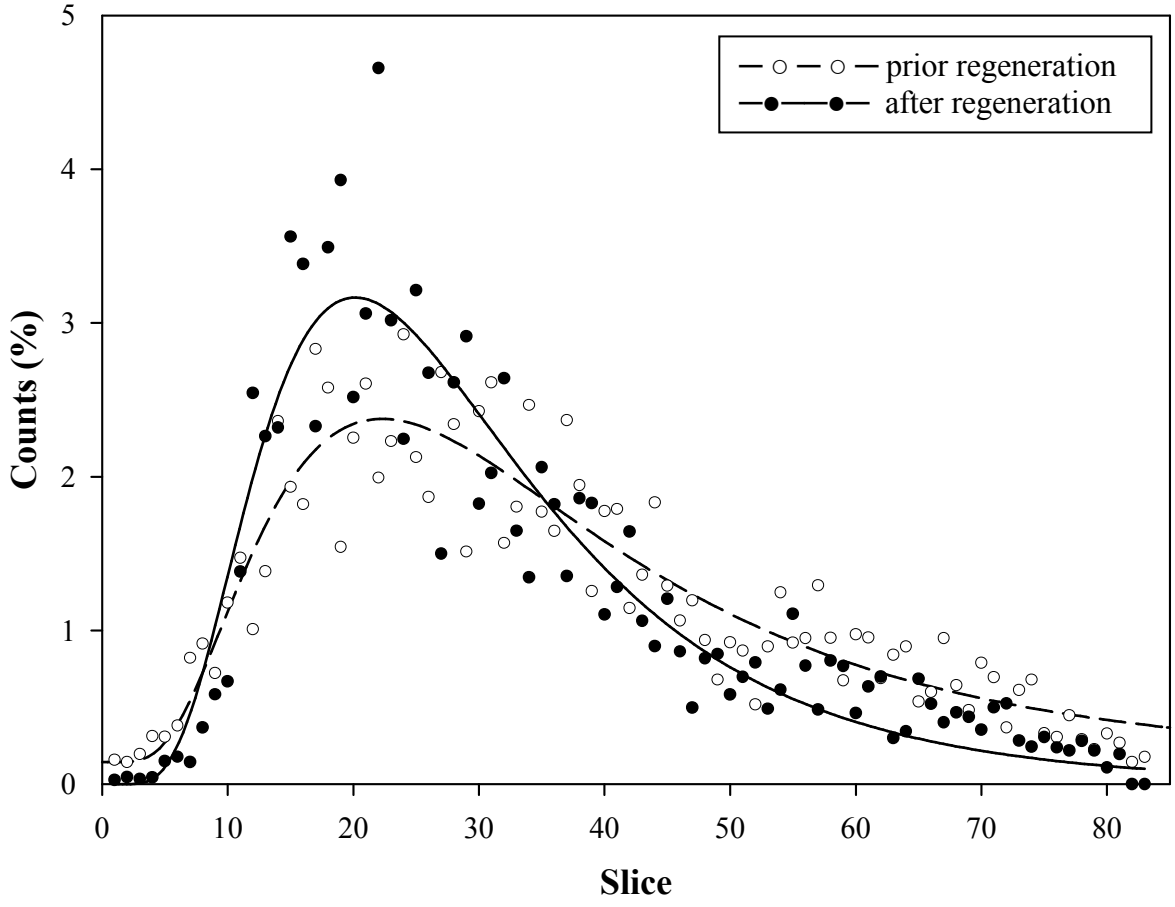

Figure 5. Distribution profile in percent for 44Ti prior and after "regeneration" as measured by PET.

3.6. Preparation of 5 mCi ^{44}Ti / ^{44}Sc radionuclide generator

Typical ^{44}Ti / ^{44}Sc radionuclide generator elution profiles of ^{44}Sc is shown in Fig. 6 for the initial experiments (mean data for the elutions 4-7) as well as a typical result for an elution done after one year (elution number 54). The corresponding activities of ^{44}Ti in the individual fractions are given in Fig. 7.

After the first seven elutions of the ^{44}Ti/^{44}Sc radionuclide generator, the profile of ^{44}Sc elutions indicates that the aliquots 4-7 contain 85±2 % of the total ^{44}Sc activity. The breakthrough of ^{44}Ti (γ-spectroscopy) in all 10 aliquots (20 mL) is less than 10^{-4} % (150 Bq).

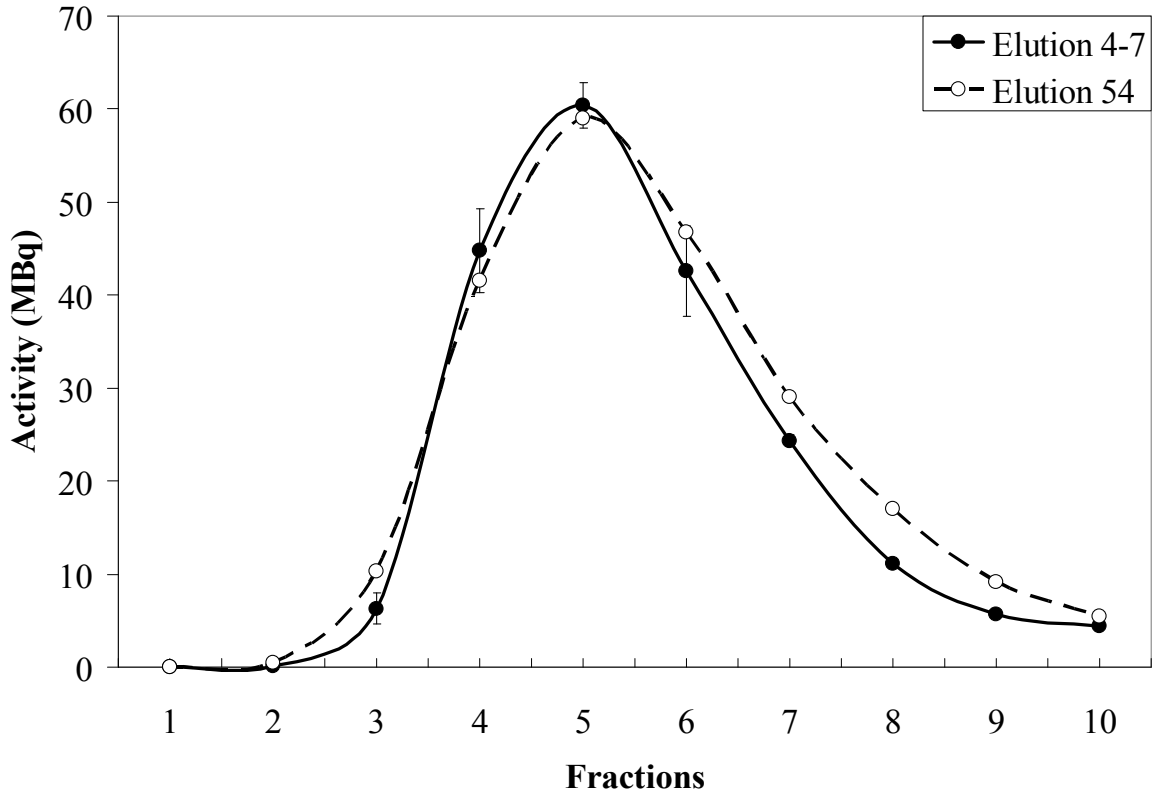

Figure 6. Elution profile of ^{44}Sc (mean of elutions 4-7) and 54 (after 1 year). Curie-meter measurements. Each fraction contains 2 mL.

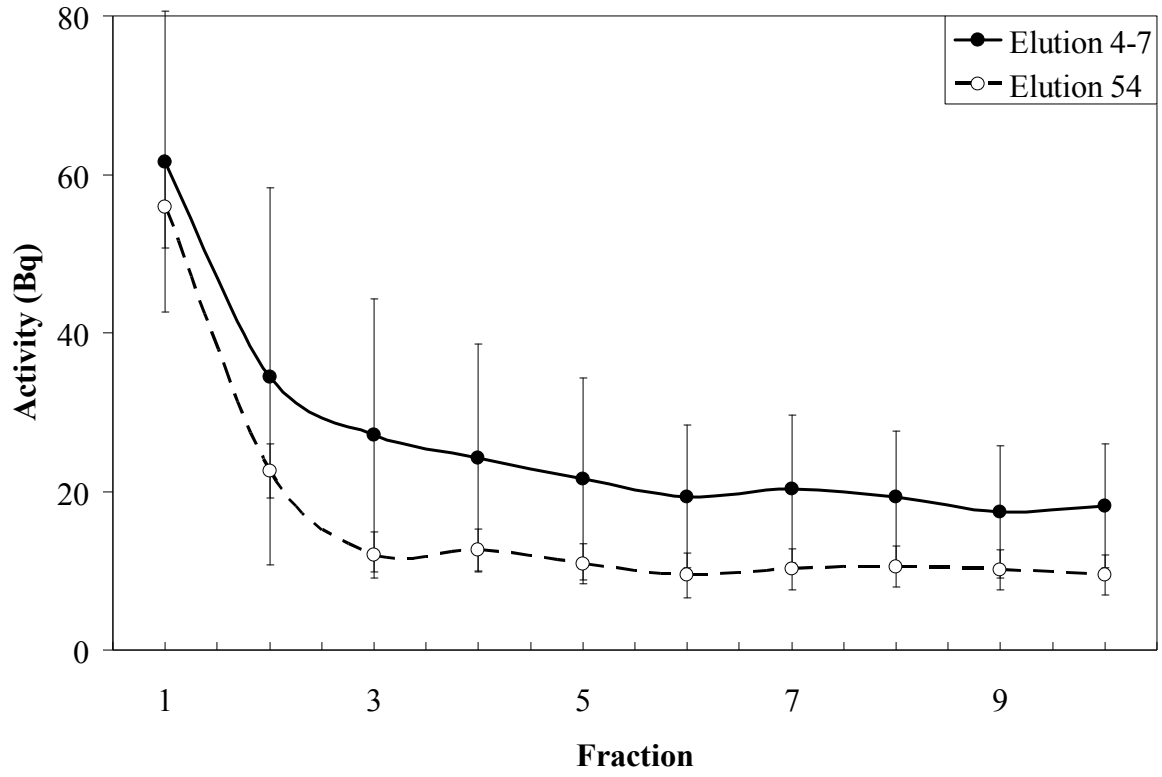

Figure 7. Breakthrough of ^{44}Ti (mean for the elutions 4-7) and 54 (after 1 year). γ-spectroscopy. Each fraction contains 2 mL.

Figure 8 illustrates the overall breakthrough of ^{44}Ti in the total 20 mL eluate volume for the elutions 3 to 7. It indicates an improvement of the breakthrough with increasing number of elutions. While about 1 kBq of ^{44}Ti was found for the third elution, this value is 0.1 kBq only for the 7th elution.

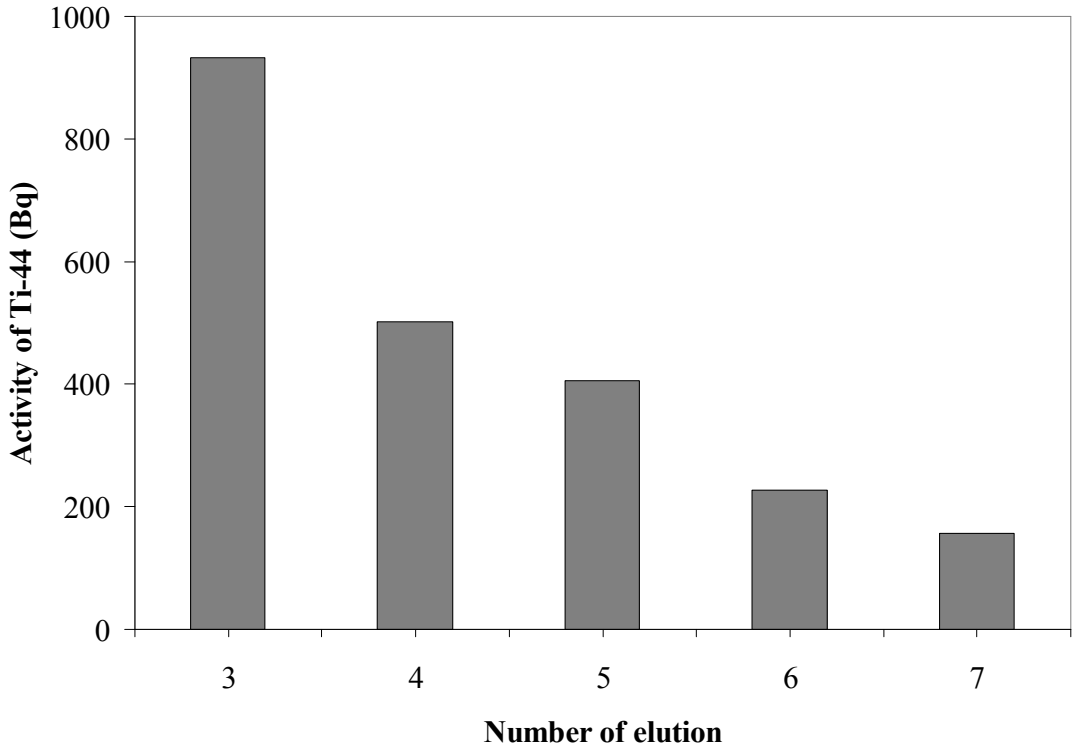

Figure 8. Total breakthrough of ^{44}Ti for the elutions 3-7. Each fraction contains 20 mL.

3.7. Final apparative scheme of the "reverse" ^{44}Ti/^{44}Sc-generator

A modular system of the 5 mCi generator was constructed. It presents the central generator column in a horizontal position (II). Two reservoirs for the eluate solutions are connected to the inlet (I) and the outlet (III) position of the generator column. The reservoirs II and III are connected to an air pressure via filter F to avoid contaminations of eluate composition with metals from the air. Transfer of eluates from the reservoirs through the generator is achieved by air pressure (elution of ^{44}Sc) or vacuum ("reverse" elution) using empty syringe S.

All parts of the 5 mCi ^{44}Ti/^{44}Sc-generator are connected via tubing and 3-way valves. The generator works in a "reverse" scheme of elutions. The initial elution is organized by transferring 20 mL of the eluate solution of reservoir (I) through the generator into the ^{44}Sc vial (IV). After each elution the generator is eluted with the same eluate composition in a reverse way using reservoir (III). While the eluate in reservoir (III) is refreshed routinely, the eluate in bottle (I) can be used for next elution of the generator. Eluted ^{44}Sc in collecting vial (IV) can be used for further experiments.

The scheme guarantees for save handing, as it represents an inherently closed system with respect to ^{44}Ti.

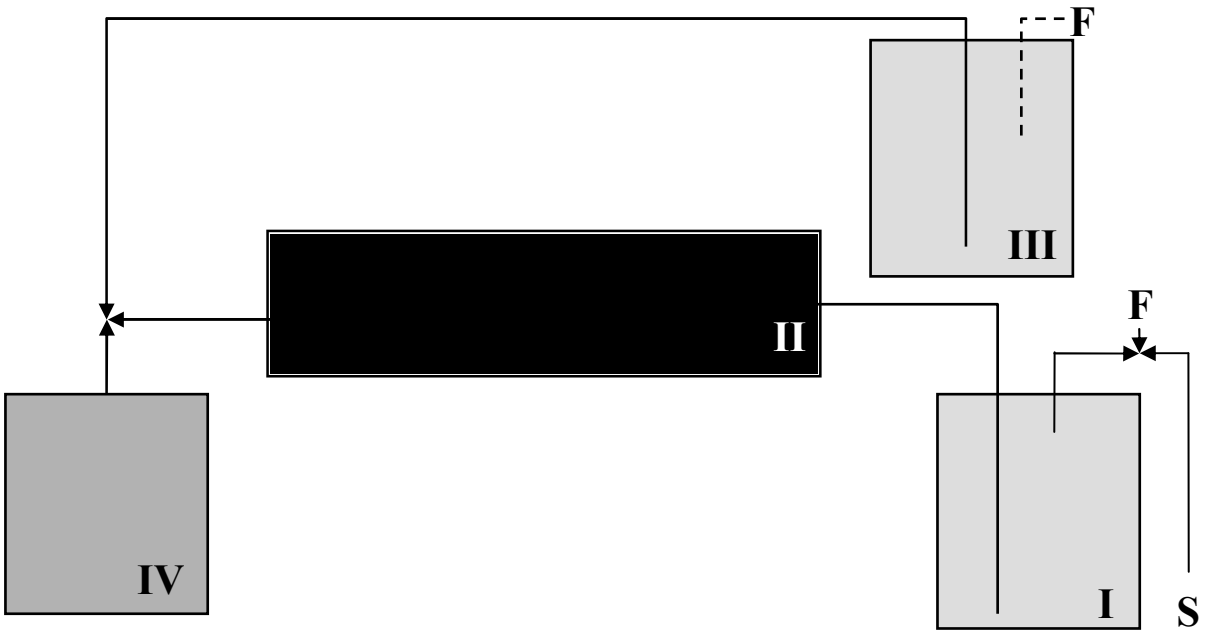

Figure 9. The final scheme of the "reverse" ^{44}Ti/^{44}Sc-generator. I – 20 mL reservoir, II – generator, III – 500 mL reservoir with 0.005 M $H_2C_2O_4$ / 0.07 M HCl mixture, IV – collecting vial, F – filter, S – syringe

4. Conclusions

Using optimum K_d values of Ti(IV) and Sc(III) for HCl/$H_2C_2O_4$ mixtures, *i.e.* 0.2 M HCl/0.1 M $H_2C_2O_4$ and 0.07 M HCl/0.005 M $H_2C_2O_4$, two low-activity pilot and a 5 mCi ^{44}Ti/^{44}Sc generators, respectively, were constructed and evaluated.

After one year of regular elution of 5 mCi ^{44}Ti/^{44}Sc radionuclide generator, the yield of ^{44}Sc and ^{44}Ti is stable and the breakthrough of ^{44}Ti is very low. The system achieves elution of 97 % (180 MBq) ^{44}Sc in 20 mL of eluate solution. The breakthrough of ^{44}Ti is $5 \cdot 10^{-5}$ % (90 Bq). This corresponds to an excellent separation factor of $2 \cdot 10^{6}$. Compared to other ^{44}Ti/^{44}Sc radionuclide generators, higher elution yields of ^{44}Sc in less volume and a higher separation factor were achieved, at higher ^{44}Ti activity compared to only kBq in other studies (Table 4).

Table 4. Comparison of different ^{44}Ti/Sc radionuclide generators

Year of study	Activity of ^{44}Ti (MBq)	Yield of ^{44}Sc (%)	Eluate volume (ml)	Separation factor
1967[14]	not given	60-70	30-50	$2 \cdot 10^4$
	not given	60-70	50	10^3 (after 40 elution)
1973[16]	0.037	42-46	30	$5 \cdot 10^4$
This work	185	97	20	$2 \cdot 10^6$

Regarding of long-term stability of ^{44}Ti/^{44}Sc generators, “direct” generator elutions may not be adequate. In comparison, a “reverse” elution strategy definitely guarantees a very low breakthrough of ^{44}Ti. This does not affect the elution yield of ^{44}Sc.

There is a possibility to even improve the distribution profile, to further reduce the ^{44}Ti breakthrough and to extend the generators shelf-life by “reverse” elution with different composition of HCl/$H_2C_2O_4$ solutions.

Finally, the new radionuclide generator design provides stable high-purity elution of significant activities of ^{44}Sc of 180 MBq per elution. In the context of medical applications of ^{44}Sc eluted from the present generator system, its absolute activities are sufficient for initial studies. However, the ^{44}Sc solution that is obtained from generator appears to be too diluted and too acidic for use in direct labelling procedures. Nevertheless, concentrating the ^{44}Sc solution and reducing the acidity in that ^{44}Sc solution may be added on line to the generator performance described.

Acknowledgement

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3.5. Post-elution processing of ^{44}Ti/^{44}Sc generator-derived ^{44}Sc for clinical application

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Keywords: ^{44}Ti/^{44}Sc Radionuclide generator, Post-processing of eluates, Cation exchange chromatography, PET/CT

Summary

The ^{44}Ti/^{44}Sc ($T_{½}$ ^{44}Ti = 60 a) generator provides cyclotron-independent access to positron-emitting ^{44}Sc ($T_{½}$ = 3.97 d) for PET imaging. This work aims to post-elutionprocessing of initial ^{44}Sc generator eluates in order to reduce its volume, HCl concentration and remove the oxalate anions. The on-line adsorption of ^{44}Sc on cationic resin AG 50W-X8 (200–400 mesh, H^+-form) is achieved with >98% efficacy. Subsequently, the purified ^{44}Sc is desorbed by using 3 mL of 0.25 M ammonium acetate (pH = 4.0). The post-processing takes 10 min. The overall yield of the post-processing reached 90%, which is referred to the ^{44}Sc obtained from the ^{44}Ti/^{44}Sc generator. In addition to the chemical purification, the content of ^{44}Ti breakthrough was further reduced by one order of magnitude. The 185 MBq generator finally provides150 MBq of ^{44}Sc containing <10 Bq of ^{44}Ti ready for labeling.

1. Introduction

Positron emission tomography (PET) is one of the rapidly developing molecular imaging technologies. The commonly used positron emitting radionuclide is ^{18}F ($T_{½}$ = 110 min), and ^{18}F-labeled fluorodeoxyglucose is the dominating PET tracer.

Another option to support hospitals with relevant PET radio-nuclides is using radionuclide generators. The decay of a long-lived parent nuclide to a short-lived PET daughter provides an inexpensive and convenient alternative (Rösch and Knapp, 2003). Recently, the $^{68}Ge/^{68}Ga$ generator is turning into an important source of new ^{68}Ga-labeled radiopharmaceuticals for routine clinical use. The generator provides ^{68}Ga ($T_{½}$ = 67.7 min) from the long-lived ^{68}Ge ($T_{½}$ = 270.8 d). While the short half-life of ^{68}Ga permits application of suitable ^{68}Ga activities maintaining an acceptable radiation dose to the patient, it limits application of ^{68}Ga-labeled tracers to the investigation of fast biological processes.

In this context, generator-derived positron emitters with longer physical half-life are needed—such as ^{72}As ($T_{½}$ = 26 h) from the $^{72}Se/^{72}As$ generator, or ^{44}Sc ($T_{½}$ = 3.97 h) from the $^{44}Ti/^{44}Sc$ generator (Rösch and Knapp, 2003). Hosain et al. (1977) has already proposed ^{44}Sc as a PET radionuclide for studying bone disease. ^{44}Sc is a positron emitter with β^+-branching (94.3%), and 99.9% photon emission of 1157.0 keV, generated by ^{44}Ti. The half-life of ^{44}Ti was measured by several groups with results varying from 46.4 to 66.6 years. The most recent studies revealed half-life of 59.2±0.6 years (Ahmad et al., 1998).

The trivalent metal Sc(III) is particularly relevant, as it may be used for labelling of radiopharmaceuticals based on bifunctional chelators, established for coordinating lanthanides such as stable Gd(III) or ^{177}Lu, as well as for radioactive rare earth metals such as ^{90}Y, or radio nuclides like ^{111}In and ^{68}Ga. Due to the increasing medical applications of trivalent radiometals in diagnosis and therapy, the $^{44}Ti/^{44}Sc$ generator could possibly provide an interesting route for PET-imaging using ^{44}Sc labelled tracers. As a β^+-emitter, it could be applied for planning and dosimetric calculations in endoradiotherapy based on the therapeutic radio-nuclides previously mentioned, but also for matching β^--emitting ^{47}Sc radiopharmaceuticals (Mausner et al., 1995).

Several strategies were used to design a $^{44}Ti/^{44}Sc$ generator (Greene and Hillman, 1967; Mirza and Aziz, 1969; Seidl and Lieser, 1973; Schumann et al., 2007). The most recent studies on a $^{44}Ti/^{44}Sc$ generator described the concept and experimental parameters of a 185 MBq (5 mCi)

generator system, utilizing the anion-exchange resin Bio-Rad AG 1-X8 (200–400 mesh, Cl^--form) (Filosofov et al., 2010). ^{44}Sc was eluted with 20 mL of a 0.005 M $H_2C_2O_4$/0.07 M HCl mixture and achieved >97% elution efficacy for ^{44}Sc. The breakthrough of ^{44}Ti, which is the ratio (percentage) of ^{44}Ti eluted referred to ^{44}Ti on the generator column, was a slow as 5×10^{-5}%. Because of its large volume (20mL) and relatively high concentration (0.07 M) of hydrochloric acid, the obtained ^{44}Sc fraction, however, appears not suitable for direct radio-pharmaceutical syntheses. In many cases, it may prevent a fast, reliable and quantitative labelling procedure.

Consequently, the aim of this work was to develop an efficient and simple method to concentrate and purify the ^{44}Sc generator eluates adequate for clinical application. Since reducing of volume of ^{44}Sc in the generator eluate by evaporation of the eluent was inadequate, more sophisticated concentration and purification steps were necessary. A strategy for such an approach relayed on the direct transfer of the initial generator eluate through a cation-exchanger. A similar approach of on-line post-processing of generator eluates was introduced recently for ^{68}Ge/^{68}Ga radio-nuclide generators and proved to be relevant for a versatile labeling strategy (Zhernosekov et al., 2007).

2. Materials and methods

2.1. Chemicals and reagents

Oxalic acid and ammonium acetate of analytical reagent grade were obtained from Merck (Darmstadt, Germany). All other chemicals were pure reagent grade and used as received unless otherwise specified. The ion exchange resins AG 50W-X4 (200– 400 mesh, H^+-form), AG 50W-X8 (200–400 mesh, H^+-form) and Chelex 100 (200–400 mesh, Na^+-form) were purchased at Bio-Rad Laboratories (Richmond, CA, USA). 1,4,7,10-tetraazacyclododе- cane-1,4,7,10-tetraaceticacid (DOTA) conjugated with D-Phe1- Tyr3-octreotide (DOTATOC), GMP-grade, was obtained from piChem R&D (Graz, Austria). Deionized Milli-Q water(18.2 MΩ cm; Millipore) was used in all experiments.

2.2. The $^{44}Ti/^{44}Sc$ radionuclidegenerator

The ^{44}Sc was available from a previously prepared 185 MBq (5 mCi) $^{44}Ti/^{44}Sc$ generator (Filosofov et al., 2010). The ^{44}Sc was eluted with a 20 mL mixture of 0.005 M $H_2C_2O_4$/0.07 M HCl with a flow rate of 1 mL/min. The elution profile was determined by fractionation and measuring of ^{44}Sc and ^{44}Ti activities in each successive fraction of the eluate.

Measurement of ^{44}Sc radioactivities was accomplished in a dose calibrator on the curiemeter M2316 (Messelektronik Dresden GmbH, Germany). The ^{18}F setting was used with a calibration factorof 0.7 to quantify ^{44}Sc on that instrument. The absolute radioactivity of ^{44}Sc and ^{44}Ti was measured by γ-spectrometry using a high-purity germanium (HPGe) well-counter detector using 1157.0 (99.9%) and 1499.5 (0.9%) keV γ-lines for both radionuclides being in decay equilibrium. Measurements of ^{44}Ti breakthrough radioactivity were performed at least 120 h more than 30 half-lives of ^{44}Sc after the elution and the ^{44}Sc measurements. ^{44}Ti radioactivity was measured also at specific photon emissions of 67.9 and 78.3 keV. All radioactive materials were handled according to approved protocols at the Institute of Nuclear Chemistry at Johannes Gutenberg University Mainz.

2.3. Concentration and purification of ^{44}Sc eluate using cation-exchange resin

Post-elution processing studies on ^{44}Sc eluate obtained from the generator were arranged into three main steps. The first step was focused on adsorbing ^{44}Sc from a 0.005 M $H_2C_2O_4$/0.07 M HCl solution on different ion exchange resins, based on K_d values of ^{44}Sc and ^{44}Ti (Filosofov et al., 2010).

Several strong acidic cation-exchange resins were investigated: AG 50W-X4 (200–400 mesh, H^+-form), AG 50W-X8 (200–400 mesh, H^+-form) and Chelex 100 (200–400 mesh, Na^+-form). Resins were suspended in 1 M NaOH for 15min, washed several times with Milli-Qwater, and re-suspended in 1 M HCl and also washed with Milli-Q water. After this procedure, resins were centrifuged and kept under Milli-Q water for further use.

Small plastic syringes were used to prepare miniaturized chromatography columns, which were plugged with polyethylene filters and packed with different amounts of wet resins. Just before use, the packed columns were conditioned by washing with 1 mL of 4 M HCl and 1 mL Milli-Q water. The $^{44}Ti/^{44}Sc$ generator was eluted according to the previously described protocol with a 20 mL mixture of 0.005 M $H_2C_2O_4$/0.07 M HCl (Filosofov et al., 2010).

Using aliquots of this eluate, the retention of the ^{44}Sc on different cation-exchangers was checked by passing 2.55–3.0 mL of the ^{44}Sc solution through pre-treated columns at a flowrate of approximately 1 mL/min at room temperature.

In the second step, different eluate systems were investigated to desorb ^{44}Sc from the optimum cation-exchange resin identified. Miniaturized chromatography columns were prepared by filling small syringes with 80 mg of wet resin AG 50W-X8 (200–400 mesh, H^+-form). The ^{44}Ti/^{44}Sc generator was eluted and ^{44}Sc was adsorbed according to procedures described above. The columns were dried by passing air through them to remove the rest of the generator eluate. Then, columns were washed by 2–4 mL Milli-Q water and dried once again. Next, several solutions at various volumes and concentrations were used to elute ^{44}Sc from the columns.

In the third step, results obtained from the experiments described above were used to on-line adsorb ^{44}Sc from the generator eluate. A miniaturized chromatography column (~2 mm inner diameter, ~5 mm length) was prepared using two 3-way valves (Cole–Palmer Instrument Co., VernonHills, IL, USA) filled with 53 mg of wet AG 50W-X8 (200–400mesh, H^+-form). The ^{44}Ti/^{44}Sc radionuclide generator was connected to the first valve via tubing. The second valve was connected with two capillary tubings directed to the reacting and waste vials, respectively. The ^{44}Sc as eluted with a 20 mL mixture of 0.005 M $H_2C_2O_4$/0.07 M HCl at a flow rate of 1 mL/min was transferred on-line through the miniaturized chromatography column. Subsequently, the column was washed by 2–4 mL Milli-Q water and dried by air. Then, 3 mL of 0.25 M ammonium acetate acidified to pH = 4.0 by drop-wise addition of acetic acid were passed slowly (0.7 mL/min) through the column.

The ^{44}Sc eluate was collected in 11 mL glass vial (Mallinckrodt). Finally, the column was reconditioned with 1 mL of 4M HCl and 1 mL of Milli-Q water. The aliquots of consecutive fractions were collected and measured according to the activity of ^{44}Sc and ^{44}Ti using the dose calibrator and γ-spectroscopy, respectively.

3. Results and discussion

3.1. Elution characteristics

The $^{44}Ti/^{44}Sc$ generator reliably provided 180 MBq of ^{44}Sc with <90 Bq of ^{44}Ti breakthroughduringa1yearperiod.The ^{44}Ti breakthrough with respect to the eluted ^{44}Sc activity was found to be 5×10^{-5}%. The initial ^{44}Sc elution profile and the ^{44}Ti breakthrough are presented in Fig. 1. The highest percentage of ^{44}Sc was eluted in fractions nos. 4–7 (8–14 mL), where as the fractions nos. 1–2 (2–4 mL) contained the highest amount of ^{44}Ti.

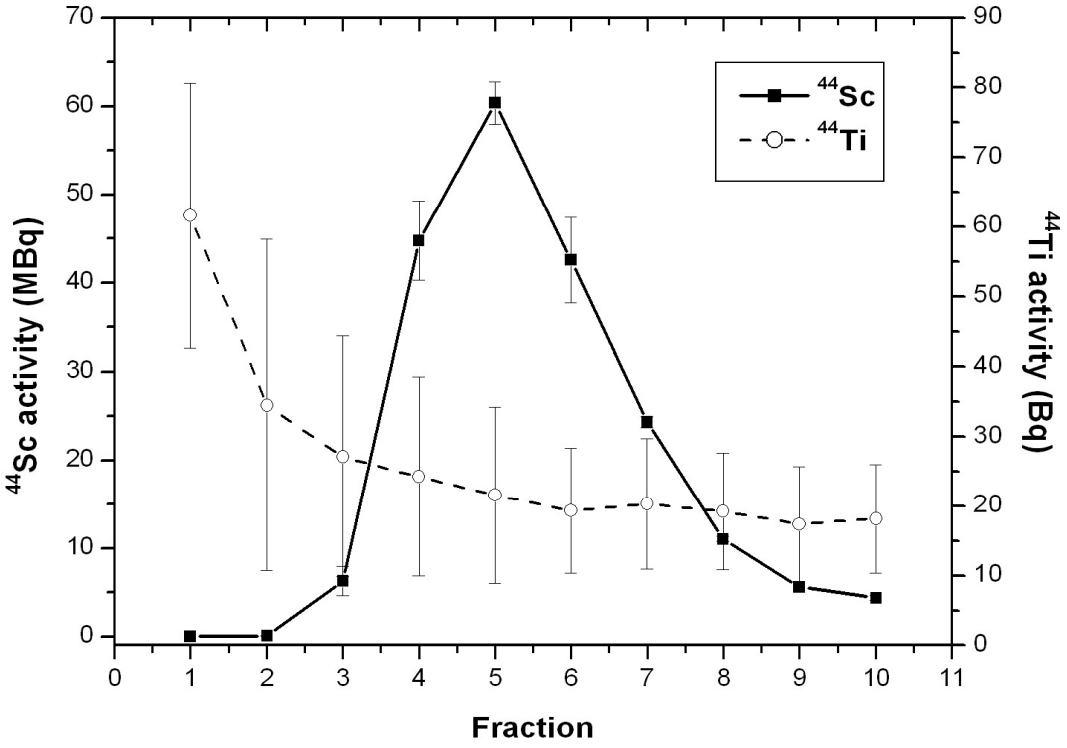

Figure 1. Elution profile of the $^{44}Ti/^{44}Sc$ generator. One fraction is 2 mL, giving a total eluted volume 20 mL of 0.005 M $H_2C_2O_4$/0.07 M HCl solution.

By fractionating the ^{44}Sc eluate, it is possible to obtain approximately 85% of the available activity in a volume of 8 mL (if only fractions nos. 4–7 are taken) of 0.005 M $H_2C_2O_4$/0.07 M

HCl. This volume appears still too large and the content of hydrochloric acid too high for labelling of , e.g., nanomoles of peptides for application in nuclear medicine. Therefore, an efficient post-elution processing with concentration of ^{44}Sc eluate on the cation-exchange resin was developed in this study.

3.2. Concentration and purification of ^{44}Sc

The first step of pre-concentration studies utilized cation-exchange resins to adsorb ^{44}Sc from the generator eluate of 0.005 M $H_2C_2O_4$/0.07 M HCl composition. The results of optimization studies concerning the capabilities of different cation-exchange resins are presented in Table 1. The lowest retention of ^{44}Sc (42%) was observed when Chelex 100 (200–400 mesh, Na^+-form) resin, containing iminodiacetate ions coupled to a styrene divinylbenzene support, was applied. Using the strong cation exchange resin AG 50W-X4 (200–400 mesh, H^+-form) with sulfonate groups on the styrene divinylbenzene matrix increased the retention of ^{44}Sc on the columns up to 51%. Utilization of the AG 50W-X8 (200–400 mesh, H^+-form) based on the same matrix with the same functional groups like AG 50W-X4 (200–400 mesh, H^+-form), but differing in the cross-linkages value, resulted in 89% retention of ^{44}Sc on the resin. Therefore, this resin was chosen for the next studies.

Table 1. Retention of the ^{44}Sc on different cation-exchange resins used in the ^{44}Sc pre-concentration and purification studies.

Resin	Amount(mg)	Retentionof 44Sc (%)
AG 50W-X4	50.0	50.5
AG 50W-X4	160	98.0
AG 50W-X8	50.0	88.7
AG 50W-X8	80.0	96.0
AG 50W-X8	200.0	99.9
Chelex 100	51.0	42.2

In the second step, several miniaturized columns were prepared and filled with 80 mg of the AG 50W-X8 (200–400 mesh, H^+-form) resin. Between 2.55 and 3.0 mL of the ^{44}Sc initial generator eluate was passed through the columns and >98% (n = 80) of ^{44}Sc was adsorbed. An additional purification with 2–4 mL Milli-Q water causes some loss of ^{44}Sc activity (<0.2%).

Then, several solutions were tested for the elution of ^{44}Sc from the column, c.f. Table 2. Addition of a water-mixable organic solvent promotes chloride complex formation of metal cations in the ‘‘outer’’ coordination sphere. Following a procedure of post-processing ^{68}Ge/^{68}Ga generator eluates (Zhernosekov et al., 2007) different mixtures of acetone (90–98%) and HCl (0.05–1.0 M) were tested initially to elute ^{44}Sc from the cation exchange columns. The obtained ^{44}Sc recovery was, however, very low (less than 1%), which is in good agreement with literature data on distribution coefficients of ^{44}Sc at these conditions (Strelow et al., 1971). It seems that only increasing the HCl concentration allows to desorb ^{44}Sc from the cation-exchange resin. Addition of acetone even at higher percentages did not change the general tendency that formation of Sc(III) complexes with chlorides appears at higher concentration of HCl (more than 1 M) (Hart, 1987).

Elution by 1 mL of 4 M HCl or 0.1 M NaOH resulted in higher recovery of ^{44}Sc, i.e. 38% and 55%, respectively. Increasing concentration of acid or base or extending the eluting volume further raised the recovery yield. On the other hand, the obtained final solution will be useless for medical application, because of very low or high pH.

Therefore, solutions containing organic complexing anions were used to remove ^{44}Sc from the cation-exchange resin. Application of 1 mL 0.1 M ethylenediaminetetraaceticacid (EDTA) resulted in 88% recovery of ^{44}Sc. EDTA forms strong complexes with Sc^{3+} ions (log K = 21.84) (Perrin, 1979). It was used, e.g. for eluting ^{90}Y from the ^{90}Sr/^{90}Y generator or ^{68}Ga from the ^{68}Ge/^{68}Ga generator (Mikheev et al., 1975; Hnatowich, 1975). The Me(III)–EDTA complexes, however, had to be destroyed prior to labelling reactions, e.g. by heating in the presence of concentrated acids, because EDTA strongly competed with other ligands for labelling (Skraba et al.,1978; Chinol and Hnatowich, 1987).

Application of only 1 mL of 0.1 M diammoniumoxalate gave the best ^{44}Sc recovery (95%) compared to the other solutions used (Table 2). However, preliminary studies to label DOTATOC with ^{44}Sc as eluted by 1 mL of 0.09 M diammoniumoxalate and added to 4 mL HEPES (4-(2-hydroxyethyl)piperazine-1-ethane-sulfonic acid) buffer (pH = 4.0) containing the peptide, resulted in low synthesis yields of less than 1%. The stability constant of the Sc(III) complex with oxalate anions is quite high (log K_1 = 7.14) and comparable to Ga(III) (log K_1 = 6.45) (Gårdhammar, 1971; Smith and Martell, 1976), and at this concentration of about 18 mM oxalates strongly may compete referred to the labeling of nanomoles of the DOTATOC. This hypothesis was later confirmed by labelling DOTATOC with ^{68}Ga in 4 mL

HEPES buffer in the presence of 1 mL of 0.1 M diammoniumoxalate at pH = 4.0. The labeling reaction yield was around 90% without oxalate solution addition and decreased to ~2% in the presence of oxalate anions (Table 3).

Table 2. Recovery of ^{44}Sc from miniaturized chromatography columns (80 mg of Bio-Rad AG 50W-X8, 200–400mesh, H^+-form) washed by different solutions.

No.	Solution	Volume (mL)	Recovery of ^{44}Sc (%)
1	Acetone (90–98%)/HCl(0.05–1.0M)	1.0	<1.0
2	4.0 M HCl	1.0	37.8
3	0.1 M NaOH	1.0	54.9
4	0.1 M Sodium tartrate	1.0	69.9
5	0.1 M EDTA	1.0	87.6
6	0.1 M Diammonium oxalate	1.0	94.7
7	1.0 M Ammonium acetate	2.0	51.7
8	0.5 M Ammonium acetate/20% EtOH	2.0	45.4
9	0.5 M Ammoniumacetate, pH=4.0	2.0	90.4
10	0.25 M Ammoniumacetate, pH=4.0	3.0	89.2

Table 3. Influence of the eluate solution composition on the labelling yield of DOTATOC with ^{44}Sc and ^{68}Ga. Labeling conditions: pH = 4.0, 95 °C, 15–25min,15–40 mg DOTATOC.

Eluate composition	Eluate volume (mL)	Buffer	Nuclide	Yield (%)
0.09 MDiammoniumoxalate	1.0	HEPES (4mL)	^{44}Sc	0.4
97.56% Acetone+0.05MHCl	0.4	HEPES (4mL)	^{68}Ga	89.1
97.56% Acetone +0.05MHCl +0.1Mdiammoniumoxalate	1.0+0.4	HEPES (4mL)	^{68}Ga	2.3
0.25 M Ammoniumacetate, pH=4.0	3.0	-	^{44}Sc	96.3

Therefore, further studies were focused on ammonium acetate solutions, despite the fact that the recovery of ^{44}Sc by 2 mL of the utilized mixture was slightly lower (Table 2). The stability constant of acetate anions with Sc(III) is much lower (log K = 3.48) (Itoh etal.,1984) than with oxalates, so acetates should not compete so strongly in the labelling reaction. Besides, literature data indicate that acetate solutions are used as buffers in labeling reactions

of radionuclides with biomolecules (Hofmann et al., 2001; Bodeietal., 2003; Buchmannetal., 2007). The ^{44}Sc elution from the miniaturized chromatography columns was optimized according to the composition of acetate solution, concentration and pH. High recovery (~90%) was obtained, when 3 mL of a 0.25 M ammonium acetate buffer acidified to pH = 4.0 by addition of acetic acid, was used.

3.3. Combined protocol: elution of the ^{44}Ti/^{44}Sc generator and post-processing in an on-line module

The performed post-elution studies resulted in the building of an on-line module system of generator post-processing (Fig. 2). The key component of the concentration system is a miniaturized chromatography column that was prepared from two 3-way valves (I and II), filled with 53 mg of AG 50W-X8 (200–400 mesh, H^+-form) resin and connected with the ^{44}Ti/^{44}Sc generator via tubing. The 20 mL of a 0.005 M $H_2C_2O_4$/0.07 M HCl solution passes the ^{44}Ti/^{44}Sc generator with a flow rate of 1 mL/min by using syringe (S) and the eluted ^{44}Sc adsorbs on-line on the small cationic cartridge (valve I in line 1).

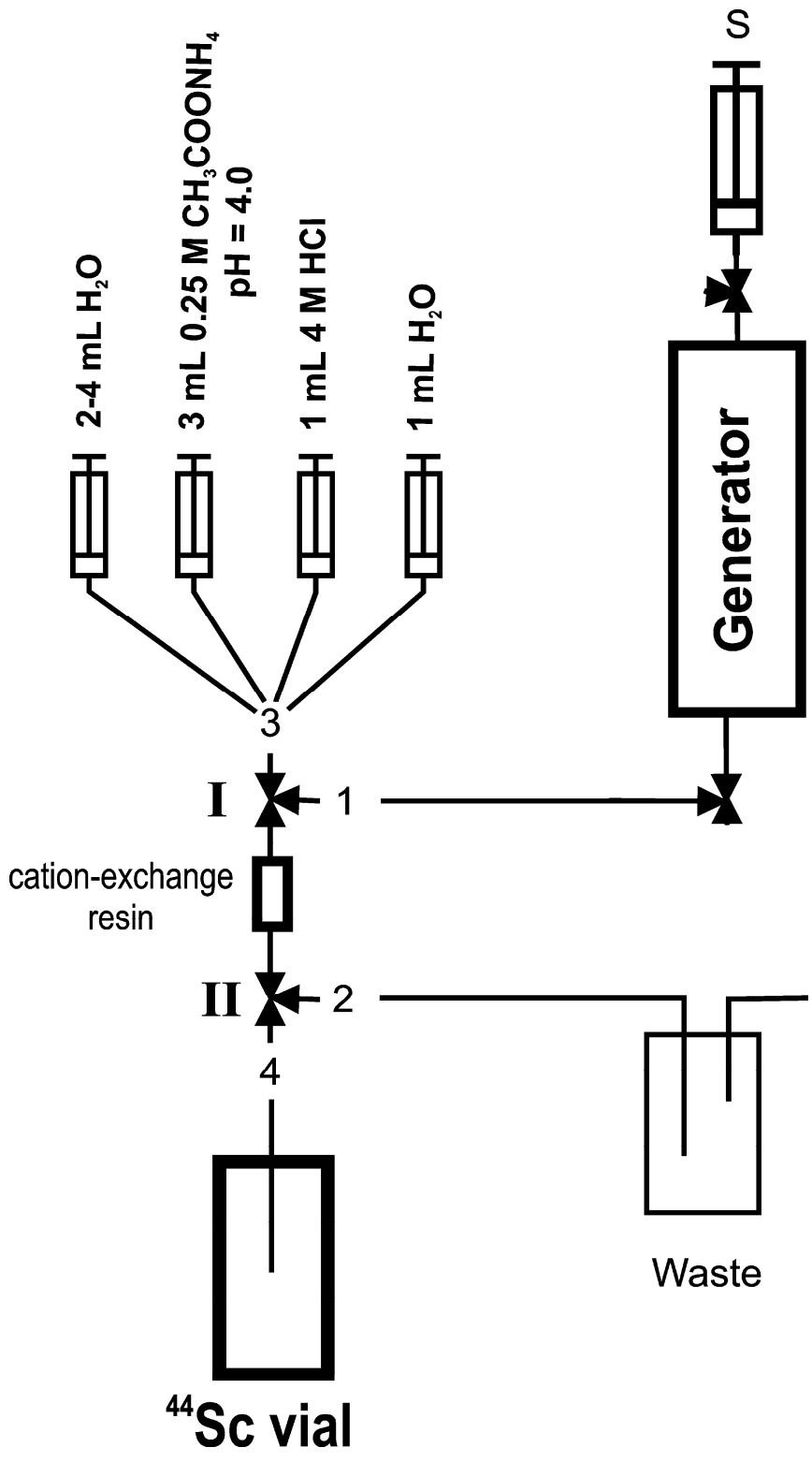

Figure 2. Scheme of the post-elution processing of ^{44}Sc-eluates.

The ^{44}Sc retains on the column, in between the two 3-way valves, whereas the generator eluate continuous to the waste vial (valve II in line 2). Next, valve I is changed to line 3. By using a standard single-use syringe the column with cation-exchange resin is washed by 2–4 mL of H_2O to remove the remaining traces of the initial eluate solution, which are collected in the waste vial as well. Finally, 5 mL air is blown through the column.

After switching the valve II to line 4, the 3 mL of 0.25 M ammonium acetate buffer, pH = 4.0, are slowly (0.7 mL/min) pressed through the column with a 2 min break after every 1 mL. Finally, air is passed through the column to remove tracer of the ammonium acetate buffer solution remainingin the dead volume of the column. The ^{44}Sc is collected in a 11 mL glass reaction vial. Profiles of the ^{44}Sc and ^{44}Ti distribution in every step of the post-processing are presented in Table 4. Changing the valve II to line 2 enables reconditioning of the column by washing with 1 mL of 4 M HCl and finally by 1 mL H_2O.

Table 4. Profile of ^{44}Sc and ^{44}Ti in successive steps of post-elution processing on the miniaturized chromatography column (53 mg of Bio-Rad AG 50W-X8, 200–400 mesh, H^+-form).

No.	Step	Eluent	Volume (mL)	Relative distribution (%)	
				^{44}Sc	^{44}Ti
1	Generator elution into waste	0.005 M Oxalic acid /0.07 M HCl	20	1.0	80.2
2	Resin purification	H_2O	2-4	0.1	2.5
3	^{44}Sc elution	0.25 M Ammoniumacetate, pH=4.0	3	88.0	7.6
4	Washing	4 M HCl	1	9.9	8.2
5	Washing	H_2O	1	0.7	1.5

In the first elution step, >98% of cationic ^{44}Sc was retained on the column, whereas most of the ^{44}Ti content of the initial generator eluate (~80%) passed the column and was transferred to waste. This is considered as an approach to further remove the amount of co-eluted ^{44}Ti breakthrough. When the H_2O fraction (2–4 mL) and air were used to remove the excess of the 0.005 M $H_2C_2O_4$/0.07 M HCl solution remaining on the cation exchanger, elution of both

radionuclides was negligible, i.e. less than 0.1% and around 3% for ^{44}Sc and ^{44}Ti, respectively. Application of 3 mL 0.25 ammonium acetate (pH = 4.0) recovered ~90% of ^{44}Sc without changing the pH of the solution. The amount of ^{44}Ti in the final ^{44}Sc fraction was less than 7 Bq. The initial breakthrough of 5×10^{-5}% was thus further reduced by a factor of 10 reaching a 5×10^{-6}% level. Reconditioning steps, i.e. washing the column with 1 mL of 4 M HCl and 1 mL Milli-Q water, consequently, removed the rest of ^{44}Sc and ^{44}Ti adsorbed on the column and pre-conditioned the resin for another elution. The obtained ^{44}Sc solution in the acetate buffer was ready for labeling chemistry.

4. Conclusions

The recently developed ^{44}Ti/^{44}Sc generator offers the fundamental requirements for radionuclide generators, namely significant differences in the distribution coefficients of mother and daughter radionuclide on the selected resin (Bio-Rad AG 1-X8, 200–400 mesh, Cl^--form) and eluent system (0.005 M $H_2C_2O_4$/0.07 M HCl), high yields of ^{44}Sc elution (>97%) and low ^{44}Ti breakthrough (5×10^{-5}%). In addition, the radiochemical design and the new process of ‘‘reverse’’ elution strategies result in a significant long-term stability of the ^{44}Ti/^{44}Sc generator (Filosofov et al., 2010). However, the large volume of ^{44}Sc initially eluted and the chemical composition of the initial generator eluate appeared in adequate in the context of radiopharmaceutical chemistry, such as the labelling of nanomoles of precursors. Comparable situations have been recently addressed by our approach to on-line post-process ^{68}Ge/^{68}Ga generator eluates, which usually are being obtained in 5±3 mL of 0.1–1.0 M HCl eluates (Zhernosekov et al., 2007).

The similar strategy applied to the ^{44}Ti/^{44}Sc generator also uses an on-line post-processing based on cation-exchanger purification. The Bio-Rad AG 50W-X8 (200–400 mesh, H^+-form) resin showed the best parameters both in terms of on-line adsorbing ^{44}Sc (>98%) and its quantitative recovery is using of 3 mL of 0.25 M ammonium acetate pH = 4.0 system (~90%). The content of ^{44}Ti co-eluted with ^{44}Sc from the ^{44}Ti/^{44}Sc generator of 5×10^{-5}% is further being reduced by a factor of 10. The final content of ^{44}Ti in the 140–160 MBq ^{44}Sc fraction ready for labeling is thus 7 Bq, representing a very low contamination of around $<2\times10^{-7}$.

This chemically efficient post-elution processing of generator-produced ^{44}Sc was adapted to a simple module, which allows a rapid and simultaneous concentration and purification of ^{44}Sc

obtained from generator. The post-elution processing of volumes and impurities is easily compatible with the synthesis of ^{44}Sc-labeled compounds. Thus, the chemically and radiochemically highly pure ^{44}Sc fraction of very high specific volume activity of around 50 MBq/mL representing 150 MBq overall activity for the first time may allow systematic research on the development and application of new ^{44}Sc-labeled compounds. Areas of interest are ^{44}Sc(III) on complex formation, labelling and radiopharmaceutical chemistry of the positron emitter ^{44}Sc, molecular imaging of ^{44}Sc-labeled tracers using PET/CT, eventually investigating even new options of ^{44}Sc molecular imaging by using new PET/3G camera based on β^+/γ decay of radionuclide (Huclier-Markai et al., 2008), and finally potential application of diagnostic ^{44}Sc tracers matching therapeutic applications of analogue compounds labeled with e.g. ^{90}Y or ^{177}Lu, but also with the β^--emitter ^{47}Sc.

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3.6. Radiolabeling of DOTATOC and DOTATATE with the longer-lived, generator-derived trivalent metallic positron emitter ^{44}Sc

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Key words: ^{44}Ti/^{44}Sc radionuclide generator, radiolabeling, DOTATOC, molecular imaging, PET/CT

Abstract

The positron-emitting radionuclide ^{44}Sc with the half-life of 3.93 h and β^+ branching of 94.3% is of potential interest for clinical positron emission tomography (PET). It is available from a ^{44}Ti/^{44}Sc generator, where long-lived ^{44}Ti decays to no-carrier-added (nca) ^{44}Sc, that would allow PET/CT imaging to be independent of a cyclotron. ^{44}Sc(III) as a metallic cation is suitable for complexation reactions with chelators alone or with bifunctional ligands conjugated to peptides or other molecular targeting vectors. As a trivalent metal cation it may be used to synthesize radiopharmaceuticals analogously to currently used radionuclides in diagnosis and therapy, such as ^{68}Ga and ^{111}In or ^{90}Y and ^{177}Lu, as well as for non-radioactive Gd(III). A key question was whether the coordination chemistry of trivalent scandium is equivalent to established tetraaza-based macrocyclic ligands. Thus, the aim of this work was to investigate, as a proof-of-principle, the potential of ^{44}Sc for labeling of DOTA-conjugated peptides. DOTA-D-Phe^1-Tyr^3-octreotide (DOTATOC, where DOTA is the macrocyclic chelator 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-1,4,7,10-tetraacetic acid) was used as the model molecule to study and optimize the labeling procedure. Reaction parameters such as buffer conditions, pH range, reaction temperature and time were optimized. Using 21 nmol of DOTATOC in 2 mL of a processed ^{44}Sc eluate in ammonium acetate buffer pH = 4.0 provided >98% labeling yields within 25 minutes of heating in an oil-bath at 95ºC. This time can be reduced to only 3 minutes by applying microwave-supported heating. The ^{44}Sc-DOTATOC was found to be stable in ethanolic solution, 0.9% NaCl, phosphate buffer with sodium chloride (PBS pH = 7.4), and also in the presence of metal cations (Fe^{3+}, Ca^{2+}, Cu^{2+}, Mg^{2+}), as well as other ligand competitors, like ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepentaacetic acid (DTPA). For comparison was taken DOTA-[Tyr^3,Thr^8]-octreotide (DOTATATE, where DOTA is the macrocyclic chelator 2-[4,7,10-Tris-(carboxymethyl)-1,4,7,10-teraazacyclododecan-1-yl]-acetyl-). This study for the first time experimentally verified that nca ^{44}Sc forms stable complexes with macrocylic ligands containing nitrogen and oxygen donor atoms. The developed method guarantees high yields and safe preparation of injectable ^{44}Sc-labeled radiopharmaceuticals for routine application and it is easy to automate. This may allow follow-up research on labeling and

radiopharmaceutical chemistry of the positron emitter ^{44}Sc(III) and molecular imaging of ^{44}Sc-labeled tracers using PET/CT, making advantage of the 3.9 hours half-life of this positron emitter.

1. Introduction

Positron emission tomography has become a powerful and widely used imaging technology. The clinical application of 2-[^{18}F]fluoro-2-deoxy-D-glucose ([^{18}F]FDG) has expanded remarkably over the last few years. In fact, PET/CT molecular imaging is dominated by the use of cyclotron-produced radionuclides such as ^{18}F and ^{11}C. In comparison, the concept of radionuclide generator-based radionuclides would allow PET/CT diagnoses to be independent of a cyclotron. Recently, the ^{68}Ge/^{68}Ga generator is turning into such an important source of new ^{68}Ga-labeled radiopharmaceuticals for routine clinical use. It provides high (85%) β^+ branching ^{68}Ga ($T_{1/2}$ = 67.7 min) from the long-lived ^{68}Ge ($T_{1/2}$ = 270.8 d) (*1–3*).

In parallel, intense studies focus on the development of new generators providing radionuclides for PET imaging with longer physical half-life, such as ^{72}As ($T_{1/2}$ = 26 h) from the ^{72}Se/^{72}As generator, or ^{44}Sc ($T_{1/2}$ = 3.93 h) from the ^{44}Ti/^{44}Sc system (*4*). Especially, the latter radionuclide generator system is of great interest for dedicated applications. ^{44}Sc decays by 94.3% through positron emission of 1.4 MeV maximum energy. Its parent, ^{44}Ti decays by electron capture and has a long half-life of 59.2 ± 0.6 years (*5*). Several approaches have been investigated in the past to develop the chemistry of ^{44}Ti/^{44}Sc generators (*6–9*). Recently, Filosofov et al. described a 185 MBq (5 mCi) system, providing excellent performance concerning the relevant radiochemical parameters, such as >97% elution efficacy for ^{44}Sc and a very low breakthrough of $<5 \times 10^{-5}$% of ^{44}Ti (*10*). This generator was further studied in the context of post-processing of the generator eluate to provide very pure, high-activity batches of ^{44}Sc in small volumes of aqueous systems applicable to subsequent labeling reactions (*11*).

The generator-derived ^{44}Sc appears to be appropriate in various directions. As a trivalent radiometal it may be used to synthesize radiopharmaceuticals based on bifunctional chelators, established to coordinate currently used other trivalent radionuclides in diagnosis and therapy, such as ^{68}Ga and ^{111}In or ^{90}Y and ^{177}Lu, as well as non-radioactive Gd(III). Due to the increasing medical applications of trivalent radiometals, the ^{44}Ti/^{44}Sc generator could possibly provide a new option for PET/CT imaging. As a relatively longer-lived β^+ emitter, it could be

applied for more accurate planning and dosimetric calculations in endoradiotherapy based on the therapeutic radionuclides mentioned above, but also for direct matching □⁻ emitting ^{47}Sc radiopharmaceuticals (*12*).

Macrocyclic chelators that form very stable complexes with metal cations are of paramount interest for radiopharmaceutical design. DOTA-conjugated peptides, such as the octreotide and octreotate somatostatin analogues are readily labeled with radionuclides such as ^{68}Ga, ^{90}Y, ^{111}In, and ^{177}Lu (*2*, *13*) being widely used for the detection and staging of neuroendocrine tumours. However, PET/CT imaging of these or other (DOTA) conjugated peptides or proteins is restricted to the rather short physical half-life of ^{68}Ga.

Consequently, the aim of this work was to determine optimal conditions for radiolabeling DOTA-peptides using the longer-lived analogue radionuclide ^{44}Sc and DOTA-D-Phe^{1}-Tyr^{3}-octreotide (DOTATOC) as a model reactant. The parameters that influence reaction kinetics: time and temperature of heating, amount of chelate-peptide addition and pH of reaction were investigated. The influence of microwave heating on the time and completeness of the complexation reaction was evaluated and compared with the conventional method of heating in an oil bath. The stability of formed conjugate was checked in pure ethanol, 0.9% NaCl, PBS (pH = 7.4), and in the presence of different metal cations as well as other competing chelators, like EDTA and DTPA.

2. Materials and Methods

2.1. Chemicals and reagents.

DOTATOC (DOTA-D-Phe^{1}-Tyr^{3}-octreotide), where the bifunctional chelator 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-1,4,7,10-tetraacetic acid (DOTA) is conjugated to the N-terminus of the 8 amino acids peptide octreotide (TOC), was obtained GMP-grade from piChem R&D (Graz, Austria) and an aqueous stock solution of 1 mg/mL was prepared. DOTATATE (DOTA-[Tyr^{3},Thr^{8}]-octreotide), where the bifunctional chelator 2-[4,7,10-Tris-(carboxymethyl)-1,4,7,10-teraazacyclododecan-1-yl]-acetyl- (DOTA) is conjugated to the N-terminus of the 8 amino acids peptide octreotide (TATE), was obtained GMP-grade from ABX (Radeberg, Germany) and an aqueous stock solution of 1 mg/mL was prepared. Ammonium acetate, sodium chloride, sodium hydroxide, sodium dihydrogen phosphate and

disodium hydrogen phosphate of analytical reagent grade were obtained from Merck. Hydrochloric acid (Fisher Scientific) and acetic acid (Merck) were of analytical grade. $FeCl_3$, $MgCl_2$, $CuCl_2$ (Merck) and $CaCl_2$ (Fluka), as well as ethylenediaminetetraacetic acid (EDTA) (VWR Prolabo) and diethylenetriamine pentaacetic acid (DTPA) (Fluka Biochemika) were of reagent grade. All other chemicals were pure reagent grade and used as received unless otherwise specified. Deionized Milli-Q water (18.2 MΩ·cm; Millipore) was used in all reactions.

2.2. ^{44}Sc and ^{44}Ti/^{44}Sc generator.

^{44}Sc ($T_{1/2}$ = 3.93 h, β^+ = 94.3%) was available from a ^{44}Ti/^{44}Sc generator system developed in Mainz, with ^{44}Ti ($T_{1/2}$ = 59.2 a) adsorbed onto a column filled with anion-exchange resin Bio-Rad AG 1-X8 (200-400 mesh, Cl^--form). The ^{44}Ti activity on the generator column was 185 MBq (5 mCi). ^{44}Sc was eluted with 20 mL of a 0.005 M $H_2C_2O_4$ / 0.07 M HCl mixture (*10*). This eluate was directly post-processed on a miniaturized column filled with the cation-exchange resin AG 50W-X8 (200-400 mesh, H^+-form) to quantitatively adsorb ^{44}Sc on-line followed by its subsequent desorption by using 2-3 mL of 0.25 M ammonium acetate buffer (pH = 4.0) (*11*). This solution was used for further labeling studies.

Measurement of ^{44}Sc radioactivities was accomplished in a dose calibrator on the Activitätmessgerät M2316 (Messelektrinik, Dresden GmbH). The ^{18}F setting was used with a multiplication factor of 0.7, to count ^{44}Sc on that instrument. The absolute radioactivity of ^{44}Sc was measured by γ-ray spectrometry using a high-purity germanium (HPGe) well counter detector using 1157.2 and 1499.4 keV γ-lines. All radioactive materials were handled according to approved protocols at the Institute of Nuclear Chemistry at Johannes Gutenberg University in Mainz.

2.3. Labeling of DOTATOC and DOTATATE with ^{44}Sc.

^{44}Sc was obtained in 2 mL of 0.25 M ammonium acetate buffer pH = 4.0. Labeling of DOTATOC or DOTATATE with ^{44}Sc was performed by mixing different volumes of DOTATOC or DOTATATE stock-solution (1 mg/mL) with the processed ^{44}Sc eluate in a glass vial and heating the mixtures in an oil bath at different temperatures. Optimization of labeling DOTATOC or DOTATATE with ^{44}Sc was performed varying time-period and temperature as well as mode of heating, addition of various amounts of DOTATOC or

DOTATATE to ^{44}Sc and screening of the pH of the reaction mixtures. Optimization of each parameter was repeated 3–4 times in separate experiments.

The effect of DOTATOC or DOTATATE precursor amounts on the labeling yield was analyzed in solutions at pH = 4.0 with the quantity of peptide being varied from 7 to 28 nmol (10–40 μL). Solutions were heated for 30 min at 95ºC. The influence of the oil bath temperature and the period of heating on the reaction yields was investigated by heating solutions containing the ^{44}Sc eluate in 2 mL 0.25 M ammonium acetate (pH = 4.0) and 21 nmol (30 μL) of DOTATOC or DOTATATE at 40º, 80º, 95º and 115ºC for up to 30 min. The effect of pH on the ^{44}Sc-DOTATOC formation was measured by addition of 100–250 μL of 4 M HCl or 4 M NaOH to the 2 mL of ^{44}Sc in 0.25 M ammonium acetate buffer (pH = 4.0). The 21 nmol (30 μL) of DOTATOC solutions were heated for 30 min at 95ºC. The pH of the reaction mixture was measured before and after the heating.

2.4. Quality control.

Radiochemical analysis of the ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE was accomplished using thin layer chromatography (TLC). The TLC plates (Silica-gel 60, Merck) were developed by three different solutions: a) 0.1 M sodium citrate pH = 4.0; b) mixture of 5% NaCl with MeOH (3:1); c) and slightly modified previous mixture of 5% NaCl with MeOH and 25% NH_3 (3:1:1). Quantitative distribution of radioactivity on TLC plates was measured using an electronic autoradiography system and associated software (Instant Imager, Packard Canberra). The R_f values for the free ^{44}Sc, ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE conjugates were determined. If the silica-TLC plates were developed with 0.1 M sodium citrate pH = 4.0, the uncomplexed ^{44}Sc moved almost with the front of developing solution with R_f = 0.8, whereas the ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE stayed on the spotted place with R_f = 0. Using of 5% NaCl with MeOH (3:1) resulted in the following R_f values: 0 and 0.3 for ^{44}Sc and ^{44}Sc-DOTATOC, respectively. Addition of 1 part 25% NH_3 aq. to 5% NaCl with MeOH (3:1:1) caused the hydrolysis of unchelated ^{44}Sc (R_f = 0), whereas ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE moved with the front of the developing solution (R_f = 0.9).

2.5. Microwave-supported labeling of DOTATOC with ^{44}Sc.

The influence of microwave heating on the time and radiolabeling of DOTATOC with ^{44}Sc was determined under the best conditions found during the chemical optimization in the oil bath-based heating studies. Thus, 21 nmol (30 μL) from the DOTATOC stock solution (1

mg/mL) was added to the 2 mL of ^{44}Sc in 0.25 M ammonium acetate buffer (pH = 4.0). The LabMate microwave unit (CEM, Matthews, NC, USA) was used for these studies. This fully-automated device provides control of the processing time, radiation power, temperature and pressure in a hermetically sealed autoclave. Samples prepared in special pressurized glass vessels (delivered with LabMate) were sealed by Teflon coated cork and put into the apparatus single-mode cavity. The apparatus was set to the PowerMax mode, with the temperature of the sample kept at the fixed value of ~95ºC during the whole period of microwave heating. This was achieved by automatically-controlled changes in the microwave power mode and simultaneously cooling of sample by the compressed-air during heating.

2.6. Purification of ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE.

^{44}Sc-labeled DOTATOC DOTATATE were purified from unreacted ^{44}Sc species by reversed-phase chromatography. The reaction mixture was passed through a small C-18 cartridge (Phenomenex Strata-X Tubes, 30 mg) providing quantitative retention of the chelated and non-chelated radionuclide. After washing the cartridge with 5 mL H_2O, the ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE were recovered with 400 μL of pure ethanol.

2.7. Stability studies.

^{44}Sc-DOTATOC of radiochemical purity >99% as obtained in the 400 μL of pure ethanol, i.e. after the C-18 purification procedure, was used for stability studies. The conjugates stability was investigated in the ethanol for 6 h at room temperature (RT) and for 7 h at 37ºC.

Stability studies of ^{44}Sc-DOTATOC in 0.9% NaCl and PBS (pH = 7.4) were performed by addition 20 μL of purified conjugate in ethanol to 500 μL of pre-warmed 0.9% NaCl or PBS. Solutions were incubated for 22 h at 37ºC.

The stability of ^{44}Sc-DOTATOC was also monitored in solutions containing different metal cations, e.g. Fe^{3+}, Ca^{2+}, Mg^{2+} and Cu^{2+} at concentration levels of 10^{-2} M each. The 20 μL ethanol containing purified ^{44}Sc-DOTATOC was added to 500 μL of an aqueous solution containing one of the metal cations. Solutions were incubated for 25 h at 37ºC.

Studies with DTPA and EDTA were also performed to check the stability of synthesized ^{44}Sc-DOTATOC in the presence of competing chelating ligands. The appropriate aliquots of purified ^{44}Sc-DOTATOC in ethanol were added to adequate aliquots of DTPA or EDTA in physiological salt solution (0.9% NaCl) in such a way that the final molar ratio of DTPA or

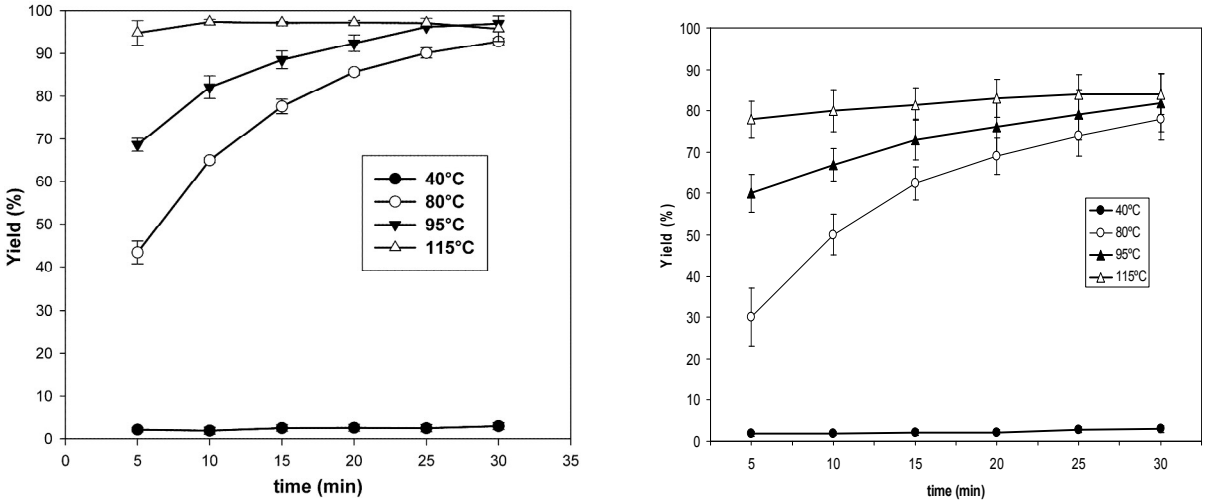
EDTA to conjugate was equal to 1:1; 10:1 and 100:1. The final volume of all solutions was 500 μL. Solutions were incubated at 37ºC up to 25 h.

From all solutions studied aliquots of 1-2 μL were taken at every one-hour time point and analyzed by silica-gel 60 TLC according to procedures described above followed by electronic autoradiography imaging of the TLC plates.

3. Results and discussions

The macrocyclic chelator DOTA, either free or coupled to targeting molecules, forms very stable complexes with many trivalent radionuclides used for molecular imaging or treatment, like ^{68}Ga, ^{90}Y, ^{225}Ac etc. (*14*, *15*), but also with non-radioactive Gd(III) for magnetic resonance imaging (MRI) (*16*, *17*). The thermodynamic stability constants (*logK*) of the complexes formed are several magnitudes higher than in the case of open-chain analogue chelators, like EDTA or DTPA. For example, the stability constants of Gd(III)-DOTA and Gd(III)-DTPA are 25.8 and 22.1, respectively (*18*). A similar situation exists also for Sc(III), with stability constant of 27.0 (*19*) and 20.99 (*20*) for Sc(III)-DOTA and Sc(III)-DTPA, respectively. However, the complex formation with macrocyclic DOTA derivatives generally requires heating at high temperatures in contrast to open-chain analogues.

Therefore, the influence of the temperature on the formation of ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE conjugate was checked by adding 21 nmol (30 μL) of DOTATOC to 2 mL of ^{44}Sc eluates and the samples were heated in an oil bath at various temperatures (40–115ºC) for 5 to 30 minutes (Figure 1a, b). Heating at 40ºC revealed less than 5% yield of the ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE complex even after 30 minutes of heating. Increasing the temperature resulted in higher yields of complex formation. After 5 minutes of heating at 100°C (oil bath temperature 115ºC) gave almost 100% yield. To avoid an intensive bubbling of the aqueous solution and its high vapouring, however, a temperature of 95ºC was selected for further studies.



a b

Figure 1. Formation of ^{44}Sc-DOTATOC (a) and ^{44}Sc-DOTATATE (b) at pH 4.0 as a function of heating at different temperatures.

The overall radiolabeling yield for ^{44}Sc-DOTATOC was >98%, when 21 nmol of DOTATOC was added to 2 mL of the ^{44}Sc eluate (pH = 4.0) and heated in the oil bath for 25 minutes at 95ºC (Figure 2a). Further increasing amounts of the peptide up to 28 nmol did not influence the reaction yield. In contrast, variable radiochemical yields were obtained if ~14 nmol of DOTATOC were used. At 7 nmol of DOTATOC the labeling yield was less than 20%.

The overall radiolabeling yield for ^{44}Sc-DOTATATE was >80%, when 21 nmol of DOTATATE was added to 2 mL of the ^{44}Sc eluate (pH = 4.0) and heated in the oil bath for 25 minutes at 95ºC (Figure 2b). Further increasing amounts of the peptide up to 28 nmol almost did not influence the reaction yield. In contrast, variable radiochemical yields were obtained if ~14 nmol of DOTATATE were used. At 7 nmol of DOTATOC the labeling yield was less than 15%.

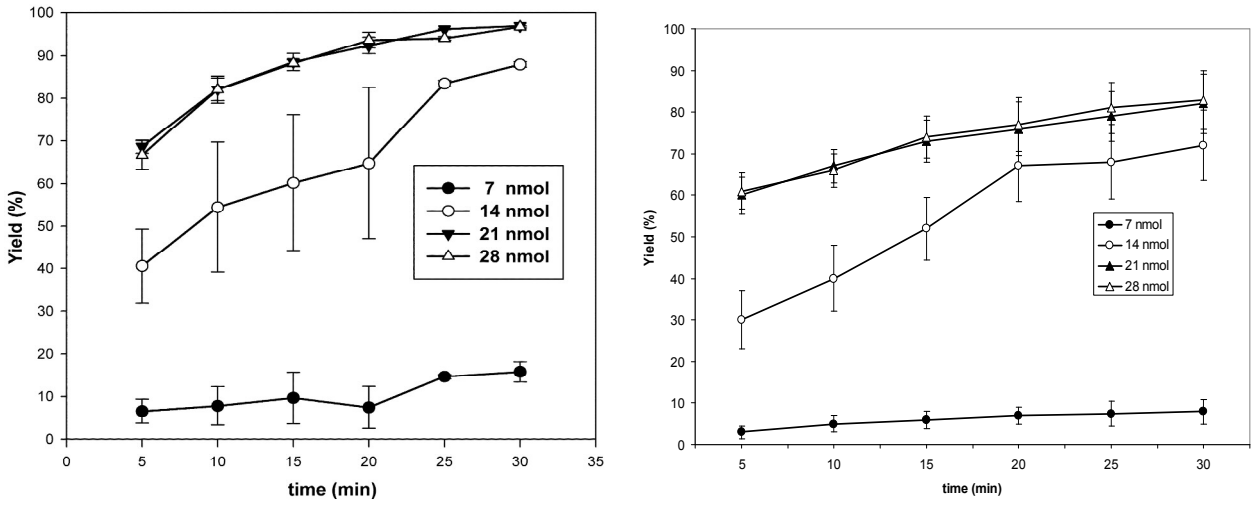


Figure 2. Time course of ^{44}Sc complexation reaction with different amounts of DOTATOC (a) and DOTATATE (b) at pH 4.0.

Labeling of DOTA and DOTA-conjugated derivatives with medically useful radionuclides is generally performed at pH = 2–6 (*13*, *21–23*). Scandium belongs to the transition metals group and its complex formation should strongly depend on the pH of the aqueous solution. Therefore, the pH was considered to be an important parameter during the optimization studies. The original pH of 4 of the 2 mL ^{44}Sc eluate derived from cation-exchange miniaturized column was thus changed in the range of pH 1 to 5 by drop-wise addition of 100–250 μL of 4 M HCl or NaOH. After addition of 21 nmol of DOTATOC, samples were heated at 95ºC for various periods. The highest reaction yield was obtained, when the pH was kept between 3 and 4 (Figure 3). Increasing the pH up to 5 caused slowly decreasing labeling yields due to the hydrolysis of ^{44}Sc(III). Acidifying the solution to pH< 2 resulted in an extreme drop of labeling yields, probably due to the protonation of the DOTA chelator. This study confirmed that the previously developed method of post-elution processing of ^{44}Sc generator eluate gave not only the best results of radionuclide desorption from miniaturized column, but also that the obtained 2–3 mL ^{44}Sc fraction in acetate buffer (pH = 4.0) is simultaneously adequate for direct labeling of DOTA-conjugated molecular tracers without a necessity of further changing its pH (*11*).

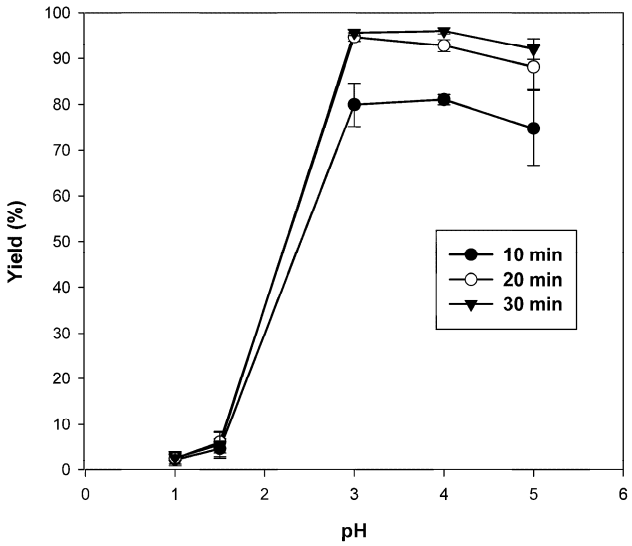


Figure 3. Formation of ^{44}Sc-DOTATOC as a function of pH (21 nmol of DOTATOC, T = 95ºC).

One of the most crucial factors for radiolabeling procedures with short-lived radionuclides is the time needed for certain processes, especially for the heating of samples. Microwave-assisted synthesis is commonly used in organic chemistry because it enhances chemical yields by reducing reaction periods without causing major degradation or introducing undesired reactions and it also improves reproducibility (*24*). This technique has been used for labeling organic molecules with ^{18}F (*25*, *26*), but also in synthesis and complex formation with other radiohalogens such as ^{123}I, ^{131}I (*27*, *28*). Microwave heating has already shown its potential in speeding up ^{68}Ga complexation with DOTA- and NOTA-conjugated oligonucleotides and peptides (*29*, *30*). The influence of microwave heating on the ^{44}Sc-DOTATOC synthesis is demonstrated in Table 1. After 1 minute of microwave heating, the reaction yield was >95% and increased up to 98% during next 2 min. It was thus confirmed that microwave heating of the reaction mixture considerably shortened the reaction time and improved the reproducibility; the relative standard deviation (RSD) values were < 2%.

Table 1. Influence of microwave heating on the yield of ^{44}Sc-DOTATOC (pH = 4.0, 95ºC, 21 nmol peptide).

Time (min)	Yield (%)	± SD (%)
1	95.7	± 2.0
3	97.1	± 1.4
5	97.9	± 0.9
10	97.6	± 1.3
15	98.2	± 0.8

Summarize the optimization studies, we identified that addition of 21 nmol of DOTATOC or DOTATATE to 2 mL ^{44}Sc eluate of 0.25 M ammonium acetate buffer at pH = 4.0 and heating the mixture at 95ºC for 25 minutes (oil bath) or 3 minutes (microwave) provided labeling yields >98% and >80%, respectively.

In both cases of ^{44}Sc-DOTATOC formations, i.e. conventional and microwave heating, the radiochemical purity was >98% and additional purification of the product was not necessary. However, in case of lower yields or studies that require removing of acetate ions, the purification can easily be performed on a reverse-phase (RP) C-18 column. In these studies, the purification of ^{44}Sc-DOTATOC was performed on the RP C-18 mini-cartridge Strata-X (30 mg) to separate the labeled peptide from unreacted ^{44}Sc and to transfer the final conjugate from 0.25 M ammonium acetate buffer to e.g. physiological salt solution. The solution of ^{44}Sc-DOTATOC was passed through a small C-18 cartridge and washed by 5 mL of water. The conjugate was recovered from the cartridge with 400 μL of pure ethanol with ~94% efficacy and containing less than 0.9% of free ^{44}Sc. Thus, a radiochemically pure product, not depending on the initial radiolabeling yield, is guaranteed. The ethanolic fraction can be either evaporated by flushing stream of nitrogen at room temperature during 10–15 minutes or directly added to physiological solutions (0.9% NaCl). Following sterilization by filtration through a 0.22 μm membrane, this ^{44}Sc-DOTA-peptide solution is available for biological or medical studies.

PET radiopharmaceuticals are particularly susceptible to self-radiolytic decomposition or autoradiolysis, because they are produced in high radioactive quantities and specific activities. The control of self-irradiative decomposition is possible e.g. by addition of ethanol, which scavenging action of reactive species is well known (*31*). Therefore, when around 140 MBq of ^{44}Sc-DOTATOC of high purity (>99%) was obtained in pure ethanol as a 400 μL fraction,

its stability was checked at room temperature and 37ºC for up to 7 h (Figure 4). The studies performed indicated that ^{44}Sc-DOTATOC is stable under these conditions. Thus, it is possible to label DOTATOC with ^{44}Sc in one place and later, after purification, to transport it to another place, where it can be accessible for further studies e.g. biological or imaging applications.

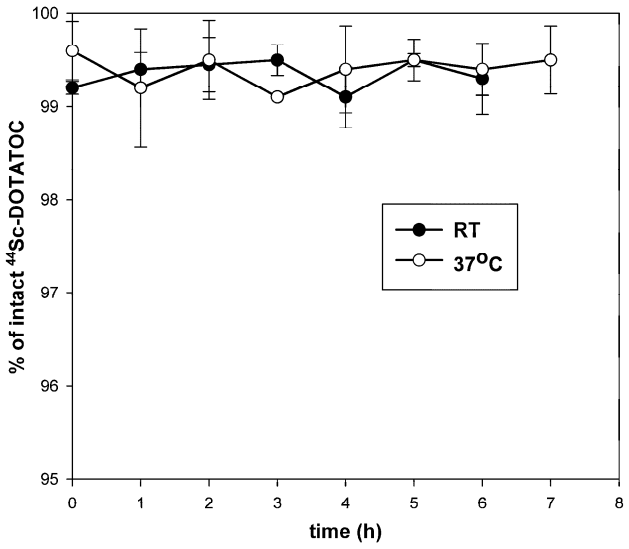


Figure 4. Stability of ^{44}Sc-DOTATOC in pure ethanol at room temperature and at 37ºC.

Stability of the labeled radiopharmaceutical *in vitro*, especially at physiological conditions, is one of the important parameters before its application *in vivo*. Therefore, the stability of ^{44}Sc-DOTATOC was analyzed in 0.9% NaCl and in PBS (pH = 7.4). The results presented in Figure 5 indicate high stability of the formed conjugate even after 22 h incubation at 37ºC.

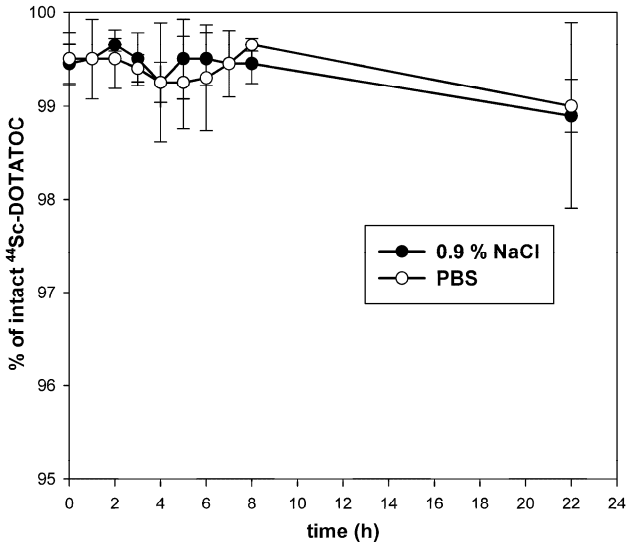


Figure 5. Stability of ^{44}Sc-DOTATOC in 0.9% NaCl and PBS (pH = 7.4) at 37ºC.

It was proved that different metals cations present in the reaction mixture (originating from chemical reagents, target materials in the case of accelerator produced radionuclides or as products of radionuclide decay) may compete strongly with medically useful radionuclides in labeling with DOTA or DOTA-conjugated derivatives (*13*, *32*). For example, ^{111}Cd formed from decay of ^{111}In is a competitor for ^{111}In incorporation in the DOTA-chelator. Analogously, $^{67/68}$Zn present from a target (^{68}Zn) or as a decay product (^{67}Zn) compete with ^{67}Ga incorporation and decrease the specific activity (SA) of the final conjugate. Amounts of some metals cations in human serum (HS) can cause transmetallation of radionuclide-conjugates and finally induce a release of the free radionuclide into the blood pool. Therefore, it was interesting to determine whether ^{44}Sc-DOTATOC is stable in the presence of relevant metal cations. The addition of Fe^{3+}, Cu^{2+}, Ca^{2+} and Mg^{2+} did not induce any removal of ^{44}Sc from the conjugate (Table 2). The ^{44}Sc-DOTATOC was stable even after 25 h incubation at 37ºC with metal cations at rather high concentration levels of around 10^{-2} M.

Table 2. Stability of ^{44}Sc-DOTATOC at 37ºC in the presence of different metals cations at 10^{-2} M concentration.

Time (h)	% of intact ^{44}Sc-DOTATOC ± SD			
	Fe^{3+}	Cu^{2+}	Ca^{2+}	Mg^{2+}
0	99.0 ± 0.3	99.5 ± 0.1	99.1 ± 0.3	99.6 ± 0.2
2	98.8 ± 0.1	99.3 ± 0.7	98.8 ± 0.2	99.3 ± 0.6
4	99.2 ± 0.1	99.6 ± 0.3	99.2 ± 0.2	99.6 ± 0.1
6	99.2 ± 0.3	99.5 ± 0.1	99.5 ± 0.2	99.7 ± 0.1
25	98.5 ± 0.7	99.2 ± 0.6	99.6 ± 0.1	99.6 ± 0.3

Similarily, the stability of ^{44}Sc-DOTATOC in the presence of other complexing agents, like EDTA and DTPA was investigated to check if ligand-ligand substitutions occur. This was not the case; the experimental studies confirmed high stability of ^{44}Sc-DOTATOC even after 25 h incubation at 37ºC with EDTA or DTPA ligands at molar ratios of competing ligand to DOTA-peptide equal to 1:1, 10:1 and 100:1 (Table 3).

Table 3. Stability of ^{44}Sc-DOTATOC at 37ºC in the presence of EDTA or DTPA at different molar ratios of competing ligand to DOTA-peptide.

Time (h)	% of intact ^{44}Sc-DOTATOC ± SD					
	EDTA			DTPA		
	1/1	10/1	100/1	1/1	10/1	100/1
0	98.6 ± 0.3	98.7 ± 0.1	98.6 ± 0.1	99.1 ± 0.2	98.9 ± 0.4	98.6 ± 0.0
0.5	98.8 ± 0.0	98.6 ± 0.1	98.7 ± 0.4	98.8 ± 0.1	98.5 ± 0.8	99.1 ± 0.0
1	99.0 ± 0.2	98.9 ± 0.3	98.6 ± 0.6	99.2 ± 0.1	98.8 ± 0.3	99.1 ± 0.2
2	99.0 ± 0.1	99.0 ± 0.1	98.8 ± 0.1	99.0 ± 0.3	98.7 ± 0.0	98.7 ± 0.4
3	98.8 ± 0.2	98.6 ± 0.4	99.0 ± 0.1	99.0 ± 0.2	98.3 ± 0.4	98.9 ± 0.0
4	98.8 ± 0.1	98.2 ± 0.8	98.9 ± 0.2	99.0 ± 0.0	98.8 ± 0.1	99.0 ± 0.0
5	98.7 ± 0.2	98.5 ± 0.1	98.8 ± 0.6	98.9 ± 0.2	98.6 ± 0.1	99.0 ± 0.0
6	98.5 ± 0.3	98.9 ± 0.1	98.4 ± 0.6	99.1 ± 0.1	98.7 ± 0.9	99.1 ± 0.3
7	99.0 ± 0.2	98.8 ± 0.3	98.5 ± 0.1	98.8 ± 0.4	99.0 ± 0.3	99.1 ± 0.1
8	99.1 ± 0.1	99.0 ± 0.1	98.7 ± 0.1	98.8 ± 0.0	99.1 ± 0.1	99.0 ± 0.2
25	98.6 ± 0.3	97.9 ± 1.8	97.9 ± 1.6	97.6 ± 0.9	97.4 ± 2.2	97.6 ± 1.6

4. Conclusions

Methods for labeling DOTA-conjugated peptides with the new PET radionuclide ^{44}Sc have been investigated systematically. Special efforts have been focused on optimization of reaction parameters and stability studies of the obtained conjugate. Incorporation of ^{44}Sc(III) into DOTATOC was almost quantitative (>98%) at pH = 4.0 after 25 minutes heating in an oil bath at 95ºC. This time can be significantly reduced to only 3 minutes when microwave heating is adopted for synthesis. Incorporation of ^{44}Sc(III) into DOTATATE was >80% at pH = 4.0 after 25 minutes heating in an oil bath at 95ºC.

Isolation of the pure ^{44}Sc-labeled product is easily performed on reverse-phase C-18 mini-cartridges (Strata-X) and results in radiochemically pure ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE in 400 µL of pure ethanol. ^{44}Sc-DOTATOC is stable in pure ethanol, 0.9% NaCl, PBS (pH = 7.4) and also in the presence of metal cations (Fe^{3+}, Cu^{2+}, Ca^{2+} and Mg^{2+}), as well as other competing ligands, like EDTA and DTPA.

The whole synthetic process guarantees safe preparation of ^{44}Sc-DOTATOC (or other ^{44}Sc-DOTA-labeled radiopharmaceutical) for routine application and can be successfully used in a clinical environment.

This radiochemically highly pure and chemically stable ^{44}Sc-DOTATOC for the first time may allow follow-up research on PET/CT imaging with this new trivalent metallic positron emitter. It may stimulate new directions on developing radiopharmaceuticals based on the longer-lived positron emitter ^{44}Sc in order to cover imaging periods of almost one day. A specific field may be the application of diagnostic ^{44}Sc tracers matching therapeutic applications of analogue compounds labeled with e.g. ^{90}Y or ^{177}Lu, but also with the β^- emitter ^{47}Sc. In addition, molecular imaging of ^{44}Sc-labeled tracers by means of a new PET/3G camera based on the β^+/γ emission of this radionuclide is discussed (*33*).

Acknowledgments

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3.7. DOTA-BN[2-14]NH_2 labeled with ^{68}Ga & ^{44}Sc PET tracers. *In vitro* and *animal studies* evaluation.

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Keywords: Scandium-44, Gallium-68, PET tracers, Bombesin analogue, GRP receptors affinity

Abstract

Aim: The *in vitro* and *in vivo* results of the labeled DOTA-Bombesin derivative DOTA-BN[2-14]NH_2 (*DOTA-QRLGNQWAVGHLM-NH2)* with either ^{90}Y or ^{177}Lu, revealed differences which could be attributed to the influence of metal ion coupled to peptide through the DOTA chelator. In this paper we have been investigating if ^{44}Sc and ^{68}Ga labeling of DOTA-BN[2-14]NH_2 has similar influence on the receptor affinity of radiolabelled peptide. ***Methods:*** The ^{68}Ga and ^{44}Sc labeling conditions were studied in order to achieve high specific activity of radiolabelled peptide. The stability of the radiolabel was also investigated. The binding affinity of ^{nat}Sc-DOTA-BN[2-14]NH_2 and ^{nat}Ga-DOTA-BN[2-14]NH_2 to GRP receptors was studied in competition to [^{125}I-Tyr^4]-Bombesin using the PC-3 cells (human prostate cancer). The internalization/efflux of ^{68}Ga- and ^{44}Sc-DOTA-BN[2-14]NH_2 was investigated in the same cell line. Biodistribution of ^{68}Ga- and ^{44}Sc-DOTA-BN[2-14]NH_2 was investigated in normal rats. Small animal PET images were assessed in male Copenhagen rats bearing the androgen-independent Dunning R-3327-AT-1 prostatic cancer tumor. ***Results:*** The labeling was carried out at 95°C for 10-20 min with yields >80%. The specific activity achieved was 7.5 GBq/μmol and 4.8 GBq/μmol for ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2, respectively. The receptor affinity of ^{nat}Ga-DOTA-BN[2-14]NH_2 (IC_{50}(nM): 0.85 ± 0.06) was higher than that of ^{nat}Sc cold complex (IC_{50}(nM): 6.49 ± 0.13*)* in PC-3 cells. The internalization rate of ^{68}Ga-DOTA-BN[2-14]NH_2 was slower than for ^{44}Sc-labeleld analogue but the percentage of internalization at the end of incubation was at comparable level, while ^{68}Ga-DOTA-BN[2-14]NH_2 was externalized faster than ^{44}Sc-DOTA-BN[2-14]NH_2. The biodistribution studies of both ^{44}Sc-DOTA-BN[2-14]NH_2 and ^{68}Ga-DOTA-BN[2-14]NH_2 in normal rats revealed a specific uptake in target organs and tissues and excretion mainly through urinary tract. MicroPET images of rat prostate carcinomas demonstrated that both tracers were accumulated in the tissue with both tracer showing similar distribution patterns. Also the uptake kinetics was not different between the ^{44}Sc- and the ^{68}Ga-labeled compound. ***Conclusions:*** The DOTA-BN[2-14]NH_2 could be labeled with ^{68}Ga and ^{44}Sc with satisfactory yields. Although Sc has been shown to form stable complexes with DOTA, the differences in receptor affinity and

internalization/externalization rate were in favor of Ga-labeled compound. This is however not limiting the future use of $^{44}Sc/^{47}Sc$ in diagnostic and therapeutic applications. The preliminary *in vivo* studies confirmed the diagnostic potential of the ^{44}Sc labeled compound.

1. Introduction

In recent years the Peptide Receptor Radionuclide Therapy (PRRT) is utilizing synthetic peptides as vectors for radionuclides such as ^{90}Y and ^{177}Lu. This has been accomplished with the aid of Positron Emission Tomography (PET) which involves the same vector molecules labeled with positron emitters, which was first demonstrated with ^{68}Ga labelled somatostatin analogues for diagnostics imaging of neuroendocrine tumors. This is turn resulted in the growing interest in other positron emitters obtained in generator systems described in the overview by Welch M.J and McCarthy T.J [1] as well as by Roesch F. and Knapp F.F [2]. So far the ^{68}Ge/^{68}Ga generator remains the most popular PET generator, it is also commercially offered by several manufacturers. It provides ^{68}Ga ($T_{½}$ = 67.71 min) from the long-lived ^{68}Ge ($T_{½}$ = 270.8 d) which subsequently decays to stable ^{68}Zn.

Recent developments have provided a high-performance, 5 mCi ^{44}Ti/^{44}Sc radionuclide generator [3]. ^{44}Sc is a positron emitter radionuclide (E_{γ} ¼ 511 keV, I_{γ} 188.68%, $E_{\beta+}$ 1475.3 keV, $I_{\beta+}$ 94.34%), with a half life of 3.97 h. The use of ^{44}Sc with half life more than 3 times longer than that of ^{68}Ga may be an useful alternative not only for diagnostic purposes but also for dosimetry studies and further therapy planning with the use peptides labeled with the β^- emitting ^{47}Sc as a radiotherapeutic agents [4].

The chemistry of Sc^{+3} is similar to that of the Lanthanides, or the "Lanthanide like" elements. Due to its small ionic radius it is also chemically similar to Aluminium and Gallium [5]. Hence chelators developed for the complexation of Gallium and the Lanthanides could be used as well for the complexation of Scandium, the thermodynamic stability constants of the above M^{+3}-DOTA complexes are of similar [6].

Generally small neuropeptides, such as Somatostatin (SST) and Gastrin Releasing Peptide (GRP)/BN analogs, labeled with gamma- and/or β^--emitting radionuclides have been, and are still being investigated for their ability to bind to receptors which are overexpressed in a variety of malignant tissues [7, 8, 9]. The affinity to these receptors of the designed chelator- peptide construct may vary depending on the metal incorporated into the complex [10].

The present study focused on the comparison of *in vitro* and *in vivo* properties of ^{44}Sc and ^{68}Ga labeled DOTA chelated Bombesin (BN) analog DOTA-BN[2-14]NH_2 (*DOTA-*

QRLGNQWAVGHLM-NH_2). This certain analog was labeled with ^{90}Y and ^{177}Lu and both complexes were compared *in vitro* and *in vivo*. The comparison revealed differences in the *in*

vitro and *in vivo* behavior of both complexes, which could be attributed to the influence of metal on the complex receptor affinity [11]. Hence, DOTA-BN[2-14]NH_2 with its affinity to GRP receptors has been identified as a suitable model molecule for in vitro assays and animal evaluation.

2. Materials and Methods

2.1. Chemicals and quality control techniques

2.1.1. Chemicals

DOTA-BN[2-14]NH_2 was synthesized by standard Fmoc solid phase synthesis on Rink Amide Resin as described previously [11, 12]. Briefly, starting from α-fluorenyl-methoxycarbonyl (Fmoc) the amino acids [Met, Gln, Arg, Leu, Gly, Asn, Trp, Ala, Val, Gly, His, Leu] were coupled and then the terminal DOTA-tris (t-Bu-ester) (Macrocyclics, USA) was conjugated. The purity and identity of the peptide was confirmed by HPLC and Electron Spray Ionization-Mass Spectroscopy (ESI-MS).

$^{68}GaCl_3$ was eluted from a commercially available $^{68}Ge/^{68}Ga$ generator (Cyclotron Co. Ltd.) using 0.05 M HCl/acetone (2:98) and the post-elution purification method [2, 13, 14].

$^{44}ScCl_3$ was eluted with 3 mL of 0.25 M ammonium acetate pH = 4 from a pilot $^{44}Ti/^{44}Sc$ generator working in “reverse” elution mode, as previously reported [2, 3].

^{125}I-[Tyr^4]-BN was purchased from Perkin-Elmer Life and Analytical Sciences. All other chemicals and materials were used as supplied and were of analytical grade unless otherwise stated.

2.1.2. High Pressure Liquid Chromatography (HPLC)

HPLC system was equipped with a UV- VIS detector (Dionex UVD170U UV-Detector), as

well as with a well-type radioactivity detector (Gabi with NaI Detector, Raytest) connected in series, the HPLC pump (Dionex P680) and a reverse phase C-18 column (Macherey Nagel ET 125/4 Nucleosil 100-5 C18 AB) were used with solvent A: 0.1% TFA/H_2O and B: Acetonitrile in the isocratic elution of 75% Solution A/ 25% Solution B at 0.6 ml/min flow rate.

2.1.3. Solid Phase Extraction (SPE)

The purification of the labeled compounds was performed using preconditioned C-18 columns (Strata-X, 1 mL tube, 30 mg resin) (1 mL ethanol and 1 mL H_2O). The sample was loaded on the cartridge followed by 2 mL H_2O (to elute non-bound radiometal) and by 400-500 μL pure ethanol (radiolabeled peptide fraction). The radioactivity of each fraction and the SPE cartridge (which retained colloidal residue) were measured in a well type γ-counter. C-18 columns (100mg resin, Sep-Pak, Waters) were used for the purification of the cold metal complexes using as eluents 0.9% NaCl (non-bound metal fraction) and methanol (cold complex fraction). The cold complexes were analyzed by HPLC before and after purification. The purified samples were also analyzed by Electron Spray Ionization-Mass Spectrum (ESI-MS). The methanol solutions were dried under vacuum giving a light yellow powder in both cases.

2.1.4. TLC:

Thin Layer Chromatography-Silica Gel strips (ITLC-SG, 60 F_{254}, Merck) and 0.1 M sodium citrate as developing solution were used. Under these conditions the radiolabeled peptide remains at the spot (R_f = 0.0), and non-bound $^{68}GaCl_3$ and $^{44}ScCl_3$ move with the solvent (R_f = 0.9-1.0). Quantitative distribution of radioactivity on TLC plates was measured using an electronic autoradiography system and associated software (Instant Imager, Packard Canberra, USA)

2.1.5. Measurements

In the *in vitro* studies the samples attained from the binding affinity studies experiments were measured using the dose calibrator LKB WALLAC 1272 CLINIC GAMMA, while the internalisation/externalisation studies were measured using the automatic gamma counter (2470 Wizard^2, Perkin Elmer). The microPET camera used for the imaging of toumor bearing rats was Siemens microPET Focus 120 Scanner.

2.2. ^{68}Ga and ^{44}Sc labeling of DOTA-BN[2-14]NH$_2$

2.2.1. ^{68}Ga-DOTA-BN[2-14]NH:

100-150 MBq of on line processed ^{68}Ga in 0.4 mL solution of 0.05 M HCl/acetone [13, 14] was added in the reaction vial containing 5 mL H_2O and the peptide (50 µL of 1 mg/mL, 26.3 nmol), pH=2. The reaction mixture was incubated at 95°C for up to 25 min to the final volume of about 3 mL, pH=2. The 5µL aliquots of reaction mixture were taken for quality control by TLC at 1, 3, 10, 15, 20 and 25 min.

2.2.2. ^{44}Sc-DOTA-BN[2-14]NH$_2$

150-200 MBq ^{44}Sc (in 3 ml of 0.25 M ammonium acetate, pH=4) was added to 26.3 nmol DOTA-BN[2-14]NH_2 (0.5 mg/mL in 0.25 M ammonium acetate, pH=4). The reaction mixture was incubated at 95 °C for up to 25 min to the final volume of about 1.5 mL, pH=4.0. The 5µL aliquots of reaction mixture were taken for quality control by TLC at 1, 3, 10, 15, 20 and 25 min.

After completing incubation the samples were purified by SPE and their radiochemical purity was checked by HPLC, TLC. Both ^{68}Ga- and ^{44}Sc-labeled DOTA-BN[2-14]NH_2 were used in further studies after SPE purification.

2.2.3. ^{nat}Ga and ^{nat}Sc cold complexes with DOTA-BN[2-14]NH$_2$

The cold metal complexes of the peptide were synthesized and identified according to the method previously described [11, 15]. Briefly 100µg of peptide was dissolved in 250 µL ammonium acetate 0.4 M, pH=5. Then 250 µL of ascorbic acid (100mg/ml) was added. The suitable volume of either $ScCl_3$ or Ga_2Cl_4 solution (1mg/mL 0.05 M HCl) was added to obtain the molar ratio of peptide to metal of 1:5. The samples were incubated at 95°C for 25 min and at the end of incubation left to cool down to room temperature. The identification and purity were checked by analytical HPLC and MS analysis.

2.3. In vitro studies

The *in vitro* studies were performed according to standard protocols and followed those used in comparative evaluation of ^{90}Y and ^{177}Lu labeled DOTA-BN[2-14]NH_2 [11].

2.3.1. Serum stability study

After adding 50 µl of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 each to 450 µL of freshly separated human serum, the mixture was incubated at 37°C. Samples for radiochemical purity assessment were taken at 30 min, 1 h and 2 h. At each time point a 50 µL aliquot was added in 50 µL pure ethanol and the sample was centrifuged for 3 min at 14000 rpm. 50 µL of the supernatant was diluted with 50 µL of water. The radiochemical purity of such obtained sample was assessed by TLC.

2.3.2. Cell culture

The human androgen–independent prostate carcinoma cell line PC-3 (provided by ATCC, Cat. No: CRL-1435) was used for the *in vitro* experiments. This specific cell line expresses the GRP receptor subtype known as BB2 [16]. PC-3 cells were cultured in DMEM (Gibco Invitrogen) supplemented with 10% fetal calf serum, FCS (Gibco Invitrogen), a mixture of antibiotics (streptomycin 100 µg/ml, penicillin100 U/ml, Sigma Aldrich) and glutamax (Gibco Invitrogen). The cells were kept in humidified atmosphere at 37°C in 5% CO_2. The cells were fed every two days and sub-cultured by trypsinization (0.5%Trypsin-EDTA, Gibco Invitrogen) when the cells have covered about 80% of the surface in the flask.

2.3.3. In vitro cell binding studies

The *in vitro* receptor binding affinities of the ^{nat}Ga-DOTA-BN[2-14]NH_2 and ^{nat}Sc-DOTA-BN[2-14]NH_2 were assessed in PC-3 cell line in a competitive cell-binding assay by displacement of ^{125}I-[Tyr^4]-BN. Briefly, the cells were incubated at 37°C for 1 h in the presence of 30,000-35,000 cpm ^{125}I-[Tyr^4]-BN(2-14) and increasing concentrations of the nonradioactive DOTA-BN[2-14]NH_2 complexes with ^{nat}Ga and ^{nat}Sc (each in triplicate). The experiment was performed in triplicate.

2.3.4. Internalization studies

Approximately 200 fmol/100 µl of each ^{44}Sc-DOTA-BN[2-14]NH_2 and ^{68}Ga-DOTA-BN[2-14]NH_2 were subjected to *in vitro* internalization assay using the PC-3 cell line. Non specific internalization was determined by adding an excess of 1 µM DOTA-BN[2-14]NH_2. The internalization was studied at appropriate time points (5, 15, 30, 60 for ^{68}Ga and 5, 15, 30, 60 and 120 min for ^{44}Sc). The experiment was performed in triplicate. Results were expressed as the percentage of internalization and the percentage of cell surface binding of total administered radioactivity and as the percentage of internalization of total bound

radioactivity (% relative internalization, % r.i).

2.3.5. Externalization studies (efflux studies)

The *in vitro* externalization rates of the radiolabeled compounds ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 were determined at appropriate time points (5, 15, 30, 60 for ^{68}Ga and ^{44}Sc, additionally at 120 min for ^{44}Sc only). The experiment was performed in triplicate. The results were presented as the percentage of the internalized tracer (% relative internalization, % r.i) and as the percentage of externalization of total bound radioactivity (%relative externalization, % r.e).

2.3.6. Statistical methods

The binding affinity and the internalization/efflux studies results were evaluated by non-linear regression analysis using the GraphPad Prism TM computer fitting program.

2.4. Animal studies

2.4.1. Biodistribution studies

Biodistribution studies were performed in male Sprague-Dawley rats (weight 190-230 g) under pentobarbital anesthesia (40 mg/kg body weight, Narcoren, Merial, Hallbergmoos, Germany), after intravenous injection (i.v.) of the radioactive sample into the jugular vein. *Ex vivo* organ distribution of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 was evaluated at 1 h and 2 h post injection (p.i.). The injected dose was 11 MBq (7.4 MBq/nmol) for ^{68}Ga-DOTA-BN[2-14]NH_2 and 3 MBq (2.9 MBq/nmol) for ^{44}Sc-DOTA-BN[2-14]NH_2. Two rats were used for each time point.

In the blocking experiments the native BN (100 μg/100 μl) was intravenously injected to the normal rat 15 min prior to administration of the ^{68}Ga or ^{44}Sc-labeled peptide. Biodistribution was evaluated at 1 h p.i., in comparison to the control group of rats, which was injected with radiolabeled analog only. Two rats were used for each group.

The radioactivity of the collected blood pool and samples of weighed tissues were measured using a dose calibrator. The results were calculated as percentage of the dose per gram of tissue (%ID/g). During the experiments, the animals were housed in metabolic cages allowing urine collection. All animal experiments were performed after approval and were carried out

in accordance with the principals of Good Laboratory Practice (GLP).

2.4.2. Small animal PET imaging studies

The dynamic micro-PET imaging was performed in male Copenhagen rats bearing the androgen-independent Dunning R-3327-AT-1 prostatic cancer tumor, which has been identified to express high affinity binding sites for GRP/BN analogs [17]. Solid carcinomas of were heterotopically induced by injection of AT1 cells (0.4 ml approx. 10^4 cells/µl) subcutaneously into the dorsum of the hind foot. Tumors grew as flat, spherical segments and replaced the subcutis and corium completely. Tumors were used when they reached a volume of between 1.0 to 2.0 mL approx. 10 to 14 days after tumor cell inoculation.The µ-PET imaging was performed on a microPET Focus 120 small animal PET (Siemens/Concorde, Knoxville, USA). During PET measurements the animals were placed in supine position and breathed room air spontaneously through a tracheal tube. After a 15 min transmission scan with an external ^{57}Co source, dynamic PET studies were acquired in 2D mode. The radiotracer was administered as a bolus injection of 0.4-0.7 mL via a catheter placed in the left jugular vein. For the blocking of the GRP receptors native BN was injected intravenously (100 µg/100 µl), 15 min prior to administration of the labeled derivative. The injected radioactivity in the control and blocked specimen was 30-50 MBq of the radiolabeled compound (^{68}Ga-DOTA-BN[2-14]NH_2: 8.2 MBq/nmol, ^{44}Sc-DOTA-BN[2-14]NH_2: 2.9 MBq/nmol).

3. Results

3.1. Labeling studies

3.1.1. ^{68}Ge/^{68}Ga and ^{44}Ti/^{44}Sc generators processing.

The eluted radioactivity of ^{68}Ga ranged from 100 to 150 MBq in 0.4 mL 0.05 M HCl/acetone (2:98), pH=2, the respective eluted radioactivity for ^{44}Sc varied from 150 to 200 MBq in 3 mL ammonium acetate 0.25 M, pH=4.

3.1.2. Labeling of ^{68}Ga-DOTA-BN[2–14]NH_2 and ^{44}Sc-DOTA-BN[2–14]NH_2

The labeling yield was higher than 80% for both ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-

DOTA- BN[2-14]NH_2. Good agreement was found between the TLC and HPLC results. The HPLC analysis revealed one peak of non-bound ^{44}Sc at 2.82 min while the retention time for ^{44}Sc- DOTA-BN[2-14]NH_2 was about 5.55 min (confirmed in UV spectrum, data not shown). In the case presented in Figure 1A. the HPLC revealed 6.0% of non-bound ^{44}Sc and 94.0% radiochemical yield of ^{44}Sc-DOTA-BN[2-14]NH_2. The equivalent TLC result is presented in figure 1B as well. The specific activity (As) achieved for ^{68}Ga-DOTA-BN[2-14]NH_2 was in the range from 7.5 to 8.0 GBq/μmol DOTA-BN[2-14]NH_2 when incubated at 95° C for 15 min. The As achieved for ^{44}Sc-DOTA-BN[2-14]NH_2 was at the level of 4.8 GBq/μmol DOTA-BN[2-14]NH_2 with longer incubation for 20 min at 95°C.

3.1.3. DOTA-BN[2-14]NH_2 cold complexes with ^{nat}Ga and ^{nat}Sc

The ESI-MS analysis of ^{nat}Ga-DOTA-BN[2-14]NH_2 confirmed the presence of single main complex at 982.1 (m/z^{+2}) which is in agreement with the calculated value (MW=1964.173) (figure 2A). The respective ESI-MS analysis of ^{nat}Sc-DOTA-BN[2-14]NH_2 also confirmed the presence of single main peak at 967.3 (m/z^{+2}), being in agreement with the calculated value (MW=1939.406) (figure 2B).

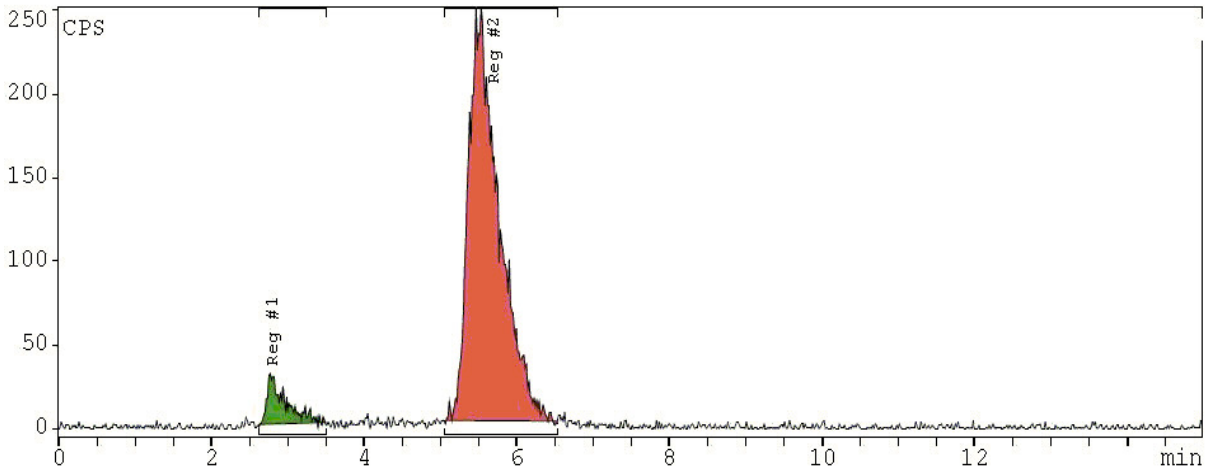
3.2. In vitro studies

3.2.1. Serum Stability

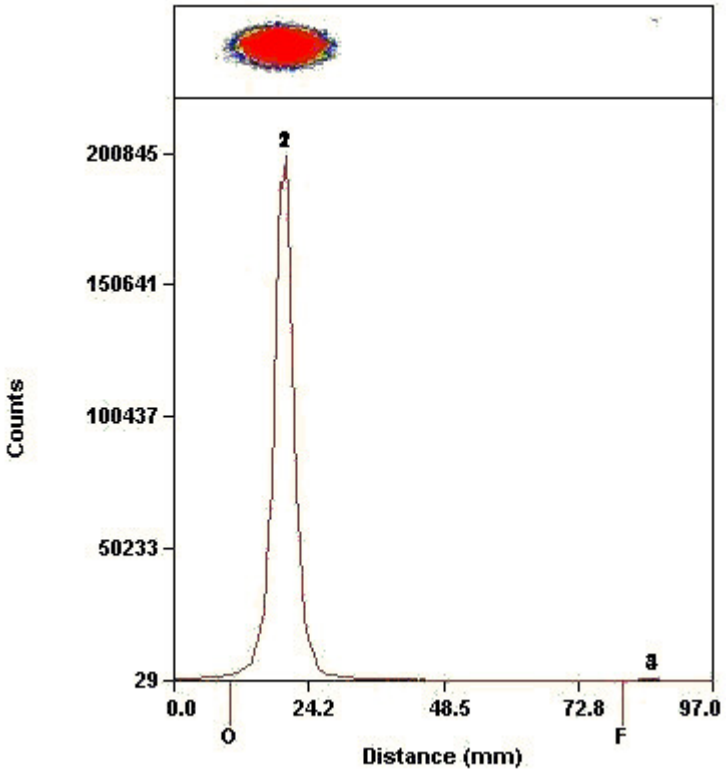
^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 were stable up to 2 h incubation to human serum at 37°C as assessed by TLC.

3.2.2. Binding Affinity

The IC_{50} values for ^{nat}Ga and ^{nat}Sc labeled analogs were derived from the displacement curve of ^{125}I-[Tyr^4]-BN presented in figure 3. The calculated values are listed in Table 1. For comparison, the IC_{50} values obtained in our previous study [11] for DOTA-BN[2-14]NH_2, ^{nat}Y-DOTA-BN[2-14]NH_2 and ^{nat}Lu-DOTA-BN[2-14]NH_2 were added to Table 1. The ^{nat}Ga- DOTA-BN[2-14]NH_2 had the highest affinity, while the affinity of ^{nat}Sc complex was the lowest.

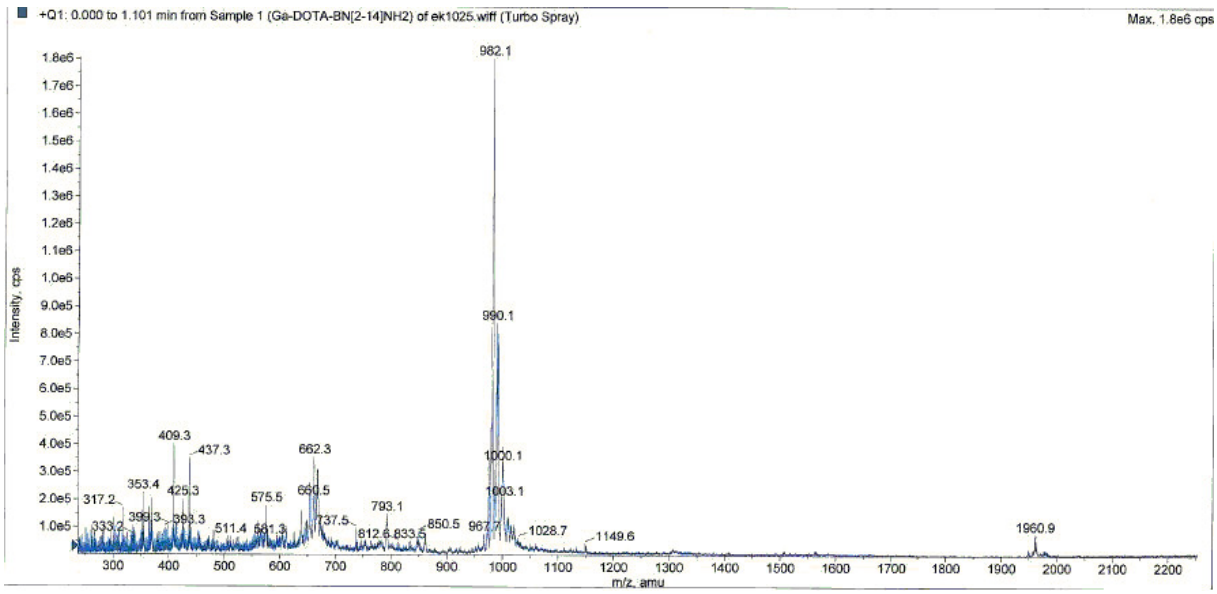


A

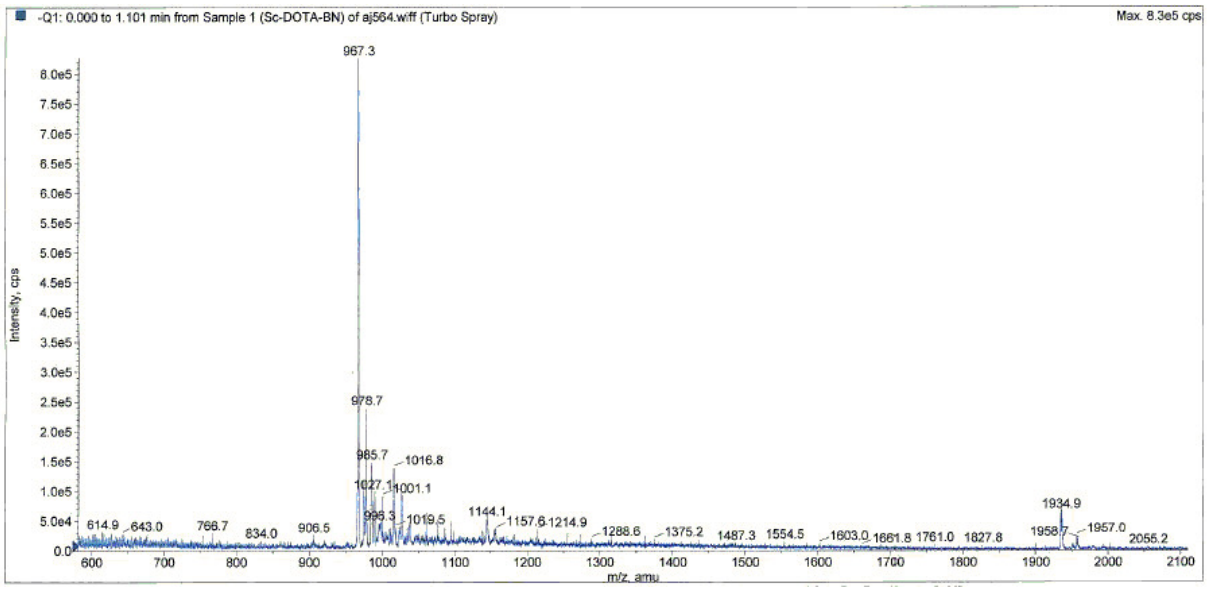


B

Figure 1. ^{44}Sc-DOTA-BN[2-14]NH_2 in ***(A)*** HPLC analysis (radioactivity detector) and ***(B)*** TLC analysis



A



B

Figure 2. ESI-MS spectra of ^{nat}Ga-DOTA-BN[2-14]NH_2 ***(A)*** and ^{nat}Sc-DOTA-BN[2-14]NH_2

(B)

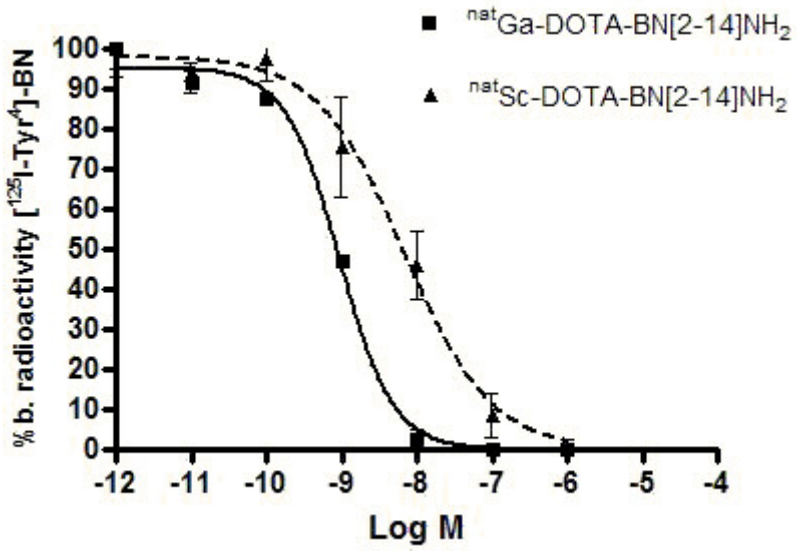


Figure 3. Displacement curves of ^{125}I-[Tyr^4]-BN from the competitive binding studies for ^{nat}Ga-DOTA-BN[2-14]NH$_2$ and ^{nat}Sc-DOTA-BN[2-14]NH$_2$

Table 1. IC$_{50}$ values vs. ^{125}I-[Tyr^4]-BN from competitive binding assays in PC-3 cells

Derivative	IC50 value (nM) (Mean±SD)
^{nat}Ga-DOTA-BN[2-14]NH$_2$	0.85± 0.06
^{nat}Sc-DOTA-BN[2-14]NH$_2$	6.49± 0.13
DOTA-BN[2-14]NH$_2$	1.78± 0.12§
^{nat}Y-DOTA-BN[2-14]NH$_2$	1.99± 0.06§
^{nat}Lu-DOTA-BN[2-14]NH$_2$	1.34± 0.11§

§: IC$_{50}$ values reported by Koumarianou *et al.* [11]

3.2.3. Internalization

The results of the internalization of ^{68}Ga-DOTA-BN[2-14]NH$_2$ and ^{44}Sc-DOTA-BN[2-14]NH$_2$ to PC-3 cells, expressing BB2 receptors, are shown in figure 4. The maximum internalization for ^{44}Sc-DOTA-BN[2-14]NH$_2$ was observed at 30 min with a 85% of relative internalization while the maximum internalization for ^{68}Ga-DOTA-BN[2-14]NH$_2$ was 80% at 60 min.

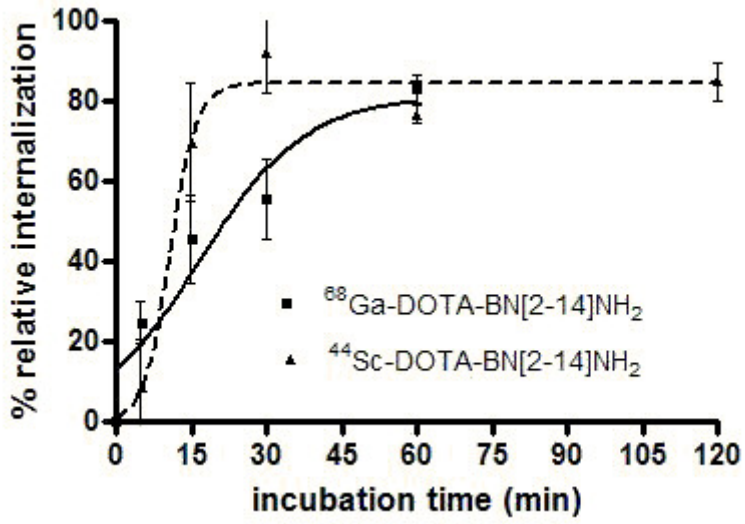


Figure 4. Internalization yield of total bound radioactivity (% r.i) of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 in PC-3 cells at 37°C

3.2.4. Externalization

The relative externalization rate of ^{68}Ga-DOTA-BN[2-14]NH_2 was found to be faster than the ^{44}Sc labelled compound at early time points. The percentage of externalization of ^{68}Ga- DOTA-BN[2-14]NH_2 was 39% after 60 min incubation while the respective value for ^{44}Sc- DOTA-BN[2-14]NH_2 was 60%. ^{44}Sc-DOTA-BN[2-14]NH_2 finally reached at 38% of relative externalization at 120 min of incubation (figure 5).

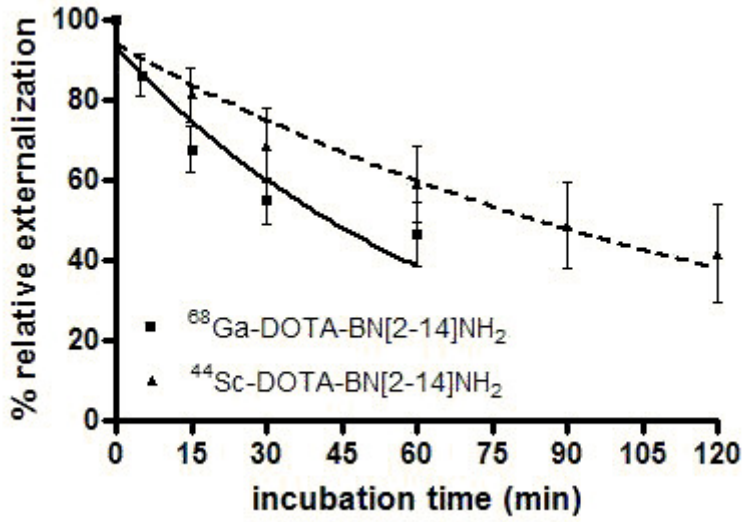


Figure 5. Externalization yield of total bound radioactivity (% r.i) of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 in PC-3 cells at 37°C

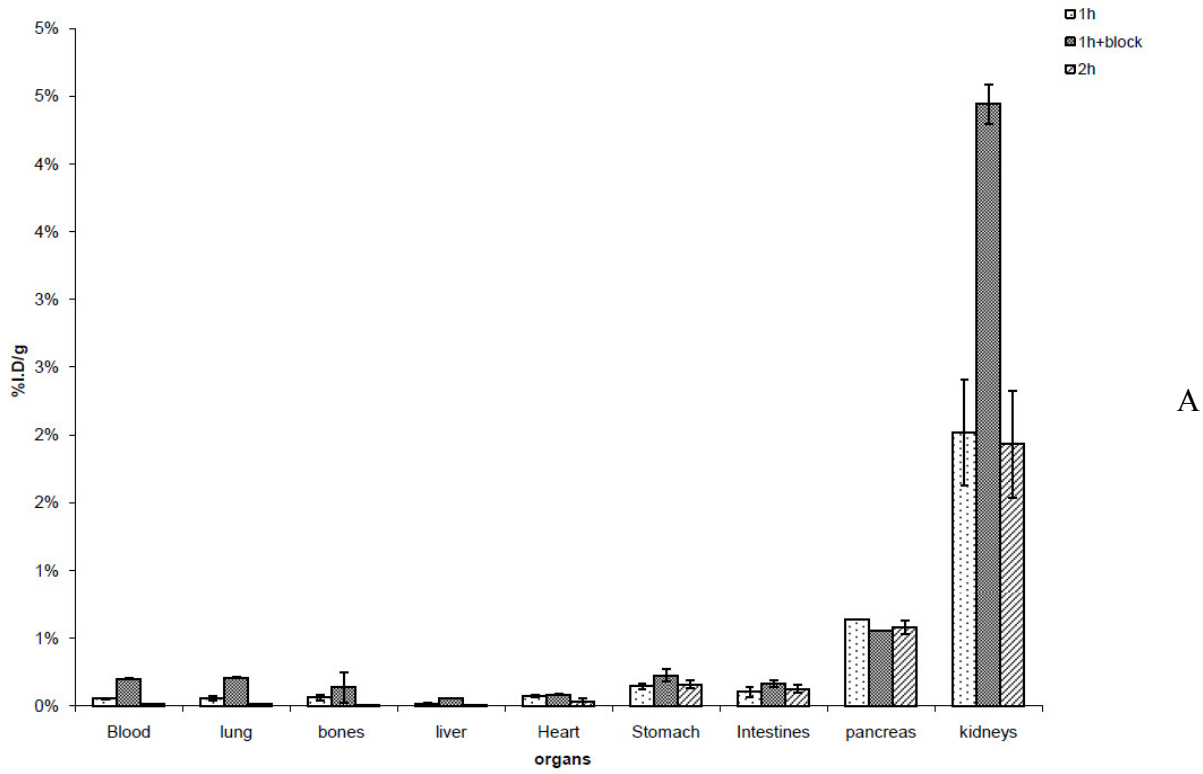
3.3 Animal studies

3.3.1. Ex vivo organ distribution

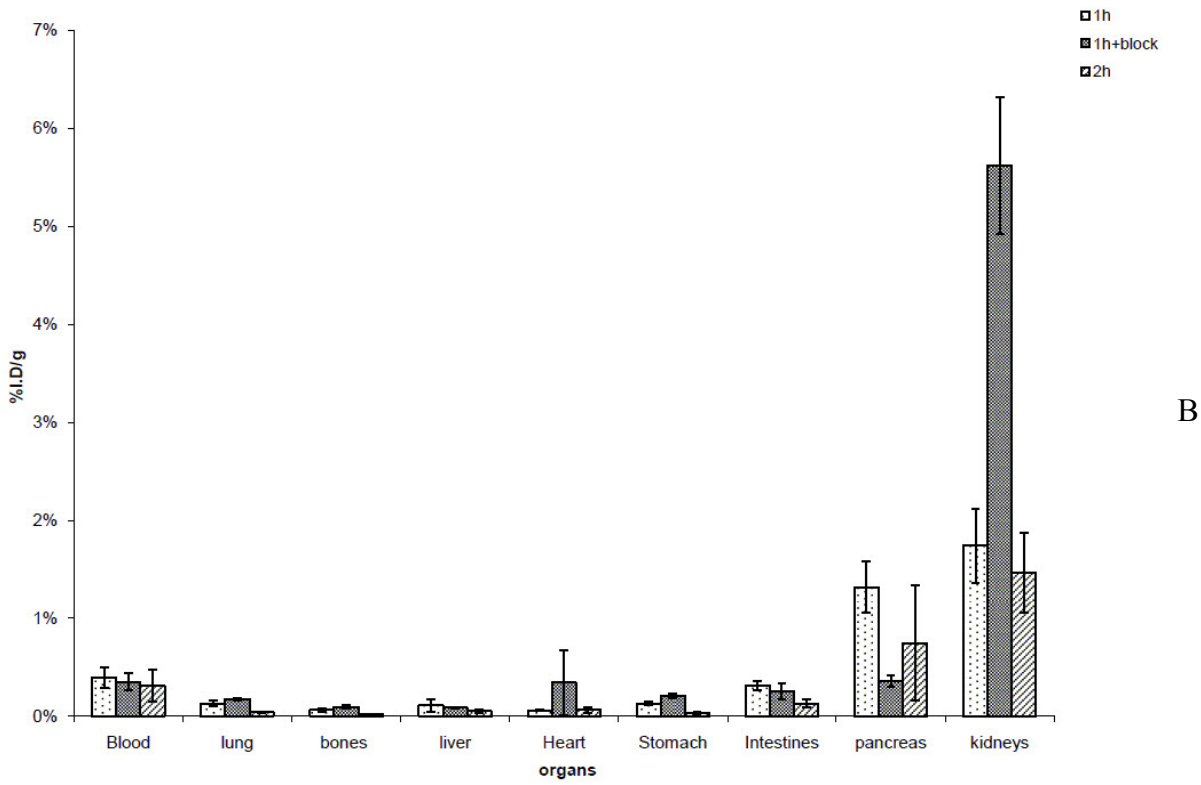
Table 2 lists the %I.D/g±S.D values of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 derived from the ex vivo organ distribution in normal male Wistar rats. Both complexes showed fast blood clearance and mainly renal excretion. The main organ of uptake was pancreas due to the naturally expressed GRP receptors. The uptake of ^{68}Ga-DOTA-BN[2-14]NH_2 in pancreas was 0.64±0.00% I.D/g at 1 h p.i and 0.58±0.05% I.D/g at 2 h p.i. (figuer 6A). The organ distribution of both complexes is essentially similar to the previously published data for ^{177}Lu-DOTA-BN[2-14]NH_2 and ^{90}Y-DOTA-BN[2-14]NH_2 [11]. The difference between the uptake in pancreas of ^{44}Sc-DOTA-BN[2-14]NH_2 in the animal group with non-blocked compared to blocked GRP receptors is significant, while it is not that obviousin case of ^{68}Ga-DOTA-BN[2-14]NH_2. ^{44}Sc-DOTA-BN[2-14]NH_2 showed 2.67±0.53% I.D/g at 1 h p.i and 1.51±1.19% I.D/g at 2 h p.i, uptake in pancreas. The specificity of uptake was verified at 1 h p.i (0.73±0.13% I.D/g) with 73% blocking of GRP receptors in pancreas (figure 6B).

Table 2. *Ex vivo* organ distribution of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 in normal male rats (%ID/g±S.D., n=2)

Organ	^{68}Ga-DOTA-BN[2-14]NH_2 %ID/g±S.D			^{44}Sc-DOTA-BN[2-14]NH_2 %ID/g±S.D		
	1 h pi	1 h pi blocking	2 h pi	1 h pi	1 h pi blocking	2 h pi
Blood	0.05±0.01	0.20±0.00	0.01±0.00	0.79±0.21	0.71±0.17	0.63±0.32
Lung	0.05±0.02	0.21±0.01	0.01±0.00	0.26±0.06	0.35±0.03	0.08±0.01
Bones	0.06±0.02	014±0.11	0.01±0.00	0.13±0.04	0.19±0.03	0.04±0.00
Liver	0.02±*0.01*	0.06±*0.00*	0.01±*0.00*	0.22±*0.12*	0.17±*0.02*	0.10±*0.03*
Heart	0.07±*0.01*	0.09±*0.00*	0.03±*0.03*	0.12±*0.01*	0.69±*0.66*	0.13±*0.06*
Stomach	0.15±*0.02*	0.22±*0.05*	0.16±*0.03*	0.26±*0.03*	0.42±*0.04*	0.07±*0.02*
Intestines	0.10±*0.04*	0.17±*0.02*	0.13±*0.03*	0.63±*0.10*	0.52±*0.16*	0.26±*0.08*
Pancreas	0.64±*0.00*	0.56±*0.00*	0.58±*0.05*	2.67±*0.53*	0.73±*0.13*	1.51±*1.19*
Kidneys	2.02±*0.39*	4.44±*0.15*	1.93±*0.40*	3.53±*0.77*	11.39±*1.42*	2.97±*0.82*



A



B

Figure 6. *Ex vivo* organ distribution of ^{68}Ga-DOTA-BN[2–14]NH_2 ***(A)*** and ^{44}Sc-DOTA- BN[2-14]NH_2 **(B)** in male rats (%ID/g ± S.D; n =2)

3.3.2. In vivo studies

The *in vivo* PET imaging of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2 was performed in male Copenhagen rats bearing the R-3327-AT-1 prostatic cancer tumor (Figure 7). The tracer was accumulated preferentially in the peripheral regions of the tumors whereas the more central part showed slightly lower concentration. However, the principal distribution pattern was not different between ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA-BN[2-14]NH_2. The tumor uptake kinetics of both tracers showed a rapid increase within the first minutes after injection followed by a slow decrease over the whole observation period (data not shown). However, the kinetics did not show profound differences between the ^{68}Ga and the ^{44}Sc labeled compound.

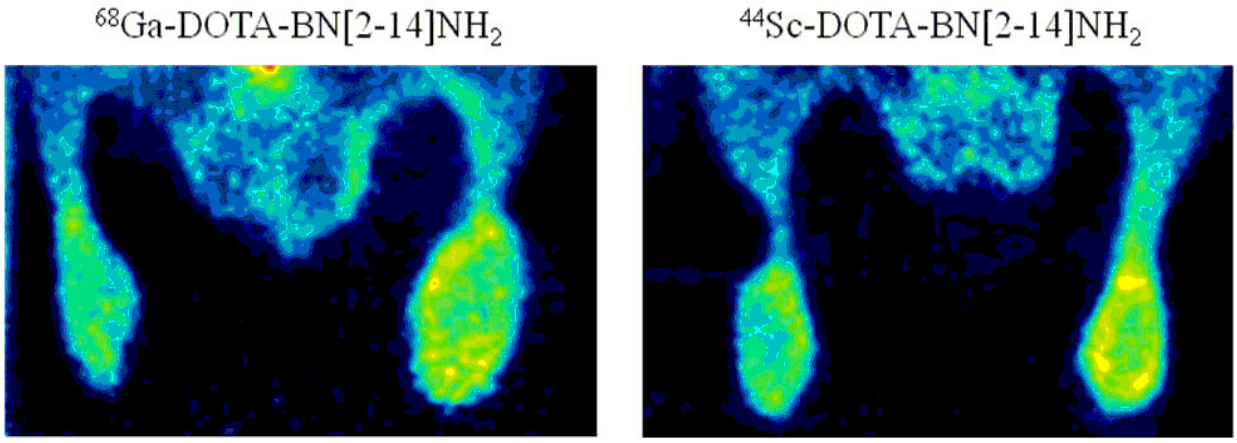


Figure 7. Cumulative µ-PET images (15 to 60 min post injection) of subcutaneous R-3327-AT-1 tumors after injection of ^{68}Ga-DOTA-BN[2-14]NH_2 or ^{44}Sc-DOTA-BN[2-14]NH_2

4. Discussion

The in vitro comparison of several somatostatin analogues indicated that not only the peptide sequence and conjugated chelator but to a large extent also the metal involved in complex formation influences the affinity of the molecule to the somatostatin receptor subtypes [10]. Our previously published comparison of the *in vitro* and *in vivo* properties of ^{90}Y-DOTA-BN[2-14]NH_2 and ^{177}Lu-DOTA-BN[2-14]NH_2 revealed differences in the receptor affinity of these two analogs [11], both in terms of *in vitro* and *in vivo* behavior. This differences may attributed to the small structural changes in the radioligand molecule which

result in significant difference in interaction with receptor. The introduction of a certain metal or its replacement by another one may provoke considerable alterations in the *in vivo* binding affinity of a peptide to cell receptors and may have an important impact on the quality of the *in vivo* biodistribution of these radiopharmaceuticals [10]. The main goal of this study was to evaluate the influence of two trivalent positron emitters ^{68}Ga and ^{44}Sc when conjugated to DOTA-BN[2-14]NH_2 in comparison to earlier published data on the same molecule labeled with ^{90}Y and ^{177}Lu. The selection of these two positron emitters was based on the increasing demand for PET radiotracers which are easily accessible, since they can be generator-produced, in contrast to the cyclotron produced ones. Considering the rather short half-life of ^{68}Ga, the ^{44}Sc can be an ideal alternative for conjugation with biomolecules of longer metabolic half-life and for acquiring extensive scintigraphic images when it is required.

Both ^{68}Ga and ^{44}Sc can label DOTA-BN[2-14]NH_2 in a fast and efficient way based on the method established prior on the labeling of DOTA-TOC [5, 14]. Initial synthesis yields were higher than 80%. The radiolabeled compounds were of low specific activity in terms of activity per peptide (GBq/μmol peptide) but of adequate radioactive concentration for further *in vitro* and animal studies. Serum stability of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTA- BN[2-14]NH_2 indicated that were stable for at least 2 h when assessed by TLC.

^{nat}Ga-DOTA-BN[2-14]NH_2 had superior binding affinity to GRP receptors comparing to the other three metals complexes and to the peptide itself with respective IC_{50} values of 0.85, 6.49, 1.50, 1.78, 1.99 and 1.34 for ^{nat}Ga, ^{nat}Sc, DOTA-BN[2-14]NH_2, ^{nat}Y and ^{nat}Lu complexes (see Table 1). However, the internalization rate of ^{68}Ga-DOTA-BN[2-14]NH_2 was lower and the washout faster than for ^{44}Sc labeled compound.

^{44}Sc-DOTA-BN[2-14]NH_2 and ^{68}Ga-DOTA-BN[2-14]NH_2 revealed a high uptake in pancreas, which is the main organ expressing GRP receptors, a fast blood clearance and an excretion mainly through kidneys. Blocking studies of ^{44}Sc-DOTA-BN[2-14]NH_2 confirmed the specificity of uptake in the main GRP receptor positive tissue by 73% blocking in pancreas (pancreas: non blocked: 2.67±0.53%I.D/g, blocked: 0.73±0.13%I.D/g and kidneys: non blocked: 3.53±0.77 % I.D/g, blocked: 11.39±1.42 % I.D/g), whereas for ^{68}Ga-DOTA-BN[2-14]NH_2 the same uptake tendency was observed (pancreas: non blocked: 0.64±0.00% I.D/g, blocked: 0.56±0.00 % I.D/g and kidneys: non blocked: 2.02±0.39 % I.D/g, blocked: 4.4±0.15 % I.D/g).

Micro PET imaging of tumors with high affinity binding sites for GRP/BN analogs show a clear accumulation of the tracer within the tissue. The tracer accumulation was slightly higher in the peripheral regions of the tumor. However, this heterogeneity was not the result of morphological differences within the tumor which was shown by histological staining of cryosections. The regional differences might be the result of differences in GRP receptor expression within the tissue or differences in functional binding capacities due to differences in the metabolic microenvironment of the tumor.

Comparing the ^{68}Ga- and the ^{44}Sc-labeled compounds no differences in the tumor accumulation (neither in the overall uptake nor in the dynamics) were seen. Both tracer show comparable distribution patterns and similar time constants of uptake and elimination. For these reasons the use of either of these compounds for detecting GPR-binding tumors is equivalent. However, one important problem independent from the tracer used is the fact that human gastrointestinal stromal tumors show GRP binding in approximately 40% [18]. For this reason radiolabeled bombesin (either with Ga or Sc) could be helpful only in a subgroup of patients with gastrointestinal tumors.

Acknowledgements

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4. Conclusions

This thesis describes the implementation of radioactive gallium-68 and scandium-44 for molecular imaging and nuclear medical diagnosis, beginning with chemical separation and purification of ^{44}Ti as a radionuclide mother, investigation of pilot generators with different elution mode, building a prototype generator, development and evaluation of post-processing of the generator eluate and the labeling chemistry, *in vitro* and *in vivo* studies of labeled compounds and, finally, *in vivo* imaging experiments.

The major achievements related to the ^{68}Ge/^{68}Ga generator system are:

- New approaches towards on line-processing of the generator itself:

  A combined protocol of processing ^{68}Ge/^{68}Ga generator eluate has been developed. It utilises the significant advantages of cation-exchanger based processing of ^{68}Ga. To avoid any presence of acetone in radiolabelling mixtures processing, ^{68}Ga was transferred from the cation-exchanger to the supplementary micro-column filled with strong anion exchanger or a novel extraction chromatographic TODGA resin. An effective processing of generator produced $^{68}Ga^{III}$ could be performed within 5 min only. The $^{68}Ga^{III}$ preparation could be obtained in a reduced volume with high chemical and radiochemical purity in a form useful for radiolabelling reactions. Overall yields of ^{68}Ga for anion exchanger and TODGA resins are 87% and 96%, respectively.

- The labeling of new ligands under acidic milieu:

  The Ga^{III} complex with PrP9, a new phosphine-based (instead of carboxylate-based) macrocyclic ligand, is quantitatively formed even below pH = 1. However, it means that for PrP9 there is no strict necessity to keep the pH in a very narrow range from 2.5–4 during the labeling procedures (as required, for example, for DOTA peptides). This might render the fully automated syntheses of ^{68}Ga radiotracers more robust, in which adjustment of labeling pH is often a crucial issue. In addition, the synthesis of PrP9 itself is fast, simple, and scalable; particularly in comparison with other ligand systems bearing additional carboxylic groups suitable for conjugation like biphosphonates, DOTA(tBu)$_3$, NOTA(tBu)$_3$,

NODAPA(tBu)$_3$ or NODAGA(tBu)$_3$. PrP9 may find potential to facilitate the application of ^{68}Ga-based nuclear medicine and molecular imaging by providing facile and cost-efficient access to ^{68}Ga radiopharmaceuticals with superior properties.

The major achievements related to the ^{44}Ti/^{44}Sc generator system are:

- Development of the ^{44}Ti/^{44}Sc generator model:

  Using optimum K_d values of Ti(IV) and Sc(III) for HCl/$H_2C_2O_4$ mixtures, *i.e.* 0.2 M HCl/0.1 M $H_2C_2O_4$ and 0.07 M HCl/0.005 M $H_2C_2O_4$, two low-activity pilot and a 5 mCi ^{44}Ti/^{44}Sc generators, respectively, were constructed and evaluated. After one year of regular elution of 5 mCi ^{44}Ti/^{44}Sc radionuclide generator, the yield of ^{44}Sc and ^{44}Ti is stable and the breakthrough of ^{44}Ti is very low. The system achieves elution of 97 % (180 MBq) ^{44}Sc in 20 mL of eluate solution. The breakthrough of ^{44}Ti is $5 \cdot 10^{-5}$ % (90 Bq). This corresponds to an excellent separation factor of $2 \cdot 10^{6}$.

- "reverse" elution of the ^{44}Ti/^{44}Sc radionuclide generator:

  Regarding of long-term stability of ^{44}Ti/^{44}Sc generators, "direct" generator elutions may not be adequate. In comparison, a "reverse" elution strategy definitely guarantees a very low breakthrough of ^{44}Ti. This does not affect the elution yield of ^{44}Sc. However, the ^{44}Sc solution that is obtained from generator appears to be too diluted and too acidic for use in direct labelling procedures. Nevertheless, concentrating the ^{44}Sc solution and reducing the acidity in that ^{44}Sc solution may be added on line to the generator performance described.

- Post-processing of ^{44}Ti/^{44}Sc generator's eluate, its concentration and purification: The chemically efficient an on-line post-elution processing of generator-produced ^{44}Sc based on cation-exchanger purification was adapted to a simple module, which allows a rapid and simultaneous concentration and purification of ^{44}Sc obtained from generator. The Bio-Rad AG 50W-X8 (200–400 mesh, H^+-form) resin was used for on-line adsorbing ^{44}Sc (>98%) and its quantitative recovery is using of 3 mL of 0.25 M ammonium acetate pH = 4.0 system (~90%). The content of ^{44}Ti co-eluted with ^{44}Sc from the ^{44}Ti/^{44}Sc generator of 5×10^{-5}% is further being reduced by a factor of 10. The final content of ^{44}Ti in the 140–160 MBq ^{44}Sc

fraction ready for labeling is thus 7 Bq, representing a very low contamination of around $<2\times10^{-7}$.

- Labeling of ^{44}Sc with DOTATOC and DOTATATE, stability studies of labeled products: The post-elution processing of volumes and impurities is easily compatible with the synthesis of ^{44}Sc-labeled compounds. Thus, the chemically and radiochemically highly pure ^{44}Sc fraction of very high specific volume activity of around 50 MBq/mL representing 150 MBq overall activity for the first time may allow systematic research on the development and application of new ^{44}Sc-labeled compounds. Areas of interest are ^{44}Sc(III) on complex formation, labeling and radiopharmaceutical chemistry of the positron emitter ^{44}Sc, molecular imaging of ^{44}Sc-labeled tracers using PET/CT.

- Methods for labeling DOTA-conjugated peptides with the new PET radionuclide ^{44}Sc have been investigated systematically. Special efforts have been focused on optimization of reaction parameters and stability studies of the obtained conjugate. Incorporation of ^{44}Sc(III) into DOTATOC was almost quantitative (>98%) at pH = 4.0 after 25 minutes heating in an oil bath at 95ºC. This time can be significantly reduced to only 3 minutes when microwave heating is adopted for radiolabeling. Incorporation of ^{44}Sc(III) into DOTATATE was >80% at pH = 4.0 after 25 minutes heating in an oil bath at 95ºC. Isolation of the pure ^{44}Sc-labeled product is easily performed on reverse-phase C-18 mini-cartridges (Strata-X) and results in radiochemically pure ^{44}Sc-DOTATOC and ^{44}Sc-DOTATATE in 400 μL of pure ethanol. ^{44}Sc-DOTATOC is stable in pure ethanol, 0.9% NaCl, PBS (pH = 7.4) and also in the presence of metal cations (Fe^{3+}, Cu^{2+}, Ca^{2+} and Mg^{2+}), as well as other competing ligands, like EDTA and DTPA.

The major achievements related to the pre-clinical evaluation of new ^{68}Ga and ^{44}Sc-radiopharmaceuticals are:

- *in vivo* and *in vitro* studies of ^{68}Ga and ^{44}Sc conjugated to DOTA-BN[2-14]NH_2 :

The influence of two trivalent positron emitters ^{68}Ga and ^{44}Sc conjugated to DOTA-BN[2-14]NH_2 in comparison to earlier published data on the same molecule labeled with ^{90}Y and ^{177}Lu was evaluated. Both ^{68}Ga and ^{44}Sc can label DOTA-BN[2-14]NH_2 in a fast and efficient way based on the method established prior on the labeling of DOTA-TOC. Initial synthesis

yields were higher than 80%. The radiolabeled compounds were of low specific activity in terms of activity per peptide (GBq/μmol peptide) but of adequate radioactive concentration for further *in vivo* and *in vitro* animal studies. Serum stability of ^{68}Ga-DOTA-BN[2-14]NH_2 and ^{44}Sc-DOTABN[2-14]NH_2 indicated that were stable for at least 2 h when assessed by TLC. MicroPET imaging of tumors with high affinity binding sites for GRP/BN analogs show a clear accumulation of the tracer within the tissue. The tracer accumulation was slightly higher in the peripheral regions of the tumor, which is a commonly observed radiotracer accumulation pattern in xenograft tumor models and is assumed to be due to a higher intertumoral pressure in the center of such fast growing xenograft tumors.

Erklärung

Hiermit erkläre ich, dass ich die hier als Dissertation vorgelegte Arbeit selbst angefertigt und alle benutzen Hilfsmittel in der Arbeit angegeben habe. Die aus Quellen übernommenen Daten wurden unter Angabe der Quellen gekennzeichnet.

Die Dissertation habe ich weder als Arbeit für eine staatliche oder andere wissenschftliche Prüfung eingerecht noch ist sie oder ein Teil dieser als Dissertation bei einer anderen Fakultät oder einem anderem Fachbereich eingereicht worden.

Mainz, Dezember 2010