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Practical Aspects of labeling DTPA- and DOTA-Peptides with ^{90}Y, ^{111}In, ^{177}Lu, and ^{68}Ga for Peptide-Receptor Scintigraphy and Peptide-Receptor Radionuclide Therapy in Preclinical and Clinical Applications

Continuing Education for Nuclear Pharmacists
And
Nuclear Medicine Professionals

By

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PRACTICAL ASPECTS OF LABELING DTPA- AND DOTA-PEPTIDES WITH ^{90}Y, ^{111}IN, ^{177}LU, AND ^{68}GA FOR PEPTIDE-RECEPTOR SCINTIGRAPHY AND PEPTIDE-RECEPTOR RADIONUCLIDE THERAPY IN PRECLINICAL AND CLINICAL APPLICATIONS

STATEMENT OF LEARNING OBJECTIVES:

Upon successful completion of this lesson, the reader should be able to:

1. Explain the physical chemistry (including solubility) of the a radionuclide of interest .
2. Discuss the kinetics of the radiolabeling reaction.
3. Compare kinetic versus thermodynamic stability.
4. List contaminants commonly found in the radiochemical-containing solution.
5. Describe the effects of metals, arising from radioisotope production target materials and/or associated decay products, on the labeling of peptides with radiometals.
6. Discuss the stability, specific activity, incorporation yield, and radiochemical purity of the final labeled DTPA- and DOTA-conjugated peptides.
7. Calculate the mass and concentration of peptides and radionuclides in radiolabeling reactions, and the specific activity of the resultant radiolabeled compounds.
8. List the methods of end-product quality control procedures to determine radionuclide incorporation yield and radiochemical purity.
9. Describe the specifications of a radiolabeled peptide product formulation.

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INTRODUCTION

Applications of Radiolabeled DTPA- and DOTA-peptides

Peptide receptor scintigraphy (PRS) with radiolabeled regulatory peptides has been shown to be a sensitive and specific technique to demonstrate the presence of somatostatin receptors on various tumors *in vivo* [1, 2]. With this technique, somatostatin receptor-positive primary tumors and metastases can be visualised. As soon as the success of PRS for tumor visualisation became clear, the next logical step was to label these peptides with radionuclides emitting α- or β-particles, including Auger or conversion electrons, and to perform peptide receptor radionuclide therapy (PRRT). Since somatostatin-14 (somatostatin) and its analogues are the most frequently used for PRS and PRRT, these peptides are used as models in this manuscript. Extensive general reviews on the application of these peptides have been recently published, as well as reviews focused on the somatostatin receptor [3-15].

^{111}In-DTPA-peptides, such as [DTPA^{0}]octreotide (see Figure 1) have proven to be very useful in PRS. While ^{90}Y and ^{177}Lu are more suitable radionuclides for PRRT, ^{90}Y- and ^{177}Lu-DTPA-peptides lack the requisite stability *in vivo*. Therefore, concordant DOTA-conjugated peptides, such as the stabilized somatostatin analogues [DOTA^{0}, Tyr^{3}]octreotide (DOTATOC), [DOTA^{0}, Tyr^{3}]octreotate (DOTAtate, see Figure 2) were developed, and labeled with ^{90}Y and ^{177}Lu [2, 4, 16]. DOTATOC, DOTAtate and DOTANOC ([DOTA^{0}, 1-NaI^{3}]octreotide) are different in liphophilicity and have different affinities for the 5 somatostatin receptor subtypes [17-21]. DOTATOC and DOTAtate are used as models here. Comparisons between radiolabeled DOTATOC *vs*. DOTAtate *vs*. DOTANOC in patients are ongoing and have been widely reported [17-21].

There are many aspects that influence the interaction of a radioligand with its receptor. As evidenced through *in vitro* techniques such as radioimmunoassay and receptor binding, the signal to background ratio is often improved by increasing the specific radioactivity of the ligand for saturable regulatory peptide binding processes, However, increasing the specific activity, has resulted in reduced uptake of radioactivity in receptor-positive targets *in vivo*. Contrary to what was expected, the percent uptake of these radiolabeled regulatory peptides, such as radiolabeled [DTPA^{0}]octreotide, is not optimal in octreotide receptor-positive tissues at the highest maximum specific activity [4, 17].

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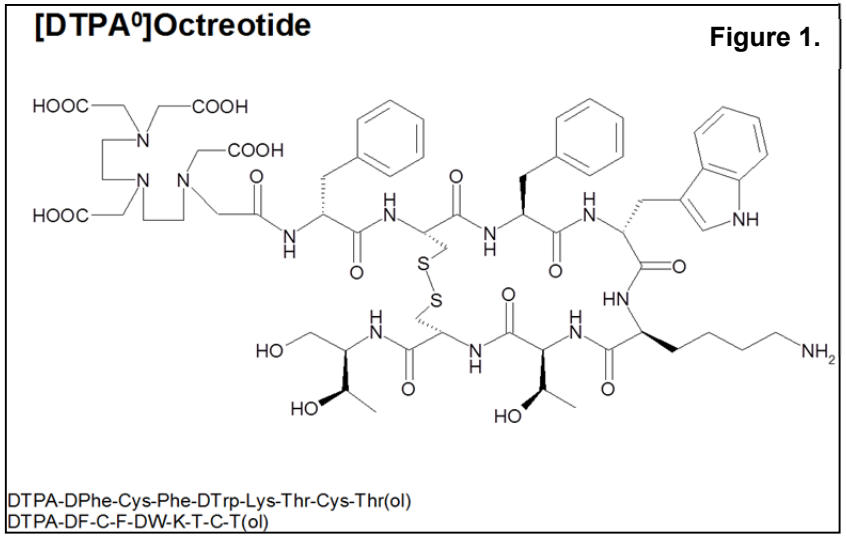

Figure 1. Structural formulae [DTPA^{0}]octreotide (OctreoScan) and [DOTA^{0}, Tyr^{3}]octreotate (DOTAtate). [DTPA^{0}]octreotide is an octreotide analogue conjugated with the chelator DTPA at the N-terminal side of octreotide(Fig. 1). It was designed for scintigraphy with ^{111}In. However, DTPA is not suitable for incorporation of therapeutic β-emitting radionuclides such as ^{90}Y and ^{177}Lu, while the macrocyclic DOTA is more suitable, forming kinetically and thermodynamically stable complexes. In addition, the amino acid phenylalanine at the third position (Phe^{3}) in octreotide is replaced by another amino acid tyrosine (Tyr^{3}), which resulted in higher binding affinity for the somatostatin receptor subtypes 2, 3 and 5, see Table 1. DOTATOC is thus the abbreviation of DOTA-Tyr^{3}-Octreotide.

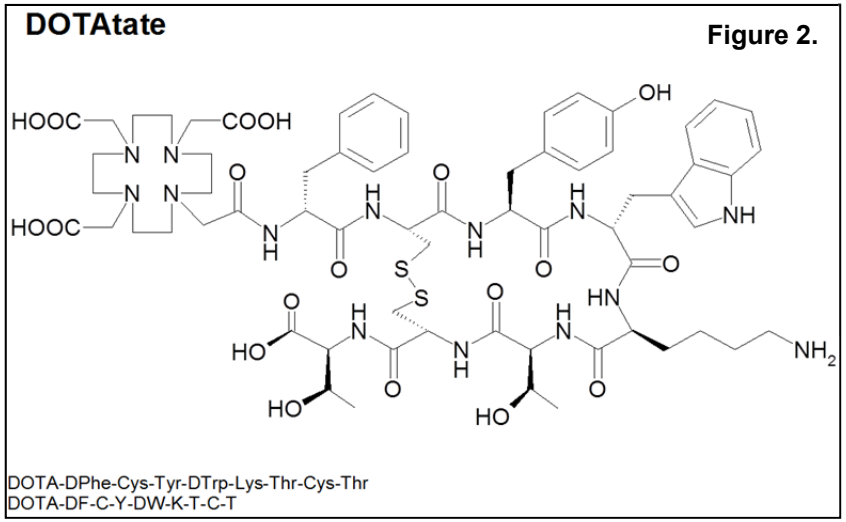

Figure 2. In DOTAtate, the alcohol group at the COOH-terminus of octreotide is replaced by a carboxylic acid group, and led to increased affinity and selectivity for somatostatin receptor subtype 2 [22]. The affinities of these analogues varies for the different somatostatin receptor subtypes, but are also influenced by the incorporation of different radiometals [2, 8]. The uptake of ^{68}Ga-DOTATOC in somatostatin receptor-positive tissues, such as pancreas and adrenals was the highest encountered in a rat model, while uptake in kidney was lowest [9]. These results confirm the data in mice [23] and in patients [24-25] . The explanation for this phenomenon lies in the coordination of the metal in the chelator, thus effecting the charge within the molecule, the IC_{50}, and also its biodistribution.

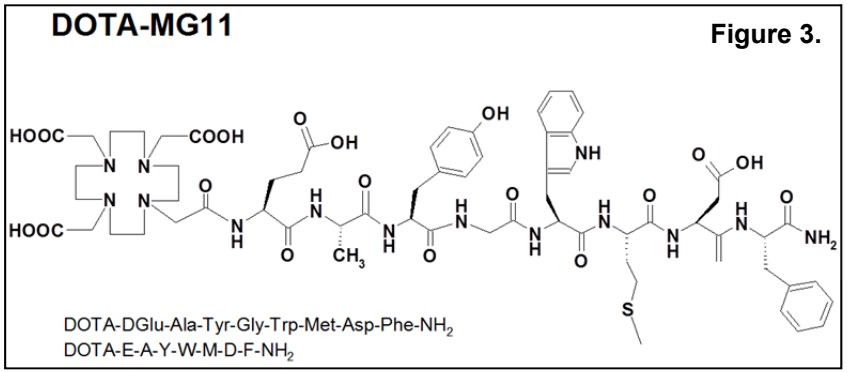

Figure 3. DOTA=[1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid];

DTPA= DiethyleneTriaminePentaAcetic Acid.

DOTA-MG11 (MW is 1392 D) was a gift from Prof. Dr. Mäcke, Freiburg, Germany as part of a joint project within the EU COST. Radiolabeling of DOTA-MG11 (12µg) in 50 mM sodium acetate with 260 MBq ^{111}In (Covedien, Petten, The Netherlands) required a heating procedure and was optimised by varying reaction time (range 5-30 min) and temperature (range 80°-100°C) at pH 4-4.5 after modification of a published protocol [26].

One explanation for this decreased binding is that *in vivo,* regulatory peptides have high affinity receptors that are expressed at low capacity [26, 27]. The uptake expressed as percentage of the administered dose is a bell-shaped function of the injected mass. These findings might be the result of two opposing effects, (1) a negative effect due to saturation of the receptor at increasing ligand concentrations and (2) a positive effect of increasing ligand concentrations on the rate of internalization by ligand-induced receptor clustering [4]. This implies that the sensitivity of detection of somatostatin receptor-positive tumors by PRS might be improved by administration of an optimized mass dose of radioligand, similar to that found for other radioligands and confirmed in patients [28-30].

Clinical data indicate that the receptors of regulatory peptides can be rapidly saturated, implying that the range for optimal uptake versus injected mass of ligand is rather small [31]. From the referenced data it can be concluded that high specific activities are necessary. However, further preclinical and clinical studies are needed to optimize the mass dose of ligand to be administered.

Specific Activity and Mass Dose

There are a number of biological factors that dictate the need for high specific activity radiolabeled peptides. The first two factors are typical for receptor-mediated processes and even more specific for the high affinity and low capacity receptor-mediated systems [4, 27].

1. For *in vivo* use, the affinity of the ligand for its receptor and the amount of available receptors limits the mass dose of ligand or radioligand that can be administered. Increasing the mass dose of ligand or radioligand will eventually result in a saturation of the receptor. As a consequence, less of the administered radioactivity will bind to receptors and become internalized by receptor-expressing tissues and tumors [4].

2. Peptides such as DOTA-substance P or DOTA-bombesin are tolerated in only very small mass quantities due to their pharmacological effects [28, 33-36]. With an increase in specific activity of the radioligand and at equal dose (in radioactivity), the total peptide mass dose to be administered can be reduced. Currently, clinical studies are under investigation with concordant antagonists of bombesin that display minimal pharmacological side effects, in contrast to the concordant agonists.

3. There is an apparent imbalance between sensitivity and resolution in current PRS animal imaging protocols. While the latter is optimized, the current sensitivity of the cameras or detectors requires high amounts of radioactivity to be administered to the animals with corresponding high mass doses of peptides. This is in conflict with arguments 1 and 2 above [36].

4. Endocytotic mechanisms that affect the cellular internalization of peptides may become desensitized at high peptide concentrations [37], which results in lower uptake of radioactivity into target tissues.

5. In addition, for *in vitro* use, the investigations aimed at measuring receptor-binding affinities require low concentrations of these radioligands (e.g. 10^{-10} M) in order to measure receptor ligand interactions accurately. Translation from these *in* vitro studies to successful *in vivo* mass dose-ranging studies has proven difficult. In short, when a high radioactivity dose is required, in combination with limited total mass doses of ligand and radioligand, the need to investigate the significance of specific activity is clear.

The optimal mass dose range of the injected radioligand *versus* target uptake is small. High specific activity radioligands are often required for these radiolabeled peptides to be used successfully in PRS and PRRT. This data indicates that PRS requires less radioactivity in comparison to PRRT.

High specific activities used with DOTA-peptides radiolabeled with ^{90}Y, ^{111}In, ^{68}Ga, and ^{177}Lu are achievable. Even higher specific activities can be achieved when radiolabeling DTPA-peptides. A major drawback of using DOTA-peptides is that the incorporation of the radiometal requires heating and time [38], while DTPA-peptides can be radiolabeled at room temperature [39]. However, as mentioned above, ^{90}Y- and ^{177}Lu-DTPA-peptides lack sufficient *in vivo* stability. While a certain amount of radioactivity is necessary for the detection of target tissue uptakes by imaging systems, concordant low mass doses of DTPA-peptides can be administered.

Maximal Achievable Specific Activity of ^{177}Lu- or ^{90}Y-labeled DOTA-peptides

Several physical factors influence the highest achievable specific activity of radioligands. For example, ^{177}Lu-DOTAtate can be radiolabeled, in theory, at 0.72 GBq ^{177}Lu per nmol DOTA-peptide . However, production contaminants reduce the theoretical maximum specific activity. Because ^{177}Lu is reactor-

produced (n,γ) from enriched ^{176}Lu , the presence of ^{175} Lu and ^{176}Lu reduce the maximum achievable specific activity to 0.15 GBq ^{177}Lu per nmol DOTA-peptide. In many instances ^{177}Lu-DOTAtate is labeled at a specific activity of 40 - 80 MBq per nmol DOTA-peptide. Higher specific activities were achievable using, 0.5 GBq ^{177}Lu per nmol DOTA-peptide prepared using high-specific activity ^{177}Lu, such as that obtained from the High Flux Isotope Reactor at Oak Ridge National Laboratory (See Table 1). Recently, reactor-produced (n, γ) ^{177} Lu from enriched ^{176}Yb has demonstrated higher achievable specific activity: 0.42 GBq ^{177}Lu per nmol DOTA-peptide (Breeman, unpublished results). High specific activities of 0.5 GBq ^{90}Y per nmol DOTA-peptide have also been achieved (Table 1). In order to achieve higher specific activities, the rigorous elimination of metal impurities is necessary, not only during radiolabeling, but also during synthesis of the DTPA- and DOTA-conjugated peptides [38, 40]. Maximal achievable specific activities with $^{67/68}$Ga and ^{111}In are discussed separately, see below and Table 1.

Bifunctional Chelators for Metals

Brechbiel [38] made an overview of many bifunctional chelating agents, including the described DTPA and DOTA. It is worth noting that there are several forms of DTPA and DOTA, each having different kinetics and stability constants. The bifunctional chelating agents can bind metals and still possess a chemically reactive functional group for covalent attachment to peptides, such as octreotide.

From the numerous combinations of different chelators with different peptides, it becomes very complicated to select the best. There are many criteria for the chelate selection, one of which is stability *in vivo*, and although the complexes are exceptionally stable for their use, the question becomes “What is good enough?” [41].

To illustrate the difference in reaction kinetics between DOTA-peptides and DTPA-peptides consider the following example: ^{111}In-DTPA-peptides, such as [DTPA^{0}]octreotide can be labeled with up to 1.7 GBq.nmol^{-1} which is close to its theoretical maximum (Table 1) even with ^{111}In days post-production. In contrast, the theoretical maximum for DOTAtate is 0.82 GBq ^{111}In per nmol but is only achievable immediately after production and with purification of the ^{111}In, since $^{111/112}$Cd also incorporates in DOTA (see Table 1, and Figures 4-6).

Table 1

Physical Characteristics of a Selection of Radionuclides					
	^{67}Ga	**^{68}Ga**	**^{90}Y**	**^{111}In**	**^{177}Lu**
Production Method	Cyclotron (p, 2n)	Generator	Generator	Cyclotron (p, 2n)	Reactor (n, γ)
Target/Parent	^{68}Zn	^{68}Ge	^{90}Sr	^{112}Cd	^{176}Lu
Decay Product	^{67}Zn	^{68}Zn	^{90}Zr	^{111}Cd	^{177}Hf
t½ [days]	3.26	4.7E-2	2.67	2.81	6.71
nmol metal per GBq	0.68	0.01	0.55	0.58	1.39
Maximal Specific Activity [GBq.nmol^{-1}]					
In Theory[a]	1.48	102	1.81	1.72	0.72
In Practice	0.37	>1.0	0.5	1.7[b] ; 0.82[c]	0.15[d], 0.5[e], 0.42[f]

a: In theory, 1 nmol of a DTPA- or DOTA-peptide can incorporate 1 nmol radionuclide, and this number indicates the maximal theoretical specific activity of the radiolabeled DTPA- or DOTA-peptides, expressed in [GBq.nmol^{-1}].

b: 1.7 GBq per nmol DTPA-peptide,

c: 0.82 GBq per nmol DOTA-peptide,

d: data from (n, γ) reactor produced ^{177}Lu from enriched ^{176}Lu [40].

e: at very high thermal neutron flux (1.5 10^{15} neutrons cm^{-2} s^{-1}) as in High Flux Isotope Reactor at Oak Ridge National Laboratory, 80% of all the Lu atoms can be in the form of ^{177}Lu [40]. DOTAtate was successfully radiolabeled with this material, up to a specific activity of 0.5 GBq nmol^{-1} (Breeman, unpublished results).

f: ^{177}Lu reactor-produced via (n, γ) from enriched ^{176}Yb [42 -42]. Maximal achieved specific activity was 0.42 GBq ^{177}Lu per nmol of DOTA-peptide (Breeman, unpublished results). See also Figures 10AB for the formation and decay schemes of $^{67/68}$Ga, ^{90}Y, ^{111}In and ^{177}Lu

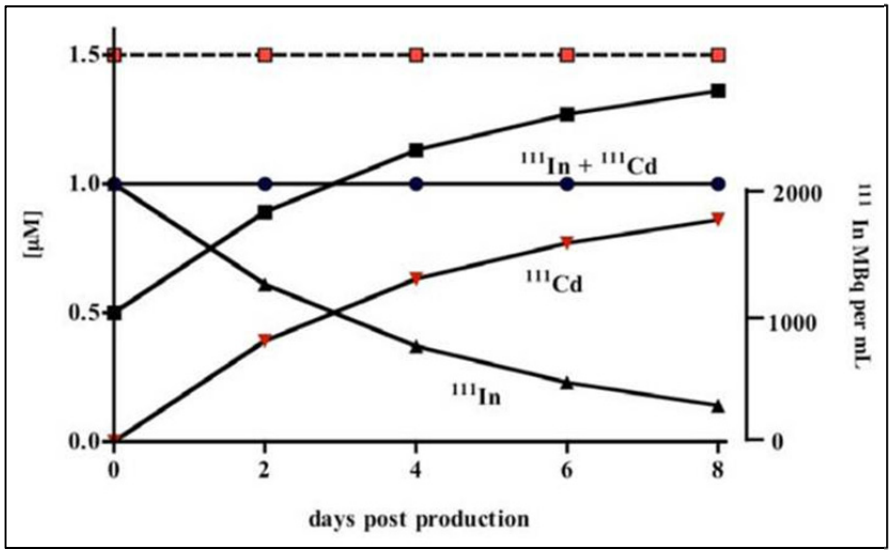

Figure 4. ^{111}In MBq per mL days post production The concentration of ^{111}In (▲, measured), ^{111}Cd (▼, calculated) and Σ(^{111}In+ ^{111}Cd)(•) are presented as a function of time. The concentration of Cd determined by ICP at t=0 (i.e. directly after production) was already 0.5 µM, indicating the presence of target material (=^{112}Cd, ■). The cumulated concentration of ^{111}In, ^{111}Cd, and ^{112}Cd is presented (□). Cd competes with ^{111}In for the incorporation in the DOTA-chelator (see Figure 6), therefore, the maximal achievable specific activity of ^{111}In-DOTA-peptides will depend on the concentration of competing contaminants such as Cd. In practice, the theoretical maximal achievable specific activity of 1.72 GBq per nmol DOTA-peptide (see Table 1), will never be reached. If, for instance, only in-growing ^{111}Cd is present: the maximal achievable

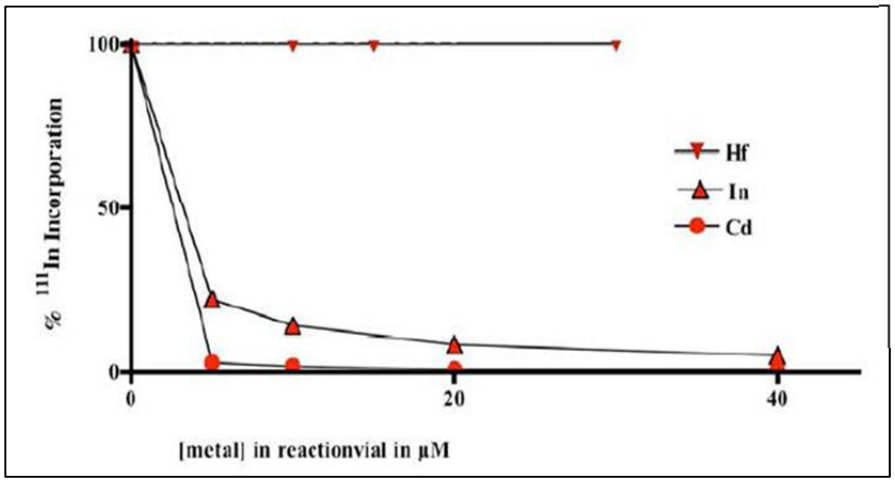

Figure 5. Effects of Contaminants on ^{111}In Labeling. Effects of contaminants on the incorporation of ^{111}In in a DOTA-peptide by the controlled addition of non-radioactive ions of In^{3+} and Cd^{2+} [26]. This illustrates that the ever present Cd^{2+} ions compete with In^{3+}-ions for the incorporation in DOTA, while the addition of Hf^{4+} ions (hafnium, the decay product of ^{177}Lu, see Figure 10A) has no effect on the % incorporation of ^{111}In and indicates that Hf^{4+} is no competitor for $^{111}In^{3+}$.

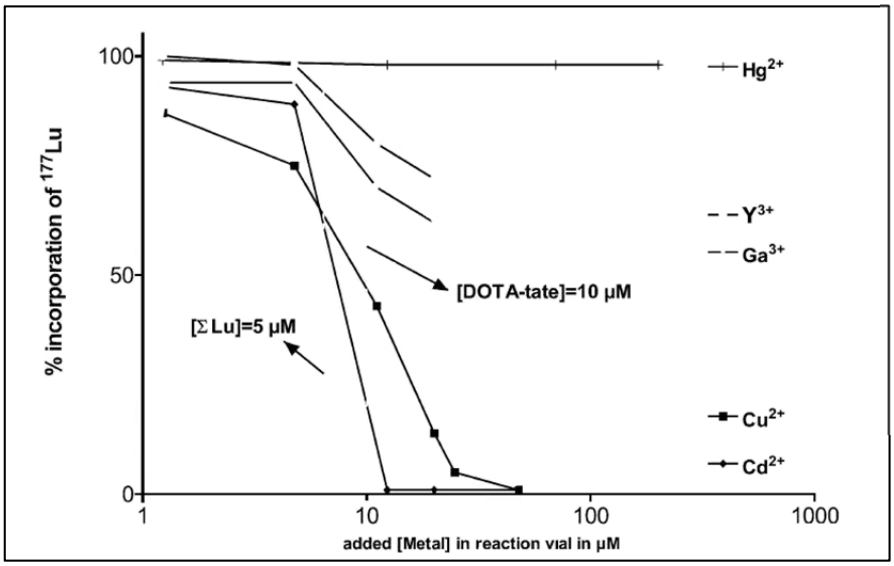

Figure 6. Effects of Contaminants on ^{177}Lu Labeling. Effects of contaminants on the incorporation of ^{177}Lu in DOTAtate by the controlled addition of non-radioactive nuclides. This model was applied to screen the possible introduction of contaminants in the reaction solution during radiolabeling. Test labeling was performed with/without addition of ascorbic acid, acetate, new glassware, rubber stoppers etc. With this technique we found significant differences between different brands or batches of Na-acetate, Na-ascorbate and ascorbic acid, and thus concordant sources of contaminants.

Specific Activities of Radiometals, Radionuclides and Radioligands

The various expressions of specific activities of radiometals, radionuclides and radioligands are a source of potential confusion. The definitions and terminologies used by scientists in different disciplines are listed below. For physicists, specific activity is radioactivity per mass of the same nuclide, thus radioactivity ^{177}Lu per mass of ^{177}Lu (A); for reactor physicists it is the radioactivity of ^{177}Lu per mass of target material (=^{175} Lu + ^{176}Lu) (B); and for radiochemists the radioactivity ^{177}Lu per mass of Lu (C); or even better, with correction for the mass of formed ^{177}Hf , thus decayed ^{177} Lu + ^{177m}Lu (D) [45]. In biochemistry and in nuclear medicine specific radioactivity is also in use for the activity of ^{177}Lu per mass of ligand or peptide (E).

$$Specific\ activity = \frac{^{177}Lu \quad activity}{\sum mass\ ^{177}Lu}$$ A: Physicists

$$Specific\ activity = \frac{^{177}Lu \quad activity}{\sum mass\ ^{175+176}Lu}$$ B: Reactor Physicists

$$Specific\ activity = \frac{^{177}Lu \quad activity}{\sum mass\ (^{175+176+176m+177+177m}Lu)}$$ C: Radiochemist

$$Specific\ activity = \frac{^{177}Lu \quad activity}{\sum mass\ (^{175+176+176m+177+177m}Lu - ^{177}Hf)}$$ D: Biochemist

$$Specific\ activity = \frac{^{177}Lu \quad activity}{\sum mass\ (DTPA-or\,DOTA-peptide)}$$ E: Nuclear Medicine

In this document we use the terminology for specific activities (C, D and E) with two different dimensions. The units of specific activity (C and D) are commonly expressed in Ci.mmol^{-1}, mCi.µmol^{-1} or MBq.nmol^{-1}. Nuclear medicine uses E, including the DTPA- or DOTA-peptides.

Peptide Characterisation, Peptide Purity and Peptide Content

Characterization, purity and content of peptide need to be determined. Characterization of the peptide can be done by ^{13}C Nuclear Magnetic Resonance (NMR) or by determining the amino acid sequence by controlled degradation with carbo-peptidase. Identification can also be performed in biological assays, especially receptor-binding techniques. For purity and content of the peptide chromatographic

techniques, such as HPLC are frequently applied. In short, it requires specific techniques and equipment, and is beyond the scope of this manuscript.

Recommended literature: UNM CE for Radiopharmacists and Nuclear Medicine Professionals 1999; Volume 8, number 1, by Dr Geert Ensing and [38].

Solubility of the Radiometals, pH and Reaction Kinetics

Unfortunately, the need for high specific activity is often compromised by conflicting parameters such as temperature (Figure 7a), pH (Figure 7b) and solubility of the radionuclide during radiolabeling. The pH determines both reaction rates and yields. The rate of formation of the metal-DTPA- and metal-DOTA complexes increases with higher pH, however the solubility of In^{3+}, Y^{3+} and Lu^{3+} decreases when pH is increased [39, 46]. Reaction kinetics of metal-DOTA complexes differ for each radionuclide and reactions can be influenced (or even hampered) by contaminants, including contaminants from target material and decay products [26].

Reaction kinetics with ^{90}Y, ^{111}In and ^{177}Lu were found to be optimal at pH 4–4.5, with a steep decrease at lower pH and a slow decrease at higher pH (Figure 7b). In addition, the observed percent incorporation of ^{111}In and ^{177}Lu at pH $\geq$5 is non-reproducible. Following reactions at pH $\geq$5, we have also found precipitates after centrifugation of the reaction vials (Figure 7c). A method to mimic the pH in the reaction mixture is shown in Figure 7c, demonstrating a titration curve of the radiochemical-containing solution and the required addition of Na-acetate to control pH. Most important, this titration curve can be performed on a small scale, without radioactivity, to aid in scaling up to larger reactions.

Reaction kinetics have also been found to be time- and temperature-dependent. Complete incorporation has been observed with ^{90}Y and ^{177}Lu after 20 min at 80°C, and with ^{111}In after 30 min at 100°C (Figure 7a). Table 1 shows the highest achieved specific activities of $^{67/68}Ga$, ^{90}Y, ^{111}In and ^{177}Lu [26].

To illustrate the kinetics and purity of the DOTA-labeling with Lu, full incorporation was achieved with a ratio of 1.2 mol DOTA-peptide per mol of Lu. In addition, high specific activity can still be achieved two weeks after the production of ^{177}Lu, confirming that Hf^{4+} is not a competitor for Lu^{3+} in the incorporation into DOTA. The formation of metal-DTPA complexes follows the “laws” of thermodynamic stability, eventually the metal-DTPA complex with the highest thermodynamic

stability will be formed. It is important to differentiate between the concepts of thermodynamic and kinetic stability. Thermodynamics (and log *K* values) indicate where the equilibrium lies. Kinetics, on the other hand, indicates how quickly equilibrium is achieved. Thermodynamic stability is usually described in terms of the association constant between the ligand and the metal.

Recommended literature: UNM CE for Radiopharmacists and Nuclear Medicine Professionals 2010;Volume 13, number 5, by Dr Alan Packard.

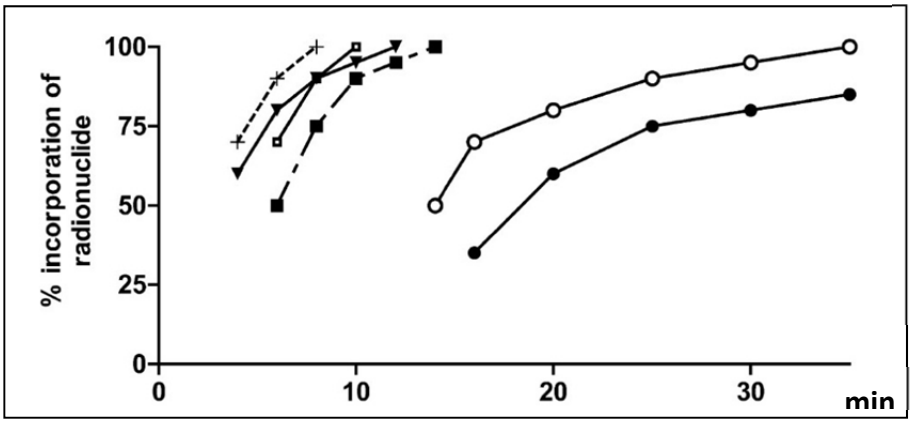

Figure 7a.
Incorporation of radionuclide as function of time (min).
Formation of radiolabeled DOTATOC at pH 4.
▼: ^{177}Lu at 80°C, +: ^{177}Lu at 100°C
■: ^{90}Y at 80°C, □: ^{90}Y at 100°C,
●: ^{111}In at 80°C and ○: ^{111}In at 100°C.
Similar results were found with DOTAtate.

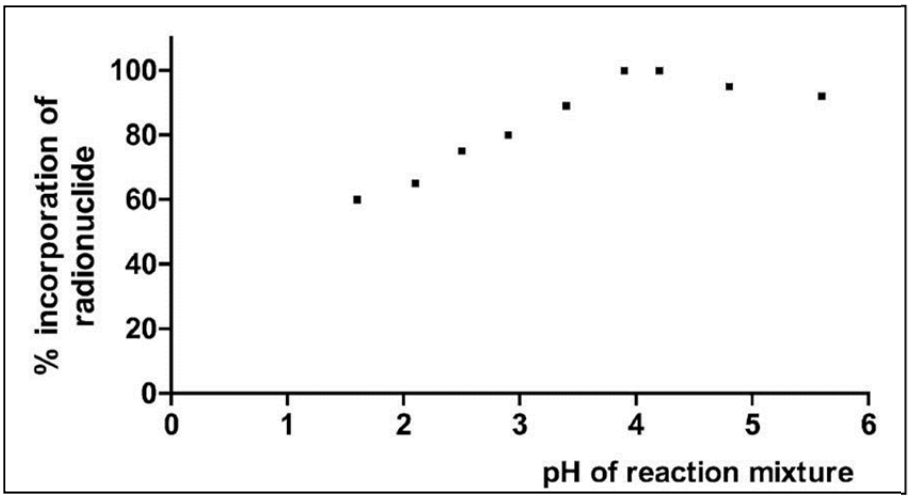

Figure 7b.
Incorporation of radionuclide as function of pH.
Formation of radiolabeled DOTATOC at pH 4 as a function of time of incubation, as measured by the % incorporation of the radionuclide. Similar results were found with DOTAtate.

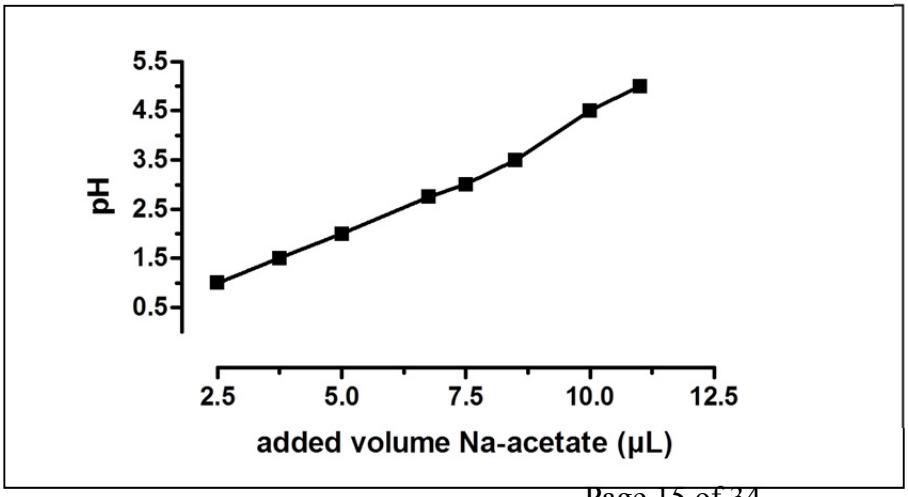

Figure 7c.
pH Curve of 100 µL 0.1 M HCl and 1.25 M Na-acetate.
pH curve of 100 µL 0.1 M HCl titrated with 1.25 M Na-acetate. The here described radionuclides are frequently provided in HCl. To optimise the reaction kinetics during radiolabeling the smallest addition of buffer to control pH is recommended.
Recommended literature:[38]

Incorporation of the Radionuclide and Radiochemical Purity of the Radioligand

The expressions of successful radionuclidic labeling and radiochemical purity of radiolabeled DTPA- and DOTA-peptides are another source of potential confusion.

Radionuclide incorporation yields can be determined by Instant Thin Layer Chromatography-Silica Gel (ITLC-SG), while radiochemical purity is used to express the portion of radioligand that is present in the desired chemical form [38]. Radiochemical purity cannot be determined with ITLC-SG, but rather requires techniques such as HPLC (High Pressure Liquid Chromatography) – see Quality Control below. Radiochemical purity methods must effectively separate the different forms of desired (intact) and undesired (non-intact) forms of radiolabeled DTPA- and DOTA-peptides. An example is the radiolabeling of DOTA-MG11 (Figure 3) an analogue with affinity for the CCK2 receptor. DOTA-MG11 contains a methionine, which is vulnerable for oxidation and/or radiolysis.

Many, if not all of radiolabeled DTPA- and DOTA-peptides are vulnerable to radiolysis and require radioprotection with quenchers such as ascorbic and gentisic acids. As illustrated in Figure 8A-F maintaining the radiochemical purity of the radioligand is a challenge for the radiochemist or the radiopharmacist.

There are many documents describing the relevant parameters of quality control by HPLC, including accuracy, linearity, precision, repeatability, detection limit, etc. However, there are no known criteria to qualify a HPLC separation method as perfect, good, or even good-enough. The tools and analytical equipment in

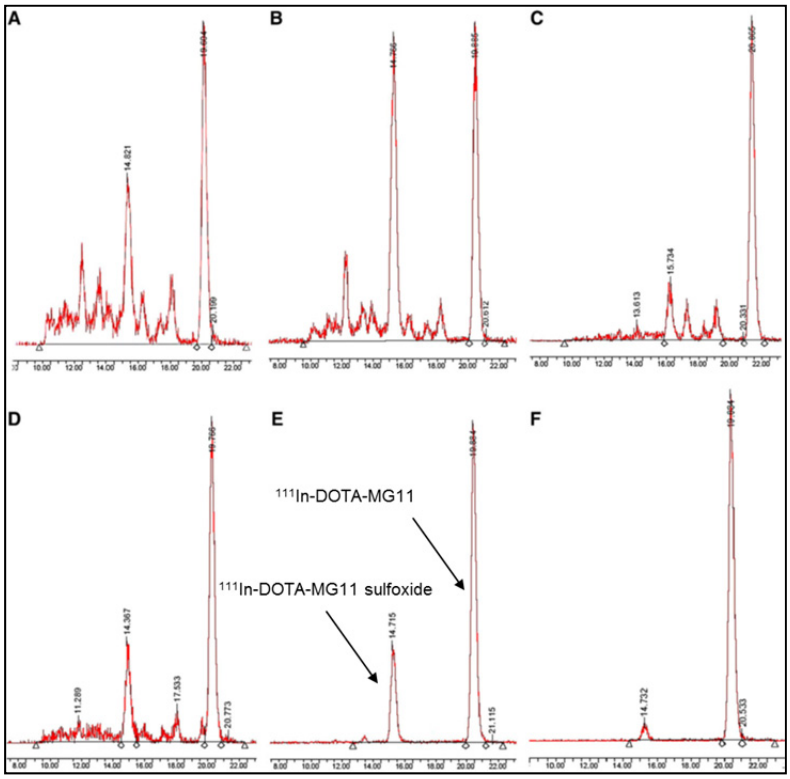

Figure 8. RP C_{18} HPLC chromatograms of ^{111}In-DOTA-MG11 at 24 h after the start of the radiolabeling (5 min at 80°C) with the effects of a single, and of combinations of quenchers, versus control (A=without quenchers). Or in the presence of (B): 10% (v/v) ethanol, (C): 4 mM L-methionine, (D): 4 mM ascorbic acid, (E): 50 mM ascorbic acid + 10% (v/v) ethanol, (F): 50 mM ascorbic acid + 10% (v/v) ethanol + 50 mM L-methionine. Radiochemical purity of (A) and of (F) were ≈60% and 94%, resp. Oxidation product (RT≈14 min) was HPLC-purified to test the biological activity in *in vitro* autoradiography studies and revealed the oxidized form has lost its CCK2 receptor-binding characteristics[40]. It should be noted that ITLC-SG will gave incorporations of radionuclide of 99+%, and clearly illustrates the short coming of the ITLC technique and also the "power" of HPLC.

radiochemistry are improving rapidly. For example, a recent report by Asti *et al* [47], discussed the separation by UPLC (Ultra PLC) of DOTAtate labeled with different metals. How this may impact the detection and sensitivity of radiolabeled DOTAtate and DOTA-peptides is uncertain. The current expressions of "radiochemical purity in %" are actually expressed in Arbitrary Units.

Although arguable, the minimal requirements for radiochemical purity by HPLC and radiodetection for radiolabeled DTPA- or DOTA-peptides are:

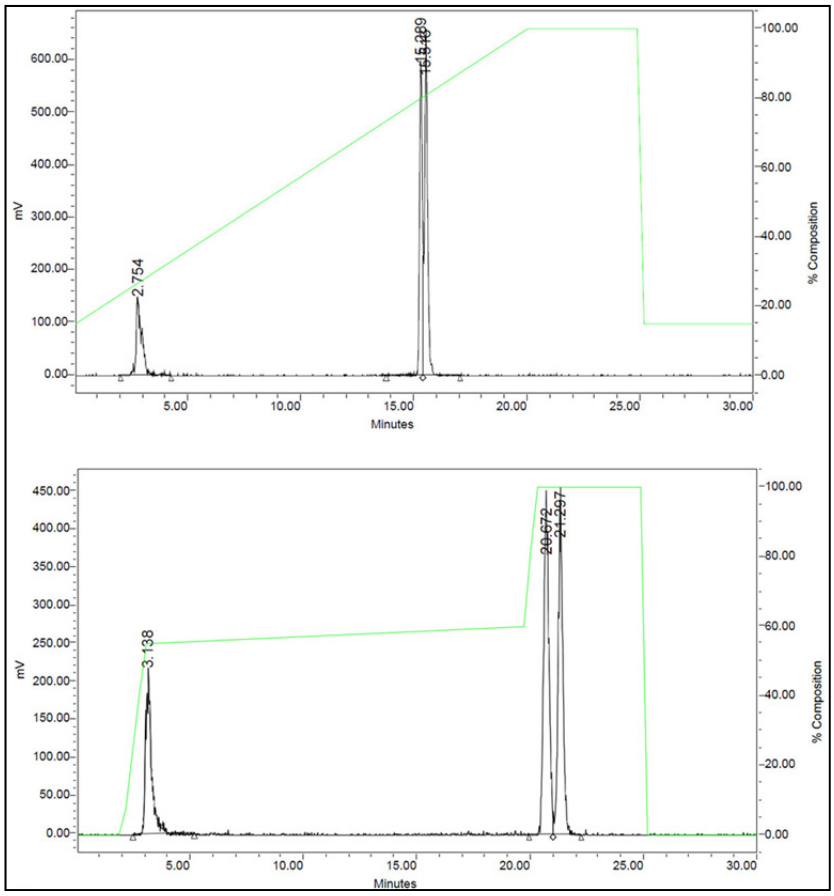

Figure 9. **Minimal Requirements for HPLC Radiochemical purity**
Two examples of a HPLC (Symmetry C_{18}) run of a radiolabeled DTPA-peptide (here with [^{111}In-DTPA^{0}]octreotide, OctreoScan, see also Figure 1). Here we present a HPLC run with a base-to-base separation of different conformations of [^{111}In-DTPA^{0}]octreotide .The peaks of these conformations must be present in all HPLC run of all radiolabeled DTPA-peptides. Varying conditions (including column, gradients etc) can be used to optimize gradient. The green line indicates the gradient, see right Y-axis. With an even steeper gradient, the two peaks disappear. Asti *et al*. recently reported the separation of different cations and DOTAtate on UPLC [47], which indicates the possibility to have base-to-base separations for DOTA-peptides. It also implies the need to further improve standards for HPLC separations. The current expression "radiochemical purity in %" are actually expressed in Arbitrary Units.

a. A base-to-base separation with 2 peaks of the DTPA conformations of a radiolabeled DTPA-peptide (Figure 9),

b. A base-to-base separation of metal-peptide *vs.* peptide for DOTA-peptide [47]. (Figure 9)

Moreover, the comparison of radiochemical purity of compounds between different HPLC or UPLC systems (including columns, conditions, gradient, detector etc), and between laboratories is difficult, if not impossible.

If radioactive impurities are found with a content of > 1% of total radioactivity, an effort should be made to characterize the impurities. This can be performed by HPLC purification, as described in the legend of Figure 8 for DOTA-MG11.

Impurities can be separated from the principle fraction, and collected from HPLC for investigation or identification using mass spectroscopy. It should be noted that the mass of these fractions are frequently on the order of nmoles or even pmoles. If the fraction is carrier free and radioactivity measured, the concordant mass can be calculated from the data provided in Table 1.

Recommended literature: UNM CE for Radiopharmacists and Nuclear Medicine Professionals 1999;Volume 8, number 1, by Dr Geert Ensing and [38]

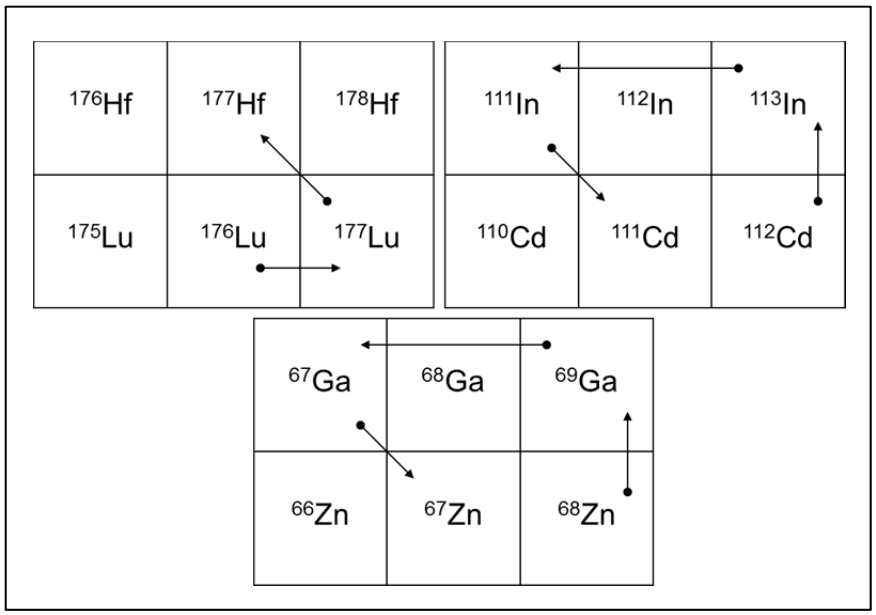

Figure 10A. Schematic presentation of formation and decay of ^{67}Ga, ^{111}In and ^{177}Lu., see also Table 1.

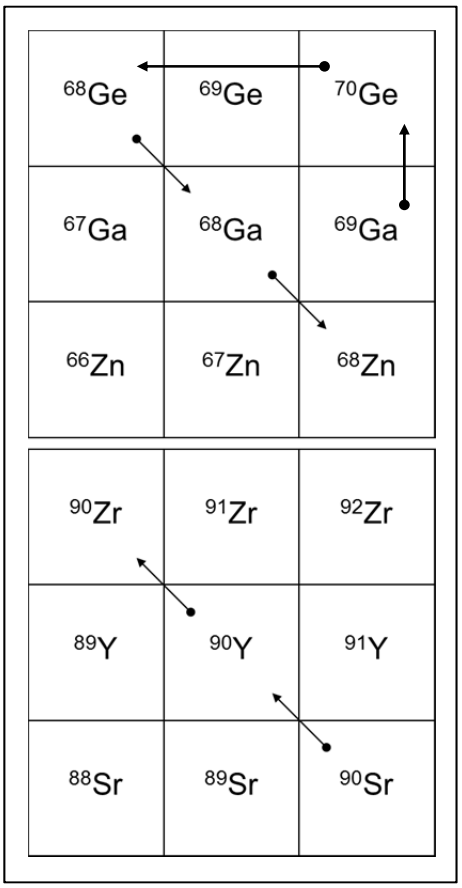

Figure 10B. Schematic presentation of formation and decay of ^{68}Ga and ^{90}Y, see also Table 1.

Quality Control

Quality control, including the addition of 4 mM DTPA post radiolabeling has been performed as described earlier [26]. In summary, incorporation of radionuclide was measured by ITLC-SG, eluents: 0.1 M Na-citrate (pH 5) and/or with 1M ammonium acetate/methanol (1:1 v/v) [5, 48]. Radioactivity on the ITLC strips was measured with a dose calibrator (e.g. Veenstra VDC-405, Joure, The Netherlands) or Packard Cyclone Phosphor Imaging system with OptiQuant software (Perkin Elmer, Groningen, The Netherlands).

Radiochemical purity was measured by HPLC (e.g. a Waters Breeze HPLC system, 1525 binair pump). A Symmetry C_{18} column (5 mm,*4.6mm 250mm,Waters, Etten- Leur, The Netherlands),

mobile phase 0.1%TFA (A) methanol (B). Gradient profile was: 0–2 min 100% A (flow 1mL/min), 2–3 min 55% B (flow 0.5mL/min),3.01–20 min 60% B (flow 0.5mL/min), 20–20.01 min 100% B (flow 1mL/min), 20.01–25 min 100% A (flow 1mL/min), 25.01–30 min 100% A (flow1 mL/min). HPLC injections were performed via a Waters 717 autosampler (injection volume 100-200 µL). Radioactivity was monitored with a Unispec MCA γ-detector (MetorX, Goedereede, The Netherlands).

An important consideration is the "sample recovery", meaning the actual radioactivity injected onto the HPLC column *vs*. the recovered radioactivity. This is especially important with colloids, which are often retained on the HPLC column and not available for detection, thus giving false positive results of the radiolabeled peptide.

Addition of DTPA Promotes Renal Elimination of non-incorporated $^{90}Y^{3+}$, $^{111}In^{3+}$ or $^{177}Lu^{3+}$ in ^{90}Y -, ^{111}In- or ^{177}Lu -labeled DOTA-Peptides for Preclinical and Clinical Application

There are several reasons for this addition. First, the free ionic fraction of radionuclide in radiolabeled DOTA-peptides can effectively be complexed by the addition of chelator *in vitro.* This results in an efficient complexation of the free ionic fraction of radionuclide resulting in rapid excretion by the kidneys [16, 26]. Since the free ionic fraction of radionuclide in radiolabeled DOTA-peptides can be complexed and effectively rerouted *in vivo,* the specification for the % of incorporation is now lowered to 97%. For example: After radiolabeling the DOTA-peptide and cooling the reaction mixture to room temperature, a chelator such as DTPA is added to react with non-incorporated radionuclide. Typically 5 µL of a 4 mM DTPA solution is added to a 100 µL reaction volume. After radiolabeling DOTA-peptides with $^{67/68}Ga$ the addition of EDTA is preferred [5], see below, **Radiolabeling DOTA-peptides with ^{67}Ga and ^{68}Ga.**

Second, it is hard to take a representative sample from a solution containing DOTA-conjugated analogues labeled with radionuclides that are known to form colloids. For example, in the accurate determination of non-incorporated ^{90}Y, ^{111}In and ^{177}Lu during the standard quality control by ITLC or HPLC, the non-incorporated ^{90}Y, ^{111}In and ^{177}Lu will rapidly bind to the origin of the ITLC or to HPLC column. This will result in a false identification of the incorporation or radiochemical purity, respectively [26, 49]. The addition of a chelator (DTPA) can solve this problem, and the addition is therefore necessary.

Effects of Contaminants on Radiolabeling

An example of the effect of contaminants, including contaminants from target material and decay products, is the presence of Cd in ^{111}In. [26]. Figure 4 shows the concentration of ^{111}In and the intrinsically present ^{111}Cd (calculated) in the ^{111}In-solution presented as a function of time post the production of ^{111}In. The increasing concentration of ever-present contaminants such as Cd^{2+} ions, a competitor for $^{111}In^{3+}$, alters the incorporation of ^{111}In in DOTA-peptides. Immediately after production (t=0) the concentration of ^{111}In is 1 µM, as shown in Figure 4. The concentration of Cd^{2+} immediately after production (t=0) was already 0.5 µM (range 0.4-0.6 µM, n=5 batches), indicating the presence of target material ^{112}Cd (See Table 1 and Figure 10A).

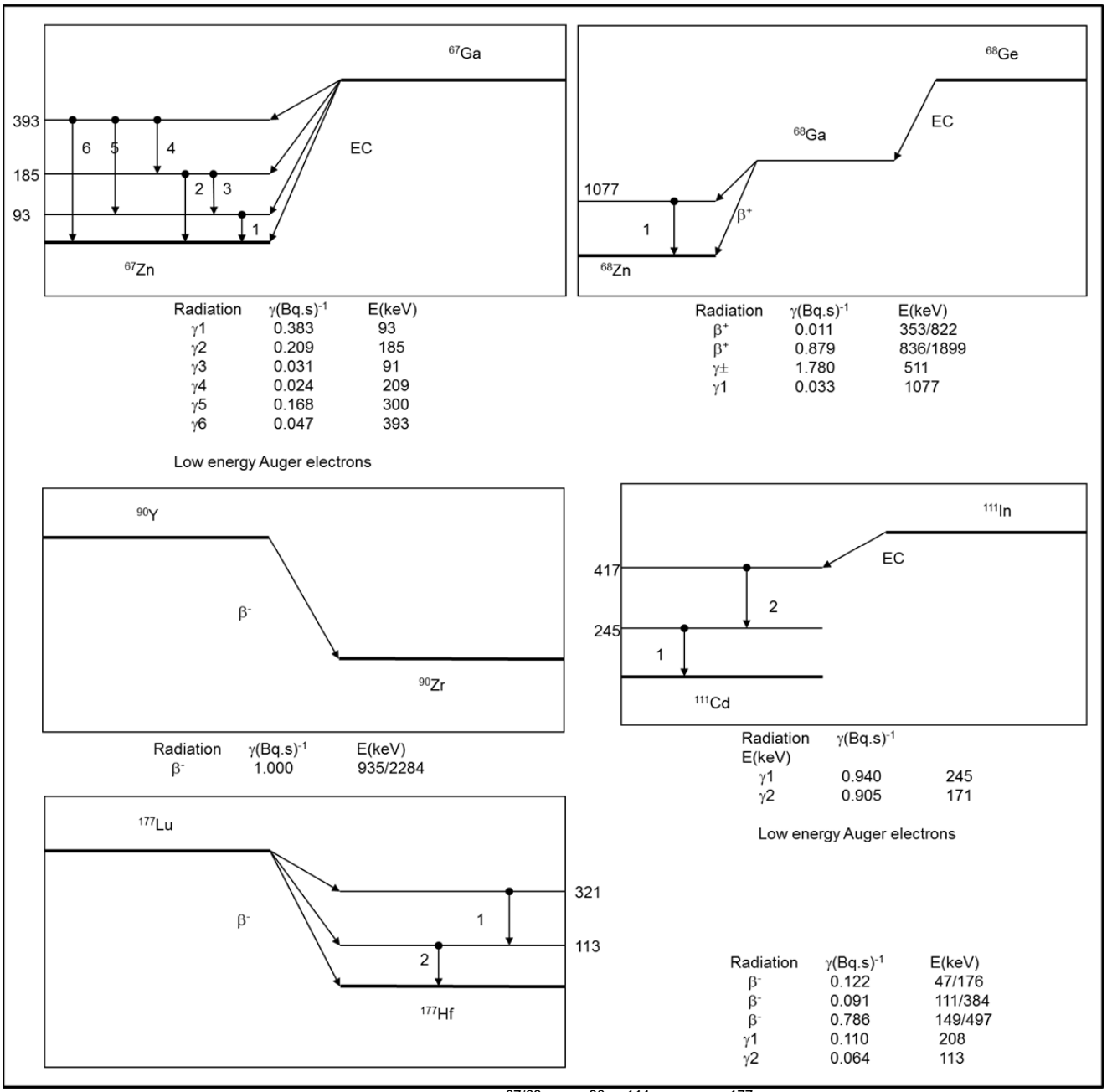

Figure 11. Schematic presentation of decay of $^{67/68}$Ga, ^{90}Y, ^{111}In and ^{177}Lu with concordant energies and abundancies.

Contaminant analysis was studied with controlled addition of ^{115}In (natural indium, a mixture of 4.23% "non-radioactive indium" ^{113}In and ^{115}In, t½ = $6*10^{14}$ years), which lowers the specific activity of ^{111}In. The added ^{115}In competes with ^{111}In for incorporation in the DOTA-chelator. As a negative control, the addition of Hf^{4+} ions (hafnium, the decay product of ^{177}Lu) has no effect on the percent incorporation of ^{111}In and indicates that Hf^{4+} is not a competitor for $^{111}In^{3+}$. In contrast, the addition of ions of Cd^{2+} or $^{115}In^{3+}$ clearly shows that these ions are strong competitors for $^{111}In^{3+}$ for the incorporation in the DOTA-chelator (Figure 6).

To illustrate the effects of target material and decay products of ^{111}In in the $^{111}InCl_3$ stock solution, the radiolabeling of DOTA-peptides with ^{111}In was assessed, while measuring the highest achievable specific activity as a function of time over a week of use of $^{111}InCl_3$ [26]. As a consequence, the maximal achievable specific activity with DOTA-peptides decreases as a function of time after the production of ^{111}In, as the concentration of ingrowing ^{111}Cd increases (See Table 2, Figure 6 and 10A for details). These results indicate that Cd^{2+} has similar, or even higher reaction kinetics under these reaction conditions. Although the stability constants of DOTA for In^{3+} and Cd^{2+} differ considerably, 23.9 and 20.9, respectively, at pH 7 the described incorporations and competitions are determined by kinetics. A thorough search of the literature for the stability constants of DOTA with Hf^{4+} and of DOTA-peptides with In^{3+}, Cd^{2+} or Hf^{4+} yielded no results.

Table 2

^{111}In: Method of production, physical constants, specific activity of ^{111}In and the maximal achievable SA of ^{111}In-labeled DOTA-peptides. Theory *vs.* Practice
^{111}In is a cyclotron-produced radiometal, formed from ^{112}Cd by a (p, 2n) reaction:
$^{112}Cd \rightarrow {}^{111}In \rightarrow {}^{111}Cd$
t½= 2.83 days, specific activity of $^{111}In \equiv 2.15*10^{-11}$ mol per 37 MBq (1 mCi) ^{111}In. In theory 1 nmol DOTA-peptide can complex 1 nmol In^{3+}, corresponding to 1.7 GBq ^{111}In (Table 1). The ^{111}In-containing stock solution is diluted by the manufacturer (e.g. Covidien Medical, Petten, the Netherlands) on the day of production to a concentration of approximately 1.7 GBq ^{111}In per mL, corresponding to 1 nmol ^{111}In per mL, or 1 µM ^{111}In.. ^{111}In decays to ^{111}Cd implying that the amount of ^{111}In remaining and that of ^{111}Cd formed can be calculated (see Table 1), also as a function of time, using the t½ = 2.83 days.

The effects of contaminants on the incorporation of ^{177}Lu into DOTAtate by the controlled addition of non-radioactive nuclides are shown in Figure 6. This model was applied to investigate the possible introduction of contaminants in the reaction mixture during radiolabeling. Test radiolabelings were performed with/without the addition of ascorbic acid, acetate, using new glassware, rubber stoppers

etc. With this technique we found significant and considerable differences between different brands or batches of Na-acetate and ascorbic acid.

Labeling Conditions for PRRT with ^{90}Y or ^{177}Lu -labeled DOTA-Peptides for Preclinical or Clinical Application

A typical reaction mixture for radiolabeling therapeutic amounts of ^{90}Y- or ^{177}Lu-labeled DOTA-peptides is 37 GBq (1 Ci) $^{90}YCl_3$ – or $^{177}LuCl_3$ in 1 mL 0.04 M HCl with 1 mg DOTAtate in 2.5 mL of 50 mM sodium-ascorbate and gentisic acid and a final pH (after reconstitution) of 3.5 - 4.

Radiolabeling DOTA-peptides with ^{67}Ga and ^{68}Ga

Radiolabeling DOTA-peptides with ^{67}Ga and ^{68}Ga resembles the radiochemistry of the other trivalent radiometals described above and will only be briefly described here. The presence and ingrowth of target and decay products is similar to ^{111}In. Analogous to ^{111} Cd and ^{112}Cd present in ^{111}In, achieving high specific activities with ^{67}Ga-DOTA-peptides is very difficult owing to the presence of [^{67} Zn and ^{68}Zn] in target (^{68}Zn) and ^{67}Zn formed during decay (Table 1, Figure 10A). Even if the [^{68}Zn] from the target at the end of production and purification of ^{67}Ga is very low or even zero, while after one half-life of ^{67}Ga the [^{67}Zn] = [^{67}Ga]. There are, however, radionuclides with much higher observed specific activities.

For the positron-emitter ^{68}Ga, specific activities of > 1 GBq ^{68}Ga per nmol DOTATOC and DOTAtate are reported [5, 50, 51] (Table 1, Figure 10B). Besides gammas, ^{67}Ga also emits Auger (0.1-8 KeV) and internal conversion electrons (80-90 KeV, Figure 11A, and might therefore also be suitable for PRRT [11]. After radiolabeling DOTA-peptides with $^{67/68}$Ga the addition of EDTA is preferred over DTPA. Although the thermodynamic stability constants for both EDTA and DTPA for Ga^{3+} are very high (21.7 and 23.3, respectively)[52], the formation of ^{68}Ga-DTPA under our conditions was found to be low, in contrast to the formation of $^{67/68}$Ga-EDTA; and we performed studies with ^{6768}Ga-EDTA [5]. Therefore, the addition of DTPA prior to quality control of $^{67/68}$Ga-labeled products under acidic conditions can be replaced by addition of EDTA [5].

Further readings are recommended on the applications of ^{68}Ga chemistry and robotics for radiolabeling [5, 48, 50, 53-55].

Waste Management

There is always ^{68}Ge (t½ = 9 months) present in ^{68}Ga, ^{114m}In (t½ = 49 days) in ^{111}In, and ^{177m}Lu (t½ = 161 days) in ^{177}Lu. The presence of these radionuclidic contaminants and their concordant long half-lives are issues to seriously consider when planning for waste management [50, 56, 57].

SUMMARY

This lesson describes the practical aspects of radiolabeling DTPA- and DOTA-peptides with ^{90}Y, ^{111}In, ^{177}Lu, and ^{68}Ga for PRS and PRRT in preclinical and clinical applications. The recommended readings are intended to provide additional context and foundations for the information presented in this lesson. The rapidly increasing interest in ^{68}Ga and ^{177}Lu are bringing renewed interest in the fundamentals of inorganic chemistry applied to bifunctional chelate-conjugated peptides. Additional studies to rigorously evaluate the effects of specific activity, radiochemical purity, and mass on the preclinical performance of PRS and PRRT are needed to inform regarding the optimal clinical dose of DTPA- and DOTA-peptides.

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QUESTIONS

1. Are the solubilities of radiometals such as Ga, In, Y, and Lu pH-dependent?

    a. True
    b. Only In and Y
    c. Only Y and Lu
    d. None are dependent

2. Is the maximum achievable specific activity of In-or Y-labeled DTPA- and DOTA-peptides always higher for DTPA-peptides?

    a. True
    b. False, it is always higher for In
    c. False, it is always higher for Y
    d. False

3. What is the effect of ^{177}Lu decay (and thus the in-growth of Hf) products on the incorporation of ^{177}Lu in DOTA-peptides?

    a. Hf decay products will decrease incorporation yields of ^{177}Lu-DOTA-peptides
    b. Hf decay products will NOT interfere
    c. Hf decay products will only interfere with in vivo studies
    d. Hf decay products will decrease radiochemical purity of ^{177}Lu-DOTA-peptides

4. Do ^{111}Cd and ^{112}Cd contaminants effect the radiolabeling of DOTA-conjugated peptides with ^{111}In?

    a. True, both have an effect
    b. False, only as ^{112}Cd has an effect
    c. False, only as ^{111}Cd has an effect
    d. False, neither one has an effect

5. Does the specific activity and mass dose influence receptor-mediated processes in vivo?

    a. Yes, these processes are well documented
    b. No, these processes are only observed in vitro as low pH
    c. No, these process are only observed with ^{111}Cd present
    d. No, there are no such in vivo processes

6. The specific activity of the radioligand is the only parameter influencing receptor-mediated processes in vivo.

    a. True
    b. Only in combination with dose
    c. Only in vitro
    d. False

7. In order to ensure complete incorporation of the radionuclide, DTPA should be added to the reaction mixture **before** the radionuclide is added and the radiolabeling reaction is initiated.

    a. True
    b. Only in minimal amounts
    c. Only with ^{90}Y
    d. False

8. The radiochemical purity of radioligands is preferably determined using HPLC.

    a. True
    b. Only as ^{111}In-labeled
    c. False
    d. Only as ^{90}Y-labeled

9. Radiochemical purity testing of radioligands by HPLC is a widely used and well-defined quality control parameter.

    a. True
    b. False
    c. It is widely used but not well-defined
    d. It is well-defined but not widely used

10. The addition of DTPA to radiolabeled DOTA-peptides will complex unincorporated or "free" radiometals and promote their renal elimination in vivo.

    a. True
    b. Only as ^{111}In-labeled
    c. Only as ^{90}Y-labeled
    d. False

11. When developing radiolabeling methods, radiometals are required to mimic pH in the reaction mixture for radiolabeling.

    a. True
    b. False
    c. Only as ^{111}In-labeled
    d. Only as ^{90}Y-labeled

12. Radiometals in the form of colloids can be used for radiolabeling DTPA-peptides

    a. True
    b. False
    c. Only as ^{111}In-labeled
    d. Only as ^{90}Y-labeled

13. The term “Log K” is a parameter used to describe chemical reaction kinetics.

    a. True
    b. False
    c. Only as ^{177}Lu-labeled
    d. Only as ^{90}Y-labeled

14. A high Log K value is an important parameter for rapid receptor-ligand interactions.

    a. True
    b. Only as ^{111}In-labeled
    c. Only as ^{90}Y-labeled
    d. False

15. Regulatory peptides without methionine, such as octreotide, are not vulnerable to radiolysis.

    a. True
    b. False
    c. Only as ^{177}Lu-labeled
    d. Only as ^{90}Y-labeled

16. The theoretical specific activity of a radioligand is dependent on the half-life of the radionuclide.

    a. True
    b. False
    c. Only as ^{90}Y-labeled
    d. Only as ^{111}In-labeled

17. Higher specific activity can be achieved for all ^{67}Ga-labeled radioligands vs. their ^{68}Ga-labeled counterparts.

    a. True
    b. False
    c. False, depends on the peptide
    d. False, depends on the chelator

18. Radionuclides with shortest half-life have the highest theoretical specific activity.

    a. True
    b. False
    c. True, and depends on the molecular weight of the peptide
    d. True, and depends on the molecular weight of the chelator

19. In practice, the maximum achievable specific activity of a radioligand is dependent upon the characteristics of the radionuclide (e.g. half-life, contaminants, target and decay products in the radionuclide-containing solution).

    a. True
    b. Only on the half-life
    c. Only on contaminants
    d. On both half-life and contaminants

20. The difference in the specific activities of radionuclides is only dependent on their difference in atomic mass.

    a. True
    b. False
    c. Only as ^{90}Y-labeled
    d. Only as ^{111}In-labeled

21. The in vivo stability of radiolabeled regulatory peptides conjugated with a DOTA is greater than the concordant DTPA-peptides.

    a. True
    b. Only as ^{90}Y-labeled
    c. Only as ^{111}In-labeled
    d. True, and depends on the molecular weight of the peptide

22. When an impurity $\geq$ 1% of total radioactivity is detected, an effort should be made to characterize the impurity.

    a. True
    b. Preferably not
    c. Only ^{90}Y-labeled impurities
    d. Only when $\geq$10%

23. A 1.5 mL solution containing >1.7 GBq of ^{111}In-labeled DOTA-peptide (molecular weight = 1500 D) is purified by HPLC. After purification, the radiolabeled peptide is carrier-free, meaning that all of the peptide present is labeled with ^{111}In. This fraction is in 1 mL and contains 170 MBq. Assume the ^{111}In radiolabeled peptide was labeled at the maximum theoretical specific activity.

    What is the peptide concentration?

    a. 0.1 nmoles/L
    b. 10 nmoles/L
    c. 1 nmoles/L
    d. 0.1 μmoles/L

24. A 10 mL solution containing >1.8 GBq ^{111}In-labeled DOTA-peptides (molecular weight = 1500 D) is purified by HPLC. After purification the radiolabeled peptide is carrier-free, that means all peptide is labeled with ^{111}In. This fraction is in 1 mL and contains 17 MBq . Assume the ^{111}In radiolabeled peptide was labeled at the maximum theoretical specific activity.

What is the peptide content?

a. 0.01 nmoles
b. 10 nM
c. 0.1 nmoles
d. 0.01 μmoles

25. For a preclinical experiment, 1 nmole of a DOTA-peptide (molecular weight = 1000 D) must be labeled with $^{90}YCl_3$. Assume complete incorporation under stoichiometrically-balanced conditions. $^{90}YCl_3$ is available with a specific activity of 452 MBq per nmol Y per mL. Assume the peptide will be labled at the maximum achievable specific activity.

How many mL's of the $^{90}YCl_3$ are required?

a. 1 mL
b. 2 mL
c. 4 mL
d. 10 mL

26. The stability of radiolabeled peptides in vivo can be measured in vitro, using serum or saline simulations

a. True
b. Only by HPLC
c. Only at 37C
d. False

Please use the following information to answer questions 27-34

Suppose a 1 mL solution containing 72 MBq ^{177}Lu-labeled DOTA-peptide (molecular weight = 1000 D) is purified by HPLC. After purification, the radiolabeled peptide is carrier-free, that is all of the peptide present is radiolabeled. Assume the isotopic abundance of ^{177}Lu is 100%. The HPLC-purified fraction contains 7.2 MBq. Assume the ^{111}In radiolabeled peptide was labeled at the maximum theoretical specific activity.

27. What is the total amount of Lu in the 1 mL starting solution?

    a. 10 nmoles
    b. 1 nmoles
    c. 0.1 nmoles
    d. 0.1 μM

28. What is the Lu concentration in the starting solution?

    a. 10 nmoles/L
    b. 1 nmoles/L
    c. 0.1 nmoles/L
    d. 0.1 μmoles/L

29. What is the DOTA-peptide concentration in the starting solution?

    a. Uncertain, but > 0.1 μmoles/L
    b. 1 nmoles/L
    c. 0.1 nmoles/L
    d. 1 μmoles/L

30. What is the DOTA-peptide content after HPLC-purification?

    a. 10 pmoles
    b. 100 pmoles
    c. 1 pmoles
    d. 1000 pmoles

31. What is the Total Lu content in pmoles after HPLC-purification?

    a. 50 pmoles
    b. 5 pmoles
    c. 0.5 pmoles
    d. 500 pmoles

32. Suppose the 7.2 MBq containing HPLC-purified solution is diluted to 10 mL. What is the DOTA-peptide concentration?

a. 100 nmoles/L
b. 10 nmoles/L
c. 1 nmoles/L
d. 0.01 μmoles/L

33. What is the concentration of ^{177}Lu?

a. 5 nmoles/L
b. 1 nmoles/L
c. 0.5 nmoles/L
d. 0.01 μmoles/L

34. Suppose the isotopic abundance of ^{177}Lu is 20%. What is the total concentration of Lu?

a. 5 nmoles/L
b. 1 nmoles/L
c. 0.1 nmoles/L
d. 0.05 μmoles/L