Organometallic ^{99m}Tc-Aquaion Labels Peptide to an Unprecedented High Specific Activity

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A new peptide labeling method that uses the organometallic aquaion $[^{99m}Tc(H_2O)_3(CO)_3]^+$ has been developed. **Methods:** A selection of amino acids was labeled at different concentrations with the organometallic aquaion, and the labeling yield was determined by high-performance liquid chromatography. This investigation has shown histidine to be a very potent ligand, with specific activities of up to 6 TBq/μmol (160 Ci/μmol) ligand. Histidine derivatives have been coupled to neurotensin(8–13) (NT[8–13]) and have been labeled with the aquaion, resulting in high specific activities with (N_α-histidinyl)acetic acid-NT(8–13) similar to those with histidine. **Results:** Histidine derivatives of NT(8–13) labeled using this approach fully retained their receptor affinity, showing K_D values of all investigated NT analogs below 1 nmol/L on colon carcinoma HT29 cells. Biodistribution experiments in BALB/c mice showed complete clearance of (N_α-histidinyl)acetic acid-NT(8–13) from the blood after 24 h and no unwanted accumulation in any tissue. **Conclusion:** The novel labeling method using the organometallic ^{99m}Tc-aquaion combines the advantage of highest specific activities with minimal functionalization of proteins and peptides under retention of biologic affinity.

Key Words: peptides; ^{99m}Tc; neurotensin; histidine

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Over the last 30 y, noninvasive radiographic methods have become a standard diagnostic method for a variety of diseases. During this period, targeting vehicles have developed from nonspecific molecules to antigen-specific antibodies and very recently to receptor-specific regulatory peptides, which have emerged as one of the most promising substance classes for scintigraphic applications and for therapeutic purposes. This emergence is attributed primarily to their favorable pharmacokinetics characterized by short retention time in the blood and high uptake in the target tissue. Indications of targeting by means of biologically active peptides include, for example, thrombosis and inflammatory diseases (*1*). However, after the discovery that neoplastic cells express neuropeptide-specific receptors with both high affinity and density, research has focused predominantly on tumor targeting, which is illustrated by numerous reports on the imaging of peptide receptor-positive carcinomas (*2–4*). The most prominent examples are represented by the routinely used ^{111}In-labeled somatostatin analog diethylenetriamine pentaacetic acid-octreotide and ^{123}I-labeled vasoactive intestinal peptide (*5,6*). Great effort has been undertaken for the labeling of small peptides with ^{99m}Tc, the most popular nuclide in nuclear medicine (*7,8*). However, these approaches were of limited success: Direct labeling is unspecific, occurs in the binding region and thus leads to the loss of the peptide's receptor affinity. The alternative approach that uses bifunctional chelates, in which one functionality is designated for the attachment to the bioactive peptide and the other stabilizes the nuclide, often suffers from insufficient labeling yields and complicated labeling and purification protocols. This finding is attributed primarily to the lack of a stable but substitution-labile technetium precursor, namely, an aquaion. Thus, after reduction of pertechnetate to Tc(V), a huge excess of ligand compared with ^{99m}Tc has to be applied to prevent reoxidation before complexation has taken place.

It is unlikely that significant improvements in the labeling with technetium will be associated with improved ligand systems because they have been optimized during the last 20 y. Thus, further advances can be expected only if the easily accessible but oxidation-sensitive +V oxidation state is abandoned. Actually, investigations on technetium chemistry in all imaginable oxidation states resulted in some powerful imaging agents, such as the well-known Tc(I) hexakisisonitrile complexes (*9*), but the objective of a substitutionable, oxidation-stable and easily accessible precursor for a generally applicable labeling has not been reached.

These investigations, which have focused on Tc/Re(I) carbonyls, have shown that the *fac*-$[M(CO)_3]^+$ moiety can be obtained by direct carbonylation of permetallates, solely by the action of borohydrides and atmospheric carbon monoxide (CO) pressure (*10*). This procedure circumvents the most common starting materials for triscarbonyl compounds, decacarbonyl and halopentacarbonyls. The only product of the normal pressure carbonylation of $^{99}TcO_4^-$ in tetrahydrofuran and in the presence of chloride has been $[TcCl_3(CO)_3]^{2-}$ that forms, when placed in water, quantitatively the carbonylic aquaion $[Tc(H_2O)_3(CO)_3]^+$. Hydrolytic

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studies on the rhenium aquaion have shown that these carbonyls are not sensitive toward oxygen or water but form typical condensation products as is known from aquaions of the first transition row (*11,12*). Substitution properties of both, technetium and rhenium, have been investigated with a variety of ligands and have shown that stable complexes are formed with moderately slow kinetics (*13,14*). Thus, thermodynamics and kinetics appear to be optimal for an application in nuclear medicine.

MATERIALS AND METHODS

Chemicals were purchased from Fluka (Buchs, Switzerland) and used as received. The ^{99m}Tc-pertechnetate was obtained from a ^{99}Mo-^{99m}Tc generator (Mallinckrodt, Inc., Petten, The Netherlands). Peptide solid-phase syntheses were performed on a Merrifield resin (Peninsula Laboratory, Merseyside, United Kingdom), with a semiautomatic Labortec Peptide Synthesizer SP640B (Bachem AG, Bubendorf, Switzerland). Boc-amino acids with the side chain protecting groups tosyl for arginine and 2-BrZ for tyrosine were used. Purification was effected by semipreparative reversed-phase high-performance liquid chromatography (HPLC). The fractions containing the product were concentrated in vacuo, and the remaining aqueous solution was lyophilized. Electrospray mass spectra were measured on a Fisons VG Trio 2000 Quadrupole (Micromass, Cheshire, United Kingdom) instrument with direct inlet (capillary voltage 2.85 kV, cone voltage 12 V and ion voltage 2.3 V) with water as solvent (100 μL/min flow rate).

Synthesis of $[^{99m}Tc(H_2O)_3(CO)_3]^+$

A penicillin vial (10 mL) with 3 mg $NaBH_4$, 4 mg Na_2CO_3 and 15 mg NaK-tartrate was equipped with a stopper, sealed and flushed for 30 s with CO gas. Three milliliters Tc-generator eluate (50 MBq–30 GBq) were added by a syringe, and the solution was heated for 25 min at 75°C. Pressure from the evolving H_2 gas was balanced with a 20-mL syringe. After cooling on ice, 0.3 mL phosphate-buffered saline ([PBS] 0.1 mol/L NaCl/0.05 mol/L sodium phosphate buffer, pH 7.4) were added. Quality control was performed by gradient HPLC. Yield > 95% (*15*).

Radiolabeling

The ligand concentration that is required for complexation was determined by mixing four different concentrations of each ligand in PBS, pH 7.4, with freshly prepared technetium carbonyl. The mixtures were incubated at 37°C for 1 h. The resulting sigmoid curves can be understood as simplified species distribution curves, which correspond to conditional stability constants of the complexes between the applied ligands and the technetium carbonyl at the chosen conditions (K_{cond} = [TcL]/[Tc][L]). Most radiolabelings require far higher ligand concentrations than technetium concentrations. Only a very small amount of the applied ligand is actually consumed for the labeling, and [L] can therefore be assumed to be constant. At 50% labeling yield, the concentration of free technetium is equal to the technetium complex ([TcL] = [Tc]), and the ligand concentration that is required for this is exactly inverse to the conditional stability constant (K_{cond} = 1/[L]). On this thermodynamic base, a direct comparison between different ligands can be made, which allows a reliable selection of the most promising ligands for the labeling of biomolecules. In this approach, however, some ligands can be applied in stoichiometric ratios compared with the total technetium, and the system becomes very sensitive to both the ligand and the technetium concentrations. The reduction of the ligand concentration in these cases is significant, but below an order of magnitude, and these changes have therefore been neglected. The obtained stability constants are clearly incorrect but are still good enough for a rough estimation of the labeling capacity of a ligand. In contrast, the molarity of technetium, which depends on the age and elution frequency of the generator, varies to a far higher extent and must be considered. To minimize this systematic error, the generator was eluted daily, and the experimentally obtained curves were normalized according to the total technetium in a sample of 1 mL and 3.7 GBq (100 mCi).

The labeling for the biologic experiments was performed by mixing 3.7 GBq (100 mCi) freshly prepared technetium carbonyl (volume dependent on the age of the generator) with 100 μL of a 1 mmol/L ligand solution in a glass vial. After dilution with PBS to a total volume of 1 mL and sealing of the vial, the reaction mixture was heated to 75°C for 30 min. After this time, the labeling was analyzed by HPLC. Yield > 95%. No purification was needed.

Stability Studies

Approximately 100 μL of a 0.1 mmol/L solution of the radiolabeled peptide was added to 1 mL fresh human serum and incubated at 37°C. Aliquots were removed occasionally and were checked by silica gel thin-layer chromatography with 99.5/0.5 MeOH/concentrated HCl as the mobile phase. Mobile fractions were the labeled peptide, whereas radioactivity at the origin was assumed to be associated with plasma proteins; this was verified by gel filtration HPLC (Superose 12; Pharmacia, Uppsala, Sweden).

Affinity Studies

Neurotensin (NT) receptors are expressed on the human colon adenocarcinoma cell line HT29, which was chosen for the K_D determination. The cells were grown in 48-well plates in McCoys 5A culture medium (GIBCO Laboratories, Grand Island, NY) and incubated (N-[2-hydroxyethyl]piperazine-N′-[2-ethane sulfonic acid] buffer, pH 7.4, with chymostatin, soybean trypsin inhibitor and bacitracin as protease inhibitors) at 37°C for 1 h with different concentrations of the labeled peptide (0.1–10 nmol/L). After washing with PBS at 4°C, the cells were solubilized in 200 μL NaOH at 37°C. Along with two ^{99m}Tc standards, the samples were counted in a gamma well counter, and the data were fitted by least squares.

Biodistribution

Biodistribution studies have been performed with BALB/c mice, which were injected with 3.7 MBq labeled ligand per mouse (0.1 mCi) through the tail vein. Mice were killed 24 h after administration of the radiolabeled ligands. Selected tissue samples were excised, weighed and, along with an aliquot of the injected dose, counted in a gamma well counter.

RESULTS

Synthesis of ^{99m}Tc-Aquaion

With a view to medical applications, a convenient synthesis of the carbonylic ^{99m}Tc-aquaion had to be found. The aquaion is actually formed quantitatively by the carbonylation of a generator eluate directly in saline as described (Fig. 1). The ^{99m}Tc-aquaion is stable at neutral pH and can be used without purification.

Labeling of Amino Acids

Thioethers, thiolates and N-heterocycles have shown to be good binding ligands for the ^{99m}Tc-aquaion. These

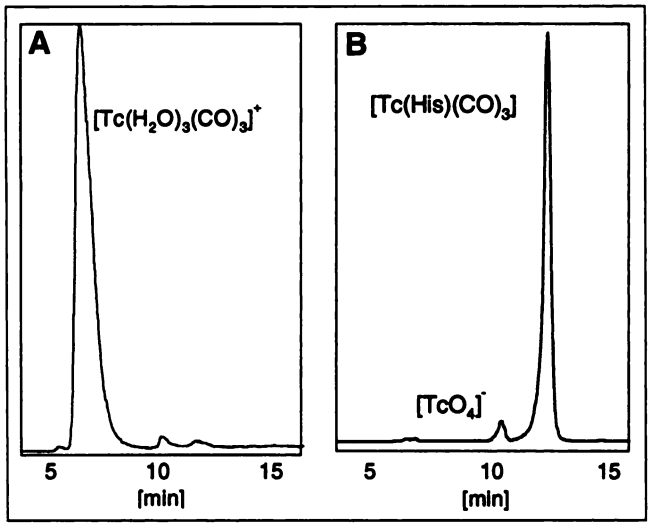

FIGURE 1. HPLC gamma traces (RP-18) of ^{99m}Tc-aquaion (A) and after addition of histidine (B).

functionalities correspond to side chains of methionine, cysteine and histidine and are therefore of particular interest in terms of labeling of peptides.

To explore the amino acid within a given peptide sequence to which the aquaion will attach, representative amino acids and amino acid fragments were labeled at various concentrations under standardized conditions, simply by adding the ligand solutions to technetium-carbonyl samples. This procedure gives rise to steep sigmoid curves for each applied ligand representing the minimal concentration at which complex formation occurs (Fig. 2). The most impressive finding of this study concerns histidine, which forms complexes at concentrations as low as 1 μmol/L. Most other amino acids had to be applied 1000 times more concentrated to obtain the corresponding ^{99m}Tc compounds. Only cysteine is able to bind the aquaion comparatively well; yet, 10 times higher concentrations than with histidine are still required. However, the oxidized form of cysteine, cystine, which is the most common form in proteins, is not a better ligand than any other amino acid and can therefore be neglected as a potential labeling site. With respect to known complexes of $[Re(CO)_3]^+$ with thioethers, it is surprising that methionine binds only slightly better to the triscarbonyl moiety than do amino acids with aliphatic side chains. The difference in labeling yield between Cys and Met suggests that complex formation depends strongly on the protic nature of the ligand, at least in aqueous solution. Elevation of the temperature to 75°C results in the same labeling yield using two powers of magnitude less of any applied ligand. This actually means that stochiometric amounts of histidine can be applied compared with the total technetium present in a generator eluate. In this way, specific activities of 6 TBq/μmol (160 Ci/μmol) histidine have been achieved.

Within a peptide chain, histidine can be positioned either at the N-terminus or C-terminus or within the peptide backbone. An estimation of the binding properties of the corresponding coordination motifs can be made using imidazole for internal, histamine for N-terminal and N_α-BOC-protected histidine for C-terminal histidine, assuming that the urethane of the latter makes no significant contributions to complex formation. As expected, the decrease in denticity of the applied ligand results in a successive increase in the required concentration. Yet, all histidine fragments still bind better than any other amino acid (excluding cysteine), although the difference between imidazole and methionine is only slight. The direct comparison of the monodentate thioether ligand thioxane with imidazole, however, reconstitutes the gap between these two functional groups.

Synthesis and Mass Spectroscopy of $Re(CO)_3$-NT(8–13) Analogs

Clearly, histidine will be the site where the labeling occurs, independently of its position in the peptide, but with a preference for N-terminal residues. Histidine can be introduced conveniently by conventional solid-phase peptide synthesis at different sites, thereby allowing direction of the technetium label away from sensitive sites that are

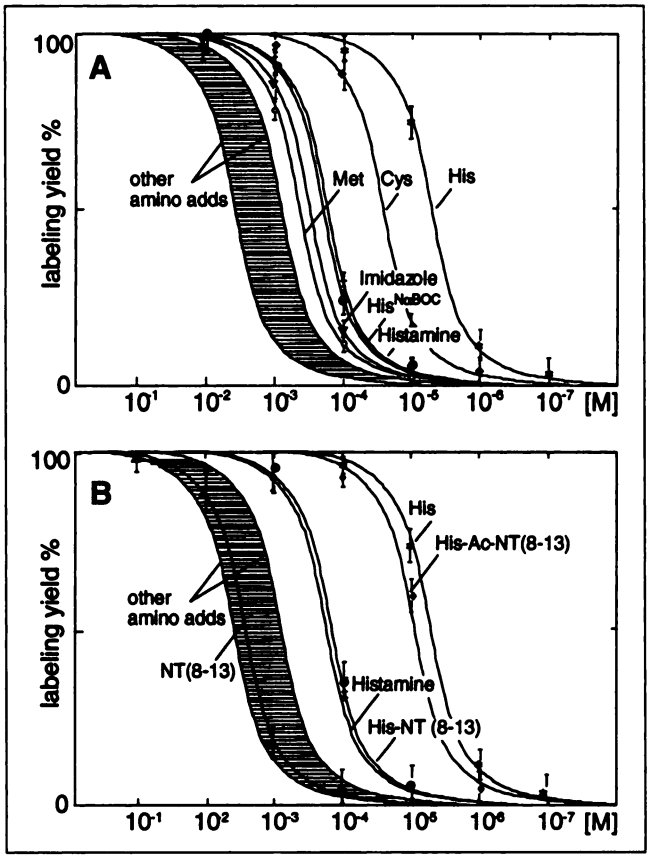

FIGURE 2. Labeling yields as function of (ligand) in PBS (0.1 mol/L NaCl/0.05 mol/L sodium phosphate buffer, pH 7.4) after 1 h at 37°C. Yields were determined by HPLC. (A) Amino acids and histidine (His) fragments. Met = methionine; Cys = cysteine. (B) Neurotensin (NT) analogs in comparison with their corresponding coordination groups. Ac = acetyl.

responsible for the biologic activity of the peptide. To test this novel peptide labeling approach, we chose the NT fragment NT(8–13). The interest in NT arises from the knowledge that its receptors are expressed on a variety of carcinomas in high density (*16*). NT(8–13) is the shortest binding analog but contains no histidine residue. Histidine was attached at the N-terminus, and both analogs, NT(8–13) and His-NT(8–13), have been reacted with an equimolar amount of $[Re(H_2O)_3(CO)_3]^+$. Several differences were found between the two compounds. As evident from HPLC, one species is formed with His-NT(8–13), but a mixture of products is formed with unmodified NT(8–13). Moreover, electrospray mass spectroscopy (ESMS) revealed dissociation of the metal center from NT(8–13), indicating a low stability of the corresponding complexes. In contrast, the rhenium complex of His-NT(8–13) showed only the complex peak. A common technique in ESMS for the determination of structural features of a molecule consists of inducing fragmentation processes by increasing the cone voltage. With the rhenium complex of His-NT(8–13), this results in the cleavage of peptide bonds, not in the dissociation of the metal center from the peptide as in the case of NT(8–13). In addition to the intact peptide complex, the only species with rhenium that can be observed is $[Re(His)(CO)_3]^+$, confirming the preference of the metal carbonyl for histidine.

DISCUSSION

^{99m}Tc Labeling of NT(8–13) Analogs

The labeling efficiency of the unmodified NT(8–13) analog was in the range of the amino acids bearing only weakly coordinating functional groups (Fig. 2B), and therefore low. Furthermore, multiple species were produced, indicating unspecific binding of $^{99m}Tc(CO)_3$. In contrast with these observations, the two N-terminally modified analogs His-NT(8–13) and (N_α-His)Ac-NT(8–13) (Fig. 3) produced efficiently uniform products when labeled with $^{99m}Tc(CO)_3$. The corresponding labeling curve followed essentially the one of histamine (His-NT[8–13]) and histidine ((N_α-His)Ac-NT[8–13]), respectively (Fig. 2B). This is a clear indication

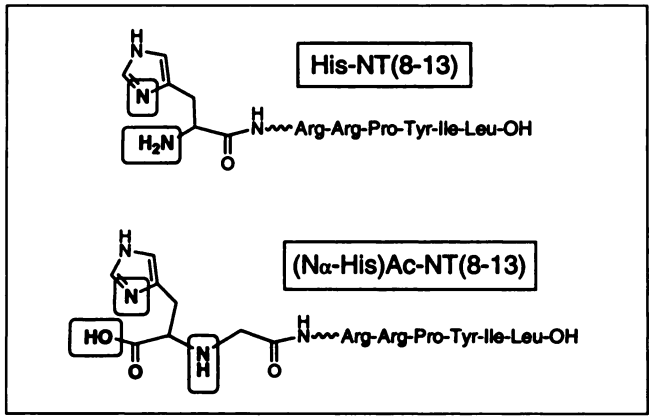

FIGURE 3. Histidine-neurotensin (His-NT) analogs labeled with $^{99m}Tc(CO)_3$. Coordinating groups are in boxes. Ac = acetyl.

that $^{99m}Tc(CO)_3$ adopts the same coordination mode (bidentate in the case of His-NT[8–13] and tridentate in the case of [N_α-His]Ac-NT[8–13]) as in the corresponding model complexes with histamine and histidine. The specific activities achieved were as high as 5.2 TBq/μmol peptide (140 Ci/μmol), which is by a factor 5–10 higher than generally observed with peptides functionalized with tetradentate, nitrogen- and sulfur-containing ligands or the HYNIC ligand systems (*8*). These high specific activities were obtained only because of the unique labeling characteristics of $^{99m}Tc(CO)_3$. The peptide requires only a minor modification of the sequence by the introduction of either a commercially available His or a (N_α-His)Ac functionality at the N-terminus by standard peptide-synthetic procedures.

Affinity Measurements

The K_D of the ^{99m}Tc-labeled peptides was determined on colon carcinoma HT29 cells. The values at 37°C were found to be 0.63 nmol/L for His-NT(8–13) and 0.25 nmol/L for (N_α-His)Ac-NT(8–13). These values are somewhat decreased compared with NT(8–13) (K_D = 1 nmol/L) (*17*).

Stability Studies and Biodistribution

In vitro stability studies in human sera and in vivo biodistribution studies in BALB/c mice have been performed with the ^{99m}Tc aquaion, ^{99m}Tc-labeled histidine, histamine, the innocent dipeptide His-Gly, His-NT(8–13) and (N_α-His)Ac-NT(8–13), and His-Arg, a possible metabolite of His-NT(8–13). Whereas the aquaion associates immediately and histamine associates completely within 3 h to high-molecular-weight plasma proteins, no such interaction is observed with histidine and (N_α-His)Ac-NT(8–13) in human sera. His-Gly, His-Arg and His-NT(8–13) do interact with plasma proteins but only slowly and to a small extent (20% after 3 h). In all these experiments, no pertechnetate has been detected. The results obtained from the biodistribution studies fit well with the results of in vitro experiments in human sera. Thus, histidine and (N_α-His)Ac-NT(8–13) are fully cleared within 24 h. Only faint amounts of (N_α-His)Ac-NT(8–13) are retained in organs that are known to bear NT receptors. These findings contrast sharply with those of the aquaion and histamine, which persist in the blood circulation (Table 1). His-Gly, His-Arg and His-NT(8–13) show similar biodistributions with a fast clearance from the blood but some accumulation in organs responsible for the breakdown and excretion. Obviously, the extent to which the labeled compounds associate with plasma proteins directs the rate of blood clearance. This can be understood in terms of the coordination properties at the metal center, where the aquaion offers three, the histamine complex one, and the histidine and (N_α-His)Ac-NT(8–13) complex no free coordination site for incoming ligands. In principle, His-Gly, His-Arg and His-NT(8–13) are expected to coordinate like histamine, which should result in a more pronounced association with plasma proteins and the same unfavorable biodistribution. However, the ^{1}H nuclear magnetic reso-

TABLE 1
Biodistribution of ^{99m}Tc-Labeled Ligands in BALB/c Mice After 24 Hours

Tissue	Ligand						
	$Tc(CO)_3$	Histamine	His-Gly	His-Arg	His-NT	His	(His)Ac-NT
Blood	3.6 ± 0.8	2.4 ± 0.4	0.13 ± 0.01	0.17 ± 0.02	0.19 ± 0.07	0.02 ± 0.01	0.06 ± 0.01
Heart	2.6 ± 0.5	2.0 ± 0.3	0.09 ± 0.01	0.19 ± 0.02	0.23 ± 0.14	0.01 ± 0.01	0.04 ± 0.01
Lung	7.9 ± 6.5	2.2 ± 0.5	0.19 ± 0.01	0.56 ± 0.04	0.6 ± 0.4	0.05 ± 0.01	0.14 ± 0.01
Spleen	2.8 ± 0.4	2.2 ± 0.4	0.18 ± 0.04	0.52 ± 0.13	1.7 ± 1.3	0.03 ± 0.01	0.12 ± 0.02
Kidneys	13.5 ± 2.9	4.1 ± 0.6	2.7 ± 0.1	7.7 ± 0.1	2.8 ± 1.1	0.22 ± 0.02	0.5 ± 0.05
Stomach	1.1 ± 0.5	4.2 ± 5.0	2.6 ± 3.9	0.6 ± 0.5	3.2 ± 2.3	0.19 ± 0.08	0.9 ± 0.2
Intestines	1.6 ± 0.3	2.6 ± 0.3	0.6 ± 0.3	0.43 ± 0.15	1.5 ± 1.5	0.06 ± 0.03	0.12 ± 0.07
Liver	10.2 ± 2.3	20.6 ± 2.8	5.4 ± 1.8	5.1 ±0.4	4.8 ± 2.0	0.16 ± 0.01	0.7 ± 0.1
Muscle	1.2 ± 0.1	1.1 ± 0.1	0.04 ± 0.01	ND	0.06 ± 3	0.01 ± 0.01	ND

His-Gly = histidine-glycine; Arg = arginine; NT = neurotensin; (His)Ac-NT = (histidinyl)acetic acid-neurotensin; ND = not done.
Data are mean ± SD of three animals and are expressed in percentage injected dose per gram tissue.

nance spectrum of the His-Gly complex shows a fixed conformation of the C_α protons of the Gly residue (^{2}J-40 Hz [complex)] versus 12 Hz [ligand]). This indicates that the C-terminal carboxylic acid of the peptide is involved in the coordination of the metal center and therefore protects it from nucleophilic attacks. Because of the lack of an efficient additional chelate effect, this protection is not as powerful as with histidine, resulting in the observed slow attachment of the small peptides to plasma proteins.

CONCLUSION

The new peptide labeling approach with $^{99m}Tc(CO)_3$ combines the highest possible specific activities with a minimal influence on the biologic properties of the peptide, including receptor affinity and metabolism. The labeling procedure is very simple, usually requires no purification step and can be transferred to other peptides of choice.

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