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Research Article

Biological evaluation and comparison of three different procedures for labelling of amino acids tyrosine and lysine with technetium-99m

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Abstract: The ^{99m}Tc-labelling of the amino acids tyrosine (Tyr) and lysine (Lys), fundamental building blocks of proteins and peptides, as well as biological properties of the labelled compounds are investigated. Three different approaches for the labelling with ^{99m}Tc have been investigated: direct reduction with stannous tin in the presence of the amino acids, the preformed chelate approach through polydentate chelates DTPA and GH, and the 'organometallic approach' using $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor. The direct labelling approach was not successful and the yield was poor. In the presence of DTPA and GH, the labelling efficiency was between 43.6 and 97.8%, depending on the amino acid and the polydentate chelate. The use of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor point at the formation of ^{99m}Tc(I) co-ordinated complexes with high yield. In this approach, pH and heating influenced the yields. The results of organ distribution study for $[^{99m}Tc(Tyr)(H_2O)(CO)_3]$ and $[^{99m}Tc(Lys)(H_2O)(CO)_3]$ show accumulation in liver, kidneys and intestine. Copyright © 2007 John Wiley & Sons, Ltd.

Keywords: tyrosine; lysine; ^{99m}Tc; $[^{99m}Tc(CO)_3(H_2O)_3]^+$; labeling; radiopharmacy

Introduction

Development of peptides and amino acids labelled with single photon gamma emitters has promoted a large number of investigations in nuclear medicine, especially for tumour imaging. Radiolabelled peptides have several advantages over antibodies mainly related to immunogenicity and size. Tumour localization and total body clearance of peptides are more rapid compared to antibodies.[1] On the other hand, labelled artificial amino acids would have advantages, as they enter cells mainly by transport system L and increased uptake into malignant cells in comparison with normal cells can be expected.[2] Labelled amino acids would facilitate research on tumours and cerebral functions and to provide a clue to the research for diagnosis of membrane transport of amino acids.[3,4]

^{99m}Tc has ideal physical properties for many applications in nuclear medicine and it is therefore still the radionuclide of choice. A large number of techniques for radiolabelling with ^{99m}Tc have been developed. They are classified as: direct labelling, the preformed chelate approach and the bifunctional chelating or indirect labelling approach.[3-6] The study of technetium radiopharmaceuticals has been stimulated by the availability of radioactive ^{99m}Tc(I) precursor $[Tc(CO)_3(H_2O)_3]^+$. $[^{99m}Tc(CO)_3(H_2O)_3]^+$ is formed in high yield directly, from ^{99}Mo/^{99m}Tc generator eluted pertechnetate ($[^{99m}TcO_4]^-$), by reduction of TcO_4^- in the presence of CO in water. As the three water ligands are labile, they can be readily exchanged with different mono-, bi- and tridentate ligands with different donors like phosphines, thioethers and aromatic amines.[7] Aliphatic amine and carboxylate groups have also been shown to form stable complexes with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor.[8] Hydrophilic organometallic $[^{99m}Tc(H_2O)_3(CO)_3]^+$ precursor allows the formation of Tc (I) radiopharmaceuticals based on the *fac*-$[^{99m}Tc(CO)_3]$+ (I) core.[7–12] This way is known as 'organometallic labelling approach'.[13,14]

We present in this paper three different approaches for the preparation of ^{99m}Tc complexes with tyrosine and lysine in different oxidation states. The amino acids are labelled either directly or by ligand exchange

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of polydentate ligands diethylen triamine-pentaacetic acid (DTPA) and glucoheptonate (GH). These results are compared to the complexation of α-amino acids with $[^{99m}Tc(CO)_3(H_2O)_3]^+$. The radiochemical purity as well as biodistribution and pharmacokinetic parameters are presented.

Results and discussion

Radiochemical purity

When $^{99m}TcO_4^-$ is reduced, the oxidation state of technetium depends on the reducing agent, ligands and reaction conditions. A series of experiments were carried out to determine the optimal ^{99m}Tc-labelling conditions for investigated amino acids by varying all the reaction parameters. The aim was to reduce Tc(VII) in $^{99m}TcO_4^-$ to a lower oxidation state, in order to produce a stable ^{99m}Tc-amino acid complex. The attempt of the direct ^{99m}Tc-labelling of Tyr and Lys gave very low yields (8.1–26.5% and 12.5–23.1%, respectively). Altering the reaction parameters did not improve the yield substantially. Amino acids are known to be weak-chelating agents, so Tyr and Lys are not able to stabilize Tc(V) to a sufficient extent and further reduction probably to Tc(IV), as witnessed by the high TcO_2 yield, occurs.

Labelling efficiencies of ^{99m}Tc-labelled Tyr and Lys, through the preformed chelate approach with DTPA and GH 30 min after preparation, were measured by ITLC-SG in acetone and 0.9% NaCl. The radiolabelling results of Tyr through DTPA and GH were found to be higher than 99%. The labelling yield of 76.9% and 68.5% were obtained for ^{99m}Tc-labelled Lys through DTPA and GH, respectively.

ITLC did not allow differentiating between Tc-DTPA or Tc-GH and ^{99m}Tc complexes of tyrosine or lysine. Only HPLC (see next section) showed that trans-chelation to Tyr and Lys indeed took place to a certain extent.

HPLC analysis

The retention times together with the yield for each of Tc-labelled compound are given in Table 1. HPLC analyses of ^{99m} TcO_2 show that its retention time is 3.503 min. The new small peaks at R_f values between 5.535 and 5.679 min in HPLC analysis of ^{99m}Tc reduction in presence of Tyr and Lys showed that there were some complexion of investigated amino acids.

Typical HPLC traces for ^{99m}Tc-DTPA, ^{99m}Tc-DTPA-Tyr or ^{99m}Tc-DTPA-Lys and for ^{99m}Tc-GH, ^{99m}Tc-GH-Tyr and ^{99m}Tc-GH-Lys are presented in Figures 1 and 2, respectively. The retention times together with the yield for each of Tc-labelled compound are given in Table 2. The retention time for ^{99m}Tc-DTPA is in accordance with literature data.[15] As is obvious from the Figures 1 and 2 and Table 2, new complexes are formed upon reaction with the amino acids Tyr and Lys. The peaks at values between 5.535 and 5.679 min, in HPLC analysis in previous section, could support that the new peaks do not contain DTPA or GH anymore.

According to the HPLC results, trans-chelation takes place through polydentate chelates DTPA and GH. The radiolabelling can be achieved along two pathways: by direct reduction of $^{99m}TcO_4^-$ in the presence of a Sn-BFCA (BFCA—bifunctional chelating agent) and formation of the ^{99m}Tc-BFCA complex and subsequent substitution of DTPA or GH in ^{99m}Tc-BFCA complex by an amino acid in a subsequent step, or by direct

Table 1 Dependence of retention times and labelling yield of ^{99m}Tc-labelled tyrosine and lysine on pH and heating

Labelled compound	^{99m}Tc-species	pH=3.5		pH=7.5	
		R_t (min)	Labelling yield (%)	R_t (min)	Labelling yield (%)
$^{99m}TcO_2$	$^{99m}TcO_2$	3.503	100.0	3.503	100.0
^{99m}Tc+Tyr	$^{99m}TcO_2$	3.550	91.54	3.503	91.27
At room temperature	^{99m}Tc-impurities	6.004	3.18	—	—
	^{99m}Tc–Tyr	6.178	5.28	5.535	8.37
^{99m}Tc+Tyr	$^{99m}TcO_2$	3.581	94.17	3.491	78.30
With heating at 100°C	^{99m}Tc-impurities	—	—	—	—
	^{99m}Tc–Tyr	6.335	5.83	5.602	21.70
^{99m}Tc+Lys	$^{99m}TcO_2$	3.576	98.77	3.517	91.31
At room temperature	^{99m}Tc-impurities	—	—	—	—
	^{99m}Tc–Lys	6.467	1.23	5.642	8.69
^{99m}Tc+Lys	$^{99m}TcO_2$	3.524	96.79	3.491	84.85
With heating at 100°C	^{99m}Tc-impurities	—	—	—	—
	^{99m}Tc–Lys	6.378	3.21	5.679	15.15

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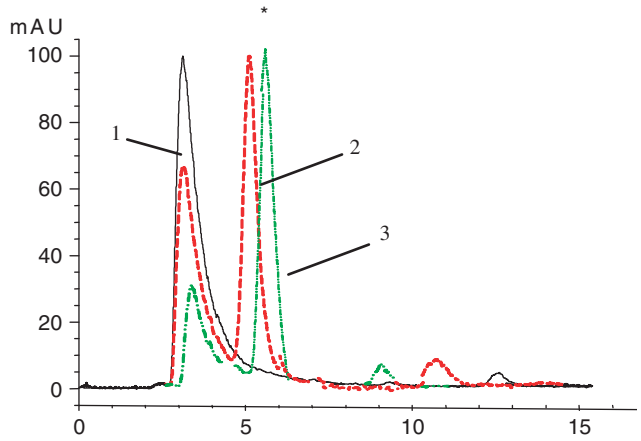

Figure 1 HPLC radiochromatograms of ^{99m}Tc- labelled Tyr and Lys through chelating agent DTPA: ^{99m}Tc-DTPA (1), ^{99m}Tc-DTPA-Tyr (2), ^{99m}Tc-DTPA-Lys (3).

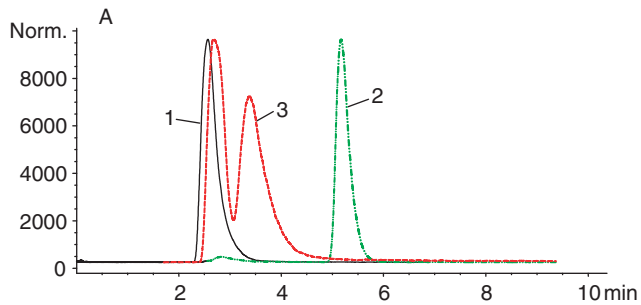

Figure 2 HPLC radiochromatograms of ^{99m}Tc- labelled Tyr and Lys through chelating agent GH: ^{99m}Tc-GH (1), ^{99m}Tc-GH-Tyr (2), ^{99m}Tc-GH-Lys (3).

reduction and coordination to the amino acid. Considering the low yields from the complex preparation in the absence of DTPA or GH (earlier section) we conclude that the second possibility is of minor importance. As is obvious from Figure 2, transchelation is quantitative with tyrosine, giving a single complex of higher lipophilicity than with lysine, for which transchelation is not complete under identical conditions. The similar retention times of the peaks received from direct labelling and labelling via trans-chelation also indicates that the products for both reactions are equal, albeit for the first method of much too low radiochemical purity to be useful in labelling experiments.

The organometallic aqua complex $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was prepared starting from pertechnetate ($^{99m}TcO_4^-$) according to literature. HPLC quality control show radiochemical purity higher than 95%. The retention time (R_t) for $[^{99m}Tc(CO)_3(H_2O)_3]^+$ and $^{99m}TcO_4^-$ are 3.151 and 12.624 min, respectively (see exp. part).

The reaction of the bidentate ligands Tyr and Lys were studied with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ in water. HPLC chromatograms of the reaction solutions under different conditions (pH and temperature) are presented in Figures 3(a) and (b) and 4(a) and (b). ^{99m}Tc-carbonyl amino acid complexes are obtained, which is clearly shown by the new peaks appearing at 8 min (Tyr) and 6 min (Lys), respectively.

The retention times together with the labelling yield for each of amino acid complex are summarized in Table 3. The HPLC results confirm that complexation of

Table 2 Retention times and labelling yield of ^{99m}Tc-DTPA and ^{99m}Tc-GH as well as tyrosine and lysine labelled with ^{99m}Tc through DTPA or GH

Labelled compound	^{99m}Tc-species	R_t (min)	Labelling yield (%)
^{99m}Tc–DTPA	^{99m}Tc-DTPA	3.124	96.7
	^{99m}Tc-impurities	6.235	1.8
	$^{99m}TcO_4^-$	12.601	1.5
^{99m}Tc–DTPA-Tyr	^{99m}Tc-DTPA	3.150	23.5
	^{99m}Tc-DTPA+Tyr	5.219	74.3
	$^{99m}TcO_4^-$	10.668	2.2
^{99m}Tc–DTPA-Lys	^{99m}Tc-DTPA	3.099	52.5
	^{99m}Tc-DTPA+Lys	5.179	43.6
	^{99m}Tc-impurities	7.311	2.3
	$^{99m}TcO_4^-$	12.385	1.7
^{99m}Tc–GH	^{99m}Tc-GH	2.668	100.0
	$^{99m}TcO_4^-$	—	—
^{99m}Tc–GH-Tyr	^{99m}Tc-GH	2.820	2.2
	^{99m}Tc-GH+Tyr	5.172	97.8
	$^{99m}TcO_4^-$	—	—
^{99m}Tc–GH-Lys	^{99m}Tc-GH	2.724	52.4
	^{99m}Tc-GH+Lys	3.378	47.6
	$^{99m}TcO_4^-$	—	—

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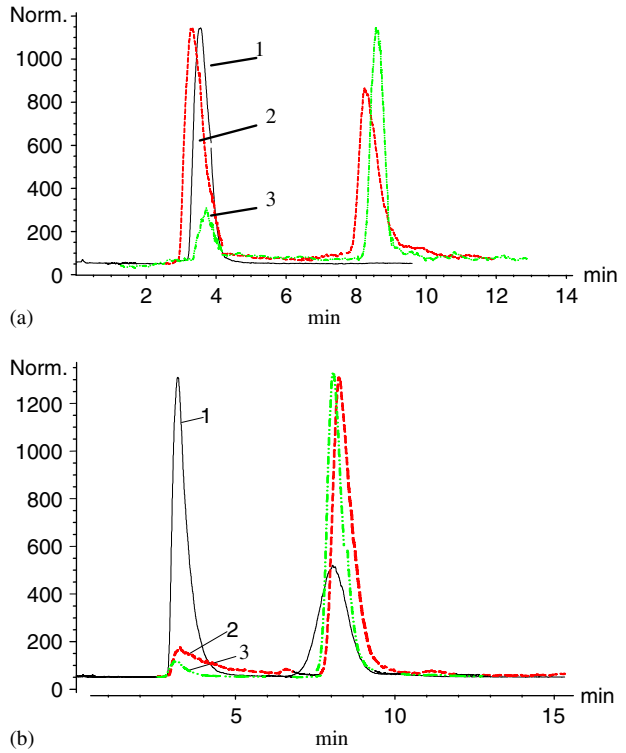

Figure 3 HPLC radiochromatograms of [^{99m}Tc(Tyr)(H_2O)($CO)_3$], labelling: (a) at room temperature, pH=3.5 (1); 5.5 (2) and 7.5 (3); (b) with heating at 100°C, pH=3.5 (1), 5.5 (2) and 7.5 (3).

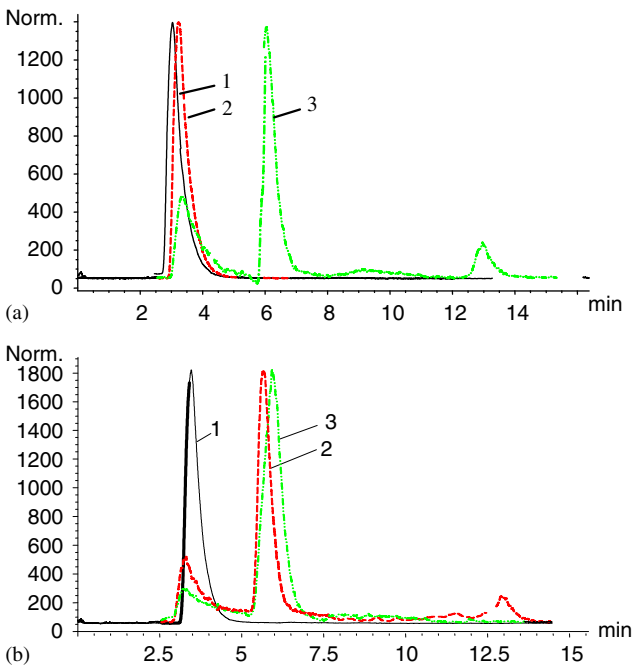

Figure 4 HPLC radiochromatograms of [^{99m}Tc(Lys)(H_2O)($CO)_3$], labeling: (a) at room temperature, pH=3.5 (1);5.5 (2) and 7.5 (3); (b) with heating at 100°C, pH=3.5 (1); 5.5 (2) and 7.5 (3).

Tyr and Lys with [^{99m}Tc$(CO)_3(H_2O)_3]^+$ depends on the conditions such as temperature or pH. If the labelling was performed at room temperature, satisfying results were obtained only for tyrosine (38.6 and 77.6% at pH 5.5 and 7.5, respectively). At a pH, 3.5 tyrosine could not be labelled with ^{99m}Tc (I) at room temperature. If the amino acids and [^{99m}Tc$(CO)_3$ $(H_2O)_3]^+$ are heated in boiling water for 30 min, tyrosine gave the highest yield at pH=7.5. Lysine (as tyrosine) shows higher labelling yield at pH=7.5 than at pH=5.5. There was no labelling at pH=3.5. The highest labelling yield for tyrosine as well as for lysine are, thus, obtained at pH=7.5 and at 100°C. At pH=3.5 the amino acids are completly protonated and not at all prone for coordination. At pH=7.5, the zwitterions is the dominant species in aqueous solutions, thus, both the amino and the carboxylic group are completely ionized.[16] Since the amino acids are bidentate ligands, one water molecule is left at the ^{99m}Tc-center and the overall charge of the complex is neutral. We propose in Scheme 1 possible structures of the ^{99m}Tc complexes with tyrosine and lysine. These results are in accordance with literature data which have shown that aliphatic amine and carboxylate groups form stable complexes with [^{99m}Tc$(CO)_3$ $(H_2O)_3]^+$ precursor.[17] The coordinating protion for Lys and Tyr are the same and it is possible that steric effects from the side chains are responsible for the different yields and stabilities of the complexes.

Protein binding measurements

Since the labelling yield of investigated amino acids labelled by Sn (II) reduced method was poor, protein binding determinations are not performed for these complexes. In Table 4 the percentage of protein binding for both tyrosine and lysine labelled through polydentate ligands DTPA or GH, as well as the results for protein binding for $^{99m}TcO_4^-$, ^{99m}Tc-DTPA or ^{99m}Tc-GH are presented. From these results it can be seen that protein binding of lysine, labelled through DTPA or GH, is higher then the ones for ^{99m}Tc-DTPA or ^{99m}Tc-GH. The values of protein binding for tyrosine, labelled through GH are similar as the results for Tyr labelled through DTPA. In comparison with the results for ^{99m}Tc-DTPA, there is a significant change of protein binding percentages of ^{99m}Tc-DTPA-Tyr: the values were almost twofold.

As the highest labelling yields for tyrosine and lysine are obtained at pH=7.5 at 100°C, these conditions were used for all further biological studies.

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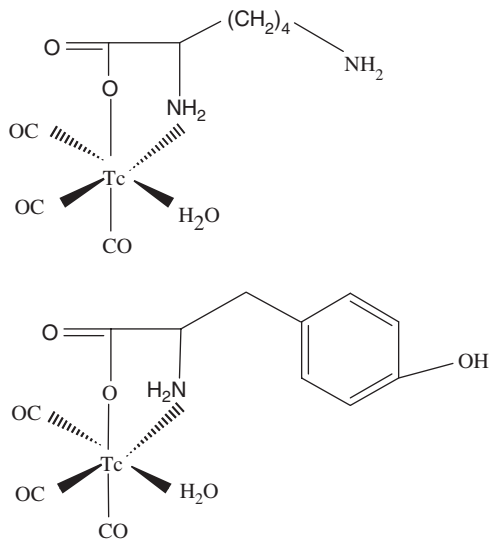
Table 3 Dependence of retention times and labelling yield for tyrosine and lysine coordinated to $[^{99m}Tc(CO)_3(H_2O)_3]^+$ on pH and heating

Samples	PH	3.5		5.5		7.5	
	^{99m}Tc-species	R_t (min)	Labelling yield (%)	R_t (min)	Labelling yield (%)	R_t (min)	Labelling yield (%)
$[^{99m}Tc(CO)_3(H_2O)_3]^+$+Tyr	$^{99m}Tc(CO)_3$	3.141	100	3.209	61.4	3.179	22.4
At room temperature	$[^{99m}Tc(Tyr)(H_2O)(CO)_3]$	—	—	8.357	38.6	8.359	77.6
	$^{99m}TcO_4^-$	—	—	—	—	—	—
$[^{99m}Tc(CO)_3(H_2O)_3]^+$+Tyr	$^{99m}Tc(CO)_3$	3.211	72.6	3.205	10.6	3.193	4.5
With heating at 100°C	$[^{99m}Tc(Tyr)(H_2O)(CO)_3]$	7.985	27.4	8.007	89.4	8.011	95.5
	$^{99m}TcO_4^-$	—	—	—	—	—	—
$[^{99m}Tc(CO)_3(H_2O)_3]^+$+Lys	$^{99m}Tc(CO)_3$	3.140	100	3.147	100	3.176	18.3
At room temperature	$[^{99m}Tc(Lys)(H_2O)(CO)_3]$	—	—	—	—	6.143	78.1
	$^{99m}TcO_4^-$	—	—	—	—	12.967	3.6
	$^{99m}Tc(CO)_3$	3.195	100	3.183	21.1	3.174	7.4
$[^{99m}Tc(CO)_3(H_2O)_3]^+$+Lys	$[^{99m}Tc(Lys)(H_2O)(CO)_3]$	—	—	6.212	72.3	6.139	89.5
With heating at 100°C	^{99m}Tc-impurities	—	—	—	—	8.99	3.1
	$^{99m}TcO_4^-$	—	—	12.911	6.6	—	—



Scheme 1 The possible structures of ^{99m}Tc carbonyl complexes of Tyr and Lys: $[^{99m}Tc(Tyr)(H_2O)(CO)_3]$ and $[^{99m}Tc(Lys)(H_2O)(CO)_3]$.

Table 5 shows the percentage of protein binding for both ^{99m}Tc-carbonyl complexes $[^{99m}Tc(Tyr)(H_2O)(CO)_3]$ and $[^{99m}Tc(Lys)(H_2O)(CO)_3]$ together with the result for $^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor.

The protein binding for ^{99m}Tc-carbonyl tagged amino acids tyrosine and lysine is much higher as compared to $^{99m}TcO_4^-$ but lower then the values of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor. $[^{99m}Tc(CO)_3(H_2O)_3]$ binds easily to proteins since it has three coordination sites left. As $[^{99m}Tc(Tyr)(H_2O)(CO)_3]$ has only one site left, through which it can bind to proteins, its possibilities for binding is lower.

Table 4 The percentage of protein binding results for Tyr and Lys labelled with ^{99m}Tc through polydentate chelating agents DTPA and GH (12% HA, TCA-method, 37°C)

Preparation	Time (min)	
	20	60
$^{99m}TcO_4^-$	3.5 ± 0.3	3.1 ± 0.4
^{99m}Tc-DTPA	29.2 ± 0.1	38.4 ± 0.5
^{99m}Tc-DTPA-Tyr	54.8 ± 0.2	54.8 ± 0.1
^{99m}Tc–DTPA-Lys	61.5 ± 0.3	62.6 ± 0.2
^{99m}Tc–GH	57.7 ± 0.2	54.4 ± 0.1
^{99m}Tc–GH-Tyr	52.4 ± 0.3	53.6 ± 0.2
^{99m}Tc–GH-Lys	59.1 ± 0.3	58.2 ± 0.4

The percentage of protein binding is the mean values ± SD from six measurements.

Table 5 The percentage of protein binding results for $[^{99m}Tc(CO)_3(H_2O)_3]^+$ coordinated Tyr or Lys (12% HA, TCA-method, 37°C)

Preparation	Time (min)	
	20	60
$[^{99m}Tc(CO)_3(H_2O)_3]^+$	85.0 ± 1.2	83.0 ± 0.8
$[^{99m}Tc(Tyr)(H_2O)(CO)_3]$	67.2 ± 0.5	63.9 ± 0.3
$[^{99m}Tc(Lys)(H_2O)(CO)_3]$	61.1 ± 0.6	60.3 ± 0.5

The percentage of protein binding is the mean values ± SD from six measurements.

Lipophilicity measurements

The results of lipophilicity measurements for Tyr and Lys labelled through DTPA or GH show the hydrophilic

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character of these compounds: $-\log P_{O/W}$ values are: 1.31; 1.17; 1.05 and 0.91 for ^{99m}Tc-DTPA-Tyr, ^{99m}Tc-GH-Tyr, ^{99m}Tc-DTPA-Lys, ^{99m}Tc-GH-Lys, respectively. These results are in accordance with the same one for ^{99m}Tc-DTPA and ^{99m}Tc-GH.

The hydrophilicity of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor ($-\log P_{O/W}=0.43 \pm 0.05$) was found to be slightly lower than for $[^{99m}Tc(Tyr)(OH_2)(CO)_3]$ ($-\log P_{O/W}=0.66 \pm 0.07$) and $[^{99m}Tc(Lys)(OH_2)(CO)_3]$ ($-\log P_{O/W}=1.27 \pm 0.09$). Each of them possessed hydrophilic character. Obtained *n*-octanol-water partition coefficients expressed as $\log P_{O/W}$ for Tyr or $\log P_{O/W}$ for Lys were lower than for hystidine ($-\log P_{O/W}=0.04$), but in correlation with Schibli's coments about values of $\log P_{O/W}$ expected for amino acids.[18]

Animal biodistribution

As the labelling yield for direct tin (II) method of ^{99m}Tc-labelling of tyrosine and lysine was poor, no biodistribution was performed with these complexes. The best results for ^{99m}Tc of the amino acids labelled through polydentate ligands were obtained for tyrosine with GH. Consequently, the organ biodistribution study for tyrosine labelled with ^{99m}Tc through GH was done and the results are presented in Figure 5. In comparison with the results for radiopharmaceutical ^{99m}Tc-GH, the biological behaviour for tyrosine labelled through GH was different. Almost all organs uptake of ^{99m}Tc-GH-Tyr was similar to the same ones for ^{99m}Tc-GH, excepte for intestines and kidneys: the uptake in intestine was higher, but in kidneys was lower as the consecuence of slower kidneys clierance. It could be expected as the proteine binding for HA was much stronger for ^{99m}Tc-GH-Tyr in comparison with ^{99m}Tc–GH.

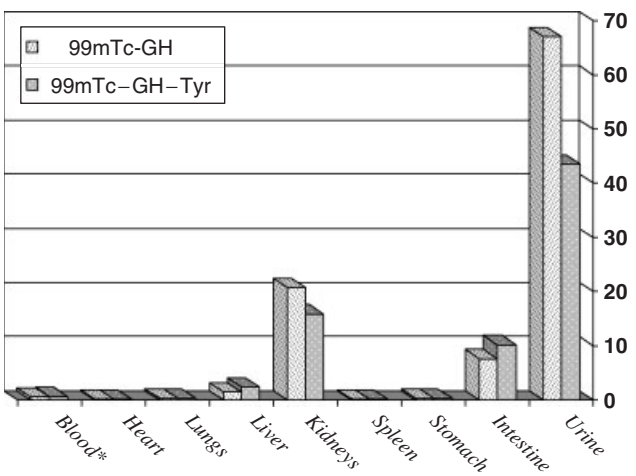

Figure 5 Biodistribution results for tyrosine labelled with ^{99m}Tc through GH ($n = 6$, values represent the means of the percent injected dose per organ or per g).

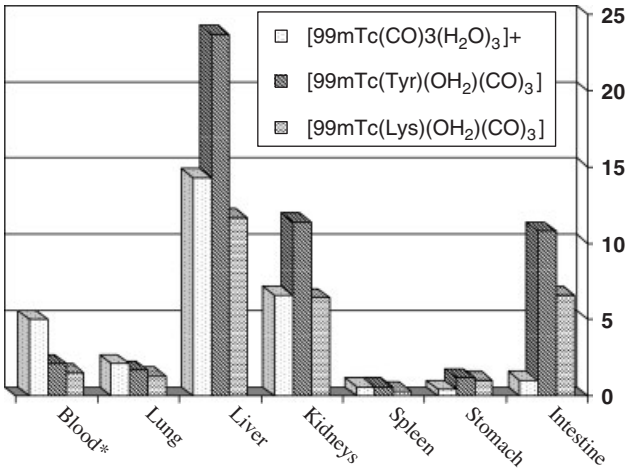

Figure 6 Biodistribution results for $[^{99m}Tc(CO)_3(H_2O)_3]^+$, $[^{99m}Tc(Tyr)(H_2O)(CO)_3]$and $[^{99m}Tc(Lys)(H_2O)(CO)_3]$ ($n = 6$, values represent the means of the percent injected dose per organ or per g).

In Figure 6 the biodistribution for $[^{99m}Tc(CO)_3(H_2O)_3]^+$ and $^{99m}Tc(CO)_3$-coordinated amino acids tyrosine and lysine are presented. The animals were sacrificed one hour after application of 0.1 ml of ^{99m}Tc-labelled compound. From the values of radioactivity measured per organ of interest, the percentages of radioactivity related to administrated dose were determined.

The comparison of $[^{99m}Tc(Lys)(H_2O)(CO)_3]$ with $[^{99m}Tc(Tyr)(H_2O)(CO)_3]^+$ show that radioactivity in liver, kidneys as well as intestines in health animals were twofold lower for $[^{99m}Tc(Lys)(H_2O)(CO)_3]$. The results for other organs of interest were almost equal. The obtained values are similar to the same ones for $^{99m}Tc(CO)_3$-hystidine for all organs, except for liver, kidneys and intestines, e.g. they were higher for tyrosine and lysine.[18] The reason for these differences could be the differences in used concentrations for tyrosine or lysine (10^{-3} mol dm^{-3}) and histidine (10^{-4} mol dm^{-3}).

The organ distribution results have shown that the biological behaviour of labelled amino acids are different since different complexes were applied. The percentages of $[^{99m}Tc(Tyr)(H_2O)(CO)_3]$ uptake in almost all organs of interest were higher in comparison with the results for ^{99m}Tc-GH-Tyr, except for liver and kidneys: the percentage of radioactivity in liver was significantly higher, but in kidneys lower.

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Experimental

Chemicals were purchased from Merck, Germany and were used without further purification. The direct labelling method was investigated performing different experiments by varying amino acids amount, the reducing agent amount, the labelling mixture pH (3.5–7.5), the reaction temperature and reaction time (5–240 min). An aqueous solution of tyrosine and lysine (0.5–5.0 mg/ml) was prepared. To a 10 ml vial 0.5 ml of amino acids solution in H_2O was added and followed by 50 μl of $SnCl_2.2H_2O$ solution (0.5–5.0 mg/ml) in 0.1 M HCl, thus molar ratios Sn(II):amino acid were 1:10, 1:25 and 1:50. pH of these mixtures was adjusted at 3.5, 5.5 and 7.5. To the total volume of 5.0 ml, 18.5 MBq $^{99m}TcO_4^-$ elute in 0.9% NaCl, from ^{99m}Tc-generator (Vinča) were added. The reaction mixtures were allowed to stand at room temperature or heated at different temperatures for different time. In the same time, the 5 ml sample of $SnCl_2.2H_2O$ (1×10^{-4} mol dm^{-3}) with 18.5 MBq $^{99m}TcO_4^-$ elute in 0.9% NaCl was prepared.

^{99m}Tc-glucoheptonate (^{99m}Tc-GH) and ^{99m}Tc-calciumtrisodium salt of diethylentriamine-pentaacetic acid (^{99m}Tc-DTPA) were prepared by reconstituting their instant kit formulations (Vinča) with 3.5 ml of sodium ^{99m}Tc-pertechnetate containing up to 37 MBq. These kits contain stannous chloride as a reducing agent and glucoheptonate and diethylentriamine-pentaacetic acid as calciumthrisodium salt as polydentate chelating agents. The vials were allowed to stand at room temperature for 30 min. A 1.5 ml aliquot of aqueous solution of tyrosine and lysine with molar concentration 10^{-2} mol dm^{-3} were added to the ^{99m}Tc-GH and ^{99m}Tc-DTPA solution above and the reaction allowed proceeding at 100°C in boiling water bath for 30 min. Molar ratio of GH or DTPA and amino acids were 1: 10 pH of prepared samples was 5.5 and 4.5, respectively.

$[^{99m}Tc(CO)_3(H_2O)_3]^+$ ion was prepared by addition of 1 ml of ^{99m}Tc-pertechnetate (740–1110 MBq $^{99m}TcO_4^-$ elute in 0.9% NaCl, from ^{99m}Tc-generator, Vinča Institute) to a penicillin vial with lyophilized form of 7.15 mg sodium carbonate, 4.5 mg sodium boranocarbonate, 2.85 mg sodium tetraborate and 8.5 mg sodium tartarate (IsoLink™, Mallinckrodt Medical B.V., The Netherlands). After heating for 30 min in boiling water bath and cooling, pH of solution was adjusted to desired value with 1 mol dm^{-3} HCl. ^{99m}Tc-carbonyl targeted tyrosine or lysine were prepared by addition of 0.1 ml of investigated amino acid solutions (10^{-3} mol dm^{-3}) to 0.9 ml of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor. The samples were labelled with ^{99m}Tc (I), without and with heating (30 min in boiling water bath). The dependance of labelling efficiency on pH was investigated too.

Radiochemical purity

Standard instant thin layer chromatography (ITLC-SG) with two solvents (acetone/0.9% NaCl) was used for determination of radiochemical purity of ^{99m}Tc-labelled tyrosine and lysine.

HPLC analysis

The HPLC analyses of $SnCl_2.2H_2O$ solution, Tyr and Lys labelled by TcO_4^- were performed using Liquid Chromatograph, Hewlett Packard 1050, S/N with UV and Raytest gamma flow detector, with RP C18 column (250 × 4.6 mm). As a mobile phase acetonitrile-buffers in different ratios, prepared from HPLC grade water and KH_2PO_4, pH=4.5 were used.[15]

The HPLC analyses of ^{99m}Tc-DTPA or ^{99m}Tc-GH as well as tyrosine and lysine, labelled through ^{99m}Tc-DTPA and ^{99m}Tc-GH, were performed in the same way, using Liquid Chromatograph with UV and Raytest gamma flow detector, with RP C18 column (250 × 4.6 mm). As a mobile phase acetonitrile-buffers in different ratios, prepared from HPLC grade water and KH_2PO_4, pH=4.5 were used too.

The quality control of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor (pH=10–11), was performed by gradient HPLC. The solution of 0.05 mol dm^{-3} triethylammonium phosphate (TEAP) with pH=2.25 and methanol were used like a mobile phase. The investigations of amino acids labelled with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor were performed as isocratic HPLC, with 80% H_2O:20% TEAP, pH=3.0, as a mobile phase, with flow rate 0.7 ml/min.

Protein binding measurements

For this measurements standard precipitation method with trichloroacetic acid (TCA - method) was used. 0.1 ml aliquots of the ^{99m}Tc- or ^{99m}Tc(I)-labelled compound were added to 1.5 ml of 12% human serum albumin (12% HA, National Blood Transfusion Institute, Belgrade) and incubated at 37°C for different time intervals. After incubation, 3 ml 20% w/v trichloroacetic acid (Merck, Germany) was added. Precipitate was separated from the solution by centrifugation for three times (5 min, 3000 *g*) and rinsed with saline. The activities of both phases were measured separately by NaI(Tl) detector.[19]

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Lipophilicity measurements

All lipophilicity measurements of amino acids were done by solvent extraction method with *n*-octanol equilibrated with 0.15 mol dm^{-3} phosphate buffers.[20] *n*-octanol was equilibrated with 0.15 M phosphate buffers (pH=3.5–7.5) before used. The samples of ^{99m}Tc- or ^{99m}Tc(I)-labelled amino acids, as well as ^{99m}Tc-DTPA, ^{99m}Tc-GH, $^{99m}TcO_4^-$ and $[^{99m}Tc(CO)_3(H_2O)_3]^+$ (50 µl, 740 kBq) were added to each of duplicate test tubes containing 1050 µl phosphate buffer. After mixing, 100 µl was removed from each test tube (labelled 'A_0'). Than exactly 1000 µl *n*-octanol was added and after wortexing of each tube for 1 min and centrifugation at 3000 *g* for 5 min, 100 µl sample from aqueous phase was removed (labelled 'A_1'). Samples A_0 and A_1 were counted by NaI(Tl) detector and distribution coefficients were calculated as

$$\frac{\text{cpm organic}}{\text{total cpm}} = \frac{A_0 - A_1}{A_0}$$

where cpm is counts per minute. All measurements were performed at room temperature.

Animal biodistribution

Organ biodistribution studies of ^{99m}Tc-labelled compounds were carried out on health white male Wistar rats (4 weeks-old). The anaesthesed animals (anaesthesia: pentobarbital 60 mg^{-1} kg i.p.) were directly injected with 0.1 ml bolus of ^{99m}Tc-labelled compounds (~74 kBq) into the tail vein ($n = 6$). The animals were sacrificed one hour after application and the radioactivity per organ of interest (or per g) was measured in a NaI (Tl) well type detector. The percentage of radioactivity related to administrated dose was determined.

Conclusion

The results presented in this paper reveal that complexation of the amino acids Tyr or Lys and ^{99m}Tc are highly dependent on the labelling approach and the oxidation state of ^{99m}Tc. Direct ^{99m}Tc-labelling of these amino acids, known to be weak-chelating agents, gave high concentrations of $^{99m}TcO_2$ and free $^{99m}TcO_4$. More defined complexes were received from the preformed chelate approach with DTPA or GH with much lower percentages of radiochemical impurities. In this procedure, amino acids are used without modification, by introduction of DTPA or GH solutions in which ^{99m}Tc was previously added.

The best results for labelling yield was obtained with ^{99m}Tc (I) carbonyl precursor when labelling was done at pH=7.5 at 100°C. As the coordinating portion for Lys and Tyr are the same, it is possible that the different yields and stabilities of the complexes of these amino acids could be the consequence of steric effects from the side chains.

All complexes are distinctly hydrophilic. The percentage of protein binding for labelled Tyr is lower for labelling through ^{99m}Tc-DTPA than through ^{99m}Tc-GH, while the results obtained for $[^{99m}Tc(Tyr)(H_2O)(CO)_3]$ are situated between them. The percentage of protein bound to labelled Lys is independent on the way of labelling.

The biodistribution results (% ID/organ) obtained in health Wistar rats show the dependence of accumulation in organ of interest on the labelling approach as the consequence of forming different complexes. These observations confirmed that the oxidation state of technetium-99m used for labelling, as well as the bidentate chelating agents, has important influence on *in vitro* and *in vivo* behaviour of ^{99m}Tc-labelled amino acids.[17] The future investigations on the animals with tumours would confirm if amino acids, labelled with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ precursor, could be used in diagnostic purpose.

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