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EFFECT OF TIN(II) CHLORIDE AMOUNT ON ^{99m}Tc-LABELING OF ONE 6-HYDRAZINONICOTINAMIDE-CONJUGATED PEPTIDE IN THE PRESENCE OF TRICINE/NICOTINIC ACID

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The use of tin(II) chloride in amount above its required mole ratio as reducing agent can lead to instability of peptide-based radiopharmaceuticals. In this work, we optimize the radiolabeling conditions, especially tin(II) chloride amount for ^{99m}Tc-labeling of one 6-hydrazinonicotinamide (HYNIC) conjugated peptide in the presence of tricine and nicotinic acid (NA). Implementation of this procedure leads to ^{99m}Tc-labeling of this HYNIC-conjugated peptide in high radiochemical yield (>95%). By comparison, our experimental results can provide an efficacious strategy for ^{99m}Tc-labeling of HYNIC-conjugated biomolecules in the presence of tricine/NA. The resulting ^{99m}Tc-labeled peptide, without the need for any chemical modification and pretreatment, shows good stability in saline and human serum. This work can provide a new perspective for ^{99m}Tc-labeling of HYNIC-conjugated biomolecules.

Keywords: ^{99m}Tc; tin(II) chloride; optimizing conditions; 6-hydrazinonicotinamide; HYNIC-conjugated peptide; tricine/nicotinic acid.

1. INTRODUCTION

Today, peptide-based radiopharmaceuticals are the spotlight of comprehensive research area for tumor diagnostics and therapy because of their surprising traits and eye-catching nature [1 – 3]. Peptides are small biomolecules that penetrate rapidly in target tissues and clear easily from non-target tissues. Peptides have low toxicity and show little immunogenicity [4, 5]. Recent literature survey shows that peptide-based radiopharmaceuticals have been introduced into clinical work more often than in the past [6]. Despite fantastic raising of positron emission tomography (PET) and availability of radionuclides, peptide-based radiopharmaceuticals are mostly labeled with technetium-99m (^{99m}Tc). Technetium-99m is the most commonly used radioisotope in nuclear medicine [7, 8]. It can also be claimed that 6-hydrazinonicotinamide (HYNIC) is the ideal bifunctional coupling agent (BFCA) for ^{99m}Tc-labeling of biomolecules, because of its rapid and efficient labeling capability [9, 10]. In addition, specific tumor uptake and appropriate pharmacokinetics have been achieved by modifying various co-ligands [11].

According to previously published data, in most conventional ^{99m}Tc-labeling method of HYNIC-conjugated biomolecules, the used mole ratio of tin(II) chloride is significantly higher than the mole ratio of biomolecules. However, the additional amount of tin(II) chloride can lead to radiopharmaceutical instability [12, 13]. Furthermore, ^{99m}Tc-labeled biomolecules have been obtained by using some pretreatment and chemical modification such as addition of *ex situ* buffers [14, 15]. To the best of our knowledge, there have been no reports on the optimized (minimized) amount of tin(II) chloride for ^{99m}Tc-labeling of HYNIC-conjugated biomolecules in the presence of tricine and nicotinic acid (NA) to date. Therefore, optimized and simplified ^{99m}Tc-labeling procedure of HYNIC-conjugated peptides in the presence of tricine/NA can be of great interest. In this research article, we introduce optimized conditions, particularly in respect of the amount of tin(II) chloride and consequently simplified ^{99m}Tc-labeling strategy for obtaining one HYNIC-conjugated

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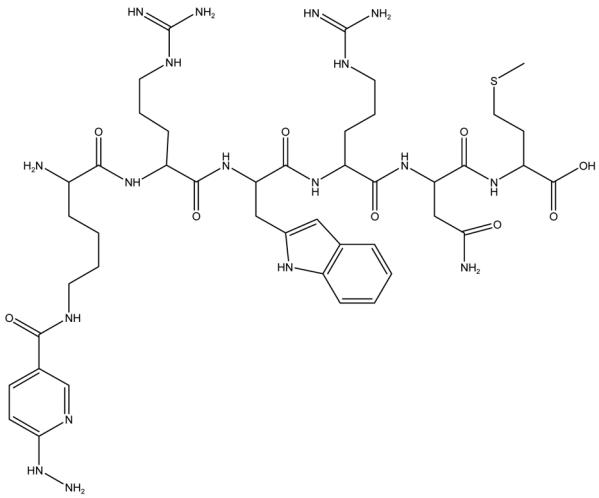

Fig. 1. Chemical structure of **M-6**.

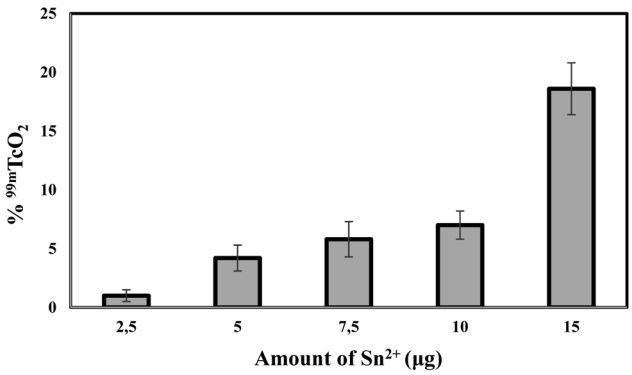

Fig. 2. Variation of $^{99m}TcO_2$ formation percentage vs. amount of $SnCl_2$ used in ^{99m}Tc-labeling of **M-6** as studied by TLC (n = 3).

peptide in the presence of tricine/NA that can be appropriate for any HYNIC-conjugated biomolecules.

2. EXPERIMENTAL

2.1. Reagents and Instrumentation

The HYNIC-conjugated peptide KRWrNM (**M-6**) (Fig. 1), with a purity of >95% was purchased from PepmicCo Ltd. (China). Sodium pertechnetate was eluted from $^{99}Mo/^{99m}Tc$ radionuclide generator (Pars Isotope, Tehran, Iran). All reagents were made using analytical grade chemicals (Sigma-Aldrich, Munich, Germany) and doubly deionized (dd) water through standard protocols. After labeling, the radiochemical yield was evaluated through aluminum supported thin-layer chromatography (TLC) on silica gel (TLC SG; Merck) strips and confirmed by reverse phase high performance liquid chromatography (RP-HPLC). The distribution of radioactivity on the silica gel strips was scanned using a Lablogic Mini-Scan TLC scanner and analyzed with Lura image analysis software (Sheffield, UK). Analytical RP-HPLC was performed on a Knauer HPLC system (Germany). The HPLC analyses of radiolabeled peptide were performed on Lablogic system equipped with radioactivity gamma and UV detector set at 240 nm, and Eurospher 100–5 C_{18} (4.6 × 250 mm) column with precolumn. The gradient eluent consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) in the following gradient program: 0–10 min, 90% solvent A; 10–15 min, 30% solvent A; 15–20 min, 100% solvent B; 20 – 30 min, 100% solvent B for a total time of 25 or 30 min. The flow rate was 1.0 mL/min. All solvents were filtered and degassed before entering the column. Under these HPLC conditions, pertechnetate and ^{99m}Tc co-ligands had a retention time of 4–6 min. Radioactivity in the samples was measured using a gamma counter with NaI(Tl) detector (Delshid, Iran).

2.2. ^{99m}Tc-Labelling of HYNIC-Peptide with Tricine/NA

Various parameters influencing the radiochemical yield (RCY) including pH (Table 1), amount of peptide added (Table 2), co-ligand mole ratios (Table 3) and amount of stannous ions added (Table 4) were analyzed. The only parameter having great effect on the RCY was the amount of stannous ions added (Fig. 2 and Table 4). For this purpose, 20 µL of HYNIC-**M-6** (1 mg/mL in H_2O) was added to a vial containing 15 mg of tricine and 10 mg of NA in 0.25 mL of ddH_2O. To this solution was added 1.25 µL (~12.5 nmol) of tin chloride solution (2 mg/mL $SnCl_2{\cdot}2H_2O$ in nitrogen-purged 0.1 m HCl). Finally, (5 – 10) mCi of $Na^{99m}TcO_4$ was added to the solution and the vial was heated for 15 min at 95°C in a shielded container. The pH vakue of the reaction mixture was adjusted at 5. After cooling to room tempera-

TABLE 1. Conditions of **M-6** Radiolabeling at Various pH (n = 3)

Peptide (µg)	Tricine (mg)	NA(mg)	^{99m}Tc activity (MBq)	Sn^{2+}(µg)	Adjusted pH	T (°C)	Time (min)	% Radiochemical purity (RCP)
20	40	3	200 – 370	40	4	95	15	72.2 ± 3.5
20	40	3	200 – 370	40	5	95	15	80.8 ± 4.7
20	40	3	200 – 370	40	6	95	15	55.4 ± 5.2
20	40	3	200 – 370	40	7	95	15	70.3 ± 5.4

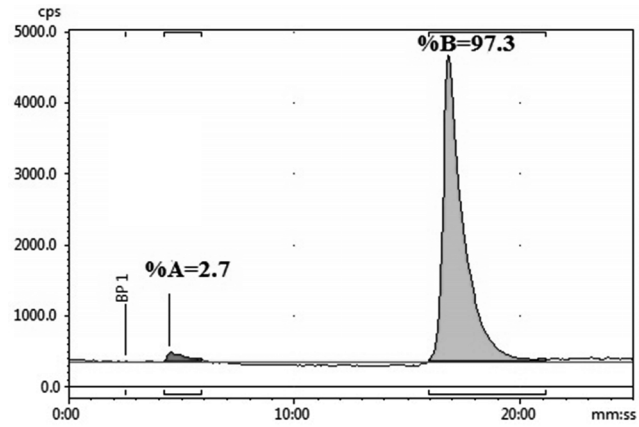

Fig. 3. RP-HPLC γ-chromatogram of [^{99m}Tc]Tc-**M-6**. A: $^{99m}TcO_4$ and ^{99m}Tc-coligands. B: [^{99m}Tc]Tc-**M-6**.

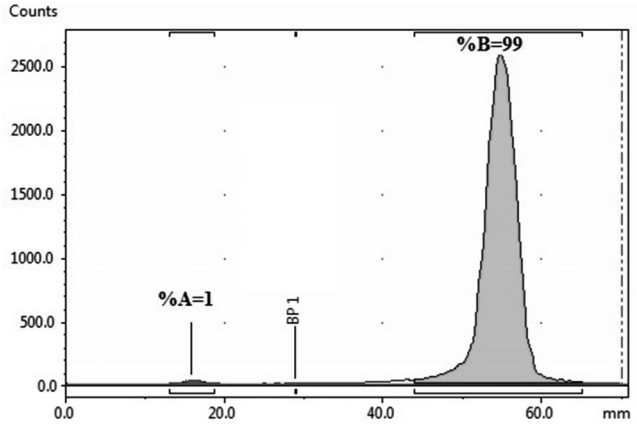

Fig. 4. TLC γ-chromatogram of [^{99m}Tc]Tc-**M-6** in C_2H_5OH:NH_4OH:H_2O (1:1:1 v/v) solution. A: ^{99m}Tc-colloid. B: [^{99m}Tc]Tc-**M-6**.

ture, the reaction mixture was analyzed by TLC and HPLC. TLC was performed using three systems: 2- butanone for determination of free $^{99m}TcO_4$ (R_f 1), 0.1 M citric buffer (pH 5) for detection of $^{99m}TcO_4$ and non-peptide-bound ^{99m}Tc-coligands (R_f 1), and C_2H_5OH:NH_4OH:H_2O (1:1:1 v/v) for detection of ^{99m}Tc-colloid (R_f 0). The radiolabeled solution was then subjected to quality control by analytic RP-HPLC using gradient elution as stated in previous section.

2.3. Stability in Solution

The *in vitro* stability of [^{99m}Tc]Tc-**M-6** was tested by incubating 100 μL of labeled peptide with 1 mL of the normal saline solution for 1, 4 and 24 h at 25°C. After incubation, [^{99m}Tc]Tc-**M-6** was assessed by TLC. All experiments were performed in triplicate.

2.4. Protein Binding and Stability in Serum

The percentage of [^{99m}Tc]Tc-**M-6** bound to plasma proteins and the stability of these in human serum was evaluated by incubating 100 μL of labeled peptide with 500 μL of fresh human serum at 37°C. At 1 and 4 h time points, 100 μL aliquots were treated with 200 μL of ethanol. The samples were centrifuged for 10 min at 4000*g* to precipitate serum proteins. Supernatants were passed through 0.22 μm filter and then analyzed by RP-HPLC. The total activity of the supernatant and sediment was measured by well-type NaI γ-counter to determine the percentage of [^{99m}Tc]Tc-**M-6** bound or transferred to serum proteins.

2.5. Calculation of Log P

Aliquot (100 μL) of [^{99m}Tc]Tc-**M-6** was added to a tube containing 1 mL *n*-octanol and 1 mL water. The tube was vortexed vigorously for 10 min and then centrifuged for 5 min at 4000*g*. Three aliquots of 100 μL were sampled from each layer and counted by γ-counter. The partition ratios were calculated by dividing the counts in the organic phase by that in the aqueous phase per unit volume.

3. RESULTS

3.1. Radiolabeling

Radiolabeling of **M-6** was performed efficiently with technetium-99m using tricine/NA as co-ligand system. The effects of various parameters on the radiochemical yield (RCY) are shown in tables including pH (Table 1), amount of peptide (Table 2), co-ligand mole ratio (Table 3) and amount of Sn(II) chloride (Table 4). The only parameter having great effect on RCY was the amount of stannous ions added. The

TABLE 2. Conditions of **M-6** Radiolabeling in the Presence of Various Amounts of HYNIC-Peptide (n = 3)

Peptide (μg)	Tricine (mg)	NA(mg)	^{99m}Tc activity (MBq)	Sn^{2+}(μg)	Adjusted pH	T (°C)	Time (min)	% Radiochemical purity (RCP)
15	40	3	200 – 370	40	5	95	15	55.1 ± 5.6
20	40	3	200 – 370	40	5	95	15	80.8 ± 4.7
35	40	3	200 – 370	40	5	95	15	75.7 ± 3.6
65	40	3	200 – 370	40	5	95	15	65.9 ± 4.5

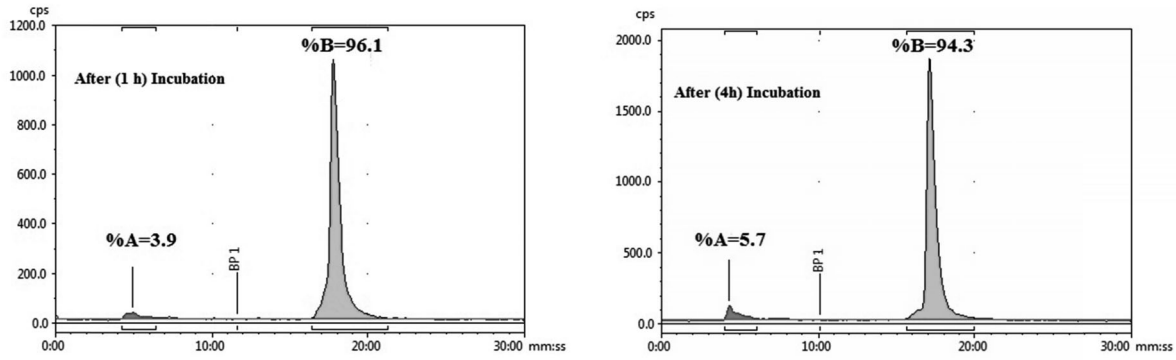

Fig. 5. RP-HPLC γ-chromatograms of [^{99m}Tc]Tc-**M-6** after 1 h and 4 h incubation in human serum. A: 99m-technetium not attached to the peptide. B: [^{99m}Tc]Tc-**M-6**.

effect of Sn(II) chloride amount on the RCY is illustrated in Fig. 2 and Table 4. The labeling yield of [^{99m}Tc]Tc-**M-6** was >95% (n = 3). The γ-chromatogram displays a single peak with a retention time of 17.05 min for [^{99m}Tc]Tc-**M-6** (Fig. 3). The percentage of ^{99m}Tc-colloid in this radiolabeled sample was detected with TLC scanner (Fig. 4).

3.2. Stability in Solution and Log P

The experimental stability in saline was monitored during 24 h. The lipophilicity of [^{99m}Tc]Tc-**M-6** was evaluated by partition between *n*-octanol and water. The log P value of [^{99m}Tc]Tc-**M-6** was –3.71 ± 0.02 (Table 5).

3.3. Stability in Serum and Protein Binding

The stability of [^{99m}Tc]Tc-**M-6** in human serum for up to 4 h was examined by RP-HPLC analysis. Figure 5 shows γ-chromatograms of **M-6** in human serum measured after 1 h and 4 h. [^{99m}Tc]Tc-**M-6** showed stability of approximately >94% over 4 h. The protein binding after 4 h incubation of [^{99m}Tc]Tc-**M-6** in serum was 32.82 ± 0.03% (Table 5).

4. DISCUSSION

In this work, we have optimized the minimum amount of tin(II) chloride and other conditions for ^{99m}Tc-labeling procedure of one HYNIC-conjugated peptide in the presence of tricine/NA. As shown in Fig. 6, the proposed procedure involves dissolving adequate amount of tricine and nicotinic acid as co-ligand system, adequate amount of HYNIC-conjugated peptide as a potential tracer, trace and unique amount of stannous ion as a reducing agent and adequate activity of technetium-99m as a radionuclide, in pure water. *In situ* heating of the solution for 15 min at 95°C in a shielded container is an essential requirement in this method. In this work, tricine/NA can have a dual role as a co-ligand system and pH stabilizing. In connection with the second role, it should be noted that function of buffer (weak acid) is best when the pK_a of the weak acid is close to the desired working pH (pH = pK_a) [16 – 18]. Thus, nicotinic acid (pK_a = 4.85 at 25°C) approximately have a maximum buffer capacity in reaction cocktail (pH 5). Application of this issue can be a key factor for *ex situ* buffer removing in ^{99m}Tc-labeling of HYNIC-conjugated biomolecules.

The most significant advantage of this procedure is to optimize the least amount of tin(II) chloride for ^{99m}Tc-labeling of HYNIC-conjugated peptide in the presence of Tricine/NA. This optimization of tin(II) chloride can be helpful because it can increase the stability of HYNIC-peptide solution without having impressive effect on the polarity or pharmacokinetics of HYNIC-peptide [12, 13]. According to Fig. 2 and Table 4, when the amount of $SnCl_2$ was changed from 2.5 to 15 μg (i.e., peptide:Sn^{2+}mole ratio changed from 2:1 to 1:3), the 99m-technetium in colloid increased, resulting in less RCY.

TABLE 3. Conditions of **M-6** Radiolabeling in the Presence of Various Amount of Coligands (n = 3)

Peptide (μg)	Tricine (mg)	NA(mg)	^{99m}Tc activity (MBq)	Sn^{2+}(μg)	Adjusted pH	T (°C)	Time (min)	% Radiochemical purity (RCP)
20	40	3	200 – 370	40	5	95	15	80.8 ± 4.7
20	15	10	200 – 370	40	5	95	15	82.3 ± 2.1
20	15	5	200 – 370	40	5	95	15	60.6 ± 9.2
20	10	10	200 – 370	40	5	95	15	50.1 ± 7.1

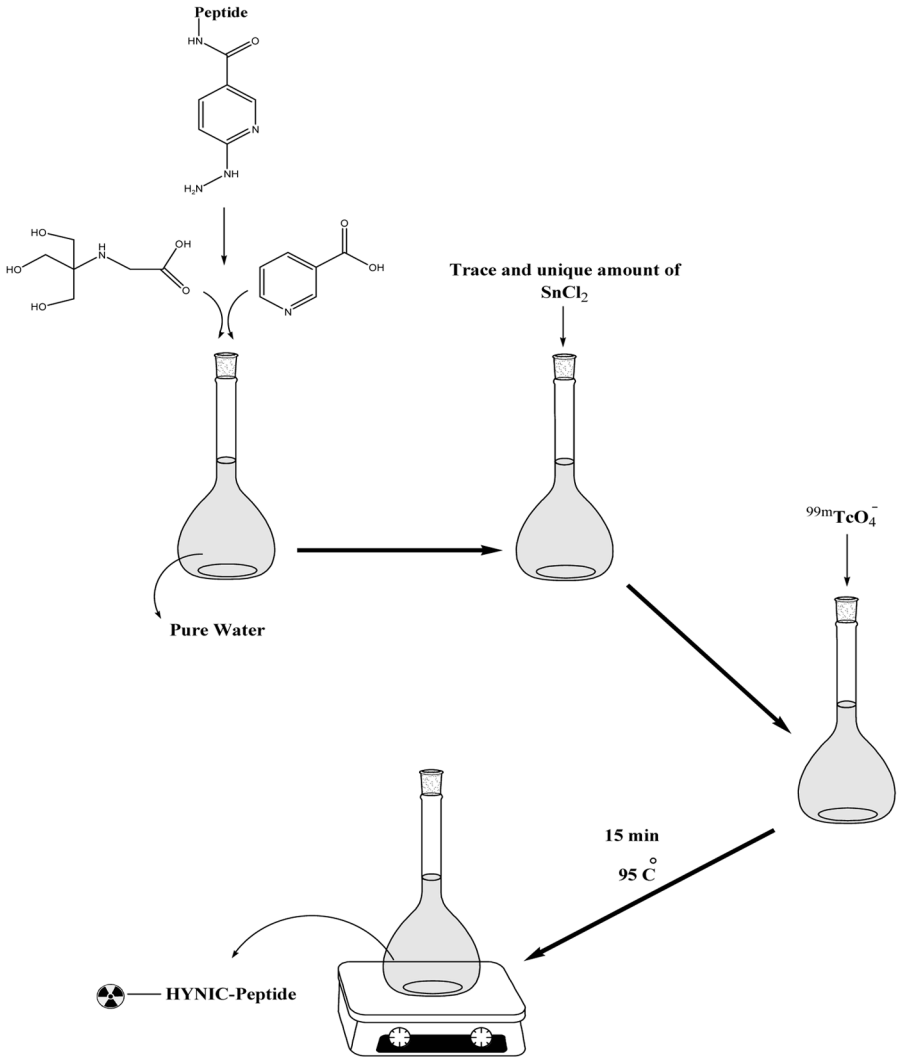

Fig. 6. Ideal scheme proposed for ^{99m}Tc-labeling procedure of **M-6**.

Generally, Table 6 summarizes the proposed ^{99m}Tc-labeling conditions in comparison to other conventional ^{99m}Tc-labeling methods of HYNIC-conjugated peptides in the presence of tricine/NA [10, 19 – 21]. As can be seen from Table 6, the prominent features of these conditions are the ^{99m}Tc-labeling of peptide without conventional (high) amount of $SnCl_2$, *ex situ* buffer and pretreatment, in pure water (ddH_2O).

The radiochemical yield of labeled HYNIC-conjugated peptide was calculated by RP-HPLC and TLC. Fig. 3 and

TABLE 4. Condition of **M-6** Radiolabeling in the Presence of Various Amounts of Stannous Ions (n =3)

Peptide (μg)	Tricine (mg)	NA(mg)	Sn^{2+}(μg)	Peptide:Sn^{2+} Mole ratio	^{99m}Tc activity (MBq)	Obtained pH	% $^{99m}TcO_2$	% Radiochemical purity (RCP)
20	15	10	2.5	2:1	200 – 370	5	1.2 ± 0.5	98.3 ± 1.1
20	15	10	5	1:1	200 – 370	5	4.2 ± 1.1	93.4 ± 2.3
20	15	10	7.5	2:3	200 – 370	5	5.8 ± 1.5	90.8 ± 2.1
20	15	10	10	1:2	200 – 370	5	7.0 ± 1.2	88.1 ± 3.3
20	15	10	15	1:3	200 – 370	5	18.6 ± 2.2	81.6 ± 2.6

TABLE 5. *In vitro* Characterization of Radiolabeled Peptide (n = 3)

Co-ligand system	Stability in saline			Stability in serum		Protein binding		Log P
	1 h	4 h	24 h	1 h	4 h	1 h	4 h	
Tricine/NA	96.40 ± 0.97%	95.77 ± 0.56%	≥95%	96.1% ± 0.81	94.3% ± 0.1.1	26.35 ± 0.08%	32.82 ± 0.03%	–3.71 ± 0.02

TABLE 6. Summary of Proposed ^{99m}Tc-Labeling Conditions in Comparison to Other Conventional ^{99m}Tc-Labeling Methods

Peptide	Co-ligand (mg)	HYNIC-peptide (μg)	$SnCl_2 \cdot 2H_2O$ (μg)	$^{99m}TcO_4^-$ (MBq)	Usedorganic solvents	pH	Time (min)	T (C°)	RCY (%)	Stability in serum (%)		Buffer	Ref.
										1 h	4 h		
HYNIC-RC160	Tricine(40) + NA(2)	10	25	100 – 500	–	5	15	100	97.7	stable	stable	Succinate	[19]
c(RGDyK (HYNIC)	Tricine (35) + NA (4)	5	20	>200	–	n.a	15	100	99.8	stable	stable	–	[20]
HYNIC-nsCCK8	Tricine (40) + NA (2)	5	25	100 – 370	–	n.a	30	75	93	n.a	n.a	PBS	[21]
HYNIC-sCCK8	Tricine (40) + NA (2)	5	25	100 – 370	–	n.a	30	75	96	n.a	n.a	PBS	[21]
HYNIC-nanogastrin	Tricine (40) + NA (9)	10	30	>200	Ethanol	n.a	30	95	97	58	55	–	[10]
HYNIC-**M-6**	Tricine (15) + NA (10)	20	**2.5**	200 – 370	–	5	15	95	>96	96	94	–	–

Fig. 4 show RP-HPLC and TLC γ-chromatogram of [^{99m}Tc]Tc-**M-6** in the presence of Tricine/NA, respectively. Accordingly, the first peak (A) correspond to $^{99m}TcO_4$ and ^{99m}Tc-coligands formation at the retention time of 4–6 min and the second peak (B) correspond to [^{99m}Tc]Tc-**M-6** formation at a retention time of 17.05 min in Fig. 3. In Fig. 4, the first peak (A) corresponds to ^{99m}Tc-colloid formation and the second peak (B) corresponds to [^{99m}Tc]Tc-**M-6** formation. The %RCY was calculated by the following equation:

$$\%RCY = 100 - (\%^{99m}TcO_4 + \%^{99m}\text{Tc-coligands} + \%^{99m}\text{Tc-colloid}). \quad (1)$$

Subsequently, the stability of [^{99m}Tc]Tc-**M-6** in saline and human serum was examined up to 24 and 4 h by TLC and RP-HPLC analysis, respectively. As can be seen from data in Table 5 and Fig. 5, [^{99m}Tc]Tc-**M-6** is stable in saline and human serum (approximately >94%) for up to 24 and 4 h, respectively. Thus, [^{99m}Tc]Tc-**M-6**, shows good stability in both saline and human serum. We profoundly believe that the use of this strategy, along with consideration of the above-mentioned advantages, can make a new challenging outlook and good foundation for the ^{99m}Tc-labeling of many HYNIC-conjugated biomolecules.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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