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Technical note

Confirmation of hydrazone formation in HYNIC-peptide conjugate preparation, and its hydrolysis during labeling with ^{99m}Tc

M. Gandomkar[a,*], R. Najafi[a], M. Shafiei[a], S.E.S. Ebrahimi[b]

[a]Radioisotope Division, Nuclear Research Center, Atomic Energy Organization of Iran (AEOI), Tehran, Iran
[b]Department of Medicinal Chemistry, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

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Abstract

Because of its monodenticity, 6-hydrazinopyridine-3-carboxylic acid (HYNIC) is of interest as a bifunctional chelator for labeling peptide with ^{99m}Tc. Here, we confirm the formation of hydrazone in HYNIC-conjugated peptide. The preparative HPLC was used to purify the HYNIC conjugated somatostatin-based peptide and the result showed two peaks, even after two consecutive purifications. Analysis of these peaks by mass spectrometry indicated the presence of hydrazone, produced during preparation conjugate. Further, we have shown that presence of hydrazone really does not matter because under ^{99m}Tc-labeling conditions, hydrazone is hydrolyzed back to HYNIC that then chelates ^{99m}Tc. A HYNIC-peptide conjugate freeze-dried kit was also prepared in a mildly acidic or neutral condition with a final pH of 6–7. The kit was then labeled by ^{99m}Tc and incubated in 100 °C for 10 min, and a labeling yield of >95% was obtained.

Keywords: Technetium-99m; HYNIC; Peptides; Hydrazones; Labeling

1. Introduction

In recent years, a variety of BFCA have been evaluated upon conjugation to peptides and among them 6-hydrazinopyridine-3-carboxylic acid (HYNIC), is more attractive because of its monodenticity which allows to use a variety of coligands altering the biodistribution in the desired way (Babich et al., 1995). In order to prepare a lyophilized peptide kit formulation with purity >98%, after conjugation of peptide with HYNIC a further preparative HPLC purification is essential. This is due to the fact that the HYNIC-peptide conjugate is not stable in aqueous solution (Edwards et al., 1999). We prepared a conjugate of HYNIC with a somatostatin analog peptide, which is then labeled with ^{99m}Tc. This compound may be used as a peptide-based radiopharmaceutical for tumor diagnosis in nuclear medicine. The aim of this study was to confirm the formation of hydrazone during conjugation and to suggest its subsequent hydrolysis to hydrazine, which then participates in labeling process influencing the labeling yield.

*Corresponding author. Tel.: +98 21 88020887; fax: +98 21 88021412.
E-mail address: msgandomkar@yahoo.com (M. Gandomkar).

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2. Experimental

2.1. Materials

Tritylchloride resin and most 9-fluorenylmethoxy carbonyl (fmoc)-protected amino acids were purchased from NovaBiochem (Germany) and Neosystem (France). All other reagents were purchased from Fluka chemical Co. The prochelator 6-Boc-HYNIC was synthesized as described by (Abrams et al., 1990). Na $^{99m}TcO_4$ obtained from commercial ^{99}Mo/ ^{99m}Tc generator (Radioisotope Division, AEOI).

2.2. Synthesis of HYNIC-peptide conjugate

The peptide was synthesized by standard Fmoc solid-phase synthesis on tritylchloride resin (substitution: 0.8 mmol/g) on an Applied Biosystems 433A peptide synthesizer. Fmoc was removed by adding 20% piperidine in *N*,*N*-dimethylformamide (DMF), coupling reagents were 3 eq *N*-hydroxybenzotriazole (HOBt), 3 eq diisopropylcarbodiimide (DIC), and 5 eq *N*-ethyldiisopropylamine (DIPEA) as base, and standard reaction time was

120 min.The fully protected peptide was cleaved from the resin with 20% acetic acid and after oxidative cyclization using 10 eq iodine, coupling of HYNIC was done in solution. Two equivalent Boc-HYNIC was coupled with 2 eq *O*-(7-azabenzotriazol-1-yl)-1,1,3,3,tetramethyluronium hexafluorophosphate (HATU) and 3 eq DIPEA to the *N*-terminus of the peptide. After deprotection and precipitation with ether, the compound was purified and characterized by preparative and analytical HPLC as described previously (Gandomkar et al., 2006). After HPLC separations, the individual components were colleted separately, dissolved in methanol and they were analyzed with ESI-MS [Waters ZMD (Micromass) with a HP1100 Quaternary LC pump (Germany)].

2.3. Kit formulation and radiolabeling

One mL of a solution containing 5 mg ethylenediamine-*N,N′*-diacetic acid (EDDA), 15 mg tricine, 40 μg $SnCl_2$ (20 μL of 2 mg/L $SnCl_2$, $2H_2O$ in nitrogen purged 0.1 M HCl) and 20 μg purified HYNIC-peptide conjugate with the final pH 6–7, was filtrated into a glass vial and freeze dried. Radiolabeling of kit was performed by adding 0.5 mL 0.9% saline in an evacuated vial and the mixtures were allowed to preincubate for 5 min. Then, 1 GBq of $^{99m}TcO_4^-$ in 0.5 mL saline was added to the vial and incubated for 10 min at 100 °C. After cooling to room temperature, the labeled peptide was analyzed by analytical HPLC and ITLC on silica gel 60 (Merck) with 1/1 methanol/1 M ammonium acetate as eluent. Under this condition, radiolabeled peptide, free $^{99m}TcO_4^-$ and non-peptide-bound ^{99m}Tc coligands migrate while ^{99m}Tc-colloid remains at the origin.

3. Results and discussion

Peptide with amino acid sequence (*D*)Phe-Cys-1-Nal-(*D*)Trp-Lys-Thr-Cys-Thr-OH was obtained by solid phase synthesis. The coupling of Boc-HYNIC was done in solution in an overall yield of 30%. HYNIC-peptide conjugate was purified twice by preparative HPLC, collecting at 10.0 min retention time. The HPLC chromatogram (Fig. 1) indicates that despite of two consecutive purifications, a second peak (14.20 min) appearing immediately after the main peak for HYNIC-peptide conjugate (13.05 min). The mass analysis of the two components indicated that the main peak was HYNIC-peptide with a molecular weight of 1219 Da $[M+H]^+$ and the second peak, corresponded to a molecular weight of 1231 Da $[M+H]^+$, was hydrazone (Fig. 2). These results confirm formation of hydrazone-peptide in preparation of purified HYNIC-peptide conjugate.

In labeled peptide kit, the results from ITLC tests indicated $<1\%$ of radioactivity at the origin, and ruling out any major formation of ^{99m}Tc-colloid. The HPLC result shows, a main peak at 12.86 min corresponding to radiolabeled peptide with very small signals at 4.43 and 5.90 min for free $^{99m}TcO_4^-$ and nonpeptide-bound ^{99m}Tc coligand. Correcting for contribution from ^{99m}Tc-colloid, the labeling yield was $>95\%$ at a specific activity of 50 MBq/nmol.

In the ^{99m}Tc-labeling of conjugated cyclic peptide Edwards et al. (1999) found that in manufacturing process, the final intermediate HYNIC conjugate itself is not stable in aqueous solution and reacts readily with aldehydes or ketones to form various impurities. These authors tried to overcome this problem by using specific hydrazones instead

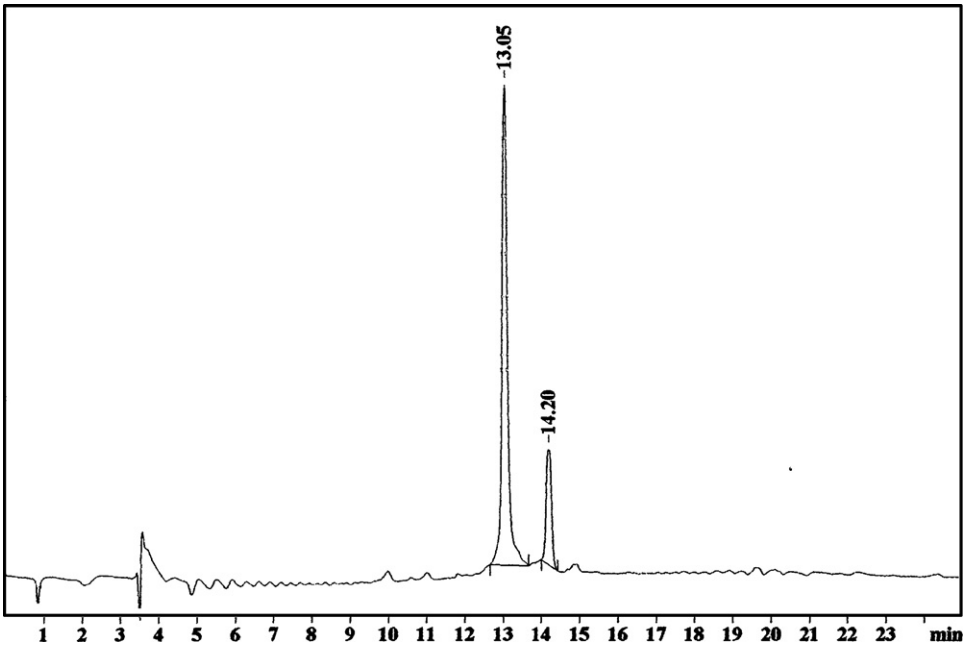

Fig. 1. HPLC analysis of HYNIC-peptide conjugate after two times purification.