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^{99m}Tc-labeling and molecular modeling of short dipeptide glycyl-L-proline

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Abstract Glycyl-L-proline (Gly-L-Pro) is the main degradation product of collagen and is a good diagnostic tool in various pathological conditions. The aim of this work was to prepare dipeptide Gly-L-Pro labeled with ^{99m}Tc. Complex preparation was carried out under alkaline reaction conditions and its stability was assessed 10 and 120 min after preparation. The formation of two types of complex compounds was observed. High-performance liquid chromatography, paper electrophoresis, paper chromatography and thin layer chromatography were employed to monitor the formation of different complexes. Molecular modeling (semi-empirical method) was used to design their structure and composition. First complex cI with formula $[TcO(Gly\text{-}L\text{-}Pro)]^{-1}$ is unstable. After 120 min cI is completely transformed to complex cII with formula $Tc(Gly\text{-}L\text{-}Pro)_3$.

Keywords ^{99m}Tc · Dipeptide · Glycyl-L-proline · Molecular modeling

Introduction

Short peptides have a great potential as radiopharmaceuticals in nuclear medicine. They have low molecular weight, low antigenicity, fast plasma clearance and good targeting properties. They are labeled with different isotopes directly or by a linker [1–3]. HYNIC (hydrazinonicotinamide) and aminethiol groups are often used as pre-conjugation molecules (groups) for the peptide radiolabeling with ^{99m}Tc [4–7]. Direct labeling is easier and less time consuming method, however labeled compound can be unstable, non-specific and quite complex for transition metals, like ^{99m}Tc [1].

Polypeptide collagen (type I) is occurred in whole human body: in bones, skin, tendons, ligaments, dentine, arteries and granulation tissues as the main locations. The importance of collagen type I for medical research is that it is involved in many human diseases, including fibrosis, osteoporosis, cancer, atherosclerosis, Marfan syndrome, systematic lupus erythematosus, rheumatoid arthritis [8–11]. Degradation products of type I collagen molecule are frequently utilized to monitor physiological changes in tissues as well as diagnostic tool in various pathological conditions [8]. Glycine and proline are the main building units of collagen while dipeptide Gly-L-Pro belongs to frequent degradation fragments of type I collagen [12].

Gly-L-Pro was directly labeled with Cu(II), Ni(II), ^{99m}Tc (under acidic reaction condition) [13–17]. Stability of coordination compounds is strongly influenced by reaction conditions and exact identification of the complex structure is often difficult [18]. The amount of ^{99m}Tc is extremely low and the preparation of the complex with inactive ^{99}Tc is impossible without changes in molar ratios and concentrations of the reactants [19]. Therefore, molecular modeling and computational chemistry can be useful for formulating the complexes structures.

The aim of the present work was to prepare ^{99m}Tc-Gly-L-Pro complex under alkali reaction condition and to establish formation and design its structure using molecular modeling approach.

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Reaction conditions

All the used chemicals were of analytical grade, obtained from commercial sources.

Gly-L-Pro was labeled with ^{99m}Tc using tin chloride ($SnCl_2$) as reducing agent. 1 mL of barbital buffer pH 9.7 was added to 2 mg of Gly-L-Pro, 20 μL of 0.02 mol L^{-1} $SnCl_2{\cdot}2H_2O$ solution (this $SnCl_2{\cdot}2H_2O$ water solution was prepared before the experiment from 2 mol L^{-1} $SnCl_2{\cdot}2H_2O$ solution in concentrated HCl) and 1 mL of $Na^{99m}TcO_4$ (the activity 100 MBq) freshly eluted from ^{99}Mo/^{99m}Tc generator. The mixture was incubated for 10 and 120 min at laboratory temperature. The quality of the reaction mixture was assessed at these two times after preparation.

To confirm the complex preparation two blank experiments were done. The first blank experiment had the same composition as mentioned above except for 20 μL of 0.02 mol L^{-1} $SnCl_2{\cdot}2H_2O$ (it was the proof the free pertechnetate anion does not interact with components of reaction mixture). The second blank experiment had the same composition as reaction mixture except for ligand (we confirmed, that reduced form of ^{99m}Tc does not react with components of reaction mixture).

The quality control methods

Quality control of ^{99m}Tc-labeled Gly-L-Pro was performed by:

- Thin-layer chromatography (TLC) using strips of Merck Silufol UV_{254} and acetone as mobile phase.
- Paper chromatography (PC) in Whatman paper No. 3 using normal saline solution as developer.
- Paper electrophoresis (PE) on the Whatman paper No. 3. 2.5 × 25 cm paper strips were impregnated with barbital buffer pH 9.7. Sample was applied on 5th cm (the starting position). The time of electrophoresis was 2 h at a constant voltage of 450 V.
- High performance liquid chromatography (HPLC), using reversed-phase C18 column (length 3 cm), with in-line radiometric detection. The mobile phase was water: barbital buffer pH 9.7 (ratio 9:1) and it was used flow rate of 1 mL/min.

The quantification of different separated species was carried out by measuring the activity of each centimeter segment of chromatograms and electrophoreograms. The activity of each centimeter segment was expressed as percentage of the total activity on the strip.

Computational methods

All herein reported calculations were performed with the HYPERCHEM 7.5 program package. We used the semi-empirical method with ZINDO/1 parameterization [17]. All molecules are computed in vacuum with RMS gradient less than 1.

Results and discussion

Blank experiments results

In preliminary experiments the mobility of reduced forms of ^{99m}Tc and free pertechnetate anion was observed.

Hydrolyzed form of ^{99m}Tc remains at the starting position ($R_f = 0$) while free pertechnetate anion ($^{99m}TcO_4^-$) moves with the solvent front ($R_f = 1$) when TLC is used as separation method [20–22]. Colloidal ^{99m}Tc remains at the point of spotting ($R_f = 0$) and free pertechnetate anion migrates to $R_f = 0.7$ on paper chromatogram developed in normal saline solution. One separated spot was identified on the starting position of the electrophoreogram (hydrolyzed form of ^{99m}Tc) [23]. Free pertechnetate anion flew over the whole length of the paper strip. It was possible to detect only $^{99m}TcO_4^-$ by HPLC (retention time = 120 s). Particles of hydrolyzed forms of ^{99m}Tc were too big and were unable to pass through the column.

^{99m}Tc-Labeling of Gly-L-Pro

Preparation of Gly-L-Pro complex with ^{99m}Tc by the procedure described previously gave an unstable product, as demonstrated PC and PE. PC analysis of sample 10 min after preparation showed wide area of radioactivity with maximum at $R_f = 0.4$ and after 120 min the whole activity remains at the origin of chromatogram (Fig. 1). 10 min after preparation the radiolabeling yield was 99% (for the complex with $R_f = 0.4$) and after 120 min 96% (for the complex remaining at the origin). Probably in the short time after mixing of the reactants, unstable ^{99m}Tc-Gly-L-Pro complex (cI) is formed and subsequently it is transformed into stable and more preferable complex compound (cII).

Paper electrophoresis supported our assumption as shown in Fig. 2. Sample applied on the paper strip 10 min after preparation was separated in two radioactive spots with positions at 1st (complex cI, radiochemical yield 30%) and 5th cm (radiochemical yield 63%). Only one radioactive spot was isolated by PE 120 after preparation. Its position was 1st cm (complex cII) and labeling yield was 94%.

Differences in the number of separated complexes by PC and EP 10 min after preparation is apparently caused

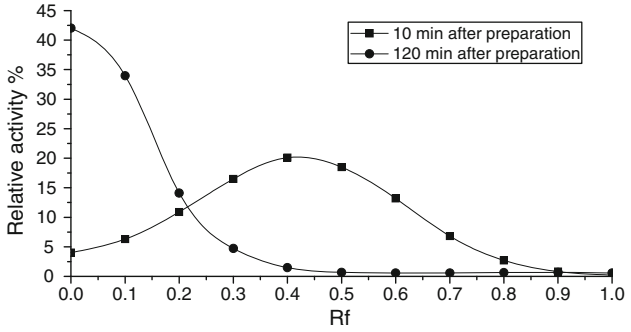

Fig. 1 PC profile of ^{99m}Tc-Gly-L-Pro complex 10 and 120 min after preparation

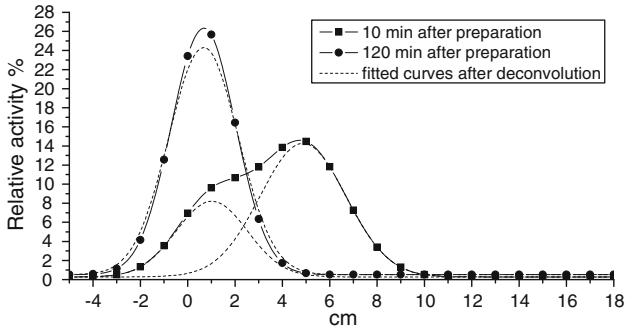

Fig. 2 PE profile ^{99m}Tc-Gly-L-Pro complex 10 and 120 min after preparation

by the duration of the separation process. PC separation took approximately 15 min and electrophoresis 2 h. During both separation methods two processes are presented simultaneously: process of separation and process of complex transformation from complex (cI) to complex (cII). Small increase of activity is on the origin of the paper chromatogram (sample applied 10 min after preparation), due to the effect of complex transformation (Fig. 1). More significant impact of transformation process is shown on the electrophoreogram (two incompletely separated spots).

TLC and acetone as mobile phase was not a suitable separation method to distinguish two complexes. Sample applied on strip 10 and 120 min after preparation remains in both cases on the origin ($R_f = 0$ and labeling efficiency 97% in both events). This method unambiguously confirmed the absence of free pertechnetate anion in both measured samples. The solvent front of these two chromatograms was without any activity.

If colloidal hydrolyzed form of ^{99m}Tc is presented in reaction mixture, it is expected to remain at the point of spotting for all three mentioned separation methods (PC, PE, and TLC). For that reason, results from HPLC were needed to determine the presence of complexes because hydrolyzed forms of ^{99m}Tc are eliminated by the column. Both samples (10 and 120 min after preparation) had the same retention time = 162 s. The injected volume (20 μL) and detected activity was identical with the blank experiment (only free pertechnetate anion, buffer solution and ligand). According to this result hydrolyzed form of technetium is not present in reaction mixture 10 and 120 min after preparation and the whole activity belongs to complexes.

Design of ^{99m}Tc-Gly-L-Pro complex

Separation methods from previous part showed that the reaction mixture contains two types of ^{99m}Tc complex compounds with dipeptide Gly-L-Pro. It is impossible to identify the exact structure and composition of these two reaction products because the amount of ^{99m}Tc eluted from ^{99}Mo/^{99m}Tc generator is approximately 10^{-9} mol/L [24]. We decided to use theoretical background and methods of molecular modeling to design the reaction products.

Reduction of ^{99m}Tc by tin chloride leads to decrease of oxidation state of technetium from IIV → V → III [25]. These two successive reactions should be rapid in the low

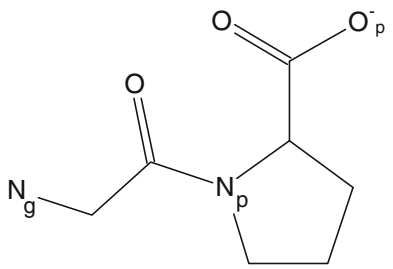

Fig. 3 Gly-L-Pro molecule with marked donor atoms

Table 1 Comparison of total energies of different types of ^{99m}TcO(Gly-L-Pro)$_2$ complexes

Complex ^{99m}TcO(Gly-L-Pro)$_2$	
Donor atoms	Total energy (kJ mol^{-1})
O_pN_p	−735664.1
O_pN_g	−735795.3
N_pN_g	***−756499.0***

The most convenient complex is *bold* and *italic*

Table 2 Comparison of total energies of different types of ^{99m}Tc(Gly-L-Pro)$_3$ complexes

Complex ^{99m}Tc(Gly-L-Pro)$_3$	
Donor atoms	Total energy (kJ mol^{-1})
N_pN_g–N_pN_g–O_pN_p	***−1022710.2***
N_pN_g–N_pN_g–O_pN_g	−1022285.9
N_pN_g–N_pN_g–N_pN_g	−1022298.0

The most convenient complex is *bold* and *italic*

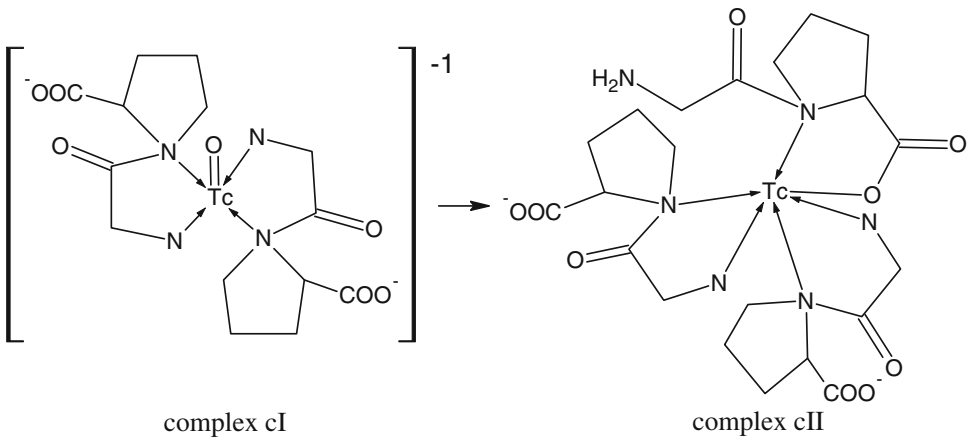

Fig. 4 Scheme of complex cI to cII transformation

complex cI complex cII

concentrations at radiopharmaceutical level [24]. It means that the first complex cI can reveal ^{99m}Tc in oxidation degree III. Ligand Gly-L-Pro provides three donor atoms: O_p, N_p and N_g (Fig. 3).

Nitrogen donors are neutral and do not dissociate [26]. Table 1 presents the formation energies for three possible combinations of donor atoms.

The most energetically preferable combination of donor atoms is N_pN_g with typical 5-ring size in technetium coordination compounds [27]. Total charge of this compound is −1 and therefore it is possible to separate this complex by paper electrophoresis (Fig. 2). Six coordinated complexes without oxygen are typical for the technetium with oxidation number III [28]. It could be the reason for transformation of complex cI into complex cII (Figs. 1, 2). During this transformation complex cI has to lose negative charge. This is caused by interchange of the oxygen atom with ligand molecule. The new Gly-L-Pro molecule can form several types of coordination bonds with complex cI. Energetically most preferable are donor atoms O_p and N_p to compensate the loss of oxygen (Table 2).

Bond lengths in complex cI and cII are in good agreement with published works (in parenthesis is deviation from literary data): Tc=O 1.64 Å (compared with the value for Tc^V, 1.2%) [28–30], Tc–O_p 2.12 Å (3%) [31, 32] and Tc–$N_{p,q}$ 2.13 (2%) [33–35]. Figure 4 shows the schematic structure of both (cI and cII) ^{99m}Tc-Gly-L-Pro complexes.

Conclusions

Our results show that ^{99m}Tc forms two types of complexes with dipeptide Gly-L-Pro under alkali reaction conditions. The stability was assessed 10 and 120 min after preparation by different separation methods. It was established that the first complex cI is transformed to second complex cII showing different position on paper chromatogram and electrophoreogram. Molecular structure of these two complexes was designed by using molecular modeling methods. Complex cI has composition $[TcO(Gly\text{-}L\text{-}Pro)]^{-1}$ and after 120 min is completely transformed to complex cII with formula $Tc(Gly\text{-}L\text{-}Pro)_3$.

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