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Synthesis, radiochemistry and stability of the conjugates of technetium-99m complexes with Substance P

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Abstract Two types of technetium-99m complexes: (i) with the Hynic ligand linked to Substance P(1–11) and (ii) of the type '4 + 1' consisting of tetradentate tripodal chelator tris(2-mercaptoethyl)-amine and monodentate isocyanide ligand previously coupled with Substance P(1–11), have been prepared on the n.c.a. scale. The obtained conjugates exhibit different lipophilicity and high stability in neutral aqueous solutions, even in the presence of excess concentration of histidine/cysteine competitive standard ligands. The conjugate $(^{99m}Tc(NS_3)(CN))_2$–SP containing two technetium-99m species in the molecule may be expected to be an extremely good diagnostic radiopharmaceutical.

Keywords Technetium-99m · Substance P · Complexes · Diagnostic agents · Stability · Lipophilicity

Introduction

Coordination compounds of technetium (the radioactive element with $Z = 43$) are well established in diagnostic nuclear medicine, and various complexes of the γ-emitting nuclide Tc-99m are commonly used for organs imaging. Its half-life time of 6 h is optimal for diagnostic medicine and the 140 keV energy of emitted γ-rays is sufficiently low to prevent a high dose delivered to the patient, but high enough to penetrate biological tissues and emerge from internal organs [1]. Modern trends in the radiopharmaceutical chemistry of technetium focus on labelling of biologically active molecules such as peptides, steroids or other receptor-seeking units.

Substance P(1–11), SP, is an undecapeptide belonging to the family of neurokinins termed tachykinins (NKs). It is a mediator responsible for the neural-immune/hematopoietic cross-talk. SP is the preferential endogenous ligand for the neurokinin type 1 receptors (NK-1), which are overexpressed in malignant gliomas [2]. The sequence of the five amino acids Phe^7, Phe^8, Gly^9, Leu^{10} and Met^{11} located at the C-terminus of the SP peptide is responsible for its affinity towards the NK1 receptor (the N-terminal region of SP is not essential) [2]. SP has been already used in diagnostic/therapeutic receptor radiopharmaceuticals as a peptidic vector leading a diagnostic/therapeutic nuclide to the receptors located on the tumor cell surface [3, 4].

The aim of this work was to synthesize conjugates containing Substance P as a biologically active peptide and different mixed-ligand technetium-99m complexes (Fig. 1), and to determine and compare their physico-chemical properties, important from the radiopharmaceutical point of view.

Experimental

Four conjugates—^{99m}Tc-SP complexes—have been prepared on the n.c.a. (no-carrier-added) scale (Fig. 1): compound **1** containing Hynic ligand bound to SP, i.e. Hynic-SP, and compounds **2a/b**, **3a/b** and **4** containing ^{99m}Tc-complexes (type '4 + 1') with a tetradentate tripodal chelator tris(2-mercaptoethyl)-amine, NS_3, and a monodentate

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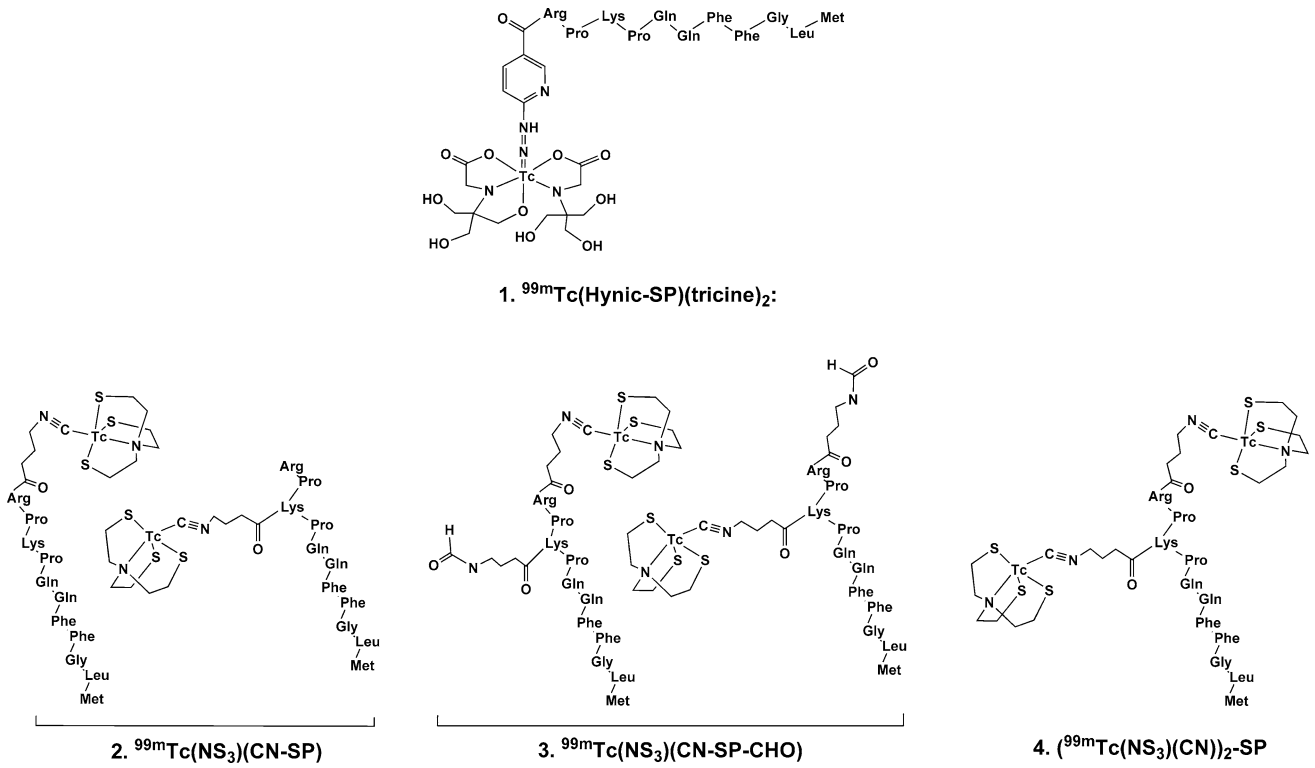


Fig. 1 Conjugates studied in this work

isocyanide ligand previously coupled with Substance P, CN–SP.

Materials and methods

Substance P and Hynic ligand combined with Substance P, Hynic-SP, were purchased from GeneCust Europe, and tricine—from Sigma-Aldrich. All chemicals were used as obtained.

Solvents used in syntheses and in the HPLC analyses were purchased from Polish Chemical Reagents S.A. as pure p.a. and used without further purification. Deionized water was prepared in a Hydrolab water purification system (Hydrolab, Poland).

The tetradentate NS_3 ligand (tris(2-mercaptoethyl)-amine; 2,2′,2″-nitrilotriethanethiol) was prepared by the reaction of tris(2-chloroethyl)amine hydrochloride with potassium thioacetate, followed by reduction with $LiAlH_4$. The final product was precipitated as the oxalate salt and applied as such in forthcoming reactions. Details of the synthesis are given in [5].

Analytical data

Elemental analysis: Calcd: C 36,51 %, H 7,66 %, N 7,10 %; Found: C 36,52 %, H 7,27 %, N 7,62 %.

^{1}H NMR ($CDCl_3$) δ (ppm): 1,80 (s, 3H, S**H**); 2,72 (m, 12H, NC$\mathbf{H}_2$ i SC$\mathbf{H}_2$).

^{13}C NMR ($CDCl_3$) δ (ppm): 822,81 (H_2**C**S); 57,01 (N**C**H_2).

IR (KBr plate), cm^{-1}: 2,987 (**CH**); 2,425 (**SH**).

The monodentate isocyanide ligand (isocyanobutyric succinimidyl ester) used as aliphatic linker CN–BFCA (**Bif**unctional **C**oupling **A**gent) was synthesized according to the procedure described in Ref. [6].

Analytical data

MS, m/z: 213,1 [M + H$^+$]; teoret. 212,21 g/mol.

IR: (KBr plate), cm^{-1}: 2,153 (C≡N), 1,814, 1,785, 1,729 (succinimidyl ester).

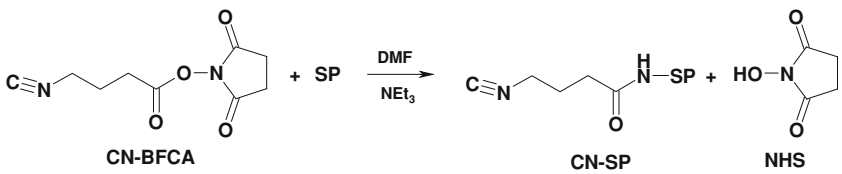
Syntheses

CN–SP

Coupling reaction of the isocyanide linker CN–BFCA with Substance P (Scheme 1) was performed according to the procedure described in Ref. [7].

0.39 mg (1.86 μmol) of CN–BFCA dissolved in 100 μL of DMF and 0.34 μL (2.45 μmol) of triethylamine were added to 1.64 mg (1.22 μmol) of Substance P. The mixture

Scheme 1 Coupling reaction of CN–BFCA linker with Substance P



was allowed to stay overnight at room temperature and then the solvent was removed under vacuum. Crude residue was dissolved in a 200 μL mixture of acetonitrile and water (1:1), purified by the semi-preparative HPLC, then alkalized and lyophilized. The conditions of HPLC analyses (system 1) were: Phenomenex Jupiter Proteo semi-preparative column (4 μm, 90 Å, 250 × 10 mm) and the UV/Vis detector (220 nm). Elution conditions: solvent A—water with 0.1 % TFA (v/v); solvent B—acetonitrile with 0.1 % TFA (v/v); gradient: 0–20 min 20–80 % of B, 20–35 min 80 % solvent B; 2 ml/min.

^{99m}Tc(Hynic-SP)(tricine)$_2$

To the solution containing 10 μg (6.7×10^{-3} μmol) of Hynic-SP, 20 mg (1.1×10^{-4} mol) of tricine and 5 mg of EDDA (2.8×10^{-5} mol) in 500 μL of the 0.1 M PBS buffer we simultaneously added 500 μL of the $Na^{99m}TcO_4$ solution (0.9 % NaCl eluate from the ^{99}Mo/^{99m}Tc generator, 50–100 MBq) and 5 μL of $SnCl_2$ (5.3×10^{-3} M in 0.1 M HCl). The reaction mixture was incubated for 20 min at 90 °C, and the reaction progress was checked by HPLC (system 2). The radiochemical yield of the ^{99m}Tc(Hynic-SP)(tricine)$_2$ conjugate was determined to be approximately 98 %. The HPLC analyses were performed using system 2: Supelcosil^{TM} LC-18 HPLC analytical column (250 × 4.6 mm), γ-radiation detector. Elution conditions: solvent A—water with 0.1 % TFA (v/v); solvent B—acetonitrile with 0.1 % TFA (v/v); gradient: 0–20 min 5–70 % solvent B, 20–25 min 70–95 % solvent B; 1 ml/min.

^{99m}Tc(NS$_3$)(CN–SP)

In order to label various forms of Substance P with the '4 + 1' ^{99m}Tc complex, two-step procedure was applied (Scheme 2) according to Ref. [7].

In the first step, 1 ml of $Na^{99m}TcO_4$ in the 0.9 % NaCl aqueous solution (^{99}Mo/^{99m}Tc generator eluate, 50–100 MBq) was added to a vial containing 5 mg of EDTA, 5 mg of mannitol and 0.08 mg of $SnCl_2$. Next, the reaction mixture was incubated for 20 min at room temperature. Progress of the reaction was checked by TLC (silica gel 60 WF_{254}, Merck) using water or acetonitrile as developing solvents. Purity of the intermediate complex (^{99m}Tc (EDTA/mannitol) was checked by HPLC. Conditions of HPLC analyses (system 3) were: analytical column Phenomenex Jupiter Proteo (4 μm, 90 Å, 250 × 4.6 mm) and γ-detector. Gradient: solvent A—water with 0.1 % TFA (v/v); solvent B—acetonitrile with 0.1 % TFA (v/v); 0–20 min 20–80 % solvent B, 20–35 min 80 % solvent B; 1 ml/min.

In the second step, the intermediate ^{99m}Tc-EDTA compound reacted with 300 μg of the NS_3 ligand and with about 50 μg of the isocyanide-modified peptide CN–SP. The reaction mixture was incubated for 30 min at 70 °C. The reaction progress was checked, as in the first step, by TLC and HPLC methods. In particular, the formation of neither $^{99m}TcO_4^-$ nor TcO_2 was found in HPLC and TLC experiments, respectively. The overall radiochemical yields of ^{99m}Tc(NS$_3$)(CN–SP) syntheses were between 85 and 95 %.

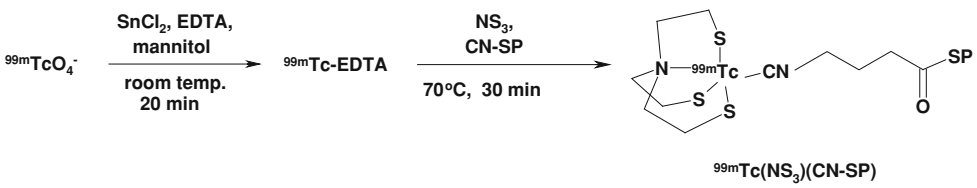
Thin layer chromatography was performed using silica gel 60 F_{254} TLC aluminum plates (Merck). All radioactive substances were placed on the strips, developed with appropriate solutions and dried. Distribution of radioactivity on the strips was determined using an automatic TLC analyzer SC-05 (home-made, INCT, Warsaw).

Lipophilicity studies

Lipophilicity of the compounds was characterised by determination of the logarithm of their partition coefficients, logD, in the *n*-octanol/PBS (pH 7.40) system, which mimics the physiological conditions [8].

Appropriate HPLC fractions containing the technetium-99m conjugates (**1**–**4**) were evaporated in the argon stream, dissolved in 0.5 mL of the PBS solution (pH 7.4) and

Scheme 2 Substance P labelling process with technetium-99m complex of the type '4 + 1'



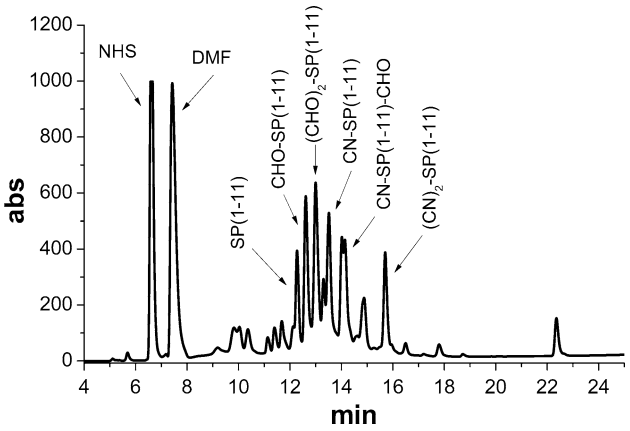


Fig. 2 HPLC chromatogram of the reaction mixture resulting from coupling the isocyanide linker CN–BFCA with Substance P

mixed with equal volume of *n*-octanol at 25 °C for 1 min. Prior to the experiments, it was found that this time was sufficient for reaching equilibrium. After separating the phases (centrifuging for 5 min with 14,000 rpm), concentration of the ^{99m}Tc compound in each phase was determined by γ-radiation counting using the well-type NaI(Tl) detector. Immediately after partition experiments, the aqueous phase was analysed by HPLC to check whether the studied complexes were not decomposed during the experiments. The logD was determined as a average value from three independent measurements.

Ligand exchange experiments

Aqueous solutions of the conjugates **1–4** (isolated from the reaction mixtures using semi-preparative HPLC) in concentrations of about 10^{-4} mM were incubated at 37 °C in the PBS buffer (pH 7.4), after adding 500 μL of 10 mM of the strongly competing standard ligands histidine or cysteine. Such experiments, called challenge experiments, are performed in order to study transchelation of the ligands by the thione and/or nitrogen donor compounds usually present in the biological fluids in excess to the examined species. HPLC analyses of the incubated solutions were performed at different time periods, up to 24 h after starting the incubation.

Results and discussion

HPLC chromatogram of the mixture resulting from coupling of the isocyanide linker CN–BFCA with Substance P is shown in Fig. 2.

The reaction products (species corresponding to the peaks at R_T of 12.3, 12.7, 13.1, 13.4, 13.6, 14.2, and 15.8 min) have been collected separately and characterized by MS analyses. Basing on these results (Table 1) all species have been identified (the compounds containing at least one isocyanide group in molecule are shown in Fig. 3).

Due to the presence of two primary amine groups in the molecule of Substance P (α-amine group of Arg^1 and ε-amine group of Lys^3), the CN–BFCA linker may react either with one of them or with both amine groups simultaneously [9]. Because of higher reactivity of the α-amine group of Arg^1 than of the ε-amine group of Lys^3 [10, 11] the CN–SP.a product is formed with higher yield (Fig. 3, peak at $R_T = 13.6$ min) than the product CN–SP.b (Fig. 3, peak at $R_T = 13.4$ min). Reaction mixture may also contain certain amounts of the CHO-BFCA compound due to low stability of isocyanide group CN– under acidic and

Table 1 Results of the MS analyses of the products obtained in the course of coupling of the isocyanide linker CN–BFCA with Substance P

Compound	R_T (min)	MS analyses		Compound structure
		Exact mass (g/mol)	Found M + H$^+$ (g/mol)	
Substance P	12.3	1346.73	1347.34	R–P–K–P–Q–Q–F–F–G–L–M
R–P–K–P–Q–Q–F–F–G–L–M				Unreacted peptide
CHO–SP	12.7	1459.78	1460.65	R*(CHO)*–P–K–P–Q–Q–F–F–G–L–M[a] or R–P–K*(CHO)*–P–Q–Q–F–F–G–L–M[a]
$(CHO)_2$–SP	13.1	1572.82	1573.56	R*(CHO)*–P–K*(CHO)*–P–Q–Q–F–F–G–L–M[a]
CN–SP	13.4	1441.77	1442.33	R–P–K*(CN)*–P–Q–Q–F–F–G–L–M or R*(CN)*–P–K–P–Q–Q–F–F–G–L–M
CN–SP	13.6	1441.77	1442.28	R*(CN)*–P–K–P–Q–Q–F–F–G–L–M or R–P–K*(CN)*–P–Q–Q–F–F–G–L–M
CN–SP–CHO	14.2	1554.81	1555.60	R*(CN)*–P–K*(CHO)*–P–Q–Q–F–F–G–L–M or R*(CHO)*–P–K*(CN)*–P–Q–Q–F–F–G–L–M
$(CN)_2$–SP	15.8	1536.80	1537.59	R*(CN)*–P–K*(CN)*–P–Q–Q–F–F–G–L–M

[a] The species inactive in technetium-99m chelating reaction

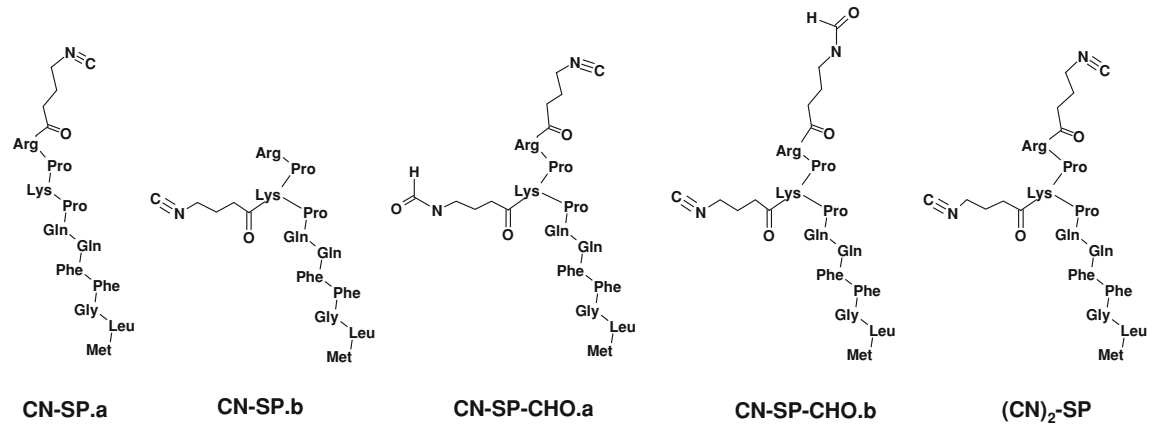


Fig. 3 Different forms of the CN–Substance P compounds

neutral conditions and its easy conversion into the aldehyde group CHO– (isocyanides react with water to give the corresponding formamide and finally the amine). As a result, the species CHO–SP, CN–SP–CHO and $(CHO)_2$–SP have been formed. However, the two aldehydes, namely CHO–SP ($R_T = 12.7$ min) and $(CHO)_2$–SP, ($R_T = 13.1$ min), which do not contain the isocyanide group in the molecule, are inactive in respect to labelling with technetium-99m.

The compounds containing one or two isocyanide groups in the molecule, which are necessary for technetium-99m chelation, are shown in Fig. 3.

Because the CN–SP.a and CN–SP.b species seem to be similar compounds (each of them contains only one isocyanide group), the difference in their HPLC retention time is only about 0.2 min. Both these species make possible to chelate technetium-99m, i.e. to synthesize the conjugate technetium-99 m complexes with the Substance P. Therefore, it is not important, which amine group of Substance P (*N*-terminal α-amine group of Arg^1 or ε-amine group of Lys^3) couples with the CN–BFCA linker. Because both amino acids suitable for complex formation are located outside the biologically active fragment of the peptide, we did not try to solve the problem of peak assignment to the particular species (i.e. which of the peaks with the retention time of 13.4 and 13.6 min belongs to CN–SP.a and which to CN–SP.b). The peak at $R_T = 14.2$ min corresponds either to CN–SP–CHO.a or to CN–SP–CHO.b, i.e. to the species containing one isocyanide group and one inactive aldehyde group. The two latter forms, both being suitable for further reactions, are practically undistinguishable in HPLC analysis, although the peak at $R_T = 14.2$ min is a doublet, see Fig. 2. The compound $(CN)_2$-SP, corresponding to the peak at $R_T = 15.8$ min, contains two isocyanide groups (Table 1) and is also suitable for subsequent technetium-99m chelating.

Chromatogram of the reaction mixture, recorded after completing synthesis of the conjugate ^{99m}Tc(Hynic-SP)(tricine)$_2$, is shown in Fig. 4. Lipophilicity (expressed in terms of the logD value) and stability of the isolated conjugate are presented in Table 2.

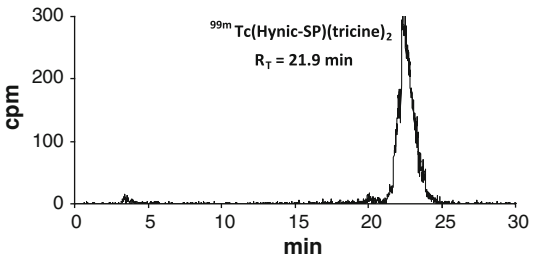


Fig. 4 Chromatogram of the reaction mixture, recorded after formation of the ^{99m}Tc(Hynic-SP)(tricine)$_2$ conjugate had been completed

For the syntheses of the conjugates of the '4 + 1" technetium-99m complex with the Substance P, the tetradentate tripodal chelator (tris(2-mercaptoethyl)-amine, NS_3) and the monodentate isocyanide CN–BFCA ligand, previously coupled with the SP biomolecule, were used. The NS_3 molecule coordinates the ^{99m}Tc(III) cation and leaves the fifth coordination site available for one monodentate isocyanide ligand: CN–SP, CN–SP–CHO or $(CN)_2$–SP. Chromatograms of the reaction mixtures, recorded after syntheses of the conjugates containing the ^{99m}Tc(III) cation, NS_3 species and one isocyanide ligand, are presented in Fig. 5. Lipophilicity and stability of the isolated conjugates are presented in Table 2.

The peaks recorded at $R_T = 3.4$ min and 7.9 min (Fig. 5) corresponds to the intermediate complexes ^{99m}Tc(EDTA/mannitol) and ^{99m}Tc(NS_3), respectively.

The data presented in Table 2 show that the conjugate ^{99m}Tc(Hynic-SP)(tricine)$_2$ (**1**) is much more hydrophilic than the conjugates containing the ^{99m}Tc(NS_3) core (**2**–**4**). Among the last three conjugates, the logD value for compound **4** (which contains two technetium(III)-99m complexes in the molecule) is significantly higher than the

Table 2 Lipophilicity and stability of the conjugates ^{99m}Tc(Hynic-SP)(tricine)$_2$, ^{99m}Tc(NS$_3$)(CN–SP), ^{99m}Tc(NS$_3$)(CN–SP–CHO) and (^{99m}Tc(NS$_3$)(CN))$_2$–SP

Conjugate	R_T (min)	logD	% of intact complexes after 20 h		
		oct/PBS (pH 7.40)	PBS (pH 7.4)	Cysteine 10^{-2} M	Histidine 10^{-2} M
^{99m}Tc(Hynic-SP)(tricine)$_2$	21.9	-3.7 ± 0.3	~97	~99	~100
1	(system 2)				
^{99m}Tc(NS$_3$)(CN–SP)	12.7	-0.24 ± 0.02	~99	~99	~100
2	(system 3)				
^{99m}Tc(NS$_3$)(CN–SP–CHO)	12.9	-0.20 ± 0.01	~100	~100	~100
3	(system 3)				
(^{99m}Tc(NS$_3$)(CN))$_2$–SP	13.1	0.89 ± 0.02	~98	~98	~100
4	(system 3)				

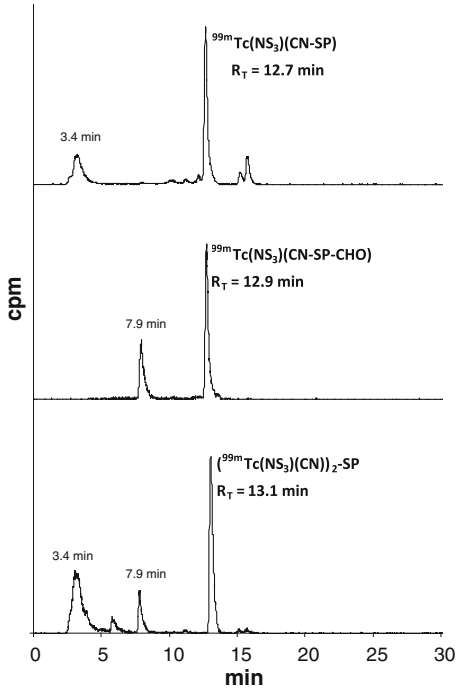


Fig. 5 Chromatograms of reaction mixtures, recorded after syntheses of the conjugates ^{99m}Tc(NS$_3$)(CN–SP), ^{99m}Tc(NS$_3$)(CN–SP-CHO) and (^{99m}Tc(NS$_3$)(CN))$_2$-SP

corresponding values for **2** and **3**. Moreover, lipohilicity of ^{99m}Tc(NS$_3$)(CN–SP) (**2**), ^{99m}Tc(NS$_3$)(CN–SP-CHO) (**3**) and (^{99m}Tc(NS$_3$)(CN))$_2$-SP (**4**) conjugates can easily be modified by introduction of either hydrophilic or hydrophobic group at the periphery of the NS$_3$ ligand [12].

Studies on stability of the isolated conjugates **1**–**4** show that despite comparatively high concentrations of interfering ligands (10 mM), the investigated technetium conjugates (present in solution in concentration of about 10^{-4} mM) do not undergo a detectable ligand exchange, neither by histidine nor by cysteine. After 16 h of incubation only trace amounts ($\leq$2 %) of transchelation products were observed on the HPLC chromatograms.

Conclusions

All synthesized conjugates of technetium-99m complexes with Substance P are formed with high yields (>85 %) and are very stable in the presence of competitive cysteine/histidine ligands. Comparing the physicochemical properties of the conjugate containing ^{99m}Tc-Hynic complex and conjugates containing technetium-99m complexes of the '4 + 1' type (with tetradentate tripodal chelator tris (2-mercaptoethyl)-amine and monodentate isocyanide ligands) one can conclude that technetium-99 m complexes of type '4 + 1' are good radiopharmaceutical precursors. It also follows from this study that the new conjugates ^{99m}Tc(NS$_3$)(CN–SP), ^{99m}Tc(NS$_3$)(CN–SP–CHO) and (^{99m}Tc(NS$_3$)(CN))$_2$–SP can be considered to be promising candidates for diagnostic radiopharmaceuticals. Moreover, the conjugate (^{99m}Tc(NS$_3$)(CN))$_2$–SP containing two technetium-99m complexes in the molecule can be expected to be a very efficient receptor radiopharmaceutical.

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