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Novel 3+1 mixed-ligand Technetium-99m complexes carrying dipeptides as monodentate ligands

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Abstract

Novel mixed ligand oxotechnetium complexes of the type [^{99m}TcO(SSS)(SR)], in which the SR monodentate ligand is derived from dipeptides gly-gly, phe-gly and ala-gly, have been synthesized. These complexes, which have a molecular weight above 300, a lipophilic moiety, $[TcO(SSS)]^+$, and an ionizable group separated from the lipophilic moiety by a spacer, have been obtained in 70-95% radiochemical yield. These compounds were prepared using ^{99m}Tc-tartrate as the precursor and Sn^{2+} as the reducing agent. The identity of the [^{99m}TcO(SSS)(SR)] complexes has been established by HPLC comparison with the analogous oxorhenium complexes. The nature of the monodentate co-ligand strongly affects the stability of the ^{99m}Tc-complexes and their biodistribution. Complex 3b is the most stable *in vitro* presenting the highest blood clearance, a high liver uptake and a selective hepatobiliary excretion (54.5% ID at 15 min post-injection, and 69.3% ID at 60 min post injection). The results obtained show that 3b have reasonable stability and *in vivo* properties that may be useful for peptide labeling.

Keywords: ^{99m}Tc; 3+1 approach; Re; Dipeptides; Biodistribution studies

1. Introduction

The development of radiopharmaceuticals designed to bind specific receptors, including membrane transport systems, is currently receiving much interest due to their potential to achieve improved *in vivo* monitoring of biochemical and physiological functions [15]. Technetium-99m (^{99m}Tc) is the radionuclide of choice for diagnostic imaging with single photon emission computed tomography (SPECT) due to its ideal nuclear properties ($E\gamma = 140$ keV, $T_{1/2} = 6$h, no β-emission) and availability from a ^{99}Mo/^{99m}Tc generator [15]. These properties have led to the search of novel ^{99m}Tc-based radiopharmaceuticals incorporating ligands specifically designed to probe protein receptors and transporters [6,9,10].

Recently, a new ′3 + 1′ mixed-ligand approach has been proposed as a new strategy for developing novel neutral ^{99m}Tc-radiopharmaceuticals containing the TcO^{3+} core [16,20]. This approach, which consists of a tridentate ligand and a monodentate co-ligand surrounding the TcO^{3+} core, has evolved successfully to generate novel lipophilic potential radiopharmaceuticals with high brain uptake and retention [12,17,21]. This strategy also offers an easy access to ^{99m}Tc-based probes with affinity and selectivity to protein receptors, in which the receptor ligand is appended either into the tridentate or the monodentate ligand [8,13,18].

To evaluate the possibility of labeling peptides with the moiety $[TcO(SSS)]^+$ we studied some model complexes. We report herein on the synthesis and characterization of these new ′3+1′ complexes, [MO(SSS)(SR)] (M = Re, **2**; M = ^{99m}Tc, **3**), in which the co-ligands consist of dipeptides coupled, *via* the corresponding terminal amino group, to a mercaptoacetyl spacer. The oxorhenium complexes **2** were synthesized for structural identification since rhenium and technetium display similar coordination chemistry. We also report biodistribution studies in mice, which clearly show how the nature of the monodentate co-ligand affects the biological behavior of the complexes.

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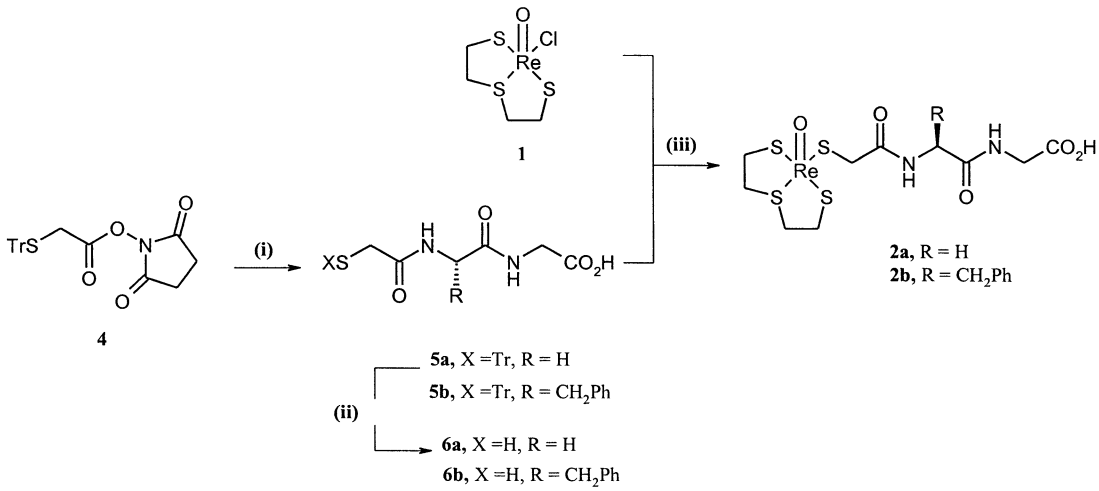


(i) glyglyOH or pheglyOH, H_2O, THF, $NaHCO_3$, r.t.; (ii) Et_3SiH, TFA, 0ºC; (iii) **6**, NEt_3, $MeCN/H_2O$ (1:1), reflux

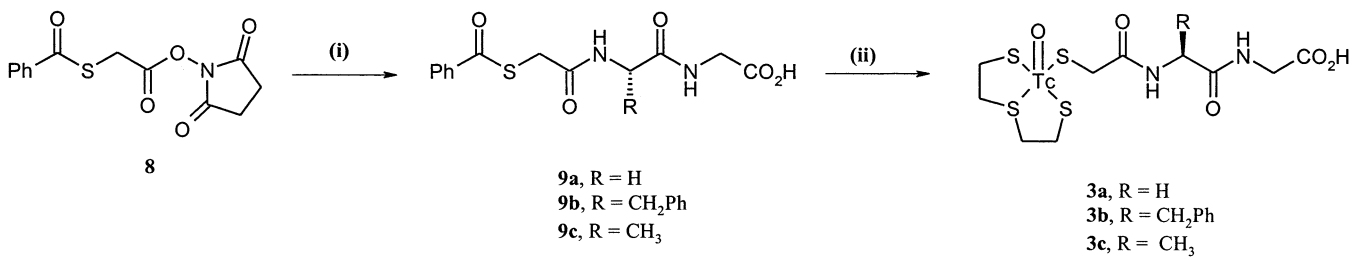
Scheme 1.

2. Results and discussion

2.1. Chemistry

As shown in Scheme 1, the synthetic route for the oxorhenium complexes **2** is based on the reaction of chloro(3-thiapentane-1,5-dithiolato)-oxorhenium(V) (ReO(SSS)(Cl), **1**, [3] with the monodentate ligands **6**. Coupling of succinimido 2-(triphenylmethyl)thioacetate **4** with glyglyOH or pheglyOH [2] in a molar ratio of 1:1 gave the corresponding triphenylmethylmercaptoacetyl dipeptides, **5a** and **5b**, respectively. Deprotection of **5a** and **5b** with triethylsilane in trifluoroacetic acid yielded the corresponding mercaptoacetyl dipeptides **6a** and **6b** (Scheme 1) [2]. By refluxing **6a-b** with [ReO(SSS)(Cl)], **1**, and triethylamine (molar ratio 1:1:1), in CH_3CN, the new complexes [ReO(SSS)(SR)], **2a** and **2b**, were obtained in relative high yield (80%). These reddish-brown complexes, which have been characterized by the normal techniques in inorganic chemistry, are air and moisture stable, very soluble in DMSO, soluble in basic aqueous solution, slightly soluble in MeOH and insoluble in chlorinated solvents.

The IR spectra of **2a** and **2b** exhibit Re=O stretching vibrations at 960 cm^{-1}. These values are in the normal range found for other Re monooxo-complexes (945-1067 cm^{-1}), [23] and are only slightly lowered in energy (by 8 cm^{-1}) relatively to **1**. The IR spectra of these compounds also exhibit several broad and intense bands in the range 1500-1730 cm^{-1}, which can be attributed to the amide and to the carboxylate C=O stretching vibrations of the monodentate co-ligand [14]. The ^{1}H-NMR spectra of **2a** and **2b** were run in DMSO-d_6 or in MeOD-d_4. In MeOD-d_4, the methylenic protons of the 3-thiapentane-1,5-dithiolato ligand appear as four signals of equal intensity integrating for two protons each. The pattern obtained for these signals indicates that all the protons are diastereotopic and is in agreement with the expected symmetry for **2a** and **2b**. In the ^{1}H-NMR spectrum of **2a** we also observed three resonances integrating for two protons each due to the methylenic protons of the monodentate co-ligand at 4.51, 3.91, and 3.76



(i) glyglyOH, alaglyOH or pheglyOH, H_2O, THF, NEt_3, 0ºC; (ii) a) ^{99m}Tc(V)O-tartrate, 100 ºC, b) H-SSS-H, Me_2CO, r.t.

Scheme 2.

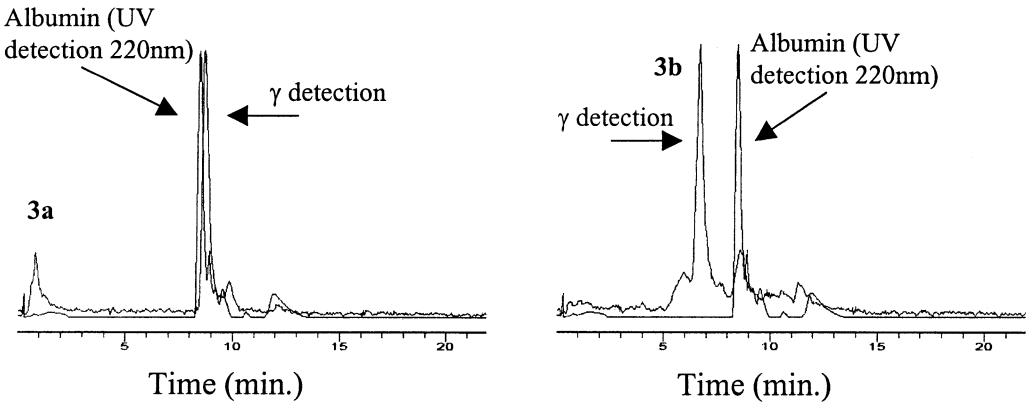


Fig. 1. HPLC analyses of complexes **3a** (left) and **3b** (right) after incubation with rat plasma 1 hour at 37°C. The plasmatic proteins were detected at 220 nm (R_t between 8 and 13 min.). Complex **3a** (R_t 0.9 min.) is bounded to plasmatic proteins (specially albumin) but 84% of complex **3b** (R_t 6.7 min.) remains intact in the same conditions.

ppm. These signals are all shifted down field relative to the signals in the free co-ligand **6a**. The amide protons of the ligand **6a** can only be detected when the spectra are run in a mixture of DMSO-d_6 and $CDCl_3$, appearing at 7.05 and at 7.15 ppm. In the ^{1}H-NMR spectrum of **2b** in DMSO-d_6 all the protons of the monodentate co-ligand are observed, including the two resonances due to the N-H protons of the amide groups, appearing at 8.15 and 8.36 ppm. The proton coordinated to the chiral carbon appears at 4.54 ppm, the two methylenic protons of the benzyl group are diastereotopic and appear at 2.99 and 2.78 ppm.

The mixed ^{99m}TcO-(SSS/SR) complexes, **3a–c**, were synthesized by a ligand exchange reaction using ^{99m}Tc(V)O-tartrate as the precursor and stannous chloride as the reducing agent (Scheme 2).

The *S*-benzoylprotected monodentate ligands, **9a–c**, prepared from **8**, [1] were used directly in the labeling reactions, to prevent thiol oxidation and to allow prolonged storage in a lyophilized kit [1,5]. The deprotection of the appropriate monodentate ligand, 9, was accomplished by heating the alkaline (pH = 9) reaction mixture at 100 °C for 15 min, before adding the tridentate 2-mercaptoethyl sulfide ligand dissolved in acetonitrile. To minimize the presence of free pertechnetate and hydrolyzed ^{99m}Tc species the amount of sodium tartrate and stannous chloride in the labeling mixture were optimized. However, we observed the formation of another radiochemical impurity (RT=6.5 min), which was minimized by decreasing the amount of the tridentate ligand. After extraction of the reaction mixture with CH_2Cl_2, complexes **3a–c** remain in the aqueous solution and were obtained with high radiochemical purity (>95%). The identity of **3a–c** were confirmed by comparison of their HPLC behavior with that of the analogous nonradioactive rhenium complexes (**3a**, RT=1.9 min; **3b**, RT=2.0 min;. **3c**, RT = 2.0 min).

The lipophilicity of the ^{99m}Tc-complexes was evaluated by determining the partition coefficients between 1-octanol and pH 7.4 buffer (log $D_{7.4}$). The log $D_{7.4}$ values for compounds **3a–c** are -2.3, -2.5 and -2.5, respectively, and contrast sharply with those of neutral ^{99m}Tc-′3+1′ complexes, which usually are higher than **1** [7]. The high hydrophilicity of complexes **3a-c** can be ascribed to the complete ionization, at pH 7.4, of the carboxylic acid group in the monodentate ligand.

2.2. Stability studies

Knowing from the literature that the ′3+1′ mixed-ligand complexes exchange quite easily the monodentate ligand with SH group-containing components, we studied the stability of **3a–c** in PBS and in rat plasma [19].

We found that **3a–c** complexes are stable for more than 4 hours in 0.1 M PBS pH 7.4 at 37°C (radiochemical purity by HPLC > 93%).

In freshly prepared rat plasma at 37°C the complexes present a quite remarkably different stability. Complex **3a** is the most unstable, **3b** is the most stable and **3c** presents an intermediate behavior. In Figure 1 are presented the results for the most (**3b**) and less stable (**3a**) compounds after 1hour incubation. As can be seen, complex **3a** is unstable in the presence of the plasmatic proteins, labeling especially albumin and less significantly the globulins (26% of **3a** remains unchanged, while 65% is bound to albumin). In contrast, 84% of **3b** remains unchanged and only 16% is bound to the proteins after 1 hour of incubation (RT in the range 8–13 min. UV detection at 220 nm) (Figure 1). Complex **3c** has an intermediate behavior, with 50% of the complex still remaining unchanged after 1 hour of incubation. Labeling of albumin has also been reported by Seifert *et al.* for other 3+1 complexes, which found that the nature of both tridentate and monodentate ligands influence the stability of complexes in plasma [19].

Table 1
Biodistribution of 3a-c in mice, at various times post-injection. The results are the average of at least three experiments

Organ	% ID/Organ											
	3a				**3b**				**3c**			
	2 min	15 min	30 min	60 min	2 min	15 min	30 min	60 min	2 min	15 min	30 min	60 min
Blood	54.7 ± 5.9	38.8 ± 6.0	37.0 ± 11.6	31.6 ± 16.2	34.8 ± 6.6	9.2 ± 2.2	5.0 ± 1.1	4.4 ± 0.9	34.4 ± 3.9	19.9 ± 3.2	12.4 ± 4.2	17.8 ± 4.2
Liver	15.9 ± 3.0	8.3 ± 1.4	6.4 ± 1.7	5.2 ± 1.3	28.2 ± 1.0	9.0 ± 2.3	1.9 ± 0.5	1.6 ± 0.6	23.6 ± 2.4	5.9 ± 1.0	5.5 ± 1.3	3.6 ± 0.3
Gall Bladder	0.4 ± 0.3	1.9 ± 0.8	0.6 ± 0.1	0.8 ± 0.3	2.0 ± 0.5	1.7 ± 0.7	3.9 ± 0.3	2.1 ± 2.1	0.7 ± 0.5	0.6 ± 0.2	1.3 ± 1.0	0.3 ± 0.0
Intestine	5.8 ± 0.6	18.6 ± 2.8	12.2 ± 1.2	16.3 ± 2.1	15.2 ± 6.2	54.5 ± 5.0	65.7 ± 5.9	69.3 ± 1.4	7.1 ± 0.2	37.1 ± 3.7	36.9 ± 8.2	49.3 ± 0.4
Heart	1.1 ± 0.2	0.7 ± 0.1	0.4 ± 0.0	0.7 ± 0.4	0.5 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0.1 ± 0.0	0.7 ± 0.0	0.4 ± 0.2	0.2 ± 0.1	0.3 ± 0.0
Lungs	3.1 ± 0.2	2.2 ± 0.2	1.4 ± 0.3	1.4 ± 0.3	2.1 ± 0.0	0.6 ± 0.2	0.8 ± 0.7	0.3 ± 0.1	1.7 ± 0.3	0.9 ± 0.0	0.9 ± 0.3	0.3 ± 0.0
Kidneys	12.5 ± 0.2	8.4 ± 1.8	4.4 ± 0.3	3.3 ± 0.1	6.8 ± 0.2	2.5 ± 0.5	1.3 ± 0.4	0.6 ± 0.1	15.0 ± 0.4	6.3 ± 1.5	3.8 ± 0.4	2.0 ± 0.6
Stomach	1.4 ± 0.5	1.6 ± 0.3	0.6 ± 0.2	1.0 ± 0.2	0.3 ± 0.2	1.3 ± 1.7	1.0 ± 0.6	0.3 ± 0.2	0.8 ± 0.0	1.3 ± 0.6	0.7 ± 0.4	0.3 ± 0.0
Brain	0.4 ± 0.2	0.5 ± 0.2	0.5 ± 0.1	0.4 ± 0.3	1.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.3 ± 0.0
Urine	0.0	9.8 ± 3.9	37.2 ± 4.0	42.2 ± 1.2	0.0	4.0 ± 1.2	8.9 ± 1.5	12.4 ± 2.9	0.0	13.3 ± 0.9	21.7 ± 2.3	23.7 ± 2.7

2.3. Biodistribution studies

Biodistribution studies of ^{99m}Tc-complexes **3a–c** were performed in normal female Charles River mice. The results are expressed as percent of injected dose per organ (%ID/organ) and as percent of injected dose per gram (%ID/g) at 2, 15, 30 and 60 min post-injection, and are shown in Tables 1 and 2.

Complex **3a**, which is derived from gly-gly, shows a reasonably high liver uptake (16% ID at 2 min post-injection), but is preferentially eliminated by the urinary tract, as revealed by the 42% ID in urine at 60 min post-injection. Moreover, this complex presents a low blood clearance, probably because the complex is bound to the blood proteins as found in the *in vitro* stability studies in rat plasma. In contrast, complex **3b**, which is derived from phe-gly, shows the highest blood clearance (from 35% ID at 2 min post-injection to 4% ID at 60 min post-injection) and initial liver uptake (28% ID at 2 min post-injection), and is rapidly eliminated by the hepatobiliary system. Quite remarkably, 15 min after injection of **3b**, 54.5% of the injected dose was found in the intestine, while 60 min after injection the value was 69.3% ID. The elimination of **3b** by the kidney is not significant, and 60 min post-injection only 12.4% ID was detected in the urine. Complex **3b** compares well with ^{99m}Tc-HIDA, [11] which is an hepatobiliary agent currently used in diagnostic Nuclear Medicine. For this commercially available radiopharmaceutical, 68.3% ID is found in the intestine 60 min post-injection [11]. Complex **3c** has no preferential elimination being excreted by the hepatobiliary system (49.3%ID, 60 min post-injection) and by the kidney (23.7%ID in the urine, 60 min post-injection).

It has been suggested that a structural requirement that a ^{99m}Tc-labeled radiopharmaceutical must fulfill in order to be excreted through the hepatobiliary system includes a molecular weight above 300, which contributes to reduced urinary excretion [4]. Indeed, all ^{99m}Tc-complexes **3a–c** have a molecular weight well above 300 (471.5, 561.6 and 485.5, respectively), but only complex **3b** presents high specificity for biliary excretion as well as a high rate of biliary excretion [22]. The larges differences observed in the biological properties of compounds **3a-c** might be related with different reactivities towards plasma proteins. A planar imaging study in mice with the ^{99m}Tc-complex was also carried out. The ^{99m}Tc-complex **3b** shows biological characteristics suitable for imaging of the hepatobiliary system (Figure 2). As can be seen, 60 min post-injection the scintigraphic image of the mice shows clearly the gall bladder

Table 2
Biodistribution of 3a-c in mice, at various times post-injection. The results are the average of at least three experiments

Organ	% ID/gOrgan											
	3a				**3b**				**3c**			
	2 min	15 min	30 min	60 min	2 min	15 min	30 min	60 min	2 min	15 min	30 min	60 min
Blood	27.4 ± 2.3	18.7 ± 2.3	19.3 ± 4.8	15.9 ± 8.4	18.5 ± 4.4	4.1 ± 1.0	3.7 ± 1.1	2.7 ± 0.7	18.3 ± 3.0	10.7 ± 1.4	7.0 ± 2.1	9.6 ± 7.8
Liver	9.5 ± 2.2	4.5 ± 0.8	3.7 ± 0.2	3.0 ± 0.5	17.0 ± 2.7	5.4 ± 1.6	1.4 ± 0.2	1.1 ± 0.4	15.0 ± 1.1	3.7 ± 0.5	3.4 ± 0.8	1.9 ± 0.2
Intestine	1.8 ± 0.2	5.3 ± 0.8	3.9 ± 0.6	5.8 ± 1.1	4.8 ± 1.9	15.3 ± 1.1	26.2 ± 1.4	27.2 ± 2.2	2.4 ± 0.1	12.2 ± 2.0	13.1 ± 1.6	15.7 ± 0.3
Heart	7.8 ± 0.4	4.1 ± 0.6	3.6 ± 0.4	4.7 ± 2.7	3.1 ± 1.31	0.8 ± 0.1	2.5 ± 1.4	1.0 ± 0.5	4.2 ± 1.0	3.1 ± 1.5	1.8 ± 0.8	1.7 ± 0.6
Lungs	10.8 ± 0.5	6.6 ± 0.8	5.4 ± 1.8	4.1 ± 0.5	5.6 ± 0.7	1.8 ± 0.7	3.5 ± 2.8	1.0 ± 0.3	5.4 ± 0.4	2.9 ± 0.2	3.3 ± 1.3	0.9 ± 0.2
Kidneys	26.8 ± 0.4	17.5 ± 2.8	10.2 ± 0.8	7.4 ± 0.5	15.1 ± 1.7	5.3 ± 1.4	3.3 ± 0.8	1.4 ± 0.1	32.6 ± 2.2	15.4 ± 2.4	8.6 ± 1.4	4.4 ± 1.6
Stomach	1.6 ± 0.5	2.1 ± 0.5	1.1 ± 0.4	2.3 ± 0.5	0.3 ± 0.2	1.9 ± 2.8	2.0 ± 0.9	0.8 ± 0.6	0.7 ± 0.0	1.8 ± 1.2	0.9 ± 0.4	0.3 ± 0.1
Brain	2.0 ± 0.2	1.3 ± 0.6	1.2 ± 0.5	0.9 ± 0.7	3.4 ± 0.5	0.2 ± 0.0	0.4 ± 0.2	0.2 ± 0.1	0.9 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	0.7 ± 0.1

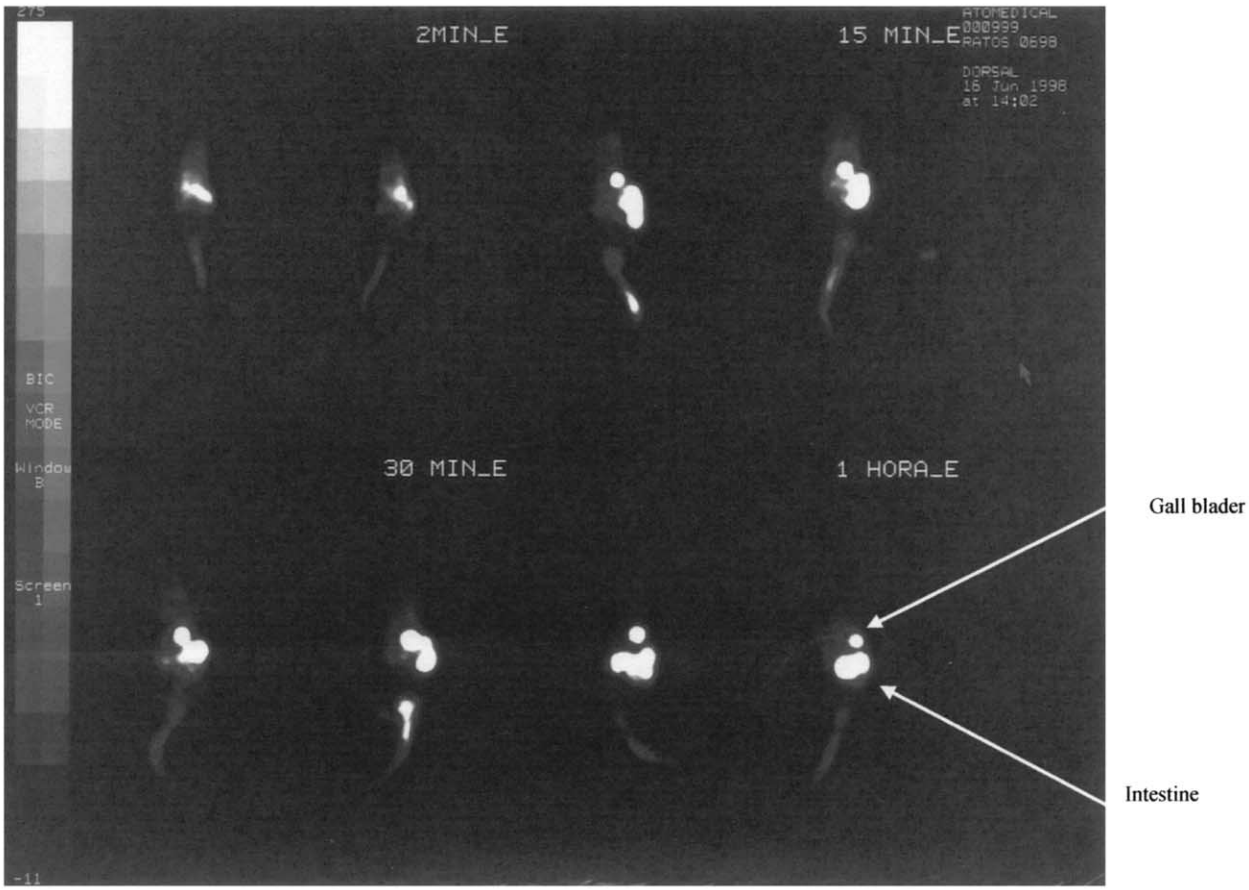


Fig. 2. Scintigraphic images in mice at 2, 15, 30 and 60 min. pos-injection of **3b**.

and the intestine. No specific uptake in different organs and tissue was found.

3. Conclusions

The ′3+1′ mixed-ligand approach has been useful for preparing ^{99m}Tc-dipeptide radiopharmaceuticals containing the TcO^{3+} core, with high radiochemical purity. These ′3+1′ mixed ligand complexes present the general structure [^{99m}Tc O(SSS)(SR)], in which the co-ligands are the dipeptides coupled, *via* the corresponding terminal amino group, to a mercaptoacetyl spacer. *In vitro* studies in rat plasma have shown that the stability of this type of complexes depends very much on the nature of the monodentate ligand, which also affects the *in vivo* behavior, as shown by the biodistribution studies. The blood clearance changes in the order **3a**<**3c**<**3b**, which seems to be in agreement with the protein binding found *in vitro*. All the complexes are quickly eliminated by the hepatobiliary or urinary tract. Complex **3a** is preferentially eliminated by the urinary tract and complex **3b** is extracted by liver (28% ID at 2 min post-injection) and excreted into bile at 30 min post-injection (66% ID). Compound **3b** might be considered useful for peptide labeling.

4. Experimental section

Chemicals and solvents were of reagent grade and were used without further purification, unless otherwise indicated. $^{99m}TcO_4^-$ was eluated from a $^{99}Mo/^{99m}Tc$ generator (Amersham). [ReO(SSS)Cl], succinimido 2-((Triphenylmethyl)thio)acetate and Succinimidyl S-benzoylthioglycolate were prepared according to literature methods [3,2,1].

Elemental analyses were performed on a Perkin-Elmer 240 or on an automatic EA110 CE Instruments automatic analyser. Melting points were obtained with a Gallenkamp apparatus and are uncorrected. IR spectra were recorded as KBr pellets on a Nicolet 5DXC spectrometer. ^{1}H-NMR spectra were recorded on a Varian Unity 300 MHz spectrometer. Proton chemical shifts, δ_H, were referenced with the residual solvent resonances relative to tetramethylsilane.

TLC and HPLC analyses were used to determine the radiochemical purity and stability of the preparations. TLC analyses were performed using silica gel plates (Merck 60 F254) developed with methylethylketone (method 1), saline (method 2) or Butanol/methanol/water/ammonium (60/20/20/1) (method 3). HPLC analysis was carried out on a Shimadzu C-R4A chromatography system coupled to a Berthold-LB 505 γ- detector and to a UV Shimadzu SPD-

10AV detector. Separations were achieved on a Perkin-Elmer C-18, RP-HPLC, 10 μm, 3.9 x 300 mm column, and the eluent was an isocratic mixture of acetonitrile/TFA (in H_2O) 0.1%, 60/40, at a flow rate of 1.0 mL/min.

(Triphenylmethyl)mercaptoacetylglycylglycine (**5a**). To a solution of active ester **4**, 2-succinimido 2-(triphenylmethyl)thioacetate (431 mg,1 mmol), in THF (15 ml) was added a solution of glycylglycine (glyglyOH) (132.2 mg, 1 mmol) and $NaHCO_3$ (168 mg, 2 mmol) in water (3 ml). After stirring at room temperature for 2h, the THF was removed and 3 mL of 50% aqueous citric acid were added. The oil obtained was extracted with ethyl acetate yielding, after drying, a white solid (78%, 420 mg). Anal. Calcd (found) for $C_{25}H_{24}N_2O_4S$: C, 66.95 (66.30); H, 5.39 (6.45); N, 6.25 (5.95), S, 7.15 (6.45). IR ν_{max}: 3360; 3310, 1720, 1650, 1540, 1440 cm^{-1}. ^1H NMR- (300 MHz, DMSO-d_6-$CDCl_3$): δd_H 2,89 (2H, d, COCH_2S), 3,62 (2H, d, NCH_2CO), 3,75 (2H, d, NCH_2COOH), 7,11-7,34 (m, 15H, aryl), 7,67 (1H, t, -N-*H*), 7,88 (1H, -N-*H*).

(Triphenylmethyl)mercaptoacetylphenylalanylglycine (**5b**). This compound has been synthesized as above described for **5a**, using 862 mg (2 mmol) of **4**, 444.4 mg (2mmol) of phegly and 336 mg (4 mmol) of $NaHCO_3$. Yield, 89%, 956 mg. Anal. Calcd (found) for $C_{32}H_{30}N_2O_4S$: C, 71.37 (70.61); H, 5.58 (6.13); N, 5.20 (4.22). ^1H NMR- (300 MHz, $CDCl_3$): δd_H 2,88 (2H, d, CH_2), 3,03 (2H, d, CH_2), 3,79-3.94 (2H, m, CH_2), 4,46-4.49 (m, 1H, -C*H*), 6.39 (1H, t, -N-*H*(Gly)), 6.68 (1H, -N-*H*(Phe)).

Mercaptoacetyl-glycylglycine (**6a**) and Mercaptoacetyl-phenylglycine (**6b**). These two compounds were obtained by deprotection of **5a** and **5b** using trifluoracetic acid and triethyl silane. The reactions were run at 0°C, yielding white precipitates. These precipitates were dissolved in a mixture of hexane/water (1:1). The aqueous phase was separated, washed several times with hexane, filtered through celite and vacuum dried, leading to a colorless oil, which was recrystallized from 2-propanol.

Mercaptoacetyl-glycylglycine (**6a**) Yield 91% (126mg). Mp: 131-133 °C. Anal. Calcd (found) for $C_6H_{10}N_2O_4S$: C, 34.95 (34.35); H, 4.89 (5.45); N, 13.58 (13.22);. IR ν_{max}: 3330, 3280, 1725, 1630, 1210 cm^{-1}. ^1H NMR- (300 MHz, DMSO-d_6): δd_H 2,77(1H, t, S*H*), 3,33(2H, d, CH_2-COOH) 3.49(2H, s, CH_2-SH), 3.73 (2H, d,NH-C)H$_{2),\ 8.19(1H,\ t,\ N}$*H*) 8.28(1H, t, N*H*), 12.57(1H, sl, COO*H*).

Mercaptoacetyl-phenylglycine (**6b**). Mp: 149-150 °C. Anal. Calcd (found) for $C_{13}H_{16}N_2O_4S$: C, 52.70 (52.35); H, 5.44 (6.41); N, 9.45 (9.22); S, 10.82 (10.91). ^1H NMR- (300 MHz, DMSO-d_6): δd_H 2,74 (1H, dd, J = 10,0; 14,0 Hz, -CH_2-Ph), 3,05 (1H, dd, J = 4,5; 14,0 Hz, -CH_2-Ph), 3,42 (2H, d, J = 5,9 Hz, -NH-CH_2), 3.76 (2H, s, HS-CH_2 -), 4.35 (t, 1H, S*H*) 4,54 (m, 1H, -C*H*), 7,20-7,25 (m, 5H, Ar*H*-Phe), 8,26 (1H, d, J = 6,0 Hz, - N-*H*(Gly)), 8,43 (1H, t, J = 8,4 Hz, -N-*H*(Phe)), 12,59 (sl, 1H, -COO*H*).

*Synthesis of (mercaptocetylglycylglycine) (3-thiapentane-1,5-dithiolato)oxorhenium [ReO(SSS)(S-GlyGly)] (**2a**).* To a solution of [ReO(SSS)Cl] (52.8 mg, 0.135 mmol) in acetonitrile (7mL) a solution of **6a** (40 mg, 0.135 mmol) and triethylamine (0.135 mmol) in a mixture acetonitrile/water (1:1) was added and stirred while refluxing. After one hour, the reddish-brown solution was vacuum dried, the solid obtained washed several times with water and vacuum dried, yielding a reddish-brown solid formulated as **2a**.

(**2a**):Yield, 80%, (60 mg). IR ν (Re=O): 960 cm^{-1}. ^1H NMR- (300 MHz, CD_3OD): δd_H 2.19 (m, 2H, SCH_2), 3.10 (m, 2H, SCH_2), 3.77 (s, 2H, NHCH_2COOH), 3.9 (s, 2H, NHCH_2CO), 4.04 (dd, 2H, SCH_2), 4.30 (dd, 2H, SCH_2), 4.51 (s, 2H, SCH_2CO).

*Synthesis of (mercaptocetylphenylalanylglycine) (3-thiapentane-1,5-dithiolato)oxorhenium [ReO(SSS)(S-PheGly)] (**2b**).* This compound has been synthesized as above described for **2a**. Using 52.8 mg (0.135 mmol) of [ReO(SSS)Cl], and 40 mg (0.135 mmol) of **6b** and 0.135 mmol of triethylamine, 70 mg of compound **2b** were obtained.

(**2b**): Yield 80% (70 mg), Anal. Calcd (found) $C_{17}H_{23}N_2O_5S_4Re$: C, 31.42 (31.49); H, 3.57 (5.40); N, 4.31 (3.78); S, 19.73 (20.0). IR ν (Re=O): 960 cm^{-1}. ^1H NMR- (300 MHz, DMSO): δd_H 2.24 (m,2H,SCH_2), 2.78 (m, 1H,CH_2-Ph), 2.99 (m, 1H,CH_2-Ph), 3.07 (m,2H,SCH_2), 3.75 (d,2H,NHCH_2COOH, J= 5.7Hz), 4.07 (m,2H,SCH_2), 4.18 (d,2H, SCH_2CO, J= 9.9Hz), 4.28 (m,2H,SCH_2), 4.54 (m, 1H, C*H*CO), 7.24 (m, 5H, Ar-*H*), 8.15 (d,1H,N*H*CH$_2$, J= 8.4Hz), 8.36 (t,1H,N*H*CH, J= 6Hz).

*Synthesis of N-[(S-Benzoylmercapto)acetyl]glycylglycine (**9a**), N-[(S-Benzoylmercapto)acetyl]alanylglycine (**9c**) and N-[(S-Benzoylmercapto)acetyl]phenylalanylglycine (**9b**).* A solution of the dipeptide in water (glycylglycine, alanylglycine or phenylalanylglycine: 1 mmol in 1 mL of water) and 2 mmol of triethylamine were added to a cooled solution (0 °C) of succinimidyl S-benzoylthioglycolate (1 mmol in 5 mL of THF) [8]. After 30 min, under stirring, the THF was evaporated and HCl (1N) added. The precipitate formed was filtered, washed with water and dried, leading to white solids, which were formulated as **9a**, **9b** and **9c**.

*N[(S-Benzoylmercapto)acetyl]glycylglycine (**9a**): Yield, 78,1%, 242 mg.* Mp:191-192 °C. Anal. Calcd (found) for $C_{13}H_{14}N_2O_5S$: C, 50.32 (50,54); H, 4.52 (4,74); N, 9.03 (8,89). IR ν_{max}: 3380; 1740; 1671; 1621 cm^{-1}. ^1H NMR - (300 MHz, DMSO-d_6): δd_H 3,76 (2H, d, J = 5,9 Hz, -NH-CH_2), 3,78 (2H, d, J = 5,9 Hz, -NH-CH_2), 3,89 (2H, s, -S-CH_2-CO-), 7,55-7,95 (5H, m, Ar*H*), 8,23 (1H, t, J = 5,9 Hz, -N-*H*), 8,53 (1H, t, J = 5,9 Hz, -N-*H*).

*N-[(S-Benzoylmercapto)acetyl]alanylglycine (**9c**).* Yield, 87.0%, 282 mg. Mp: 134-135 °C. Anal. Calcd (found) for $C_{14}H_{16}N_2O_5S$: C, 51.85 (51.63); H, 4.94 (5.14); N, 8.64 (8.41). IR ν_{max}: 3380; 1740; 1671; 1621 cm^{-1}. ^1H NMR- (300 MHz, DMSO-d_6): δd_H (ppm) 1,25 (3H, d, J = 7,2 Hz, -CH-CH_3), 3,77 (2H, d, J = 6,0 Hz, -NH-CH_2), 3,88 (2H, s, -S-CH_2-CO-), 4,34 (1H, m, -C*H*), 7,55-7,96 (m, 5H, Ar*H*), 8,27 (1H, t, J = 6,0 Hz, -N-*H*(Gly)), 8,51 (1H, d, J = 7,7 Hz, -N-*H*(Ala)), 12,49 (1H, sl, -COO*H*).

*N-[(S-Benzoylmercapto)acetyl]phenylalanylglycine (**9b**).*

Yield, 67.5%, 135 mg. Mp: 141-143 °C. Anal. Calcd (found) for $C_{20}H_{20}N_2O_5S$: C, 60.00 (60.38); H, 5.00 (5.20); N, 7.00 (6.61). IR ν_{max}: 3380; 1740; 1671; 1621 cm^{-1}. ^1H NMR- (300 MHz, DMSO-d_6): δd_H 2,82 (1H, dd, J = 10,0; 14,0 Hz, -CH_2-Ph), 3,09 (1H, dd, J = 4,5; 14,0 Hz, -CH_2-Ph), 3,79 (2H, d, J = 5,9 Hz, -NH-CH_2), 3,90 (2H, s, -S-CH_2-CO-), 4,34 (m, 1H, -C*H*), 7,20-7,25 (m, 5H, Ar*H*-Phe), 7,55-7,95 (m, 5H, Ar*H*), 8,45 (1H, t, J = 6,0 Hz, -N-*H*(Gly)), 8,50 (1H, d, J = 8,4 Hz, -N-*H*(Phe)), 12,49 (s, 1H, -COO*H*).

*4.1. Synthesis of ^{99m}Tc complexes **3a-c***

150 μL of sodium tartrate in nitrogen purged buffer, (pH= 9.0; 0,5 M sodium bicarbonate, 0,25 M ammonium acetate, 0,18 M ammonium hydroxide) (50 mg/mL), 50 μL stannous chloride solution (1mg/mL 0,1 N HCl) and 500 μL of $^{99m}TcO_4^-$ (37–200 MBq) were reacted. The ^{99m}TcO(V) tartrate solution was added to the functionalized dipeptide (1.0 mg –1.3 mg; 3.2 $\times$ 10^{-3} mmole) and the mixture heated at 100 °C for 15 minutes. The tridentate ligand (HSSSH) diluted in acetone was added (50μL, 4,0 $\times$ 10^{-4} mmoles). The mixture reacted at room temperature for 15 minutes. The complexes were purified by extraction with CH_2Cl_2 (1.5 mL) to remove by-products and the excess of tridentate ligand. The characterization of ^{99m}Tc complexes was based on HPLC by comparison with the corresponding Re complexes.

4.2. Stability in rat plasma

To 500 μL of fresh rat plasma, 50 μL of the ^{99m}Tc complex solution was added and the mixture incubated at 37°C in a thermoshaker. At appropriate periods of time (5, 30 min, 1 and 2 h) the mixture was analyzed by HPLC in a Supelguard column (20 x 4.6 mm, 10 μm, flow rate 1.0 mL/min) with a linear gradient 95-40% A in 15 min., hold 5 min., then to 95% A in 2 min. [A: isopropanol/0.1% TFA (10/90), B: isopropanol/0.1% TFA (90/10)]. The proteins were detected by UV at 220 nm (R_t values between 8 and 13 min.) and the complexes were γ detected (**3a**, Rt=0.9 min; **3b**, Rt=6.7 min; **3c**, Rt=1.5 min).

4.3. Biodistribution studies

The animal studies were carried out in compliance with the Portuguese laws. Biodistribution studies were made in normal female Charles River mice. Groups of 4 animals with 30-35 days were intravenously injected with 50 μL of a saline solution containing the ^{99m}Tc complexes (3-5 MBq), *via* the tail vein. The radioactive dose injected into each mouse was measured in a dose calibrator. The animals were sacrificed by cervical dislocation at 2, 15, 30 and 60 min post-injection. The relevant organs were dissected, weighed and counted. The results were expressed as percentage of injected dose per organ (%ID/organ). For blood, the activity was calculated assuming 7% of the total weight body. Whole body gamma camera images at the same times post-injection were also acquired.

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References

[1] Brandau W, Bubeck B, Eisenhut M, Taylor DM. Technetium-99m Labeled Renal Function and Imaging Agents: III. Synthesis of ^{99m}Tc-MAG_3 and Biodistribution of By-products. Int J Appl Rad Isot 1988; 39:121–9.

[2] Brenner D, Davison A, Lister-James J, Jones AG. Synthesis and characterization of a series of isomeric oxotechnetium(v) diamido dithiolates. Inorg Chem 1984;23:3793–7.

[3] Fietz T, Spies H, Pietzch H-J, Leibnitz P. Synthesis and molecular structure of chloro(3-thiapentane-1,5-dithiolato)oxorhenium(V). Inorg Chim Acta 1995;231:233–6.

[4] Freeman LM, Lan JA. Radiopharmaceutical evaluation of the hepatobiliary pathway. Nucl Med Biol 1990;17:129–39.

[5] Fritzberg AR, Kasina S, Eshima D, Johnson DL. Synthesis and biological evaluation of technetium-99m MAG_3 as a Hippuran Replacement. J Nucl Med 1986;27:111–6.

[6] Hom RK, Katzenellenbogen JA. Technetium-99m-labeled receptor-specific small-molecule radiopharmaceuticals: recent developments and encouraging results. Nucl Med Biol 1997;24:485–98.

[7] Johannsen B, Pietzsch H-J. Development of Technetium-99m-based CNS receptor ligands: have there been any advances? Eur J Nucl Med 2002;29:263–75.

[8] Johannsen B, Scheunemann M, Spies H, Brust P, Wober J, Syhre R, Pietzch H-J. Technetium(V) and Rhenium(V) complexes for 5-HT_{2A} serotonin receptor binding: structure-affinity considerations. Nucl Med Biol 1996;23:429–38.

[9] Jurisson SS, Lydon JD. Potential technetium small molecule radiopharmaceuticals. Chem Rev 1999;99:2205–18.

[10] Liu S, Edwards DS. ^{99m}Tc-labeled small peptides as diagnostic radiopharmaceuticals. Chem Rev 1999;99:2235–68.

[11] Loberg MD, Fields A. Chemical structure of technetium-99m-labeled N-(2,6-dimethylphenylcarbamoylmethyl)-iminodiacetic acid (Tc-HIDA). Int J Appl Rad Isot 1978;29:167–73.

[12] Mastrostamis SG, Papadopoulos MS, Pirmettis IC, Paschali E, Varvarigou AD, Stassinopoulou CI, Raptopoulou CP, Terzis A, Chiotellis E. Tridentate ligands containing the SNS donor atom set as a novel backbone for the development of technetium brain-imaging agents. J Med Chem 1994;37:3212–8.

[13] Meegalla S, Plossl K, Kung M-P, Stevenson DA. First example of a ^{99m}Tc complex as a dopamine transporter imaging agent. J Am Chem Soc 1995;117:11037–8.

[14] Nakamoto K. Infrared spectra of inorganic and coordination compounds, 2nd ed. New York: Wiley-Interscience, 1970, Ch. 3-9.

[15] Nunn AD. Radiopharmaceuticals. Chemistry and pharmacology. New York: Marcel Dekker, Inc, 1992.

[16] Pietzch H-J, Spies H, Hoffmann S. Lipophilic technetium complexes VI. Neutral oxotechnetium(V) complexes with monothiole/tridentate dithiole coordination. Inorg Chim Acta 1989;165:163–6.

[17] Pirmettis IC, Papadopoulos MS, Chiotellis E. Novel ^{99m}Tc aminobisthiolato/monothiolato "3 + 1" mixed ligand complexes: structure-activity relationships and preliminary in vivo validation as brain blood flow imaging agents. J Med Chem 1997;40:2539–49.

[18] Pirmettis IC, Patsis G, Pelecanou M, Tsoukalas C, Papadopoulos A, Raptopoulou CP, Terzis A, Papadopoulos M, Chiotellis E. Synthesis of oxorhenium(V) and oxotechnetium(V) [SN(R)S/S] mixed ligand complexes containing a phenothiazine moiety on the tridentate SN(R)S ligand. Bioorg Med Chem Lett 2001;11:1859–62.

[19] Seifert S, Gupta A, Syhre R, Spies H, Johannsen B. Ligand-exchange reaction of labile "3+1" ^{99m}Tc(V) complexes with SH group-containing proteins. Appl Radiat Isot 2001;54:637–44.

[20] Spies H, Fietz T, Pietzch H-J, Johannsen B, Leibnitz P, Reck G, Scheller D, Klostermann K. Neutral oxorhenium(V) complexes with tridentate dithiolates and monodentate alkane- or arene-thiolate coligands. J Chem Soc Dalton Trans 1995:2277–80.

[21] Spies H, Pietzch HJ, Johannsen B. The "n + 1" mixed-ligand approach in the design of specific technetium radiopharmaceuticals: potencials and problems. in: technetium, rhenium and other metals in chemistry and nuclear medicine, Padova, 1999; Vol. 5, pp. 101-108.

[22] Ullrich KJ. Membrane Transporters as Drug Targets. Amidon GL, Sadée W, eds. Kluwer Academic/Plenum, New York, 1999, p. 159.

[23] Nugent WA, Mayer JM. Metal-ligand multiple bonds. New York: Wiley-Interscience, Wiley & Sons, 1998.