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SCIENCE DIRECT
Tetrahedron Letters
Tetrahedron Letters 47 (2006) 4641–4645

Structure–biodistribution relation of neutral $^{99m}Tc(CO)_3$-complexes with tridentate N-substituted derivatives of aminoethylglycine and phenylenediamine

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Received 28 March 2006; revised 21 April 2006; accepted 26 April 2006

Abstract—Derivatives of ethylenediamine-*N*-acetic acid (EDAA = *N*-aminoethylglycine = AEG) and *ortho*-phenylenediamine-*N*-acetic acid (PDAA) with uncharged substituents on one or both of the amines form neutral complexes with a $[^{99m}Tc(CO)_3]^+$-moiety. We studied the influence of different modifications at the amines (e.g., with methyl, ethyl, butyl or benzyl groups) on the behaviour of the $^{99m}Tc(CO)_3$-complexes in vivo in mice, with special focus on blood–brain barrier (BBB) passage. The complexes have been characterised by reversed phase HPLC, log *P*, electrophoresis and some of them also by LC–MS. Log *P* values of the ^{99m}Tc–tricarbonyl complexes varied from −0.52 (AEG) to 2.5 (*N*,*N*′-dibenzyl-EDAA). With increasing lipophilicity, more of the activity was found in liver and intestines as compared to kidneys and urine for the more polar complexes. Brain uptake was found for the $^{99m}Tc(CO)_3$-complexes with *N*,*N*′-dibutyl-ethylenediamine-*N*-acetic acid (0.34% of I.D. after 2 min) and *ortho*-phenylenediamine-*N*-acetic acid (0.22% of I.D. after 2 min).
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Technetium–tricarbonyl complexes with a *fac*-$[^{99m}Tc(CO)_3]$-moiety have been the focus of interest since Alberto et al. developed a convenient low pressure synthesis in aqueous media for the precursor *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$.[1,2] A recently developed kit for preparation of this precursor complex (IsoLink™, Tyco-Mallinckrodt, Petten, The Netherlands), which contains the solid sodium boranocarbonate as both source of CO and reducing agent, made its use even more attractive.[3] Major characteristics of this Tc–tricarbonyl complex are the presence of three stable CO-groups and three labile water molecules. The latter can be replaced by a variety of donating ligands.[4] Especially tridentate amine containing ligands form complexes of high stability when labelled with a $[^{99m}Tc(CO)_3]^+$-moiety. Depending on the type of ligand and heteroatoms, the resulting complexes can be neutral (e.g., with ligands of N–N–O type such as aminoethylglycine), positively charged (e.g., with ligands of N–N–N type such as diethylenetriamine) or negatively charged (e.g., with ligands of O–N–O type such as iminodiacetic acid).[5–7] In the current study, we have investigated derivatives of ethylenediamine-*N*-acetic acid (EDAA) and *ortho*-phenylenediamine-*N*-acetic acid (PDAA), which form neutral complexes with the $[^{99m}Tc(CO)_3]^+$-moiety. The influence of uncharged substituents on one or both of the amines on the biodistribution of the resulting ^{99m}Tc–tricarbonyl complexes in mice was investigated, with a special focus on the ability of the ^{99m}Tc-tracers to pass the blood–brain barrier (BBB). The clinical usefulness of, for example, ^{99m}Tc-TRODAT-1 for diagnosis of Parkinson's disease has shown that there is still a place for new ^{99m}Tc-based radiopharmaceuticals, especially in the field of neuroreceptor-targeting molecules.[8,9] The availability of ^{99m}Tc–tricarbonyl complexes with significant brain uptake could result in a new generation of ^{99m}Tc-labelled agents for diagnosis and/or follow up of neurological diseases.

The model compound AEG offers a primary amine, a secondary amine and a carboxylic acid group for

Keywords: Technetium; Tricarbonyl; Imaging agents; Biodistribution; Brain uptake; LC–MS.
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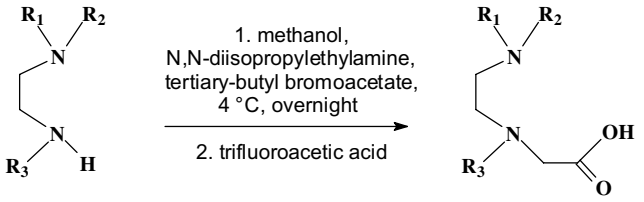
doi:10.1016/j.tetlet.2006.04.136



Scheme 1. Preparation of *N*-alkyl substituted derivatives of aminoethylglycine. N-alkylated derivatives of ethylenediamine reacted with *tert*-butyl bromoacetate to tridentate N–N–O type ligands. Deprotection of the resulting tertiary butyl esters was achieved with trifluoroacetic acid.

complex formation with the $[^{99m}Tc(CO)_3]^+$-moiety, resulting in the neutral complex $[^{99m}Tc(CO)_3(AEG)]$. Previous studies have shown excellent complex forming properties of AEG as tridentate ligand, but no brain uptake of the formed ^{99m}Tc-complex.[10] A log*P* value of −0.52 further characterised the complex as probably too hydrophilic to cross the blood brain barrier.

In the present study, we have investigated the preparation and biological characteristics of a series of more lipophilic derivatives of $[^{99m}Tc(CO)_3(AEG)]$, modified with respect to AEG by a gradually increasing number of methyl, ethyl or benzyl substituents on one or both of the amines. In addition, two derivatives of phenylenediamine have been included. The ligands were prepared by reacting the bidentate N-alkylated starting materials *N*,*N*-dimethyl-ethylenediamine, *N*,*N*,*N*′-trimethyl-ethylenediamine, *N*,*N*-diethyl-ethylenediamine, *N*,*N*′-dibutyl-ethylenediamine and *N*,*N*′-dibenzyl-ethylenediamine (starting materials commercially available from Fluka or Aldrich) with *tert*-butyl protected bromoacetic acid,[†] to introduce a single acetic acid substituent at one of the amines (Scheme 1). In the same way we modified *ortho*-phenylenediamine and *N*-methyl-*ortho*-phenylenediamine.

The reaction products with only one acetic acid group substituent were isolated by HPLC using a mobile phase containing 0.1% trifluoroacetic acid. This concentration of trifluoroacetic acid was sufficient for a complete removal of the tertiary butyl protective group after incubation at room temperature for 10 min (see footnote[†]). Figure 1 shows the structures of the obtained tridentate N–N–O type derivatives of ethylenediamine-*N*-acetic acid (EDAA) and *o*-phenylenediamine-*N*-acetic acid

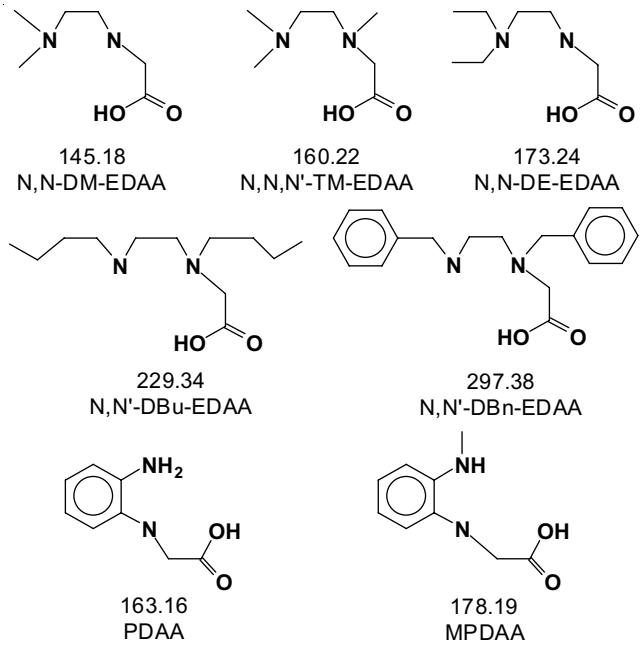


Figure 1. Tridentate N–N–O type ligands for labelling with $[^{99m}Tc(CO)_3]^+$and their molecular masses (EDAA = ethylenediamine-*N*-acetic acid; PDAA = *ortho*-phenylenediamine-*N*-acetic acid).

(PDAA) after deprotection. The identity of the deprotected ligands was confirmed by high-resolution mass spectrometry.

The Tc–tricarbonyl precursor $[^{99m}Tc(CO)_3(H_2O)_3]^+$ was prepared by addition of a solution of $[^{99m}TcO_4]^-$ (eluate from a commercially available Ultratechnekow FM ^{99m}Tc-generator; Tyco-Mallinckrodt, Petten, The Netherlands; activities up to 1850 MBq in 1 ml saline) to a vial containing a mixture of $NaBH_4$ (25 mg), Na_2CO_3 (4.5 mg) and K–Na–tartrate (20 mg) under an atmosphere of CO and heating for 20 min at 70 °C.[1] For labelling of the ligands, 0.3 ml of the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ solution was added to a solution of the HPLC-isolated ligands, the mixture was then adjusted to pH 10 and heated at 70 °C for 10 min. The reaction mixtures were then analysed by RP-HPLC. All tested aminoethylglycine derivatives afforded a main radiolabelled reaction product in high yield (80–98%), although *N*,*N*′-dibutyl-EDAA and *N*,*N*′-dibenzyl-EDAA showed lower labelling yields than the other ligands. Labelling of PDAA with a ^{99m}Tc–tricarbonyl moiety was also realised with an excellent labelling yield (>90%), while the additional alkyl group in MPDAA significantly decreased the yield (30–40%). For some of the ligands the formation of diastereomers was observed, namely for *N*,*N*′-dibutyl-EDAA, *N*,*N*′-dibenzyl-EDAA and MPDAA. For each of these ligands, the diastereomer eluting last during HPLC analysis on a reversed phase column was formed in a predominant way (70–90%). For example, $[^{99m}Tc(CO)_3(DBu)]$ showed two peaks on HPLC (t_R = 18.57 min and t_R = 19.08 min) in a relative ratio of 30:70. LC–MS measurements confirmed that both labelled compounds had the same mass, corresponding to the assumed $^{99m}Tc(CO)_3$-complex. Biodis-

[†] Typical procedure: equimolar amounts (1 mmol) of *N*,*N*-diisopropyl-*N*-ethylamine (DIEA), *tert*-butyl-bromoacetate and an ethylenediamine derivative, for example, *N*,*N*,*N*′-trimethyl-ethylenediamine, were dissolved in methanol (3 ml) and kept at 4 °C overnight. The resulting protected ethylenediamine-*N*-acetic acid derivatives were isolated by HPLC (X-Terra RP-18 column; 4.6 mm × 250 mm; Waters, Brussels, Belgium; gradient elution from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile in 20 min; flow rate 1 ml/min). Incubation of the collected HPLC-eluate fractures containing the desired product for 10 min at room temperature afforded the fully deprotected ligands under these conditions (by the trifluoroacetic acid in the mobile phase).

tribution experiments have always been performed with the predominant compound. To elucidate which conformation was present in the predominant isomer, crystallisation of the corresponding rhenium complex followed by X-ray crystallography would be necessary, but this was not part of the study.

All ^{99m}Tc-complexes were isolated by HPLC and analysed for charge by paper electrophoresis and for lipophilicity by log *P* determination. Paper electrophoresis of the ^{99m}Tc-complexes was performed on Whatman 4 paper strips (13 cm × 4 cm) using 0.05 M ammonium acetate buffer pH 7.4 as electrolyte solution and a voltage of 400 V for 20 min. Pertechnetate was used as reference compound to prove migration of the charged ^{99m}Tc-complexes. As expected, none of the tested compounds showed any migration during electrophoresis, indicating that the complexes are neutral. Log *P* values have been determined according to a literature procedure.[11,12] For the ^{99m}Tc(CO)$_3$-complexes with the following ligands they were found to be 0.15 for *N*,*N*-dimethyl-EDAA, 0.21 for *N*,*N*,*N*′-trimethyl-EDAA, 0.98 for *N*,*N*-diethyl-EDAA, 1.65 for *N*,*N*′-dibutyl-EDAA, 2.5 for *N*,*N*′-dibenzyl-EDAA and 0.87 for *ortho*-PDAA. Dischino et al. suggested a log *P* range of 1–2.5 as ideal for a potential brain uptake.[13]

To support the supposed structures of tridentate ^{99m}Tc(CO)$_3$-complexes with the studied ligands, we performed LC–MS measurements at the example of [^{99m}Tc(CO)$_3$(DBu)], which turned out to be the key compound of the study (DBu = *N*,*N*′-dibutyl-EDA-*N*-acetic acid). Mass spectra were recorded using LC–MS (Waters separation module, XTerra MS C18 column 50 mm × 2.1 mm, 3-in. NaI(Tl) radiation detector, Micromass LCT mass spectrometer and MassLynx software, Waters-Micromass, Manchester, UK). The column was eluted at a flow rate of 300 μl/min with gradient mixtures of water (containing 0.01% formic acid) and acetonitrile (linear gradient from 0% acetonitrile at 0 min to 50% at 20 min, then kept at 50% till 30 min). A solution of 0.01% kryptofix 2.2.2 in CH_3CN–H_2O (50:50) was added to the mobile phase at a flow rate of 1 μl/min and served as lock mass (377.26 Da) for accurate mass determination. Generator eluate with a high relative content of ^{99}Tc was used for a carrier-added synthesis of the complexes to enhance the signal in mass spectrometry. The solution with the complex was concentrated to obtain a detectable signal, but this remained close to the detection limit of the mass spectrometer. An alternative procedure was the addition of ^{99}Tc to the ^{99m}Tc generator eluate for the carbonylation reaction (200–300 μl of an aqueous ^{99}Tc solution, containing 15 μg/ml NH_4TcO_4). Mass spectrometry supported the supposed structure of [^{99m}Tc(CO)$_3$(DBu)]. In Figure 2, the single ion mass chromatogram shows the complex at a retention time of 21.41 min and the detected mass of 414.98 Da corresponds with the theoretical value of 415.11 Da $[M+H^+]^+$ for this complex.

Biodistribution experiments of each of the isolated ^{99m}Tc(CO)$_3$-complexes was determined in eight normal male NMRI mice (body mass 28–42 g). A ^{99m}Tc-activity of approximately 40 kBq/mouse was injected via a tail vein. Four mice were sacrificed at 2 min p.i., the other four at 60 min p.i. and the organs and body parts were dissected and weighed. The activity in the dissected organs and body parts was measured using a Wallac 1480 WIZARD 3″ automatic gamma counter (Perkin–Elmer, Boston, USA). For calculation of total blood radioactivity, blood mass was assumed to be 7% of the body mass.[14] Table 1 shows the results of the biodistribution experiments for selected organs as percentage of injected dose (% of I.D.). As a general tendency we found an increasing liver uptake and a decreasing renal excretion with increasing lipophilicity (and increasing log *P*) of the compounds. Heart, lungs, stomach and spleen did not show any significant uptake. For [^{99m}Tc(CO)$_3$(DBu)] and [^{99m}Tc(CO)$_3$(PDAA)], brain uptake was found at 2 min after injection, namely 0.34% of I.D. and 0.22% of I.D., respectively. [^{99m}Tc(CO)$_3$(MPDAA)] was not yet fully characterised, but the additional methyl group did not change the brain uptake significantly (0.19% of I.D. after 2 min) as compared to [^{99m}Tc(CO)$_3$(PDAA)].

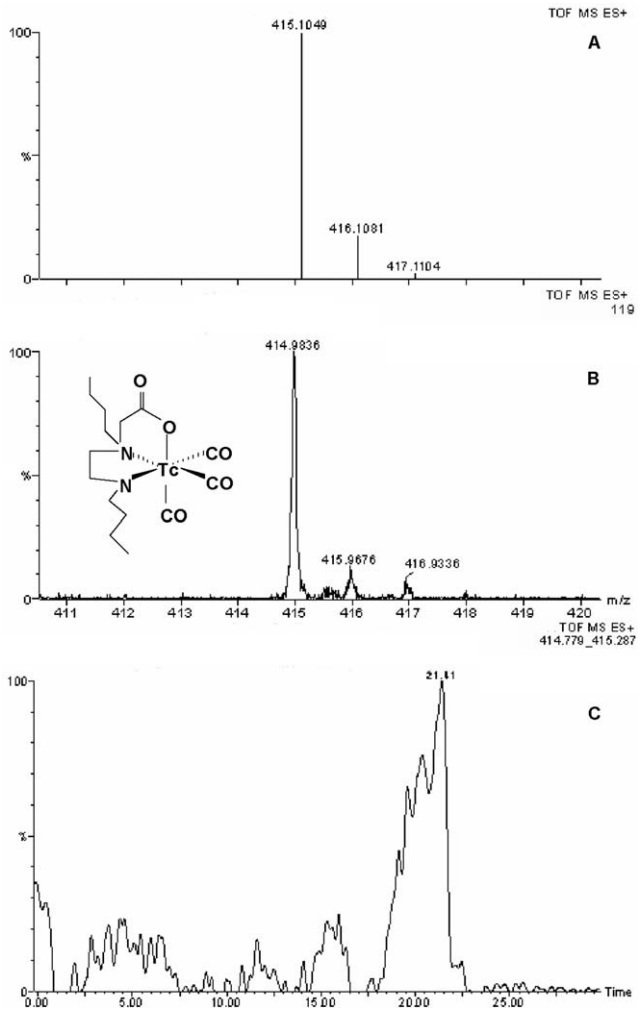


Figure 2. LC–MS analysis of the Tc–tricarbonyl complex [$^{99/99m}$Tc(CO)$_3$(DBu)]°: (a) calculated mass spectrum, (b) detected mass spectrum and (c) single ion mass chromatogram.

Table 1. Results of biodistribution experimets of ^{99m}Tc-labelled compounds in mice (activity in % of injected dose; $n = 4$ at each time point)

	^{99m}Tc(CO)$_3$-complexes with					
	DM–EDAA	TM–EDAA	DE–EDAA	DBu–EDAA	DBn–EDAA	PDAA
% I.D. 2 min p.i.						
Urine + kidneys	10.0	7.5	6.6	23.9	8.7	9.3
Liver	22.1	22.9	36.8	33.5	39.7	24.4
Intestines	10.6	9.3	14.3	9.4	11.7	9.0
Stomach	1.4	1.3	1.1	0.9	1.8	1.2
Spleen	1.1	1.4	0.9	0.9	1.9	0.9
Lungs	1.5	1.2	0.6	2.5	1.2	1.6
Heart	0.5	0.9	0.5	0.9	0.9	0.6
Blood	23.0	17.9	8.6	18.0	8.1	23.0
Brain	0.0	0.01	0.0	0.34	0.0	0.23
% I.D. 60 min p.i.						
Urine + kidneys	26.4	20.9	15.3	19.4	4.7	24.8
Liver	17.3	36.7	36.4	41.1	26.9	20.6
Intestines	15.1	13.3	24.0	14.3	56.8	15.4
Stomach	0.7	0.6	0.9	0.8	0.3	1.1
Spleen	0.4	0.2	0.5	1.0	0.2	0.7
Lungs	0.5	0.4	0.4	0.9	0.3	0.7
Heart	0.3	0.2	0.2	0.7	0.1	0.4
Blood	5.5	7.0	4.0	3.4	3.0	13.0
Brain	0.0	0.0	0.0	0.22	0.0	0.12

Increased liver uptake, enhanced intestinal excretion and reduced renal excretion can be expected in case of increasing lipophilicity of (similar) compounds.[15] None of the tested compounds showed a significant activity in the stomach, indicating stable ^{99m}Tc–tricarbonyl complexes and no re-oxidation to pertechnetate. From the ^{99m}Tc(CO)$_3$-labelled complexes with *N*,*N*-dimethyl-EDAA (DME), *N*,*N*,*N*′-trimethyl-EDAA (TME), *N*,*N*-diethyl-EDAA (DET), *N*,*N*′-dibutyl-EDAA (DBu) and *N*,*N*′-dibenzyl-EDAA (DBZ) only [^{99m}Tc(CO)$_3$(DBu)] showed a clear brain uptake. This seems to support the theory of an 'ideal log *P* range' of 1–2.5 for potential brain uptake of radiopharmaceuticals suggested in the literature.[13] The log *P* of [^{99m}Tc(CO)$_3$(DE)] (0.98) and [^{99m}Tc(CO)$_3$(DBn)] (2.5) slightly falls outside this range and these complexes do not show brain uptake. Other effects seem to play a role in case of the phenylenediamine complex [^{99m}Tc(CO)$_3$(PDAA)], as it showed brain uptake, but had a log *P* of only 0.87.

Brain uptake of the tested compounds is lower as compared to that of classic brain perfusion agents such as ^{99m}Tc–HMPAO, which showed a brain uptake in mice of 2.9% of I.D. for the *d*,*l*-isomer and 1.2% of I.D for the *meso* isomer after 1 min.[16] Other examples, for example, ^{99m}Tc–(SNS/S) mixed ligand complexes as described by Tsoukalas et al.,[17] are with 0.41% of I.D. in brain after 5 min in the same order of magnitude as the complex [^{99m}Tc(CO)$_3$(DBu)] presented here.

In conclusion, potential and usefulness of the Tc–tricarbonyl chemistry has been described in numerous articles.[2,4,18] Despite the use of various ligands, especially designed for a labelling with the ^{99m}Tc(CO)$_3$-moiety, brain uptake of tridentate ligands labelled with the ^{99m}Tc–tricarbonyl moiety has—to our knowledge—not yet been reported. A rare example of a ^{99m}Tc(CO)$_3$-complex with brain uptake is the ^{99m}Tc(CO)$_3$-cyclopentadienyl derivative 'cytectrene'.[19]

Of the ^{99m}Tc(CO)$_3$-complexes investigated in this study, [^{99m}Tc(CO)$_3$(AEG)], [^{99m}Tc(CO)$_3$(DM)], [^{99m}Tc(CO)$_3$(TM)], [^{99m}Tc(CO)$_3$(DE)] and [^{99m}Tc(CO)$_3$(DBn)] showed no brain uptake in mice. On the other hand, the dibutyl-aminoethylglycine derivative [^{99m}Tc(CO)$_3$(DBu)] and the phenylenediamine derivative [^{99m}Tc(CO)$_3$(PDAA)] are able to pass the blood–brain barrier and showed a clear brain uptake. With these compounds as examples, ^{99m}Tc–tricarbonyl complexes with tridentate ligands may also be considered as potential brain imaging agents. By further altering chain length or nature of the N-substituents of ethylenediamine and phenylenediamine, derivatives with an optimised uptake will be explored in future experiments.

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