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Evaluation Of ^{99m}Tc-Complexes With Novel Monoamine Diamide (MADA) Thiolate Tetraligands: *L*-Cysteine Acetyldiglycine Diethyl Ester And *L*-β-Methylhomocysteinate Acetyldiglycine Ethyl Ester

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Summary

Compounds with a monoamine diamide (MADA) thiolate tetraligand structure (*L*-cysteine acetyldiglycine diethyl ester, *L*-CAG2D and *L*-β-methylhomocysteinate acetyldiglycine ethyl ester, *L*-HAG2ME) were synthesized and labelled with ^{99m}Tc to investigate the ^{99m}Tc-complexation characteristics of these tetraligands. On reversed-phase HPLC, ^{99m}Tc-*L*-CAG2D and ^{99m}Tc-*L*-HAG2ME each give two peaks, probably isomers. During electrophoresis, all isomers migrate towards the anode indicating a net anionic charge. Octanol-buffer partition coefficients were respectively –0.47 and –0.05 for the isomers of ^{99m}Tc-*L*-CAG2D and–0.51 and –0.32 for the isomers of ^{99m}Tc-*L*-HAG2ME. In addition, brain uptake of all HPLC-isolated isomers was low and did not exceed 0.1% of injected dose at 2 min, 10 min or 30 min post injection. It may be concluded that the MADA-type ligands lose their amine proton upon complexation with ^{99m}Tc resulting in hydrophilic, negatively charged ^{99m}Tc-complexes which do not exhibit significant brain uptake in mice.

Key-words: *L,L*-ECD, Technetium-99m, homocysteine, MADA-tetraligands

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Introduction

A number of tetraligands forming ^{99m}Tc-complexes which are neutral at physiological pH have previously been developed and are reported in the literature (1-4). By virtue of their neutral character, these ^{99m}Tc-complexes are, in general, able to cross the blood-brain barrier by passive diffusion and to cross cell membranes. An example of such a tetraligand is the *bis*-amine *bis*-thiolate (BAT) *L,L*-ethylene cysteine diethylester (*L,L*-ethyl cysteinate dimer, *L,L*-ECD) which is commercially available as a lyophilised labelling kit (Neurolite®, Du Pont Pharma, Billerica, MA) for the preparation of ^{99m}Tc-bicisate (^{99m}Tc-L,L-ECD), a radiopharmaceutical for the assessment of regional cerebral perfusion. X-ray diffraction analysis has indicated that upon complexation of ECD with ^{99m}Tc, one of its amine protons is retained (5) resulting in a neutral ^{99m}Tc-complex at physiological pH (Fig. 1). ^{99m}Tc-*L,L*-ECD owes its ability to cross the blood-brain barrier (BBB) to this neutrality and to its lipophilic nature. If both amine protons were split off during complexation, the ^{99m}Tc-*L,L*-ECD complex would be anionic.

Similarly, the ^{99m}Tc-complexes of acetyl-*bis*-cysteamine (Fig. 1) and other monoamine monoamide (MAMA) *bis*-thiolate tetraligands have been reported to retain the amine proton after complexation with ^{99m}Tc, resulting in neutral complexes (6, 7).

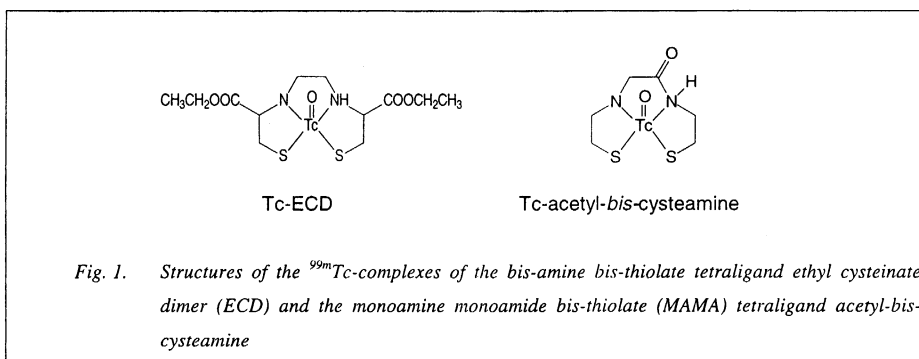

Fig. 1. Structures of the ^{99m}Tc-complexes of the bis-amine bis-thiolate tetraligand ethyl cysteinate dimer (ECD) and the monoamine monoamide bis-thiolate (MAMA) tetraligand acetyl-bis-cysteamine

The objective of this study was to develop compounds bearing a monoamine diamide (MADA) thiolate tetraligand structure and to evaluate their complexation characteristics.

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Therefore, two MADA-type tetraligands namely L-cysteine acetyldiglycine diethyl ester (L-CAG2D, Fig. 2) and L-β-methylhomocysteinate acetyldiglycine ethyl ester (L-HAG2ME, Fig. 2) were synthesised and labelled with ^{99m}Tc.

CAG2D HAG2ME

Fig. 2. Structures of cysteine acetyldiglycine diethyl ester and of β-methylhomocysteinate acetyldiglycine ethyl ester

Techniques such as high pressure liquid chromatography (HPLC), determination of 1-octanol/buffer coefficient and electrophoresis were used to evaluate the lipophilicity and charge of the obtained ^{99m}Tc-complexes and in this way, to estimate indirectly the structure of the ^{99m}Tc-MADA complexes, i.e. to determine whether the amine proton is retained. In addition, the biological behaviour of the ^{99m}Tc-complexes of L-CAG2D and L-HAGME was evaluated with special attention for brain uptake.

Materials and methods

General

All reagents obtained from commercial sources were of analytical grade.

S-benzyl L-CAG2D and S-benzyl L-HAG2ME were synthesized from the building blocks S-benzyl L-cysteine ethyl ester and S-benzyl L-β-homocysteine methyl ester, respectively, as intermediates of their diacid derivatives. Their synthesis has been reported previously (8).

Technetium-99m in the form of [^{99m}Tc]-TcO_4^- solution in normal saline was obtained from a commercially available ^{99m}Tc-generator (Mallinckrodt Medical, Petten, the Netherlands).

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Labelling with ^{99m}Tc

S-Benzyl-protected *L*-CAG2D and *L*-HAG2ME were labelled with ^{99m}Tc using an exchange labelling procedure. Potassium sodium tartrate dihydrate (0.4 ml of a 60 mg/ml aqueous solution) was added to a solution of 3 mg of *S*-benzyl-*L*-CAG2D or *S*-benzyl-*L*-HAG2ME in water. Consequently, the pH of the solution was adjusted to the desired pH value by addition of 0.2 ml 0.5 M phosphate buffer pH 6-9. Fifty µg stannous chloride dihydrate dissolved in 12.5 µl 0.05 M HCl was added, followed by 1 ml ^{99m}Tc-pertechnetate solution (0.4–1 GBq) and the reaction mixture was heated at 100 °C for 10 min.

Analysis

The radiolabelled reaction mixtures were analysed by instant thin-layer chromatography (ITLC) on ITLC-SG strips (Gelman Sciences, Ann Arbor, MI) eluted with acetone to rule out the presence of technetium-99m in colloidal form (R_f =0, R_f ^{99m}Tc-complexes >0.5).

HPLC analysis of the labelled reaction mixtures was performed using a system consisting of a Merck-Hitachi ternary gradient pump (L-6200 intelligent pump, Merck, Darmstadt, Germany), a Valco N6 injector (Alltech, Deerfield, IL) and a Hypersil C-18 BDS 5U column (250 mm x 4.6 mm) (Alltech) which was eluted with linear gradient mixtures of (a) 0.025 M phosphate buffer pH 2.5, (b) 30% V/V ethanol in 0.025 M phosphate buffer pH 2.5 and (c) ethanol, at a flow rate of 1 ml/min. The gradient mixtures at given time points are: at 0 min, 100% solvent (a), at 20 min, 100% solvent (b), and at 20.1 min to 35 min, a mixture of 57% solvent (b) and 43% solvent (c). The radioactivity in the eluent was measured with a 2-in. NaI(Tl) scintillation detector connected to a single-channel analyser and a Rachel integration system (Lablogic, Sheffield, UK) installed on a personal computer.

To determine the stability of the complexes, the peaks obtained during HPLC analysis were isolated and re-analysed on the same HPLC-system after incubation at ambient conditions for up to 6 h. The stability of the isolated species and the degree of conversion to other species were calculated by integration of the area-under-the curve of the resulting chromatograms.

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Electrophoresis

Aliquots (5 µl) of HPLC-isolated ^{99m}Tc-*L*-CAG2D and ^{99m}Tc-*L*-HAG2ME peaks were applied in the middle of Whatman #1 chromatographic paper strips (2.54 cm x 17 cm, Whatman International, Maidstone, U.K.) and electrophoresis was performed at 300 V for 20 min using 50 % methanol in 0.025 M phosphate buffer pH 7.4 as electrolyte solution.

Partition coefficient of ^{99m}Tc-complexes

The two main radiochemical species in the preparations of both ^{99m}Tc-*L*-CAG2D and ^{99m}Tc-*L*-HAG2ME were isolated by HPLC and their 1-octanol-buffer partition coefficient was determined according to the method of Yamauchi and co-workers (9). A volume of 10 µl of either complex was added to a mixture of 2 ml of 1-octanol and 2 ml of 0.025 M phosphate buffer pH 7.4 in a testtube. The testtube was vortexed at room temperature for 1 min and centrifuged at 1600 g for 10 min. A 0.2-ml aliquot of both phases was pipetted into another testtube with adequate care to avoid cross contamination between the phases and the radioactivity in each tube was counted in an automated sample counter (1480 Wizard™3", Wallac, Turku, Finland). The results were corrected for background activity and decay during counting. The partition coefficient, P, was calculated using the following equation:

$$P = \frac{\text{radioactivity in 1-octanol}}{\text{radioactivity in buffer}}$$

Biological evaluation in mice

Male NMRI mice (body mass 25-35 g) were sedated by intramuscular injection of 0.25 mg fluanisone and 0.005 mg fentanyl (0.1 ml of a 1 to 4 diluted solution of Hypnorm®, Duphar, The Netherlands). They were weighed and 0.1 ml of a HPLC-purified tracer solution, diluted with saline to a radioactive concentration of 148 kBq/ml was injected via a tail vein. Five mice were sacrificed by decapitation at each of the time points 2 min, 10 min and 30 min p.i., respectively. Blood was collected in a tared tube and weighed. The mice were dissected and the activity in all organs and body parts was determined using an automated sample counter (Wallac).

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Results were corrected for background radiation and physical decay during. The activity in each organ was expressed as a percentage of the injected dose (ID), equal to the sum of net counts in all organs. For calculation of radioactivity in total blood, blood mass was assumed to be 7 % of total body mass (10).

Results and Discussion

Labelling with technetium-99m and HPLC analysis

S-Benzyl *L*-CAG2D and S-benzyl *L*-HAG2ME could be labelled with ^{99m}Tc with reasonable radiochemical yields using a standard exchange labelling procedure at pH 6-9. The amounts of colloidal ^{99m}Tc (as determined on ITLC) were negligible for all preparations.

HPLC-analysis of the ^{99m}Tc-labelled preparations revealed for both *L*-CAG2D and *L*-HAG2ME two peaks, referred to respectively, as isomer A and isomer B in the order of elution. In the described HPLC conditions, the isomers A and B of ^{99m}Tc-*L*-CAG2D and those of ^{99m}Tc-*L*-HAG2ME all eluted from the column with retention times between 24 and 26 min (Fig. 3).

As expected, labelling efficiency increased with elevation of the pH of the reaction mixtures. This is due to the higher binding ability of the tetraligands as a result of the higher anionic character of the metal-complexing atoms (nitrogen and sulphur). When labelling was performed at pH 6, higher amounts of pertechnetate (peak **p**, Fig. 3) and ^{99m}Tc-tartrate (**t**) were observed, indicating some degree of incomplete ^{99m}Tc-labelling or product degradation. Of course, when increasing the pH of the labelling mixture, the risk of hydrolysis of one or both of the ester groups increases as well. Evidence of some product hydrolysis can be seen in the chromatograms of ^{99m}Tc-*L*-CAG2D and ^{99m}Tc-*L*-HAG2ME obtained after labelling at pH 8-9 (Fig. 3). In these chromatograms peaks eluting with retention times intermediate between those of the diester complexes and those of the respective ^{99m}Tc-diacid complexes were observed. These peaks are most probably the monoester monoacid forms of the complexes (11).

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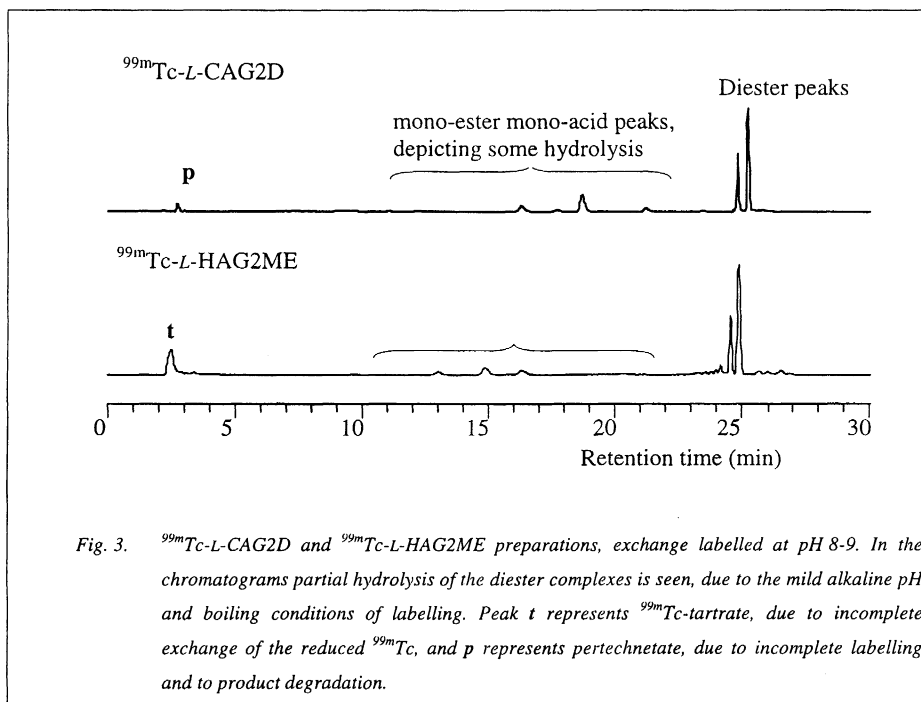

Fig. 3. *^{99m}Tc-L-CAG2D and ^{99m}Tc-L-HAG2ME preparations, exchange labelled at pH 8-9. In the chromatograms partial hydrolysis of the diester complexes is seen, due to the mild alkaline pH and boiling conditions of labelling. Peak t represents ^{99m}Tc-tartrate, due to incomplete exchange of the reduced ^{99m}Tc, and p represents pertechnetate, due to incomplete labelling and to product degradation.*

Isomers A and B of ^{99m}Tc-*L*-CAG2D or ^{99m}Tc-*L*-HAG2ME are presumably diastereomers, with the oxo-technetium core of the complex in *syn*- or *anti*-orientation with respect to the cysteinyl or β-homocysteinyl carboxyl group. After isolation, isomer A of ^{99m}Tc-CAG2D does not convert to an appreciable extent to isomer B, and the same is true vice versa for isomer B. Similarly, the isomers of ^{99m}Tc-*L*-HAG2ME do not interconvert after isolation from the column and incubation at ambient conditions.

Electrophoresis

During electrophoresis, both isomers A and B of ^{99m}Tc-*L*-CAG2D and ^{99m}Tc-*L*-HAG2ME migrated towards the anode, which implies that these complexes are not neutral. This anionic character of the complexes at pH 7.4 is somewhat surprising and is different from the situation shown by ^{99m}Tc-complexes with BAT tetraligands (e.g. ^{99m}Tc-*L,L*-ECD) and the ^{99m}Tc-complexes with monoamine monoamide bis thiol (MAMA) tetraligands (6, 12). Both

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^{99m}Tc-BAT and ^{99m}Tc-MAMA complexes are neutral since they retain an amine proton after complexation with ^{99m}Tc. As a consequence, they do not migrate during electrophoresis. Therefore, the results obtained from the electrophoresis experiments indicate that L-CAG2D and L-HAG2ME lose their amine proton during complexation with ^{99m}Tc resulting in complexes carrying a net anionic charge (probably a –1 charge).

Octanol-buffer partition coefficient

Confirmation of the hydrophilic nature of ^{99m}Tc-L-CAG2D and ^{99m}Tc-L-HAG2ME was obtained in the values of the octanol-buffer partition coefficients for the isomers of these complexes. The log P values of the HPLC-isolated isomers A and B of ^{99m}Tc-L-CAG2D were, respectively, -0.47 and -0.05 (n=3), and the values for HPLC-isolated isomers A and B of ^{99m}Tc-L-HAG2ME were -0.51 and -0.32 (n=3), respectively. The isomers of these complexes seem to distribute more into the aqueous layer and clearly can not be considered to be lipophilic.

Obviously, in the case of the MADA tetraligands, which carry only one thiol as opposed to two thiols for the MAMA tetraligands, the constituent groups are not able to promote the retention of the amine proton. It is possible that the presence of an extra amide group instead of a thiol group makes the pK_a value for the amine group in the MADA tetraligands sufficiently low so that the molecule is deprotonated around or below physiological pH. Alternatively, it is possible that based on the affinities of the metal-binding atoms or on the constitutional make-up of the MADA tetraligand system, the mechanisms of complexation of these tetraligands with ^{99m}Tc require deprotonation of the amine group.

Evaluation of biological behaviour

Results obtained for biodistribution in mice for HPLC-isolated isomers of ^{99m}Tc-L-CAG2D and ^{99m}Tc-L-HAG2ME are presented in Table 1. ^{99m}Tc-L-CAG2D levels in brain were low and did not exceed 0.1% of ID at 2 min, 10 min or 30 min p.i. Evidently, these low brain uptake values are a consequence of the anionic nature of the complexes and their fairly rapid

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clearance from the blood. Neither of the isomers of ^{99m}Tc-HAG2ME exhibits significant brain uptake in mice at 2 min, 10 min, or 30 min p.i.

Evidently, the inability of ^{99m}Tc-*L*-CAG2D and ^{99m}Tc-*L*-HAG2ME to retain an amine proton after complexation impairs their brain uptake because neutrality of a complex is an essential requirement to cross the blood brain barrier. In addition, Oya and co-workers have reported that the presence of amide groups instead of amine groups in ^{99m}Tc-complexes causes significant decreases in brain uptake, even when the corresponding amide and amine complexes show similar octanol-water partition coefficient values (6). Therefore, the presence of two amide functions in MADA tetraligands versus zero in BAT ligands and one in MAMA ligands may be an additional reason for the low brain uptake.

Table 1. Biodistribution in mice of HPLC-isolated isomers of ^{99m}Tc-L-CAG2D and of ^{99m}Tc-L-HAG2ME (% of ID ±standard deviation, n=5)

Time p.i. (min)	urine	kidneys	liver	intestines	brain	blood
^{99m}Tc-*L*-CAG2D isomer A						
2	5.4 ±0.4	15.3 ±6.6	50.8 ±5.2	4.8 ±0.6	0.07 ±0.0	7.5 ±1.6
10	15.7 ±2.5	4.5 ±1.1	52.3 ±4.0	9.0 ±1.3	0.05 ±0.0	4.4 ±0.1
30	31.3 ±0.5	2.5 ±0.2	34.8 ±0.8	16.8 ±0.2	0.05 ±0.0	3.7 ±0.4
^{99m}Tc-*L*-CAG2D isomer B						
2	5.4 ±2.9	17.5 ±1.3	51.3 ±0.7	4.1 ±0.6	0.06 ±0.0	9.4 ±2.6
10	36.4 ±2.8	7.6 ±3.3	36.4 ±0.8	6.1 ±1.3	0.05 ±0.0	3.9 ±1.1
30	62.0 ±4.8	2.5 ±0.9	16.9 ±3.1	11.2 ±0.2	0.03 ±0.0	1.8 ±0.3
^{99m}Tc-*L*-HAG2ME isomer A						
2	1.6 ±0.5	14.4 ±1.8	56.5 ±0.5	4.0 ±0.3	0.06 ±0.0	6.4 ±0.3
10	7.8 ±3.8	5.4 ±3.1	69.4 ±2.4	8.7 ±0.6	0.03 ±0.0	1.8 ±0.1
30	19.4 ±1.8	2.0 ±0.6	52.1 ±2.5	18.1 ±4.1	0.04 ±0.0	1.3 ±0.2
^{99m}Tc-*L*-HAG2ME isomer B						
2	1.3 ±0.4	14.7 ±1.1	55.5 ±1.7	8.4 ±1.0	0.06 ±0.0	3.7 ±0.4
10	19.6 ±2.3	1.9 ±0.1	45.1 ±3.2	26.0 ±4.4	0.02 ±0.0	1.2 ±0.5
30	23.6 ±3.3	1.1 ±0.0	24.1 ±4.4	46.8 ±2.4	0.01 ±0.0	1.0 ±0.3

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Conclusions

From the results of this study, it can be concluded that MADA-type tetraligands can be efficiently labelled with ^{99m}Tc using an exchange labelling procedure at pH 6-9. The resulting ^{99m}Tc-complexes are anionic, relatively hydrophilic and do not show significant brain uptake in mice. As a consequence, it is postulated that MADA-ligands loose their amine proton upon complexation with ^{99m}Tc.

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