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Comparative Evaluation of ^{99m}Tc-Ethylene bis-l-Cysteine and ^{99m}Tc-Ethylene bis-l-ß-Homocysteine During Reversed Phase HPLC Analysis and Electrophoresis at Various pH Conditions

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SUMMARY

Ethylene bis-*L*-ß-homocysteine (*L,L*-EH) differs from ethylene bis-*L*-cysteine (*L,L*-EC) in having an extra methylene group between each pair of amine and carboxyl groups. The objective of this study was to determine the effect of the extra methylene groups on the characteristics of the complex of these compounds with technetium-99m during analysis by reversed phase HPLC and by electrophoresis at various pH values. Up to pH 5.5, ^{99m}Tc-*L,L*-EH exhibits a substantially longer retention time during reversed phase HPLC than ^{99m}Tc-*L,L*-EC, suggesting a more lipophilic character for ^{99m}Tc-*L,L*-EH under these conditions. On the other hand, ^{99m}Tc-*L,L*-EH clearly possesses a higher negative charge in the pH range 3-6.5 as shown by the markedly greater migration towards the anode in electrophoresis experiments. A rational explanation for these seemingly opposing observations can not yet be offered.

INTRODUCTION

The ^{99m}Tc-complex of the *bis*-amine *bis*-thiol tetraligand ethylene bis-*L*-cysteine (^{99m}Tc-*L,L*-EC, Fig. 1) is rapidly and efficiently excreted by the kidneys and has been proposed as a tracer agent for the evaluation of renal function by Verbruggen and co-workers [1]. Attempts have also been made to use *L,L*-EC as a bifunctional chelating agent (BCA) for the labelling of bioactive molecules [2,3] Conjugation of the bioactive molecule to *L,L*-EC can be done at one of the amine groups of *L,L*-EC or at one or both of its carboxyl groups. It was with the aim of making derivatives of *L,L*-EC with improved conjugation properties that we previously synthesised ethylene bis-*L*-β-homocysteine (*L,L*-EH) and compared the biological characteristics of its ^{99m}Tc-complex (Fig. 1) with those of ^{99m}Tc-*L,L*-EC [4,5]. *L,L*-EH differs from *L,L*-EC in having an extra methylene group between each pair of amine and carboxyl groups.

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In the context of its use as a BCA, the longer side-groups of *L,L*-EH offer carboxyl groups that are in principle less sterically hindered to conjugation than the carboxyl groups of *L,L*-EC.

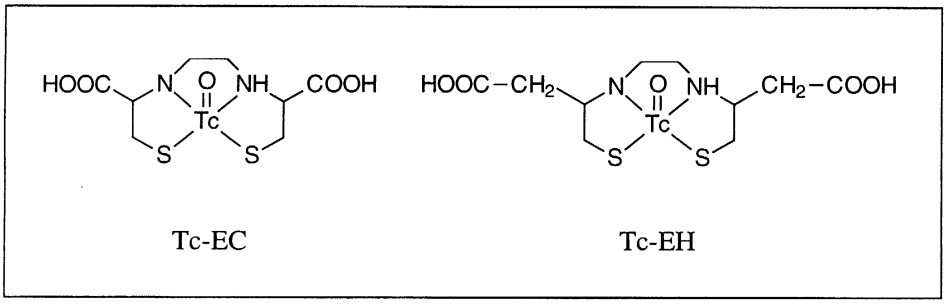

Fig. 1. Structures of the complexes of L,L-EC and L,L-EH with ^{99m}Tc

The objective of this study was to evaluate the effect of the extra methylene groups of *L,L*-EH on its physicochemical characteristics by comparison of the behaviour of ^{99m}Tc-*L,L*-EH and ^{99m}Tc-*L,L*-EC during analysis by reversed phase HPLC and electrophoresis. Electrophoresis and reversed phase HPLC analyses were performed for the two complexes over a range of pH values.

MATERIALS AND METHODS

General

The syntheses and characterisation of *L,L*-EC and *L,L*-EH have been previously described [1,4]. All reagents obtained from commercial sources were of analytical grade. Proportions of solvents are expressed on a volume/volume basis. Na[^{99m}TcO$_4$] in normal saline was eluted from a commercially available ^{99m}Tc generator (UltraTechnekow™ generator, Mallinckrodt Medical, Petten, The Netherlands).

Labelling with ^{99m}Tc

[^{99m}Tc]pertechnetate solution was added to a mixture containing 1 mg of one of the compounds in 1 ml water, 0.2 ml phosphate buffer pH 11-12 (0.5 M) and 50 μg $SnCl_2.2H_2O$ (12.5 μl of a 4 mg/ml solution in 0.05 M HCl). The mixtures were incubated at room temperature for 10 min and neutralised by addition of 0.1 M HCl. Prior to application on the HPLC column, the pH of the ^{99m}Tc-*L,L*-EC and ^{99m}Tc-*L,L*-EH preparations was adjusted to the pH of the mobile phase by addition of 0.1 M HCl.

HPLC analysis of ^{99m}Tc-complexes

Reversed-phase HPLC analysis was performed on a system consisting of a Merck-Hitachi L-6200 gradient pump (Merck, Darmstadt, Germany), a Valco N6 injector (Alltech, Deerfield, IL) and a 250-mm x 4.6-mm column filled with Hypersil C-18 BDS 5 µm (Alltech). Radioactivity in the column effluent was monitored using a 2-in. NaI(Tl) scintillation detector connected to a single channel analyser and a Rachel analysis program (version 1.40, Lablogic, Sheffield, England). Gradient mixtures of solvent (a), solvent (b) and solvent (c) as given in Table 1 were used for elution at a flow rate of 1 ml/min. A linear gradient was applied between the different time points.

Table 1. *Gradient mixtures used for reversed-phase HPLC analysis. Solvent (a) is 0.0125 M phosphate buffer, solvent (b) is a 30 % V/V solution of ethanol in solvent (a), and solvent (c) is absolute ethanol.*

Time (min)	0	20	20.1	30
% Solvent (a)	100	0	0	0
% Solvent (b)	0	100	57	57
% Solvent (c)	0	0	43	43

For the determination of the behaviour of the complexes during HPLC analysis at different pH values, the pH of solvent (a) was varied. The HPLC analyses were performed at pH values of 2.63, 3.66, 4.38, 4.9, 5.5, 6.08 and 6.85.

Behaviour during electrophoresis

HPLC-purified ^{99m}Tc-EC and ^{99m}Tc-EH preparations were evaluated concurrently for behaviour during electrophoresis under different pH conditions. Electrophoresis was performed on Whatman #1 chromatographic paper strips (Whatman International, Maidstone, U.K.) (17 cm x 3 cm) using an Elvi 22 equipment (Elvi, Milan, Italy) at a potential difference of 300 V. The analyses were performed at pH values varying from pH 2.17 to pH 11.52 using 0.025 M phosphate buffer of the appropriate pH value as the electrolyte solution. Prior to application of the HPLC-purified preparations on the electrophoresis strips, the pH of the preparations was adjusted to the pH of the electrolyte solution using 0.2 M HCl or 0.2 M NaOH. The sample solution (5 µl) was then applied on to the centre of a chromatographic paper strip previously moistened with electrolyte solution and

electrophoresis was performed during 20 min. Each time, analyses of a ^{99m}Tc-*L,L*-EC and a ^{99m}Tc-*L,L*-EH preparation were run concurrently. The developed electrophoresis strips were left to dry and the migration distance was determined using a thin layer chromatography scanner (model LB2723, Berthold, Bad Wildbad, Germany) provided with a collimated 1-in. NaI(Tl) scintillation detector connected to a single channel analyser and a Rachel analysis program (Lablogic).

RESULTS AND DISCUSSION

L,L-EC and *L,L*-EH were readily and efficiently labelled with ^{99m}Tc following a direct labelling procedure at alkaline pH to give the respective ^{99m}Tc-complexes with a radiochemical purity of at least 95 % (Fig. 2). Retention times of ^{99m}Tc-*L,L*-EC and ^{99m}Tc-*L,L*-EH during reversed phase HPLC analysis under different pH conditions are presented graphically in Fig. 3. At pH 2.63, retention times for ^{99m}Tc-*L,L*-EC and ^{99m}Tc-*L,L*-EH are, respectively, 12.4 min and 23.9 min. The large differences in retention time between ^{99m}Tc-*L,L*-EH and ^{99m}Tc-*L,L*-EC at pH 2.5 to pH 5 can not solely be attributed to the increase in hydrophobicity in ^{99m}Tc-*L,L*-EH because of the presence of the two extra methylene groups. As a consequence, it is suggested on the basis of these results that at low pH values, ^{99m}Tc-*L,L*-EC exists in a more ionised state compared to ^{99m}Tc-*L,L*-EH and therefore is retained to a lesser extent on the column.

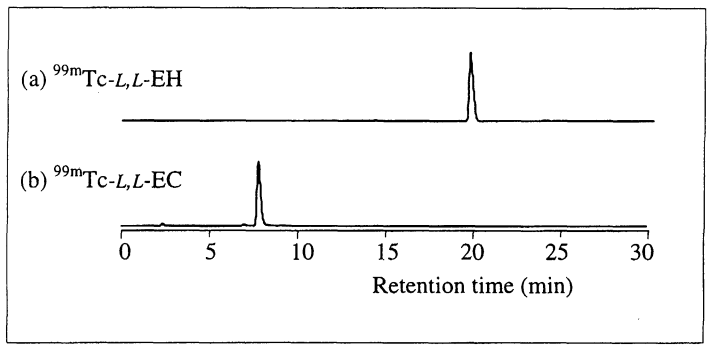

Fig. 2. Reversed-phase HPLC chromatograms of (a) ^{99m}Tc-L,L-EH and (b) ^{99m}Tc-L,L-EC at pH 3.66

As expected, with increasing pH of the mobile phase both ^{99m}Tc-*L,L*-EC and ^{99m}Tc-*L,L*-EH are eluted faster from the column because increased pH of the solvents leads to a higher degree of ionisation and a higher hydrophilicity of the complexes. It is notable that at higher pH values, the retention times for ^{99m}Tc-*L,L*-EC and ^{99m}Tc-*L,L*-EH tend to converge, such that at pH values greater than 6, the two complexes exhibit similar retention times. At alkaline pH conditions, the two complexes elute very early from the column (< 3.5 min).

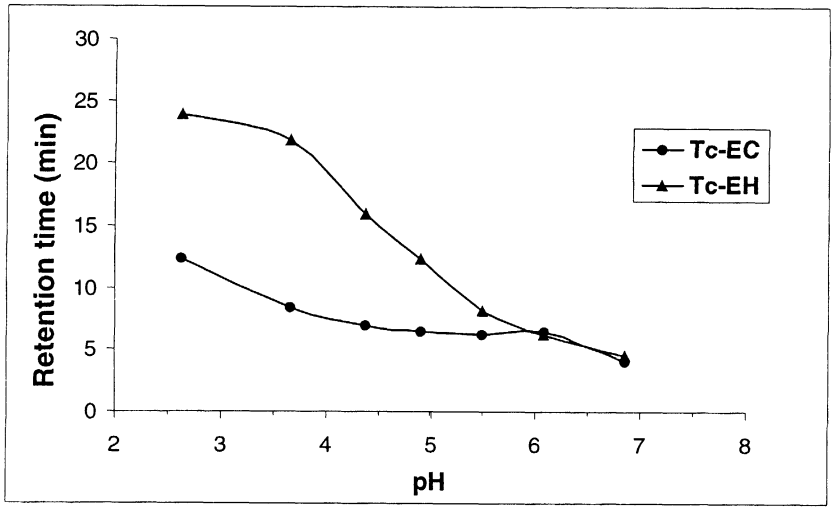

Fig. 3. Effect of the pH of the mobile phase on the retention times of ^{99m}Tc-L,L-EC and ^{99m}Tc-L,L-EH during analysis by reversed phase HPLC

Marzilli and co-workers have reported that during potentiometric titration in water, the rhenium(V)oxo analogue Re-*L,L*-EC exhibits pK_a values of 3.8, 6.64 and 10.2 [6]. Using crystallography, they found that these pK_a values correspond to deprotonation of the *syn*-carboxyl and to the amine groups on the *anti*- and the *syn*-sides, respectively (Fig. 4). It is generally accepted that the chemistry of complexation of rhenium is similar to that of technetium, and that characteristics of rhenium complexes are a reflection and indication of the nature of the corresponding technetium complexes[7-9]. The deprotonations suggested by Marzilli and co-workers at pH 3.8 and pH 6.64 correspond well with the retention times of both studied ^{99m}Tc-complexes (Fig. 3) during HPLC at different pH values.

Results obtained during electrophoresis of ^{99m}Tc-*L,L*-EC and ^{99m}Tc-*L,L*-EH at different pH values are presented in Fig. 5. These results indicate two values for the pK_a's for ^{99m}Tc-*L,L*-EC, in the 2.5-4.0 range and 6.0-7.5, which agrees with the results obtained by Marzilli and co-workers who found pKa values for Re-*L,L*-EC of 3.8, 6.64 and 10.2. Based on their results, the migration characteristics for ^{99m}Tc-*L,L*-EC at pH 3 to pH 6 as shown in Fig. 5 are consistent with the predominant existence of the mono-anionic form. Transformation of the mono-anionic to the di-anionic form of ^{99m}Tc-*L,L*-EC occurs at pH 6 to pH 7.5.

The results of the electrophoresis experiments indicate that, remarkably, the transformation of ^{99m}Tc-*L,L*-EH from a tetraprotonated mono-cationic form, which can be assumed to exist at pH values less than 2.5, to the apparently di-anionic form occurs rapidly. The three dissociation steps all occur between pH 2 and pH 5. Between pH 4 and pH 5, the ^{99m}Tc-*L,L*-EH complex is assumed to

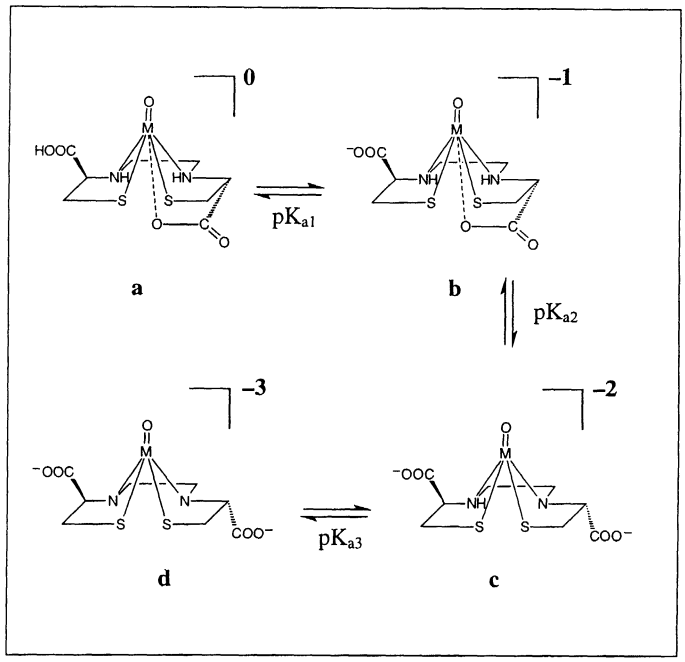

Fig. 4. Possible equilibria at different pH values for complexes of ^{99m}Tc with L,L-EC (M = Tc, pKa_1 = 3.8, pKa_2 = 6.64 , pKa_3 = 10.2)[6].

exist predominantly in the di-anionic form on the basis of its migration to the anode (Fig. 5) to a distance comparable to di-anionic ^{99m}Tc-*L,L*-EC. As a consequence, it seems possible to deprotonate the *anti*-amine group in ^{99m}Tc-*L,L*-EH under more acidic pH conditions than in the case of ^{99m}Tc-*L,L*-EC.

The reason for this marked difference between two closely related complexes is not clear. However, Marzilli and co-workers reported that the process of deprotonation of the *anti*-amine group of the complex with *L,L*-EC occurs simultaneously with the process of de-ligation of the *anti*-carboxyl group from the core (transformation of **b** to **c**, Fig. 4). They propose that protonation of the anti-amine group facilitates coordination of the *anti*-carboxyl group by positioning one oxygen atom at a favourable co-ordination position. It is probable, therefore, that in the case of ^{99m}Tc-*L,L*-EH, the extra methylene moiety between the carboxyl side-groups and the core of the complex impairs the ability of the carboxyl group to co-ordinate to the ^{99m}Tc(V)O core. This would then lead to the existence of ^{99m}Tc-*L,L*-EH at pH 3 to pH 6 with the carboxyl group de-ligated. In that case, it is possible that this protonated de-ligated form of ^{99m}Tc-*L,L*-EH and the equivalent protonated-ligated form of ^{99m}Tc-*L,L*-EC possess different dissociation characteristics, leading to the differences observed between pH 3 and pH 7 (Fig. 5).

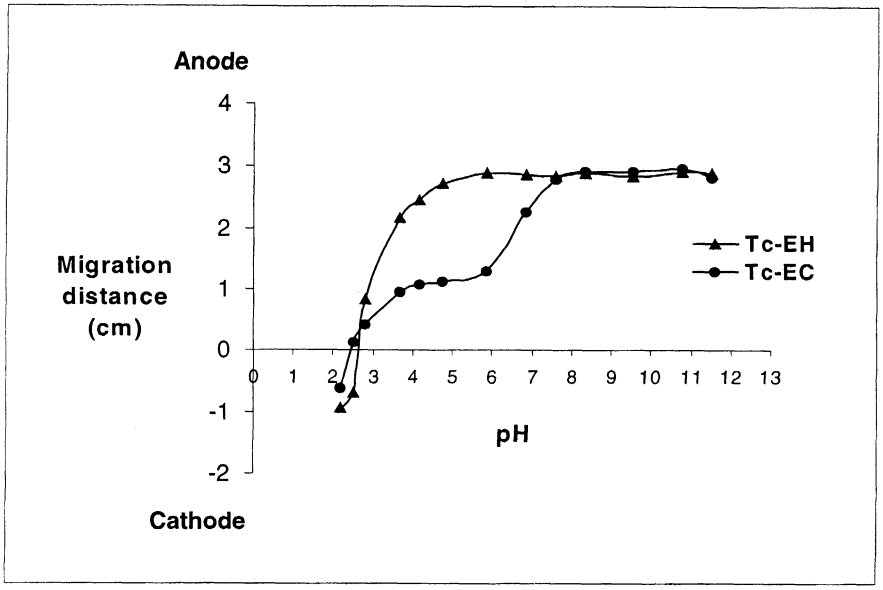

Fig. 5. Effect of the pH of the electrolyte solution on the migration distance of ^{99m}Tc-L,L-EC and ^{99m}Tc-L,L-EH during electrophoresis

Based on the electrophoresis results, ^{99m}Tc-*L,L*-EH is clearly more ionised than ^{99m}Tc-*L,L*-EC between pH 3 and pH 6.5. These results contradict the pattern obtained during analysis by reversed phase HPLC (Fig. 3). Although electrophoresis shows ^{99m}Tc-*L,L*-EH to be more ionised than ^{99m}Tc-*L,L*-EC at pH 3 to pH 6 it is not clear why, on the other hand, ^{99m}Tc-*L,L*-EH elutes later from the column than ^{99m}Tc-*L,L*-EC during HPLC analysis under these pH conditions. An explanation for the apparent contradictory patterns observed during electrophoresis and during reversed phase HPLC analysis is not clear. It is, however, possible that elucidation of the structures of the ^{99m}Tc-*L,L*-EH and of the ^{99m}Tc-*L,L*-EC complexes could shed some light on the observed discrepancy. It would also become clearer whether the two tetraligands complex with ^{99m}Tc in the same way. However, both HPLC and electrophoresis results indicate that ^{99m}Tc-*L,L*-EH and ^{99m}Tc-*L,L*-EC exist in similar ionic forms at physiological pH. This is significant because it is the ionic state of the complexes *in vivo* that determines their biological behaviour.

CONCLUSIONS

In this study, a comparative evaluation was performed of the characteristics of ^{99m}Tc-*L,L*-EC and ^{99m}Tc-*L,L*-EH, two molecules with structures that differ by the presence of an extra methylene group between the carboxyl and the amino groups of ^{99m}Tc-*L,L*-EH. This limited structural change markedly alters some of the characteristics and behaviour of the resulting ^{99m}Tc-complex.

The results of the electrophoresis experiments show that the extent of ionisation of ^{99m}Tc-*L,L*-EC and ^{99m}Tc-*L,L*-EH is clearly different between pH 3 and pH 6.5. ^{99m}Tc-*L,L*-EH possesses in this range a higher negative charge which suggests that in this pH range, one of the two amine groups of ^{99m}Tc-*L,L*-EH is already deprotonated and that the anti-carboxyl group is probably de-ligated from the oxo-technetium core of the complex. On the other hand, it was observed that up to pH 5.5, ^{99m}Tc-*L,L*-EH exhibits a substantially longer retention time during reversed phase HPLC than ^{99m}Tc-*L,L*-EC, indicating a more lipophilic character for ^{99m}Tc-*L,L*-EH. This is in contrast to the results of the electrophoresis experiments, and a rational explanation for these opposing observations can not yet be offered.

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