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Short communication

HPLC separation of ^{99m}Tc-*L*-cysteine acetyldiglycine diethylester and its monoester-monoacid and diacid derivatives ☆

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1. Introduction

Nuclear medicine is a relatively new field of medical practice that involves the administration of a radiopharmaceutical, i.e. a radionuclide or a biologically interesting molecule (ligand) labelled with a radionuclide for the diagnosis or treatment of disease. Diagnostic nuclear medicine techniques rely on the imaging of physiological function and are therefore usually able to visualise pathologies not yet detectable by other imaging techniques that generally rely on the detection of anatomical abnormalities [1]. For diagnostic uses, ligands are most commonly labelled with metastable technetium-99 (^{99m}Tc), a γ-radiation emitting radionuclide that is recognised as possessing the most ideal characteristics [2]. The γ-rays emitted by ^{99m}Tc can penetrate human tissue relatively well and therefore offer a means of externally monitoring the in vivo handling of the radiopharmaceutical. In this way, pathologies can be studied and even quantified.

During the last decade, considerable progress has been made in the development of radiopharmaceuticals, especially those labelled with technetium-99m. In search of ligands that can be useful for the study of brain and renal abnormalities, we developed the complex of ^{99m}Tc with L-cysteine acetyldiglycine (CAG2) diethylester (**I**, Fig. 1) and the ^{99m}Tc complex with its diacid derivative *L*-CAG2 (**III**, Fig. 1).

I can exist in two diastereomeric forms, with the TcO core at an orientation either *syn*- or *anti*- with respect to the carboxylester group at R_1 (Fig. 1). Similarly, in the case of the diacid analogue ^{99m}Tc-CAG2, two diastereomers are possible, depending on the orientation of the carboxyl group at R_1 (**III**, Fig. 1).

In addition, during the labelling of CAG2-diethylester with ^{99m}Tc it is possible for hydrolysis

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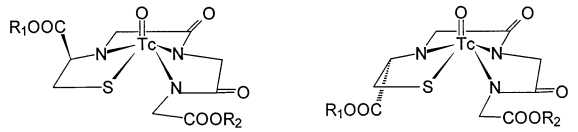

I: R_1, R_2=Et II: R_1=H, R_2=Et or R_1=Et, R_2=H III: R_1, R_2=H

Fig. 1. Structures of the *syn*- and *anti*-diastereomers of ^{99m}Tc-CAG2–diethylester (**I**), ^{99m} Tc with CAG2–monoester-monoacid (**II**), and ^{99m}Tc-CAG2 (**III**).

of one of the esters to occur, with formation of ^{99m}Tc-monoester-monoacids (**II**, Fig. 1). **II** can exist as four isomers i.e. two pairs of diastereomers, with the carboxyl group ($R_1 = H$, $R_2 = Et$) at a *syn*- or at an *anti*- orientation or the carboxylester group ($R_1 = Et$, $R_2 = H$) at a *syn*- or at an *anti*-orientation with respect to the TcO core.

The various isomers of **I**, **II** and **III** show divergent biological characteristics. Therefore, it is critical that a method of analysis is developed that can separate **I**, **II** and **III**, and also their different isomeric forms. The method would allow for the monitoring, analysis and even preparative isolation of preparations of ^{99m}Tc-CAG2–diethylester and ^{99m}Tc-CAG2.

In this study, we developed a reversed-phase HPLC method that allows for the separation and quantification of all the expected isomers of **I** (two diastereomers), **II** (four isomers) and **III** (two diastereomers).

2. Materials and methods

Chemical synthesis of *L*-cysteine acetyl-diglycine diethylester was achieved following a modified procedure of classical peptide synthesis [3]. Alkaline hydrolysis of the *L*-CAG2-diethylester yielded the diacid *L*-CAG2. Technetium-99m, as sodium pertechnetate, was eluted from a ^{99}Mo/^{99m}Tc generator ('Ultratechnekow', Mallinckrockt Medical, Petten, The Netherlands). All reagents and solvents were proanalysis grade.

2.1. Labelling of L-CAG2 and L-CAG2-diethylester with ^{99m}Tc

To form ^{99m}Tc-CAG2 or ^{99m}Tc-CAG2–diethylester complexes, an exchange labelling procedure is followed. In the procedure, a weak complex of ^{99m}Tc with tartrate is initially formed in the reaction mixture, and this weak complex then undergoes exchange in which CAG2 or CAG2–diethylester replaces the tartrate. These two ligands form a more stable complex with ^{99m}Tc than tartrate does.

The exchange labelling procedure involves addition of sodium pertechnetate ($^{99m}TcO_4^-$) solution (about 740 MBq) in 1 ml normal saline to 1.5 ml of an aqueous solution containing 2 mg *L*-CAG2 or the *L*-CAG2–diethylester, 16 mg sodium potassium tartrate, 50 μg $SnCl_2.2H_2O$ and 0.1 ml 0.5 M phosphate buffer pH 9 (also pH 6 in the case of *L*-CAG2–diethylester). The mixture is then boiled in a water bath for 10 min.

2.2. Hydrolysis experiments

Hydrolysis experiments were carried out on the ^{99m}Tc-complexes with CAG2–diethylester and with CAG2–monoester-monoacids. The hydrolysis experiments are performed in a basic and not in an acidic environment because most ^{99m}Tc-complexes are unstable in the drastic conditions of acid hydrolysis. Generally, partial alkaline hydrolysis was achieved by incubating an aliquot of the preparation with NaOH (0.2 ml, 0.1 M) at room temperature for 5 min, at which time-point HCl (0.22 ml, 0.1 M) was added to neutralise.

An attempt was made to identify which *L*-CAG2–diethylester isomers yield particular monoester-monoacid and diacid forms. This was done by collecting either of the two ^{99m}Tc-CAG2–diethylester isomers on HPLC, partially hydrolysing either isomer separately and then re-analysing the mixture on the same reversed-phase HPLC system. The four ^{99m}Tc-monoacid-monoester isomers were also isolated and similarly hydrolysed and re-analysed.

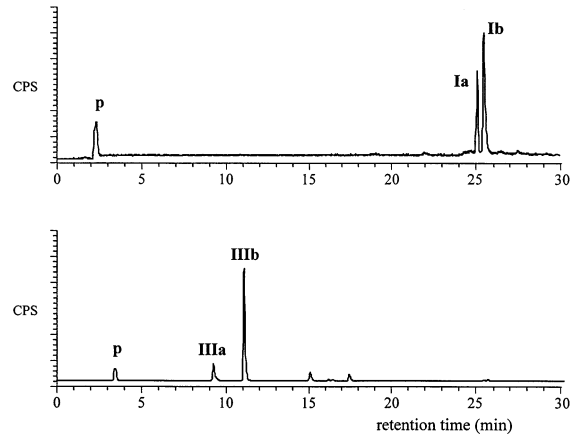

Fig. 2. Reversed phase HPLC chromatogram of ^{99m}Tc-CAG2–diethylester showing its two isomers (**Ia**, **Ib**). Similarly, ^{99m}Tc-CAG2 exists as two isomers (**IIIa**, **IIIb**). Peak **p** represents pertechnetate, which is an impurity of incomplete labelling and of product degradation. The *y*-axis represents the level of radioactivity detected, measured as counts per s (CPS).

2.3. Reversed-phase HPLC analysis

Analysis equipment consisted of a Merck-Hitachi ternary gradient pump (model L-6200 intelligent pump, Merck, Overijse, Belgium), a Valco N6 injector (Alltech, Laarne, Belgium) fitted with a 50-μl loop, and a 250×4.6 mm column filled with Hypersil BDS 5 μm (Shandon Scientific, Cheshire, UK). Elution was done at ambient temperature using gradient mixtures of (a) phosphate buffer (pH 2.5; 0.025 M), (b) 30% v/v ethanol in phosphate buffer (pH 2.5; 0.025 M), and (c) ethanol. The relative respective proportions (v/v/v) of the elution solvents at specific time points are: 0 min, 100:0:0; 20 min, 0:100:0; and 20.1 to 35 min, 0:53:47. Between two consecutive time-points, a linear gradient was applied.

The choice of ethanol in the mobile phase as an organic modifier stems from the fact that HPLC is often used as an analytical and purification technique prior to study of the samples in biological models. The collected sample is directly administered into animals after appropriate dilution, and the low concentrations of ethanol present in the samples are not expected to affect the study or to be harmful to the animals. The use of other organic modifiers, e.g. methanol, tetrahydrofuran or acetonitrile is less convenient because these organic solvents have to be eliminated from the radioactive samples prior to further biological evaluation.

Elution of the complexes was monitored by detection of the γ-radiation emitted by the ^{99m}Tc 'label' as the column effluent passed through a 2-in. NaI(Tl) scintillation γ-ray detector coupled to a single channel analyser and a Rachel analysis program (version 1.40, Lablogic, Sheffield, UK).

Prior to application on the HPLC column, all the preparations were adjusted to pH 5–6, and 50 μl of sample was applied for analysis. The analyses were performed over a period of about 2 months without change of the column. Fresh mobile phase was regularly prepared.

3. Results

When ^{99m}Tc-CAG2–diethylester (**I**) is prepared at pH 6.0 and 100°C, and analysed under the HPLC conditions described, two peaks (**Ia**, **Ib**, Fig. 2) are obtained, eluting with retention times of 24.6 ± 0.125 min ($n = 5$) and 25.4 ± 0.443 ($n = 5$) min, respectively. Similarly, ^{99m}Tc-CAG2 (**III**) exists as two isomers (**IIIa**, **IIIb**, Fig. 2) that elute after 9.5 ± 0.418 min ($n = 10$) and 11.4 ± 0.633 min ($n = 10$), respectively. Under these HPLC conditions, ^{99m}Tc-pertechnetate, which is a possible impurity of the labelling process or of product degradation, elutes after 4 min (peak **p**, Fig. 2).

When **I** is prepared at pH 9.0 and 100°C, partial hydrolysis is observed, yielding four peaks eluting at retention times intermediate between those of **I** and those of **III** (Fig. 3). These four peaks (**IIa**, **IIb**, **IIc**, **IId**) represent isomers of ^{99m}Tc-monoester-monoacid complexes and elute after 16.1 ± 0.459 min ($n = 10$), 17.6 ± 0.253 min ($n = 10$), 18.6 ± 0.267 min ($n = 10$) and 20.9 ± 0.202 ($n = 6$) min, respectively.

Isolation of diester isomer **Ia** and subsequent hydrolysis yields principally the monoester–monoacid isomers **IIa** and **IIb**. Hydrolysis of **Ib** yields principally **IIc** and **IId**. Hydrolysis of HPLC-isolated monoester-monoacid isomers **IIa** and **IIb** give the diacid isomer **IIIa** as product. Conversely, hydrolysis of **IIc** yields **IIIb**.

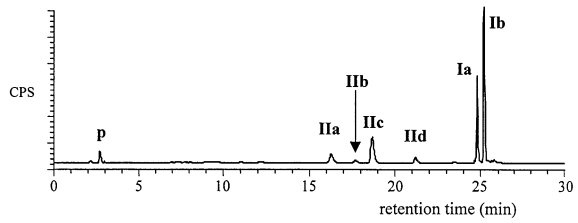

Fig. 3. Reversed phase HPLC chromatogram of ^{99m}Tc-CAG2–diethylester, prepared by exchange labelling at pH 9, showing partial hydrolysis of the diester complexes (**Ia**, **Ib**) to the monoester-monoacid analogues (**IIa**, **IIb**, **IIc**, **IId**). The *y*-axis represents the level of radioactivity detected, measured as counts per s (CPS).

Unfortunately, isolation and subsequent hydrolysis could not be done on **IId** because it was always formed in very small amounts. Even when **Ib** was isolated and hydrolysed, the proportion of **IId** formed was still too low, constituting less than 4% of the mixture. Apparently, formation of **IIc** is greatly favoured when **Ib** is hydrolysed.

The ^{99m}Tc-complexes with CAG2 and CAG2–diethylester were found to be stable during the reversed-phase HPLC analytical procedure. This was shown by collecting the different peaks as they eluted from the column, letting the solutions stand in the HPLC solvent mixture at ambient conditions for up to 6 h and then re-applying the solutions for analysis. The re-applied ^{99m}Tc-CAG2 and ^{99m}Tc-CAG2–diethylester samples elute as single peaks and with retention times consistent with retention times given by the parent preparation.

4. Discussion and conclusion

The described reversed-phase HPLC method is able to separate the eight different species efficiently from each other and from the potential impurity pertechnetate.

It is not possible, based on HPLC characteristics, to conclusively and directly assign the different HPLC peaks to particular diastereomers or isomers. In the case of the ^{99m}Tc-CAG2–diethylester complexes and also for the ^{99m}Tc-CAG2 (diacid) complexes, there exist two isomers on HPLC, either of which could be the *syn*- or the *anti*-diastereomer. However, Rao and co-workers performed X-ray crystal diffraction analysis experiments on the diastereomeric complexes of *n*,*n*-bis(mercaptoacetyl)-2,3-diaminopropionate with the long-lived radionuclide technetium-99. They concluded that for this complex the diastereomer that elutes first has the *syn*-configuration [4]. An extrapolation of their finding to the ^{99m}Tc-CAG2–diethylester and ^{99m}Tc-CAG2 (diacid) complexes would indicate that **Ia**, **IIa**, **IIb** and **IIIa** are *syn*-isomers (Figs. 1–3).

It appears that in the hydrolysis of the total ^{99m}Tc-CAG2–diethylester preparation, or even of HPLC-isolated isomer **Ib**, minimal amounts of **IId** are obtained. This indicates that for **Ib** (presumably the diester diastereomer in *anti*-configuration) one of the ester groups is much more accessible to hydrolysis and is preferentially hydrolysed. This most likely is the R_2-ester group, which is more accessible by virtue of being less sterically hindered and less rigidly attached to the skeleton. It could be presumed that **IIc** is the product of the hydrolysis of the R_2-ester group as it is obtained in the highest proportion in all hydrolysed diethylester preparations.

Tentatively, the above considerations suggest that **Ia**, **IIa**, **IIb** and **IIIa** are *syn*-diastereomers and that **Ib**, **IIc**, **IId** and **IIIb** are in *anti*-configuration. It also appears that in the case of **IIa** and **IIc**, the R_1-ester group is retained and the R_2-ester group is hydrolysed, and that the inverse is true for **IIb** and **IId**, where the R_2-ester is retained and the R_1-ester is hydrolysed. However, for definitive assignation of structure and configuration to specific isomers, X-ray diffraction studies need to be done on corresponding complexes labelled with the long-lived nuclide ^{99}Tc.

In conclusion, the method developed and reported here has been shown to be appropriate for reliable qualitative analysis of preparations of ^{99m}Tc-CAG2–diethylester and its acid analogues. The method is also useful for preparative separation of the different isomers.

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