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RESEARCH ARTICLE

Oxidant and Antioxidant Effects of Gentisic Acid in a ^{177}Lu-Labelled Methionine-Containing Minigastrin Analogue

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Abstract: ***Background:*** The radiolabelling of receptor-binding peptides for therapy is a challenge since the peptide itself is exposed (during labelling, storage and transport) to radiation-induced damage, directly or indirectly, in aqueous solution. Hence, the use of radiostabilizers seems to be mandatory, especially in peptide molecules that contain radiation-sensitive amino acids.

Objective: The aim of this study was to investigate the effect of two stabilizers, gentisic acid and methionine, to delve into how each of them affects the radiolabelling and stability of the minigastrin analogue [^{177}Lu]Lu-DOTA-His-His-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 through the analysis of the 22 species distinguished over time by an optimized HPLC system.

Methods: The stabilizers, in different combinations, were present from the beginning of the labelling process carried out at 96 °C for 15 min. The stability was studied for up to 7 days.

Results: The unexpected selective oxidation of the methionine residue of the radiolabelled peptide, promoted by gentisic acid, led to studying the effect of pH, from 3.5 to 6.0, in the presence of only this stabilizer. A pH-dependent antioxidant behaviour was revealed, showing a decrease in peptide impurities but an increase in the selective oxidation as the pH was increased.

Conclusion: The selective oxidation of the methionine residue could be induced by oxidizing species probably produced in the reaction between gentisic acid and free radicals of water, during the protection of the radiolabelled peptide from the attack of these harmful species. Therefore, the addition of methionine becomes necessary to effectively decrease this selective oxidation in the methionine-containing peptide.

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1. INTRODUCTION

Radiolabelled receptor-binding peptides are powerful tools for both imaging and therapy of tumours expressing receptors specifically binding these peptides. For therapy, these peptides are mainly radiolabelled with β$^-$ or α-emitting radionuclides and are a promising treatment modality for patients with inoperable or metastasized tumours [1].

Medullary thyroid carcinoma (MTC) is still one of the most challenging cancers. Epidemiological studies have shown that during the past 40 years, neither a change in stage at diagnosis nor improvement in survival has occurred for MTC patients [2, 3]. In particular, patients with metastatic MTC are left with only a few ineffective therapeutic options [3, 4]. One of the receptors that is overexpressed in MTC with high density and incidence (92%) is the cholecystokinin subtype 2 (CCK-2) receptors. CCK-2 receptors were also expressed in stromal ovarian cancers (100%), astrocytomas (65%) and small cell lung cancers (57%) [5]. Different peptides related to gastrin and cholecystokinin families were studied due to the great affinity that they presented with these CCK-2 receptors. Specifically, peptides based on human minigastrin (Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2) were investigated and conjugated with 1,4,7,10-tetraazacyclododecane-N,N′,N′′,N′′′-tetraacetic acid (DOTA) for radiolabelling with different radionuclides giving promising results [4].

In this study, we investigated the radiolabelling of a DOTA conjugated-minigastrin analogue: DOTA-His-His-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 = DOTA-H2MG11 (Fig. **1**) with lutetium-177 (^{177}Lu). This radiometal is a low-energy β-emitting radionuclide (average energy: 133 keV) with an average tissue penetration range of 0.67 mm and a $T_{1/2}$ of 6.7 days, thus having suitable characteristics for use in non-bulky tumour therapy or small metastases [6]. The β$^-$ particles released from this radionuclide can damage cellular components in the tumour tissue either directly or indirectly

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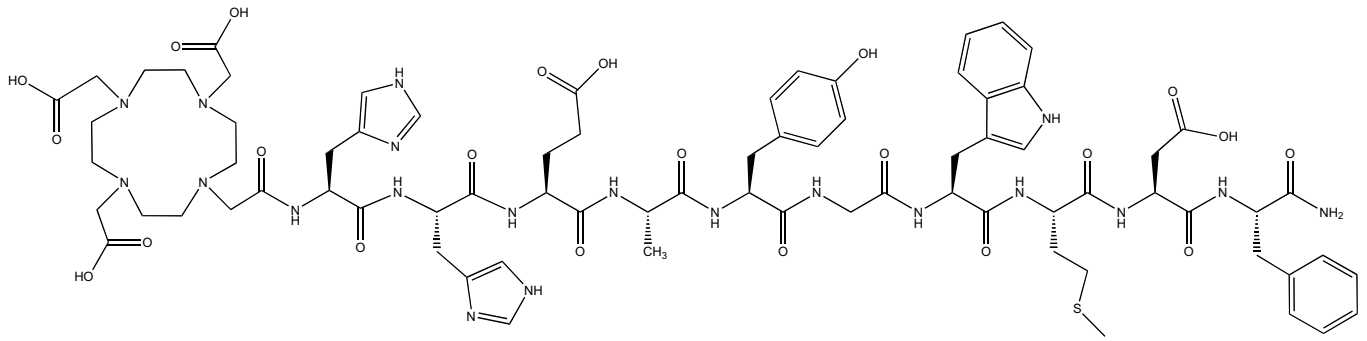


Fig. (1). Chemical structure of DOTA-His-His-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2 (DOTA-H2MG11), molecular weight: 1677.8 $g.mol^{-1}$.

via the free radicals formed by the interaction of ionizing radiation with water in the tumour tissue. However, this damage is not limited to the tumour, the radiolabelled peptide itself is exposed (during labelling, storage and transport) to radiation-induced damage directly or indirectly in aqueous solution [7]. This damage may include oxidation, hydroxylation, aggregation and/or bond scission [8]. Particularly, the methionine residue (Met) of different peptides was found to be readily oxidized to its methionine sulfoxide and/or sulfone forms (Met(O)), being the major by-products of the labelling process [4, 7, 9, 10]. In the case of minigastrin analogues, the Met oxidized compounds can lose its CCK-2 receptor affinity [10], so the replacement of Met with oxidation-resistant amino acid analogues, like norleucine or methoxinine, has been studied with encouraging results [11]. In addition, it is well established that aromatic amino acids (Tyr, Trp and Phe) react with hydroxyl radicals in aqueous solution, which would cause a greater sensitivity of the minigastrin molecule due to the radiolysis of water molecules [9]. Hence, use of radical scavengers as radiostabilizers seems to be mandatory in the radiolabelling of the minigastrin analogues. Among the stabilizers, most commonly used in radiochemistry are ascorbic acid (vitamin C) and gentisic acid [4, 7, 9, 10, 12, 13], with the second being the least studied.

The purpose of this study was to investigate the effect of two stabilizers, gentisic acid and methionine, to delve into how each of them affects the radiolabelling and the stability of the ^{177}Lu-labelled methionine-containing minigastrin analogue [^{177}Lu]Lu-DOTA-H2MG11. This was possible through the stability study carried out up to 7 days, where 22 species were detected and analyzed. The unexpected selective oxidation of the Met residue of the radiolabelled peptide, promoted by gentisic acid, led to a more extensive investigation of this stabilizer. A pH-dependent antioxidant behaviour of the gentisic acid was revealed, allowing us to propose a hypothesis to explain the selective oxidation of the Met residue.

2. MATERIALS AND METHODS

2.1. Chemicals

Sodium acetate (NaOAc) (Suprapur), glacial acetic acid (Suprapur), hydrochloric acid 30% (Suprapur), gentisic acid (2, 5-dihydroxybenzoic acid), sodium hydroxide, ammonia solution 25% (NH_4) and ethanol (EtOH) were purchased from Merck. L-methionine was obtained from Sigma-Aldrich. Hydrogen peroxide (H_2O_2) 30 volume was bought from DI (Uruguay). Acetonitrile (HPLC grade) was obtained from Carlo Erba. Trifluoroacetic acid (TFA) was purchased from J.T. Baker. DOTA APH070 Minigastrin (DOTA-H2MG11) = (DOTA-His-His-Glu-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH_2) as its TFA salt was obtained from piCHEM. DOTA.$6H_2O$ was bought from Macrocyclics. [^{177}Lu]$LuCl_3$ no-carried-added in HCl 0.04 M was bought from Isotope Technologies Garching GmbH (ITG). Ultrapure water (Milli-Q) was used for all solutions containing water, including HPLC and TLC mobile phases. None of the solutions used for the labelling reactions were degassed nor inert atmosphere was incorporated in any part of the experiments. All the reactions were performed in 2 mL conical bottom polypropylene microcentrifuge tubes (T332-7) obtained from Simport.

2.2. Evaluation and Comparison of Different Stabilizers

2.2.1. Radiolabelling

To evaluate the performance of gentisic acid and L-methionine as stabilizers of the labelled molecule, four radiolabelling procedures were carried out with different combinations of them.

The labelling reactions were performed by addition of 605 µL of the corresponding buffer-stabilizer mixture and 15 µL of the 1 $mg.mL^{-1}$ aqueous solution of DOTA-H2MG11 (8.9 nmol). To each tube, 5 µL of [^{177}Lu]$LuCl_3$ in HCl 0.04 M (137-140 MBq) with a specific activity of 2770 $GBq.mg^{-1}$ was incorporated, giving an average activity concentration of 221 $MBq.mL^{-1}$. A pH of 4.5 was verified in the reaction mixtures and they were immediately incubated at 96 °C for 15 min in a heating block.

The buffer-stabilizer mixtures were the following:

- M1 (gentisic acid and L-methionine as stabilizers):

  230 µL of the buffer NaOAc 0.8 M – gentisic acid 0.3 M and 375 µL of L-methionine 0.13 M

- M2 (L-methionine as stabilizer):

  230 µL of the buffer NaOAc 0.8 M – acetic acid 0.9 M and 375 µL of L-methionine 0.13 M

- M3 (gentisic acid as stabilizer):

230 µL of the buffer NaOAc 0.8 M – gentisic acid 0.3 M and 375 µL of water

- M4 (without stabilizers):

  230 µL of the buffer NaOAc 0.8 M – acetic acid 0.9 M and 375 µL of water

From the end of the heating (t=0), the tubes were kept at room temperature (RT) and samples of the labelling solutions were analyzed by HPLC and TLC.

2.2.2. In vitro Stability

The four radiolabelling solutions were kept at RT for 7 days in the original and closed reaction tubes. No other solution was added in order not to diminish the radiolysis by dilution and, therefore, not to diminish the formation of impurities for its better analysis. The reaction solutions were analyzed by HPLC and TLC at three different times post-labelling: 0.8, 3.9 and 6.9 days.

2.3. Effect of pH in the Presence of Gentisic Acid

2.3.1. Radiolabelling

For pH effect evaluation of the radiolabelling reaction in the presence of only gentisic acid as the stabilizer of the labelled molecule, seven labelling procedures were carried out adjusting the pH of the buffer in a range from 3.5 to 6.0. The starting material to prepare each buffer solution was 200 µL of the buffer NaOAc 0.8 M – gentisic acid 0.3 M (pH 4.9) to which HCl 5 M or NaOH 2 M solutions and sufficient water were added to complete 225 µL of the final buffer solution; in the case of pH 4.9, only 25 µL of water was added. The pH was measured with a pH meter EquipsLab PH220.

The labelling reactions were achieved by the addition of 115 µL of the corresponding buffer (pH 3.5, 4.0, 4.5, 4.9, 5.0, 5.5 or 6.0) and 7 µL of the 1 $mg.mL^{-1}$ aqueous solution of DOTA-H2MG11 (4.2 nmol). To each tube, 2.5 µL of [^{177}Lu]$LuCl_3$ in HCl 0.04 M (58-61 MBq) with a specific activity of 2498 $GBq.mg^{-1}$ was incorporated, giving an average activity concentration of 476 $MBq.mL^{-1}$. The desired pH was verified in the reaction mixtures and they were immediately incubated at 96 °C for 15 min in a heating block. At the end of the heating (t=0), the tubes were kept at RT and samples of the labelling solutions were analyzed by HPLC and TLC.

2.3.2. In vitro Stability

The seven radiolabelling solutions were analyzed according to Section 2.2.2 at 2.9 and 6.7 days post-labelling.

2.3.3. Oxidation of the Precursor

To evaluate the oxidation products of the peptide and their identification in the HPLC system, a non-radioactive oxidation reaction was carried out.

The oxidation reaction was performed by the addition of 10 µL of the 1 $mg.mL^{-1}$ aqueous solution of DOTA-H2MG11 (6.0 nmol) and 10 µL of H_2O_2 30 vol. It was immediately incubated for 7 min in a heating block at 90 °C. At the end of the heating, the tube was kept at RT and then 15 µL of the solution was analyzed by HPLC at 214 nm.

2.4. Radiolabelling of the DOTA Ring

The labelling reaction was performed by the addition of 230 µL of the buffer NaOAc 0.8 M – gentisic acid 0.3 M and 5 µL of the 1 $mg.mL^{-1}$ aqueous solution of DOTA.$6H_2O$ (9.8 nmol). A volume of 7 µL of [^{177}Lu]$LuCl_3$ in HCl 0.04 M (179 MBq) with a specific activity of 2366 $GBq.mg^{-1}$ was incorporated, giving an activity concentration of 740 $MBq.mL^{-1}$. A pH of 4.5 was verified in the reaction mixture and it was immediately incubated at 96 °C for 15 min in a heating block. At the end of the heating, the tube was kept at RT and then the radiolabelling solution was analyzed by HPLC and TLC.

2.5. Analytical Methods

2.5.1. HPLC Analysis

HPLC analysis was performed in a Shimadzu LCsolution system equipped with a photodiode array detector (UV-Visible) connected in series with a gamma detector (Lablogic). As the stationary phase a C18 column, 5 µm, 11 nm pore size, 150 x 4.6 mm, Macherey-Nagel (Nucleodur 100-5 C18 ec) was used. It was eluted with a solvent system consisting of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile (B) with the following optimized gradient: 0-18 min (20-38% B), 18-20 min (38-20% B), 20-25 min (20% B) at a flow rate of 1 $mL.min^{-1}$. The activity percentage of each peak was calculated as the percentage of the area present under each peak relative to the total integrated area (all combined radioactive peaks plus any segments of elevated baseline).

2.5.2. Radiocolloid Determination

[^{177}Lu]Lu-colloid was monitored by thin-layer chromatography (TLC) using iTLC-SG strips (Agilent Technologies) developed with NH_4:EtOH:H_2O (1:5:10 = v:v:v) and subsequently measured in a EZ-SCAN (Carroll and Ramsey Associates) using a PeakSimple 2000 chromatography integration software (SRI Instruments). [^{177}Lu]Lu-colloid and free ^{177}Lu (*i.e.* not incorporated into the DOTA-peptide) remained at the origin while [^{177}Lu]Lu-DOTA-H2MG11 migrated with the solvent front. [^{177}Lu]Lu-colloid percentage was calculated by subtracting the percentage of free ^{177}Lu determined by HPLC from the percentage remained at the origin in the TLC.

3. RESULTS AND DISCUSSION

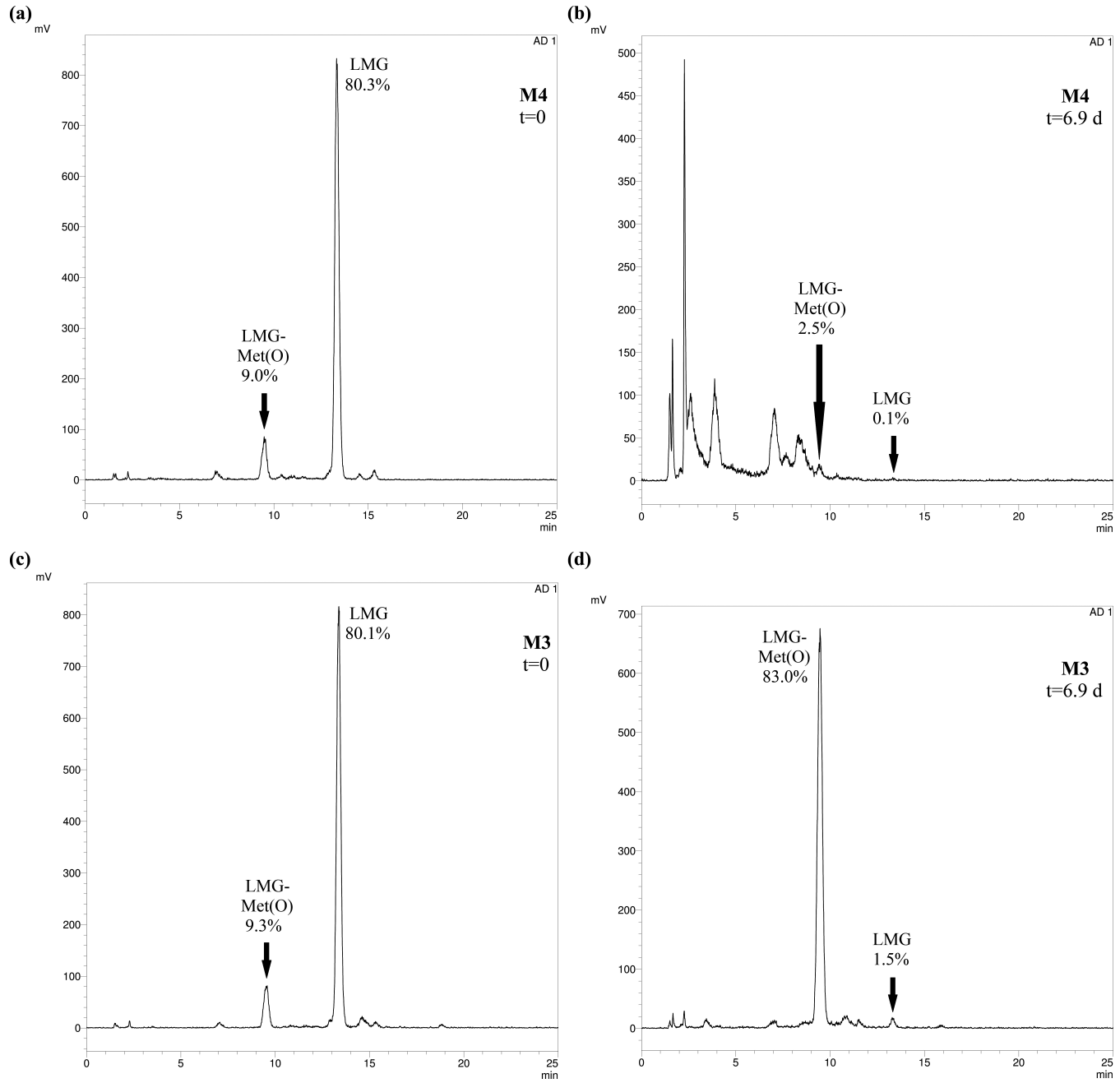
3.1. Evaluation and Comparison of Different Stabilizers

3.1.1. Radiolabelling

A high incorporation of ^{177}Lu (99%) was achieved at initial time (t=0) for the different conditions of radiolabelling and no [^{177}Lu]Lu-colloid was found in any of them in spite of the relatively high molar ratios of gentisic acid / peptide (7700) and L-methionine / peptide (5600). This represented a low competition between the two stabilizers with the chelating agent DOTA for its binding with ^{177}Lu, being 1/47 the average molar ratio of ^{177}Lu / peptide. As can be seen in Fig. (**2a**), the HPLC analysis reveals that ^{177}Lu was incorporated by the following species: the radiotracer of interest [^{177}Lu]Lu-DOTA-H2MG11 (LMG) with retention time (t_R)

of 13.3 min, the main impurity [^{177}Lu]Lu-DOTA-H2MG11-Met(O) (LMG-Met(O)) with t_R=9.4 min and other molecules with t_R from 3.4 to 18.8 min. These latter molecules were assigned to peptide impurities because they were not found in the blank of the M1 reaction. This was performed using gentisic acid and L-methionine as stabilizers, while the precursor (DOTA-H2MG11) was substituted by water. The species that were present in this blank of reaction had t_R between 1.5 and 2.6 min and corresponded to free ^{177}Lu (not incorporated into the DOTA-peptide).

Similar results were obtained at t=0 for M4 (performed without antioxidants) and M3 (with gentisic acid) being 80% of LMG, 9% of LMG-Met(O) and 10% of peptide impurities as shown in Figs. (**2a** and **2c**). However, better and similar results were obtained for M2 (performed with L-methionine) and M1 (with gentisic acid + L-methionine) reaching 90% of LMG, 1-2% of LMG-Met(O) and 8-7% of peptide impurities as can be seen in Figs. (**2e** and **2g**). This indicates that the presence of L-methionine during labelling allows the decrease of the main impurity (LMG-Met(O)), which is formed by the oxidation of the Met residue of the amino acidic sequence of the precursor. Consequently, is there not only an increase in the percentage of the radiotracer of interest at the initial time by avoiding the oxidation of the Met residue but also by slightly decreasing other peptide impurities whose presence is probably associated with oxidation processes.



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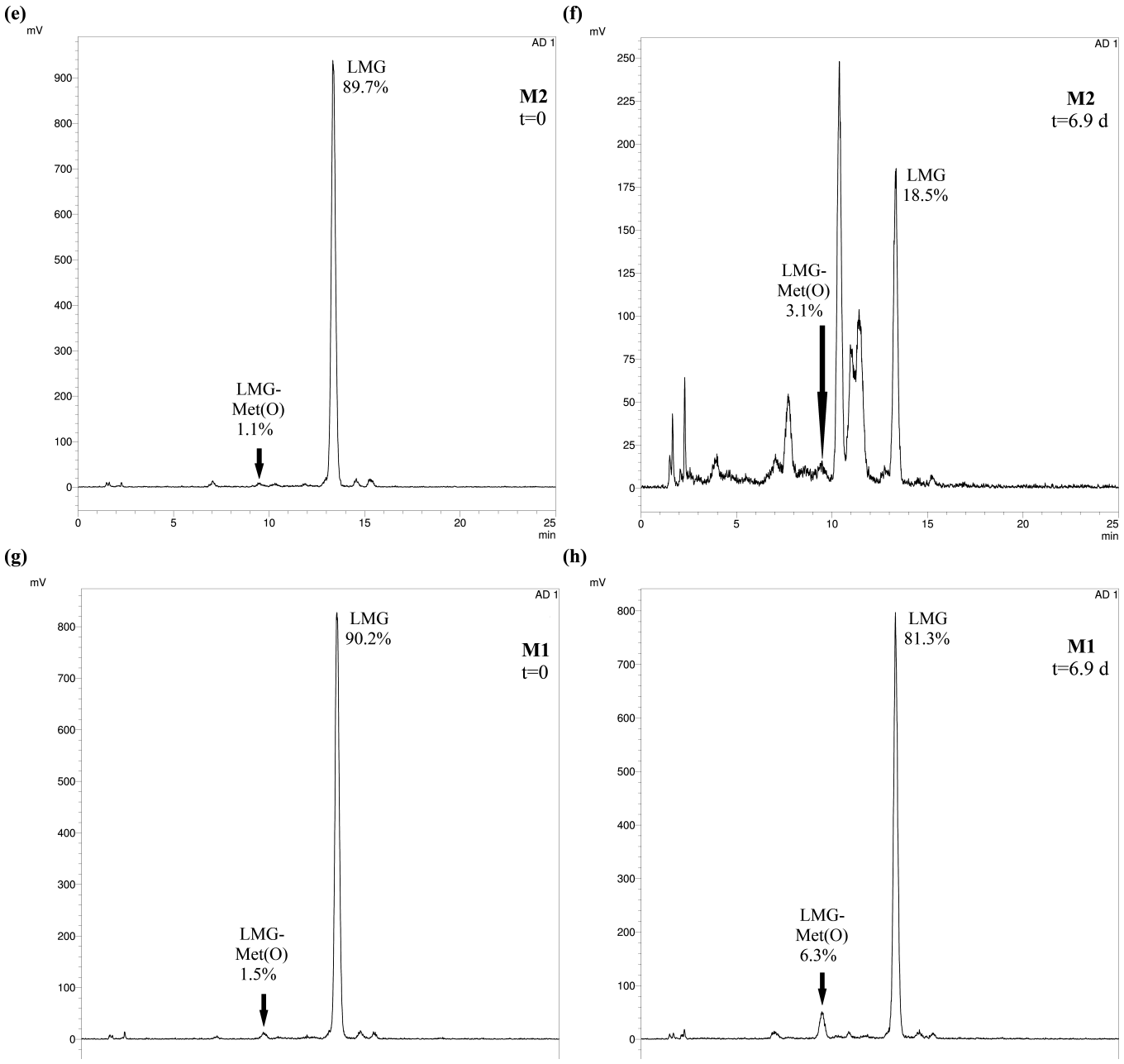

Fig. (2). HPLC radiochromatograms at t=0 and t=6.9 days for different radiolabelling conditions: (**a**, **b**) without antioxidants, (**c**, **d**) with gentisic acid, (**e**, **f**) with L-methionine and (**g**, **h**) with gentisic acid + L-methionine.

3.1.2. In vitro Stability

The exhaustive analysis of the HPLC profiles over time revealed six peaks corresponding to free ^{177}Lu (t_R: 1.5-2.6 min), and its sum was designated as total free ^{177}Lu. It reached ~1% in the four labelling conditions at t=0 and slightly increased to 2-3% in the two experiments containing gentisic acid at 6.9 days (Figs. **2d** and **2h**), while in the test containing L-methionine it increased up to 5% (Fig. **2f**). However, in the experiment without antioxidants total free ^{177}Lu increased up to 43% (Fig. **2b**) due to the instability of the labelled peptide molecules, resulting in the release of ^{177}Lu over time (Fig. **3a**). In spite of these processes, [^{177}Lu]Lu-colloid was not detected in any of the tests over time.

The first peak with t_R=1.5 min increased in detectable amounts only in M4, from 0.3% to 4% in 6.9 days. It was first assigned as [^{177}Lu]$LuCl_3$ because of its retention time determined with the original solution of the radionuclide in HCl 0.04 M (as blank of reference), but as it was not detected by TLC (with R_f=0) its presence could be discarded in all the tests. Nevertheless, another species that presented the same retention time in this HPLC system but an R_f=1 in the TLC system was [^{177}Lu]Lu-DOTA, so that it could be present in M4 as a radiolysis product. The species with t_R=1.7 min increased slightly over time, with the higher increment in M4 (0.3% to 4% in 6.9 days). The fact that it was the main peak in the blank of reaction, made us presume that would be [^{177}Lu]Lu-acetate, a weak complex that keeps the ^{177}Lu in

solution when it is released from the DOTA ring. The peak with t_R=2.3 min was one of the main species of free ^{177}Lu, with a higher increase over time in the absence of antioxidants, reaching 16% at 6.9 days in M4. Also, the peak with t_R=2.6 min was predominant in M4, increasing over time and reaching 18% at 6.9 days. These two components (2.3 and 2.6 min) had an R_f=1 by TLC and they could be controlled by the addition of antioxidants, with gentisic acid being the most effective. This fact suggests that these species were generated when ^{177}Lu was released from the radiotracer due to important damage caused by radiolysis.

The molecule of interest (LMG) decreased over time for all the radiolabelling conditions but with different kinetics, reaching the maximum stability in the presence of antioxidants according to the following order: M1 > M2 > M3 > M4. The following percentages remained at 6.9 days: 81 > 18 > 2 > 0.1 % respectively (Figs. **3a**-**d**).

The radiochemical impurity LMG-Met(O) increased slightly in M1 and M2, reaching 6% and 3% respectively, at 6.9 days post-labelling (Figs. **3d** and **3c**). Surprisingly, in M3 the increase was so dramatic that it reached 83% at that time, being the predominant species that neared the percentage corresponding to the sum of LMG and LMG-Met(O) obtained at t=0 (89%) (Fig. **3b**). While for M4 the maximum was 20% at 0.8 days, decreasing later to 2% at 6.9 days, indicating that this impurity was generated in the first hours but later destroyed (Fig. **3a**).

With regard to peptide impurities, there was a small increase in M1 and M3 up to 6.9 days, as can be seen in Fig. (**3d**) and Fig. (**3b**), reaching 10% and 13% respectively. However, in M2 they were predominant from 3.9 days post-labelling, reaching 73% at 6.9 days (Fig. **3c**). Meanwhile, for M4 these impurities became preponderant at 0.8 days, with a maximum at 3.9 days (58%) and remaining almost constant at 6.9 days (Fig. **3a**). Therefore, peptide impurities had some similar growth pattern in M2 and M4 over time, whereas in M1 and M3 they did not show pronounced changes. Nevertheless, if they are studied in more detail some differences can be found. For this purpose, these impurities were classified in three groups according to their retention times by HPLC:

- t_R 3.4-8.5 min (prior to the LMG-Met(O) peak);
- t_R 10.3-12.8 min (between LMG-Met(O) and LMG peaks) and
- t_R 14.5-18.8 min (after LMG peak).

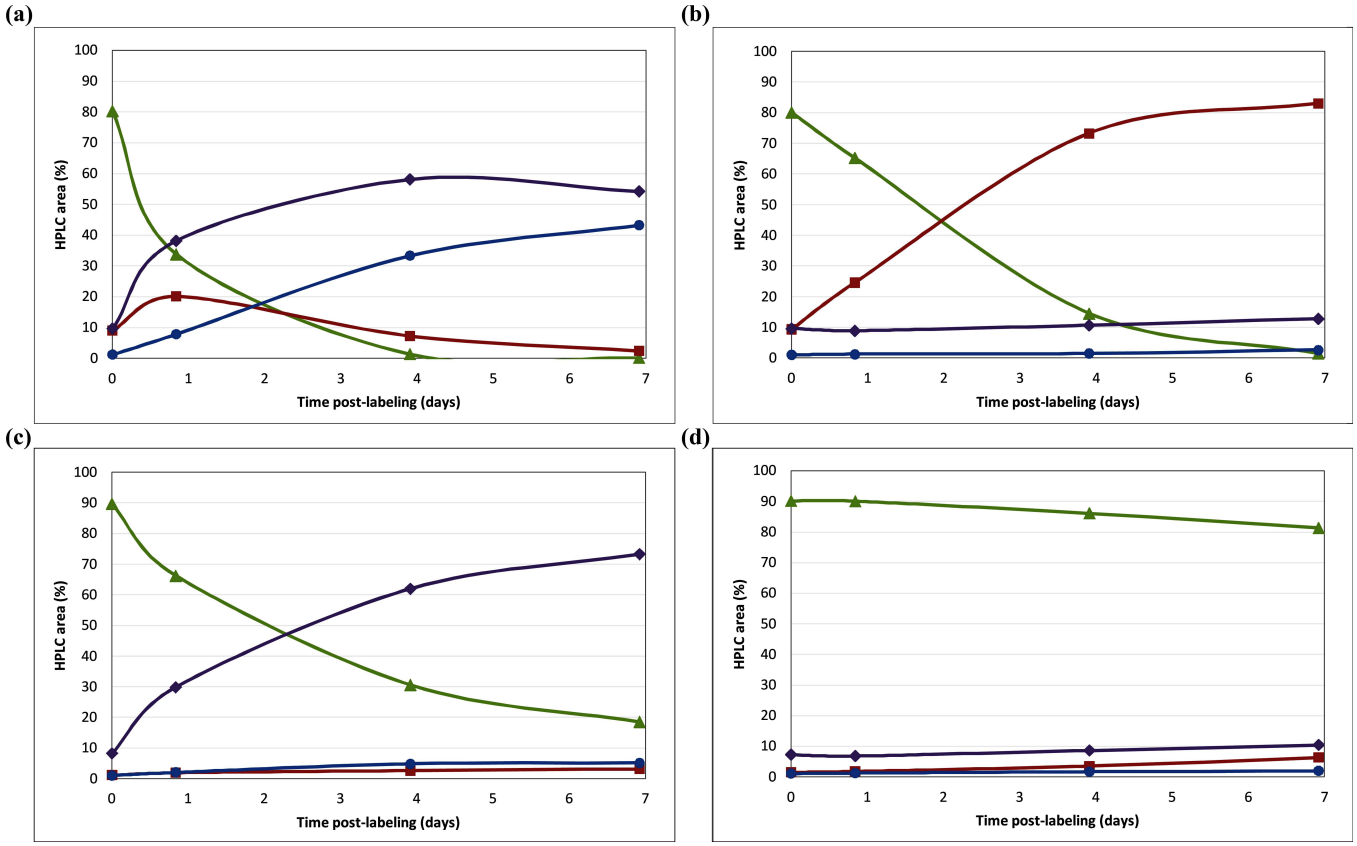

Fig. (3). Evolution of [^{177}Lu]Lu-DOTA-H2MG11 (green ▲), [^{177}Lu]Lu-DOTA-H2MG11-Met(O) (red ■), [^{177}Lu]Lu-peptide impurities (purple ♦) and total free ^{177}Lu (blue ●) for the different radiolabelling conditions over time: (**a**) without antioxidants (M4), (**b**) with gentisic acid (M3), (**c**) with L-methionine (M2) and (**d**) with gentisic acid + L-methionine (M1).

The group with lower t_R (3.4-8.5 min) was predominant over time in M4 (Fig. **2b**), increasing up to 53% in 6.9 days (Fig. **4a**), being almost the total of peptide impurities (54%). These hydrophilic impurities could be produced by major damage to the main molecules present at initial time (LMG and LMG-Met(O)) probably caused by oxidation, peroxidation, hydroxylation and/or cleavage of both the side chains and the peptide skeleton itself. These destructive reactions could occur both, through the interaction with free radicals (generated by several reactions between ionizing radiation and the aqueous media) as well as by the direct interaction of the radiation emitted by the radiolabelled molecules themselves [7, 8, 14, 15].

A thorough analysis of the HPLC profiles over time revealed five main peaks in this group. The species with t_R 3.9, 7.0 and 8.5 min were remarkable in M4, reaching 16%, 13% and 12% respectively, at 6.9 days, with gentisic acid being the most effective stabilizer to control these impurities. At last, the component with t_R=7.7 min was the predominant of this group in M2, reaching 8% at 6.9 days, but it was also reduced with the addition of gentisic acid in M1.

The group of peptide impurities with intermediate t_R (10.3-12.8 min) predominated over time in M2 (Fig. **2f**), increasing up to 53% in 6.9 days (Fig. **4b**), a high value considering the total of peptide impurities (73%). These species could be molecules with less damage than the previous group, originating from the oxidation, peroxidation and/or cleavage of different side chains of the LMG, not considering the Met residue, which was preserved by the addition of the same amino acid during the radiolabelling procedure. If it is considered that amino acids with aromatic groups act as radical-scavengers and, therefore, are susceptible to oxidation [8, 14, 16, 17], the amino acids tyrosine (Tyr), tryptophan (Trp), phenylalanine (Phe) and both histidines (His) from H2MG11, would be subject of diverse modifications that could explain several of the impurities detected in this group.

In this second group of impurities, the five most relevant peaks were examined. The species with t_R 10.3, 10.8 and 11.5 min had similar pattern over time within each experiment. They increased to the highest proportion in M2 reaching 25%, 12% and 14% respectively, at 6.9 days. A certain protection provided by L-methionine could explain these molecules, probably as a result of minor damage such as the oxidation of amino acids other than Met. However, in M4 these three impurities showed a maximum at 0.8 days

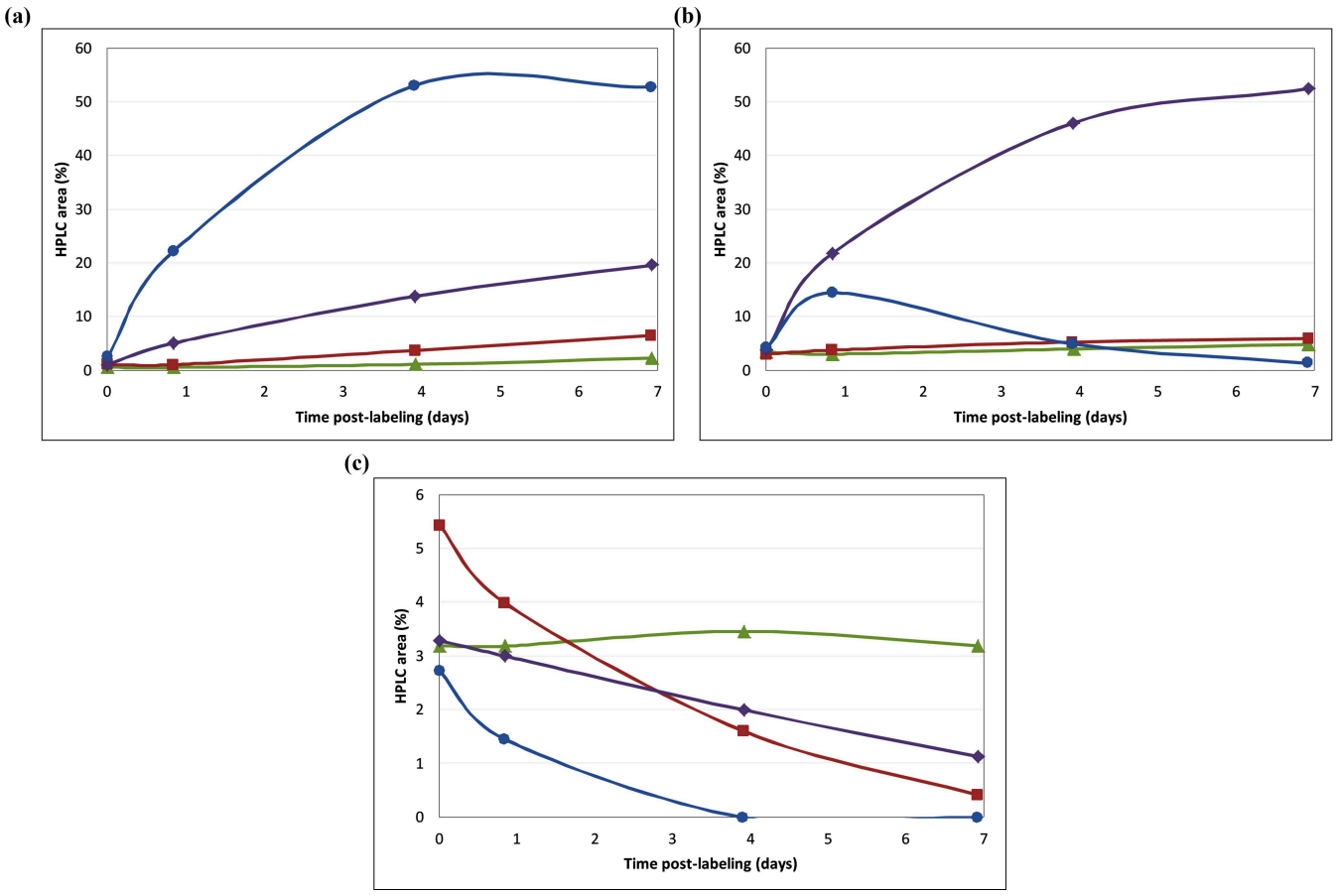

Fig. (4). Evolution of the different groups of peptide impurities over time according to their t_R (**a**) 3.4-8.5 min, (**b**) 10.3-12.8 min and (**c**) 14.5-18.8 min for the different radiolabelling conditions: (M4) without antioxidants (blue ●), (M3) with gentisic acid (red ■), (M2) with L-methionine (purple ♦) and (M1) with gentisic acid + L-methionine (green ▲).

(5-4%), decreasing to less than 1% at 6.9 days. Indicating the generation of these impurities in the first hours followed by a rapid decrease, just as it happened with the close impurity LMG-Met(O) in the same radiolabelling reaction (Fig. **3a**). This would reinforce the hypothesis that these species are produced by slight changes in LMG. In turn, in M3 and M1, these three species increased very slightly, confirming the efficacy of the gentisic acid to control these impurities.

Finally, peptide impurities with higher t_R (14.5-18.8 min), were slightly accentuated in the presence of gentisic acid at t=0, reaching 5% in M3 (Fig. **2c**). In spite of their low amount, they presented a similar pattern over time respect to LMG (Fig. **4c**). So, they could be molecules with low damage maintaining some similarities with LMG, and its higher lipophilicity could be explained by aggregation via covalent crosslinking or by hydrophobic interactions of peptide molecules [8, 14].

Therefore, the addition of gentisic acid greatly impeded the generation of most peptide impurities over time. An exception to this was the main impurity that was unexpectedly increased due to a selective oxidation of the Met residue of the radiolabelled peptide, which was effectively decreased by the addition of L-methionine. The unexpected behaviour of the gentisic acid, led us to a more extensive investigation of only this stabilizer in the radiolabelling of this highly radiation-sensitive molecule at different pH.

3.2. Effect of pH in the Presence of Gentisic Acid

3.2.1. Radiolabelling

A notorious increment in the amount of LMG-Met(O) was observed when the pH increased from 3.5 to 6.0 (9% to 35%) at t=0 in the presence of gentisic acid as stabilizer, while the LMG decreased from 78% to 55% (Fig. **5a** and Table **1**). Because of that, better results were obtained at pH values of 3.5-4.0, when the LMG reached a maximum of 78-79%, assuming in principle a higher antioxidant effect of the gentisic acid at pH≤4.0. However, peptide impurities (t_R 3.4-18.8 min) reached a maximum at pH 3.5 (11%), showing a decrease as the pH increased, obtaining a minimum of 7% at pH 5.5. Also a higher amount of total free ^{177}Lu (t_R 1.5-2.6 min) was obtained at pH 3.5 (3%), which is consistent with the fact that lower pH stimulates the protonation of carboxylic acid and amino groups from the DOTA macrocycle, thus slowing the incorporation kinetics of the radiometal into the polyaminocarboxylic ligand [18-21]. The lower amount of total free ^{177}Lu was found at pH≥4.5, (1-2%), and [^{177}Lu]Lu-colloid was not detected in the pH range studied. Therefore, as the pH was increased we found an increase in the main impurity (with the Met residue oxidized) but a decrease in the rest of the impurities, facts that go against each other.

3.2.2. In vitro Stability

A high instability of the LMG radiotracer was found over time, consistent with the results obtained in M3 of the previous study (Section 3.1). As can be seen in Table **1** and Fig. (**5b**), a large decrease of this molecule was observed at 6.7 days throughout the pH range under evaluation, remaining only between 0% and 13%, with the maximum value obtained at pH 4.0. Whereas at that time, LMG-Met(O) was mainly produced (73-85%) and to a lesser extent did the remaining peptide impurities (12-17%). Also, a low release of ^{177}Lu from the different molecular species was maintained over time (<5%) and [^{177}Lu]Lu-colloid was not detected in any of the samples examined.

Table 1. Percentage of areas obtained by HPLC for [^{177}Lu]Lu-DOTA-H2MG11, [^{177}Lu]Lu-DOTA-H2MG11-Met(O), total [^{177}Lu]Lu-peptide impurities and total free ^{177}Lu in the radiolabelling reactions at different pH values at initial time, 2.9 and 6.7 days post-labelling.

-	pH							Time
	3.5	4.0	4.5	4.9	5.0	5.5	6.0	(days)
LMG	78	79	71	66	62	65	55	0
LMG-Met(O)	9	10	19	23	27	27	35	
Peptide imp.	11	9	9	9	9	7	8	
Free ^{177}Lu	3	2	1	1	2	1	2	
LMG	17	43	5	3	1	0.3	0.2	2.9
LMG-Met(O)	66	45	83	86	87	89	88	
Peptide imp.	13	10	11	9	10	9	11	
Free ^{177}Lu	4	2	2	1	2	1	1	
LMG	0.3	13	1	0	0	0.2	0.3	6.7
LMG-Met(O)	79	73	84	84	85	84	82	
Peptide imp.	17	12	13	14	13	13	16	
Free ^{177}Lu	4	2	2	2	2	2	2	

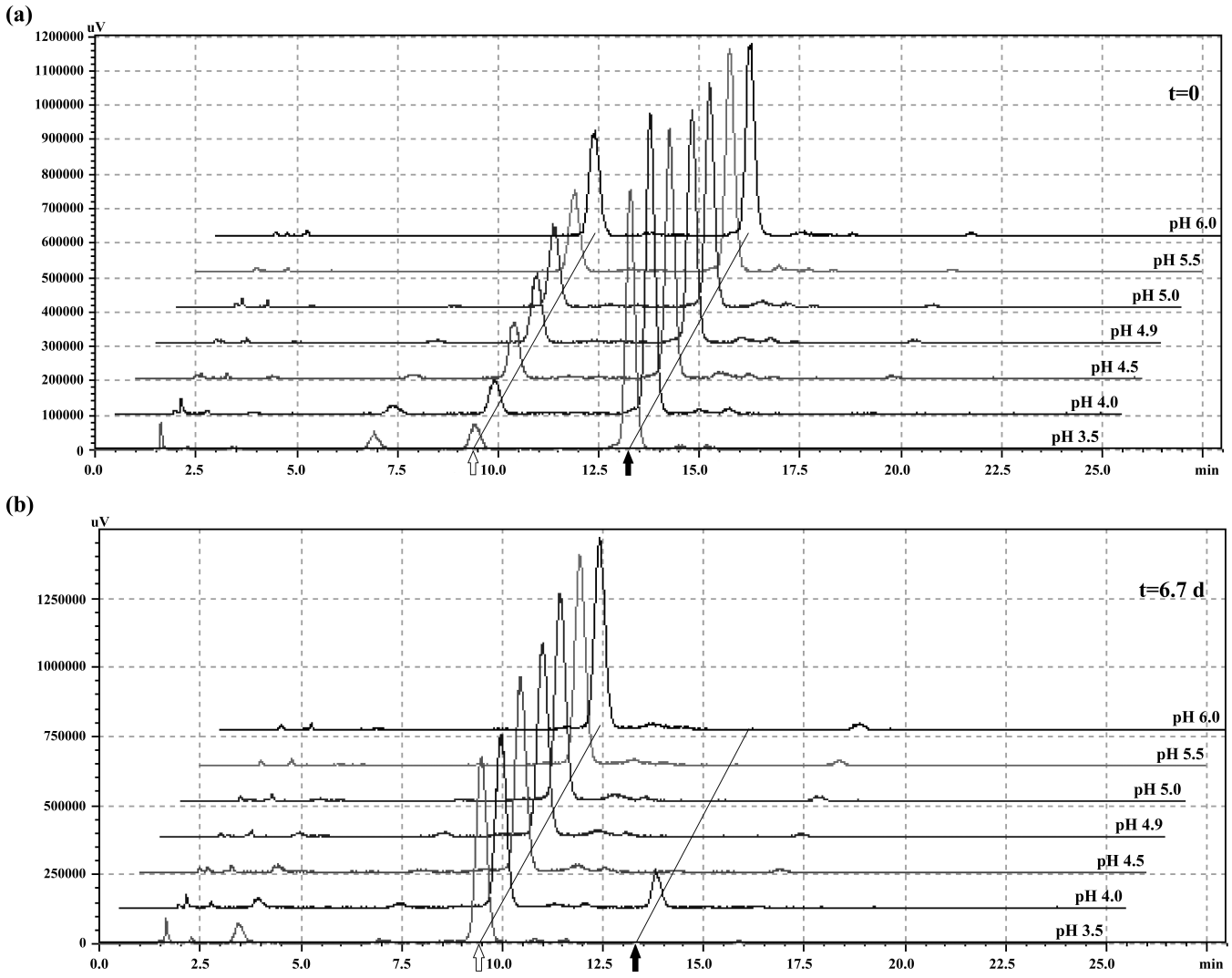

Fig. (5). HPLC radiochromatograms of the labelling reactions at different pH values at (**a**) t=0 and (**b**) t=6.7 days. [^{177}Lu]Lu-DOTA-H2MG11-Met(O) with t_R=9.4 min and [^{177}Lu]Lu-DOTA-H2MG11 with t_R=13.3 min.

Although total free ^{177}Lu was present in low amount, it slightly increased over time for all pH values. The highest percentages were obtained at pH 3.5, reaching the maximum at 6.7 days (4%) and decreasing as the pH increased (Table **1**). The main component within the free ^{177}Lu was the peak with t_R=1.7 min previously assigned to [^{177}Lu]Lu-acetate (also detected by TLC with R_f=0), with the highest values for pH 3.5 (3%) and pH 4.0 (1%) that remained almost unchanged since t=0. This behaviour reinforces the presumption that this species could be [^{177}Lu]Lu-acetate, a weak complex rapidly generated which stabilizes the Lu in aqueous solution while the slower and pH-dependent incorporation reaction of this metal into the DOTA ring takes place. Therefore, from the point of view of the ^{177}Lu incorporation into the peptide molecule, it would be more convenient to carry out the labelling reaction at pH≥4.5.

The LMG molecule decreased extensively over time for the entire pH range studied, but it became more pronounced at pH≥4.5 where it nearly disappeared at 2.9 days (Fig. **6a**). However, at pH values of 3.5 and 4.0, it did so more gradually up to 6.7 days. This instability occurred in concert with the increase (near symmetrical) of its main impurity LMG-Met(O), which was greatly increased over time for all pH values but in a larger proportion at pH≥4.5 (Fig. **6b**). Therefore, at pH 3.5-4.0, this impurity increased progressively achieving 79-73% at 6.7 days, while at pH≥4.5 it increased even more, reaching 83-89% at 2.9 days, maintaining these values at 6.7 days. Considering that this oxidation took place concomitantly with the increase of pH, it could be presumed that the antioxidant power of gentisic acid is greater at lower pH values.

Total peptide impurities (t_R 3.4-18.8 min) showed a slight decrease with the increase in pH at t=0, but did not exhibit a clear trend at t=6.7 days (Table **1**), so they were divided according to the three groups of the previous study (Section 3.1.2) for meticulous analysis.

The first group of peptide impurities with t_R 3.4-8.5 min increased over time for all pH values. Its growth was boosted by the decrease in pH, being predominant at pH 3.5 where it raised from 6% to 11% in 6.7 days (Fig. **7a**). In accordance

with Section 3.1, these more hydrophilic peptide impurities could be the result of greater damage to the main species LMG and LMG-Met(O). This was more evident in the experiment where no antioxidant agents were added (Fig. **4a**), suggesting at this point that gentisic acid (a phenolic acid) would not have a protective effect against free radicals at pH≤4.0. This proposal, although contradicts our previous discussion, would be in agreement with Choe *et al* [22] and Amorati *et al* [23]. They propose that the antioxidant activity of phenolic acids is dependent on the pH, since they are not efficient radical scavengers under acidic pH, but very good scavengers above pH 7 to 8. This is because the phenolic acids are ionized to the phenolated form at basic pH, due to their pK_a of 8 to 9. The phenolated antioxidant has a higher electron-donating capacity than the parent species and activates the phenolic group to give higher free radical scavenging activity [23]. In our conditions, the gentisic acid has pK_{a1}= 2.77 (carboxylic acid), pK_{a2}= 10.01 (phenolic group 1) and pK_{a3}= 10.80 (phenolic group 2) [24]. Probably, at pH 4.5-6.0, this high pK_{a2} value (~10) yields a very low percentage of the phenolate ion to give a higher free radical scavenging activity. Nevertheless, the electrochemical behaviour of the gentisic acid was studied by Sazou *et al* [25]. At pH range 2-5, it did not give any oxidation wave at the mercury electrode, but at pH 5-6, one poorly-defined oxidation-reduction wave appeared, while at pH 6, a well-defined wave was obtained. Therefore, perhaps from a pH as low as 5, we could appreciate an increase in the antioxidant power of gentisic acid.

Moreover, all the different pH solutions underwent a change in colour over time, starting with colourless solutions at t=0 and ending at 6.7 days with yellowish-brown solutions for pH 3.5-4.5 and dark brown for pH 5.0-6-0. According to [26, 27], the quinones formed by the oxidation of the phenols tend to dimerise and polymerise producing brown pigments (browning of the solution) due to a shift in the visible spectrum. Considering this, the greater browning of the solutions observed with the increase in pH could be related to greater oxidation of gentisic acid, which would be in accordance with a higher antioxidant power of this phenol at higher pH.

The second group of peptide impurities, with t_R 10.3-12.8 min, also increased over time for all pH values. However, its formation was favored with the increase of pH, standing out at pH 6.0 with an increment from 4% to 9% in 6.7 days (Fig. **7b**). In accordance with Section 3.1.2, these peptide impurities with intermediate t_R could be the product of minor damage to the LMG radiotracer, probably due to some modification of certain amino acids. Therefore, the increase of this less damaged group of impurities at pH≥4.5 would be in agreement with a minor destruction of the main molecule due to a more effective action of gentisic acid against free radicals when pH is raised. In this second group, the component with t_R of 10.8 min presented the most notable change. It increased over time for all pH values, in a higher proportion at pH≥4.5 and standing out at pH 6.0, rising from 1% to 5% in 6.7 days. Assuming that this impurity was caused by the modification of one or more amino acids, an increase of this species with the pH could be expected due to a greater action of gentisic acid instead of producing other more damaged molecules. This behaviour is quite similar but in a smaller proportion to the nearby impurity LMG-Met(O) (Fig. **6b**) that also presents a minor damage.

At this point, it would be necessary to explain the large increase of LMG-Met(O) at pH≥4.5, when the gentisic acid increases its antioxidant power. If there were the possibility of producing *in situ,* some oxidizing agent that would oxidize the Met residue of the molecule of interest almost selectively, this peculiarity would be explained. It is reported that L-ascorbic acid, widely used as an antioxidant, has a great capacity to neutralize reactive oxygen species [28, 29]. Precisely, in these oxidative processes resulting from the transfer of two electrons, where different molecular radicals intervene and originate, oxidized ascorbic acid (L-dehydroascorbic acid) and H_2O_2 are finally produced. Furthermore, ascorbate can also act as a pro-oxidant, and this behaviour was observed in the presence of iron, producing an effective oxidation of Met to methionine sulfoxide in small peptides [30]. This oxidation of ascorbate catalyzed by metals produces electrons that react with the oxygen contained in the solvent to form superoxide radicals, hydroxyl radicals and H_2O_2. Being the latter, the major responsible for

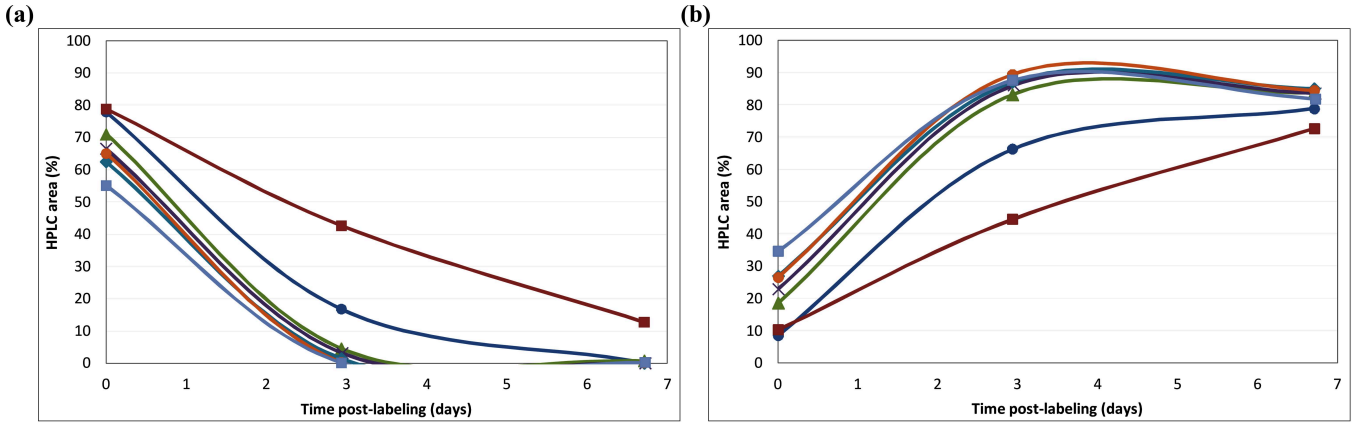

Fig. (6). Evolution of (**a**) [^{177}Lu]Lu-DOTA-H2MG11 and (**b**) [^{177}Lu]Lu-DOTA-H2MG11-Met(O) in the radiolabelling reactions at different pH values over time. pH 3.5 (blue ●), pH 4.0 (red ■), pH 4.5 (green ▲), pH 4.9 (purple ×), pH 5.0 (aquamarine ♦), pH 5.5 (orange ●) and pH 6.0 (light blue ■).

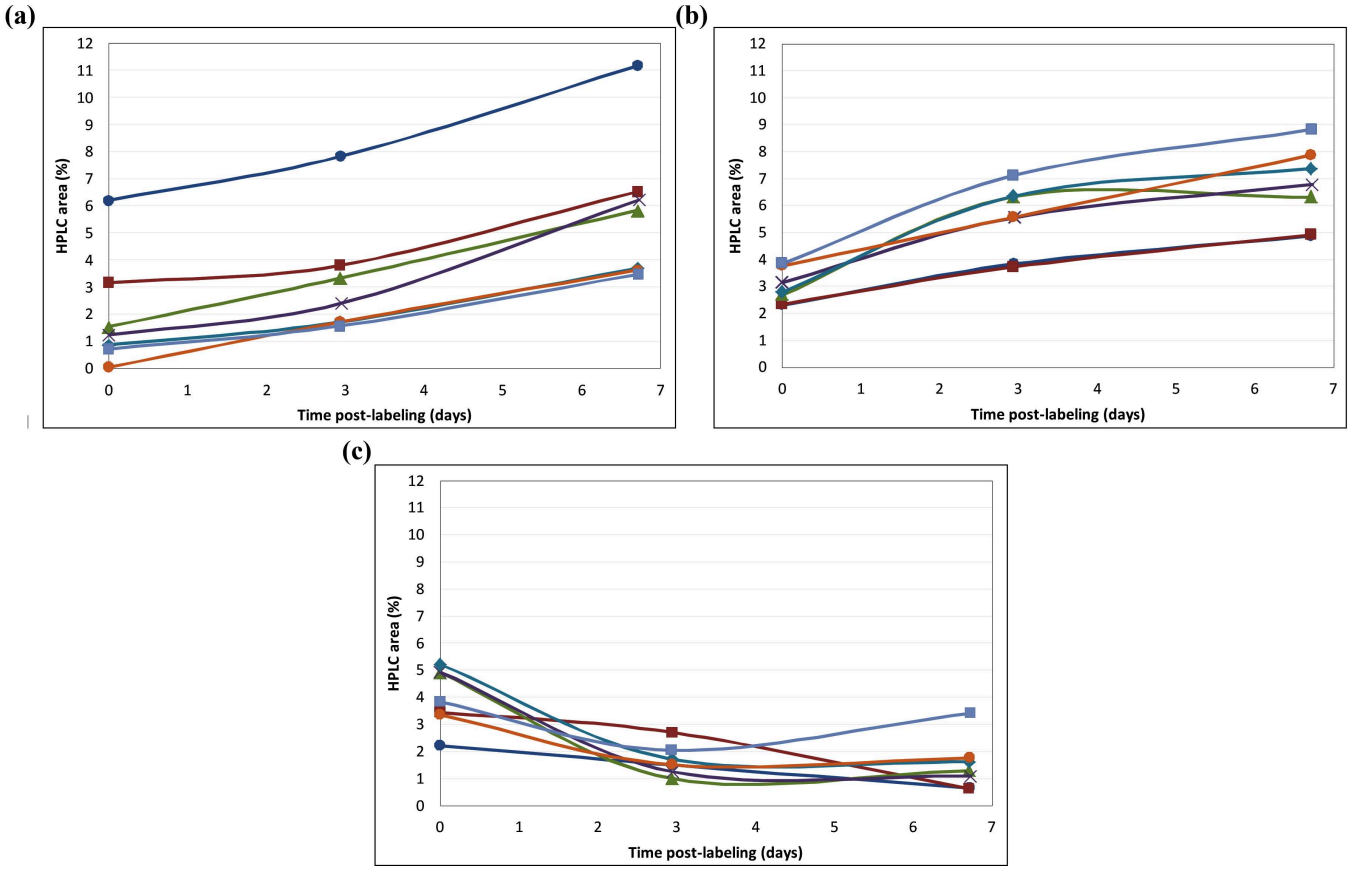

Fig. (7). Evolution of the different groups of peptide impurities over time according to their t_R (a) 3.4-8.5 min, (b) 10.3-12.8 min and (c) 14.5-18.8 min in the radiolabelling reactions at different pH values. pH 3.5 (blue ●), pH 4.0 (red ■), pH 4.5 (green ▲), pH 4.9 (purple ×), pH 5.0 (aquamarine ♦), pH 5.5 (orange ●) and pH 6.0 (light blue ■).

the oxidation of Met to methionine sulfoxide contained in the peptide without the formation of other products of degradation at pH≤7 [31]. It is reported that gentisic acid reacts efficiently with the $^{\bullet}OH$ radical, one of the most deleterious free radicals, with a high reaction rate constant ($k= 1.1x10^{10}$ $M^{-1}.s^{-1}$) measured at pH 7. In the reaction, a hydroxyl radical adduct of gentisic acid (**I**) and a phenoxyl radical of gentisic acid (**II**) are produced:

$C_6H_3(OH)_2COOH + {}^{\bullet}OH \rightarrow {}^{\bullet}C_6H_3(OH)_3COOH$ (**I**), $C_6H_3(OH)(O^{\bullet})COOH$ (**II**) + H_2O

They also found that the hydroxyl radical adduct (**I**) was a reducing radical but the phenoxyl radical (**II**) was an oxidizing radical. Further, the reducing radical (**I**) has been found to transform into the oxidizing radical (**II**) [32]. Therefore, in our labelling reactions, this oxidizing radical could be produced *in situ*, and thus oxidize the Met residue of the LMG molecule while decreases the occurrence of major damage to the rest of the peptide molecule. In addition, the increase in pH would enhance the antioxidant activity of gentisic acid, thus producing more oxidizing radical (**II**) that would oxidize more Met residues, what could explain the large increase of the main impurity (LMG-Met(O)) with the pH.

On the other hand, the radiolysis of water itself produces H_2O_2 *in situ* [33-35] which could oxidize selectively the Met residue of the LMG, as was achieved by incubation of the non-labelling peptide with H_2O_2 (Section 2.3.3). This could explain the initial formation of LMG-Met(O) in the previous section without the addition of antioxidant agents (Fig. **3a**), with subsequent transformation into more damaged molecules due to the destructive non-stabilized free radicals. While in the presence of gentisic acid, the LMG-Met(O) could be continuously produced and preserved due to the free radical scavenging activity. Perhaps, the selective oxidation of the Met residue by the H_2O_2 could also increase with the pH in our conditions, making our system even more complex.

Finally, the impurities of the third group with a longer t_R than the LMG peak (14.5-18.8 min) decreased over time for all pH values but without a clear tendency with respect to this parameter. The maximum value at t=0 was presented at pH 5.0 (5%) while at t=6.7 days was at pH 6.0 (3%) (Fig. **7c**). According to the previous section, it was found that peptide impurities of higher t_R were in the lowest amount, and because of their behaviour, they would most likely be similar to the molecule of interest, being probably modified by aggregation via covalent crosslinking or by hydrophobic interactions.

To conclude, from the point of view of the total peptide impurities (not considering LMG-Met(O)), it would be more convenient to carry out the radiolabelling at pH 5.0-5.5.

The highly radiation-sensitive molecule studied probably requires the addition of a third antioxidant to the labelling such as ascorbic acid, since it has a synergistic effect with certain phenols. Therefore, the quinones formed by oxidation can react with ascorbic acid, reducing them to their original phenols, regenerating them and thus contributing to a greater antioxidant effect [22, 27, 32].

CONCLUSION

The gentisic acid greatly impeded the generation of the most part of peptide impurities over time, except for the main impurity, that was unexpectedly increased due to selective oxidation of the Met residue of the radiolabelled peptide, which was effectively decreased by the addition of L-methionine. This selective oxidation could be induced by the oxidizing species produced in the reaction between gentisic acid and free radicals of water, during the protection of the radiolabelled peptide from the attack of these harmful species. The antioxidant capacity of the gentisic acid turned out to be pH-dependent, being a better radical scavenger at pH>4.5. Higher incorporation of ^{177}Lu into the molecule was achieved at pH 5.0-5.5 in addition to a lower formation of peptide impurities due to gentisic acid. Therefore, the pH would not only be an important factor for the radiometal incorporation into the molecule, but also for the efficiency of the stabilizer necessary for its protection, a fact not generally considered.

Due to the high sensitivity to radiation presented by this peptide molecule, where 6 out of a total of 10 amino acids are susceptible to oxidation, including the Met residue, it was possible to observe the antioxidant and oxidant effects of the gentisic acid.

AUTHOR CONTRIBUTIONS

Victoria Trindade designed and carried out the experiments, analyzed the data and prepared the original draft. Henia Balter and Victoria Trindade reviewed and edited the article.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are the bases of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and analyzed during the current study are available from the corresponding author, [V.T.], on reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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