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Il Farmaco 57 (2002) 845–852

IL FARMACO

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Advanced glycation end-products/peptides: a preliminary investigation by LC and LC/MS

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Received 7 June 2002; accepted 20 July 2002

Abstract

An investigation on AGE-peptides, originating by proteolysis of in vitro glycated proteins, was carried out by LC methods with different detection applied to the mixture produced by proteinase K digestion of in vitro glycated human serum albumin (HSA). Classical approaches, like spectroscopic (UV, fluorescence) and mass spectrometric methods (MALDI, LC/ESI/MS), show that the digestion mixture is highly complex. However, there are clearcut differences between the digestion mixtures of glycated and unglycated HSA, in the former case allowing identification of possible glycated peptides belonging to the AGE-peptide class. MS/MS experiments on selected species seem to be promising as regards structural information.

Keywords: Non-enzymatic glycation; LC/ESI/MS; LC/ESI/MS/MS; MALDI/MS

1. Introduction

The Maillard reaction [1] occurring between sugars and amino groups is relevant in living systems. When amino groups belonging to protein chains are involved, the Maillard reaction has been invoked as responsible for protein crosslinking and the production of 'toxic' compounds [2]. The reaction leads to the production of a heterogeneous group of substances, usually called advanced glycation end-products (AGE) [3]. For example, small carbohydrate moieties obtained by oxidation and peptides (arising from protein digestion) containing sugar-derived substructures may be considered as AGE. Some AGE compounds have been structurally identified e.g. *N*-carboxymethyllysine (CML) [4], pentosidine [5,6] as well as some AGE precursors (3-deoxyglucosone [7], methylglyoxal and its metabolites [8]).

Oxidation through the Maillard reaction pattern has also been proposed as a source of irreversible damage to proteins leading to AGE-modified proteins [9–12].

As AGE-modified proteins are chemically altered, biological mechanisms are consequently required for their recognition and turnover [13]. In fact, as proven by the researches of Gugliucci and Bendayan [14], they are not usually excreted at the kidney level. Their digestion by macrophages, lysosome-containing cells or extracellular proteolitic systems [15,16] leads to the production of the so-called *AGE-peptides*, which are only partially and selectively excreted by the kidney. In cases of renal failure, their excretion is impaired, so that AGE-peptides accumulate in plasma, leading to toxin production, strong interactions with basal membrane, and bonding to lipoprotein and collagen. The two last processes may be responsible for functional alterations (atherosclerosis) and crosslinks, respectively [17].

However, AGE-peptides have not yet been structurally defined, and the term AGE-peptides is currently applied to AGE products present among the circulating peptides isolated by ultrafiltration of plasma with

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0014-827X/02/$ - see front matter
PII: S0014-827X(02)01305-8

membranes at a molecular weight cut-off of 10 000 Da [14,18–20].

In order to elucidate the fate of AGE proteins and AGE-peptides in renal tissues, Gugliucci and Bendayan [14] undertook a study based on in vitro glycation of BSA and its further digestion by proteinase K. In these conditions, it was proposed that AGE-peptides similar to those observed in in vivo conditions would be produced. The digestion products, possibly glycated peptides, were characterized by spectrophotometry, spectrofluorimetry, chromatography, SDS-PAGE and RIA. This research highlighted the production of AGE-peptides, but only little information on their structure was gained by the analytical approaches employed. It is to emphasize that this experimental design lead to albumin heavily modified, about 100 times greater than the average albumin molecule in plasma. However, even if the peptide maps so obtained are surely not the same coming from in vivo AGE-peptides, this may be considered a valid model to achieve preliminary information.

In order to increase the amount of information available on AGE-peptides, and to verify whether in vitro produced AGE-peptides (i.e. glycated BSA digested with proteinase K) represents a valid model for in vivo AGE-peptides as described to date, we undertook a previous investigation [21] based on highly specific techniques to determine definitely the molecular weight distribution of AGE-peptides obtained by in vitro glycation of BSA followed by digestion with proteinase K, according to the procedure of Gugliucci and Bendayan [14].

In view of the valid results obtained by Matrix-Assisted Laser Desorption Ionization/Mass Spectrometry (MALDI/MS) and Liquid Chromatography/Electrospray Ionization/Mass Spectrometry (LC/ESI/MS) in the study of glycation levels in both in vitro and in vivo glycated proteins [22–28], and considering that LC and LC/ESI/MS are useful in the identification of AGE compounds of very low molecular weight in both in vitro and in vivo conditions [27,28], the present investigation was based on both LC, LC/ESI/MS and MALDI of in vitro glycated human serum albumin (HSA) followed by digestion with proteinase K. The data used to evaluate in vitro AGE-peptides were compared with those deriving from commonly employed UV–Vis and fluorimetric detectors.

2. Experimental

All reagents and solvents were used as purchased without further purification.

2.1. Glycation of human serum albumin

In vitro glycation of pure HSA was carried out with glucose in pseudophysiological conditions according to a published procedure [21]. Briefly, HSA (Sigma, St. Louis, MO, USA) (100 mg ml^{-1} in 0.005 M phosphate buffer, pH 7.4, containing 5 mM sodium azide as a bacteriostatic) was incubated with 0.5 M D-glucose (Sigma) at 37 °C for 0 and 90 days (samples **1** and **2**, respectively). After incubation, unreacted glucose and buffer were voided by dialysis against bidistilled water, and samples were lyophilised and stored at −20 °C. Control HSA was incubated in the same conditions without the addition of glucose, providing compounds **1a** and **2a**.

2.2. Preparation of AGE-peptides

AGE-peptides were prepared by proteolysis of in vitro glycated HSA, according to the literature [14]. Glycated HSA (5 mg, sample **2**) were incubated with 0.1 mg of proteinase K (EC 3.4.21.64, Sigma) at 37 °C for 20 h [on phosphate buffer saline (PBS) at pH 7.4]. The digested material was centrifuged through a Centricon-10 membrane (Millipore Corporation, Bedford, MA, USA; MW cut-off 10 000 Da) at $4000 \times g$ for 3 h, in order to separate the high molecular weight fraction (retentate, MW > 10 000 Da, undigested HSA and proteinase K) from the low molecular weight one (ultrafiltrate, MW < 10 000 Da, AGE-peptides, sample **3**). Control peptide samples (samples **3a**) were obtained by identical proteolysis of control HSA.

2.3. LC

Ultrafiltrate (10 μl) obtained after centrifugation through the Centricon 10 membrane were applied via loop to a Jupiter C18 (Phenomenex, Torrance, CA, USA) 250 × 2 mm, 5 μm, 300 Å column, protected by a Widebore C18 (Phenomenex) 4 × 2 mm guard cartridge. Separation was achieved via gradient elution of two solvents (A: LC grade water with 0.1% trifluoroacetic acid; B: LC grade MeCN with 0.1% trifluoroacetic acid) at a flow rate of 0.2 ml min^{-1}. The gradient profile for solvent B was as follows: 15%, 1 min; 15–45% in 34 min, 45%, 5 min; 45–95% in 10 min, 95%, 1 min. The LC pump was a Spectra Series P4000 (Thermo Separation Products, San Jose', CA, USA).

In order to perform on-line LC/UV and LC/MS, after elution from the column the flow was split into two aliquotae, of which one supplied to the ESI source of the mass spectrometer (see below) and the other to a programmable UV detector (UV 1000, Thermo Separation Products). The UV was read at 280 nm for peptides or 350 nm for browning products.

2.4. ESI/MS

Half the flow eluting from the LC column was supplied to the electrospray ion source of an LCQ (ThermoQuest Corporations, San Jose', CA, USA) ion trap mass spectrometer operating in positive ion mode. ESI parameters were: source voltage: 3.3 kV; sheath gas (nitrogen) flow: 40 (arbitrary units); auxiliary gas (nitrogen) flow: 10; capillary temperature: 220 °C; capillary voltage 3 V.

The ion trap mass spectrometer was scanned in full scan mode from 220 to 2000 Th at a rate of 2.64 s × scan.

2.5. ESI/MS/MS

To elucidate the structure of some ionic species detected in the LC/MS runs, LC/MS/MS experiments were performed, exploiting the MS^n potentials of the ion trap mass spectrometer. Specific ion species produced by ESI in the source were isolated in the ion trap by application of appropriate rf voltages. These selected species (generally MH^+) were made to collide with He by application of a supplementary rf voltage, in order to obtain collisionally induced dissociations. The resulting spectra were then acquired by scanning the main rf voltage. In the present case, the ion species of interest were detected in a LC/MS run with full scan acquisition; subsequently, a further LC run was performed for the same samples with the instrument set to operate in MS/MS mode (isolation width: 4 a.m.u., collision energy: 35% of maximum available collision energy, corresponding to a tickle voltage of 1.75 V).

2.6. MALDI

MALDI measurements on samples **1**, **2**, **1a**, **2a**, **3** and **3a** were made by a REFLEX time-of-flight mass spectrometer (Bruker, Bremen, Germany) equipped with a SCOUT ion source, operating in the linear positive ion mode. Ions formed by a pulsed UV laser beam (nitrogen laser, $\lambda = 337$ nm) were accelerated to 25 keV. The UV laser light (energy about 50 μJ) was focused on the sample, using a focal diameter of 100–150 μm and sinapic acid as matrix.

For MALDI measurements, samples were dissolved in 0.1% trifluoroacetic acid at a concentration of about 6 mg ml^{-1} and diluted fivefold with the same solvent. A 5-μl volume of the final sample solution was added to 5 μl of the matrix solution, and about 1 μl of this mixture was deposited on the stainless steel probe and allowed to dry before being introduced into the mass spectrometer. Mass spectra were obtained and averaged over 10 shots; three independent MALDI measurements were made for each sample, to evaluate reproducibility. A mass accuracy ranging from 0.01 to 0.1% was always obtained. External mass calibration was performed using pure BSA MH^+ (66 431 Th) and $[M+2H]^{2+}$ species (33 216 Th) to determine intact HSA and glycated HSA.

All the experimental procedures have been repeated for at least five times, leading to results practically superimposable.

3. Results and discussion

In a previous investigation [21], based on in vitro glycation of BSA followed by proteinase K digestion of glycated and unglycated protein, LC and LC/ESI/MS proved to be valid analytical tools, able to highlight differences in composition of the very complex mixtures originating after enzymatic digestion.

In the present study, the same analytical approaches was used to evaluate AGE-peptides deriving from enzymatic digestion of HSA which, necessarily, are structurally close to those present in humans. The glycation procedure proposed and employed by Gugliucci and Bendayan [14] was carefully followed and the glycated protein and the related digestion products were analysed with highly specific techniques.

The analytical strategy is summarised in the flow chart of Fig. 1: HSA was glycated, as in the previous study of Gugliucci and Bendayan, in pseudophysiological conditions with glucose 0.5 M. The choiche of this high glucose concentration, quite far from the physiopatological ones (50–20 mM) was done in order to enhance the yield of glycation processes. Firstly, a sample was drawn after 90 days of incubation and

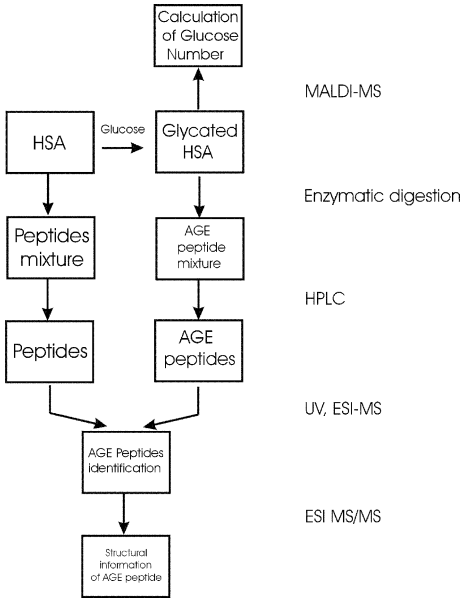

Fig. 1. Flow chart of the analytical strategy followed in the present investigation.

analysed by MALDI/MS. This technique yielded the molecular weight of glycated HSA (sample **2**, *m/z* 71 350), which was compared with that of untreated HSA (sample **1**, *m/z* 66 680). As the addition of a glucose molecule to the protein leads to a mass increase of 162 Da, it was possible to calculate the number of glucose molecules condensing on the protein, simply by dividing the mass difference between the two proteins (71 350–66 680 = 4670) by 162: 4670/162 = 28.8. This simple calculation shows that 29 glucose molecules condensed on HSA. This datum is in agreement with those previously obtained in the case of BSA (showing the condensation of 33 glucose molecules after 90 days of incubation with glucose): in other words, BSA and HSA exhibit a similar final degree of glycation, fitting the small primary structural differences between the two proteins. The procedure, repeated for further four times, led to a mean value of 71 312 ± 50 Da for molecular weight of glycated HSA and 66 654 ± 52 Da for genuine HSA.

UV data support the MALDI ones, showing an increase in absorbance at 350 nm, typical of glycation products.

The two sets of proteins were digested by proteinase K, following the procedure described in Section 2, and the digestion mixtures were subsequently analysed by LC. The MALDI spectra of the two mixtures do not show the presence of the protonated proteins, indicating the occurrence of extensive digestion processes. The chromatograms of the digestion products of one sample of untreated HSA obtained by MS and UV detection systems are shown in Fig. 2. Detection by UV at 280 nm, typical for tyrosine-tryptophan residues, was choosen in order to obtain a simpler LC run and it leads to a well-defined chromatogram (see Fig. 2b), revealing many chromophoric species with retention times lower than 30 min. Absorbance at 350 nm, typical of browning products (Fig. 2c), gives a poorly significant chromatogram, most species detected being in the retention time range of 15–25 min.

Lastly, ESI mass spectrometry gave the chromatogram shown in Fig. 2a, which clearly highlights the great complexity of the digestion mixture.

The other four samples led to chromatograms superimposable with those reported in Fig. 2, proving the good reproducibility of the procedure.

The same approaches, applied to the digestion products of one sample of glycated HSA, give rise to the chromatograms shown in Fig. 3. Worth noting is the great increase in absorbance at 350 nm, proving the production of large amounts of browning products (see Fig. 3c; cf. Fig. 2c). Although the chromatographic trace at 280 nm (Fig. 3b) is very similar to that obtained for unglycated proteins (Fig. 2b), it has significant differences, proving that glycation promotes the formation of various digestion products and suggesting that the enzyme activity on glycated HSA is different from that observed for unglycated proteins. Mass spectrometry clearly describes the complexity of the digestion mixture: the number of digestion products is so high that, apart from a few well-defined peaks, most of the products are unresolved, leading to a general rise in the base-line. Again the other four samples led to analogous results.

For more information on AGE-peptides, the mass spectra obtained at various retention times for both glycated and unglycated samples were compared. This comparison shows the presence of a wide number of components in both sample, and allows to put in evidence analogies and differences between the digestion products of genuine and glycated HSA. Those detected only in the digestion mixture of the latter could be, in principle, considered as possible AGE-peptides.

The most important results are listed in Table 1, in which the relative abundances (RA) of the different species are subdivided in three classes: +, 3–10%; ++, 11–60%; +++, 61–100%. The digestion products detected for both samples are not reported and only the species characteristic for glycated and unglycated HSA are listed. Peptides with molecular weight ranging from 455 to 1918 Da have been detected. Interestingly, some components detected in the unglycated sample are not found in the glycated one. At first sight it could be hypothesised that these digestion products could be substituted, in the case of glycated HSA, by the glycated analogs. But, looking at the related molecular weights reported in Table 1, this explanation results to be wrong. Consequently, the data reported in Table 1 can be rationalised considering that the enzymatic digestion processes of glycated HSA are, in some extent, different from those of the genuine protein. However, the situation is even more complicated than that described until now. In fact it should also be emphasized that, in some cases, isobaric species are present in both glycated and unglycated samples, but at very different retention times, proving that they are structurally different molecules. As examples, Figs. 4 and 5 show the case of ions at *m/z* 573 and 1201. In the former (unglycated sample), the reconstructed ion chromatogram shows two main peaks at retention times of 5.50 and 22.35 min; in the latter, these peaks practically disappear and two others (retention times of 10.02 and 11.57 min) are detected.

The ion at *m/z* 1201 behaves in a similar fashion (Fig. 5). In the case of glycated HSA, peaks at retention times of 28 and 29 min disappear, and a new peak appears at a lower retention time (7.25 min).

These results clearly exemplify the great complexity of the digestion mixtures; however, some general considerations may be made.

Summarising, the differences observed in the digestion products of glycated and unglycated HSA may be explained by at least two mechanisms:

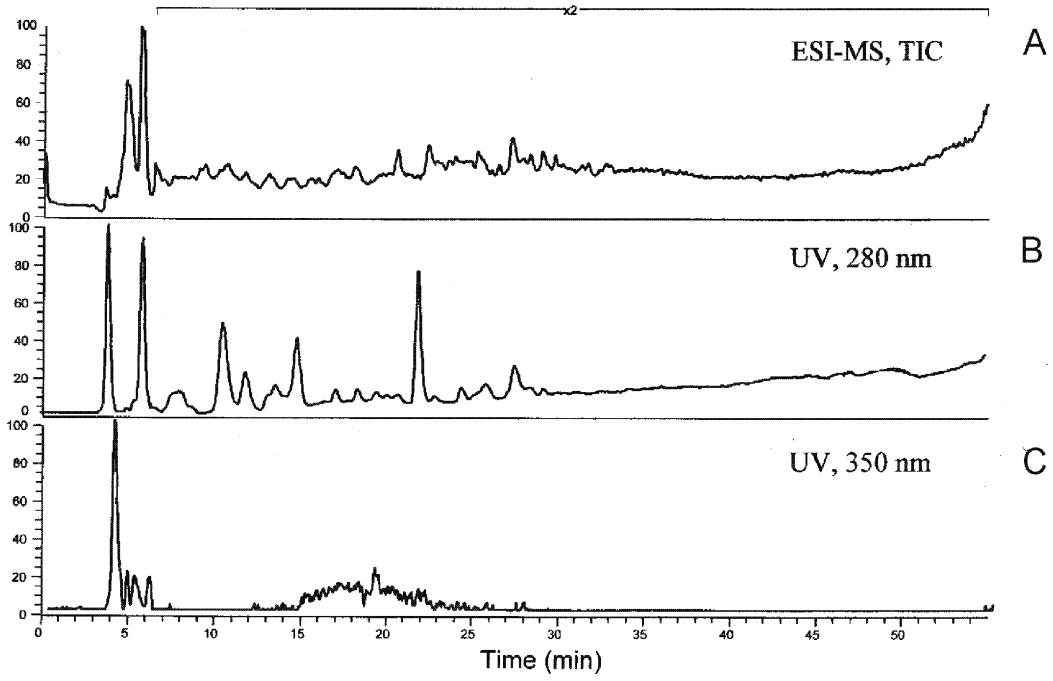

Fig. 2. Chromatograms of the products obtained by digestion with proteinase K of untreated HSA, as detected by: (a) ESI/MS; (b) UV detection at 280 nm; (c) UV detection at 350 nm.

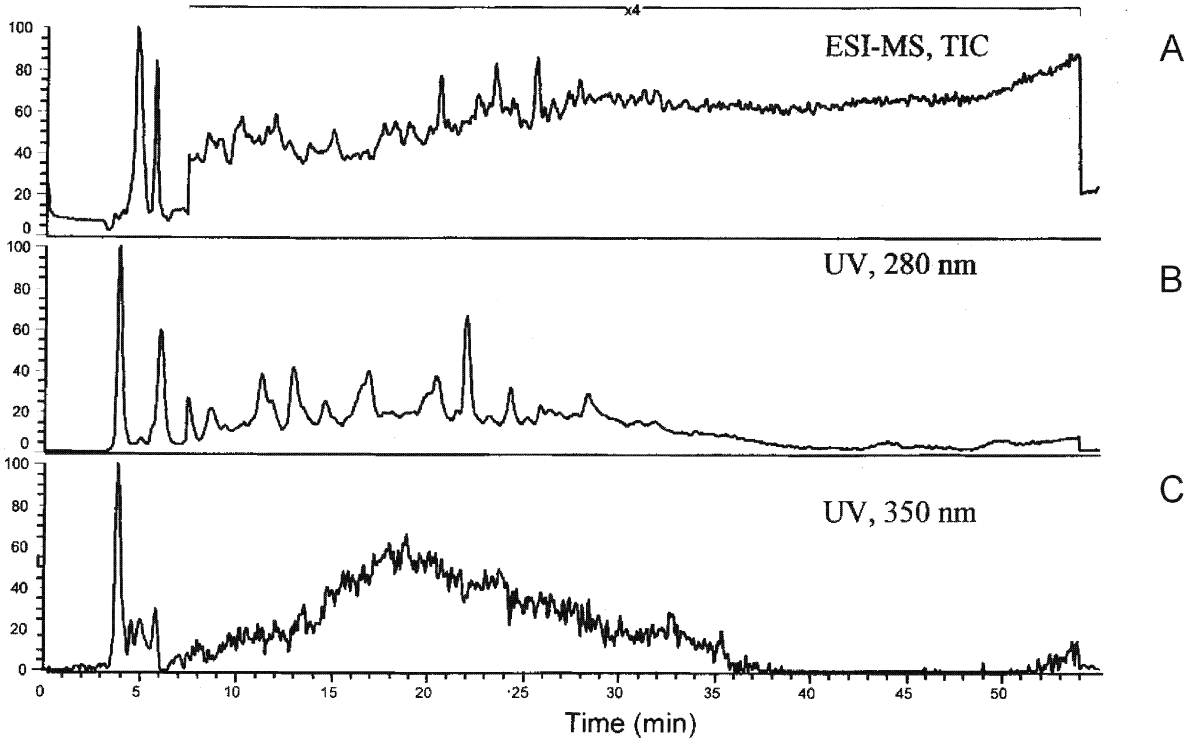

Fig. 3. Chromatograms of the products obtained by digestion with proteinase K of in vitro glycated HSA, as detected by: (a) ESI/MS; (b) UV detection at 280 nm; (c) UV detection at 350 nm.

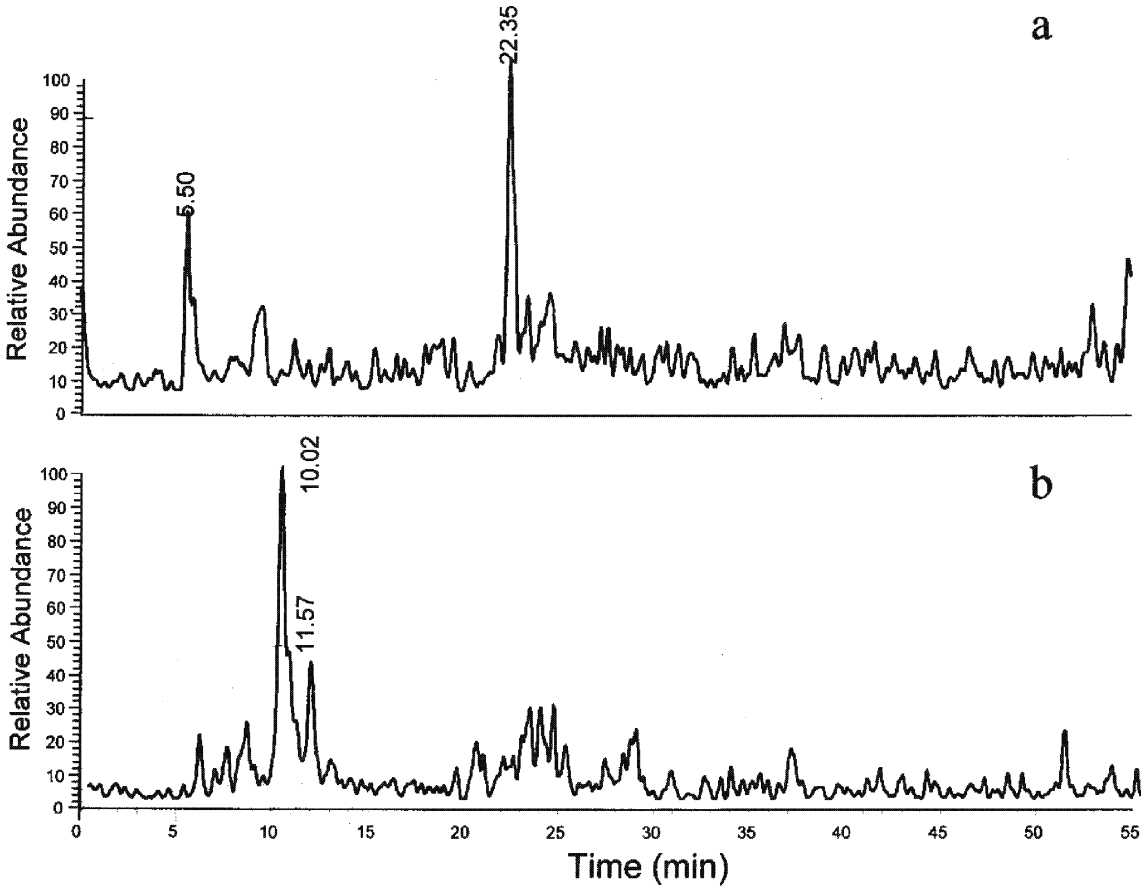

Fig. 4. Reconstructed ion chromatograms of the species at *m/z* 573 detected among the digestion products of: (a) untreated HSA; (b) glycated HSA.

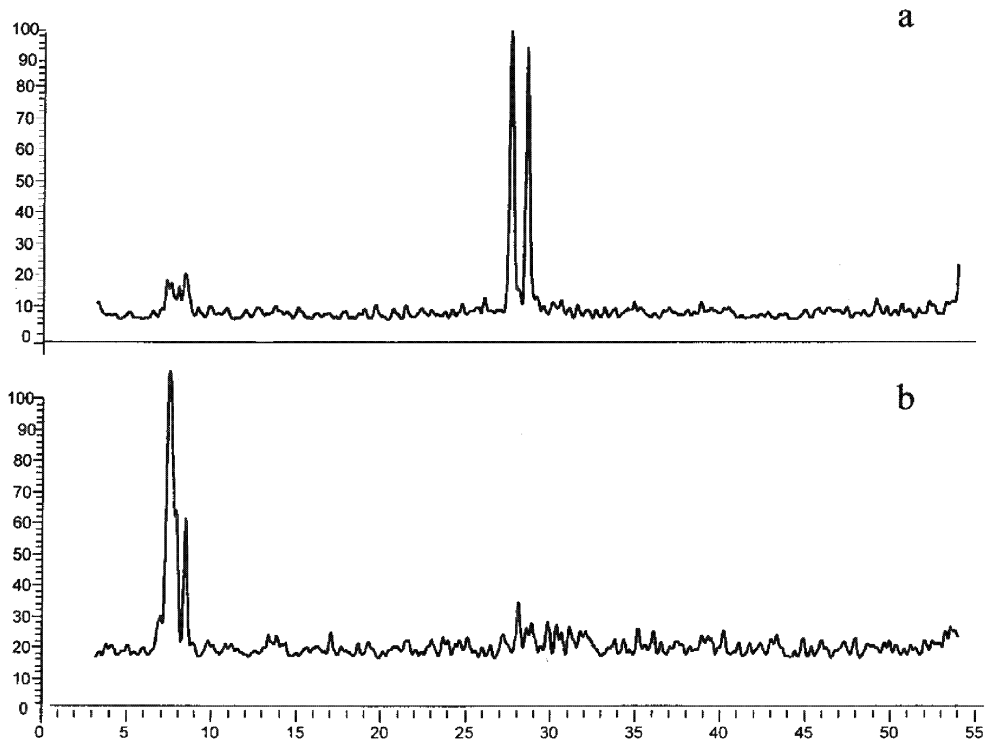

Fig. 5. Reconstructed ion chromatograms of the species at *m/z* 1201 detected among the digestion products of: (a) untreated HSA; (b) glycated HSA.

Table 1
Relative abundances of the different species detected in the total ion chromatograms of the digestion mixtures of glycated and control HSA

Retention times (min)	Ionic species	Glycated HSA	Control HSA
5.99	1423		+
10.02	573	+	
10.55	761		+
12.55	463	+	
13.15	668	++	
16.63	613		+++
16.83	613		+
16.86	1088		++
16.86	1088		++
18.26	613	++	
18.85	622	++	
20.63	649	+	
22.35	573		+
22.41	1068		+
25.24	748		+++
25.24	603	+	
25.57	910	++	
25.57	456	++	
27.23	779	+	
27.42	1201		+++
27.85	1180	+	
28.5	913	+	
29.75	1171		+
32.61	1149	+	
33.52	1077		+
35.35	1179		+
39.21	1919		++
41.6	721		++
45.17	767	++	

+, RA 3–10%; ++, RA 11–60%; +++, RA 61–100%.

i) assuming that the reactivity of proteinase K is the same against both HSA and glycated HSA, the differences in mass between the two sets of peptides (see Table 1) must be ascribed to glyco-oxidation processes occurring at the aminoacid side-chains. In other words, proteinase K reacts by hydrolysing the peptide bonds of glycated HSA, producing oligopeptides structurally different from the similar ones obtained by enzymatic hydrolysis of unglycated HSA;
ii) the presence of glycooxidation products on the side-chains of basic aminoacids somehow alters the reactivity of proteinase K towards HSA peptide bonds, inhibiting or favouring some specific peptide bond cleavages, unlike those present in the case of unglycated proteins.

Of course, both mechanisms (i) and (ii) may act simultaneously, thus hindering the task of identifying oligopeptides on the basis of their molecular weight only.

Attempts were made by MS/MS to identify the species present only in the digested mixture from glycated HSA, and some information was obtained by this approach. As an example, the ion at *m/z* 463, detected among the digestion products of glycated HSA (Fig. 6), shows a fragmentation pattern in agreement with a glycated peptide nature. In fact on one hand the favoured formation of an ion at *m/z* 337, corresponding to the loss of a neutral species of 126 u is observed. This substructure cannot be explained by a peptide residue

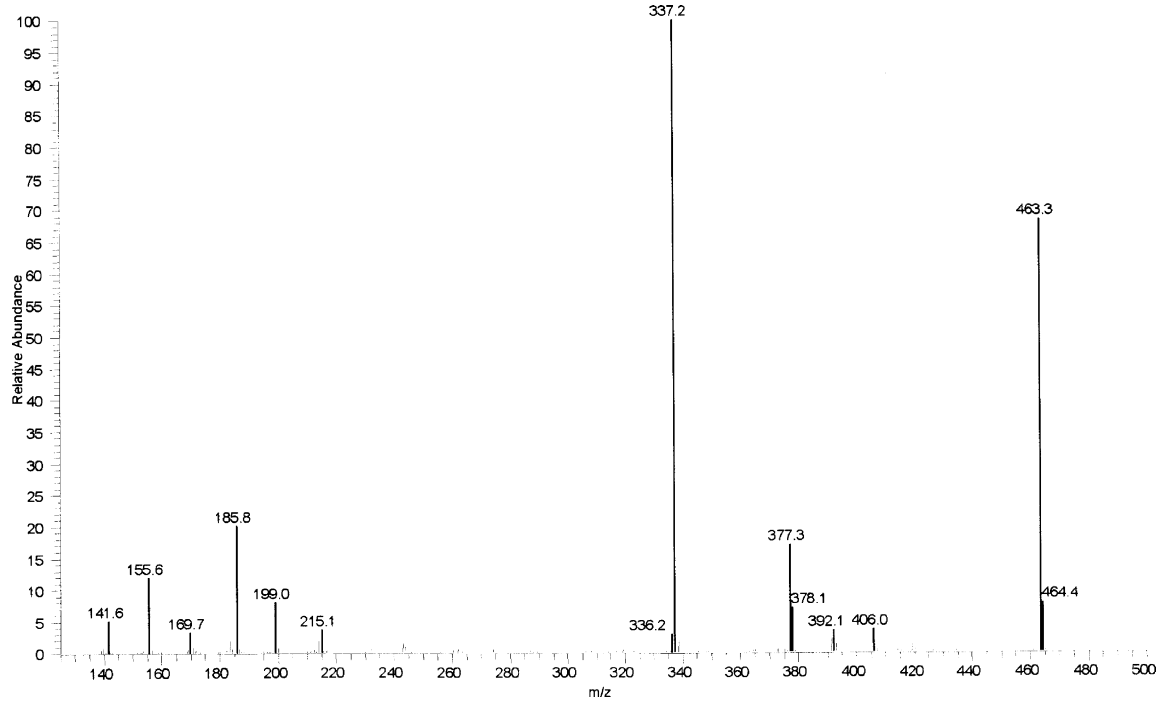

Fig. 6. MS/MS spectrum of the ionic species at *m/z* 463 dedected in the ESI spectrum of the hydrolysis products of glycated HSA.

but by the presence of a dehydrated glucose molecule ($180-3\times18=126$). On the other hand the loss of a species of 86 u, leading to the ion at m/z 377, indicating the presence of a terminal serine, is a good evidence of the peptide nature of this molecule.

The loss of the species of 126 u has been observed also in the collisional spectra of the abundant ions at m/z 668, 613 and 622, detected in the digestion mixture of glycated HSA. On the contrary, it was never observed among the collisionally induced processes of the ionic species characteristic for the control HSA.

The results obtained by LC and LC/ESI mass spectrometry on the mixture originating from enzymatic digestion of glycated HSA, described and discussed here, show that, although some structural information can be obtained, a more specific approach must necessarily be employed for more definitive structural information on AGE-peptides. For this aim, accurate mass measurements to determine elemental formulae seem to be highly promising and work is in progress along these lines.

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