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Mass spectrometric analysis of N-carboxymethylamino acids as periodate oxidation derivatives of Amadori compounds Application to glycosylated haemoglobin

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Summary. Periodate oxidation of free and protein-bound Amadori compounds formed by the condensation of reducing sugars with primary amino groups generates, on acid hydrolysis, N-carboxymethyl derivatives of amino acids. The analysis of these modified amino acids may be used to estimate both the extent and the site of protein glycosylation. The present study describes the use of gas chromatography-mass spectrometry (GC/MS) and gas chromatography-tandem mass spectrometry (GC/MS/MS) for the identification of the various N-carboxymethylamino acids. Application of this approach to the quantitation of N-carboxymethylvaline and N^{ε}-carboxymethyllysine resulting from the oxidation of glycosylated haemoglobin is presented.

Keywords: Amino acids – Amadori compound – Glycosylation – Haemoglobin – Tandem mass spectrometry

Introduction

Non-enzymatically glycosylated (or glycated) proteins are formed by the condensation of free amino groups with reducing sugars and subsequent Amadori rearrangement (Hodge, 1953; Mauron, 1981; Furth, 1988a,b). The periodate oxidation of these modified proteins result in the formation, after acid hydrolysis, of N-carboxymethylamino acids (CM-AAs) derivatives (Badoud et al., 1990).

The analysis of CM-AAs permits investigation of both the extent and the site of protein glycosylation. In food for instance, the formation of N^{ε}-carboxymethyllysine (ε-CM-Lys) as a result of the oxidative cleavage of N^{ε}-(deoxyketosyl)-lysine formed by the reaction of ε-lysino groups with glucose is of special concern. It gives a measure of the degree of blockage of the essential amino acid lysine and, at the same time, the decrease of the nutritive value of the protein (Mauron, 1981).

Apart from lysine, free and N-terminal amino acids may also be glycosylated. This may lead to modifications of the functional properties of proteins, which can have important consequences in biological systems (Furth, 1988a,b; Finot et al., 1990).

As an example, the level of glycosylation of certain proteins (haemoglobin, glycogen, lens crystallin, albumin, etc.) is higher in diabetic patients (Monnier and Cerami, 1982). For these reasons there is a great interest in a straightforward method to evaluate the extent of non-enzymatic protein glycosylation.

In a previous publication we proposed a method to measure the mono- and bis-carboxymethyl derivatives of lysine in milk products (Badoud et al., 1991). These compounds were analysed by gas chromatography after formation of the ethyl ester N-ethoxycarbonyl derivatives using very mild derivatization conditions, i.e. reaction with ethyl chloroformate at room temperature (Husek, 1991). Unfortunately these derivatives were difficult to analyse in complex mixtures even using a selective mass spectrometric detection due to their low molecular weights.

In this communication we describe the use of gas chromatography-mass spectrometry (GC/MS) and gas chromatography-tandem mass spectrometry (GC/MS/MS) for an unambiguous characterisation of the CM-AAs using the isobutyl ester N,O-pentafluoropropionyl (iBu-PFP) derivatives (Desgres et al., 1979; Moodie et al., 1989). Advantages and drawbacks of this type of derivative are discussed. Application of the method to the determination of glycosylated amino acids in normal and abnormal haemoglobin is presented.

Experimental

Materials

N-Carboxymethyl derivatives of alanine (CM-Ala), valine (CM-Val), leucine (CM-Leu), isoleucine (CM-Ile), phenylalanine (CM-Phe) and the mono and bis derivatives of lysine (α-CM-Lys, ε-CM-Lys and bis-CM-Lys) were synthesised according to the procedure of Kihlberg et al. by the reaction of the corresponding amino acids with glyoxylic acid (Kihlberg et al., 1983). All compounds were fully characterised by NMR and mass spectrometry and gave satisfactory elemental analyses. N-Carboxymethylglycine (CM-Gly, iminodiacetic acid), periodic acid and pentafluoropropionic anhydride were purchased from Fluka (Buchs, Switzerland).

Gas chromatography/mass spectrometry

Mass spectra were acquired with a Finnigan MAT 8430 double focusing mass spectrometer (Bremen, Germany) interfaced with an HP-5890 gas chromatograph (Hewlett Packard, Geneva, Switzerland) with an HP-7673A autosampler. The instrument was equipped with capillary columns DB-5 or DB-1701 (30 m × 0.32 mm I.D., J&W Scientific, Folsom, CA, U.S.A.) which were operated in the splitless injection mode at 250°C. The oven was programmed from 50°C (1 min.) to 200°C at 30°C/min. and from 200 to 300 at 5°C/min. Helium was used as carrier gas (10 psi). Electron impact mass spectra were recorded at 70 eV and positive chemical ionisation mass spectra were obtained with ammonia as reagent gas.

GC/MS/MS experiments were performed on a Finnigan TSQ-700 triple quadrupole mass spectrometer. Positive chemical ionisation using ammonia as the reagent gas was used to generate $[M + NH_4]^+$ ions, analysed by collision-induced dissociation (CID) experi-

ments. A collision energy of 6 eV in the laboratory frame was used, with argon, the collision gas, set to 1.0 mtorr.

Sample treatment

Lyophilized glycosylated haemoglobin, normal and abnormal controls, supplied by Pierce (Rockford, IL, U.S.A., prod. #41005), were reconstituted in 1.0 ml of water. An aliquot (100 μl) was placed in a 5 ml hydrolysis tube followed by 100 μl of 100 mM periodic acid and the mixture was well homogenised. After 2 h. at room temperature, 50 μl of 2 N sodium thiosulphate was added followed by 37% HCl and water to make the final concentration 6 N in HCl. The mixture was then hydrolysed at 110°C for 23 h. After evaporation, the residue was reconstituted in water and the solution applied to a small column (20 × 4 mm I.D.) of Dowex 50W-X4. The resin was washed with 10 ml of water and the compounds eluted with 2 ml of 3 M ammonia solution. After evaporation of the solvent under a stream of nitrogen, the samples were ready for the derivatization. For comparison, another series of samples including normal and abnormal controls, were only hydrolysed, missing out the oxidation step.

Derivatization

The iBu-PFP derivatives were prepared by treating the purified extract described above at 100°C for 45 min. with a mixture of 50 μl of acetic anhydride and 200 μl of isobutyl alcohol. After cooling, the reaction mixture was concentrated *in vacuo*, then 200 μl of pentafluoropropionic anhydride were added and the mixture heated at 110°C for 20 min. After cooling and evaporation to dryness, the residue was dissolved in ethyl acetate:hexane (1:10, v/v) and the solution analysed by GC/MS.

Results and discussion

Characterization of the CM-AAs by mass spectrometry

The formation of the pentafluoropropionyl isobutyl derivatives is a two step derivatization procedure carried out at high temperature (110°C). Under such conditions some degradations products corresponding to the loss of one molecule of alcohol were observed for all the CM-AAs studied and for all the alcohols used for the esterification (isopropanol, propanol and isobutanol). This point had to be taken into account before using the iBu-PFP for quantitative analysis. Nevertheless, in our preliminary experiments good linearity was always observed.

The two steps of the derivatization must be performed under strictly anhydrous conditions. Therefore, the samples obtained after the ion-exchange resin purification stage must be thoroughly dried, either by lyophilisation or by drying under a nitrogen stream for a long period of time, and the acylation reaction must be done after complete evaporation of the alcoholic solution.

The iBu-PFP derivatives are efficiently analysed using a medium polar column. A good separation of the studied compounds was achieved using a OV-1701 capillary column as shown in Fig. 1. Although CM-Gly and CM-Ala are not separated by GC they can easily be identified using the mass spectrometric data.

As an example, the electron impact mass spectrum of CM-Val is presented in Fig. 2. Table 1 summarizes the characteristic ions of the electron impact mass

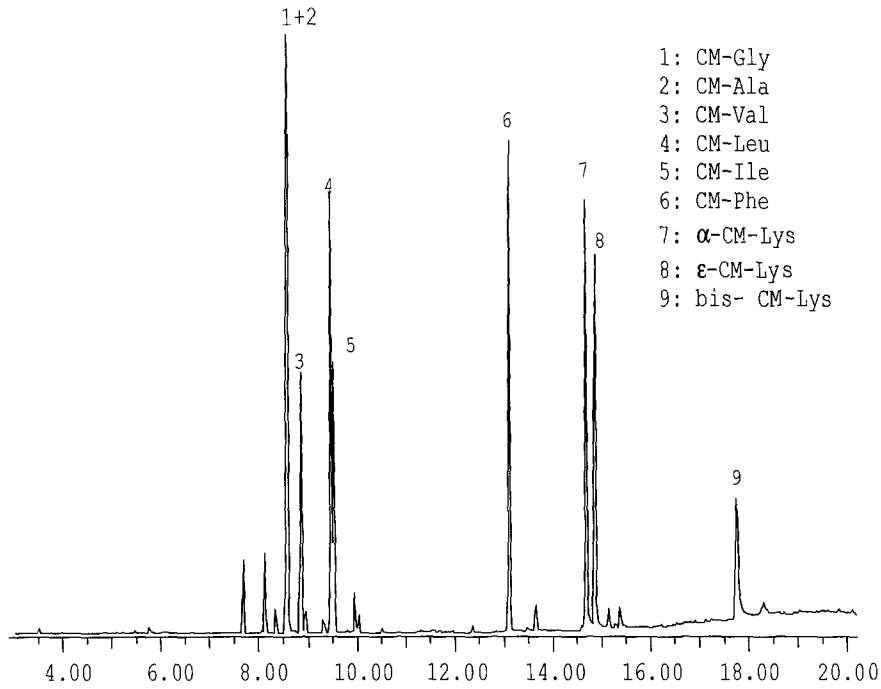

Fig. 1. Separation of the isobutyl ester N,O-pentafluoropropionyl derivatives of 9 N-carboxymethylamino acids

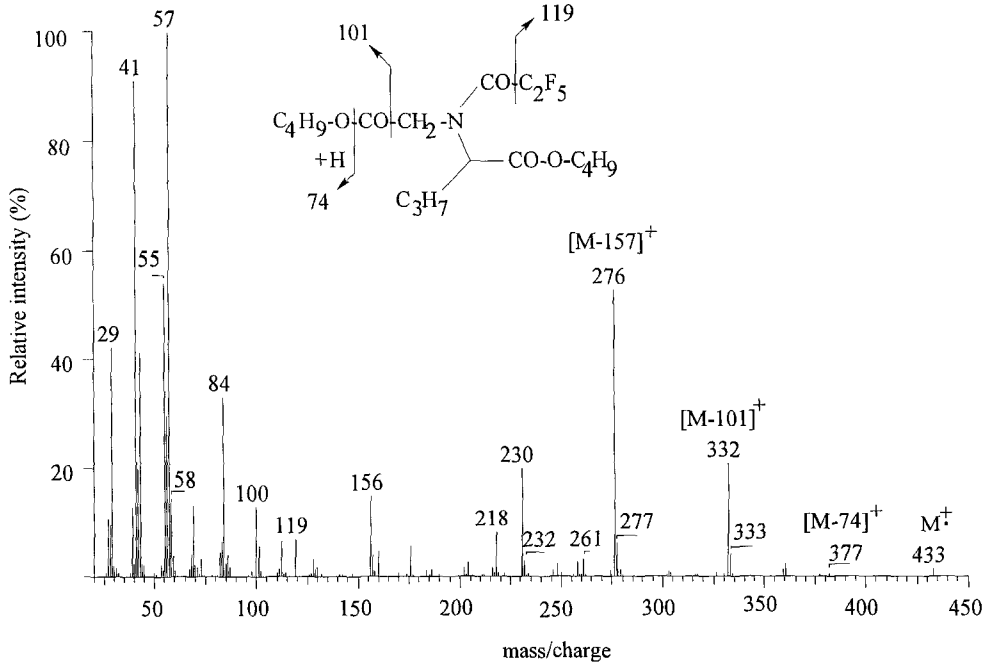

Fig. 2. Electron impact mass spectrum of the isobutyl ester N,O-pentafluoropropionyl derivatives of N-carboxymethylvaline

Table 1. Characteristic ions in the electron impact mass spectra of the N-carboxymethyl amino acid isobutyl N,O-pentafluoropropionyl derivatives

CM-AA	M^+	$[M\text{-butanol}]^+$	$[M\text{-}101]^+$	$[M\text{-}29\text{-}101]^+$	$[M\text{-}56\text{-}101]^+$	Other major ions			
CM-Gly	391(16)	317(20)	290(5)	261(80)	234(35)	335(14)	279(60)	190(9)	176(14)
						119(4)	57(100)		
CM-Ala	405(16)	331(21)	304(46)	275(28)	248(90)	349(15)	230(25)	202(62)	190(34)
						176(35)	119(19)	57(100)	
CM-Val	433(1)	359(2)	332(21)	303(1)	276(53)	230(20)	218(8)	176(6)	156(15)
						119(7)	57(100)		
CM-Leu	447(1)	374(2)	346(10)	317(1)	290(16)	391(5)	244(8)	176(2)	119(1)
						57(100)			
CM-Ile	447(1)	374(1)	346(10)	317(0.5)	290(9)	391(0.5)	244(2)	176(1)	119(0.4)
						57(100)			
CM-Phe	481(4)		380(6)		324(19)	278(37)	250(3)	204(23)	176(4)
						148(100)	91(11)	57(23)	
α-CM-Lys	608(6)	534(6)	507(8)	478(4)	451(19)	433(18)	405(31)	259(18)	248(21)
						242(31)	230(99)	190(1)	176(31)
						119(16)	57(100)		
ε-CM-Lys	608(4)	534(4)	507(1)	478(10)	451(4)	405(3)	274(4)	259(3)	242(5)
						230(6)	190(5)	176(1)	119(1)
						57(100)			
bis-CM-Lys	722(12)	648(10)	621(9)		565(12)	509(15)	491(22)	463(12)	242(12)
						190(10)	176(5)	119(3)	57(100)

spectra obtained after iBu-PFP derivatization of the nine CM-AAS investigated. The main advantage of using the iBu-PFP derivatization is that derivatives with high molecular weights are formed, which are easier to detect in complex mixtures. The positive chemical ionization of each compound leads to the formation of a $[M + NH_4]^+$ ion, the base peak of the spectrum, which can be selectively detected in a complex environment such as food or biological matrices. The identity of this ion can be confirmed by tandem mass spectrometry. The collision induced dissociation spectrum obtained allows the unambiguous identification of the corresponding CM-AA.

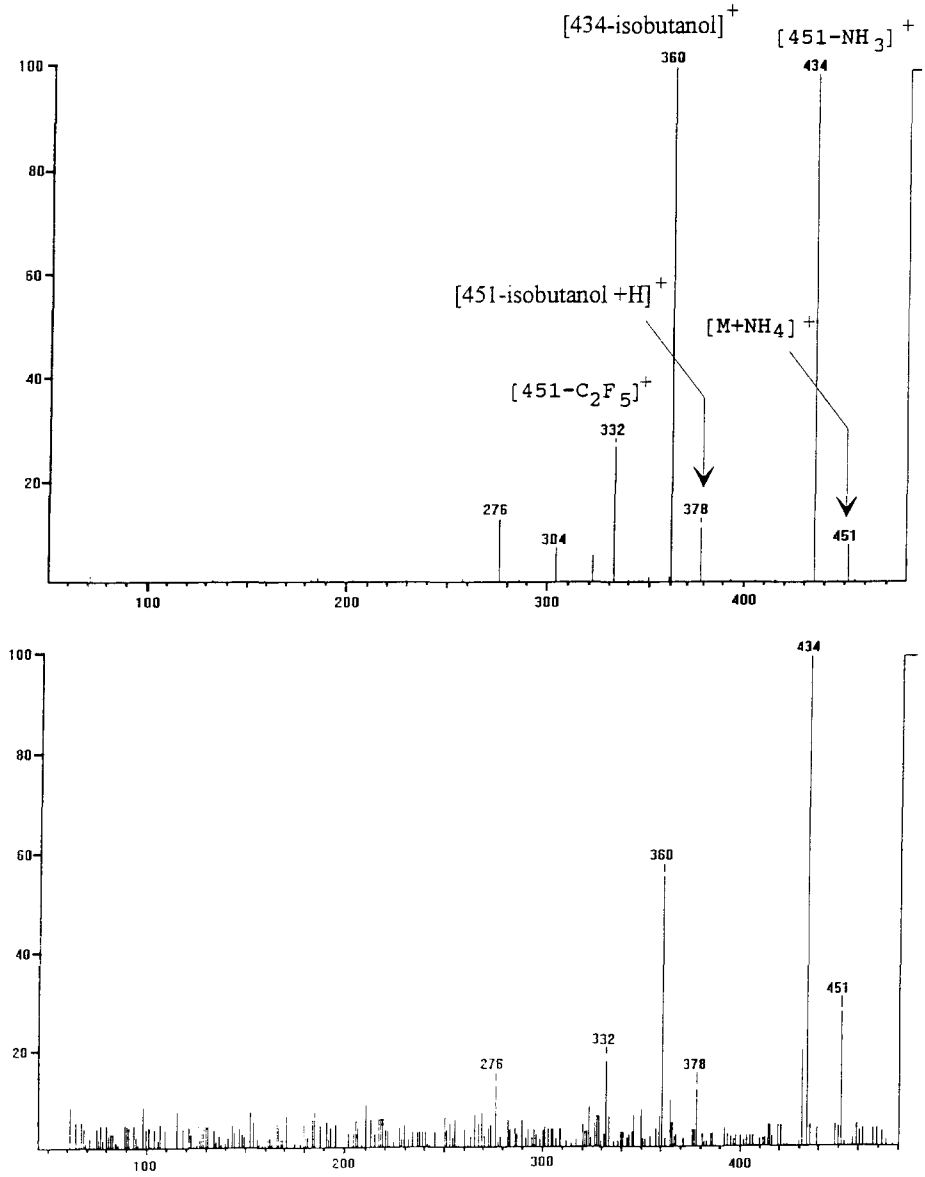

Fig. 3. Product ions after collision induced dissociation (6 eV) of the ion $[M + NH_4]^+$ formed after positive chemical ionisation using ammonia as reagent gas. Upper trace: standard of N-carboxymethylvaline. Lower trace: N-carboxymethylvaline identified in oxidised and hydrolysed abnormal haemoglobin

Applications

As far as the glycosylation is concerned, the ε-lysino groups are clearly the most abundant reactive sites in a protein. In addition, free and N-terminal amino acids may also be glycosylated. Therefore, the methodology described above should allow to identify which amino groups in a protein are blocked by a reducing sugar.

One of the characteristics of diabetes mellitus is associated with elevated levels of glycosylated haemoglobin. Recent studies (Shapiro et al., 1980; Olufemi et al., 1987; Garlick et al., 1983; Acharya et al., 1991; Nacharaju and Acharya, 1992) showed that the major glycosylation sites are on the ε-amino group of lysine and on the α-amino group of valine. In an experiment aimed at testing the feasibility of our approach, samples of normal and abnormal haemoglobin were oxidised with periodic acid, hydrolysed, derivatised and assayed by GC/MS and GC/MS/MS. Two amino acid derivatives, CM-Val and ε-CM-Lys, were identified in the reaction mixtures. As an example Fig. 3 illustrates the identification of CM-Val by gas chromatography-tandem mass spectrometry. The proportion of glycosylated valine and lysine in both normal and abnormal samples are reported in Table 2. The amount of CM-Val found in the abnormal sample is approximately double the amount found in the control, while that of ε-CM-Lys is 4-fold.

Table 2. Determination of CM-Val and ε-CM-Lys in two glycosylated haemoglobin controls from Pierce. The results are given in μg/ml of reconstituted sample

Pierce glycosylated haemoglobin controls	CM-Val	ε-CM-Lys	% Glycosylated haemoglobin acceptable range
Normal Control	3.1	6.9	5.1–6.1%
Abnormal Control	6.7	29.3	16.3–18.3%

Moreover, the ratio of glycosylated to non-glycosylated haemoglobin as given by the supplier of the test material (Pierce Glycosylated Hemoglobin, Normal and Abnormal Controls) is also shown. These data compare very well with recent figures published by Acharya et al. (Acharya et al., 1991; Nacharaju and Acharya, 1992) where the authors observed that, in model non-enzymatic glycosylation reactions using glyceraldehyde as the carbonyl compound and haemoglobin A as the substrate, the ratio of overall glycosyated Val to glycosylated Lys was roughly 3 to 8. The results obtained in the present study on abnormal haemoglobin glycosylated by glucose, indicate a ratio of modified Val to modified Lys of approximately 1 to 4, while the ratio was 1 to 2 in the normal sample.

Conclusion

For nutritional, biochemical or toxicological interests, a simple method for investigating the CM-AAs resulting from the periodate oxidation of protein-

bound Amadori compounds in food or biological samples is required. The iBu-PFP derivatives have shown good properties for an unambiguous identification of CM-AAs in complex mixtures, and gas chromatography-tandem mass spectrometry seems to be the most promising technique for the investigation of CM-AAs in food or biological samples.

The usefulness of this approach for the determination of non-enzymatic glycosylation of proteins was demonstrated by the identification of both CM-Val and ε-CM-Lys as the modified amino acids in both normal and abnormal haemoglobin. However, the potential of this oxidative degradation has not been fully exploited yet. We believe that through amino acid sequence analysis or enzymatic hydrolysis of periodate treated proteins, it should be possible to more readily identify the various sites of glycosylation particularly in model systems of glycosylation where glucose itself could be used as the reducing sugar.

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