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Liquid chromatographic method for the quantitative determination of N^{ϵ}-carboxymethyllysine in human plasma proteins

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Abstract

The modification of the lysine moieties of proteins to N^{ϵ}-carboxymethyllysine (CML) is supposed to play a major role in the development of long-term complications in patients with diabetes mellitus. This paper presents an analytical method for the quantitative determination of CML in plasma proteins, which could be used for studying the development of diabetic complications. The method is based on isolating proteins from plasma by precipitation with trichloroacetic acid and hydrolysing these under acidic conditions (6 M hydrochloric acid at 110 °C for 20 h) to the individual amino acids. After hydrolysis, CML is derivatised along with the other amino acids to 9-fluorenylmethoxycarbonyl (FMOC) derivatives, which are subsequently separated by reversed-phase column liquid chromatography using a 150 mm × 4.6 mm C8 column and a mobile phase of 25 mM potassium phosphate buffer (pH 2.0) and acetonitrile (80:20 (v/v)) and detected using fluorescence detection (excitation at 260 nm and emission at 310 nm). Quantification of the protein-bound CML content of a plasma sample is achieved using standard addition. The impact of several aspects of the sample preparation and chromatography on method performance is discussed. Method evaluation results are reported and show that this method is capable of determining CML with good accuracy and precision (below 10%) in the relevant concentration range (1–10 μg/ml), with a limit of detection of 0.2 μg/ml.

Keywords: Derivatization, LC; N^{ϵ}-Carboxymethyllysine; Proteins

1. Introduction

Advanced glycation end-products (AGEs) are a heterogeneous group of endogenous compounds, which are associated with various long-term complications of diabetes, such as atherosclerosis and renal failure [1–3]. The first step in the formation of an AGE is the non-enzymatic reaction of a reducing carbohydrate such as glucose with an amino group (usually a lysine side chain) of a protein or other macromolecule. This is followed by cleavage and/or intra-molecular rearrangements to an irreversibly glycated compound, which can form covalent cross-links within a tissue, thus altering its functional properties [4,5]. Probably the most important (yet non-cross-tinking) AGE currently known is N^{ϵ}-carboxymethyllysine (CML, Fig. 1). Accumulation of CML in various tissues is promoted by hyperglycaemic and oxidative conditions and has been suggested as a major cause of diabetic complications [3,6,7].

For a better understanding of the underlying processes that cause tissue damage during diabetes, a reliable analytical method, capable of determining the CML content of proteins, would be of great significance. Presently, however, in the field of diabetes research it is felt that no universally applicable method is available [8]. Frequently used techniques such as fluorescence spectroscopy and immunoassays lack specificity and the different sources of anti-CML antibodies show a large variability in their affinity towards CML, which results in inaccurate and irreproducible results and makes comparison of results between laboratories difficult, if not impossible [8].

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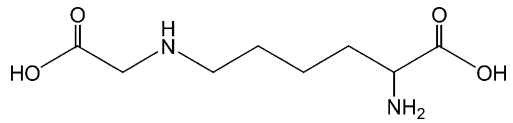

Fig. 1. Structural formula of N^{ϵ}-carboxymethyllysine (CML).

It has been demonstrated that method performance can be improved by chromatographically separating CML from other amino acids in a sample. In a number of cases, CML has been determined with a classical amino acid analyser, which is based on ion-exchange liquid chromatography (LC) and fluorescence detection after post-column derivatisation [9,10], but the analytical performance of these methods was not evaluated. In addition, CML has been determined with good reproducibility in urine [11] and hydrolysates of milk products [12], both using reversed-phase LC with pre-column derivatisation and fluorescence detection. A truly quantitative method for the determination of the CML content of tissue or plasma proteins has, however, never been described. In this paper, we present such a method; it is based on pre-column derivatisation of CML and other amino acids in protein hydrolysate samples with 9-fluorenylmethoxycarbonyl (FMOC) chloride, followed by separation and detection with reversed-phase LC and fluorescence detection. Special attention is paid to the accuracy and precision of the method, which are of key importance for its universal applicability, and have been rather neglected in previously published methods.

2. Experimental

2.1. Chemicals

N^{ϵ}-Carboxymethyllysine was purchased from SyMO-Chem (Eindhoven, The Netherlands). Trichloroacetic acid, amino acid solution (1 mM each) and FMOC were obtained from Fluka (Buch, Switzerland). Phosphoric acid, hydrochloric acid, sodium hydroxide, sodium tetraborate decahydrate, potassium dihydrogen phosphate and acetonitrile came from Merck (Darmstadt, Germany). HPLC grade water was prepared using a Milli-Q (Millipore, Bedford, MA, USA) purification system.

2.2. Chromatography

The chromatographic system consisted of a Waters (Milford, MA, USA) Separations Module Alliance 2690 for injection and pumping of the mobile phase, a Zorbax Stablebond (Agilent, Palo Alto, CA, USA) C8 column (150 mm × 4.6 mm, 3.5 μm), conditioned at 45 °C in a Julabo (Seelbach, Germany) water bath, and a Jasco (Tokyo, Japan) FP 920 fluorescence detector, set at an excitation wavelength of 260 nm and an emission wavelength of 310 nm. The mobile phase, a mixture of 25 mM potassium phosphate buffer (pH 2.0) as eluent A and acetonitrile as eluent B, was pumped at a flow-rate of 1.3 ml/min. Elution of the analyte was achieved using 80% A and 20% B for 17.5 min, after which a step gradient was applied with 15% A and 85% B until 22.5 min; finally the column was re-equilibrated at 80% A/20% B until 30 min.

2.3. Sample preparation

An aliquot of 200 μl of human plasma was mixed with 1000 μl of 10% aqueous trichloroacetic acid in a 10 ml glass hydrolysis tube (Organon Teknika, Boxtel, The Netherlands) and left to stand for 10 min. The sample was centrifuged at 3300 × *g* for 10 min and the supernatant was discarded. The remaining protein pellet was dissolved in 1000 μl of 6 M hydrochloric acid, thoroughly purged with nitrogen and hydrolysed at 110 °C for 20 h. After cooling the sample to ambient temperature, 1000 μl of 5 M sodium hydroxide was added and the mixture was centrifuged at 3300 × *g* for 10 min. A 20 μl aliquot of the supernatant was added to 180 μl of 0.4 M sodium borate buffer (pH 10.4). Subsequently, 10 μl of water or an aqueous 5.00 μg/ml CML solution (for standard addition, see below) were added. After mixing, 20 μl of this mixture were added to 180 μl of 0.4 M sodium borate buffer (pH 10.4) in a polypropylene injection vial and derivatisation was performed by adding 10 μl of a 1 mg/ml FMOC solution in acetonitrile. After vortex-mixing, the vial was transferred to the autosampler and an aliquot of 40 μl was injected into the chromatographic system.

2.4. Quantitation

In order to quantitatively determine the CML concentration in a sample, the method of standard addition was applied. To this end, two aliquots of each individual protein hydrolysate were analysed, one without and one with a known amount (50.0 ng) of CML added, and the CML peak heights were determined. As the added amount of CML corresponds to a concentration of 25.0 μg/ml plasma, the original CML concentration (in μg/ml) was subsequently calculated by multiplying 25 with the ratio of the original peak height over the difference between the two peak heights.

2.5. Method evaluation

The precision of the entire procedure at a relatively low CML concentration (established as 4.13 μg/ml) was assessed by the repeated analysis of a single human plasma sample. Five aliquots were separately hydrolysed, after which the resulting hydrolysates were divided into three portions and independently derivatised and analysed. The 15 CML peak heights obtained were subjected to analysis of variance to determine the coefficients of variation originating from the hydrolysis and derivatisation steps, respectively. For the determination of the precision at a relatively high

CML concentration, the experiment was repeated, but just before derivatisation an amount of CML corresponding to 25.0 μg/ml in plasma was added.

The accuracy of the method was determined by analysing calibration lines consisting of five calibrators (0.500, 1.00, 2.50, 10.0 and 25.0 μg/ml CML) prepared in amino acid solution and three different lots of human plasma. The slopes of the calibration lines were compared to assess the possibility of accurately determining known amounts of CML by reference to a calibration line.

The stability of CML during the hydrolysis procedure was determined by diluting a 1.00 mg/ml solution of CML in 1 mM amino acid mixture to 5.00 μg/ml with 6 M hydrochloric acid and comparing the CML peak heights obtained by immediate analysis to those obtained by analysis after heating at 110 °C for 20 h.

The stability of the FMOC-derivatives of CML during storage in the autosampler was assessed at two concentrations. One plasma sample was hydrolysed, after which 15 aliquots of the hydrolysate were derivatised without any CML added and 15 after the addition of the equivalent of 25.0 μg/ml CML. The derivatised samples were pooled per concentration, each divided over 60 injection vials and injected every 30 min for a period of 60 h, during which the samples were stored in the autosampler at 10 °C.

3. Results and discussion

3.1. Separation and detection

The major challenge in the determination of the CML content of proteins is the need to distinguish the analyte from a large variety of other amino acids, many of which are present in the hydrolysate at much (typically 1000-fold) higher concentrations. This implies that an efficient chromatographic separation prior to the detection of CML is essential. Two chromatographic approaches for amino acid analysis have been widely employed: (i) pre-column derivatisation to fluorescent and more hydrophobic derivatives, which are separated by reversed-phase LC and (ii) separation of the native amino acids by ion-exchange LC, followed by their post-column derivatisation [13]. Both approaches have their advantages and disadvantages, but for the present application pre-column derivatisation was selected, its main benefits being technical simplicity, speed and the relative ease to manipulate the separation of the amino acids by tuning the mobile phase composition.

Pre-column derivatisation of amino acids has been achieved with a wide variety of reagents [13]. For the sake of robustness and sensitivity, FMOC chloride was employed in the present work, because this reagent forms highly fluorescent and very stable derivatives with both primary and secondary amines in a matter of seconds. It was deemed superior to the well-known and probably most frequently used o-phthalaldehyde (OPA)/2-mercaptoethanol combination, which has the major disadvantage of forming relatively unstable derivatives.

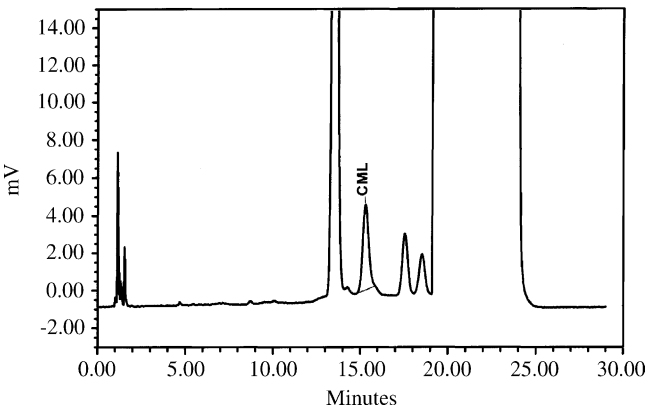

Fig. 2. Chromatogram of a standard solution of CML (10 μg/ml) in a 1 mM mixture of amino acids.

The most efficient separation of the FMOC derivative of CML from those of the other amino acids could be obtained with an acidic mobile phase, probably because of the increased retention of the (protonated) derivatives on the octadecylsilane phase at a lower pH. This is another reason to prefer FMOC over OPA for derivatisation, since the fluorescence intensity of OPA derivatives is greatly reduced at low pH. Using a 15 cm column and a mobile phase containing 20% acetonitrile, CML could be efficiently separated from other amino acids, with a retention time of about 15.5 min. To speed up the analysis, a step gradient of 85% acetonitrile was applied between 17.5 and 22.5 min to simultaneously elute the more hydrophobic amino acid derivatives, which are of no importance for the present application, and the fluorescent hydrolysis products of FMOC. Altogether, the analytical run, including the step gradient and subsequent re-equilibration, was completed in 30 min, which compares favourably with existing methods, especially those based on post-column derivatisation with its run times in the order of 60 min [9,10].

Fig. 2 shows a chromatogram of CML in a standard amino acid solution. It illustrates that CML is well separated from the other amino acids; the major of part of which elutes together in the step gradient.

3.2. Sample preparation

In order to hydrolyse the plasma proteins to their individual amino acids, the common approach of acid hydrolysis in 6 M hydrochloric acid at 110 °C was taken. It was found that CML concentrations were significantly lower in case of hydrolysis in the absence of oxygen. Human plasma samples from a single source were hydrolysed without precautions and after thorough purging with nitrogen. The mean ($n = 3$) response found in the former case was 62% higher than in the latter case, with coefficients of variation of each set of three measurements not exceeding 11%. This confirms

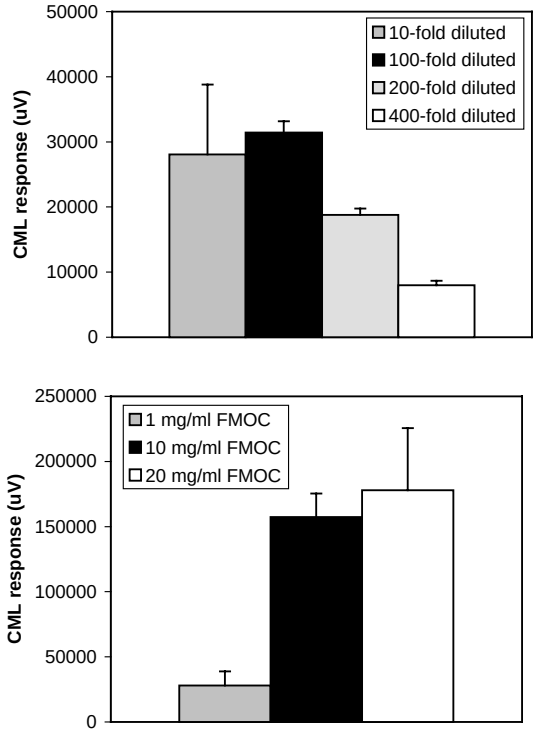

Fig. 3. Influence of sample dilution (upper panel) and FMOC concentration (lower panel) on the response and precision of the CML determination.

earlier findings with milk samples that CML is formed in vitro during hydrolysis in oxidative conditions [12]. Removing oxygen prior to hydrolysis is therefore key to obtaining accurate results, as the oxidative formation of CML would otherwise lead to a substantial overestimation of the actual CML concentration.

The performance of the derivatisation also turned out to be of importance, especially for the precision of the method. It was first attempted to dilute the neutralised hydrolysate 10-fold with borate buffer prior to the addition of the FMOC derivatisation reagent, but a very poor precision was obtained (CV of 38.2%, $n = 12$). Apparently, the derivatisation reaction does not proceed in a controlled way under these conditions. This was suspected to be due to the high abundance of amino acids in the hydrolysate, which compete with CML for the reaction with FMOC. Higher dilution factors were tested prior to derivatisation (see Fig. 3A) and it was found that 100-fold dilution resulted in a mean CML detector response that was even higher than in case of 10-fold dilution, but with a much better precision (CV of 5.5%, $n = 6$). Apparently, diluting out the amino acids in the sample resulted in a better proportion of FMOC to amino acids and improved reaction kinetics. Increasing the dilution factor to 200 or 400 did not further improve precision, but did lead to an approximately proportional decrease in response. It was also tried to increase the proportion of FMOC over amino acids by adding more concentrated FMOC solutions (Fig. 3B). Increasing the concentration 10-fold (to 10 mg/ml) or 20-fold (to 20 mg/ml) caused a five-fold increase in the response, but the precision remained poor: a CV of 11.5% for 10-fold and of 26.9% for 20-fold higher FMOC concentrations ($n = 6$). Altogether, this indicates that 100-fold dilution prior to derivatisation with a 1 mg/ml FMOC reagent solution is optimal.

3.3. Quantitation and method evaluation

Although the analytical procedure consisted of a rather large number of steps and no internal standard was used, the precision of the method was fully satisfactory. An overall CV of only 8.9% ($n = 15$) was found for human plasma with a physiological concentration of CML (4.13 μg/ml). Since the plasma was hydrolysed in five separate aliquots, which were each derivatised in triplicate, the contribution of the hydrolysis and derivatisation steps could be calculated by analysis of variance. These were 7.1 and 5.8%, respectively, and indicate that hydrolysis contributes slightly more to method imprecision, although the role of the derivatisation step is not insignificant. The experiment was repeated with the same plasma sample, but before derivatisation an amount of CML was added to each of the 15 aliquots in order to assess the precision at a higher concentration (29.1 μg/ml). The overall CV was now 9.1% and the contributions of hydrolysis and derivatisation were 8.7 and 2.8%, respectively. This suggests that a better precision for the derivatisation is obtained at higher CML concentrations. Altogether, method precision was found to be adequate over a relevant concentration range: upon application of this method it was found that the majority of the CML concentrations were around 4 μg/ml or above.

Ideally, it should be possible to calculate the concentration of CML in a study sample by substitution of the response found in a calibration line constructed of the responses found in calibrators of known concentration. Generally, for endogenous compounds these calibrators are standard solutions in e.g. water (external calibration), but accurate results can only be obtained in this way if the response is independent of the matrix. This was tested by analysing calibration lines of CML in a standard amino acid mixture and in three different lots of human plasma. Although the calibration lines were linear in all cases ($r > 0.99$ and back-calculated concentrations within 15% from nominal), it was found that the slope of the calibration line in the amino acid mixture (451) was typically 30% higher than in the plasma samples (297, 279 and 354, respectively). External calibration is, therefore, unsuitable for accurate quantification of CML. In addition, the variation between the slopes of the calibration lines in different lots of plasma (a difference of up to 20%) was deemed too large to justify the use of calibration lines spiked in plasma. The reason behind this observation is probably related to differences in the composition of plasma from different sources, an effect which has been observed before for physiological amino acids [14]. Therefore, it was decided to calculate the concentrations by the method of standard addition, which completely rules out matrix effects. In none

of the methods described so far has attention ever been paid to this phenomenon, which seems to indicate that the accuracy of the results reported using these methods is at least questionable. The limit of detection of the method, defined as the mean plus three times the standard deviation of the baseline noise ($n = 6$) and derived from CML in an amino acid mixture, was 0.2 μg/ml. The practical limit of quantitation was set at the lowest CML calibration standard that was investigated and showed accuracy and precision below 20%: 0.5 μg/ml.

The stability of CML itself during hydrolysis was investigated and found to be satisfactory. The mean response ($n = 9$) obtained for CML after heating at 110 °C for 20 h in 6 M hydrochloric acid in the presence of a standard mixture of amino acids was not statistically different from that of the same sample that had not been heated (difference of 4%).

The stability of the FMOC derivatives of CML in the autosampler was investigated over a period of 60 h, by injecting pretreated samples every hour. Regression analysis of the responses over time showed an increase of 4.1% for CML at the low level (5.27 μg/ml) and a decrease of 5.2% at the high level (30.3 μg/ml) over the first 15 h. Over 30 h, these results were a decrease of 1.2% and a decrease of 7.9%, respectively; over the total of 60 h, a decrease of 8.0% and a decrease of 14.6% were found, respectively. Thus, the responses were typically found to decrease over time, which was most pronounced at the highest concentration. Still, if a deviation of no more than 10% is considered acceptable, samples can be stored in the autosampler at 10 °C for up to 30 h without problems.

3.4. Application

The described method has been applied to several hundreds of human plasma samples originating from a variety of clinical trials. CML concentrations found in plasma typically ranged from 1 to 4 μg/ml in healthy subjects and from 2 to 10 μg/ml in diabetic patients. When related to the amount of plasma protein, a significant difference can be seen between the two populations: in type 1 diabetics, the CML concentrations (95% confidence interval) range from 56 to 73 ng/mg protein, while in (age-matched) healthy subjects, they range between 15 and 42 ng/mg protein. This shows that CML accumulates in plasma proteins during diabetes and that the determination of the CML concentration in plasma proteins is potentially useful to investigate the progress of the disease and the development of diabetic complications.

As an illustration, Fig. 4 shows chromatograms of a plasma sample of a diabetic patient without (Fig 4A) and with (Fig. 4B) standard addition. Comparison of these chromatograms with the one in Fig. 2 clearly illustrates that separation of CML from the standard amino acids alone is not sufficient: upon hydrolysis of plasma proteins, a variety of less abundant amino acids is set free and separation from these is also crucial for a proper quantitation of CML. As is shown in Fig. 4, the separation efficiency as obtained in the present method is sufficient to achieve this.

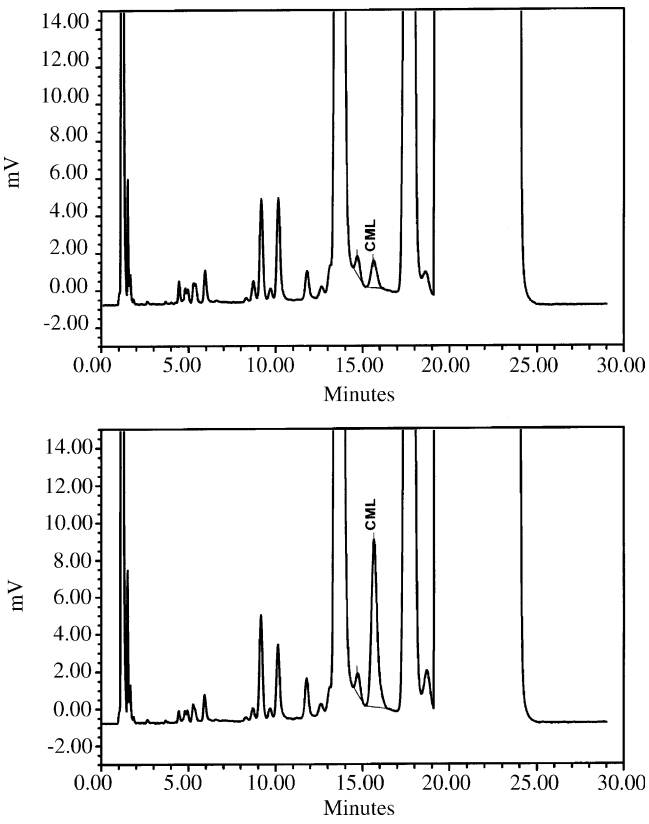

Fig. 4. Chromatograms of a plasma sample taken from a diabetic patient; upper panel: without standard addition (CML concentration 4.82 μg/ml); lower panel: with standard addition (CML concentration 29.8 μg/ml).

4. Conclusion

A reliable LC method has been developed and evaluated for the determination of the protein-bound CML concentration in human plasma. It allows the accurate quantitation of CML in the physiological range (typically 1–10 μg/ml), which is adequate for the use as a marker for diabetic complications. In comparison with other methods, it is equally sensitive, shows a favourable run time (30 min) and, especially compared to ELISA methods, a much better accuracy and precision. So far, the method has only been applied to plasma proteins; more research is underway to investigate the suitability of the method for proteins from other tissues.

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