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Liquid chromatography-based determination of urinary free and total *N*(epsilon)-(carboxymethyl)lysine excretion in normal and diabetic subjects

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Abstract

We propose a specific, reproducible and sensitive HPLC method for the determination of *N*(epsilon)-(carboxymethyl)lysine (CML) excreted in urine. Total CML was measured in acid hydrolysates of urine samples, while free CML was measured in acetonitrile-deproteinised urine samples using a RP-HPLC method with *ortho*-phtaldialdehyde (OPA)-derivatisation and fluorescence detection suited for automation. We compared the CML excretion of 51 non-proteinuric patients with diabetes mellitus (DM) (age 57±14 years, HbA1c 8.0±1.8%) to 42 non-diabetic controls (C) (age 45±17 years). The urinary excretion of total CML in diabetic patients was increased by ~30% (DM: 0.58±0.21; C: 0.45±0.14 μmol/mmol creatinine; $P<0.001$). While urinary excretion of free CML was not significantly different, excretion of bound CML was increased (DM: 0.36±0.17; C: 0.27±0.14; $P<0.05$) in diabetic patients. CML excretion was correlated with protein and albumin excretion, but did not correlate with HbA1c, duration of DM or diabetic complications such as neuropathy or retinopathy. Furthermore, no age-dependent change of total CML excretion was found, while free CML excretion was lower in younger subjects. The specific and sensitive determination of CML by RP-HPLC of its OPA-derivative is well suited for automation and better than that of less defined glycoxidation products (AGEs).

Keywords: *N*(ϵ)-(Carboxymethyl)lysine

1. Introduction

N(epsilon)-(carboxymethyl)lysine (CML) is the most abundant advanced glycation endproduct (AGE). Among different AGEs, CML is unique because it is, besides pentosidine, the only AGE known to increase with age and to accumulate at an accelerated rate in diabetes [1]. Previous reports have shown that CML is formed in vitro by oxidative cleavage of the carbohydrate moiety bound to the ε-amino group of lysine [2,3]. In vivo CML modification of proteins occurs post-translationally and

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proceeds under hyperglycemic and oxidative conditions. Therefore, it has been suggested that CML may serve as a biomarker for oxidative stress in diabetes mellitus (DM) or in chronic inflammatory or degenerative diseases [4]. In DM the deposition of CML in a wide variety of tissues has been linked to the development of diabetic complications. Increased levels of the glycoxidation product CML have been found in skin collagen of diabetic patients [5]. Histological deposition of CML is associated with the severity of retinopathy [6] and nephropathy [7]. CML or other AGEs are found in the expanded mesangial cell matrix and the thickened glomerular capillary walls of diabetic nephropathy [8], in artherosclerotic plaques [9] and in human diabetic peripheral nerves [10]. Furthermore, it has been shown that the CML content of the skin and of intervertebral discs increases with increasing age [11,12]. Recent results from our laboratory show that CML is also formed upon action of substances which increase cellular oxidative stress, e.g. tumor necrosis factor or angiotensin II (Schleicher et al., unpublished findings). As shown by Degenhardt and coworkers [13], intracellular proteins may also be modified by methylglyoxal leading to carboxyethyllysine (CEL). Methylglyoxal is physiologically derived from glycolysis intermediates.

The carboxymethyl-lysyl groups are not metabolised and are chemically inert. However, it is of note that CML modification leads to a zwitter ion of the originally positive charged ε-amino group of lysine and may therefore alter the physicochemical properties and the function of proteins. After protein catabolism either the free modified amino acid or small molecular mass CML peptides are excreted in the urine. Analysis of AGEs or glycoxidation products has been difficult because commonly used methodologies, i.e. immunoassays or fluorescence measurements, reflect group reactivity and were not specific for chemically well-defined substances [14]. Previously, CML has been determined by SIM-GC–MS [12] or in tissue hydrolysates by an HPLC procedure involving two sequential HPLC columns [5]. Both procedures, although reliable and precise, require equipment which is still not widely available in research facilities. We aimed to develop a sensitive and precise method using standard HPLC equipment. This method was evaluated and applied to investigate the urinary free and peptide-bound CML excretion in non-proteinuric patients with diabetes mellitus (DM) and in normal controls.

2. Experimental

2.1. Chemicals

All solvents were of HPLC-grade and were obtained from Merck (Darmstadt, Germany). Reagents and buffer salts were of the highest grade obtainable. Amino acids, *o*-phtaldialdehyde (OPA), 3-mercaptopropionic acid (MPA) and HCl (>36.5%, w/w; 12 *M*) were from Sigma (Deisenhofen, Germany). The carboxymethyl-lysine (CML) standard was a generous gift from Dr M. Lederer (Stuttgart, Germany), and was characterised by LC–MS, NMR and elemental analysis. The carboxyethyl-lysine (CEL) used for peak identification was a gift from Professor Hammes (Giessen, Germany).

2.2. Sample collection

First morning urine samples were obtained from 51 diabetic patients (DM: age 57±14 years; HbA1c: 8.0±1.8%) and from 42 controls (C: age 45±17 years). Characteristics of patient and control groups are summarised in Table 1. As assessed from the clinical record, 72% of patients (29 out of 42 patients with data available) showed signs of diabetic neuropathy (reduced vibration sensation) and 37% (16 out of 43 patients who were examined) showed nonproliferative or proliferative retinopathy. Inclusion criteria for all participants were: absence of gross proteinuria (<300 mg/l), absence of renal impairment (serum creatinine≤1.0 mg/dl) and absence of inflammatory signs and symptoms by clinical record and by laboratory parameters (normal white blood count, no elevation of C-reactive protein). HbA1c was measured by cation-exchange HPLC (Tosoh 2.2 HLC-723, Tosoh, Tokyo, Japan), urinary protein by a modified Biuret reaction on a BM/Hitachi 747 clinical chemistry analyser and urinary albumin by nephelometry (Dade Behring BN II, Marburg, Germany). Urine was stored frozen at −20 °C for up to 2 years. Urinary creatinine, protein and albumin were measured directly prior to sample work-up.

Table 1
Characteristics of diabetic patients and control subjects

	DM (n=51)	Controls (n=42)
Sex (M/F)	27/24	19/23
Age (years)	57±14	45±17
Type of DM (I/II)	9/42	None
BMI (kg/m^2)	29.5±5.5	25.6±2.7
Duration of DM (years)	10.0±6.5	0
Diabetic neuropathy (prevalence)	72%[a]	None
Diabetic retinopathy (prevalence)	37%[b]	None
HbA1c (%)	8.0±1.8	w.n.l.
Fasting glucose (mg/dl)	172±73	N.D.
Proteinuria (mg/l)	77±66	59±45
Albuminuria (mg/l)	30±24	N.D.
Serum-creatinine (μmol/l)	79.6±8.8	70.7±8.8

Data are mean±SD. BMI, body mass index; N.D., not determined; w.n.l., within normal limits.
[a] Data on neuropathy were available from 42 patients.
[b] Examination for retinopathy was performed in 43 patients.

2.3. Sample preparation

A volume of urine equivalent to 1 mg creatinine was evaporated to dryness with a Speedvac centrifugal evaporator (Christ, Osterode, Germany). A 420-μl amount of 12 *M* HCl (>36.5%, w/w) and 300 μl H_2O was added to a final concentration of 7 *M* HCl. The solution was transferred to vacuum hydrolysis tubes (Wheaton, Milville, NJ, USA), cooled in liquid nitrogen and evacuated after the contents solidified. Samples were hydrolysed at 110 °C for 24 h under vacuum. The hydrolysate was filtered, evaporated to dryness, resuspended in 1000 μl redryness solution (H_2O/EtOH/triethylamine, 2:2:1) to remove traces of HCl, evaporated to dryness and resuspended in 200 μl 15 m*M* NaH_2PO_4, pH 7.2. For analysis of free CML, 1 vol. of urine equivalent to 0.5 mg creatinine and 2 vol. of acetonitrile were allowed to stand overnight at 4 °C for precipitation and centrifuged at 4000 rpm for 5 min. The supernatant was evaporated to dryness and resuspended in 100 μl H_2O.

2.4. Sample derivatisation and HPLC analysis

All steps of pre-column derivatisation with OPA were performed with a programmable autosampler Shimadzu SIL-10A (Shimadzu, Kyoto, Japan) using the following protocol: 200 μl of appropriately diluted sample (1:250 for urine hydrolysates and 1:100 for deproteinised urine) was incubated with 200 μl buffer 1 (0.2 *M* Na_3BO_3, 5 m*M* EDTA, pH 10) and 100 μl OPA reagent (50 mg OPA/50 μl 3-mercaptopropionic acid in 5 ml methanol) for 3 min, stopped with 200 μl buffer 2 (1 *M* KH_2PO_4, pH 4.0) and immediately injected for HPLC analysis (injection volume 10 μl). HPLC analysis was performed in an air conditioned laboratory environment with room temperature set at 21 °C using a Shimadzu series 10A HPLC device equipped with a Shimadzu RF-10AXL fluorescence detector (excitation 340 nm, emission 455 nm) and a GROM-SIL 100 ODS-0 AB RP-column (5 μm, 200×4 mm) from Grom (Herrenberg, Germany). Eluents were: A, 1% ACN/1% THF; and B, 45% ACN/5% THF in 15 m*M* NaH_2PO_4, pH 7.2. The gradient was: 0–1.25% B (0–17 min at 0.4 ml/min), 1.25–100% B (17–18 min at 0.4 ml/min), 100% B (18–28 min at 0.8 ml/min), and 100% A (29–39 min at 0.8 ml/min).

2.5. Statistical analysis

Unpaired Student's *t*-test (two-sided) was used to compare mean values among the different groups and linear regression analysis was used to assess correlations. All data were shown as the mean±SD, unless otherwise specified.

3. Results

3.1. Validation of the analytical procedure

Upon variation of the gradient on various columns filled with modified C_{18} reverse phase materials, we found conditions for base line separation of CML in hydrolysed and in deproteinised urine. Chromatograms of an urine sample after acid hydrolysis (Fig. 1A,B) and of an urine sample after acetonitrile precipitation (Fig. 1C,D) of a representative diabetic patient are shown in Fig. 1. CML eluted at RT 8.7–9.1 min (Fig. 1A,C) and peak identity was confirmed by the coelution of added CML standard (Fig. 1B,D). Throughout all samples the CML peak was found to be narrow and fully baseline separated from the chemically related homolog CEL (RT 10.5 min; Fig. 1B). With other tested columns (GromSIL ODS-3 CP, GromSIL ODS-4 HE, GromSIL ODS 5-ST, GromSIL OPA3; all from Grom, Herrenberg, Germany) CML could not be fully separated either from the preceding peak (consisting of the abundant physiological amino acid serine) or from the homolog CEL. For six-point calibration, standard solutions from 0.05 to 1.5 μmol/l (corresponding to 0.14–4.26 pmol on column) were prepared from CML standard in H_2O. A 200-μl aliquot of each standard concentration was derivatised and analysed in triplicates. Linearity of the standard calibration curve was verified in a range from 0.14 to 4.26 pmol CML on column. The calibration line was: CML (pmol)=0.009+0.318×area (10^6 au) (R=0.994).

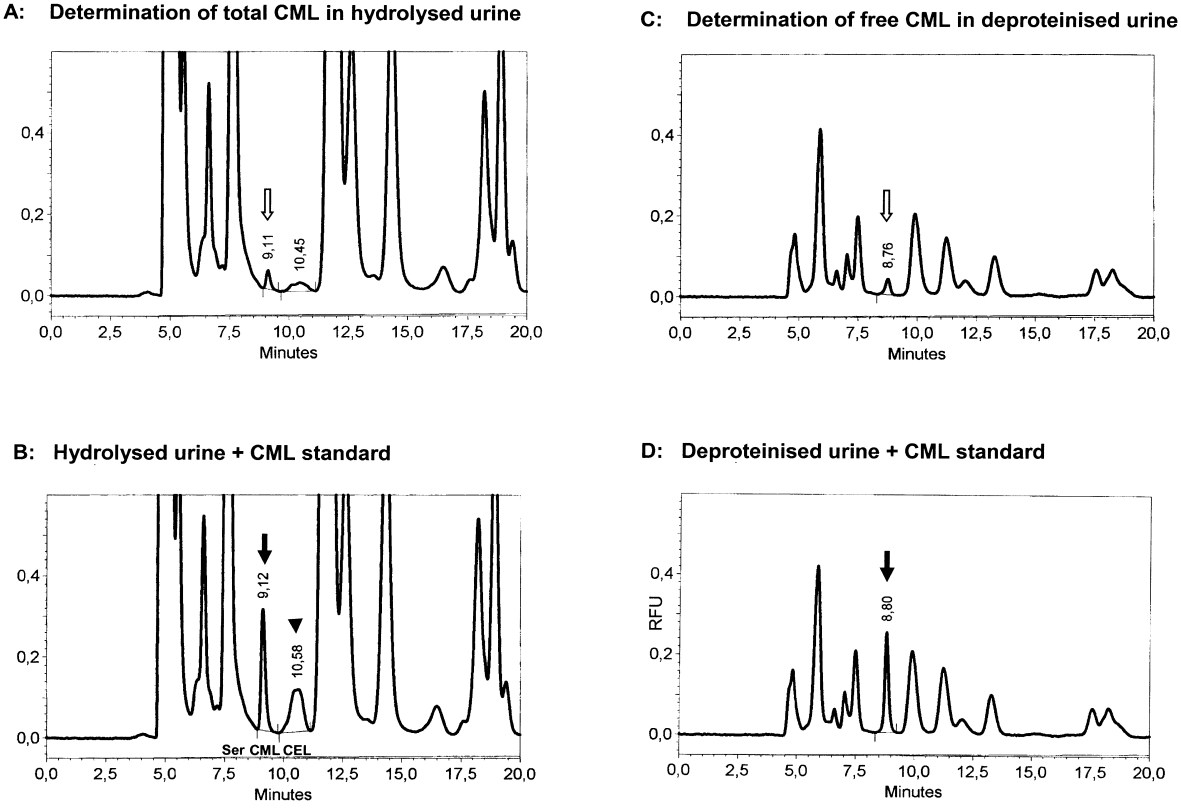

Fig. 1. Determination of urinary total and free carboxymethyllysine (CML) in a representative diabetic patient. Urine samples were either hydrolysed (A/B, dilution 1:250) or deproteinised (C/D, dilution 1:100). OPA derivatives were analysed by RP-HPLC and fluorescence detection (340 nm/455 nm). For details see text. In B and D, diluted samples were spiked with 0.5 μmol/l CML standard (filled arrows) corresponding to 1.4 pmol CML on column. Endogenous CML was identified at RT 8.7–9.1 min (A/B; open arrows) by coelution. CML was fully baseline separated from its homolog carboxyethyllysine (CEL) and from serine (Ser) as demonstrated in B, where also CEL was added (arrowhead; 1.4 pmol on column) for peak identification (RFU, rel. fluorescence unit).

For validation of the analytical procedure, a processed patient sample was diluted (1:250 for hydrolysed urine and 1:100 for deproteinized urine), spiked with 0.1 or 0.5 μmol/l CML standard (corresponding to 0.28 and 1.4 pmol CML on column), aliquotted, and measured in 8-fold replicates. The peak areas were calculated from measurements of unspiked and spiked sample pairs. The coefficient of variation (C.V.) for the peak area was 5.1% (in series) and 7.5% (from day to day) for the calibration level of 0.28 pmol CML on column and 2.17% (in series) and 6.7% (from day to day) for the calibration level of 1.4 pmol CML on column. Recovery of CML during acid hydrolysis was 90–95%. The detection limit of the method was 0.07 pmol CML on column (signal/noise 4).

3.2. Determination of urinary CML in diabetic and non-diabetic subjects

In Fig. 2 the urinary excretion of total and free CML in 51 diabetic patients (DM) is compared to 42 non-diabetic controls (C). In diabetic patients the excretion of total CML is increased by ~30% (DM: 0.58±0.21; C: 0.45±0.14 μmol/mmol creatinine; $P<0.001$). Urinary excretion of free CML was not statistically different between both groups, although it tended to be higher in diabetic patients (DM: 0.22±0.15 vs. C: 0.18±0.13 μmol/mmol creatinine; NS). To assess the peptide- and protein-bound fraction of CML, we calculated the difference between total and free CML. This portion (''bound CML'') contained ~60% of total CML and consisted of protein- and peptide-bound CML, which has not been precipitated. Patients with diabetes had a significantly higher excretion of bound CML than controls (DM: 0.36±0.17; C: 0.27±0.14; $P<0.05$). As shown in Fig. 3, this calculated fraction of bound CML was found to correlate significantly with urinary protein excretion ($R=0.46$, $P<0.001$) and with albumin excretion ($R=0.73$, $P<0.001$), whereas the correlation of total CML excretion with urinary excretion of protein ($R=0.28$, $P=0.047$) and albumin ($R=0.29$, $P=0.037$) was at the border of significance (data not shown). Excretion of free CML was not correlated with protein or albumin excretion. Neither total nor free nor bound CML was correlated with age, duration of DM or HbA1c. The urinary CML excretion in the subgroups with diabetic neuropathy or retinopathy did not differ from the total group of diabetic patients (data not shown). Since previous reports have shown that CML formation in certain tissues such as skin, vertebral discs or eye lens collagen is increased with age [11,12], we compared urinary free and total CML excretion in 19 young healthy subjects (30±6 years) to 23 older subjects (59±11 years) who were age matched to our study group of diabetic patients. Urinary excretion of total CML did not differ in both groups (0.45±0.09 vs. 0.44±0.17 μmol/mmol crea; NS). Urinary excretion of free CML was found to be lower in young controls (0.12±0.10 vs. 0.23±0.15 μmol/mmol crea; $P<0.01$) compared to old controls.

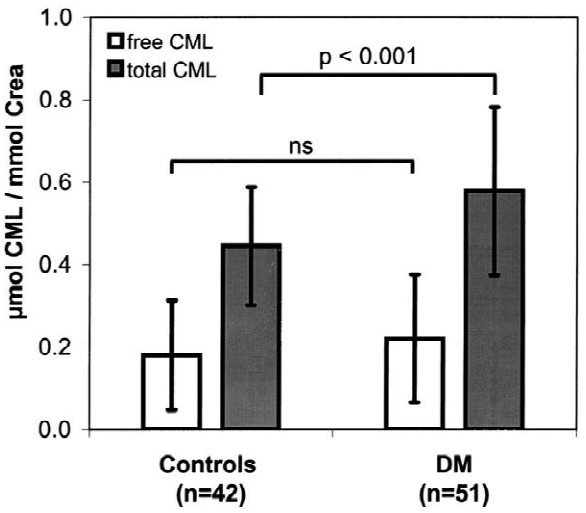

Fig. 2. Comparison of urinary free (open bars) and total (filled bars) CML excretion. Average values for free CML in controls versus diabetic subjects were 0.18±0.13 and 0.22±0.15 μmol/mmol creatinine, respectively (NS). Average values for total CML in controls versus diabetic subjects were 0.45±0.14 and 0.58±0.21 μmol/mmol creatinine, respectively ($P<0.001$) (all values±SD; DM, diabetes mellitus; NS, non-significant).

4. Discussion

Until recently, measurement of AGEs in urine was impeded by the lack of specific and sensitive assays [14]. Some AGEs exhibit fluorescence at 440 nm upon excitation at 370 nm. However, fluorescence can also result from reaction between lipid peroxida-

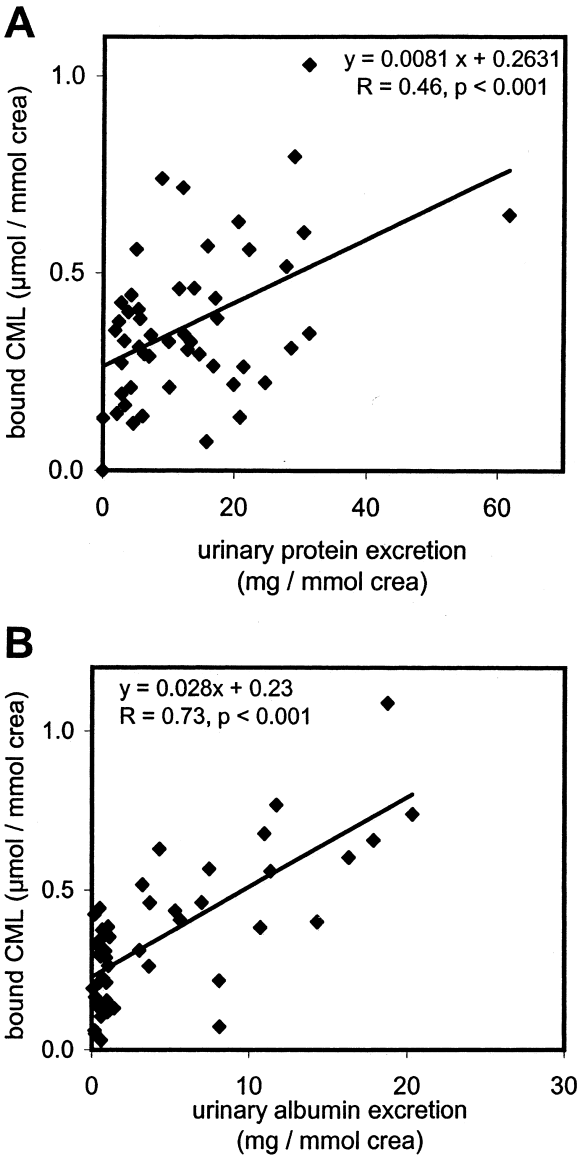

Fig. 3. Correlation between the excretion of peptide- and protein-bound CML and the excretion of protein (A) and of albumin (B) in the urine of 51 diabetic patients. The fraction of bound CML was calculated from the difference of total CML (measured in acid hydrolysates of urine) and free CML (measured in deproteinised urine samples).

tion products and proteins or from the oxidation of amino acids by free radicals [15]. CML is the chemically best defined and most abundant glycoxidation product. Its formation from early glycation products requires oxidative cleavage between C-2 and C-3 of the carbohydrate moiety, and it has been proposed that the measurement of CML in urine and other body fluids may serve as an integrative biomarker for oxidative stress [16]. CML in urine originates from structurally heterogenous compounds, ranging from the free monomer to carboxymethylated oligopeptides/polypeptides and proteins, which do not share a well defined common epitope. Several monoclonal and polyclonal antibodies have been raised against CML-modified proteins and peptides and used to measure CML. But up to now, there is no universally established standard or unit of measurement to compare results from different immunological assays [4,14]. It is still not clear which portions of monomeric CML or of carboxymethylated lysyl residues bound in different forms are recognised by a specific antibody. Therefore, the determination of the free modified amino acid using chromatographic separation techniques and synthetic CML as a defined standard provides an advantage. Previously, Knecht et al. have used SIM-GC–MS and ($^{2}H_{8}$)CML as a deuterated internal standard to determine CML in urine [17]. Although this method is specific and very precise, it is not widely available. Drusch et al. [18] described a RP-HPLC method to determine the total CML content of fresh and heat-treated food samples. By adapting this method to urine, we developed a sensitive and specific assay using standard equipment. A drawback of all the methods used, be it GC–MS or HPLC, is a low dynamic range. Excreted urine volumes and analyte concentrations (e.g. of physiological amines and amino acids, which are in ~50–500-fold excess to CML) vary widely. Therefore, a sample preparation step of evaporation, resuspension and predilution could not be omitted.

In diabetic patients, we found the urinary excretion of CML to be increased by 30% as compared to young and age-matched non-diabetic controls. This observed increase in total CML excretion in diabetes is mainly due to an increase in protein- and peptide-bound CML (Fig. 2). Our data, as well as recent findings from other studies [19], indicate that albuminuria could be the source for elevated CML excretion in diabetes. As shown in Table 1, our patients exhibited increased albumin excretion. Among the major proteins in serum and urine,

albumin is especially prone to carboxymethylation because of its low turn-over rate and high lysine content. Hamelin et al. [19] reported just recently that CML-albumin is the major excreted CML-modified protein in rats. In their study, the excretion of CML-albumin was correlated to glycoxidative tissue- and kidney damage. In our cohort of diabetic patients, we found a significant correlation of the degree of proteinuria (most of the excreted protein being albumin) and albuminuria to the urinary excretion of bound CML, whereas no such correlation could be demonstrated for total or free CML excretion (data not shown). The proportion of ~40% of the excreted CML, which has not been removed by precipitation, is the free amino acid CML. This fraction, originating from protein catabolism or from nutritional sources, was not significantly different between diabetic and non-diabetic patients. The values we obtained for urinary CML are in good agreement with the few published reports. Knecht et al. [17] using SIM-GC–MS in hydrolysed urine found CML-levels of 1.2 ± 0.5 μg/mg creatinine (equivalent to 0.67 ± 0.28 μmol/mmol crea) in diabetic subjects. Wagner et al. [20] determined urinary CML in type II diabetic patients and found CML-excretion rates of 130 ± 50 μg CML/mmol creatinine (equivalent to 0.64 ± 0.25 μmol/mmol crea) in diabetic patients with moderate renal impairment.

Our observation that CML levels in body fluids are not directly correlated to blood glucose control and to HbA1c is shared by others [6,21]. Urinary CML excretion can not be used as a glycation marker alone, because circulating and excreted CML is known to result from a variety of different metabolic or dietary sources. Formation of CML has been described from glycoxidation of glucose, but also of other carbohydrates such as fructose or ascorbate, from carbonyl intermediates such as glyoxal or glycolaldehyde or from lipid peroxidation [3]. Increased carboxymethylation of serum or tissue proteins has been described for diabetes, uremia or aging and for a wide variety of diseases such as atherosclerosis, Alzheimer's disease or rheumatoid arthritis [4,22]. Current investigation is under way as to whether the increased formation of reactive oxygen species is a common property of those conditions. Overall we propose that the sensitive and specific determination of CML by the described HPLC method gives an advantage over less defined AGEs. In diabetic patients we found urinary CML to be increased. Determination of urinary CML excretion could be a useful tool in research on oxidative stress.

5. Nomenclature

ACN	acetonitrile
AGE	advanced glycation end-product
CEL	carboxyethyl-lysine
CML	*N*(epsilon)-(carboxymethyl)lysine
crea	creatinine
HbA1c	hemoglobin A1c
GC–MS	gas chromatography–mass spectroscopy
LC–MS	liquid chromatography–mass spectroscopy
NMR	nuclear magnetic resonance spectroscopy
OPA	*ortho*-phtaldialdehyde
RP-HPLC	reverse phase high pressure liquid chromatography
SIM-GC–MS	selected ion monitoring GC–MS
THF	tetrahydrofurane

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