

Fluorescent serum and urinary advanced glycoxidation end-products in non- diabetic subjects

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ABSTRACT

Introduction: Advanced glycoxidation end-products (AGEs) are involved in age-related conditions and diabetic complications. Diet intake contributes to their circulating concentrations.

Aim: To measure serum and urinary AGEs in non-diabetic volunteers and relate their concentration to body composition, blood chemistry and dietary ingestion.

Methods: We studied 41 adult men (31 middle-aged adults and 10 elderly). A nutritional assessment including a dietary recall designed for detection of AGE ingestion (specifically carboxymethyl-lysine(CML)), and anthropometric measurements were performed. Also serum lipoproteins, insulin, glucose, leptin and C reactive protein (CRP). AGEs were measured in serum and urine samples using size exclusion chromatography and flow injection assay (FIA); the technical procedures were first employed in 11 heterogeneous diabetics, as positive controls for this methodology.

Results: Serum and urinary chromatograms indicated that areas under the curve were not different in younger compared with elderly adults. AGEs did not correlate with dietary intake, body composition, nor metabolic parameters, however they correlated significantly with renal function and CRP concentration.

Discussion: In these non-diabetic volunteers, with low CML intake, serum and urinary concentration of AGEs were not related to dietary intake. AGEs were related to renal function and CRP, but not to body composition, lipoproteins, insulin and glucose.

Key terms: Advanced glycation end-products, Fluorescent AGEs, glycoxidation.

INTRODUCTION

Advanced glycation and lipoxidation end-products (generically called advanced glycation end-products, or AGEs) are involved in the pathogenesis of several age-related conditions (Schleicher et al., 1999, Chen et al., 1999, Vitek et al., 1994), apart from their well known participation in the microvascular complications of diabetes mellitus (Vlassara et al., 1986). The main source of circulating and tissue AGEs is endogenous synthesis associated with impaired excretion, especially in diabetics and diabetic nephropathy (Zilin et al., 2001). However, several recent studies have shown the contribution of dietary ingestion of these products to their circulating levels. Dietary

AGEs are mainly originated from fatty foods and meats cooked at high temperatures, as well as some types of processed starchy foodstuffs (Goldberg et al., 2004), or elevated intake of fructose, as in vegetarians (Krajcovicová-Kudlacková et al., 2002). Therefore, serum and urinary AGEs concentrations can be lowered by dietary short-term manipulations (Uribarri et al., 2003), however the contribution of this dietetic management in the prevention of chronic age-related disease requires long-term intervention studies. In mice, energy restriction lowers collagen-associated AGE deposits (Reiser et al., 1994), effect that has been attributed to the type of carbohydrate ingested, independent of caloric restriction (Murtagh-Mark et al., 1995).

AGEs are proinflammatory molecules; they are able to generate reactive oxygen species (ROS), deplete antioxidant molecules (Cai et al., 2002) and induce production of several cytokines (mainly tumor necrosis factor alpha (TNF α), interleukins 1 β and 6), vascular adhesion molecule-1 (VCAM-1), and C reactive protein among others (Vlassara et al, 2002). These mechanisms seem to account for the enhanced cardiovascular risk associated to age, diabetes and end stage renal disease (Peppia et al., 2004). AGE binding to specific receptors generates ROS which activate nuclear factor kappa B (NF κ B), a transcription factor which stimulates the production of the mentioned cytokines by macrophages and induces changes in gene expression. The latter translates into a procoagulatory state and adhesion of inflammatory cells to vascular endothelial cells (Brownlee M, 2000). On the other hand, oxidative stress and inflammatory processes accelerate the formation of AGEs, as seen in rheumatoid arthritis (Hein et al., 2005) and alcoholism (Kalousova et al., 2004). Thus, oxidative reactions are implicated both in the synthesis of AGEs as well as in their pathogenic effects.

Oxidative stress is also associated with complications of obesity (Ferretti et al., 2005, Molnar et al., 2004, Uzun et al., 2004), however the contribution of diet-related AGEs, independent of hyperglycemia or renal failure has seldom been studied in relatively healthy subjects of differing adiposity. Kalousova et al. reported a positive correlation between AGEs, body fat and leptin among hemodialyzed diabetic patients (Kalousova et al., 2003), but negative correlations between AGE levels and leptin in alcoholics (Kalousova et al., 2004). Interestingly, calorie restriction and weight decrease lowered urinary AGEs (pentosidine) in patients with rheumatoid arthritis (Iwashige et al., 2004).

Since AGEs are heterogeneous moieties, originated from the nonenzymatic reaction of reducing sugars with proteins, lipids, and nucleic acids, there are multiple structurally identified AGEs. Among them, N-carboxymethyllysine (CML), pentosidine, pyrrolidine, imidazolone, and other glycated

proteins or peptides of dissimilar molecular weights, have been identified. These substances can be measured through various techniques, such as competitive ELISA, fluorescence and immunohistochemistry, however, specific antibodies were not commercially available for this study. Therefore we assayed serum and urinary concentrations of AGEs, by size exclusion chromatography with fluorescence detection, as described by Henle et al. (Henle et al., 1999) and fluorescence spectroscopy through a flow injection assay as described by Wrobel (Wrobel et al., 1997) and Zilin (Zilin et al., 2001), both methodologies based on fluorescence emission, a feature of most AGEs.

The aim of the present investigation was to study the relationship between serum and urinary AGE concentrations, blood chemistry, body composition and dietary ingestion of AGEs, in a group of healthy non-diabetic male volunteers.

SUBJECTS AND METHODS

This study was conducted according to the Helsinki declaration and approved by INTA's ethics committee. Fluorescent serum and urinary AGEs were first measured in a sample of 11 diabetic subjects aged 45 to 78 years, two of them with renal failure, as positive controls. The same methodology was then employed for the determination of AGEs in 41 non-diabetic volunteers, 31 middle-aged (35-52 years) and 10 elderly (66-101 years).

At inclusion, all subjects signed a written informed consent, after which a nutritional assessment was performed. The latter included a dietary recall (24 h and food frequency) designed to calculate intake of N-carboxy-methyl and ethyl-lysine (CML and CEL respectively), according to Goldberg et al. (Goldberg et al., 2004) (adapted for Chilean meals), and anthropometric parameters (weight, height, waist and hip circumference, and skinfold thickness at 4 sites to estimate body fat % according to Durnin and Womenseley) (Durnin et al., 1974). Then, a fasting blood sample was obtained for determination of

lipoproteins (total cholesterol, HDL cholesterol and triglycerides), glucose, insulin, leptin, C reactive protein (CRP) and AGE concentrations. A second morning urine sample was also obtained, to measure urinary AGEs. All samples were centrifuged and stored at -70°C until assayed.

Analytical techniques

Lipoproteins and glucose serum concentrations were measured through standard automated systems, using *in vitro* reagents from Roche Diagnostics through CHOD-PAP and hexokinase techniques respectively, and CRP through an automated immunoturbidometric assay (with standard sensitivity) at Laboratorio Vidaintegra. Insulin was measured by a Diagnostic Product Corporation RIA kit (DPC, USA), leptin by a Linco RIA kit (Linco Research Inc, USA).

For the AGE assay in serum samples, a fluorescent spectroscopy was performed, at 350 nm excitation and 430 emission wavelengths, then samples were diluted 1:25 in phosphate-buffered saline (PBS). Size-selective gel permeation chromatography was performed in a Merck-Hitachi HPLC system, using a Superdex 75 10/300 column (Amersham Bioscience, Uppsala, Sweden), eluted at 0.5 mL/min with PBS. Fluorescence signals in mV were recorded in a Merck-Hitachi detector (F-1080). A molecular weight calibrator was employed (Molecular Weight Marker kit MW-GF-70, Sigma-Aldrich, St Louis, USA), containing the following proteins: bovine serum albumin (BSA) (66 kDa), carbonic anhydrase (29 kDa), cytochrome c (12.4 kDa) and aprotinin (6.5 kDa). In the tested samples, the molecular weights of the chromatographic peaks obtained were assigned, according to their retention times, using a semi-logarithmic regression curve based on the molecular weight standard proteins. The reproducibility of the assay was ensured by injecting an AGE-Bovine Serum Albumin standard (catalogue N° 121800, EMD Biosciences, La Jolla, CA) together with the subjects' samples. For urine samples a similar procedure was

employed, except for dilution (1:5) and elution at 0.7 mL/min. As all urinary signals were beyond the separation range of the column, their molecular weight was not identified by the molecular weight standards employed. Data were expressed as area under the curve (AUC)/ total serum protein or urinary creatinine respectively.

For detection of serum and urine small-sized AGE-peptides, a flow injection assay (FIA) in the HPLC system was employed, as follows. Briefly, the samples were treated with trichloroacetic acid, then centrifuged, and the aqueous layer was injected at a flow rate of 0.5 mL/min into the flow system, driven by a Merck-Hitachi L-6200 pump to the fluorescence detector. We employed 50mg/L AGE-BSA after hydrolysis with proteinase K as a standard (intrassay error = 2,26 %). Results were expressed as fluorescence intensity/g creatinuria $\times 10^{10}$.

Statistics

Data are expressed as means \pm standard deviations. Means between parametric data were compared by Student's t test and non-parametric data by Kruskal-Wallis. Pearson correlation coefficients were performed to study associations between variables. All statistical analyses were performed with Statistica for Windows.

RESULTS

Laboratory procedures for detection of AGEs in serum and urine samples were first tested in 11 diabetic volunteers (6 men), ranging from 44 to 78 years of age; A1c haemoglobin averaged $8.3 \pm 1.7\%$ and two of them had renal failure. Serum AGEs were similar in these diabetics compared with our sample of non-diabetic subjects, however urinary AGEs were significantly higher compared with the non-diabetic volunteers.

Clinical laboratory and nutritional features of the study groups are depicted in table 1. Among the 31 middle-age subjects, body mass index (BMI) ranged from 20.3 to 45 kg/m^2 (8 were obese, $\text{BMI} > 30\text{ kg/mt}^2$).

Among the 10 elderly volunteers BMI ranged from 20.2 to 32.4 kg/m^2 , only 1 was obese. Creatinine serum levels exceeded the cutoff point of 1.3 mg/dL (114.9 $\mu\text{mol}/\text{L}$) in one elderly subject.

According to dietary recalls, daily average AGE intake in middle-age subjects was $9,754 \pm 3,936$ kU per day (2,678 to 16,713 kU/d), and $9,893 \pm 3,784$ (5,619 to 17,222 kU/d) in the elderly group.

Serum AGEs were assessed as the area under the curve (AUC) at certain molecular weight fractions, according to the calibration proteins. Fraction 1 (F1) corresponds to 44-148 kDa moieties (retention time 14-20 min), Fraction 2 (F2) corresponds to 8-19 kDa (retention time 24-28 min), Fraction 3 (F3) < 2 kDa (retention time 35-43 min) and Fraction 4 (F4) correspond to peaks beyond the separation capacity of the column (retention time 46-60 min). Figures 1 and 2 depict representative serum chromatograms of F1 and F2. No detectable peaks were found beyond F2 in the first 5 samples from diabetic positive controls, so we decided to establish a maximal analysis time of 30 minutes, and are therefore unable to inform F3 and F4. In all serum samples, a first clearly detectable fluorescent peak was

detected at 16 min retention time. Spiking of the samples indicated that this first slope was equivalent to the AGE-BSA standard. A second smaller peak at 26 min retention time was also evident among 7 positive controls, but 4 of them had no signal at this time. The two diabetics with renal failure had higher AUCs (representative chromatograms in fig. 1).

Comparison of AUCs in serum sample from the study groups is depicted in table 2. The F2 peak was not present in 2 elderly and 5 middle-aged subjects. No significant differences in F1, F2 and serum FIA were observed between groups.

In urinary samples, chromatograms were more heterogeneous, but 2 clearly identifiable peaks were observed at retention times 29-30 (urinary fraction 3, uF3) and 35-36 (urinary fraction 4, uF4), in all the studied subjects (figure 3). No significant differences were observed in urinary AGEs between young and elderly subjects.

Serum and urinary AGEs did not correlate with the anthropometric indexes measured, nor with macronutrient ingestion assessed by dietary recalls. Dietary intake of CML was not associated with serum or urinary AGE concentrations in these subjects.

TABLE 1

Clinical and Nutritional Features

Variable [Normal Range]	Elderly (n=10)	Young Adults (n = 31)	p =
Age (years)	74 \pm 10	41 \pm 5	< 0.001
Weight (kg)	67 \pm 9	82 \pm 17	0.012
Height (cm)	163 \pm 4	171 \pm 7	< 0.001
Body Mass Index (kg/m^2) [18.5 - 24.9]	25.2 \pm 4	28.1 \pm 6	0.15
Body Fat (%) [20 - 25]	25.4 \pm 4	26.7 \pm 5	0.45
Total Cholesterol (mg/dL) [< 200]	206 \pm 35	205 \pm 37	0.94
HDL Cholesterol (mg/dL) [40 -60]	45 \pm 9	46 \pm 9	0.89
Triglycerides (mg/dL) [< 150]	141 \pm 68	185 \pm 162	0.41
Creatinine (mg/dL) [0.70-1.20]	1.1 \pm 0.3	1.0 \pm 0.4	0.54
Glucose (mg/dL) [80-100]	98 \pm 10	103 \pm 35	0.66
Insulin ($\mu\text{U}/\text{mL}$) [< 20]	5.4 \pm 4	7.7 \pm 5	0.21
Leptin (ng/L) [2.2-5.4]	6.2 \pm 4	8.5 \pm 7	0.32
C Reactive Protein (mg/dL) [< 0.5]	0.4 \pm 0.3	0.16 \pm 0.12	< 0.001

p = < 0.05 according to Student's t-test.

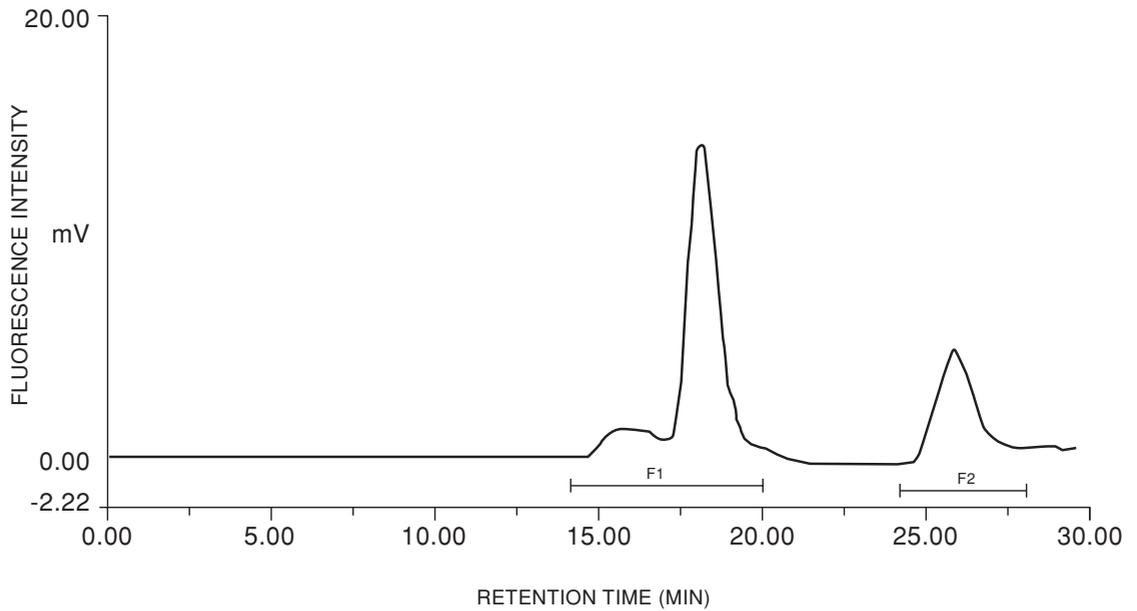
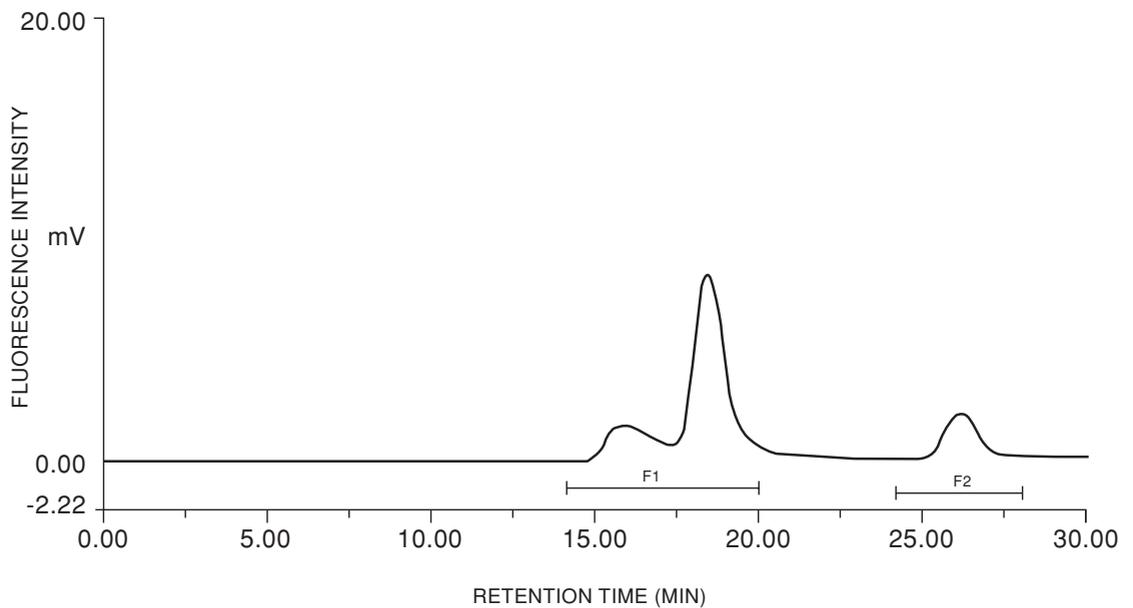
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Figure 1: Chromatograms of serum AGEs in diabetic positive control patients. A) Diabetic patient with end-stage renal disease B) Diabetic patient with normal renal function. F1 = fraction 1 (retention time 14-20 min) F2 = fraction 2 (retention time 24-28 min)

TABLE 2

Serum AGE Levels

AGEs	Elderly (n=10)	Young Adults (n = 31)	p =
F1 - Fraction 1	1.8 ± 0.7	1.6 ± 0.3	0.67
F2 - Fraction 2	6.5 ± 2.6	6.6 ± 1.6	0.46
FIA	2.3 ± 1.5	1.6 ± 0.4	0.19

Serum AGEs are expressed as Arbitrary Units (AU) = AUC /g total serum protein concentration.

(F1 = AU/g protein x 10⁸, F2 = AU/g protein x 10⁷, FIA = AU/g protein x 10⁵)

Significantly different if p < 0.05, calculated by Kruskal-Wallis.

TABLE 3

Urinary AGE Levels

Urine AGEs	Elderly (n=10)	Young adults (n = 31)	p =
uF3 - urinary Fraction 3	10.0 ± 2.6	8.7 ± 2.6	0.19
uF4 - urinary Fraction 4	7.9 ± 4.5	8.5 ± 3.5	0.46
uFIA	2.9 ± 0.5	3.2 ± 1.6	0.82

Urinary AGEs are expressed as Arbitrary Units (AU) = AUC /g urinary creatinine

(uF3 = AU/g creatininuria x 10⁹, F2 = AU/g creatininuria x 10⁹, FIA = AU/g creatininuria x 10¹⁰)

Significantly different if p < 0.05 calculated by Kruskal-Wallis.

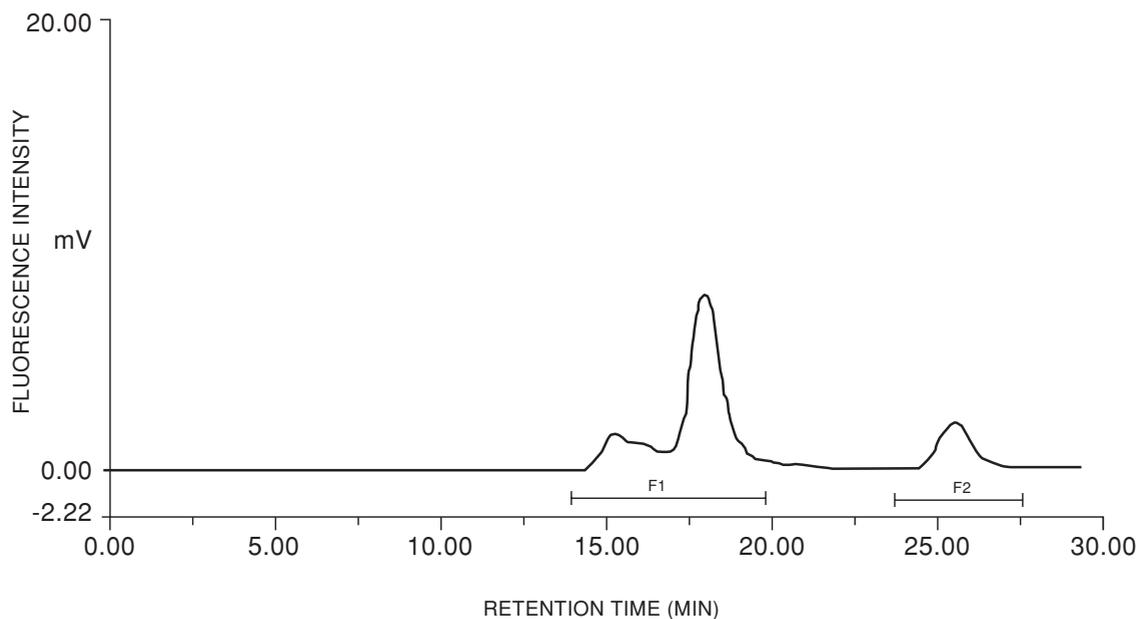


Figure 2: Chromatogram of serum AGEs in a representative non-diabetic volunteer. F1 = fraction 1 (retention time 14-20 min) F2 = fraction 2 (retention time 24-28 min)

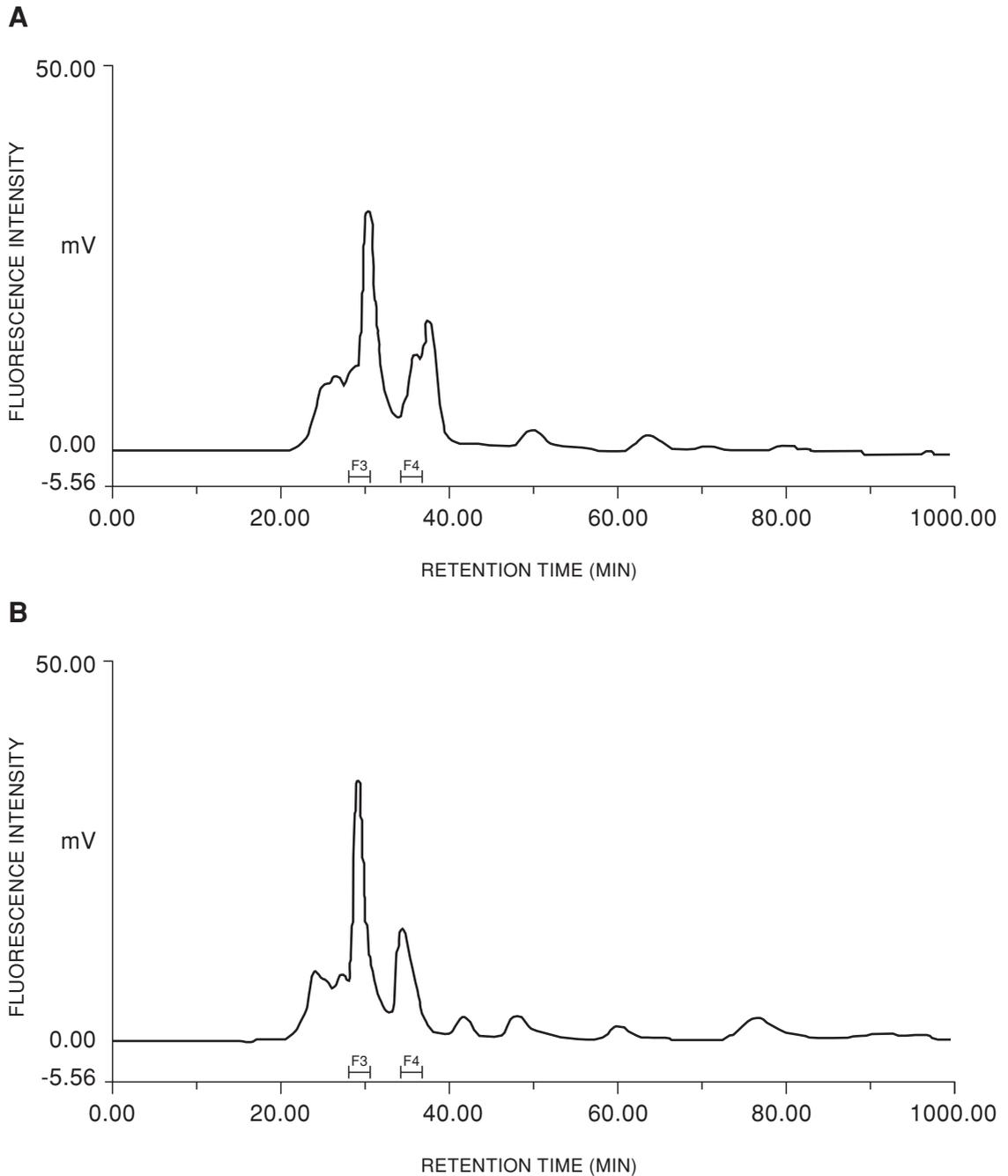


Figure 3: Representative urinary chromatograms in A) Diabetic patient B) Non-diabetic healthy volunteer. uF3 = fraction 3 (retention time 29-30 min) uF4 = fraction 4 (retention time 24-28 min)

Serum F1 did not correlate with glucose, insulin, and lipoproteins, but correlated significantly with creatinine ($r=0.57$, $p < 0.001$) and CRP ($r = 0.58$, $p < 0.001$). F2 correlated positively with HDL cholesterol ($r=0.48$, $p = 0.01$) and creatinine ($r=0.44$, $p = 0.01$). Serum FIA

was also significantly associated with creatinine ($r = 0.39$, $p = 0.014$) and CRP ($r = 0.44$, $p = 0.009$). However, the association between creatinine and serum AGEs was highly influenced by the one elderly subject with elevated creatinine concentration (1.8 mg/dL).

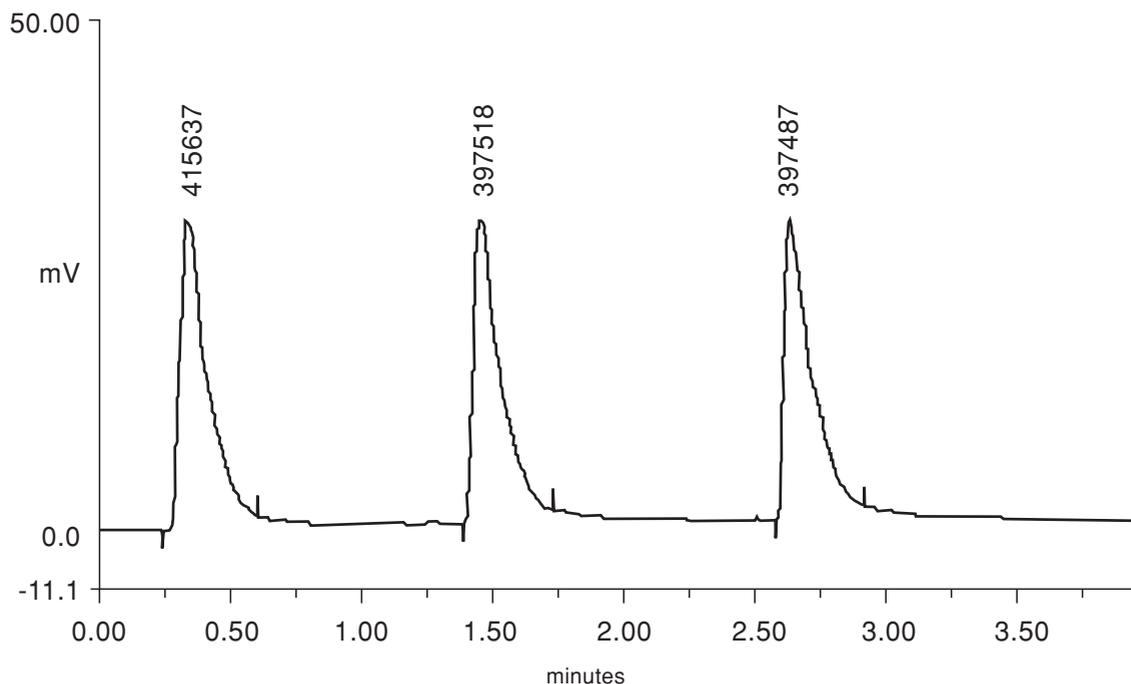


Figure 4: Flow Injection Assay (FIA) - Chromatographic readings for 3 injections of 50mg/L AGE-BSA standard.

Urinary excretion of AGEs was not related with serum lipoproteins, creatinine or glucose, but one fraction (uF3) was significantly associated with CRP ($r = 0.44$ $p = 0.02$). Urinary FIA did not correlate with anthropometric measurements nor metabolic variables.

DISCUSSION

In this study we were able to set up 2 relatively inexpensive methods for detection of AGEs through fluorescence, which is a distinguishable feature of most AGE moieties. We detected AGEs in blood and spot urine samples, first in a small group of heterogeneous diabetics, where these substances are expected to increase due to hyperglycemia and decreased glomerular filtration, and then in non diabetic male volunteers of different ages and body compositions. As recent literature has highlighted the contribution of dietary intake of AGEs to their circulating levels, associated with oxidation markers (Vlassara et al., 2002), we studied dietary ingestion of

CML and CEL (two of the most representative AGEs), using a specially adapted dietary recall, according to Goldberg et al. (Goldberg et al., 2004).

These preliminary results indicate that urinary excretion of AGEs is probably a better indicator of hyperglycemia and diabetic nephropathy than serum levels. However these findings need to be replicated in a larger sample of diabetics, with wider ranges of metabolic control and renal function, assessed through more accurate methodologies, such as creatinine clearance or albuminuria. Our aim was to study AGEs in healthy normoglycemic subjects. From our results in healthy volunteers, it appears that serum fluorescent AGEs concentration is more dependent on renal function than urinary excretion of AGEs. Similarly in diabetics, the correlation between creatinine and serum AGEs is higher than with urinary AGEs.

In our sample of 41 non-diabetic men, AGE intake was lower ($< 10,000$ kU/day) than that reported by Goldberg et al. (2004), which averaged $16,000 \pm 5,000$ kU/day. This could explain the lack of

association between intake and serum and urinary concentrations of AGEs, because it has been reported that higher CML intake correlates linearly with CML serum levels (Uribarri et al., 2003). Another plausible explanation is that Goldberg's dietary recall quantifies CML and CEL (whose detection in food or biological samples requires antibodies that were not commercially available when we performed this study), while fluorescence detects other AGE moieties.

However, even if our non-diabetic subjects consumed low AGE diets and had relatively low AGE concentrations (compared with diabetics, because normal ranges have not yet been defined) these were significantly associated with CRP, an indicator of a pro-inflammatory condition. This is consistent with published data on the association between circulating AGEs and several markers of inflammation (Vlassara et al., 2002, Peppas et al., 2004). More importantly, it has been demonstrated both in animal and human studies that lowering dietary AGEs (and thus circulating and tissue concentrations) can induce a wide range of benefits, ranging from reduction of visceral fat and improved insulin sensitivity to reduction of vascular pro-atherogenic inflammatory mediators (NF- κ B and VCAM) and recuperation of endothelial function (Uribarri et al., 2005).

This study has several limitations, such as the small sample size and the evaluation of dietary intake by only one recall. Also as mentioned, AGEs are multiple and heterogeneous moieties that can be originated from different metabolic pathways or from dietary sources, thus there is no available gold standard for their detection. Most authors employ immunologic methods, although these rely on detection of one or few epitopes, and therefore could not reflect the real AGEs load. In this study we used detection by fluorescence, which measures a variety of circulating substances, it does not however suggest which ones are predominating. Nevertheless, the concentration of small-sized AGEs detected by FIA has prognostic value in haemodialysis patients (Roberts et al., 2006).

In non-diabetic subjects dietary intake is the main source of circulating and tissue AGEs. Goldberg et al. reported the content of CML in American foodstuffs (Goldberg et al., 2004). If our volunteers are representative of the habitual Chilean diet, this would be indicating a healthier pattern compared with North America, at least regarding CML, which is one of the main circulating AGEs. However we need to measure AGEs directly in the foods regularly consumed in our country, because AGEs are mostly originated during processing and cooking. It is also necessary to study groups of people with special dietary habits such as teenagers, or with special prescriptions, such as diabetic and hypercholesterolemic patients.

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