Clinical Chemistry 43:9
1563–1569 (1997)

Novel analytical approach to monitoring advanced glycosylation end products in human serum with on-line spectrophotometric and spectrofluorometric detection in a flow system

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We proposed a simple analytical procedure for measurement of serum advanced glycosylation end products (AGEs) based on simultaneous detection of low-molecular-mass peptides and AGEs with a flow system and two detectors connected on-line: spectrophotometric for peptides (λ = 280 nm) and spectrofluorometric for AGEs (λ_{ex} = 247 nm, λ_{em} = 440 nm). Sample pretreatment was carried out in microcentrifuge tubes: Serum (20 μL) was deproteinized with trichloroacetic acid (480 μL, 0.15 mol/L) and lipids were extracted with chloroform (100 μL). Twenty microliters of the filtered aqueous layer was injected to the flow system and the relation between fluorescence and absorption signals was measured. A peptide-derived AGE calibrator was used for calibration. Within-day and between-day CVs were 6.7% and 9.1%, respectively, at an AGE concentration corresponding approximately to that in healthy individuals. Mean results (±SD) in 10 healthy individuals were 10.1% ± 1.0%, in 21 patients with diabetes without complications 18.0% ± 6.2%, in 25 patients with complications 24.1% ± 15.4%, and in 12 diabetic patients in end-stage renal disease 92% ± 30%. Comparison with an ELISA procedure (x, in arbitrary units/L) yields a regression equation $y = 0.713x + 1.24$ ($S_{y|x}$ = 6777, r = 0.8477, n = 41).

Reducing sugars react with free amino groups of proteins, first forming reversible Schiff bases and then covalently bonded Amadori rearragement products *[1, 2]*. The final, irreversible products of this process are advanced glycosylation end products (AGEs) *[3]*.[3] These products tend to form further protein–protein cross-links. AGEs accumulate slowly in long-lived proteins such as collagen, altering structure and properties of some tissues (vascular walls, kidney, etc.) *[4, 5]*. This process is an important pathogenic factor in complications of diabetes *[5–9]*. Serum AGE concentration is a marker for monitoring the treatment of diabetic patients, especially those with renal and (or) vascular complications *[7–11]*. However, use of AGE measurements in clinical practice is still limited by lack of simple and rapid analytical procedures *[9–11]*.

AGE–proteins can be determined by immunochemical methods *[6, 12–14]*. For such assays, AGE-specific antibody is required, as first described by Makita et al. *[6]* and used in later studies *[13, 14]*. However, no commercial kit is available at this time. Serum and tissue AGEs have also been measured by radioreceptor assay *[9, 15]*, for which cell cultures are needed; the procedure is labor and time consuming.

AGEs exhibit characteristic fluorescence *[6]*. With excitation in the range 350–390 nm, fluorescence emission has been measured at 440–470 nm for AGE detection *[8, 9, 16–18]*. In one study *[9]*, the protein concentration in all samples was adjusted to 1 g/L and fluorescence measurements were expressed as the percentage of relative fluorescence as compared with that of an AGE–albumin calibrator. The results obtained did not agree well with the results of radioreceptor assay *[9]*.

In the present work a simple analytical procedure is proposed for AGE measurement. The experimental conditions are discussed, results of 68 serum samples from healthy persons and diabetic patients are presented, and

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[3] Nonstandard abbreviations: AGE, advanced glycosylation end product; BSA, bovine serum albumin; TCA, trichloroacetic acid; and PMSF, phenylmethylsulfonyl fluoride.
Received December 10, 1996; revised and accepted May 16, 1997.

statistical correlation between those results and the results obtained by ELISA is evaluated.

Materials and Methods

APPARATUS

Analysis in flow system was performed with a Hewlett Packard high pressure pump (Series 1050 high-performance liquid chromatograph), with integrated sample injector (loop 20 μL) and two detectors connected on-line: multiple wavelength spectrophotometric detector (Series 1050) and spectrofluorometric detector (HP 1046A). ChemStation (Series 1050) was used for data processing.

A Spectronic 3000 Diode Array Milton Roy spectrophotometer was used for absorbance measurements of bovine serum albumin (BSA) and AGE-BSA calibrators.

REAGENTS

BSA, glucose, proteinase K, trichloroacetic acid (TCA), chloroform, phenylmethylsulfonyl fluoride (PMSF), and Bradford reagent were obtained from Sigma. Suprapur perchloric acid was from Merck.

Phosphate buffer solution (0.2 mol/L, pH 7.4) was prepared from respective sodium salts (Sigma).

AGE antibody was a kind gift from R. Bucala (The Rockefeller University, New York, NY). Stock enzyme solution (8 g/L) was prepared in Tris buffer (0.05 mol/L, pH 8) with 0.2 g/L sodium azide (Sigma) and stored at −20 °C for up to 2 weeks. Working solutions were obtained by diluting this stock solution (1:20) in 0.02 mol/L phosphate buffer.

Stock solution of 0.1 mol/L PMSF in ethanol was prepared daily. For proteinase K inhibition this solution was diluted 1:200 in phosphate buffer.

AGE–albumin was prepared by incubating albumin (50 g/L) with 0.5 mol/L glucose in 0.2 mol/L phosphate buffer (pH 7.4) for 60 days. The control samples of albumin without glucose were also incubated in these same conditions (37 °C, sterile conditions, darkness). After incubation, dialysis against PBS was carried out to remove unbound material.

AGE–peptide calibrator was obtained by hydrolysis of calibrator AGE-BSA (50 g/L) with proteinase K: 10 μL of proteinase K solution (8 g/L) was mixed with 90 μL of AGE-BSA and incubated for 24 h at 37 °C. In parallel, BSA was incubated with proteinase K to obtain a peptide calibrator. The reproducibility of AGE–peptide preparation was satisfactory: For three hydrolyzed AGE-BSA calibrators, prepared with the same proteinase K, the difference in AGE content was not greater than 4% as measured by our method.

CLINICAL SAMPLES

Serum samples from healthy individuals and 58 diabetic patients were analyzed. One control group of 10 healthy persons (1) and three experimental groups were defined: (2) 21 diabetic patients without chronic diabetes-relevant complications, (3) 25 diabetic patients with chronic complications (neuropathy, nephropathy, retinopathy, etc.), and (4) 12 diabetic patients with end-stage renal disease.

ON-LINE DOUBLE- (SPECTROPHOTOMETRIC AND SPECTROFLUOROMETRIC) DETECTION FLOW SYSTEM

Serum samples (20 μL) were mixed with 480 μL of TCA (0.15 mol/L) in microcentrifuge tubes, and 100 μL of chloroform was added. The tubes were shaken vigorously to complete the precipitation of proteins and to extract lipids to organic phase and then centrifuged (10 min, 13 000*g*), and 20 μL of the aqueous layer was injected into the flow system. Water flow rate was 0.5 mL/min (deionized water, HPLC grade, Labconco); spectrophotometric detector was set at 280 nm for detection of peptides (reference wavelength 550 nm). By using spectrofluorometric detector, AGE fluorescent signal was obtained at 440 nm with excitation at 247 nm (bandwidth 17 nm, cutoff filter 370 nm). The samples were run in triplicate and peak height mode was used for signal measurements.

IMMUNOCHEMICAL ASSAY

Serum samples (200 μL) were digested with proteinase K. To do so, samples (100 μL) were placed in the microcentrifuge tubes and 200 μL of the working proteinase K solution was added. After incubation (37 °C, 12 h) samples were centrifuged (10 min, 13 000*g*) and 200 μL of clear solution was mixed with 50 μL of PMSF solution (diluted 1:200). Volumes of 50 μL of such prepared samples were taken to competitive ELISA *[6]*. Proteinase K could contain some AGEs, so the enzyme control sample (adding 100 μL of phosphate buffer instead of serum) was prepared and run in parallel.

The method of Bradford *[19]* was used for quantification of proteins (BSA and AGE-BSA after dialysis).

STATISTICAL ANALYSIS

The detection limit for AGEs in the proposed method was evaluated as follows *[20]*:

$$\mathrm{DL} = 3\frac{S_{R,c}}{b}\sqrt{\frac{\mathrm{n}-2}{\mathrm{n}-1}}$$

where: $s_{R,c}$ is regression standard deviation of instrument signal (R) to analyte concentration (c), b is the slope of calibration line, and n is the total number of pairs used to calculate the regression line. The $s_{R,c}$ value was calculated according to equation:

$$S_{R,c} = \sqrt{\frac{1}{\mathrm{n}-2}\sum(R_\mathrm{i} - R_\mathrm{i}^c)}$$

where R_i represents the value of instrumental signal for calibration sample i, R_i^c is the value of the signal calculated on the regression line corresponding to the concentration c_i. In this work, instrumental signal was taken as

the relation between relative fluorescence ($FLD_{ex247,em440}$) and absorbance (A_{280nm}).

Within-day precision was evaluated as CV for 20 measurements of one sample, while measuring 10 other samples in between.

Between-day precision was evaluated as CV for three measurements of one sample during 20 days.

Differences between AGE results obtained in the control group and each of three groups of diabetic patients were studied with the *t*-test for unequal variances (Welch test) *[21]*. Significance was accepted with $P < 0.05$.

Results and Discussion

The most pronounced difference between the relative fluorescence (λ_{em} = 440 nm) of albumin (BSA) and AGE-BSA was seen with excitation at 247 nm (Fig. 1a). Emission fluorescence spectra (λ_{ex} = 247 nm, Fig. 1b) showed maximal spectral shift at 440 nm.

Previous work *[9]* reported that AGEs in the low-molecular-mass serum fraction showed more marked differences between healthy subjects and diabetic patients than did AGEs in total serum.

In Fig. 2, fluorescence excitation and emission spectra of hydrolyzed BSA and peptide-derived AGE calibrator (obtained by degradation of AGE-BSA with proteinase K) are presented. Comparison of Fig. 1a and Fig. 2a reveals

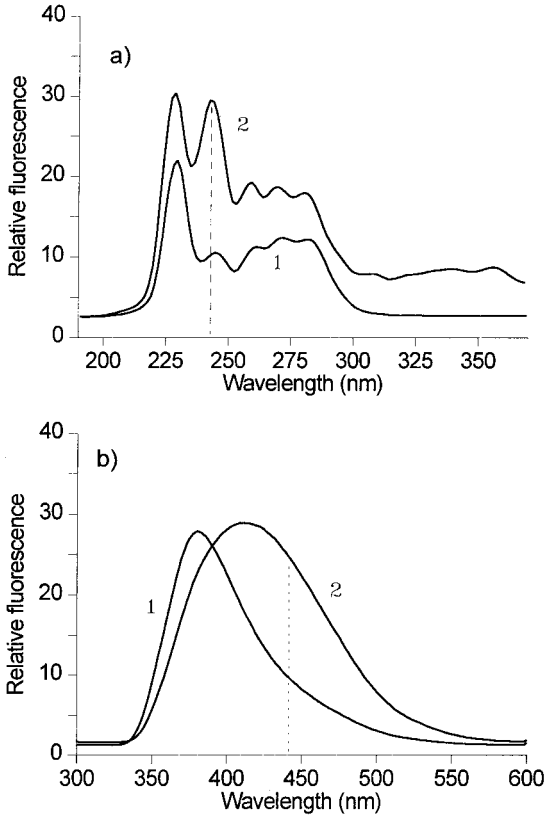

Fig. 1. Fluorescence spectra of: (*1*) albumin (0.125 mg·mL^{-1}) and (*2*) AGE–albumin calibrator at pH 7.4.
(*a*) Excitation spectra (λ_{em} = 440 nm); (*b*) emission spectra (λ_{ex} = 247 nm).

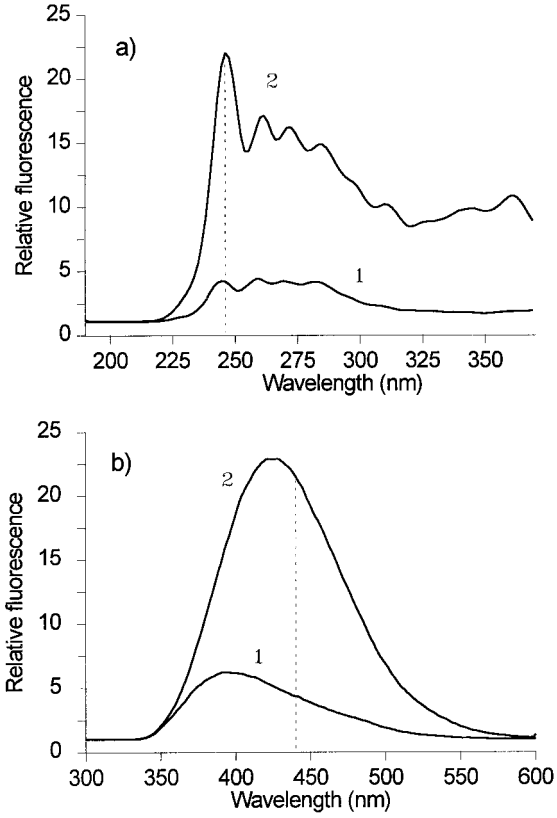

Fig. 2. Fluorescence spectra of: (*1*) hydrolyzed albumin (0.125 mg·mL^{-1}) and (*2*) hydrolyzed AGE–albumin calibrator at pH 7.4.
(*a*) Excitation spectra (λ_{em} = 440 nm); (*b*) emission spectra (λ_{ex} = 247 nm).

that fluorescence peaks for BSA and AGE-BSA with excitation at 228 nm (Fig. 1a) disappeared in excitation spectra of hydrolyzed calibrators (Fig. 2a), confirming that this peak corresponded to peptide bonds. As can be observed in Figs. 1b and 2b, emission spectra of BSA and AGE-BSA were also changed after degradation of proteins: The difference between signals for hydrolyzed calibrators (Fig. 2b) was larger than for BSA and AGE-BSA (Fig. 1b). The obtained results indicate that, after protein degradation, some interferences affecting AGE fluorescence signal while using spectrofluorometric detection conditions λ_{ex} = 247 nm and λ_{em} = 440 nm were reduced (compare Fig. 1b and Fig. 2b). We experimentally confirmed that wavelengths corresponding to excitation and emission spectral maxima of AGEs in calibrators were not affected in the pH range 1 to 8.

The idea of this work was to use these conditions of spectrofluorometric detection for determination of AGEs in serum (λ_{ex} = 247 nm, λ_{em} = 440 nm). A low-molecular-mass serum fraction was obtained by deproteinization of serum with TCA. The advantage of selected fluorescence conditions was the improved sensitivity for AGEs, which in turn enabled sample dilution and better reproducibility of protein precipitation (difference in fluorescence signal for 10 repeated precipitation procedures did not exceed 2%).

Four pooled serum samples were prepared by mixing seven serum aliquots (20 μL) from: (1) healthy persons, (2) diabetic patients without chronic complications, (3) diabetic patients with chronic complications (neuropathy, nephropathy, etc.), and (4) diabetic patients with end-stage renal disease. These pools were treated with 0.15 mol/L TCA and, in Fig. 3a and b, excitation and emission fluorescence spectra obtained for centrifuged and diluted (pools 1–3 25 times, pool 4 75 times) samples are presented. As can be observed in Fig. 3a, the fluorescence signals obtained in deproteinized serum pools (λ_{ex} = 247 nm, λ_{em} = 440 nm) corresponded to fluorescence signal obtained for calibrators of albumin- and peptide-derived AGEs (Fig. 1a, 2a). Moreover, significant differences can be observed (Fig. 3a) between relative fluorescence signals (λ_{ex} = 247 nm, λ_{em} = 440 nm) in four serum pools, in agreement with expected content of AGEs: low in healthy persons and increasing in diabetic patients depending on the possible complications. It seems possible that fluorescence observed in the excitation wavelength range 260–300 nm was emitted by degradation products of serum proteins. Indeed, the highest relative fluorescence in this range was observed for pooled serum from end-stage renal disease patients, whose serum contained peptides accumulated because of impaired renal function (Fig. 3a). Fluorescence emission spectra (λ_{ex} = 247 nm) of the four pooled serum samples (1–4) are presented in Fig. 3b. The progressive shift of these spectra (2–4) towards the position of maximum on the peptide-derived AGE spectra (λ_{max} = 420 nm, Fig. 2b) can be observed with the expected increase of AGE content in serum of diabetic patients with respect to that of healthy persons. These results confirmed that after serum deproteinization, and using 247 nm for excitation (λ_{em} = 440 nm), the AGE fluorescence signal can be measured in serum. Furthermore, in such experimental conditions, AGE content in four pools prepared from serum of different groups of patients was clearly distinguished.

In the previous reports, AGE serum content was measured as a fraction of total serum proteins *[6, 11]* or a fraction of serum peptides *[9]*. Calibration was carried out with a single calibrator of AGE-BSA (1 mmol/L AGE-BSA = 12 A_{350nm}) *[6, 9]*. In the present work, two detectors were connected on-line in a flow system to measure simultaneously signals corresponding to AGEs and to peptides in the sample. Spectrophotometric detection was used for total peptides and spectrofluorometric detection for AGEs. In Fig. 4 the example of measurement record for eight samples injected in triplicate is presented. The value of the ratio between relative fluorescence

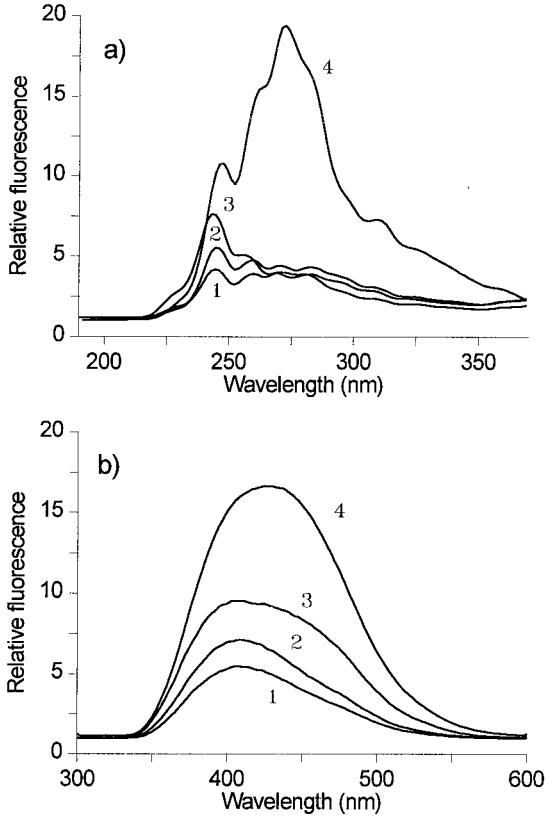

Fig. 3. Fluorescence spectra of deproteinized pooled serum samples: (*1*) healthy individuals, (*2*) diabetic patients, (*3*) diabetic patients with complications, and (*4*) end-stage renal failure (3× diluted).

(*a*) Excitation spectra (λ_{em} = 440 nm); (*b*) emission spectra (λ_{ex} = 247 nm).

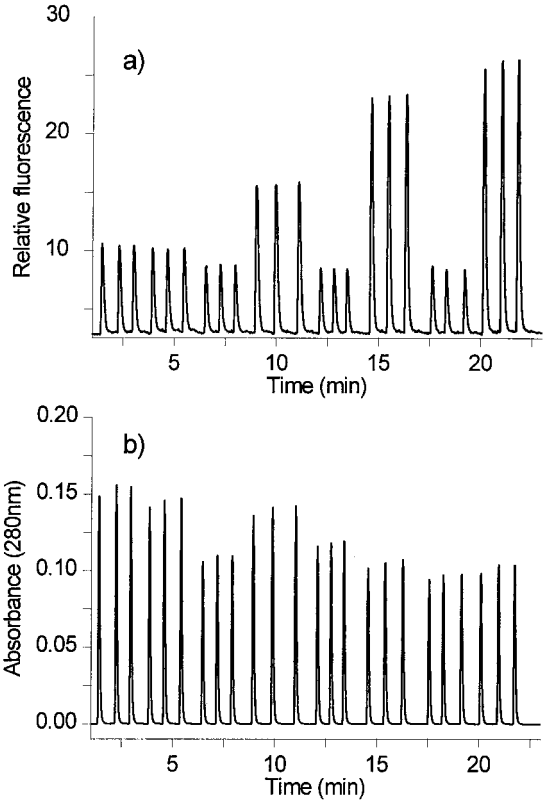

Fig. 4. Measurement record for eight serum samples obtained in the proposed flow system.

Each sample was injected in triplicate. (*a*) Spectrofluorometric detector response (λ_{ex} = 247 nm, λ_{em} = 440 nm); (*b*) spectrophotometric detector response (λ = 280 nm).

($FLD_{ex247nm,em440nm}$) and absorbance (A_{280nm}) was taken as the analytical signal for AGEs (S):

$$S = FLD_{ex247nm,em440nm}/A_{280nm}$$

The analytical signals for hydrolized calibrators of AGE-BSA ($S_{AGE\text{-}BSA}$) and BSA (S_{BSA}) were measured at physiological pH (pH 7.4). The ratio between these signals was significantly lower than the ratio between specific AGE signals measured as absorbance at 350 nm *[6, 9]* ($S_{AGE\text{-}BSA}/S_{BSA}$ = 4.8 and $A_{AGE\text{-}BSA}/A_{BSA}$ = 12.3). This indicates that in such conditions the proposed procedure did not assure measurement of signal specific for AGEs. In further development it was observed that, in acid medium, relative fluorescence ($FLD_{ex247nm,em440nm}$) was suppressed, but the ratio $S_{AGE\text{-}BSA}/S_{BSA}$ increased. This suggests that fluorescence not specific for AGEs was quenched in acid medium. Then, effect of TCA and perchloric acid, commonly used for deproteinization of serum, on the relation between AGE signals in the two hydrolized calibrators was studied.To do so, 20 μL of each calibrator was treated with different concentrations of TCA or perchloric acid (480 μL) and we determined that, after addition of acid, peptides did not precipitate (absorbance of peptide-derived AGE calibrator at 280 nm not altered after acid treatment). In the presence of TCA, relative fluorescence ($FLD_{ex247nm,em440nm}$) was always lower than in the presence of perchloric acid. For TCA concentrations up to 0.15 mol/L and for perchloric acid concentrations up to 0.4 mol/L the ratio $S_{AGE\text{-}BSA}/S_{BSA}$ increased with increasing acid concentration (Fig. 5) up to $S_{AGE\text{-}BSA}/S_{BSA}$ = 12.3, which corresponded exactly to the relation obtained between absorbances of AGE-BSA and BSA measured at 350 nm. These results clearly indicate that analytical signal (S) obtained with calibrators in the presence of 0.15 mol/L TCA or perchloric acid >0.4 mol/L was specific for AGEs, as compared with earlier reports *[6, 9]*. A very similar effect of the two acids was observed while deproteinizing four serum pools (1–4). The most marked differences between AGE signals in

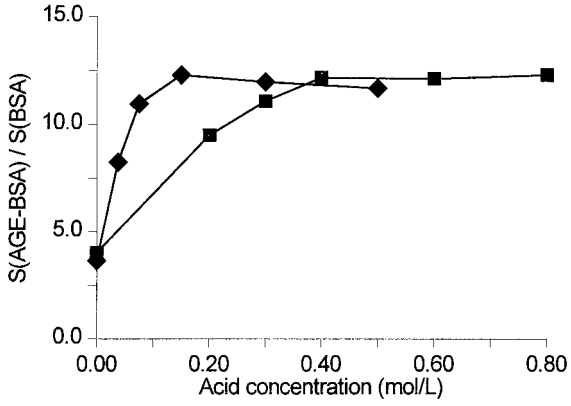

Fig. 5. Effect of acid concentration on the relation between analytical signals obtained in AGE-BSA ($S_{AGE\text{-}BSA}$) and BSA (S_{BSA}) hydrolized calibrators: ♦, TCA; ■, perchloric acid.

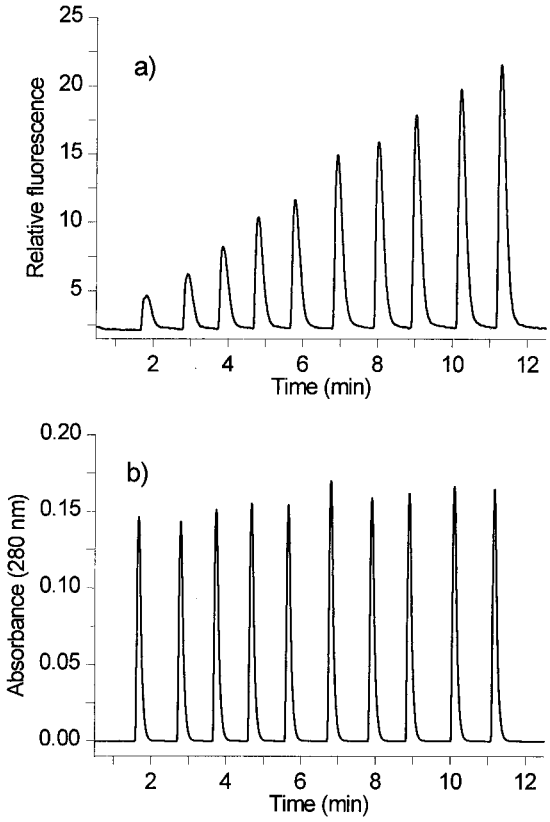

Fig. 6. Measurement record for calibration samples.
(*a*) Spectrofluorometric detector response (λ_{ex} = 247 nm, λ_{em} = 440 nm); (*b*) spectrophotometric detector response (λ = 280 nm).

diabetic (2–4) and control (1) serum pools were obtained with 0.15 mol/L TCA and 0.50 mol/L perchloric acid, and these concentrations were selected for further studies. It should be mentioned that a significant fluorescence blank was observed for perchloric acid.

As no AGE standard is available, external calibration was carried out with an AGE–peptide calibrator derived from an AGE-BSA calibrator (50 g/L) by hydrolysis with

Table 1. Analytical parameters of the proposed method with two acids for sample pretreatment.

Analytical parameter	Perchloric acid, 0.5 mol/L	TCA, 0.15 mol/L
Linear regression	S = 0.1528[AGE] + 0.0427	S = 0.1113[AGE] + 0.0143
Correlation coefficient	0.9942	0.9986
RSD for slope	0.022	0.007
RSD for intercept	0.322	0.042
Within-day precision:		
CV for 10% AGE, %	12.2	6.7
CV for 60% AGE, %	6.7	2.5
Between-day precision:		
CV for 10% AGE, %	17.8	9.1
CV for 60% AGE, %	13.1	3.4
Detection limit, %	2.9	0.4

S, measured signal; RSD, relative standard deviation; [AGE] expressed in %.

Table 2. Analytical results for AGE in four groups of patients with the proposed procedure.

Experimental group	n	AGE content, %		
		Mean ± SD	Min. value	Max. value
(1) Healthy individuals	10	10.1 ± 1.0	8.9	11.8
(2) Diabetes without diabetes-related complications	21	18.0 ± 6.2	11.2	32.2
(3) Diabetes with complications	25	24.1 ± 15.4	11.5	86.1
(4) Diabetes with end-stage renal disease	12	92.2 ± 30.1	40.2	132.9

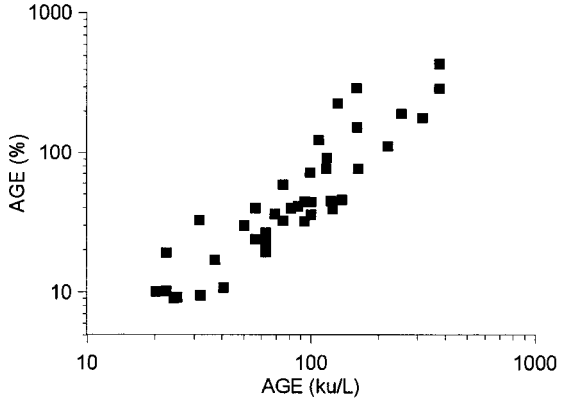

Fig. 7. Relation between AGE content in 41 serum samples from diabetic patients and healthy persons, as determined with the proposed method and with ELISA.

proteinase K. This calibrator was diluted (five times) to obtain the same peptide content as in deproteinized serum (10 g/L) and prepared for analysis in the same way as serum (see *Materials and Methods*: treatment with acid, extraction, etc.). The obtained sample corresponded to a calibrator 1 mmol/L AGE-BSA = 12 A_{350nm}, used in the previous reports *[6, 9]*. For this sample, analytical signal *S* was measured as described above, and taken as 100% of AGE-derived peptides (saturation of BSA with AGEs before hydrolysis with proteinase K). This solution was used as a "stock" AGE–peptide calibrator, and multipoint calibration was carried out in the proposed measurement system. To do so, a series of calibration solutions was prepared by appropriate dilution of stock AGE–peptide calibrator. These calibration samples contained different AGE concentrations and constant peptide concentration, achieved by addition of hydrolyzed BSA (in each sample the final peptide content was 0.4 g/L). In Fig. 6 the measurement records obtained for calibration samples (10% to 100% AGEs) with the two detectors are presented. It should be mentioned that by using the proposed procedure it was impossible to prepare a blank solution (0% AGEs) because BSA contains some natural AGEs. AGEs in the hydrolyzed BSA calibrator were determined as described previously (1 mmol/L BSA = 12 A_{350nm}) *[6, 9]*, and the evaluated content corresponded to 8.1% of AGEs in the scale proposed here. In Table 1, analytical characteristics obtained while treating samples with TCA and perchloric acid are presented. As expected, better sensitivity was obtained with perchloric acid (compare slope values of linear regression) because of quenching of AGE fluorescence in the presence of TCA. However, relative standard deviations for slope and for intercept were much higher for perchloric acid. Also the detection limit, within-day, and between-day precision were poorer for this acid, which should be ascribed to a high fluorescence blank. Thus, for analytical application 0.15 mol/L TCA was chosen.

The proposed procedure was applied to determination of AGEs in serum from (1) healthy persons, (2) diabetic patients without diabetes-relevant complications, (3) diabetic patients with chronic complications (neuropathy, nephropathy, etc.), and (4) diabetic patients with end-stage renal disease. The results obtained are given in Table 2. Variances of the results obtained in the four groups (1–4) were calculated, and the Welch test *[21]* for unequal variances was performed between results for the control group (1) and each of three groups of diabetic patients (2–4). The obtained results indicated that samples from groups (1) and (2–4) are statistically different ($P < 0.05$).

Finally, ELISA was carried out for AGE quantification in 41 serum samples selected from the samples previously analyzed by the proposed procedure to cover the observed range of AGE content. The procedure of immunochemical assay described previously *[6]* was slightly modified. Following the kind suggestions of R. Bucala, degradation of serum proteins was carried out (proteinase K, protocol given in *Materials and Methods*) to facilitate the access of specific antibody to AGEs. In Fig. 7, the relation between AGE content in serum samples obtained by using spectrofluorometric–spectrophotometric procedures and ELISA is plotted. Statistically significant correlation between the results obtained with the two methods can be observed in this Figure (regression equation $y = 0.713x + 1.24$, $S_{y|x} = 6777$, $r = 0.8477$, n = 41).

We conclude that the simplicity of the proposed method should enable its automation and use in routine clinical laboratories. Moreover, the small sample volume requirement (~1 μL of serum) may allow use of a filter-paper spot test in the future.

We are grateful to Professor Richard Bucala for his valuable suggestions and for providing AGE-specific antibody. Financial support from the CONACyT (Mexico) is

acknowledged: project 458100-5-3417 M and project 0463P-A9506.

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