

## A REVIEW ON METHODS OF ESTIMATION OF ADVANCED GLYCATION END PRODUCTS

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Article Received on  
05 July 2015,

Revised on 29 July 2015,  
Accepted on 24 Aug 2015

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### ABSTRACT

Chronic diabetes leads to the development of complications. One of the major mechanisms of development of these complications is the formation of advanced glycation end products (AGEs). These AGEs accumulate in long lived proteins of tissues causes cross linking and develops inflammation and thickening of basement membranes. This leads to the development of complications like retinopathy, neuropathy, nephropathy and atherosclerosis. Clinical studies have demonstrated that the level of circulating AGEs may be linked to various diabetes complications. Despite intensive investigation, the elucidation of the structure of specific AGE remains a problem. Till now, there is no ideal way to measure various AGE moieties. The

present review describes the various methods of detection of AGEs by using Spectrofluorimetry, ELISA, HPLC and mass spectrometry.

**KEYWORDS:** Diabetes, Advanced glycation end products, Spectrofluorimetry.

### INTRODUCTION

WHO reports Diabetes mellitus as one of the most common public health problems which will affect a total population of 220 million worldwide in the year 2020.<sup>[1,2]</sup> People with diabetes have higher levels of AGEs than nondiabetic subjects because hyperglycemia and oxidative stress both contribute to their accumulation. N-carboxymethyllysine (CML), pentosidine and Methylglyoxal (MG) are among some of the well characterized compounds that commonly are used as AGE markers. So measurement of the products of non-enzymatic glycation has a twofold meaning: on one hand, measurement of early glycation products can estimate the extent of exposure to glucose and the subject's previous metabolic control; on

the other hand, measurement of intermediate and late products of the glycation reaction is a precious instrument in verifying the relationship between glycation products and tissue modifications.<sup>[3]</sup> AGEs can be measured by a variety of techniques including Spectrofluorimetry, ELISA, HPLC or mass spectrometry.<sup>[4]</sup>

The purpose of the present study was to evaluate the different methods available to detect the advanced glycation end products.

## METHODS

### SPECTROFLUORIMETRIC METHODS

#### Determination of total advanced glycation end products (AGEs)

To 100 µl samples add 10 µl of 100 % (w/v) TCA in each tube. The supernatant containing sugar, test sample and the interfering substances was removed after agitation and centrifugation (15000 rpm, 4 °C), then the precipitate of AGEs-BSA was dissolved with 400 µl buffer (PBS) to serve for screening. The fluorescence intensity of glycated materials was measured at 370 nm excitation and 440 nm emission using Varian spectrofluorometer, Cary Eclipse model. The IC<sub>50</sub> (the concentration that resulted in 50% inhibition of the activity) was estimated for each test sample from the least-squares regression line of the logarithmic concentration plotted against the remaining activity.<sup>[5]</sup>

% inhibition =  $1 - \frac{\text{fluorescence of the solution with inhibitors}}{\text{fluorescence of the solution without inhibitors}} \times 100\%$ .

#### *In vitro* glycation of bovine serum albumin

The bovine serum albumin (BSA, 10 mg/ml) was incubated in fructose (100 mM) and sodium azide (0.02 %) with or without CuSO<sub>4</sub> (100 µM) in 0.2 M phosphate buffer (pH 7.4) 5. The samples in capped vials were protected from light and incubated at 37°C for 21 days. Samples were then dialyzed against sodium phosphate buffer at 4°C for 48 h. This removed reversibly bound and unbound sugars from the BSA solutions. Following dialysis, the samples were stored at -20 °C in small aliquots prior to analysis. The dialysis experiments were repeated three times and the results were similar. After dialysis, the protein concentration of samples was determined in triplicate by BSA assay.

#### BSA-glucose assay

To 5 g BSA and 14.4 g D-glucose were dissolved in phosphate buffer (1.5 M, pH 7.4) to obtain the control solution with 50 mg/mL BSA and 0.8 M D-glucose. 2 mL of the control

solution was incubated at 37°C for 7 days in the presence or absence of 1 mL of test sample in phosphate buffer (1.5 M, pH 7.4). The test solution also contained 0.2 g/L NaN<sub>3</sub> to assure an aseptic condition.<sup>[6,7]</sup> AG (1 mM) and rutin (100 μM) were used as positive controls. After 7 days of incubation, fluorescent intensity (excitation, 330 nm; emission, 410 nm) was measured for the test solutions. Percent inhibition of AGE formation by test compound was calculated using the following equation,

$$\% \text{ inhibition} = 1 - \left( \frac{\text{fluorescence of the solution with inhibitors}}{\text{Fluorescence of the solution without inhibitors}} \right) \times 100\%.$$

### **BSA-MGO assay**

To 40 mg BSA mixed with 31 μL MGO in phosphate buffer (0.1 M, pH 7.4) to obtain the control solution with 1 mg/mL BSA and 5 mM MGO. 2 mL of the control solution was incubated at 37°C for 6 days with/without test sample in phosphate buffer.<sup>[8]</sup> The test solution also contained 0.2 g/L NaN<sub>3</sub> to assure an aseptic condition. AG (1 mM) and rutin (100 μM) were used as positive controls.

$$\% \text{ inhibition} = 1 - \left( \frac{\text{fluorescence of the solution with inhibitors}}{\text{Fluorescence of the solution without inhibitors}} \right) \times 100\%.$$

### ***In vitro* glycation of proteins**

A 10% homogenate of goat lenses was prepared in phosphate buffer saline, pH 7.4 and centrifuged at 10,000 g for 30 min at 4°C. The lens total soluble protein (TSP) was used for *in vitro* glycation. Each 1ml reaction mixture contained 10 mg of TSP, 0.2 M phosphate buffer, pH 7.4, 0.1M-fructose, 50 mg of penicillin and streptomycin and 0.01% sodium azide. Reaction tubes were incubated in the dark at 37°C for 3 weeks. At the end of the incubation, unbound sugars were removed by dialysis against the same buffer. Protein concentration was determined by the Lowry method using BSA as standard.<sup>[9]</sup> Stock solutions of all the reaction contents were filtered through 0.20 mm syringe filters.<sup>[10,11,12]</sup>

### **Estimation of Serum AGEs**

The blood was centrifuged and the serum was diluted with 1:50 ratio with phosphate buffer saline at pH 7.4. The amount of AGE was measured in a spectrofluorometer then fluorescence intensity was measured at the excitation wavelength 350nm and emission 440nm against PBS.<sup>[13]</sup>

### Urinary AGE estimation

A fasting urine samples were centrifuged and stored at  $-80^{\circ}\text{C}$  until assayed. To measure the urinary fluorescent AGEs, the urine samples were diluted to 1:10-1:200 by phosphate buffer saline, and the fluorescence intensity was measured at 440 nm after excitation at 370 nm with a 96-plate spectrophotofluorimeter (Spectra Max Gemini EM; Molecular Devices, Sunnyvale, CA) at room temperature. The fluorescence was expressed as the relative fluorescence intensity in arbitrary units (AU).<sup>[14,15]</sup>

### Estimation of retinal AGEs

The retinal tissue was homogenized in Tween buffer  $\text{p}^{\text{H}}-8$ , followed by centrifugation at 8000g for 15 min at  $4^{\circ}\text{C}$ . Estimate the protein levels in soluble protein in homogenate. Dilute the protein up to 1mg/ml and the amount of AGE was measured in a spectrofluorometer at an excitation/ emission wavelength of 370/440 nm against buffer blank. BSA preparation (1 mg/ml in distilled water) was used as a reference, and its fluorescent intensity was defined as 1 arbitrary unit (AU).<sup>[16]</sup> The fluorescence intensities of the samples were measured and expressed as arbitrary units (AU)/mg protein.

### Estimation of renal AGEs

Kidney was minced and delipidated by shaking gently with mixture of chloroform and methanol (2:1 v/v) overnight. The delipidated tissue was homogenized in 0.1 N NaOH, followed by centrifugation at 8000g for 15 min at  $4^{\circ}\text{C}$ . Estimate soluble protein in homogenate and dilute the protein up to 1mg/ml and the amount of AGE was measured in a spectrofluorometer at an excitation/ emission wavelength of 370/440 nm against 0.1 N NaOH blank, BSA preparation (1 mg/ml in distilled water) was used as a reference, and its fluorescent intensity was defined as 1 arbitrary unit (AU). The fluorescence intensities of the samples were measured and expressed as arbitrary units (AU)/mg protein.<sup>[17]</sup>

## HPLC METHODS

### Estimation of AGEs in serum and urine

The serum samples were diluted 1:25 in phosphate-buffered saline (PBS). Size selective 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), at 350 nm excitation and 430 emission wavelengths. 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<sup>o</sup>) 121800, EMD Biosciences, La Jolla, CA) together with the subjects' samples.<sup>[18]</sup> 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**

Flow injection assay in the HPLC system was employed. The samples were treated with trichloroacetic acid, and 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. The 50mg/L of AGE-BSA after hydrolysis with proteinase K as a standard (Mp De LM, 2007). Results were expressed as fluorescence intensity/g creatininuria x 10<sup>10</sup>.

#### **Estimation of pentosidine**

Plasma or dialysate protein was subjected to acid hydrolysis. Protein was precipitated on ice with 10% TCA (Tri Chloro Acetic Acid). The pellets were washed twice with 5% cold TCA and acid hydrolyzed in 1 ml 6 N HCl for 16 h at 110<sup>0</sup> C. Acid was removed by vacuum centrifugation, The hydrolyzed pellet was dissolved in 250 ml of water/0.01 M heptafluorobutyric acid. The hydrolysate was filtered with 0.45- mm nylon microfilterfuge tube. The equivalent of 0.8 mg of plasma protein was injected onto an HPLC system, Waters Division of Millipore.<sup>[19]</sup> The column used was a 25×0.46 cm C-18 Vydac type 218 TP (10 μm). The HPLC was programmed with a linear gradient from 0 to 35 min of 10–17% acetonitrile in HPLC water and 0.1% heptafluorobutyric acid as a counter ion. Pentosidine eluted at ~30 min as monitored by fluorescence excitation at 335 nm and emission at 385 nm.<sup>[20]</sup> Results were calculated per milligram added protein or milligram collagen protein.

## ELISA TECHNIQUES

### The measurement of CML

The antigen was diluted to 10 µg/mL in 50 mM sodium carbonate buffer, pH 9.5–9.7 and loaded in a 96-well polystyrene plate (0.2 mL per well). The plate was coated overnight at 4°C. After coating, the wells were washed three times with PBS (phosphate buffer saline) and then blocked with gelatin for 2 h at 37°C. Rabbit anti-CML antibody was diluted at a titer of 1:500 in antibody diluent and loaded in each well.<sup>[21]</sup> After the overnight incubation at 4°C, wells were washed three times with NP-40. An alkaline phosphatase conjugated antibody to rabbit IgG was then added as the secondary antibody at a titer of 1:1000 in antibody diluent, incubated for 2 h at 37°C and washed with NP-40 again. The wells were developed with p-nitrophenyl phosphate substrate solution (pH 10.4). The reaction was terminated by adding 2 M sodium hydroxide and the absorbance at 405 nm was determined by a micro-plate reader.<sup>[22,23]</sup>

### Determination of CML

BSA (2mg/mL) and glyoxal (5 mM) were incubated with test sample in PBS at 37°C for 2 days, followed by the determination of CML by ELISA.<sup>[24]</sup> The 96-well microtiter plate was coated with 100 µL of PBS containing 0.05% Tween 20 (washing buffer). The wells were incubated with 0.1 mL of anti-CML antibody, 6D12 (1 µg/mL), or anti-pentosidine antibody (1 µg/mL) dissolved in washing buffer for 1 h. The wells were then washed with washing buffer three times and reacted with HRP-conjugated anti-mouse IgG antibody, followed by reaction with 1, 2-phenylenediamine dihydrochloride. The reaction was terminated by the addition of 0.1 mL of 1M sulfuric acid, and the absorbance at 492 nm was read by a micro-ELISA plate reader. The CML content of the samples were quantified by acid hydrolysis with 6N HCl for 24 h at 110°C, followed by amino acid analysis on a Hitachi L-8500A instrument equipped with an ion-exchange HPLC column (2622 SC, 4.6×80 mm; Hitachi) and a ninhydrin postcolumn detection system, as described previously.<sup>[25]</sup>

### Plasma AGE evaluation

The Hanson Advanced Glycosylation End Products immunoassay kit (Hanson Hong Biomedical Co, Ltd, Taipei, Taiwan) was used and the results are expressed as unit/mL. Anti-AGE reagent (0.25 mL) was added to each labeled colorimetric tube and the OD value (H1) at 340 nm was recorded for each tube. Each serum was diluted 1:4 after standing for 30 min. Diluted serum (15 µL) was added to the differently labeled colorimetric tubes that contained

0.25 mL of the anti -AGE reagent from which the OD value had been recorded as H1. The OD value was obtained at 340 nm immediately after 360 sec incubation; this value was recorded as the H2 value. To obtain the true OD value of the diluted serum in accordance with the standard curve, the following equation was used:  $H2 - H1 = H0$ . This value for the diluted serum was multiplied by 4 to obtain the final result.<sup>[26]</sup>

#### **Estimation of renal CML.**

The kidney was homogenized in ice-cold buffer (0.1 mmol L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub>, pH 7.0, plus 29.2 mg ethylenediamine tetraacetic acid in 100mL of distilled water and 10mg digitonin in 100mL of distilled water, final volume, and 2,000 mL) to produce a homogenate. The kidney homogenates were then centrifuged at 10,000 g for 10 min at 4°C. Then, the supernatant was tested for CML using the anti-CML rat autoantibody ELISA kit which employs the semiquantitative enzyme immunoassay technique. The absorbance of the resulting yellow product is measured at 450 nm.

#### **LC-MS TECHNIQUE**

##### **AGE analysis**

Pentosidine, GOLD, MOLD, CML were evaluated on total tissue extracts after hydrolysis with 0.6M trichloroacetic acid and 50 µl of hydrochloric acid 6M for 12h at 60°C. The chromatographic separations were run on an Ultimate 3000 HPLC (Dionex, Milan, Italy) coupled to a high-resolving-power mass spectrometer LTQ Orbitrap (Thermo Scientific, Rodano, Italy), equipped with an atmospheric pressure interface and an ESI ion source. The samples were analyzed by using a Reverse Phase C18 column (Phenomenex Synergi 150×2.1 mm, 3 µm particle size) at a flow rate of 200µl/min. A gradient mobile phase composition was adopted: 95/5 to 40/60 in 25 min, 5 mM heptafluorobutanoic acid/ acetonitrile.<sup>[27]</sup>

#### **WESTERN-BLOTTING DETECTION**

##### **AGE modified proteins in 1D-SDS gels**

3 µl test samples (20–30 µg total protein) were mixed with 1 µl of 4× sample buffer (0.125 M Tris-HCl, 2% SDS, 40% v/v glycerol, 0.8% bromophenol blue, pH 6.8). Following incubation at AT for 10 min, samples were loaded onto a pre-cast Bio-Rad 4%–12% Bis-Tris 1.0 mm mini gel. Electrophoresis was performed at 100 V in running buffer (25 mM Tris base, 0.1% SDS, 192 mM glycine, pH 8.3) until the dye front reached the end of the gel. After soaking the gel in equilibrating buffer (25 mM Tris base, 192 mM glycine, 20% methanol, pH 8.3) for 30 min, the proteins were electrotransferred to NC membrane using a

Bio-Rad mini-gel transfer apparatus in transfer buffer (250 mM Tris base, 1.92 M glycine, 20% methanol, pH 8.3) at 100 V, 4°C for 1 h. The membrane was washed twice in MilliQ water, then blocked for 2 h with protein-free blocking buffer at AT. Following three rinses with TBST, the membrane was incubated with rabbit anti-human AGE polyclonal antibody, goat anti-rabbit IgG (diluted 1: 40,000 in protein-free blocking buffer) and substrate as in dot-immunobinding assay. Bands were visualized and analyzed as described above.<sup>[28]</sup> The membranes were stained with Ponceau S, a general protein staining dye, after western blotting to visualize the major proteins in test samples.

#### **AGE modified proteins in Dot-immunobinding assay**

The test samples were diluted 1:20 in PBS (pH 7.4). The 4 µl of the diluted test samples and various concentrations of glycated BSA were dotted on nitrocellulose (NC) membrane at 1 cm intervals and allowed to dry for 1 h at ambient temperature (AT). Unreacted protein binding sites on the membrane were blocked by immersing the membranes in 3% BSA in Tris-buffer saline (TBS, 10 mM Tris base and 150 mM NaCl, pH 7.5) and incubated for 2 h at AT, followed by washing three times with 0.05% Tween-20 in TBS (TBST). NC membranes were incubated with rabbit anti-human AGE polyclonal antibody diluted 1:1,000 in blocking solution containing 0.5% BSA (BMBA) for 2 h at AT. After washing three times with TBST, the membrane was incubated with secondary antibody (goat anti-rabbit IgG peroxidase-labeled; Bio-Rad, Hercules, CA) diluted 1:10,000 in BMBA for 1 h at AT. Luminal/enhance and peroxidase buffer solutions in a 1:1 ratio were added to the membrane after another three washes and incubated for 3–5 min. The chemiluminescent spots were detected using a Versa Doc Imaging System (Bio-Rad). Quantity-one software (Bio-Rad) was used to analyze the image. Standard curves were generated using the AGE-BSA standards (1,000, 500, 250, 125, 62.5, 31.2 and 15.6 ng/ml) and were used to calculate the concentrations of AGE modified proteins in test samples. The results were also divided by total protein concentration to convert to amount (µg) of AGE modified proteins per mg of total proteins.<sup>[29]</sup>

#### **CONCLUSION**

Measurement of early glycation products has shown itself to be a precious and consolidated instrument in the metabolic monitoring of diabetic patients. The possibility of being able to monitor some intermediate and advanced glycation end-products will certainly provide important information on the pathogenesis and progression of chronic complications.



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