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Development of an automated immunoassay for advanced glycosylation end products in human serum[1]

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Abstract

Objective: Nonenzymatic reaction of protein and carbohydrate produce a series of brown fluorescent advanced glycosylation end products (AGEs). However, a convenient and rapid assay for serum AGEs level is currently unavailable.
Methods: We raised AGEs-specific polyclonal antibodies, which were used to develop a fully automated, noncompetitive, homogeneous, light-scattering immunoassay for serum AGEs.
Results: The assay requires a sample volume of 2 μL and takes a reaction time of 2 min. The coefficient of variation range from 1.8 to 6.1%, and the mean recovery rate was 98.6%. Comparative analysis shows moderate correlation with competitive ELISA ($r = 0.8209$, $n = 52$). The mean $\pm$ SD concentration of AGEs in young and in older healthy subjects were 4.6 ± 1.5 ($n = 39$) and 4.9 ± 1.4 ($n = 40$), respectively. The level of AGEs was significantly higher in serum from patients with type II DM 7.8 ± 4.8 ($n = 89$) than that from the normal subjects ($p < 0.05$).
Conclusions: The automatic immunoassay for AGEs is appropriate for clinical use.

Keywords: Advanced glycosylation end products; Light-scattering immunoassay; Competitive ELISA; Diabetes mellitus; End stage renal diseases

1. Introduction

Aging or prolonged elevation of glucose levels in diabetic patients results in a number of complications including nephropathy, arteriosclerosis, retinopathy, neuropathy, and cataracts. These complications have been related to the advanced glycosylation end products (AGEs) [1]. AGEs are a heterogeneous group of compounds that have a characteristic yellow brown fluorescence formed by the nonenzymatic "Maillard reaction". In Maillard reaction, reducing sugars react nonenzymatically with amino groups in proteins, phospholipids and nucleic acids to form Schiff base. The Schiff base continuously proceed an intra-molecular rearrangement to form stable Amadori products which will slowly rearrange, dehydrate, and aggregate that ultimately lead to the formation of AGEs [2,3]. Because the nonenzymatic glycosylation is concentration-dependent, persistent hyperglycemia in diabetic patients may enhance proteins in long-lived tissue to from AGEs. AGEs have been implicated in a wide range of pathologic conditions, which could explain many of the changes observed in diabetes related complications [4–6]. Indeed, there are evidences that serum AGEs level is elevated in diabetic patients and under certain pathologic states [7–9].

Serum AGEs have been shown to have striking reactivity with tissue matrix proteins or plasma lipoprotein and contribute to a variety of diabetic complications. Thus, circulation AGEs are the molecular markers responsible for tissue damage by reattaching to tissue or plasma proteins [6]. Furthermore, patients under long-term dialysis easily get complication of dialysis-related amyloidosis. AGEs-modi-

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[1] Nonstandard Abbreviations: AGEs, advanced glycosylation end products; CML, N′-carboxymethyl-lysine; CV, coefficient of variation; ELISA, enzyme linked immunosorbent assay; PBS, phosphate buffered saline; TBST, tris-buffered saline/Tween.

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fied β_2-microglobulin was found in histopathologic examination, which lowers down the clearance of macromolecules, accelerates the glycation, and finally makes the renal failure worse [10–11]. The accumulated AGEs in kidneys interferes with the release of cytokines and growth factors, which induce the platelet adhesion and deposition of lipids and oxidants [11–13].

Given that the serum AGEs level is closely related to diabetic complications and renal function, monitoring the AGEs level is important in diabetic control [14]. Serum AGEs level can be determined by the radio-receptor assay [15], by HPLC separation of pentosidine [8], and by online spectrophotometric and spectrofluorometric detection in a flow system [16]. Although fluorescence can be used to quantify AGEs, it has been shown to be a less specific measurement for most AGEs moieties [17]. Recently, the immunochemical assay has been developed to quantify AGEs using an AGEs-specific competitive ELISA [7,9,18,19]. However, most of these methods are tedious, time consuming and cannot be compared between different laboratories. Thus, the clinical studies have been limited to several investigative laboratories. At present, there are still many important clinical questions that are remained to be elucidated. For example, no correlation has been made between serum AGEs levels and duration of diabetes; the contribution of exogenous AGEs to serum AGEs levels as well as the diabetic sequelae remained to be clarified. However, the heterogeneous nature of AGEs' chemical moieties has hampered the development of a universal assay for the measurement of serum AGEs.

In the present study, a fully automated, convenient and rapid immuno-turbidimetry assay is proposed for the large-scale measurement of serum AGEs. The methodologies, the within-run and between-day precisions, the possible interferences, and the recovery rates of the assay were evaluated. Results of 79 serum samples from healthy control subjects, 89 samples from diabetic subjects, and 40 samples from patients with end stage renal diseases were discussed.

2. Materials and methods

2.1. Reagents

HDL-cholesterol and total bilirubin reagent kits were from Olympus Daiichi (Japan). Cholesterol and triglyceride reagent kits were from Olympus (Japan). HbA_1c reagent Kit and HbA_1c control: Lyphocheck Diabetes control was from Bio-Rad (USA). Pathonorm Low and Pathonorm High control serum were from Sero (Japan). All other chemicals were purchased from Sigma (St Louis, MO).

2.2. Preparation of RNase-derived AGEs and anti-AGE antibodies

RNase-derived AGEs were prepared by incubating 1 mol/L glucose with 25 mg/dL bovine pancreatic ribonuclease A (RNase A) in phosphate buffered saline (PBS) pH 7.4 at 37°C for at least 6 months. After incubation, un-reacted sugars were removed before assay by extensive dialysis against PBS. For preparation of anti-AGEs antibodies, RNase-AGEs (1.0 mg) were emulsified in 50% complete Freund adjuvant and injected intradermally into two New Zealand White rabbits at 6 to 10 skin sites. Rabbits were boosted 4 times with the same amount of RNase-AGEs emulsified in 50% incomplete Freund adjuvant every other week.

2.3. Preparation of antibody-coated particle reagent

Preparation of Ab-coated particles was essentially as described by Holowania *et al.*, [20] with minor modification. Briefly, polystyrene latex beads (10 μm; Sigma.) were added into the diluted antibody (in 15 mM sodium phosphate buffer, pH 7.6, 0.5 ml/L Tween-20) drop by drop slowly and shaking gently. The mixture was incubated at 37°C in an orbital shaker at 240 rpm overnight, centrifuged, and the final pellet was re-suspended in a volume of storage buffer (500 mM glycine buffer, pH 7.6, 0.5 ml/L Tween-20) equivalent to one half of the volume of the starting mixture. The stock reagent was placed in an ice water bath and sonicated (20 microns intensity; 30 s) by 1 min interval between two bursts. The working reagent was adjusted to 1.0 O.D against 380 nm with diluting buffer (5 mM glycine buffer, pH 7.6 0.005 ml/L Tween-20).

2.4. Preparation of calibrator

Standardized calibrator with universally defined AGEs units is currently unavailable due to the heterogeneous nature of AGEs' chemical moieties. Because serum AGEs levels are consistently within a narrow range, more than 500 serum samples from health subjects were collected as the calibrator for this study. One AGE unit is defined as the absorbance obtained from 1:5 diluted pooled serum in the turbidimetric assay.

2.5. Assay optimization

To determine the maximum change in signal, difference between two bichromatic readings at the active wavelength of 380 nm and reference wavelength of 700 nm were measured at 4.5 s intervals. Antibody coated particles were prepared with different protein loading (0.5–3 mg of antibody/mL particles) and at different particle concentrations. The assay protocol was optimized by minimizing the sample volume and appropriate amount of antibody within the operation framework of the autoanalyzer.

2.6. Assay validation and method comparison

The "within-run" imprecision was assessed using 20 replicate analyses of two serum pools at approximately 3 and 11 A.U. Inter-assay precision was assessed by analyzing the two serum pools across twenty working days, using new calibration curve each time. Analytical recovery was assessed by mixing a sample containing relatively high AGEs (17.6) with low AGEs (3.2) samples by factors of 0.2, 0.4, 0.6, and 0.8. The potential interference of hemoglobin, bilirubin, and lipemia were assessed by performing analytical recovery experiments in increasing concentration of interferents; hemoglobin (human hemolysate) up to 1.0 g/L, bilirubin (BILIRUBIN LIN-TROL from Sigma, USA) up to 32.0 mg/dL and triglyceride (LIPID LIN-TROL from Sigma, USA)up to 1000 mg/dL cholesterol up to 640 mg/dL.

In method comparison, a total of 52 serum samples selected from the samples cover the observed range of AGE content were analyzed by the automatic immunoassay and a standardized competitive AGE-ELISA as originally described by Makita *et al.* [18] and modified by Mitsuhashi *et al.* [19].

2.7. Collection of clinical samples

Serum samples from 89 diabetic patients (average age of 60.59 yr old) who had a final diagnosis of DM were collected for this study. Serum samples of 12 hemodialysis patient with end stage renal diseases (average age of 66.78 yr old) and those from 40 healthy elderly individuals (average age 73.48 yr) and 39 young individuals (average age of 28.15 yr old) showing normal results of physical examinations, chemistry profile and hematologic profile within normal limits were collected for this study.

2.8. Statistics analysis

Results are presented in mean ± SD (or ± SE). The *t*-test is used to identify the precision and $p < 0.05$ is used as an indicator of statistic significance. The software of *Sigma Plot* is used for all data analysis.

3. Results

3.1. Assay development and calibration

The antibody particle reagent was optimized at 0.5 mg antibody/mL of 1% particles. The sample volume was 2 μl; reagent volume was 450 μl; reaction temperature was 37°C, dual wavelengths were 380 nm (main) and 700 nm (secondary). A typical calibration curve, obtained from the diluted pool serum under the finalized assay conditions, was shown in Fig. 1. The calibration curve was constructed by using a logistic curve-fitting program and the concentrations of AGEs in unknown samples were determined by using the algorithm on the auto analyzer. The calibration curve gave a linear regression, with a regression coefficient of r = 0.9976 (Fig. 1).

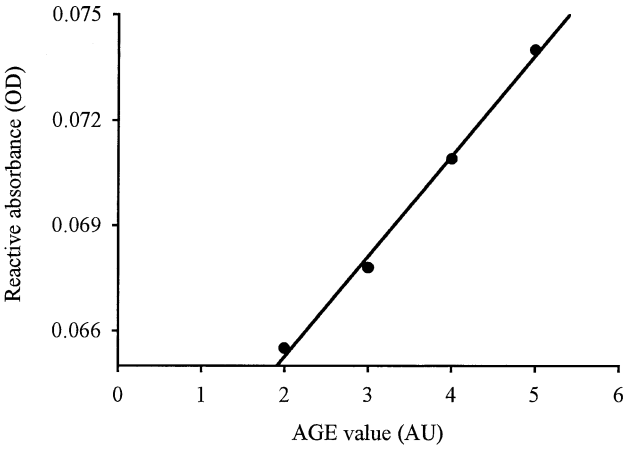

Fig. 1. Standard curve of dilution vs. absorbance using the results of the determination of AGEs on the Olympus AU-600 chemistry analyzer. Data represent means ± S.E. Diluted pool serum was used for calibration as described in METHODS. The curve is linear. $y = 0.0029x + 0.0595$; r = 0.9976.

3.2. Precision assessment

The "within-run" imprecision was calculated by the ANOVA procedure, using 20 replicate analyses of two serum pools at approximately 3 and 11 A.U. This was performed on 10 occasions through a period of 30 days, using new calibration curve each time. The imprecision of between-day was assessed by using one assay-per day design on duplicate analyses of two serum pools at across twenty working days, using the new calibration curve each time. The data of precision assessment were summarized in Table 1. The mean percentage recovery for each sample of the fixed proportions was 98.6% (Table 2).

3.3. Interferences and method comparison

To compare the possible interference from anticoagulants, triplicate blood samples from 40 diabetic patients

Table 1A
Within-run reproducibility

	Mean result (AU)	S.D.	CV%
Low	3.4	0.1	2.9
High	11.4	0.2	1.8

Table 1B
Between-day reproducibility

	Mean result (AU)	S.D.	CV%
Low	3.3	0.2	6.1
High	11.1	0.5	4.5

Table 2
Recovery rates of AGE determination

Healthy (3.2)	High (17.6)	Observed value	Expected value	Recovery rate (%)
100%	—	3.2	3.2	100
80%	20%	6.0	6.1	98.4
60%	40%	9.0	9.0	100
50%	50%	10.3	10.4	99
40%	60%	11.9	11.8	100.8
20%	80%	14.9	14.7	101.4
—	100%	17.6	17.6	100

Two samples of AGEs (3.2 and 17.6) were mixed flow from a different ratio.

The observed value is divided by the expected value to get the recovery rate.

were collected in plain tubes, in heparinized tubes, and in EDTA-3K tubes. The means ± SE were 9.82 ± 1.56, 9.99 ± 1.32, and 9.74 ± 1.46 for serum, EDTA- plasma and heparinized plasma, respectively. There were no significant differences between serum, EDTA-plasma and heparinized plasma ($p > 0.5$). To evaluate the possible interferences of hemolysis, jaundice and heperlipidemia, two samples with AGEs of 3.5 A.U and 16.1 A.U were mixed with different concentrations of hemoglobin, bilirubin, and triglyceride. There were no discernible interferences of hemoglobin up to 250 mg/dL or bilirubin up to 32.0 mg/dL. However, the AGEs level were significantly higher in specimens with >500 mg/dL added triglyceride or > 320 mg/dL added cholesterol ($p < 0.05$) (Table 3). A comparative analysis between automatic immunoassay and competitive ELISA was used to validate the proposed assay. In Fig. 2, the relationship between the results obtained with the two methods is plotted (regression equation: $y = 1.040x + 1.68$, r = 0.8215, $n = 52$).

Table 3
Interferences from additions of hemoglobin, bilirubin, triglyceride, and cholesterol to two serum sample pools, expressed as measured results and percent recoveries in the absence of the interferent

Addition	Low (3.5)		High (10.1)	
	mean (AU)	recovery (%)	mean (AU)	recovery (%)
Hemoglobin, mg/dl				
250	3.5	100	16.4	101
200	3.4	97	16.4	101
100	3.6	102	16.2	100
25	3.5	100	16.3	101
Bilirulin, mg/dl				
32	3.6	102	16.2	100
24	3.6	102	16.3	101
16	3.4	97	16.0	99
8	3.5	100	16.0	99
Triglyceride (cholesterol), mg/dl				
1000 (600)	4.5	128	17.9	111
750 (480)	4.1	117	17.3	107
500 (320)	3.4	97	16.2	100
250 (160)	3.5	100	16.0	99

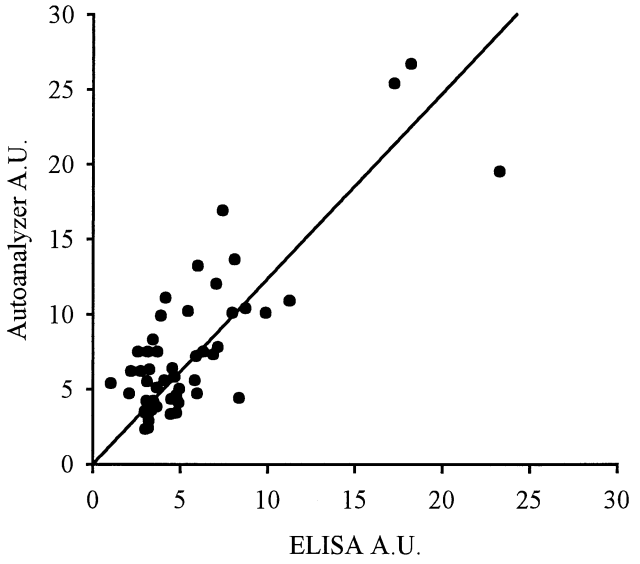

Fig. 2. Correlation of AGEs contents determined by automatic immunoassay and by competitive ELISA. Serum AGEs levels in 52 serum samples obtained from the automatic immunoassay and from the standardized competitive ELISA were compared.

3.4. Clinical significances

We examined concentrations of AGEs in serum from 79 healthy control subjects, 89 patients with type II DM and 40 patients with end-stage renal disease requiring hemodialysis. As shown in Fig. 3 the mean ± SD concentrations of AGEs in young and in older healthy subjects were 4.6 ± 1.5 ($n = 39$) and 4.9 ± 1.4 ($n = 40$), respectively. The serum

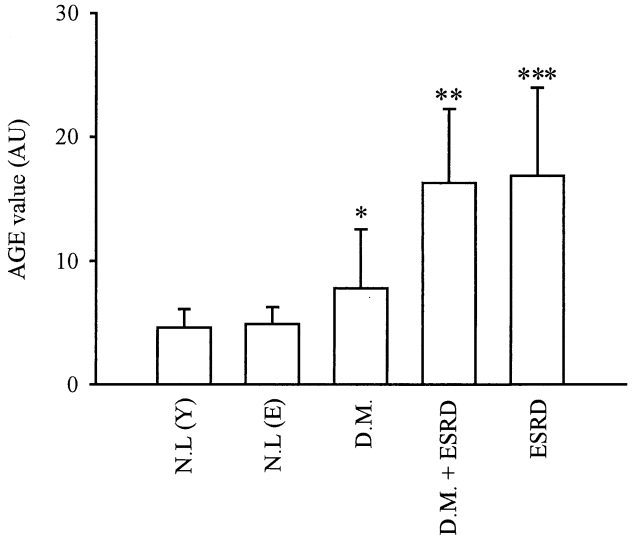

Fig. 3. The concentrations of serum AGEs in normal young group (N.L.Y) ($n = 39$), normal elderly group (N.L.E)($n = 40$), diabetes patient group (D.M) ($n = 89$), DM with end stage renal disease patient group (DM + ESRD) ($n = 12$), and end stage renal disease patient group (ESRD) ($n = 28$). Results represent means ± SD * $p < 0.025$; ** $p < 0.001$; *** $p < 0.001$.

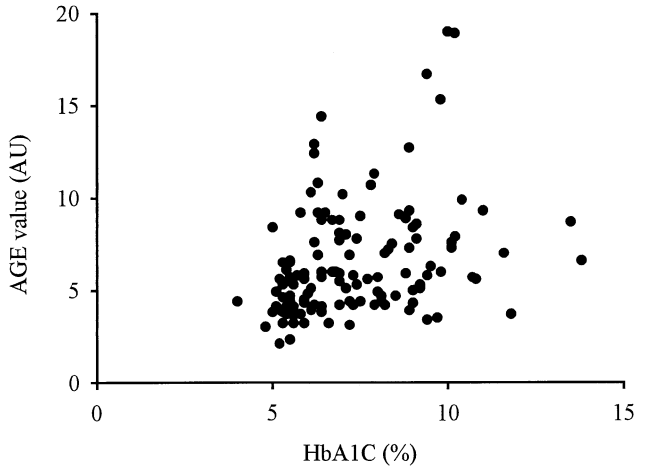

Fig. 4. Correlation between HbA1C and AGEs. The HbA1C and AGEs levels in the blood of 39 non-DM patients and 89 diabetic patients were measured as described in METHODS. Serum AGEs increased with HbA1C and AGEs ($r = 0.1265$, $p < 0.05$).

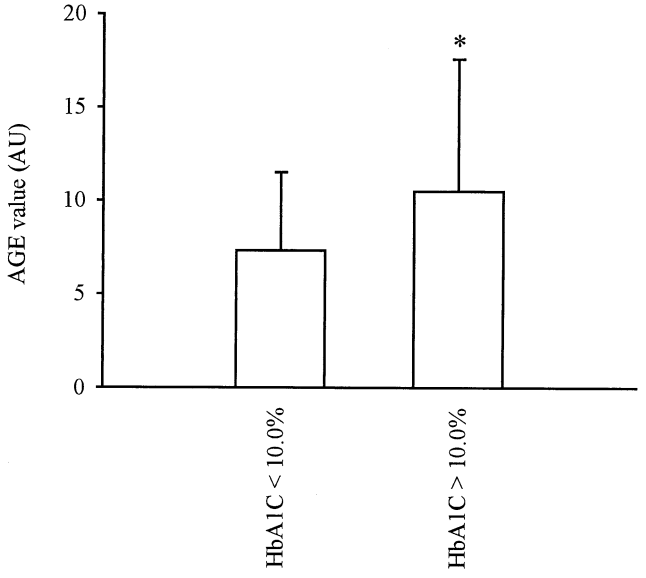

Fig. 5. Comparison of serum AGEs level of the poor-controlled with the well-controlled diabetic patients. Serum AGEs levels of the poor-controlled group ($HbA_1C>10.0\%$) ($n = 14$) were compared to the well-controlled group ($HbA_1C<10.0\%$) ($n = 75$). Results represent means ± SD $^*p < 0.005$.

AGEs level of DM patients (7.8 ± 4.8 A.U, $n = 89$) was significantly higher than that obtained from the healthy group ($p < 0.025$). The serum AGEs level of DM patients with end stage renal complication was 16.3 ± 6.0 A.U ($n = 12$) and that of end stage renal disease patients without DM was 16.9 ± 7.1 A.U ($n = 28$). Both were significantly higher than those of the young and the elder healthy subjects ($p < 0.001$). The correlation between HbA1c and AGEs level was evaluated by comparing HbA1c and AGEs data from 39 healthy subjects and 89 DM patients (Fig. 4). The serum AGEs level in the poor-controlled group was significantly higher than the well-controlled group (Fig. 5). Serum AGEs and triglyceride levels were significantly correlated with the diabetes-related vascular complications (Table 4).

4. Discussion

We have developed a simple and automated method, which provides numerous advantages for the clinical laboratory over existing methodology. The advantages include: 1) a simple automated homogeneous test format; 2) the small sample volume (2 μl), small single reagent volume (450 μl) and high throughput (800 tests/h); 3) good precision (C.V. for within-run, 1.8–2.9%; for between-day, 4.5–6.1%), and high recovery rate (98.6%). The assay was not interfered by any of the added hemoglobin, bilirubin, triglyceride (<500 mmg/dL) and cholesterol (<320 mg/dL).

AGEs are composed of a wide variety of glyoxidation products but the precise structures of most AGEs are yet to be determined. Due to their heterogeneity, the immunochemical approaches that can detect most AGEs episodes seem to be the methods of choice. However, the immunoreactivity of *in vitro* prepared AGEs- proteins depends on the incubation time, the nature of the carrier proteins and the types of reducing sugars used [19]. Mitsuhashi *et al.*, have employed the mean serum AGEs values in the normal population, which are proven to be within a narrow range, as a universal standard for AGEs measurement. The definition of the AGE unit is independent of antigens, antibodies and can be compared from laboratory to laboratory. By using the pooled sera collected from apparently normal individuals as calibrator, the automated method can clearly distinguish AGEs levels between normal and diabetic and/or ESRD. Our results are consistent with previous reports showing minor increases of serum pentosidine in diabetic subjects (2.5-fold) and to a great extent in ESRD subjects (up to 23-fold) [21]. Also in agreement with previous studies, no correlation was found between AGEs and chronological age [22].

HbA_1c reflects the Amadori content of early glycation in

Table 4
Evaluation of risk factors for the DM patients without or with complications

	DM without complications	DM with complications	*p*
n	9	52	
M/F	7/2	26/26	
Age (yr)	63.7 ± 2.4	62.3 ± 10.7	
AGEs (AU)	4.4 ± 0.7	7.7 ± 4.3	<0.025
HbA_1c (%)	8.3 ± 1.8	8.2 ± 1.8	>0.05
Cho (mg/dl)	187 ± 30	208 ± 42	>0.05
TG (mg/dl)	99 ± 39	232 ± 131	<0.005
HDL-C (mg/dl)	50 ± 15	44 ± 12	>0.05

red blood cells, which occurred in several weeks. HbA_1c has been used to evaluate the blood sugar control of the last two months. Because the life span of RBC is 120 days whereas the formation of AGEs takes several months, whether the HbA_1c values can serve as the predictors of AGEs remained to be determined. In the present study, we found significant correlation between HbA_1c and AGEs, which is consistent with the previous report that the accumulation of AGEs is significantly correlated with the blood HbA_1c levels in children and adolescents with type 1 diabetes mellitus [22,23] and patients with type 1 diabetes mellitus complicated with microalbuminuria [24]. It has been suggested that circulation AGEs are mostly the degradation products of tissue AGEs and have striking reactivity with tissue matrix proteins or plasma lipoprotein and contribute to a variety of diabetic complications. Thus, the serum AGEs level is more useful in predicting the pathologic changes of diabetic complications than HbA_1c. Indeed, the serum AGEs level can be used to predict changes in the kidney morphology of patients with insulin-dependent diabetes mellitus [24]. Nevertheless, the clinical significance of serum AGEs still isn't well concluded. It has been reported that the Hb-AGEs level did not correlate with age, diabetes or severity of retinopathy and nephropathy [25]. Some studies suggested levels of AGEs but not CML were significantly related to the development of macrovascular complications in patients with type 2 diabetes [14].

In conclusion, the automated, noncompetitive, homogeneous, light-scattering immunoassay is a convenient, precise and reliable method for the measurement of serum AGEs. The assay can be implemented in most of the autoanalyzers where multiple tests can be analyzed simultaneously. Because only minute sample volumes are required for the determination of serum AGEs levels, this allows a routine profile analysis for diabetic patients. Given the accumulation of AGEs in tissues is closely related to the pathogeneses of renal disorders and numerous diabetes and aging related complications, monitoring the serum AGEs level will be proven important in clinical evaluation of the disease status.

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