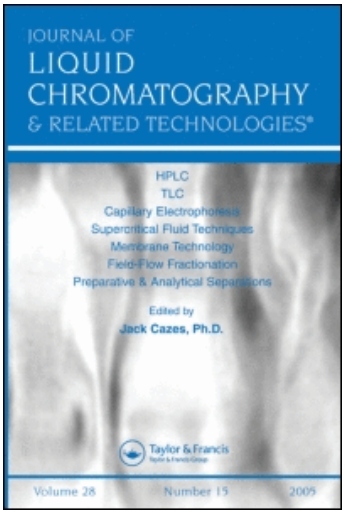

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FAST, SELECTIVE ANALYSIS OF GLYCATED ALBUMIN IN HSA

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ABSTRACT

Glycated albumin in human serum was analyzed by solid-phase extraction and selective detection. One system with one solid-phase extraction column and a chemiluminescence detector facilitated rapid analysis. A second system with two solid-phase extraction columns and a fluorescence detector was a fast and stable analytical method compared to liquid chromatographic analysis. Both analytical systems were suitable for diagnostic analysis of glycated human serum albumin. These systems require 1 μL blood serum for analysis.

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INTRODUCTION

The analysis of glycated human serum albumin in blood serum has been commonly used for diagnostic analysis for diabetes (1). The concentration of glycated human serum albumin (GHSA) in human serum albumin (HSA) indicates the mean concentration of GHSA over the previous 2–3 weeks, because glycation occurs continuously *in vivo*. However, the amount of glycated human serum albumin varies between methods and reports (2–21).

Further development of a rapid and simple method is required. Theoretically, high resolution separation on liquid chromatography requires a high theoretical plate number column, but a highly selective column does not require a highly theoretical plate number. The latter method requires simple solid-phase extraction instead of a high pressure operation system. In addition, selective detection does not require complete separation. Therefore, selective separation and detection were studied using 4 bonded phases, fluorescence and chemiluminescence detectors, and a high-pressure pump system with a column switching system and a gradient elution system.

Previously, chemiluminescence detection was applied for selective detection of glycated albumin in size-exclusion liquid chromatography of human blood serum (22). Chemiluminescence detection was based on measurement of superoxide produced in strong alkaline solution from Amadori form ketoamine, according to Maillard reaction of glycated human serum albumin (23–25). This superoxide produces chemiluminescence with lucigenin in strong alkaline solution. Chemiluminescence detection was combined with solid phase extraction to develop a fast analytical method of glycated human serum albumin. Another system using two solid-phase extraction columns was also developed for easy comparison with the present liquid chromatographic system with two columns. The newly developed systems were applied to analyze glycated human serum albumin in blood sera from diabetic patients, and the results were compared with those measured by liquid chromatography, using two columns and high-pressure gradient elution with column switching and two fluorescence detectors.

EXPERIMENTAL

Preparation of Solid-Phase Extraction Columns

Albumin Affinity Extraction Column

Albumin affinity gel was synthesized from Sepharose 4B (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the reference method described previously (26,27). The bonded phases were packed into polyethylene tubes.

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Glycated Albumin Affinity Extraction Column

Asahipak borate resin (a kind gift from Dr. Keiko Yasukawa, Asahi Chemical Co.) was packed into polyethylene tubes. The resin was 9 μm vinylalcohol polymer gel, modified using metha-aminoboronate (28).

Flow Injection and Liquid Chromatography Systems

A flow injection system was assembled with two model LC-9A liquid chromatography pumps from Shimadzu (Kyoto), a model 7125 Reodyne injector, and a model Shodex CL2 chemiluminescence detector from ShowaDenko (Tokyo). The intensity of chemiluminescence was recorded with a Chromatocorder 12 from SIC (Tokyo, Japan). A liquid chromatograph was constructed with two model LC-10AD pumps, a model SIL-10AXL auto-injector from Shimadzu (Kyoto), a model ERC-3522 degasser from ERC (Tokyo), and a model Σ02 column oven from IRICA (Kyoto). The fluorescence detector was a model RF530 from Shimadzu. The operation and chromatographic data analysis were performed with a model CLASS-LC10 workstation from Shimadzu.

Micro-Plate Analysis

Micro-plate analysis was performed using a Luminescenser-JNR AB2100 from Atto Bio-Instrument (Tokyo, Japan) and a black plate with 96 wells for ELISA, MS-8496K) from Sumitomo Bakelite (Tokyo, Japan). The Luminescenser was modified to inject equivalent-volumes of 0.02 M lucigenin aqueous solution and 1.0 N NaOH solution with a three port PEEK connector, because the instrument was equipped with only one liquid injection pump. Aliquots of 50 μL of extracts from the albumin affinity extraction column were applied to wells, then 100 μL of reagents were added before counting photons. As a calibration standard, 0.0105 M 1-deoxy-1-*p*-toluidine-D-fructose was used.

RESULTS AND DISCUSSION

The Selectivity and Loading Capacity of Albumin Affinity Columns

The selectivity and loading capacity of solid-phase extraction columns were examined using liquid chromatography. Briefly, 1 mL of albumin affinity gel was packed into a polyethylene tube, then washed with solution A containing 0.15 M

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sodium chloride and 0.02 M sodium-phosphate buffer (pH 7.50). Then, 100 μL of blood serum was applied to the column and washed 10 times with 200 μL of solution A. The column was then washed 10 times with solution B containing 200 μL of 0.075 M sodium phosphate buffer (pH 2.40) and 50 v% ethanol, and fractions of 200 μL were collected for further liquid chromatographic analysis using two columns, a column switching system, and two fluorescence detectors. These chromatograms are not shown.

The chromatograms of these fractions indicated that washing with 7 column volumes of solution A was sufficient to clean blood serum, and extraction with 4 column volumes of solution B could quantitatively recover albumin. No pure albumin is available, and GHSA is a mixture of different degrees of glycated human serum albumin. Therefore, the recovery could not be calculated with precision.

The loading capacity of the albumin affinity column was examined using a 100 μL packed column several times. When 20 μL blood serum was applied to this small column, the chromatograms of the effluent indicated that the maximum loading capacity was less than 40% of the column volume. The column could be reused several times after washing with the above solutions.

The Selectivity and Loading Capacity of Glycated Albumin Affinity Column

One mL of borate gel was packed into a polyethylene tube, then washed with solution C containing 0.25 M ammonium acetate, 0.05 M magnesium chloride, and 5% ethanol (pH 8.50 adjusted with ammonium solution). Then, 100 μL of albumin extract from the albumin affinity column was applied to the column and washed 10 times with a 200 μL of solution C. Then, the column was washed 10 times with 200 μL of solution B containing 0.20 M D-sorbitol, 0.10 M 2-amino-2-hydroxymethyl-1,3-propanediol, and 0.05 M disodium ethylenediamine tetraacetate (pH 8.50 adjusted with ammonium solution), and all fractions collected were analyzed by both modified borate column affinity liquid chromatography, and flow injection analysis equipped with a fluorescence detector.

The peak area indicated that washing with 7 column volumes of solution C was sufficient to collect nonglycated human serum albumin from the human serum albumin fraction previously extracted using the albumin column; and extraction with 4 column volumes of solution D was sufficient to extract glycated human serum albumin quantitatively.

The loading capacity of albumin affinity column was examined using a small 100 μL packed column. When 20 μL of albumin extract solution was applied to this small column several times, the chromatograms measured by

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modified affinity liquid chromatography using a borate column indicated that the maximum loading capacity was less than 40% of the column volume. The columns could be reused several times after washing with the above solutions.

Analysis of Blood Serum

Blood serum samples of 20 μL from diabetic patients were applied to 200 μL albumin affinity extraction columns, washed with 1.4 mL of solution A, then extracted 4 times with a 200 μL of solution B. The extracted fractions were further analyzed by both flow injection analysis equipped with a chemiluminescence detector, and liquid chromatography equipped with a fluorescence detector. A portion of the extracted solution was further separated to GHSA and NGHSA using the second solid-phase extraction column (100 μL borate affinity columns).

Chromatography and Flow Injection Analysis of Extracts

The purity of the HSA fraction of blood serum of diabetic patients obtained from the albumin affinity extraction column, was analyzed, first, by an ordinary chromatographic system handling ion-exchange and borate gel-affinity liquid chromatographies (15,16). Chromatograms, after ion-exchange column liquid chromatography monitored with a fluorescence detector, indicated that fractions of large and small molecular size were eliminated by the albumin affinity column. The albumin fraction, further separated by the borate column liquid chromatography, showed only two peaks. These results indicated that the solid-phase extraction by the albumin affinity extraction column is a rapid and efficient method to purify human serum albumin in blood serum. These chromatograms are not shown here.

The purified fractions of albumin were analyzed by size-exclusion liquid chromatography with a fluorescence detector and a chemiluminescence detector. Representative chromatograms are shown in Fig. 1. The chromatograms indicated that the peak of HSA was sharper than that measured by the original chromatographic system. Both the larger molecular and smaller molecular size fractions disappeared. The second large peak in the chemiluminescence chromatogram was due to contamination by impurities in ethanol used for extraction. The sharper peak shape indicated that extraction through an albumin affinity column improved the purity of albumin. The albumin fraction was also analyzed by modified affinity liquid chromatography with a borate column, and a representative chromatogram is shown in Fig. 2. The further separation of GHSA from NGHSA indicated that the concentration of GHSA in the HSA fraction was equivalent to that measured by the original system. Therefore, 10 albumin samples

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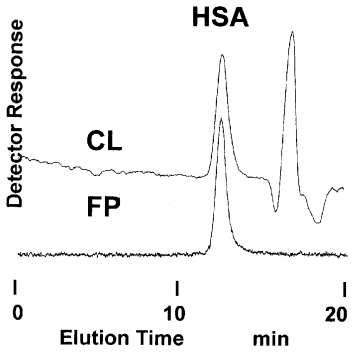

Figure 1. Size-exclusion chromatograms of blood serum after albumin affinity gel extraction. Extraction column: 200 μL of albumin affinity gel. Washing solvent: 0.15 M sodium chloride + 20 mM sodium phosphate buffer (pH 7.50). Extraction solvent: 75 mM sodium phosphate buffer (pH 2.4) + ethanol (1 + 1). Column: Asahipak 510H, 250 × 6.0 mm I.D. Eluent: 25 mM ammonium acetate + 200 mM D-sorbitol (pH = 10.0) + 15% ethanol. Flow rate: 0.5 mL/min at 37°C. Fluorescence detector: ex. 285, em. 340 nm. Chemiluminescence detector: Reagent A: 0.12 M lucigenin + 5% ethanol. Flow rate: 0.6 mL/min. Reagent B: 0.5 M sodium hydroxide + 2.5% ethanol. Flow rate: 0.3 mL/min.

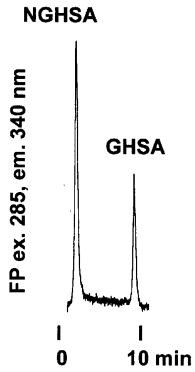

Figure 2. Separation of NGHSA and GHSA in HSA using borate affinity liquid chromatography. Column: 100 × 4.6 mm I.D. packed with Asahipak borate resin (8 μm). Eluent A: 250 mM ammonium acetate + 50 mM magnesium chloride (pH 8.50) + 5% ethanol. Eluent B: 200 mM D-Sorbitol + 100 mM 2-amino-2-hydroxymethyl-1,3-propanediol and 50 mM disodium ethylenediamine tetraacetate (pH 8.50). Flow rate: 1.0 mL/min at ambient. Gradient condition: 0–8 min: B conc 0%, 12 min: B conc 70%, 12–14 min: B conc. 70%, 15 min: B conc. 0%, 19 min: STOP.

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from diabetic patients were analyzed by both size-exclusion liquid chromatography and modified borate column-affinity liquid chromatography.

The correlations of GHSA concentration in sera of 10 diabetic patients measured by the original two-column chromatographic system (X) and those of the modified borate column-affinity liquid chromatography method ($Y1$), were linearly related as shown in Figure 3, as monitored with the fluorescence detector ($Y1 = 0.763X + 7.455$, $r = 0.972$, $n = 10$). The data are given as percentage of GHSA in total HSA from peak areas of GHSA and NGHSA, due to lack of standard compounds. The slightly higher concentration of GHSA on the modified borate column-affinity liquid chromatograms for samples containing low concentrations of GHSA, may have been due to the reduced tailing of peaks in this method.

The results obtained by flow injection analysis ($Y2$) of the same samples after two-step solid-phase extractions, are also shown in Fig. 3 ($Y2 = 0.832X + 5.385$, $r = 0.975$, $n = 10$). The latter results indicated that the samples treated using two solid-phase extractions can be analyzed by flow injection analysis within a very short time, compared to liquid chromatographic analysis. The concentration of GHSA correlated well with that measured by modified borate-column affinity liquid chromatography.

The albumin fractions of 10 different diabetic patients extracted from the albumin affinity columns were analyzed by size-exclusion liquid chromatography with chemiluminescence detection. The content of glycated albumin was

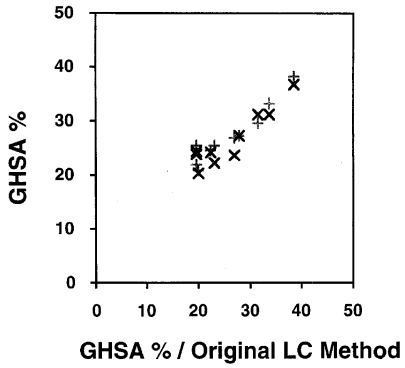

Figure 3. Comparison of GHSA concentration in HSA measured using borate resin affinity liquid chromatography, flow injection analysis, and original liquid chromatography. +: Combination of one solid-phase extraction and borate resin affinity liquid chromatography. ×: Combination of two solid-phase extractions and flow injection analysis. Detector: Fluorescence detector ex. 285, em. 340 nm. Chromatographic condition of original liquid chromatography (ref. 15).

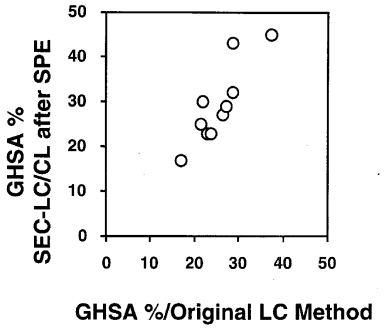

Figure 4. Comparison of GHSA concentration in HAS, measured using size-exclusion liquid chromatography and original liquid chromatography. Chromatographic conditions: see Figure 1.

determined from the calibration curve of 1-deoxy-1-*p*-toluidine-D-fructose (DTF) as the standard (22). The total amount of albumin in serum can be easily analyzed by ordinary clinical analysis. Then, the amount of GHSA was reported as relative concentration in total albumin in serum ($Y3$). The results were correlated with GHSA concentration measured by the original two-column liquid chromatographic system (X), as shown in Fig. 4 ($Y3 = 0.575X^{1.210}$, $r = 0.885$, $n = 10$). The stability and sensitivity of the chemiluminescence detector were affected by environmental changes in the laboratory. The peak area was adjusted by the background change displayed on the monitor of the instrument. The long-term monitoring of chromatograms by the chemiluminescence detector is not recommended, but chemiluminescence analysis of albumin affinity column extraction was faster than the original two column liquid chromatographic system.

The albumin fractions from the albumin affinity column had a high degree of purity, as shown in Fig. 1. Therefore, the same albumin column extracts used for experimental results given in Fig. 1, were analyzed by flow injection analysis using a chemiluminescence detector after evaporation of ethanol. The peak area was not adjusted using the background change, due to the shorter analysis time compared to liquid chromatography. The results are summarized in Fig. 5 along with the results observed by the basic two-column liquid chromatography ($Y4 = 0.324X^{1.336}$, $r = 0.888$, $n = 9$). The amount of GHSA was calculated from concentration of DTF as a standard, due to lack of standard GHSA. The content of GHSA in serum of 9 diabetic patients ranged from 1.0 to 1.6 g/dL. The total amount of HSA in these serums ranged from 3.5 to 4.8 g/dL, as determined by the standard clinical test. The concentration of GHSA in HSA ranged from 15 to 45%. A combination of albumin affinity extraction and flow injection analysis

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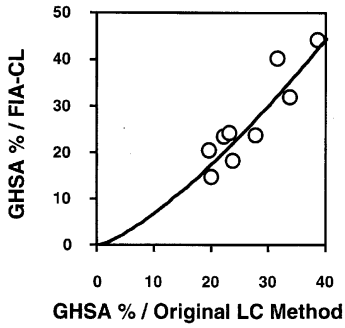

Figure 5. Comparison of GHSA concentration in HSA measured using flow injection analysis after albumin affinity gel extraction and original liquid chromatography. Extraction column: 200 μL of albumin affinity gel. Washing solvent: 0.15 M sodium chloride + 20 mM sodium phosphate buffer (pH 7.50). Extraction solvent: 75 mM sodium phosphate buffer (pH 2.4) + ethanol (1 + 1). Flow injection analysis: Chemiluminescence detector: Reagent A: 0.06 M lucigenin. Flow rate: 0.25 mL/min. Reagent B: 0.5 M sodium hydroxide. Flow rate: 0.25 mL/min.

using a chemiluminescence detector was a simple and fast analytical method for GHSA in blood serum.

The sensitivity of flow injection analysis using a fluorescence detector is dependent on the power of excitation lamp and, that using a chemiluminescence detector is influenced by the flow rate. Therefore, the total flow rate effect for chemiluminescence detection was measured. The detection limit of the standard compound 1-deoxy-1-*p*-toluidine-D-fructose calculated from the peak height was 8.5 pmol (S/N = 3) at a flow rate of 0.1 mL/min. The detection limit was improved by 5-fold at a flow rate of 0.01 mL/min. Lower flow rate was associated with higher sensitivity. The noise level decreased at a slower flow rate. These results indicated, that micro-plate analysis equipped chemiluminescence detection should be a suitable analytical system for GHSA with high sensitivity. The micro-plate analyzer can be used for measurements under the same conditions, and environmental changes will not affect the sensitivity.

Therefore, the albumin column extracts used for experimental results given in Fig. 4 were directly analyzed using the modified micro-plate analyzer equipped with chemiluminescence detection. The detection limit of DTF was 0.1 nmol (S/N = 3), and the calibration was linear from 0.1 to 10 nmol. The total count was adjusted using the extraction blank. The results ($Y5$) are summarized in Fig. 6, along with the results ($X2$) measured by affinity liquid chromatography after first solid-phase extraction ($Y5 = 0.0041 X_2^{2.5810}$, $r = 0.887$, $n = 9$). The amount of GHSA was calculated from the concentration of DTF as a standard, due to lack

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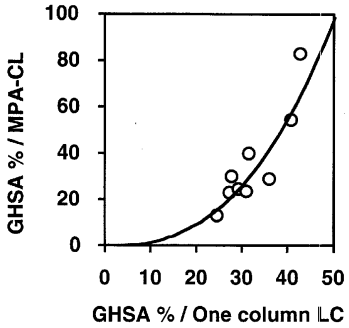

Figure 6. Comparison of GHSA concentration in HAS, measured using micro-plate analyzer equipped with a chemiluminescence detection and affinity liquid chromatography after first solid-phase extraction.

of standard GHSA. The content of GHSA in serum of 9 diabetic patients ranged from 0.45 to 3.56 g/dL. The total amount of HSA in these sera ranged from 3.73 to 4.76 g/dL, as determined by standard clinical test. The concentration of GHSA in HSA ranged from 13 to 83%. The non-linear relation observed by chemiluminescence detection was due to Mailard reaction of glycated albumin. That is, glycated albumin binds with a different number of glucose units, the chemical structure of which is dependent on Mailard reaction. The fluorescence detector indicates the concentration of albumin, and the chemiluminescence detection indicates the amount of enaminol-form glycated albumin.

Further study of GHSA analysis using a micro-plate analyzer equipped with a chemiluminescence detector, will facilitate development of the highly sensitive methods for analysis of GHSA in blood serum, and further studies using a micro-plate analyzer equipped with a fluorescence detector, will decide which analytical method is practical for clinical use. These new analytical methods may be used as standard clinical tests in the future.

REFERENCES

1. Kawamori, R.; Osonoi, K.; Miyagawa, K.; Kato, J. Medical Asahi **1999**, *1*, 1–6, Kawamori, R.; Odakiri, R.; Tahara, Y.; Miura, J. ibid *3*, 1–6. Kawamori, R.; Kadowaki, T.; Setoyama, S.; Shima, K.; Takei, I. ibid *8*, 1–6. Asahi Kasei, Tokyo.
2. Dorhofer, R.; Wieland, O.H. FEBS Letters **1979**, *103*, 282–286.
3. Guthrow, C.E.; Morris, M.A.; Day, J.F.; Thorpe, S.R.; Baynes, J.W. Proc. Natl. Acad. Sci. USA **1979**, *76*, 4258–4261.

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4. Day, J.F.; Thorpe, S.R.; Baynes, J.W. J. Biol. Chem. **1979**, *254*, 595–597.
5. Day, J.F.; Ingebretsen, C.G.; Ingebretsen, W.R.; Baynes, J.W.; Thorpe, S.R. Diabetes **1980**, *29*, 524–527.
6. Dolhofer, R.; Wieland, O.H. Diabetes **1980**, *29*, 417–422.
7. Dolhofer, R.; Renner, R.; Wieland, O.H. Diabetologia **1981**, *21*, 211–215.
8. Kim, H.J.; Kurup, I.V. Clin. Res. **1982**, *30*, 396A.
9. Jones, I.R.; Owens, D.R.; Williams, S.; Ryder, R.E.J.; Birtwell, A.J.; Jones, M.K.; Gicheru, K.; Hayes, T.M. Diabetes Care **1983**, *6*, 501–503.
10. Kemp, S.F.; Creech, R.H.; Horn, T.R.; J. Pediatr. **1984**, *105*, 394–398.
11. Leiper, J.M.; Talwar, D.; Robb, D.A.; Lunan, C.B. MacCuish, A.C. Quartery J. Med., New Series **1985**, *55*, 225–231.
12. Rendell, M.; Kao, G.; Mecherikunnel, P.; Petersen, B.; Duhaney, R.; Nierenberg, J.; Rasbold, K.; Klenk, D.; Smith, P.K. Clin. Chem. **1985**, *31*, 229–234.
13. Smith, C.H.; Nierenberg, J.; Rasbold, K.; Klenk, D.; Smith, P.K. Am. J. Med. Sci. **1986**, *292*, 11–14.
14. Ducrocq, R.; Bonniec, B.Le.; Carlier, O.; Assan, R.; Elion, J. J. Chromatogr. **1987**, *419*, 75–83.
15. Shima, K.; Ito, N.; Abe, F.; Hirota, M.; Yano, M.; Yamamoto, Y.; Uchida, T.; Noguchi, K. Diabetologia **1988**, *31*, 627–631.
16. Yasukawa, K.; Abe, F.; Shida, N.; Koizumi, Y.; Uchida, T.; Noguchi, K.; Shima, K. J. Chromatogr. **1992**, *597*, 271–275.
17. Cohen, M.P.; Hud, E. J. Immunol. Meth. **1989**, *122*, 279–283.
18. Schlkwijk, C.G.; Ligtvoet, N.; Twalfhoven, H.; Jager, A.; Blaauwgeers, H.G.; Schlingemann, R.O.; Tarnow, L.; Parving, H.H.; Stehouwer, C.D.; van Hinsbergh, V.W. Diabetes **1999**, *48*, 2446–2453.
19. Ikeda, K.; Sakamoto, Y.; Kawasaki, Y.; Miyake, T.; Tanaka, K.; Urata, T.; Yatayama, Y.; Ueda, S.; Horiuchi, S. Clin. Chem. **1998**, *44*, 256–263.
20. *Molecular Probes. Pro–Q Emerald 300 Glycoprotein Gel Stain Kit*. Product Information 15–Nox–2000, Eugene, OR. USA.
21. Silver, A.C.; Lamb, E.; Cattell, W.R.; Dawnay, A.B.St.J. Clin. Chi. Acta **1991**, *202*, 11–12.
22. Hanai, T.; Uchida, M.; Amao, M.; Ikeda, C.; Koizumi, K.; Kinoshita, T. J. Liq. Chromatogr. & Rel. Technol. **2000**, *23*, 3119–3131.
23. Baynes, J.W.; Thorpe, S.R.; Murtiashaw, M.H. Meth. Enzymol. **1984**, *106*, 88–98.
24. Monnier, V.M.; Cerame, A. Clinics Endocrinol. Metabol. **1982**, *11*, 431–452.
25. Wolf, S.P. Free Radicals, Glycation Theory. *The Maillard Reaction: Consequences for the Chemical and Life Sciences*; Ikan, R., Ed.; John Wiley and Sons Ltd.: New York, **1996**, 73–88.

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26. Peters Jr., T.; Taniuchi, H.; Anfinsen Jr., C.B. J. Biol. Chem. **1973**, *248*, 2447–2451.
27. Cuatrecasas, P.; Anfinsen, C.B. Meth. Enzymol. **1971**, *22*, 345.
28. Itoh, M.; Uchida, T. Japanese Patent HEI1–291159, **1989**.

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