

Fructosamine Assay Using Albumin Extracted from Serum

Emi OHKAWARA,^{*,a} Yukio NOHARA,^a Yoshihiko KANNO,^b Hiromichi SUZUKI,^b Gou MATSUMOTO,^c Toshio KINOSHITA,^d and Mitsuo WATANABE^a

^aFaculty of Pharmaceutical Sciences, Teikyo University; 1091-1 Suwarashi, Sagamiko-machi, Tsukui-gun, Kanagawa 199-0195, Japan; ^bDepartment of Nephrology, Saitama Medical School; 38 Morohongo, Moroyama-machi, Iruma-gun, Saitama 350-0495, Japan; ^cDepartment of Medicine, Matsumoto Hospital; 2-2-30 Hinooda-machi, Chichibu, Saitama 368-0034, Japan; and ^dSchool of Pharmaceutical Sciences, Kitasato University; 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan. Received March 15, 2002; accepted May 11, 2002

Albumin extracted from serum by a simple technique using trichloroacetic acid and ethanol has been applied to a fructosamine assay using nitroblue tetrazolium. A fructosamine assay using extracted albumin sample was carried out without interference from low molecular weight substances with reducing activities and other proteins with varying concentrations, half-lives and reducing activities. 1-Deoxy-1-*p*-toluidino-D-fructose (DTF) was selected as a calibrator for the assay because it is a stable Amadori reaction product with a constant glycation rate. Albumin fructosamine value was calculated using the calibration curve of DTF. The corrected albumin fructosamine value was expressed as the amount of albumin fructosamine per gram of extracted albumin taking into consideration the variation in albumin concentrations in sera from patients. The corrected albumin fructosamine values correlated more closely with the fasting blood glucose levels ($r=0.735$) than the serum fructosamine values corrected for albumin concentrations ($r=0.514$) ($p<0.05$).

Key words fructosamine; albumin; extraction; trichloroacetic acid; diabetic control

Glycated proteins are pathophysiologically important because they are related to chronic complications such as nephropathy, retinopathy and neuropathy.^{1–4)}

The glycation of proteins is a non-enzymatic modification dependent on the glucose concentration. Glycated proteins accumulate in patients with diabetes mellitus (DM) more than in normal subjects because plasma proteins of diabetic patients are exposed to high concentrations of glucose over a long period.^{5,6)} Glycated proteins provide information about the mean blood glucose level.^{7–9)} Glycated hemoglobin has been used widely as an index of diabetic control because it reflects blood glucose levels of the past 2–3 months;⁷⁾ fructosamine values, in contrast, reflect the status of diabetic control in the past 2–3 weeks.^{8,9)} A fructosamine value reflecting a shorter period is required to assess diabetic control more accurately and would, for example, allow for the adjustment of therapy or therapy for unstable DM.^{10,11)}

A fructosamine assay which depends on the abilities of the glycated proteins to reduce nitroblue tetrazolium (NBT) under alkaline conditions was reported by Johnson *et al.*¹²⁾ Measurements using NBT are more useful in the assessment of diabetic control and screening of DM because the method is quick, economical, and technically simple.

Conventionally, serum or plasma samples are used for fructosamine assays. However, the low molecular weight substances, uric acid, ascorbic acid *etc.*, in these samples might also reduce NBT.^{12–14)} It is impossible to correct the effects of low molecular weight substances because their concentrations in serum vary. In fact, uricase has been incorporated into commercial kits to cope with the effect of uric acid.¹⁵⁾ Nonetheless, it is difficult to completely eliminate the effects of low molecular weight substances. Also, it is unlikely that the fructosamine value can be assessed accurately because individual glycated proteins with different half-lives and reducing activities contribute unequally to the value.^{10,13,16–18)}

Although the conventional method is based on non-spe-

cific reduction, the fructosamine value reflects mainly the glycation of albumin because albumin accounts for 55–80% of the glycated proteins in serum.^{6,16)} Therefore, it is assumed that a fructosamine assay using albumin (albumin fructosamine assay) can be useful.^{10,13)}

The albumin fructosamine assay makes use of affi-gel blue¹⁰⁾ or polyethylene glycol¹³⁾ for the purification of albumin. However, the method using affi-gel blue¹⁰⁾ lacks simplicity and accuracy, and the method using polyethylene glycol¹³⁾ is likely to be affected by low molecular weight substances. We have developed an albumin fructosamine assay which copes with these problems. Serum albumin was separated from low molecular weight substances and other proteins by extraction using trichloroacetic acid (TCA) and ethanol. In addition, 1-deoxy-1-*p*-toluidino-D-fructose (DTF) was used as a calibrator instead of conventional calibrators such as 1-deoxy-1-morpholinofructose (DMF) or glycated polylysine which have problems of reducing activity and the rate of glycation, respectively.

MATERIALS AND METHODS

Chemicals A 100% (w/v) TCA solution, ethanol, albumin B-test wako, human serum albumin and NBT were purchased from Wako Pure Chemical Industries (Osaka, Japan). A 100% (w/v) TCA solution was diluted into 10% TCA with water. DMF was purchased from SIGMA Chemical (St. Louis, MO, U.S.A.). Precimat[®] fructosamine was obtained from F. Hoffmann-La Roche (Basel, Switzerland).

Patients Blood samples were provided by Saitama Medical School Hospital. Informed consent was obtained from all of the forty-five diabetic patients with renal failure.

Synthesis of DTF DTF was synthesized as reported previously.¹⁹⁾ It was obtained from a mixture of D-glucose and *p*-toluidine in water and its structure was identified by infrared and nuclear magnetic resonance spectrometry and elemental analysis.

* To whom correspondence should be addressed. e-mail: emi_rnr-1968@mtj.biglobe.ne.jp

Extraction of Albumin from Serum Serum albumin was extracted using TCA and ethanol. The methods of Campbell *et al.*²⁰⁾ and Schwert²¹⁾ were modified as follows: 200 μ l of serum was added to an equal volume of 10% (w/v) TCA at room temperature, vortexed sufficiently and allowed to stand for 10 min. After centrifugation at 10000 *g* for 2 min, the supernatant was eliminated. The precipitate of the proteins was suspended in 600 μ l of ethanol. The supernatant obtained by centrifugation at 10000 *g* for 2 min was used as the extracted albumin sample for the albumin fructosamine assay.

Assay of Albumin Concentration Measurement of the albumin concentration in the extracted albumin sample was performed with a commercial kit (albumin B-test wako). This measurement is based on the increase in absorbance caused by the binding of albumin and bromocresol green (BCG).

A 30- μ l volume of the albumin sample was added to 2.48 ml of BCG solution and absorbance was measured at 630 nm after 20 min.

Fructosamine Assay Fructosamine was quantified by a colorimetric method using NBT. The method described by Johnson *et al.*¹²⁾ was modified as follows: 450 μ l of sample was added to 2.7 ml of NBT reagent (0.2 M carbonate buffer, pH 10.3, containing 0.574 mM NBT) at 37°C. The absorbance change at 530 nm (ΔA 530 nm) in the interval 10 to 13 min after the start of the reaction was measured and com-

pared with that for DTF treated under the same conditions.

Albumin fructosamine was evaluated using the extracted albumin sample, and the corrected albumin fructosamine value was expressed as follows:

$$\text{corrected albumin fructosamine value } (\mu\text{mol/g}) = \frac{[\text{albumin fructosamine}]}{[\text{extracted albumin}]}$$

The serum fructosamine value was measured using the serum samples diluted three-fold with purified water, and the corrected serum fructosamine value was expressed as follows:

$$\text{corrected serum fructosamine value } (\mu\text{mol/g}) = \frac{[\text{serum fructosamine}]}{[\text{serum albumin}]}$$

RESULTS

Extraction of Albumin The extraction recovery for albumin in a concentration of 48 g/l was measured by comparing the concentration before and after extraction using a commercial kit. The recovery was $87.20 \pm 3.93\%$ (mean \pm S.D., $n=5$).

Comparison of Calibrators Figure 1 shows calibration curves of 3 calibrators (precimat[®] fructosamine, DTF and DMF). Precimat[®] fructosamine is the standard serum for a commercial kit. Though both DTF and DMF were Amadori reaction products, a difference in NBT-reducing activity between these compounds was observed. The reducing activity of DMF was about 50% that of DTF. On the other hand, the calibration curve of DTF was similar to that of precimat[®] fructosamine.

Fructosamine Assay Figure 2 shows the effect of the albumin level on the fructosamine value. The 45 diabetic patients with renal failure were divided into two groups based on albumin levels. One group was comprised of patients with high levels of albumin (40 g/l or greater, $n=25$) and the other those with low levels of albumin (less than 40 g/l, $n=20$). There was no significant difference between the two groups with respect to blood glucose levels (high, 8.46 ± 3.95 ; low, 7.26 ± 2.21 mM, $p>0.05$). The serum fructosamine values were significantly higher ($p<0.001$, Fig. 2A) for the patients

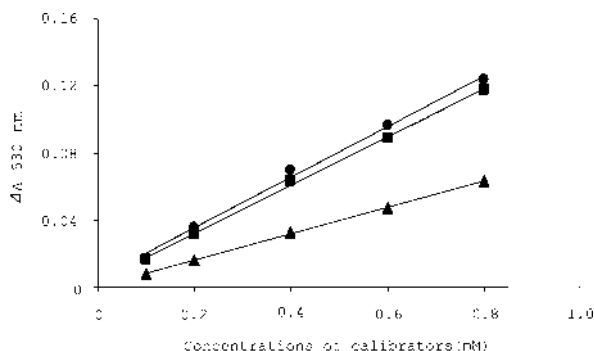


Fig. 1. Calibration Curves of Three Calibrators
●, precimat[®] fructosamine; ■, DTF; ▲, DMF.

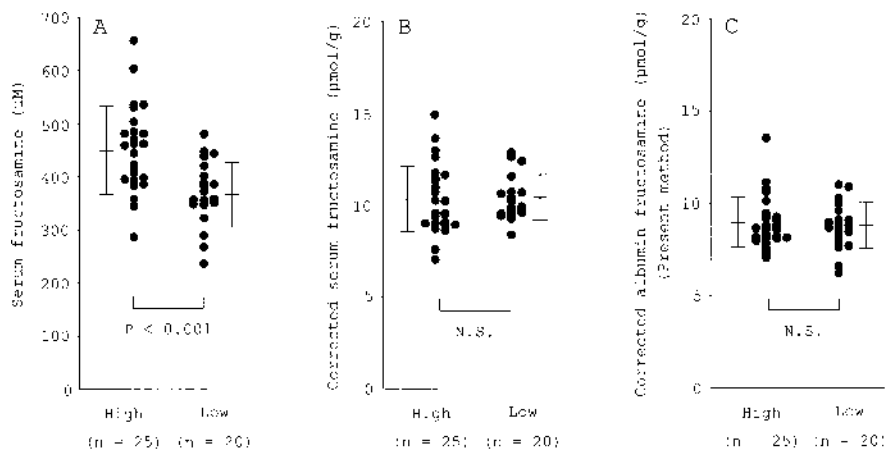


Fig. 2. Serum Fructosamine Values (A), Corrected Serum Fructosamine Values (B) and Corrected Albumin Fructosamine Values (C) in Patients with High and Low Levels of Albumin

Bars represent the mean \pm S.D. N.S.=not significant.

with high levels of albumin ($450.56 \pm 82.14 \mu\text{M}$) than those with low levels ($368.60 \pm 61.28 \mu\text{M}$), while there was no significant difference between the two groups ($p > 0.05$, Fig. 2B) with respect to the corrected serum fructosamine value (high, 10.31 ± 1.88 ; low, $10.43 \pm 1.16 \mu\text{mol/g}$). There was also no significant difference between the two groups ($p > 0.05$, Fig. 2C) in the corrected albumin fructosamine value (high, 8.91 ± 1.46 ; low, $8.73 \pm 1.25 \mu\text{mol/g}$).

Figure 3 shows the relationship between the serum fructosamine assay and the albumin fructosamine assay. The serum fructosamine values correlated with the corrected albumin fructosamine values ($r = 0.687$, $p < 0.001$).

Figure 4 shows the relationship between the fasting blood glucose level and the corrected serum fructosamine value or the corrected albumin fructosamine value. The latter value correlated more closely with the fasting blood glucose level ($r = 0.735$, $p < 0.001$, Fig. 4B) than the corrected serum fructosamine value ($r = 0.514$, $p < 0.001$, Fig. 4A). A significant difference was found at $p < 0.05$.

Table 1 shows the within-run and between-run reproducibility of the albumin fructosamine assay. The reproducibility was examined using an albumin solution diluted to six different concentrations (10–60 g/l), while the corrected albumin fructosamine value for the albumin concentration (10–60 g/l) was linear ($r = 0.99$).

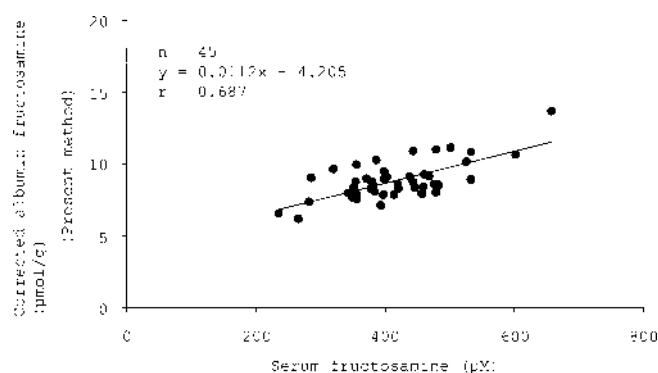


Fig. 3. Correlation between Serum Fructosamine Values and Corrected Albumin Fructosamine Values

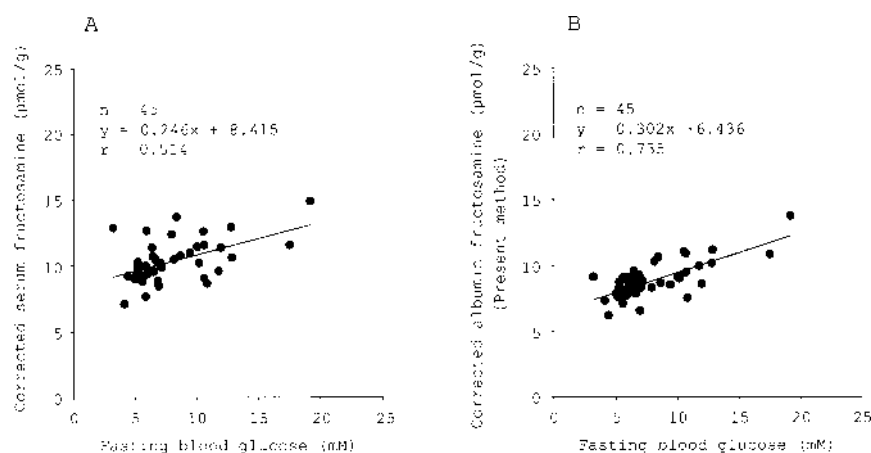


Fig. 4. Correlation between Fasting Blood Glucose Levels and Both Corrected Serum Fructosamine Values (A) and Corrected Albumin Fructosamine Values (B)

DISCUSSION

Extraction with TCA and ethanol was used to separate serum albumin from globulin.²²⁾ Albumin, after the precipitation with TCA, is soluble in organic solvents such as ethanol, while globulin is insoluble in these solvents.²¹⁾ With this extraction method, it is possible not only to eliminate other proteins but also to eliminate low molecular weight substances along with the supernatant after precipitation of the proteins. Although the concentrated TCA solution caused the precipitation of NBT, TCA contained in the supernatant was removed before the redissolution of precipitated albumin by ethanol. Therefore, the present assay was executed without the effects of these substances.

The question about the standardization of fructosamine values has been controversial. Johnson *et al.* used a calibrator solution containing DMF and albumin.¹²⁾ However, Hindle *et al.*²³⁾ and Smid *et al.*²⁴⁾ pointed out that the use of different batches of albumin in the calibrator solution influenced the calibration curve. In addition, the reducing activity of DMF differs from that of physiological fructosamine.^{25,26)} In the present study, DTF used as a calibrator was similar in reducing activity to physiological fructosamine as Fig. 1 indicates. Otherwise, Schleicher and Vogt recommended the use of a secondary serum protein standard calibrated by glycated polylysine.²⁶⁾ However, the glycation rate for glycated polylysine is not constant.²⁶⁾ Although standard serum for a commercial kit is readily available, the fructosamine value for

Table 1. The Reproducibility of Albumin Fructosamine Assay

Albumin concentration (g/l)	C. V. (%)	
	Within-run (n=5)	Between-run (n=5)
10.0	8.39	11.64
20.0	3.51	6.36
30.0	2.23	2.93
40.0	1.52	1.98
50.0	1.34	1.74
60.0	0.96	1.46

Albumin dissolved in purified water was diluted to 6 different concentrations. A 150 μl volume of each albumin solution was added to 3 ml of NBT reagent at 37°C, and the absorbance change was measured at 530 nm in the interval 10 to 13 min after start of the reaction.

standard serum is determined by a complicated method using elementary analysis of C and N and radioactivity measurements. Moreover, the standard serum seems inappropriate as a calibrator because it contains low molecular weight substances and various proteins. DTF, however, has a constant reducing activity because it does not contain other substances and has a constant glycation rate; it therefore has advantages in the standardization of fructosamine values.

Long-term hyperglycemia leads to diabetic nephropathy,^{3,4)} and the serum protein levels of these patients tend to be low due to factors including malnutrition, loss during dialysis, the alteration of protein metabolism, and so on.^{27,28)} In the present study, fructosamine values were corrected for albumin concentration, and three kinds of these values were compared between patients with renal failure divided into two groups according to serum albumin levels to evaluate the utility of correction by albumin. The reference value for the grouping was determined on the basis of reports that the risk of mortality rises dramatically as serum albumin levels decline to less than 40 g/l.^{29–31)} There was no significant difference between the two groups in blood glucose levels. However, serum fructosamine values of the group with low levels of albumin were significantly lower than those of the group with high levels of albumin. Otherwise, the corrected serum fructosamine values and the corrected albumin fructosamine values were much the same in these two groups, respectively. The fructosamine values of patients with low protein levels tend to be low.^{32–34)} Therefore, the serum fructosamine values of renal failure patients with low albumin levels may be misleading. However, it is possible to assess the fructosamine values of patients with low albumin levels accurately using the fructosamine value corrected for albumin.

The correlation between the present method and serum fructosamine value ($r=0.687$) was looser than that of a similar study ($r=0.85$).¹⁰⁾ The low albumin levels are believed to have an effect on the correlation between the corrected albumin fructosamine value and the serum fructosamine value, because the present study was carried out using serum from diabetic patients with renal failure.

Comparison of the corrected serum fructosamine values with the corrected albumin fructosamine values revealed the latter to be correlated more closely with the fasting blood glucose levels than the former. The significant difference in correlation coefficients is probably due to the existence of low molecular weight substances and various glycosylated proteins in the serum samples. These findings indicate that the corrected albumin fructosamine values are important for the accurate assessment of diabetic control. Therefore, it is conceivable that the present method is more useful for diabetic control, especially in the management of diabetic patients with renal failure.

REFERENCES

- 1) Brownlee M., Cerami A., Vlassara H., *N. Engl. J. Med.*, **318**, 1315–1321 (1988).
- 2) Vlassara H., Brownlee M., Cerami A., *Clin. Chem.*, **32**, B37–B41 (1986).
- 3) Suzuki D., Miyata T., *Intern. Med.*, **38**, 309–314 (1999).
- 4) Chen S., Cohen M. P., Ziyadeh F. N., *Kidney Int.*, **58** (Suppl. 77), S40–S44 (2000).
- 5) Macfarland K. F., Catalano E. W., Day J. F., Thorpe S. R., Baynes J. W., *Diabetes*, **28**, 1011–1014 (1979).
- 6) Dolhofer R., Wieland O. H., *FEBS Lett.*, **103**, 282–286 (1979).
- 7) Dunn P. J., Cole R. A., Soeldner J. S., Gleason R. E., Kwa E., Firoozabadi H., Younger D., Graham C. A., *Diabetologia*, **17**, 213–220 (1979).
- 8) Armbruster D. A., *Clin. Chem.*, **33**, 2153–2163 (1987).
- 9) Okumura T., Park K., Sasaki M., *Rinsho Byori*, **37**, 537–542 (1989).
- 10) Walker S. W., Howie A. F., Smith A. F., *Clin. Chim. Acta*, **156**, 197–206 (1986).
- 11) Baker J. R., Metcalf P. A., Holdaway I. M., Johnson R. N., *Br. Med. J.*, **290**, 352–355 (1985).
- 12) Johnson R. N., Metcalf P. A., Baker J. R., *Clin. Chim. Acta*, **127**, 87–95 (1982).
- 13) Mashiba S., Uchida K., Okuda S., Tomita S., *Clin. Chim. Acta*, **212**, 3–15 (1992).
- 14) Tas S., Zein el Din R. R., *Clin. Chem.*, **36**, 1825–1830 (1990).
- 15) Baker J., Metcalf P., Scragg R., Johnson R., *Clin. Chem.*, **37**, 552–556 (1991).
- 16) Seng L. Y., Staley M. J., *Clin. Chem.*, **32**, 560 (1986).
- 17) Sivas A., Unal T., Oz H., *Clin. Chem.*, **36**, 1386–1387 (1990).
- 18) Zoppi F., Mosca A., Granata S., Montalbetti N., *Clin. Chem.*, **33**, 1895–1897 (1987).
- 19) Hanai T., Uchida M., Amao M., Ikeda C., Koizumi K., Kinoshita T., *J. Liq. Chrom. & Rel. Technol.*, **23**, 3119–3131 (2000).
- 20) Campbell P. N., Greengard O., Kernot B. A., *Biochem. J.*, **74**, 107–117 (1960).
- 21) Schwert G. W., *J. Am. Chem. Soc.*, **79**, 139–141 (1957).
- 22) Debro J. R., Tarver H., Korner A., *J. Lab. Clin. Med.*, **50**, 728–732 (1957).
- 23) Hindle E. J., Rostrom G. M., Gatt J. A., *Ann. Clin. Biochem.*, **22**, 84–89 (1985).
- 24) Smid E., Ferencz A., Fodor M., *Clin. Chim. Acta*, **156**, 215–219 (1986).
- 25) Johnson R. N., Baker J. R., *Clin. Chem.*, **32**, 368–370 (1986).
- 26) Schleicher E. D., Vogt B. W., *Clin. Chem.*, **36**, 136–139 (1990).
- 27) Kopple J. D., *Kidney Int.*, **14**, 340–348 (1978).
- 28) Lim V. S., Kopple J. D., *Kidney Int.*, **58**, 1–10 (2000).
- 29) Bircher G., "Comprehensive Clinical Nephrology," Chap. 75, ed. by Johnson R. J., Feehally J., Mosby, London, 2000, pp. 75.1–75.8.
- 30) Owen W. F., Jr., Lew N. L., Liu Y., Lowrie E. G., Lazarus J. M., *N. Engl. J. Med.*, **329**, 1001–1006 (1993).
- 31) Held P. J., Port F. K., Turenne M. N., Gaylin D. S., Hamburger R. J., Wolfe R. A., *Kidney Int.*, **45**, 1163–1169 (1994).
- 32) Baker J. R., O'Connor J. P., Metcalf P. A., Lawson M. R., Johnson R. N., *Br. Med. J.*, **287**, 863–867 (1983).
- 33) Van Dieijen-Visser M. P., Seynaeve C., Brombacher P. J., *Clin. Chem.*, **32**, 1610 (1986).
- 34) Dominiczak M. H., Orrell J. M., Finlay W. E., *Clin. Chim. Acta*, **182**, 123–129 (1989).