Yonsei Medical Journal
Vol. 27, No. 1, 1986

A Simplified Colorimetric Assay of Nonenzymatic Glycosylation of Human Low Density Lipoproteins in Normal and Diabetes Mellitus

Kyung-soo Hahm, Kwang Kyun Park and Yoon Soo Kim

A simplified colorimetric method for measurement of the levels of glycosylation of proteins was developed by a modification of an existing method. Employing this method, the extent of nonenzymatic glycosylation of apolipoprotein B subspecies (B-100, B-74, B-26), LDL, VLDL, and total serum proteins in human plasma obtained from patients with diabetes mellitus and control subjects was compared. Plasma LDL ($1.019<d<1.063$) and VLDL ($d<1.006$) were separated using the sequential ultracentrifugation method, and the subspecies of apolipoprotein B were isolated by extracting them from polyacrylamide gels after they were separated by preparative SDS-polyacrylamide gel electrophoresis. Increases in the level of glycosylation of serum proteins, LDL, VLDL, and apo B subspecies obtained from diabetic patients were observed. Among them, the increases of glycosylated LDL and apo B-26 were most significant ($p<.001$). Also, good correlations were found between glycosylations of apo B-26 and LDL ($r=.88$), and glycosylation of LDL and LDL cholesterol level ($r=.79$). The results also showed an excellent correlation between levels of HbA1c and glycosylated apo B-26 ($r=.93$).

Key Words: Nonenzymatic glycosylation, atherosclerosis, LDL, apo B subspecies, diabetes mellitus.

Nonenzymatic glycosylation of proteins involves the condensation of carbohydrate and free amino groups of a protein (Mohammad *et al.* 1949; Feeney *et al.* 1964). The reaction occurs nonenzymatically through the formation of covalent linkage with glucose (Gottschalk 1972). That similar reactions can also occur under physiological conditions was brought to light more recently, when it was shown that hemoglobin undergoes nonenzymatic glycosylation in human blood, in vivo, forming the so-called minor hemoglobin factions (HbA1a-c) (Bunn *et al.* 1975; Cerami and Koening 1978; Dolhofer *et al.* 1977).

It has been suggested that the nonenzymatic glycosylation could occur at enhanced rates during hyperglycemia, and this may contribute to the progressive and accelerated atherosclerosis in chronic complications of diabetes (Crall and Roberts 1978; Christy and Clements 1982). One proposed mechanism contributing to the development of atherosclerosis involves disorders of low density lipoprotein (LDL) metabolism. Goldstein and Brown (1974, 1977) have suggested that the cellular LDL receptor pathway may play an important role in atherosclerosis and LDL is the source of most of the cholesterol present in vascular regions and cellular elements of the vessel wall. Recently it has been reported that the modification of lysine residues of LDL alters its catabolism in vivo (Weisgraber *et al.* 1978; Mahley *et al.* 1979; Brown *et al.* 1979, 1980; Fogelman *et al.* 1980; Gonen *et al.* 1983) and in vitro (Gonen *et al.* 1981; Kim and Kurup 1982; Sasaki and Cottam 1982; Witztum *et al.* 1982). It has also been demonstrated that glycosylation of LDL occurs at lysyl residues both in vitro and in vivo (Gonen *et al.* 1981; Kim and Kurup 1982). Glycosylated LDL has been reported to be elevated in diabetes (Schleicher *et al.* 1981; Witztum *et al.* 1982).

Various procedures have been used for the determination of the degree of glycosylation in proteins. These procedures include HPLC (Schleicher and Wieland 1981; Bisse *et al.* 1982; Monnier and Cerami 1983), affinity chromatography (Bannon *et al.* 1984;

Received November 25, 1985
Accepted January 20, 1986

Department of Biochemistry, Yonsei University College of Medicine, Seoul, Korea

This work was supported by YUHAN-CMB grant from Yonsei University College of Medicine (1983).

Part of this work was presented at the Third Korea-Japan Symposium on Diabetes Mellitus, 1985, and at the Thirty-seventh Annual Meeting of Korean Biochemical Society, 1985.

Fluckiger *et al.* 1984; Agarval *et al.* 1985), isoelectric focusing and electrophoresis (Spicer *et al.* 1978; Allen *et al.* 1980), and colorimetric assay (Dolhofer and Wieland 1980; Ney *et al.* 1981). However, the procedures using HPLC or affinity chromatography require either expensive equipment or reagents, and electrophoretic procedures are not well established, whereas the colorimetric assay method is time consuming. The first aim of the present study has been therefore, to modify the colorimetric assay method so that the determination of glycosylated level in proteins can be made with ease, less cost, and within a shorter length of time.

LDL contains apolipoprotein B (apo B) as its major protein constituent (Blue *et al.* 1980) and apo B is now known to consist of subspecies, apo B-100, B-74, and B-26 according to their apparent molecular weights (Kane *et al.* 1980). Apo B-100 is also a protein component of very low density lipoproteins (VLDL) (Kane *et al.* 1980; Tikkanen *et al.* 1984). Apo B is also known to be the recognition marker on lipoproteins for the cellular LDL receptors, although the roles of various apo B subspecies are unknown. Therefore, the second aim of the present study has been to measure and compare the degree of glycosylation in LDL, VLDL, and various apo B subspecies obtained from the plasmas of normal and diabetic subjects, and find meaningful correlations between various factors including cholesterol levels and HbA1c in order to locate the most significant apo B subspecies.

MATERIALS AND METHODS

Separation of Plasma Lipoproteins

Blood was obtained from 13 apparently normal, healthy subjects (mean age: 33) and 20 diabetic patients (type II) after an overnight fasting using 1 mM EDTA as an anticoagulant. The patients were admitted to Severance Hospital at Yonsei University in Seoul, Korea, from December 1984 to February 1985. After erythrocytes were spun down, each plasma was dialyzed against 1.006 EDTA-saline (1 mM Na_2EDTA-165 mM NaCl, d=1.006) containing chloramphenicol (50 mg/l), gentamycin sulfate (50 mg/l), and sodium azide (0.02%) (Kane *et al.* 1980). Apo B containing lipoproteins, VLDL ($d<1.006$) and LDL ($1.019<d<1.063$) were isolated by the density-adjusted sequential ultracentrifugation method (Schonfeld *et al.* 1974) using a Beckman L2-75B Ultracentrifuge. Density was adjusted with solid KBr and two ultracentrifugations were carried out at each density. After isolation, each lipoprotein fraction was dialyzed exhaustively against 1 mM EDTA-water, pH 8.0, containing the above antibiotics, passed through 0.45 μm membrane filter (Gelman, Ann Arbor, Mich., USA) and stored at 4°C.

Chemical Analysis

Plasma and LDL cholesterol levels were determined by the method of Leffler (1959) and the triglyceride contents were measured by the method of Biggs *et al.* (1975). Protein concentrations in separated apo B subspecies were measured according to the modified micro method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard, and protein concentrations of lipoprotein fractions and whole plasma were determined in the same way except that each sample was delipidated by extracting it with water-saturated ether before measuring the absorbance at 750 nm. Levels of fasting blood sugar were measured using YSI (model 23A) glucose analyzer.

SDS-Polyacrylamide Gel Electrophoresis

Analysis of apo B subspecies in lipoprotein fractions was carried out by 3.5% polyacrylamide gel electrophoresis containing 0.1% SDS (SDS-PAGE) (Hahm *et al.* 1983). The electrode buffer was 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS, and the sample buffer contained 20% sucrose, 2.5% SDS, 5% 2-mercaptoethanol, and 0.0075% bromophenol blue. For electrophoresis, each lipoprotein fraction (20 μg) was mixed with 40 μl of the sample buffer and treated at 100°C for 2 min. Electrophoresis was carried out at the constant current of 30 mA for 20 hrs at room temperature in an LKB 2001 vertical electrophoresis unit. The gels were stained with 0.1% Coomassie brilliant blue R-250 in 12.5% trichloroacetic acid and 50% methanol for 18 hrs at room temperature and destained in 10% acetic acid-30% metanol for 8 hrs. Cross-linked BSA (Davies and Stark 1970) was used as a molecular weight standard.

Isolation of Apo B Subspecies

For isolating various apo B subspecies of VLDL and LDL from normal and diabetic subjects, preparative 3.5% SDS-PAGE was carried out as described above except using 3 mm thickness slabs rather than 1 mm thickness slabs. On each slab, 10 sample wells were prepared in order to apply as much as 170 μl of sample in each well. After electrophoresis, gels were stained briefly (1 hr) with Coomassie blue and destained for 2 hrs. The apoprotein bands were then cut from the gel, homogenized in 2 ml of cold 10% trichloroacetic acid-30% ethanol using Teflon

homogenizer, and centrifuged at 7,500 × g for 10 min. This washing procedure was repeated 4 more times. To the final pellet, 0.1 N NaOH (1 ml) was added, incubated at 37°C for 2 hrs with gentle shaking, and then centrifuged at 10,500 × g for 10 min. The clear supernatant was used for further analysis.

Quantification of Glycosylated Proteins

The degree of glycosylation in total plalsma proteins, lipoprotein fractions, and apo B subspecies were determined according to the procedure of Ney *et al.* (1981) with the following modifications. To 1.0 ml sample, 0.5 ml of 1.0 M oxalic acid was added to make final concentration of 0.33 M oxalic acid and pH 1.0. The solution was hydrolyzed by autoclaving the samples in Teflon-capped tubes at 125°C for 60 min. After cooling the solutions to room temperature, 50% trichloroacetic acid (0.5 ml) was added and centrifuged at 1,000 × g for 10 min. To the supernatant (1.5 ml), 0.5 ml of 0.05 M thiobarbituric acid (TBA) was added, incubated at 40°C for 30 min and the amount of 5-hydroxymethylfurfural (5-HMF) produced by the reaction was measured from the absorbance readings at 443 nm using fructose as a standard (Pecoraro *et al.* (1979). The level of glycosylation was expressed as pmoles of 5-HMF/mg of protein for total serum proteins and nmoles of 5-HMF/mg of protein for lipoprotein fractions and apo B subspecies.

RESULTS

In Table 1 are shown the blood chemical analysis data for control subjects and patients with diabetes. All the patients were diagnosed as type II diabetics with a mean HDL-cholesterol of 29 mg/dl and plasma triglycerides of 207 mg/dl. The result in Table 1 further shows that total plasma cholesterol level and LDL cholesterol level as well as fasting blood sugar were greatly elevated in diabetics. LDL cholesterol level was expressed as mg/100 mg protein instead of unit volume because the concentrations of LDL in whole blood volume varied greatly with different preparations.

LDL and VLDL were separated by the sequential ultracentrifugation method from plasmas obtained from normal and diabetic subjects and were analyzed by 3.5% SDS-PAGE in order to identify and compare the apo B subspecies in lipoprotein fractions. As the result (Fig. 1) shows, all of the LDL samples showed apos B-100, B-74, and B-26, while all of the VLDL samples showed only the apo B-100. No apo B-48 was observed in any of the VLDL samples tested and there were no differences in apo B compositions between normal and diabetic lipoproteins. For the isolation of apo B subspecies, electrophoreses were run that were identical except for the fact that thicker (3 mm) gel was used with larger sample wells so that up to 170 μl of sample containing 250 μg proteins could be applied in each well. A total of 500 μg of lipoprotein was applied routinely for separating apo B subspecies. From VLDL, an average of 44 μg of apo B-100 was obtained, and from LDL (200 μg), an average of 33 μg of apos B-100 and B-74, and 23 μg of apo B-26 were obtained. Apos B-100 and B-74 were extracted together because the resolution between these two apo B subspecies was such that it was almost impossible to completely separate and isolate apo B-100 and B-74 from each other to the extent that they would be rendered free from contamination with each other.

Fig. 2 compares the glycosylated total serum proteins and total apo B containing lipoproteins between normal and diabetic subjects. In diabetics, both the glycosylated total serum proteins and total apo B containing lipoproteins were increased, but the increase in glycosylated total serum proteins (0.373±0.139 vs 0.339±0.100 pmoles 5-HMF/mg protein) was not statistically significant, while the increase in glycosylated total apo B containing lipoproteins (0.196±0.026 vs 0.080±0.004 nmoles 5-HMF/mg pro-

Table 1. Chemical analysis of control subjects and patients with diabetes mellitus

	Control	Diabetes Mellitus	p
Fasting Blood Sugar (mg/dl)	116.56±2.87 (8)	224.32±14.77 (22)	<0.001
Total Cholesterol (mg/dl)	159.54±6.18 (8)	235.60±14.32 (20)	<0.01
LDL Cholesterol (mg/100 mg Protein)	41.79±6.00 (9)	129.86±17.51 (13)	<0.001

Each value represents mean ± SE with the number of samples indicated in parenthesis.

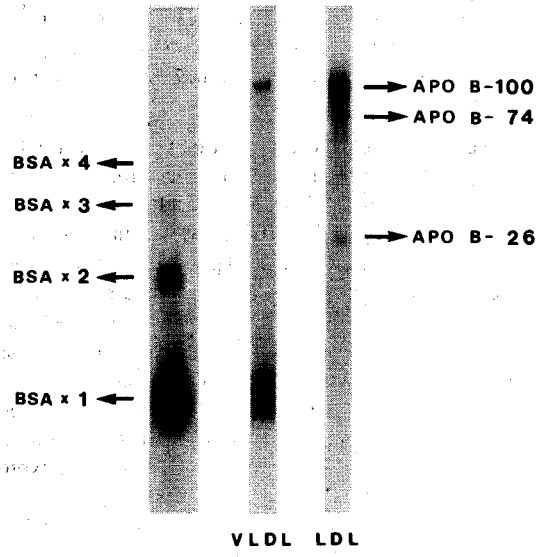

Fig. 1. Representative electrophoretogram of analytical 3.5% SDS-PAGE of VLDL and LDL. The left lane showed cross-linked BSA used as molecular weight standards. Apolipoprotein B subspecies are indicated as apo B-100, apo B-74, and apo B-26.

tein) was highly significant ($p<.001$). Since we found that the total apo B containing lipoproteins were glycosylated significantly more in diabetics as compared to normal subjects, we then measured the level of glycosylation in apo B containing lipoproteins separately. The result (Fig. 3) showed that the levels of glycosylation were increased in diabetics for both VLDL and LDL. These increases in both VLDL (0.102±0.018 vs 0.050±0.004 nmoles 5-HMF/mg protein) and LDL (0.094±0.013 vs 0.031±0.003 nmoles 5-HMF/mg protein) were statistically significant, the increase in LDL glycosylation being more significant ($p<.001$ vs $p<.02$).

Although the increase in glycosylated VLDL in diabetics was significant, there was no significant difference in the level of glycosylation of isolated VLDL apo B-100 between normal subjects and diabetics (Fig. 4). On the other hand, the result (Fig. 4) showed that the level of glycosylation in LDL apo B-100 + B-74 and in LDL apo B-26 was increased significantly in diabetics (1.418±0.104 and 1.242±0.035 nmoles 5-HMF/mg protein compared to 0.873±0.041 and 0.605±0.034 nmoles 5-HMF/mg protein, respectively). It was also noted from the scattergram (Fig. 4) that the increase in glycosylated LDL apo B-26 in diabetics was even more significant, judging from dense population of dots.

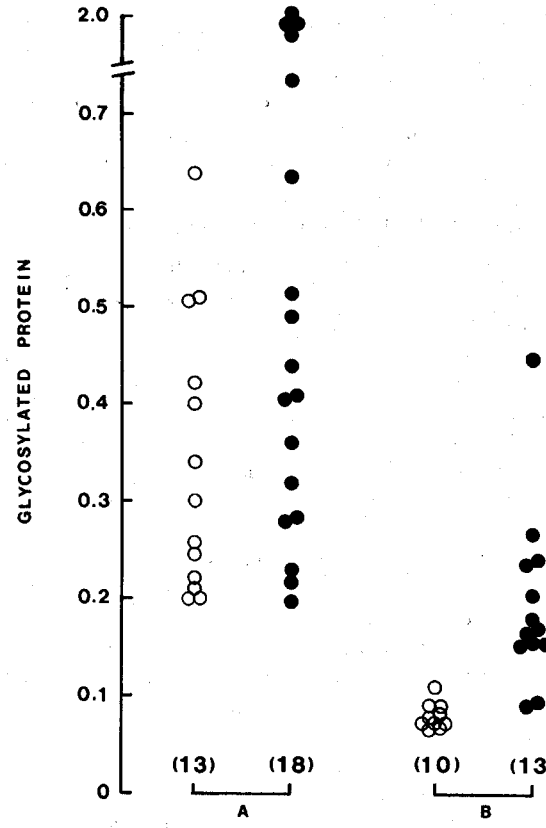

Fig. 2. Glycosylated levels of total serum proteins (A) and total apo B containing lipoproteins (B) in normal (o) and patients with diabetes mellitus (•). The degree of glycosylation was measured by the modified TBA assay as described under Materials and Methods and is expressed as pmoles 5-HMF/mg of total serum proteins and nmoles 5-HMF/mg of total apo B containing lipoproteins. Numbers in parenthesis indicate the number of subjects.

With the above information we then analyzed the results to correlate various factors related to the state of hyperglycemia. Fig. 5 shows the result of regression analysis which shows a good positive correlation ($r=.79$) between LDL cholesterol and glycosylated LDL. When we analyzed the relationship between glycosylated LDL and various apo B subspecies, we found that there is a very good linear correlation ($r=.88$) between glycosylated LDL and LDL apo B-26 (Fig. 6), which in turn showed an excellent positive correlation ($r=.93$) with HbA1c (Fig. 7). The results showed that the level of glycosylated apo B-26 could be a sensitive indicator of a hyperglycemic state as

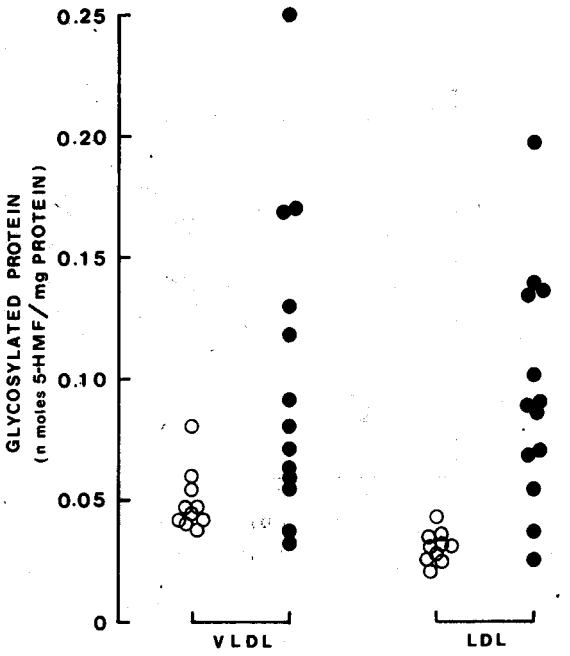

Fig. 3. Glycosylated lipoprotein levels in normal (o; n=10) and patients with diabetes mellitus (•; n=13). Apo B containing lipoproteins, VLDL and LDL, were isolated by density adjusted sequential ultracentrifugation method and the degree of glycosylatior was measured by the modified TBA assay as described under Materials and Methods.

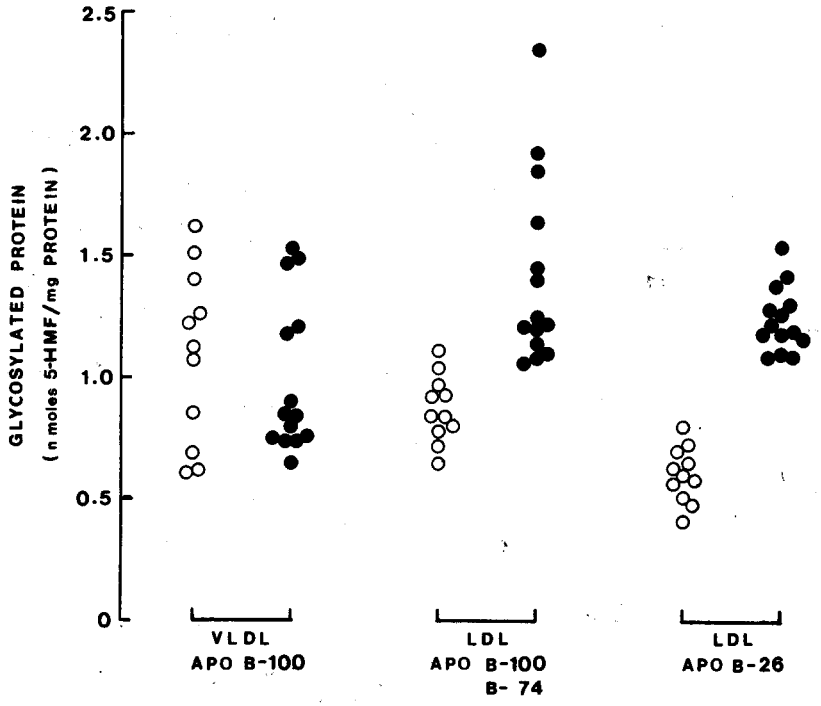

Fig. 4. Glycosylated levels of apolipoprotein B subspecies in normal (o; n=11) and patients with diabetes mellitus (•; n=14). Apolipoprotein B subspecies were separated by preparative SDS-PAGE, extracted from the gel, and the degree of glycosylation was measured by the modified TBA assay as described under Materials and Methods.

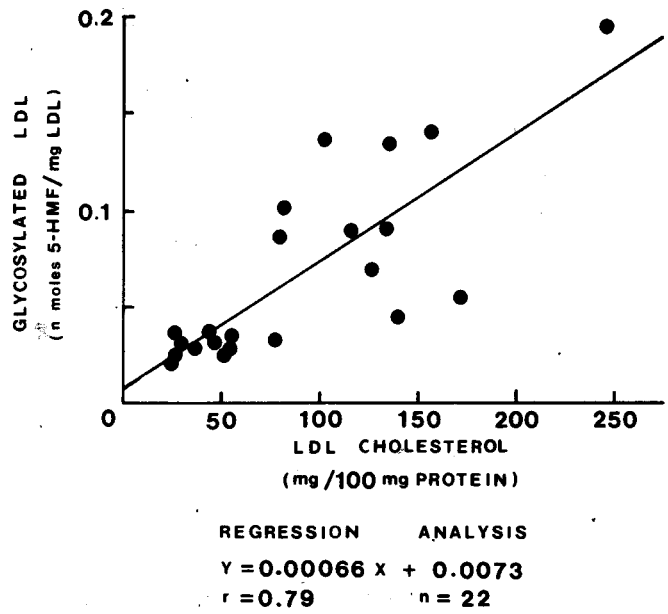

Fig. 5. The relationship between LDL cholesterol and glycosylated LDL.

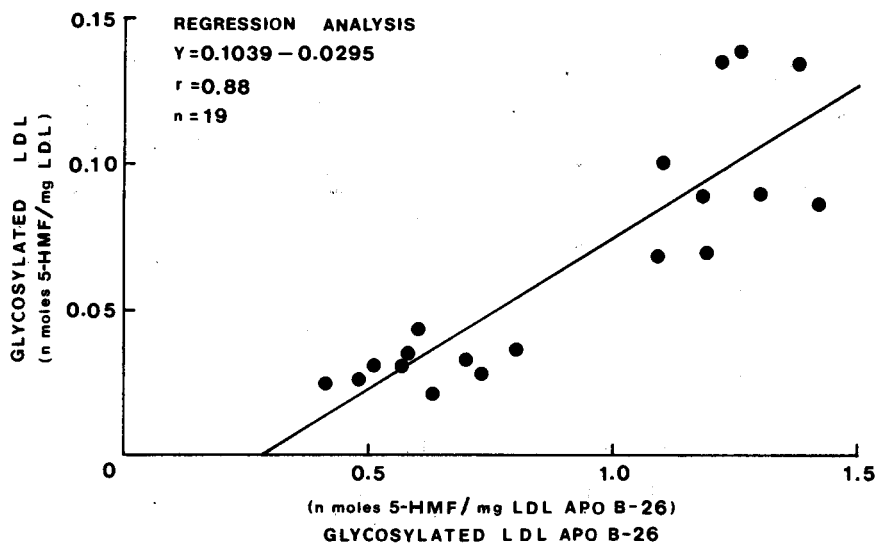

Fig. 6. The relationship between glycosylated LDL apo B-26 and glycosylated LDL.

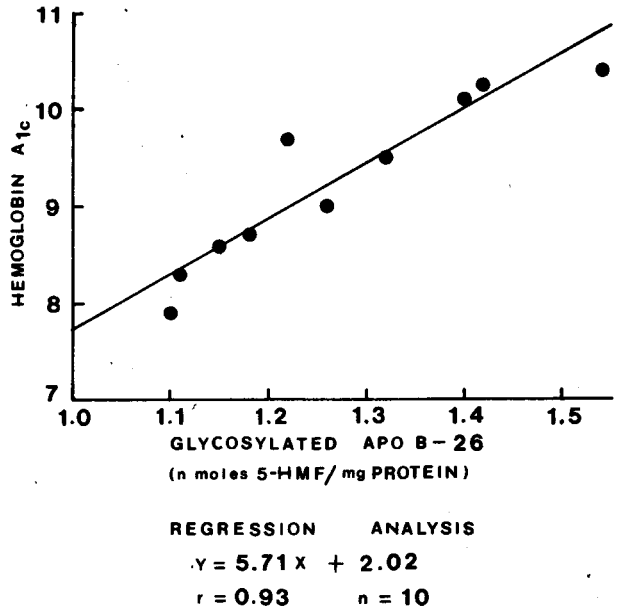

Fig. 7. *The relationship between glycosylated apo B-26 and hemoglobin A1c(%).*

HbA1c. The results showing a good correlation between LDL cholesterol, glycosylated LDL, and glycosylated LDL apo B-26 also indicate that the measurement of LDL cholesterol could be used in place of glycosylated LDL apo B-26 for indices of a hyperglycemic state.

DISCUSSION

Soon after the first description of the nonenzymatic glycosylation of serum proteins in vivo appeared in a publication (Bunn *et al.* 1975), the increased level of glycosylation was reported in patients with diabetes mellitus (Gabbey *et al.* 1977), and since then there have been many studies devoted to gaining an understanding of the functions of glycosylated proteins in diabetic complications. Several methods for measuring the degree of glycosylation have been reported. Although HPLC (Schleicher and Wieland 1981; Bisse *et al.* 1982; Monnier and Cerami 1983) and affinity chromatography (Bannon *et al.* 1984; Fluckiger *et al.* 1984; Agarwal *et al.* 1985) are efficient methods, they require expensive equipment and have drawbacks in the sense that only a few samples or one sample can be analyzed at a time. Electrophoretic methods (Spicer *et al.* 1978; Allen *et al.* 1980), on the other hand, are time consuming and also require specialized equipment. The colorimetric method using TBA (Fluckiger and Winterhalter 1976; Dolhofer and Wieland 1980; Ney *et al.* 1981) can be performed with ease requiring a minimum amount of equipment, but the latest known procedure (Ney *et al.* 1981) required at least 8 hrs for analysis. The central step of the TBA assay is the release of adducted glucose from the protein as 5-HMF by weak acid hydrolysis, a reaction which is time- and temperature-dependent. In the latest known procedure (Ney *et al.* 1981), the hydrolysis requires 6 to 8 hrs at 115°C for obtaining optimum results, and, at first in using it, we frequently lost samples by evaporating during the relatively long period of hydrolysis. However, we were able to avoid the sample loss completely by hydrolyzing samples at 125°C for 1 hr in an autoclave. As a suitable and accurate alternative standard for 5-HMF, fructose has been reported to be used as it is coverted to 5-HMF by weak acid hydrolysis (Pecoraro *et al.* 1979). By introducing these modifications (weak acid hydrolysis for 1 hr in an autoclave and fructose standard), we were able to quantitate the level of glycosylation more rapidly. We were able to compare on two occasions the reproducibility of the present method and the unmodified method. The mean±SD for 4 separate LDL apo B-26 samples was 1.43±0.512 when the unmodified method was used and 1.36±0.155 when the

present method was used, and the mean±SD for 10 separate VLDL apo B-100 samples was 1.057±0.622 and 1.042±0.343, respectively, which shows a considerable improvement over the unmodified method.

One of the known complications of diabetes mellitus is accelerated atherosclerosis. The cholesterol of atherosclerotic plaques is derived from LDL that circulates in the blood stream. The more LDL there is in blood the more rapidly atherosclerosis develops (Goldstein and Brown 1977). Plasma LDL is known to be catabolized mostly (~80%) by the high affinity LDL receptor pathway (Brown and Goldstein 1984) specifically recognizing apo B of LDL on the cell surface. LDL contains apos B-100, B-74, and B-26, and VLDL usually contains only apo B-100 as its apo B components (Hahm *et al.* 1983; Tikkanen *et al.* 1984), whereas apo B-48 is reported to be derived from the intestine and thus associated with chylomicrons (Lock *et al.* 1983; Hahm *et al.* 1985). LDL undergoes nonenzymatic glycosylation in vivo, and in vitro with glucose to yield glycosylated derivatives on its ε-lysine groups of apo B (Gonen *et al.* 1981; Witztum *et al.* 1982), and the level of glycosylation was reported to be increased in patients with diabetes mellitus (Schleicher *et al.* 1981; Witztum *et al.* 1982). However, there have been no reports on quantitative relationships either between the degree of glycosylation of LDL and the degree of severity of diabetes, or between the degree of glycosylation of LDL and various apo B subspecies. In the present study, we have measured and compared the level of glycosylation in apo B containing lipoproteins, VLDL and LDL, and various apo B subspecies as it is found to occur in normal subjects and in patients with diabetes mellitus, and found that the amounts of glycosylated LDL, glycosylated LDL apo B-100 + B-74, and apo B-26 were increased significantly in diabetic patients when compared to those found in normal subjects. Furthermore, we found a good linear relationship between glycosylated LDL apo B-26 and glycosylated LDL. The results also showed that glycosylated LDL is linearly correlated with the level of LDL cholesterol suggesting that the level of LDL cholesterol in type II diabetics reflects the degree of glycosylated LDL apo B-26. At present, the measurement of HbA1c is being used in many clinics to assess diabetic control and diabetic severity. From the results showing that there is a very good correlation between HbA1c and glycosylated LDL apo B-26, we have shown that LDL apo B-26 or LDL cholesterol could also be used as an alternative to using HbA1c values.

CONCLUSIONS

A reliable and rapid method for measuring the level of glycosylation of proteins was developed by modifying an existing method. Employing this method, the degree of glycosylation of VLDL, LDL, and apo B subspecies were compared between normal subjects and patients with diabetes mellitus. The levels of glycosylation of LDL, LDL apo B-100 + B-74, and LDL apo B-26 were found to be increased significantly in the diabetics. There was a good linear correlation between glycosylated LDL apo B-26 and glycosylated LDL, which in turn correlated well with the level of LDL cholesterol. Glycosylated LDL apo B-26 was also found to be in excellent linear relationship with hemoglobin A1c and was most significant among the glycosylated apo B subspecies.

REFERENCES

Agarwal KC, Parks RE Jr, Widness JA, Schwartz R: Nonenzymatic glycosylation of erythrocytic proteins in normal and diabetic subject. *Diabetes 34: 251-255, 1985*

Allen RC, Stastny M, Hallett D, Summons MA: A comparison of isoelectric focusing and electrochromatography for the separation and quantitation of hemoglobin Alc hemoglobin, in *Electrophoresis 1979.* Radola BJ ed. New York, W. deGruyter, 1980, pp 663-670

Bannon P, Lessard F, Lepage R, Joly JG, Dufresne L: Glycated hemoglobin in uremic patients as measured by affinity and ion-exchange chromatography. *Clin Chem 30: 485-486, 1974*

Biggs HG, Erickson JM, Moorehead WR: A manual colorimetric assay of triglycerides in serum. *Clin Chem 21: 437-441, 1975*

Bisse E, Berger W, Fluckiger R: Quantitation of glycosylated hemoglobin: Elimination of labile glycohemoglobin during sample hemolysis at pH 5. *Diabetes 31: 630-633, 1982*

Blue M-L, Protter AA, Williams DL: Biosynthesis of apolipoprotein B in rooster kidney, intestine, and liver. *J Biol Chem 255: 10048-10051, 1980*

Brown MS, Basu SK, Palck JR, Ho YK, Goldstein JL: The scavenger cell pathway for lipoprotein degradation. *J Superamol Struct 13: 67-81, 1980*

Brown MS, Goldstein JL: How LDL receptors influence cholesterol and atherosclerosis. Sci Am 251: 52-60, 1984

Brown MS, Goldstein JL, Krieger M, Ho YK, Anderson RGW: Reversible accumulation of cholesterol esters in macrophages incubated with acetylated lipoproteins. *J Cell Biol 82: 497-613, 1977*

Bunn HF, Haney DN, Gabbay KH, Gallop PM: Further iden-

tification of the nature and linkage of the carbohydrate in hemoglobin A1c. *Biochem Biophys Res Commun 67: 103-109, 1975*

Cerami A, Koenig RJ: Hemoglobin A1c as a model for the development of the sequelae of diabetes mellitus. *Trends Biochem Sci 3:73-75, 1978*

Christy JH, Clements SD: The heart and endocrine disease: *The heart.* Hurst JW ed. New York, McGraw Hill, *1982, pp 1547-1567*

Crall FV Jr, Roberts WC: The extramural and intramural coronary arteries in juvenile diabetes mellitus. *Am J Med 64: 221-230, 1978*

Davies JE, Stark JR: Use of dimethylsuberimidate, a cross-linking reagent, in studyng the subunit structure of oligomeric proteins. *Proc Natl Acad Sci USA 66: 651-656, 1970*

Dolhofer R, Stadele A, Wieland OH: Clinical and biochemical studies on the significance and formation of hemoglobins A1c and A1a+b in diabetes mellitus. *Klin Wochenscher 55: 945-954, 1977*

Dolhofer R, Wieland OH: Increase glycosylation of serum albumin in diabetes mellitus. *Diabetes 29: 417-422, 1980*

Feeney RE, Clary JJ, Clark JR: A reaction between glucose and egg white proteins in incubated eggs. *Nature 201: 192-193, 1964*

Fluckiger R, Winterhalter KH: In vitro synthesis of hemoglobin A1c. *FEBS Lett 71: 356-360, 1976*

Fluckiger R, Woodtli T, Berger W: Quantitation of glycosylated hemoglobin by boronate affinity chromatography. *Diabetes 33: 73-76, 1984*

Fogelman AM, Shechter I, Seager J, Hokom N, Child JS, Edwards PA: Malondialdehyde alteration in low density lipoproteins leads to cholesterol ester accumulation in human monocyte-macrophages. *Proc Natl Acad Sci USA 72: 2214-2218, 1980*

Gabbay KH, Hasty K, Breslow JL, Ellison RC, Bunn HF, Gallop PM: Glycosylated hemoglobins and long-term blood glucose control in diabetes mellitus. *J Clin Endocrinol Metab 44: 859-864, 1977*

Goldstein JL, Brown MS: Binding and degradation of low density lipoproteins in cultured human fibroblasts. *J Biol Chem 249: 5153-5162, 1974*

Goldstein JL, Brown MS: The low density lipoprotein pathway and its relation to atherosclerosis. *Annu Rev Biochem 46: 897-930, 1977*

Gonen B, Baenziger J, Schonfeld G, Jacobson D, Farrar P: Nonenzymatic glycosylation of low density lipoproteins in vitro: Effects on cell-interactive properties. *Diabetes 30: 875-878, 1981.*

Gonen B, Cole T, Hahm KS: The interaction of carbamylated low density lipoprotein with cultured cells. *Biochim Biophys Acta 754: 201-207, 1983*

Gottschalk A: *Glycoproteins.* Gottschalk A ed. New York, Elsevier Publishing Company, *1972, pp 141-157*

Hahm K-S, Lee YS, Lee NJ: Study on the distribution and synthesis of apolipoprotein B subspecies in rat lymph chylomicrons and plasma lipoproteins. *Korean Biochem J 18: 16-20, 1985*

Hahm K-S, Tikkanen MJ, Dargar R, Cole TG, Davie JM, Schonfeld G: Limited proteolysis selectively destroys epitopes on apolipoprotein B in low density lipoproteins. *J Lipid Res 24: 877-885, 1983*

Kane JP, Hardman DA, Paulus HE: Heterogeneity of apolipoprotein B: Isolation of a new species from human chylomicrons. *Proc Natl Acad Sci USA 77: 2465-2469, 1980*

Kim H-J, Kurup IV: Nonenzymatic glycosylation of human plasma low density lipoprotein: Evidence for in vitro and in vivo glycosylation. *Metabolism 31: 348-353, 1982*

Leffler HH: Estimation of cholesterol in serum. *AM J Clin Pathol 31: 310-313, 1959*

Lock DR, Hockenberry D, Cuningham J, Hahm K-S, Kantor O, Schonfeld G: Apolipoprotein B subspecies in chylomicrons isolated from a patient with chyluria. *Am J Med 75: 360-364, 1983*

Lowry OH, Resebrough NJ, Farr AL, Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem 193: 265-275, 1951*

Mahley RW, Innerarity TL, Weisgraber KH, Oh SY: Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective chemical modification of lysine residues of the apoproteins. *J Clin Invest 64: 743-750, 1979*

Mohammad A, Fraquenkel-Conrat H, Olcott HS: The browning reaction of proteins with glucose. *Arch Biochem 24: 157-178, 1949*

Monnier VM, Cerami A: Detection of nonenzymatic browning products in the human lens. *Biochim Biophys Acta 760: 97-103, 1983*

Ney KA, Colley KH, Pizzo SV: The standardization of the thiobarbituric acid assay for nonenzymatic glucosylation of human serum albumin. *Anal Biochem 118: 294-300, 1984*

Pecoraro RE, Graf RJ, Halter JB, Beiter H, Porte D Jr: Comparison of a colorimetric assay for glycosylated hemoglobin with ion exchange chromatography. *Diabetes 28: 1120-1125, 1979*

Sasaki J, Cottam GL: Glycosylation of human LDL and its metabolism in human skin fibroblasts. *Biochem Biophys Res Commun 104: 977-983, 1982*

Schleicher E, Deufel T, Wieland OH: Nonenzymatic glycosylation of human serum lipoproteins: Elevated ε-lysine glycosylated low density lipoprotein in diabetic patients. *FEBS Lett 129: 1-4, 1981*

Schleicher E, Wieland OH: Specific quantitation by HPLC of protein (lysine) bound glucose in human serum albumin and other glycosylated proteins. *J Clin Chem Clin Biochem 19: 81-87, 1981*

Schonfeld G, Lees RS, George PK, Pfleger B: Assay of total

plasma apolipoprotein B concentration in human subjects. *J Clin Invest 53: 1458-1467, 1974*

Spicer KM, Allen RC, Buse MG: A simplified assay of *hemoglobin A1c in diabetic patients by use of isoelectric* focusing and quantitative microdensitometry. *Diabetes 27: 383-388, 1978*

Tikkanen MJ, Cole TG, Hahm K-S, Krul ES, Schonfeld G: Expression of apolipoprotein B epitopes in very low density lipoprotein subfractions: Studies with monoclonal antibodies. *Arteriosclerosis 4: 138-146, 1984*

Weisgraber KH, Innerarity TL, Mahley RW: Role of lysine residues of plasma lipoproteins in high affinity binding to cell surface receptors on human fibroblasts. *J Biol Chem 253: 9053-9062, 1978*

Witztum JL, Mahoney EM, Branks MJ, Fisher M, Elam R, Steinberg D: Nonenzymatic glucosylation of low-density lipoprotein alters its biologic activity. *Diabetes 31: 283-291, 1982*