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An improved enzymatic assay for glycated serum protein

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Recent studies have shown that blood HbA1c levels alone may not accurately reflect serum glucose concentrations in all diabetic patients. For certain diabetic patients, there exists a glycation gap. It was reported that the glycation gap information obtained by measuring HbA1c and glycated serum protein (GSP) or glycated albumin (GA) together may improve evaluation of diabetic patients by more reliably predicting complications of diabetes than HbA1c alone. Therefore, a new GSP assay for clinical use was developed and its performance was evaluated. Diazyme's enzymatic GSP assay (trademarked GlycoGap®) is formulated with a 2-part liquid stable reagent system with a shelf-life of >15 months when stored at 2–8 °C. The assay was highly reproducible with within-run and total imprecisions of ≤1.3% CV. Method comparison studies showed good correlations with a previous powder version GSP assay ($r^2 = 0.9966$) and with Lucica® GA-L assay ($r^2 = 0.9746$). The assay was linear within the range of 21–1354 μmol L^{-1} with a reference range of 151–300 μmol L^{-1} and was not affected by substances commonly found in human specimens such as ascorbic acid, bilirubin, hemoglobin, glucose, triglycerides, or uric acid. Diazyme's GSP assay was highly accurate with no interferences from endogenous reducing substances which interfere strongly with the traditional NBT based fructosamine assay. A conversion equation was developed to allow conversions of GSP values (μmol L^{-1}) into % of GA values, and a reference range for %GA was established for the US population. The relationship between %GA and %HbA1c was also investigated by measuring both %GA and %HbA1c values of blood samples from both diabetic and non-diabetic donors.

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Introduction

The incidence of hyperglycemia is rising at an alarming rate, with the number of adults with diabetes worldwide having more than doubled over the past 30 years.[1] The number of adults with diabetes increased from 153 million in 1980 to 347 million in 2008.[1] The number further increased to 371 million in 2011 according to the International Diabetes Federation (IDF). Diabetes and its complications are imposing a severe economic burden on individuals, families, and national health systems. In 2007, diabetes and pre-diabetes related medical costs in the United States were estimated to have reached $218 billion that accounts for 10% of the total US healthcare spending for the year.[2] In 2012, the total cost increased to $245 billion according to the American Diabetes Association (www.diabetes.org). Swift action is needed to slow down the current trend, and any improvements in diabetic control, both in diagnosis and treatment, will be of significant impact to patients as well as to the reduction of the national economic burden.

In recent years, progress has been made in the improvement of clinical diagnosis of diabetes. In 2010, the American Diabetes Association (ADA) introduced HbA1c as a new diagnostic marker for diagnosis and monitoring of diabetes.[3] Though HbA1c is an effective indicator that was proven to predict the complications of diabetes clinically through randomized clinical trials including the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS),[4,5] some studies have shown that blood HbA1c levels alone may not accurately reflect serum glucose concentrations in all diabetic patients.[6–11] In a small percentage of the population, HbA1c levels do not accurately correlate with glucose levels and there exists a glycation gap, a gap between the measured HbA1c level and the HbA1c level predicted by the glycated serum protein concentration. Misdiagnosis can happen if HbA1c alone is used for diagnosis or screening of this group of the population.[7] In addition, HbA1c is not reliable for certain conditions that change the lifespan of red blood cells, such as hemolytic anemia, gestational diabetes, and renal dialysis.[8–11] Additional markers or tests are needed to improve the accuracy and reliability of HbA1c based diagnosis.

Glycated serum protein (GSP) or glycated albumin (GA) test has been recommended as a complementary test to HbA1c.[12,13] GSP or GA test is used as a short-term to medium-term index for glycemic control for average blood glucose levels over the past 2–3 weeks period in contrast to the HbA1c test that is used as a long term index for average glucose levels over the past 2–3 months period.[12,13] Glucose measurements, on the other hand, only provide transient glucose concentrations.

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In the literature, glycated serum protein is also known as fructosamine.[14,15] Fructosamine is traditionally measured by a non-specific chemical method using nitroblue tetrazolium (NBT) that is interfered by various reducing substances in the samples.[16,17] A more specific fructosamine assay was developed by Genzyme Diagnostics using an enzymatic method,[18] and the assay is referred to as GSP with a trademark of GlyPro®. GlyPro® is formulated in a lyophilized powder format, and reconstitution of reagents before use is required, which is a less user-friendly feature. A GA assay developed by Asahi Kasei Pharma Corporation, Lucica® GA-L assay, measures the percentage of glycated albumin to total albumin.[19] Recently, Diazyme Laboratories has developed a new enzymatic GSP assay (trademarked GlycoGap®) for clinical laboratory determination of GSP or GA that is formulated with ready-to-use liquid stable reagents. In this article, the performance characteristics of the Diazyme GSP assay are presented and its utility as a medium-term glycemic control, reference values in healthy population, and its relationship with HbA1c are discussed.

Materials and methods

Reagents

The Diazyme GSP assay consists of two reagents, R1 and R2. All reagents were prepared from analytical grade chemicals from Sigma Chemical Company, St. Louis, MO, unless otherwise indicated. R1 contained 25 mM Tris–HCl, pH 8.0, 4 mM 4-aminoantipyrine (4-AAP), 30 kU per L *Tritirachium* sp. proteases (Genzyme, Kent, UK), 4 mM of oxidizing agent sodium metavanadate ($NaVO_3$), and stabilizers (10 mM methyl-β-cyclodextrin, 2% trehalose). R2 contained 40 mM Tris–HCl, pH 8.65, 50 kU per L of fructosyl valine oxidase enzyme (Fructosaminase™ from Diazyme), 200 kU per L of peroxidase (Toyobo, Osaka, Japan), 10 mM of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline, sodium salt, dihydrate (TOOS, Dojindo, Kumamoto, Japan), 1 mM of redox agent ($K_4Fe(CN)_6$) and stabilizers (glycerol, 8% and sorbitol, 15%).

Assay principle

The Diazyme GSP assay uses a specific protease to digest GSP into low molecular weight glycated protein fragments (GPF) and uses Diazyme's specific Fructosaminase™, a microorganism-originated amadoriase, to catalyze the oxidative reaction of the Amadori product from GPF to yield protein fragments (PF) or amino acids, glucosone and H_2O_2. H_2O_2 released is measured by a colorimetric Trinder reaction. The absorbance generated at 546 nm is proportional to the concentration of GSP in the sample as indicated in the scheme below.

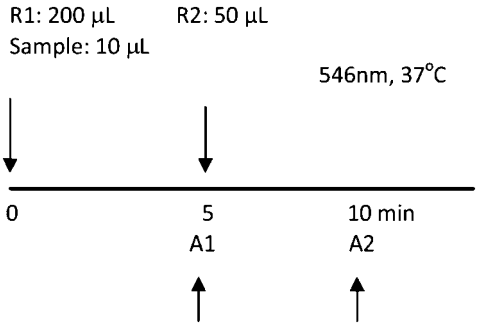
$$\text{GSP} \xrightarrow{\text{protease}} \text{glycated protein fragments(GPF)}$$

$$\text{GPF} \xrightarrow{\text{Fructosaminase™}} \text{PF or amino acids} + H_2O_2$$

$$H_2O_2 + \text{TOOS} + \text{4-AAP} \xrightarrow{\text{peroxidase}} \text{color} + H_2O$$

Assay procedure

An automated assay of Diazyme GSP was performed with a Hitachi 917 automatic clinical analyzer (Hitachi, Tokyo, Japan). The test procedure is as follows: 10 μL of serum is mixed with 200 μL of R1 and the reaction mixture is incubated at 37 °C for 5 min. Then, the first absorbance at 546 nm is measured followed by addition of 50 μL of R2. After an additional 5 min of incubation at 37 °C another absorbance measurement is taken. The difference of the two absorbance measurements (end point) is used for GSP calculation. The assay procedure is schematically depicted below.



Calibrators and controls

GSP calibrator and control materials were prepared from bovine serum albumin (Equitech Bio, Kerrville, TX). Original bovine serum albumin stock and glucose incubated stock were dialyzed and then mixed to obtain target GSP values. After addition of phosphate buffer saline and stabilizers, the calibrator and control materials were aliquoted into vials and lyophilized. Prior to usage, the vials were reconstituted with 1.0 mL sterile distilled water and allowed to equilibrate at room temperature for 30 minutes. The calibrator value was assigned based on replicate analysis using Diazyme GSP assay reagents, control materials, and patient samples with known GSP values assigned with a currently marketed fructosamine assay (Randox, Antrim, UK). Randox fructosamine assay was calibrated relative to human serum glycated with ^{14}C-glucose.

Samples

The pooled and the individual serum samples were obtained from human donors in conjunction with certified commercial source (BioChemed, Winchester, VA and ProMedDx, Norton, MA) with Institutional Review Board (IRB) certification on the handling and informed consent protocols.

Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) were determined according to the CLSI EP17-A guideline.[20] To determine the LOD, a true blank sample was tested with 20 replicates daily for three days and five diluted serum samples were tested with 4 replicates daily for three days. The LOD was calculated as the average of 3rd and 4th highest obtained true blank values plus 1.645 times standard deviation of five diluted samples. The LOQ was determined as the lowest concentration

for which CV is less than 20%. Five diluted serum samples were tested on five separate runs with eight replicates per run, and data were analyzed for LOQ.

Linearity

The linearity test was performed according to Clinical and Laboratory Standards Institute (CLSI) EP6-A.[21] Nine levels of linearity samples were prepared by diluting the spiked serum sample containing 1579 μmol L^{-1} GSP with saline.

Precision

The assay precision was evaluated according to the CLSI EP5-A guideline.[22] Two controls and two levels of the serum specimen were tested two runs per day in duplicate over 20 working days. Precision was calculated using the following formula: within-run precision $(S_r) = [((1) + (2))/4I]^{1/2}$, and within-laboratory precision $(S_T) = [(2B^2 + A^2 + S_r^2)/2]^{1/2}$, where I = number of days, $A = [(3)/2I]^{1/2}$, B = standard deviation of "Daily Means", and the numbers of (1) and (2) are sum of squares of difference between duplicates from run 1 and run 2, respectively, and the number (3) is the sum of squares of difference between means of run 1 and run 2.

Method comparison

The method comparison studies followed the CLSI EP9-A2.[23] A total of 65 serum samples were tested with both the Diazyme GSP assay kit and with the previous lyophilized GSP assay kit which is a legally marketed GSP assay product in the market. For the study, two samples were diluted with saline and four samples were spiked with fructosyl propylamine to ensure distribution across the analytical measurement range. All the results were reported in μmol L^{-1}.

The assay was also compared with the Lucica® GA-L assay (Asahi Kasei Corporation, Japan) with all results reported in % of glycated albumin (%GA). For this comparison, GSP values were determined with Diazyme GSP assay and total albumin values with the bromcresol green (BCG) method. The GSP values obtained were converted to %GA by using a conversion equation:

$$\%GA = \frac{\text{GSP } (\mu\text{mol L}^{-1}) \times 0.182 + 1.97}{\text{total albumin } (\text{g dL}^{-1})} + 2.9$$

Interference

The interference of common substances found in human serum was tested using chemicals from Sigma Chemical Company, St. Louis, MO, unless otherwise indicated. The substances tested were: ascorbic acid, bilirubin free (Frontier Scientific, Logan, UT), bilirubin conjugated (Frontier Scientific, Logan, UT), triglycerides (Indofine Chemicals, Hillsborough, NJ), glucose, uric acid, and hemoglobin. The interference test was performed in triplicate on two serum samples, one with low GSP and the other with high GSP concentrations, which were spiked with various concentrations of interfering substances following the CLSI EP7-A guideline.[24] For each substance, serum samples were spiked with an interfering substance to obtain samples with low (L) and high (H) concentrations of the interference substance. The low and high interference samples were then mixed to obtain five interference levels: C_1 = L, C_2 = (3L + H)/4, C_3 = (L + H)/2, C_4 = (L + 3H)/4 and C_5 = H. The tolerance limit for each substance was determined to be the concentration at which the interfering substance causes 10% deviation of the GSP value from the true value of the specimen.

Stability

Shelf-life of the Diazyme GSP assay reagents was determined by accelerated stability and real time stability studies on two lots of reagents, calibrators, and controls. For accelerated stability, reagents were incubated at 37 °C and removed for testing on specific days. For real time stability, reagents were stored at 2–8 °C and tested on specific months. Two levels of GSP controls and two serum samples with normal and above normal level of GSP were tested. The recovered values were compared to those collected on month 0 with reagents and calibrator stored at 2–8 °C.

For stability of the calibrator and controls, the lyophilized vials were incubated at 37 °C and removed for testing on specific days. For real time stability, the lyophilized vials were stored at 2–8 °C and tested on specific months. The recovered values from incubated vials were compared to those stored at 2–8 °C collected on month 0.

The calibration frequency was determined by comparing recovery of controls and one sample when tested with calibration on day zero *versus* without calibration on subsequent testing days. For reagent on-board stability on the Hitachi 917 system, one lot of reagent was stored on board for the length of the study. Recovered values of controls and one sample were compared between day zero and subsequent testing days. To determine open vial stability, the obtained calibrators and control values from the day of reconstitution were compared to those of subsequent testing days.

Reference interval

Blood samples from 130 apparently healthy donors (125 African American and 5 Caucasian, 21 female and 109 male, age range from 19 to 65 years old) were tested for GSP and HbA1c using the Diazyme GSP assay and Diazyme Direct Enzymatic HbA1c assay, as well as the Roche fructosamine assay (Roche Diagnostics, Basle, Switzerland). The GSP reference interval was determined using samples with HbA1c values <6.5% according to the CLSI C28-A method provided by EP8 Evaluator. The reference range for %GA was obtained from the same population of samples after converting GSP values into % of GA values with the conversion equation.

Comparison between HbA1c and GSP or %GA

Blood samples from 200 donors of diabetic and non-diabetic patients were tested for GSP or GA, and HbA1c using Diazyme GSP assay and Diazyme Direct Enzymatic HbA1c assay (Diazyme, Poway, USA). Obtained GSP or GA values were plotted against the corresponding HbA1c values.

Statistical analysis

Statistical analyses were done with Excel 2003 (Microsoft, Redmond, WA) unless otherwise indicated. The limit of quantitation, linearity and reference interval analyses were performed using EP Evaluator Version 8 Software (Data Innovations, South Burlington, VT). The Bland–Altman plot in the method comparison was obtained using MedCalc for Windows, version 5.00.020 (MedCalc Software, Ostend, Belgium).

Results

Assay development

The old version of GSP assay was developed with a lyophilized powder format. Users had to reconstitute the powders before use. The reconstituted reagents were stable only for 2 weeks, which was not a user-friendly format in clinical settings. In order to overcome this drawback of the GSP assay reagent, a systematic optimization study was conducted to identify the thermo liable components in the reagents, and an extensive search for stabilizers that can prevent thermo liable components from degradation in the liquid state was performed. It was found that the major unstable components were the oxidizing agent, $NaVO_3$ in R1, and the enzyme Fructosaminase™ in R2. Various chemicals were tested to search for compounds that have protective effects towards $NaVO_3$. Among the chemicals tested, it was found that methyl-β-cyclodextrin, a cyclic oligosaccharide, was the best in preventing $NaVO_3$ from degradation in the liquid state. When 10 mM of methyl-β-cyclodextrin was added to R1 that contains 4 mM of $NaVO_3$, the R1 reagent became stable in liquid for more than 15 months when stored at 2–8 °C. This protective effect may be attributed to the special toroidal structure of methyl-β-cyclodextrin molecules that effectively trap small molecules such as $NaVO_3$ inside their rings. On the other hand, the enzyme Fructosaminase™ was stabilized by addition of polyols and sugars into the R2 reagent. When glycerol of 8% and sorbitol of 15% were included in the R2 solution, the reagent became stable for more than 15 months when stored at 2–8 °C. A typical reaction curve of the liquid stable GSP reagents is shown in Fig. 1.

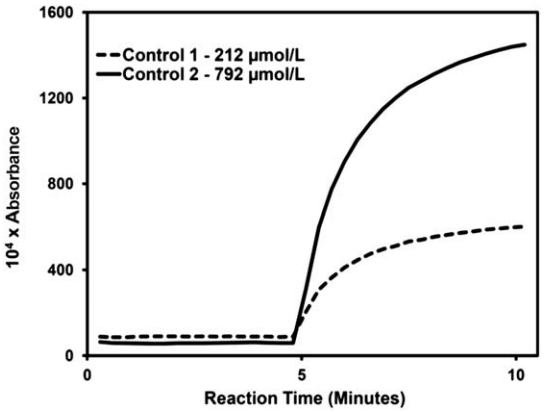


Fig. 1 Typical reaction curves of the GlycoGap® assay. Reagent 1 (R1, 200 µL) and serum sample (10 µL) were added to the reaction cuvette of a Hitachi 917 analyzer, and incubated for 5 min at 37 °C before addition of 50 µL of R2. The absorbance was monitored at 546 nm, and the absorbance difference ($A_2 - A_1$, where A_1 is the absorbance just before addition of R2, and A_2 is the absorbance 5 min after addition of R2) was used for calculation of the GSP concentration. Dashed line (---): GSP control of 212 µmol L^{-1}; solid line (—): GSP control of 792 µmol L^{-1}.

Sensitivity

The sensitivity study found that the limit of detection (LOD) was 7.2 µmol L^{-1} and the limit of quantification (LOQ) was 13 µmol L^{-1}, which was a better sensitivity than that of the powder version of the GSP assay reagent.

Linearity

Clinical linearity analysis performed with EP Evaluator showed that the assay is linear within the analytical measurement range of 21.0–1354.0 µmol L^{-1} with an allowable systematic error of 3.5%. The scatter and residual plots are shown in Fig. 2 and 3. The standard deviation of the residual was 11.4, and the bias between expected and recovered values was <10%.

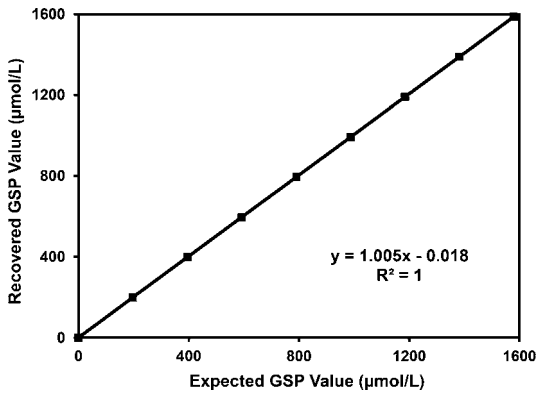


Fig. 2 Linearity scatter plot of the GlycoGap® assay. Nine levels of linearity samples were prepared by diluting a high concentration GSP serum sample (1579 µmol L^{-1}) with saline, and their GSP values were measured in triplicate. The mean values obtained were plotted against the expected GSP values.

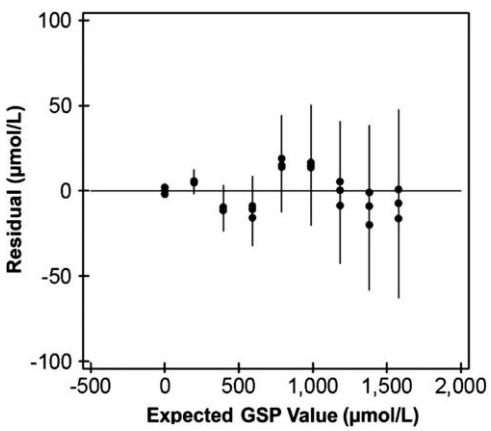


Fig. 3 Linearity residual plot of the GlycoGap® assay. The average values of the nine levels of linearity samples were plotted against the target values at each linearity level.

Table 1 Within-run precision and total precision of the Diazyme GSP assay. Two controls and two levels of the serum specimen were tested two times per day over 20 working days. Precisions were calculated using the formulas described in the materials and methods section

Specimen	*N*	Mean (μmol L^{-1})	Within-run precision SD	Within-run precision CV	Total precision SD	Total precision CV
Control 1	80	204	2.20	1.1%	2.40	1.2%
Control 2	80	751	4.90	0.7%	5.60	0.7%
Sample 1	80	251	1.90	0.8%	3.20	1.3%
Sample 2	80	373	2.40	0.6%	3.70	1.0%

Precision

The precision of the Diazyme GSP assay is summarized in Table 1. For two GSP controls and two levels of the serum specimen, 20 day reproducibility data showed that the within-run imprecision was from 0.6% to 1.1% and the within-laboratory imprecision was from 0.7% to 1.3%.

Method comparison

Method comparison studies indicated excellent accuracy with a slope of 0.954, an intercept of 14.57 and a correlation coefficient of 0.997. Sample bias between methods was ≤10%. The correlation between the results is shown in Fig. 4. A Bland–Altman analysis was also conducted as shown in Fig. 5.

A method comparison between GlycoGap® and Lucica® GA-L assay was also performed (Fig. 6). There was an excellent correlation with $r^2 = 0.975$ and an intercept value of 0.131%.

Interference

Table 2 shows the effects of common serum interfering substances on the Diazyme GSP assay. There were no significant interferences (≤10% deviation) up to the indicated concentrations which are at least five times higher than their physiological concentrations found in samples.

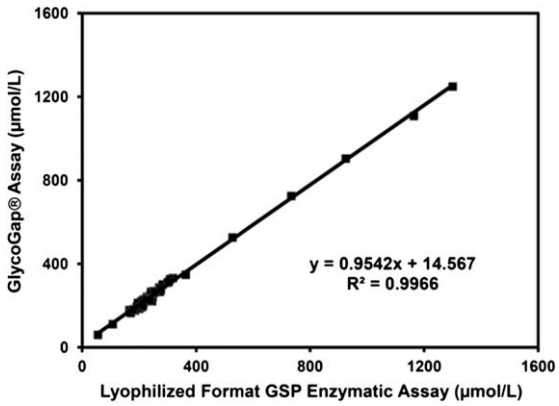


Fig. 4 Correlation between GSP values obtained with GlycoGap® and results obtained with the lyophilized GSP method. Sixty-five serum samples were tested for GSP values with both the liquid stable Diazyme GSP assay and the previous lyophilized powder version of the GSP test method. The results were compared according to EP9-A to obtain the correlation coefficient between the two methods.

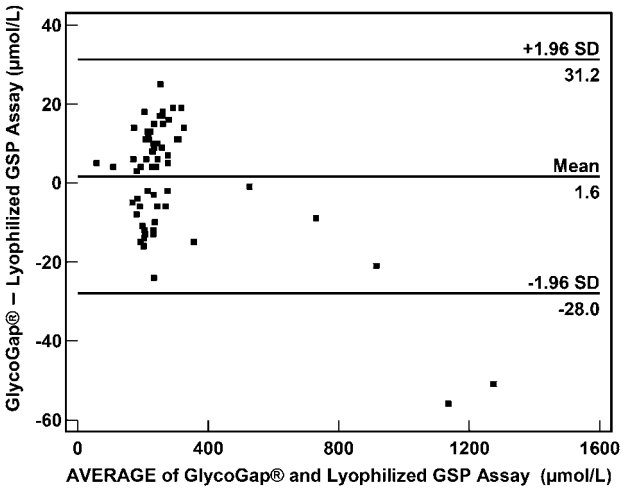


Fig. 5 Bland–Altman plot comparing GSP values obtained by the GlycoGap® method and the lyophilized GSP method. Sixty-five serum samples were measured for GSP values with both the liquid stable Diazyme GSP assay method and the previous lyophilized powder version of GSP assay method. The results were plotted according to Bland–Altman to evaluate the agreement between the two methods.

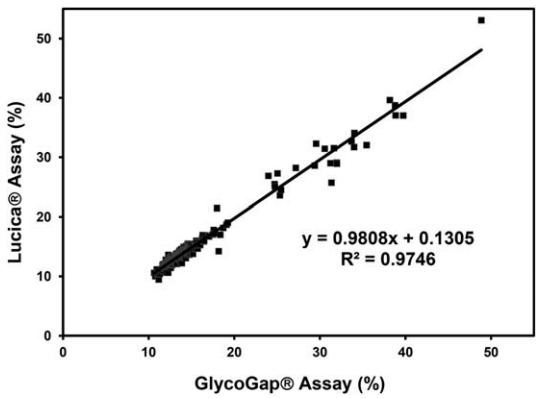


Fig. 6 Method comparison between the GlycoGap® assay (%GA calculated) and Lucica® GA-L assay. Serum samples (315) were tested for %GA values with both the liquid stable Diazyme GSP assay method and Lucia® GA-L assay method. The %GA results obtained were compared according to EP9-A to obtain the correlation coefficient between the two methods.

Stability

Table 3 summarizes the results from an accelerated reagent stability study where Diazyme GSP assay kits were stored at both 37 °C and 4 °C. The remaining reagent reactivity (Δ absorbance at 540 μmol L^{-1} of GSP) was monitored and used for predicting the shelf-life at 4 °C using Arrhenius law based stress model. After 11 days of heat stress, the liquid reagent retained approximately 90% of its reactivity or reaction window compared to the reactivity measured at day zero of the heat stress study (Table 3). The liquid stable Diazyme GSP assay reagent was predicted to have at least 15 months of shelf-life if the reagents (kits) were kept at 2–8 °C. The real time data shown in Table 4 confirmed that stability of the reagent was up to 19 months. The on-board reagent stability was determined to be at

Table 2 Effects of interfering substances on the Diazyme GSP assay. The interference test was performed in triplicate on two serum samples, one with low GSP and the other with high GSP concentrations, which were spiked with various concentrations of interfering substances according to the procedure described in the CLSI EP7-A guideline.[24] The concentrations of interfering substances indicated in the table are concentrations at which the interfering substances did not cause deviations of GSP values for more than 10% of the original value

Interfering substance	Concentration (mg dL^{-1})
Ascorbic acid	5
Bilirubin	15
Bilirubin conjugated	15
Triglyceride	2000
Glucose	2400
Uric acid	35
Hemoglobin	200

Table 3 Accelerated stability of the Diazyme GSP assay reagent. Two controls and two serum samples were tested with two lots of Diazyme GSP assay reagents that were incubated at 37 °C for indicated days. The reagent stability was monitored by measuring the reactivity or Δ of absorbance changes at the 540 μmol L^{-1} level of GSP as well as by measuring the recoveries of control and sample values. The remaining reactivity values after an indicated period of incubation time at 37 °C were used for prediction of the shelf-life at 4 °C. Data were the average of duplicated tests

		Obtained value after incubation at 37 °C (μmol L^{-1})					
	Reagent lot	Day 0	Day 3	Day 5	Day 7	Day 9	Day 11
Reactivity	1	0.0797	0.0793	0.0772	0.0756	0.0740	0.0732
(Δ O.D.)	2	0.0757	0.0745	0.0732	0.0707	0.0697	0.0680
Control 1	1	217	224	227	224	229	227
	2	225	232	231	227	233	236
Control 2	1	729	727	737	733	740	739
	2	739	735	736	729	738	740
Sample 1	1	254	255	254	252	260	254
	2	251	247	246	247	246	244
Sample 2	1	528	527	532	531	533	533
	2	530	529	532	528	529	527

Table 4 Real time stability of the Diazyme GSP assay. Two controls and two serum samples were tested with two lots of Diazyme GSP assay reagents that were stored at 2–8 °C for the indicated time periods. Controls and samples were tested at each time point, and the values were compared with the values obtained on month zero. The reagents were claimed to be stable when the values recovered had less than 10% of deviations from the values obtained at the month zero time point. Data were the average of duplicated tests

		Obtained value (μmol L^{-1})					
Specimen	Reagent lot	Month 0	Month 6	Month 9	Month 12	Month 15	Month 19
Control 1	1	217	204	207	218	204	218
	2	225	208	214	218	210	226
Control 2	1	729	715	714	737	728	726
	2	739	722	719	737	727	731
Sample 1	1	528	526	529	529	528	535
	2	530	533	535	529	540	546
Sample 2	1	254	243	253	249	246	255
	2	251	241	254	252	248	249

Table 5 On-board stability of the Diazyme GSP assay reagent. Two controls and one serum sample were tested with Diazyme GSP assay reagents that were kept on the Hitachi 917 instrument with open bottles. At each indicated time point, the controls and the sample were tested for their GSP values after calibration with the same calibrator set. Data were the average of duplicated tests

	Obtained value (μmol L^{-1})				
Specimen	Day 0	Day 7	Day 14	Day 21	Day 28
Control 1	203	202	206	202	206
Control 2	753	740	738	736	745
Sample 1	274	271	274	269	272

Table 6 Calibration frequency of the Diazyme GSP assay. Two controls and one serum sample were tested with Diazyme GSP assay reagents that were kept at 2–8 °C. The calibration curve was established at day zero, and was used for all subsequent data collections at each indicated time point. Data were the average of duplicated tests

	Obtained value after initial calibration (μmol L^{-1})				
Specimen	Day 0	Day 3	Day 5	Day 7	Day 14
Control 1	200	196	188	187	180
Control 2	744	743	732	716	711
Sample 1	265	258	256	256	252

least 4 weeks when the reagents were kept on a Hitachi 917 as indicated in Table 5. The calibration frequency was determined to be one week on a Hitachi 917 analyzer as indicated in Table 6. On the other hand, stabilities of calibrators and controls were also investigated, and it was found that there was only less than 5% deviation after 11 days of heat stress at 37 °C for calibrator and controls, and both calibrator and controls were stable for 19 months at 2–8 °C according to the real time stability study (data not shown).

Reference interval

Reference intervals for the Diazyme GSP assay are shown in Fig. 7 and 8. GSP values, %HbA1c, and Roche fructosamine values were determined for 130 blood samples from apparently healthy patients. A total of six samples out of 130 samples were excluded from the analysis. Five samples were excluded because of high HbA1c values (>6.5%). One sample was excluded because of a high fructosamine value (369 μmol L^{-1}) and a high GSP value (539 μmol L^{-1}), even though its HbA1c value was only 5.7%. Nonparametric (CLSI C28-A) 95% confidence interval for GSP was found to be 151–300 μmol L^{-1} (Fig. 7), and was 10.4–15.7% for %GA (Fig. 8).

Comparison between HbA1c and GSP or GA

The correlation studies between GSP, GA and HbA1c were performed by testing 200 samples. Results of 118 samples were in the non-diabetic range with HbA1c <5.7%, 37 samples were at

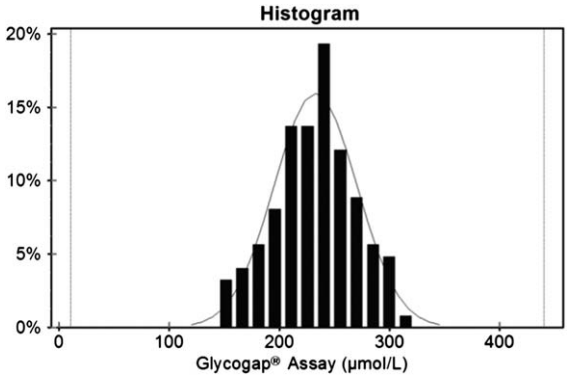


Fig. 7 Histogram for the GlycoGap® assay GSP reference interval. One hundred and twenty four (124) serum samples were tested for GSP levels and the results were analyzed for reference interval using the CLSI C28-A method provided by EP8 Evaluator software. Each black bar represents the percentage of samples within the corresponding GSP value range.

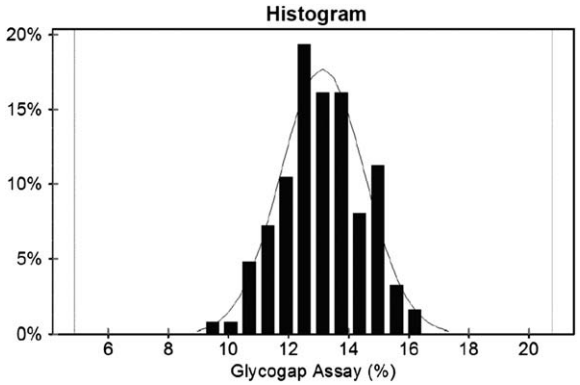


Fig. 8 Histogram for the GlycoGap® assay %GA reference interval. One hundred and twenty four (124) serum samples were tested for %GA levels and the results were analyzed for reference interval using the CLSI C28-A method provided by EP8 Evaluator software. Each black bar represents the percentage of samples within the corresponding %GA value range.

risk for developing diabetes with HbA1c values ranging from 5.7% to 6.4%, and 45 samples were in the diabetic range with HbA1c values ≥6.5%. The correlation coefficient between GSP and HbA1c was found to be 0.79. The correlation coefficient (r^2) between HbA1c and %GA was found to be 0.824 as shown in Fig. 9.

Discussion

HbA1c and glucose testing are the current standard in glycemic monitoring. However, the HbA1c value does not properly represent glycemic status in patients with renal failure,[25] red blood cell disorder,[26] hemoglobinopathy[27] and obesity.[28] In addition, caution in HbA1c interpretation has been raised due to evidence of mismatches between HbA1c and other measures of glycemia,[29] clinical variability such as in the ability to predict macrovascular disease and biological variability[30] such as age and genetics.[31–33] On the other hand, the fluctuations of glucose necessitate frequent testing. To address the need for better tools

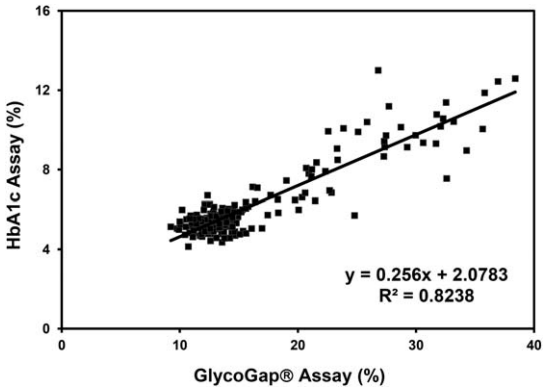


Fig. 9 Correlation between %HbA1c and %GA values measured by the Direct Enzymatic HbA1c method and by the GlycoGap® method, respectively. Two hundred (200) paired patient samples (serum and whole blood) from both diabetic and non-diabetic subjects were tested for both %GA and for %HbA1c concentrations. The values of %GA (*x*-axis) and %HbA1c (*y*-axis) from each patient were plotted, and linear regression was used for obtaining the correlation coefficient (r^2).

for glycemic monitoring, and to fill the discrepancy between HbA1c and other measures of glycemia, as well as for effectiveness of diet or medication adjustments, GSP or GA test has been recommended as a useful test complementary to HbA1c and glucose tests.[12,13,25]

The Diazyme GSP assay demonstrated an excellent assay precision having the within and total CV% values of less than 2% in both high and low GSP levels. It gave a wide dynamic range with an assay linearity ranging from 21 to 1354 µmol L^{-1} which fully covers the normal and disease conditions.

The assay correlated well with previously reported assay, a lyophilized powder form GSP assay, with an r^2 value of 0.997. Though the *y*-intercept was 14.57 µmol L^{-1}, it is still less than the low end of the analytical measurement range (21.0 µmol L^{-1}), and thus should not introduce a significant bias relative to clinical diagnosis.

Serum albumin is the most abundant plasma protein in humans. Though albumin protein accounts for 55–60% of the total serum protein concentration, glycated albumin accounts for 80% of the total glycated serum protein.[16] GSP levels in serum are present proportionally to the levels of GA. The relationship between GSP and GA can be expressed as GSP = GA + *C* (a constant factor).[16] Therefore, determination of GSP is equivalent to the determination of GA which can be obtained from the GSP value by including a factor in the calculation. The results obtained in this study showed that %GA obtained with GlycoGap® method had an excellent correlation with Lucica® GA-L (a %GA assay kit) assay with an r^2 value of 0.975, and a *y*-intercept of 0.131%. If the results are reported in %GA, the reference range is reported to be 11–16% according to the package insert of the Lucica® GA-L test and the 2008–2009 guidance from the Japanese Diabetes Society.[34] The present study found that the reference range of %GA obtained by testing 124 apparently healthy and non-diabetic patient samples from the US population was similar to the reference range reported for the Japanese population, ranging from 11 to 16%.

The GlycoGap® assay had no significant effects from common interfering substances found in serum samples and it had a better tolerance to bilirubin than the predicate assay. Among all interfering substances tested, bilirubin, ascorbic acid and hemoglobin were found to have the greatest effects (10% bias) if the concentrations were greater than 5 mg dL^{-1} for ascorbic acid, 15 mg dL^{-1} for free and conjugated bilirubin, and 200 mg dL^{-1} for hemoglobin, which are all much higher than physiological concentrations found in samples.

The most significant improvement made was the unique formulation that allows all the reagents to be stable in the liquid format. Sugar stabilizers were used in Reagent 2 to stabilize Fructosaminase™, a key enzyme involved in the assay. Methyl-β-cyclodextrin was found to be effective to prevent oxidizing agents such as $NaVO_3$ from degradation in the liquid state. This formulation resulted in significant improvement in stability of over 15 months of shelf-life when it is stored at 2–8 °C, 4 weeks of on-board stability, and 7 days of calibration curve stability.

Reference intervals for GSP values are similar but wider compared to the reference range of fructosamine listed in the Tietz Textbook of Clinical Chemistry (200–285 μmol L^{-1}).[35] This difference may be due to the systematic difference between enzymatic and nitroblue tetrazolium (NBT) colorimetric methods. A similar reference range was obtained when samples from patients who are at risk for developing diabetes (HbA1c values between 5.7 and 6.4%) were excluded.

There exists a trend of overall correlation between GSP and HbA1c or %GA and HbA1c values (r^2 = 0.791 and 0.824, respectively) though there are some variations among samples. This correlation indicates that the Diazyme GSP assay can be used to predict HbA1c levels for certain patients who are not suitable for the HbA1c test because of their conditions including hemolytic anemia, gestational diabetes and renal dialysis. Some outlier samples with discrepant values between GSP and HbA1c may have resulted from the differences in half-lives of primarily albumin for GSP and erythrocytes for HbA1c. However, some of these discrepancies may be also caused by differences in predispositions of each individual to protein glycation, which is indicated by a temporally stable glycation gap. This glycation gap information will enable more precise glycemic control and individually tailored diabetes management.

Conclusions

A liquid stable enzymatic GSP assay (GlycoGap®) has been developed for clinical determination of glycated serum protein in patient blood samples. The assay showed excellent performance when compared to a previously reported lyophilized powder form GSP assay. When used as a ratio assay normalized to serum albumin, the Diazyme GSP assay demonstrated an excellent correlation to the Lucica® GA-L assay. There is a fairly good correlation between GSP or %GA and %HbA1c, and the normal ranges were found to be 151–300 μmol L^{-1} for GSP and 10.4–15.7% for %GA. The Diazyme GSP assay may provide clinics with a better tool for glycemic monitoring.

References

1 G. Danaei, M. M. Finucane, Y. Lu, G. M. Singh, M. J. Cowan, C. J. Paciorek, J. K. Lin, F. Farzadfar, Y. Khang, G. A. Stevens, M. Rao, M. K. Ali, L. M. Riley, C. A. Robinson and M. Ezzati, National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants, *Lancet*, 2011, **378**(9785), 31–40.

2 United Health, Center for Healthcare and Modernization, Working paper 5 The United States of Diabetes: Challenges and opportunities in the decade ahead, 2010 Nov. [cited 2011 Aug 8], http://www.unitedhealthgroup.com/hrm/UNH_WorkingPaper5.pdf.

3 American Diabetes Association, Clinical practice recommendations: executive summary: standards of medical care in diabetes, *Diabetes Care*, 2010, **33**(suppl. 1), S4–S5.

4 Diabetes Control and Complications Trial Research Group, The effect of intensive treatment of diabetes on the development and progression of long-term complications of insulin-dependent diabetes mellitus, *N. Engl. J. Med.*, 1993, **329**, 977–986.

5 UK Prospective Diabetes Study Group, Intensive blood glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UK PDS 33), *Lancet*, 1998, **352**, 837–853.

6 D. B. Sacks, D. M. Nathan and J. M. Lachin, Gaps in the glycation gap hypothesis, *Clin. Chem.*, 2011, **57**, 150–152.

7 S. Rodriguez-Segade, J. Rodriguez, J. M. Cabezas-Agricola, F. F. Casanueva and F. Camina, Progression of nephropathy in type 2 diabetes: the glycation gap is a significant predictor after adjustment for glycohemoglobin (HbA1c), *Clin. Chem.*, 2011, **57**, 264–271.

8 R. M. Cohen, Y. R. Holmes, T. C. Chenier and C. H. Joiner, Discordance between HbA1c and fructosamine: evidence for a glycosylation gap and its relation to diabetic nephropathy, *Diabetes Care*, 2003, **26**, 163–167.

9 B. I. Freedman, L. Andries, Z. K. Shihabi, M. V. Rocco, J. R. Byers, C. Y. Cardona, M. A. Pickard, D. L. Henderson, M. V. Sadler, L. M. Courchene, J. R. Jordan, S. S. Balderston, A. D. Graham, V. L. Mauck, G. B. Russell and A. J. Bleyer, Glycated albumin and risk of death and hospitalizations in diabetic dialysis patients, *Clin. J. Am. Soc. Nephrol.*, 2011, **6**, 1635–1643.

10 T. P. Peacock, Z. K. Shihabi, A. J. Bleyer, E. L. Dolbare, J. R. Byers, M. A. Knovich, J. Calles-Escandon, G. B. Russell and B. I. Freedman, Comparison of glycated albumin and hemoglobin A1c levels in diabetic subjects on hemodialysis, *Kidney Int.*, 2008, **73**, 1062–1068.

11 R. Schleicher and O. H. Wieland, Protein glycation: measurement and clinical relevance, *J. Clin. Chem. Clin. Biochem.*, 1989, **27**, 577–587.

12 M. Koga and S. Kasayama, Clinical impact of glycated albumin as another glycemic control marker, *Endocr. J.*, 2010, **57**(9), 751–762.

13 H. V. Roohk and A. R. Zaidi, A review of glycated albumin as an intermediate glycation index for controlling diabetes, *J. Diabetes Sci. Technol.*, 2008, **2**(6), 1114–1121.
14 D. A. Ambruster, Fructosamine: structure, analysis, and clinical usefulness, *Clin. Chem.*, 1987, **33**, 2153–2163.
15 B. Willms and P. A. Lehmann, New fructosamine test as a routine parameter in the control of diabetes, *Wien. Klin. Wochenschr., Suppl.*, 1990, **180**, 5–10.
16 E. D. Schleicher, R. Mayer, E. M. Wagner and K. D. Gerbltz, Is serum fructosamine assay specific for determination of glycated serum protein?, *Clin. Chem.*, 1988, **34**, 320–323.
17 M. R. Lawson, Fructosamine assay, *Ann. Clin. Biochem.*, 1985, **22**, 655–656.
18 D. J. Torrens, D. P. Shipley, M. B. Holdridge, S. R. Lane, H. Kallwass, Calibration of the Genzyme GlyPro Method for the Specific Enzymatic Measurement of Glycated Protein, *Proc. ACB National Meeting*, 1998, vol. 63.
19 T. Kouzuma, T. Usami, M. Yamakoshi, M. Takahashi and S. Imamura, An enzymatic method for the measurement of glycated albumin in biological samples, *Clin. Chim. Acta*, 2002, **324**, 61–67.
20 NCCLS, *Protocols for Determination of Limits of Detection and Limits of Quantification*, Approved Guideline, NCCLS Document EP17-A, NCCLS, Wayne, PA, 2004.
21 NCCLS, *Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach*, Approved Guideline, NCCLS Document EP6-A, NCCLS, Wayne, PA, 2003.
22 NCCLS, *Evaluation of Precision Performance of Quantitative Measurement Methods*, Approved Guideline, NCCLS Document EP5-A, NCCLS, Wayne, PA, 2nd edn, 2004.
23 NCCLS, *Method Comparison and Bias Estimation Using Patient Samples*, Approved Guideline, NCCLS Document EP9-A2, NCCLS, Wayne, PA, 2nd edn, 2004.
24 NCCLS, *Interference Testing in Clinical Chemistry*, Approved Guideline, NCCLS Document EP7-A, NCCL, Wayne, PA, 2002.
25 M. Inaba, S. Okuno, Y. Kumeda, S. Yamada, Y. Imanishi, T. Tabata, M. Okamura, S. Okada, T. Yamakawa, E. Ishimura and Y. Nishizawa, Osaka CKD expert research group. Glycated albumin is a better glycemic indicator than glycated hemoglobin values in hemodialysis patients with diabetes; effect of anemia and erythropoietin injection, *J. Am. Soc. Nephrol.*, 2007, **18**, 896–903.
26 S. Panzer, G. Kronik, K. Lechner, P. Bettelheim, E. Neumann and R. Dudczak, Glycosylated hemoglobin's (GHb): an index of red cell survival, *Blood*, 1982, **59**, 1348–1350.
27 L. Bry, P. C. Chen and D. B. Sacks, Effects of hemoglobin variants and chemically modified derivatives on assays for glycohemoglobin, *Clin. Chem.*, 2001, **47**, 156–163.
28 American Association for Clinical Chemistry, HbA1c Alone not a Reliable Diagnostic Tool in Obese Adolescents, Clinical Laboratory News, 2011 July page 46, [cited Aug 08 2011], http://www.aacc.org/publications/cln/2011/July/Documents/CLN_July_11online.pdf.
29 R. M. Cohen and E. P. Smith, Frequency of HbA1c discordance in estimating blood glucose control, *Curr. Opin. Clin. Nutr. Metab. Care*, 2008, **11**, 512–517.
30 S. L. Jeffcoate, Diabetes control and complications: the role of glycated hemoglobin, 25 years on, *Diabetic Med.*, 2004, **21**, 657–665.
31 J. B. Saaddine, A. Fagot-Campagna, D. Rolka, K. M. V. Narayan, L. Geiss, M. Eberhardt and K. M. Flegal, Distribution of HbA1c levels for children and young adults in the U.S.: Third National Health and Nutrition Examination Survey, *Diabetes Care*, 2002, **25**, 1326–1330.
32 L. N. Pani, L. Korenda, J. B. Meigs, C. Driver, S. Chamany, C. S. Fox, L. Sullivan, R. B. D'Agostino and D. M. Nathan, Effect of aging on A1c levels in individuals without diabetes; evidence from the Framingham Offspring Study and the National Health and Nutrition Examination Survey 2001–2004, *Diabetes Care*, 2008, **31**, 1991–1996.
33 W. H. Herman, Y. Ma, G. Uwaifo, S. Haffner, S. E. Kahn, E. S. Horton, J. M. Lachin, M. G. Montez, T. Brenneman and E. Barrett-Connor, Diabetes prevention program research group. Differences in A1C by race and ethnicity among patients with impaired glucose tolerance in the diabetes prevention program, *Diabetes Care*, 2007, **30**, 2453–2457.
34 Part of Japan Diabetes Society, *Indicators of Diabetes B; Guide the Treatment of Diabetes 2008–2009*, Wenguang Hall, 2008, p. 9.
35 N. Tietz, *Tietz Textbook of Clinical Chemistry*, ed. C. A. Burtis and E. R. Ashwood, Elsevier Health Sciences, 2nd edn, 1993.