Analytical Performances of an Enzymatic Assay for the Measurement of Glycated Albumin

Roberto Testa,1* Elena Guerra,2 Anna Rita Bonfigli,3 Nicola Di Gaetano,4 Gabriele Santini,4 and Ferruccio Ceriotti2

Background: Short to intermediate integrated glycemic control is best determined by glycated albumin (GA). This assay is appropriate when interpretation of glycated hemoglobin (HbA1c) is critical because of hemoglobinopathies, severe anemias, or other factors that affect red blood lifespan as hemodialysis. We evaluated a new assay based on the enzymatic quantification of GA by ketoamine oxidase and an albumin-specific protease.

Methods: Limits of blank, detection, and quantification; precision; linearity; accuracy; interferences; correlation with HbA1c; and serum vs plasma study have been evaluated on ILab® systems.

Results: Limit of blank, detection, and quantification for GA (g/L) were, respectively, 0.26, 0.36, and 1.15. Repeatability and within-device precision CVs were lower than 2.11%, 1.61%, and 1.56% for GA (g/L), albumin (g/L), and GA%, respectively. Linearity for GA (g/L) and GA% was 1.2–36.8 and 5.5–92.2, respectively. Highest deviation from linearity was <11% and recovery was higher than 90%. Accuracy against the certified RecCS Japan Clinical Chemistry Reference Material (JCCRM) 611 was <1%. Classical interfering substances had no significant impact. Correlation of GA% between ILab® Taurus and ADVIA system was $y = 1.02[GA%]+0.25; R^2 = 0.994$. No difference was found in the determination of GA% in serum vs plasma.

Conclusions: GA enzymatic assay is a reliable, fully automated method allowing accurate and precise determination of GA in a routine laboratory.

IMPACT STATEMENT

This article will benefit both diabetic patients with hemoglobinopathies or reduced red blood cell lifespan that need an alternative marker for glycemic control and people at risk of developing diabetes. Data show glycated albumin (GA) assay reliability. Moreover, it is easy to use, being a clinical chemistry assay: it can be run on standard clinical chemistry automated systems. This article summarizes almost a full proof of performance report of a GA assay investigating most of the performance aspects as precision, accuracy, linearity, and interference study.

1Experimental Models in Clinical Pathology, Italian National Research Center on Aging (INRCA), Ancona, Italy; 2Service of Laboratory Medicine, Ospedale San Raffaele, Milan, Italy; 3Scientific Direction, Italian National Research Center on Aging (INRCA), Ancona, Italy; 4IL Diagnostic R&D Department Instrumentation Laboratory, SpA, A Werfen Company, Milano, Italy
*Address correspondence to this author at: Experimental Models in Clinical Pathology, INRCA-IRCCS National Institute, Ancona, Italy, Via Della Montagnola 81, 60127 Ancona, Italy. Fax +39-071-800-3556; e-mail r.testa@inrca.it.
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Nonstandard abbreviations: GA, glycated albumin; HbA1c, glycated hemoglobin; Alb, albumin; LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantification.
Clinical management of diabetes requires an accurate means of monitoring blood glucose concentrations. The indicator most widely used is glycohemoglobin, which gives a retrospective view over the preceding 60–90 days of glycemia. Short-to intermediate-term integrated glycemic control is better reflected by glycated albumin (GA)\(^5\) (1), which correlates with glucose profiles during the preceding 2–4 weeks because the turnover of human serum albumin is shorter (half-life 14 days) than that of hemoglobin (Hb half-life 60 days). This parameter is also appropriate when interpretation of glycated hemoglobin (HbA\(_1\)c) is critical (e.g., in renal failure, anemias, or other erythrocyte abnormalities) (2). Although helpful in some circumstances, GA quantification does have its limitations because it can be falsely lowered in increased albumin catabolism in obesity, because of chronic microinflammation and pathologies such as nephrotic syndrome and hyperthyroidism (3–6). In liver cirrhosis and hypothyroidism, in which albumin metabolism is reduced, GA is falsely increased.

GA belongs to the wider family of glycated serum proteins. Different methods have been developed to measure glycated serum proteins: methods that are able to separate glycated proteins from nonglycated ones on the basis of differences in chemical reactivity and methods able to separate them on the basis of different structural characteristics (e.g., affinity chromatography) (7). Although the first are suitable for routine practice in clinical chemistry analyzers, the second are less practical to use. The first group of methods includes the fructosamine assay. This method was developed by Johnson et al. (8) and it is based on the ability of ketoamines, or fructosamine, to catalyze the reduction of nitroblue tetrazolium to formazan in alkaline solution. Since the initial description of the method, there have been many reports about interference concerns, such as uremia, lipemia, bilirubin, ascorbate, and hemolysis (9). Standardization and matrix effects, dependence on buffer pH, measuring time, and protein concentration effects were also reported as further criticalities (10, 11). For these reasons, the method is not relevant for use in clinical practice.

We have evaluated an enzymatic method coupled to a colorimetric output for the quantification of GA by ketoamine oxidase and an albumin-specific protease to verify its performance and suitability for routine use on clinical chemistry automated analyzers.

**MATERIALS AND METHODS**

Reagent kits, used for the determination of GA expressed in % (GA%), as the ratio of GA in g/L to albumin (Alb) g/L, were provided by Asahi Kasei Pharma Corporation along with related calibrator and controls (Lucica\(^\)® GA-L, GA-L Calibrator, GA-L Control Serum L, H). Lucica GA-L is an in vitro diagnostic assay for the measurement of GA% based on an enzymatic method, containing GA reagents and Alb reagents. The method has been adapted on ILab\(^\)® Taurus and ILab\(^\)® Aries chemistry systems (Instrumentation Laboratory) and ADVIA 2400 (Siemens Healthcare).

HbA\(_1\)c (mmol/mol) was determined with a capillary electrophoresis method, IFCC standardized, manufactured by Sebia (Capillaries 2 Flex Piercing).

**Analytical platforms**

Performance characteristics were evaluated using the ILab Taurus.

Method comparison studies were performed between 3 automated chemistry analyzers: the ILab Taurus, the ILab Aries, and the ADVIA 2400.

**Patient samples**

Serum pools, for assay performance testing, were collected from freshly drawn leftover samples. De-identified patient serum samples (n = 99) were collected at San Raffaele Hospital to perform the method comparison studies. All samples were selected from leftover tubes collected during the
daily normal laboratory routine activity of 1 week. Serum isolated from centrifuged tubes was frozen at −20 °C for 2 weeks. Thawed samples were analyzed in a single analytical session.

**GA assay method**

Lucica GA-L assay is an enzymatic method coupled to a colorimetric output. The quantitative measurement of GA is described in Fig. 1 (12).

GA and Alb application settings were developed by Instrumentation Laboratory SpA for ILab systems. Application settings for the ADVIA 2400 were completely derived and adopted from ADVIA 1650 settings previously developed by Asahi Kasei Pharma (12).

The quantitative measurement of Alb is obtained using a bromocresol purple (BCP) method (13). Calculation of GA% in the sample is obtained as the GA/Alb ratio and corrected with the intermethod arithmetic expression designed to adhere to the GA% levels determined by the HPLC method, which uses a different measurement principle (12–14):

\[ \text{GA} = \left( \frac{\text{GA}}{\text{Alb}} \times 100 / 1.14 \right) + 2.9. \]

**Limit of blank, limit of detection, and limit of quantification**

Limit of blank (LoB), limit of detection (LoD), and limit of quantification (LoQ) were determined for both Alb and GA using a scalar dilution with saline according EP-17A CLSI protocol (15). LoD = LoB + 1.645 (SD low concentration sample); LoQ was the concentration in linearity determination for which total CV of replicates was <5%.

**Precision**

Precision was run according the following protocol. Briefly, 3 pools (high, medium, and low GA%) were analyzed for 10 days with 2 runs/day and 3 replicates/run. Runs were held at 10:00 AM and 3:00 PM, with an average time between runs of 5

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**Fig. 1. GA assay method.**

First, sample (serum or plasma) reacts with a ketoamine oxidase (KAOD) to eliminate endogenous glycated amino acids by conversion to glucosone, amino acids, and hydrogen peroxide. Second, the treated solution reacts with an Alb-specific protease, which converts GA to glycated amino acids. Third, the glycated amino acids react with a KAOD to form glucosone, amino acids, and hydrogen peroxide. Fourth, the generated hydrogen peroxide reacts with peroxidase (POD) in the presence of TODB [N,N-Bis (4-sulfobutyl)-3-methylaniline disodium salt (4-AA)], forming quantitatively a blue-purple pigment. Measurement of the absorbance of this blue-purple pigment quantifies the glycated amino acids produced by GA.
hours. Repeatability and intra-device precision of GA, Alb, and GA% were calculated as SD and CV%.

**Linearity**

Linearity assessment was performed according to CLSI document EP6-A (16) with not less than 10 concentration levels; each diluted concentration was measured in quintuplicate. Serum pools were obtained using samples from different patients mixed with a highly glycated sample obtained with 24-h exposition at very high glucose concentration. Low GA% level was obtained by dilution with a human Alb fatty acid free (Golden West Biological).

**Accuracy with reference material**

The Japan Clinical Chemistry Reference Material (JCCRM) 611 is certified reference material for measurement of GA% in human serum and is available through the ReCCS (Reference Material Institute for Clinical Chemistry Standards) according to the Japan Society of Clinical Chemistry recommendation (17). This material is titrated with isotope dilution–mass spectrometry and provided in liquid frozen vials (3 levels). It is the current highest level of traceability for GA determination according to the Committee on Diabetes Mellitus Indices of the Japan Society of Clinical Chemistry (17).

**Analytical interference**

Interference from turbidity was assessed with INTRALIPID® 10 g/100 mL (Fresenius-Kabi) and Interference Check A Plus (Sysmex). Interference from lipemia was determined with different levels of triglyceride-containing sera prepared by mixing a normal triglycerides pool with a high level one. Interference from icterus was assessed by spiking samples with concentrated solution of Bilirubin (Sigma-Aldrich, catalog number B4126). Interference from hemolysis was tested by spiking samples with Multi4 Co-Oxime-ter control (Instrumentation Laboratory). Interference from glucose was tested by spiking samples with a concentrated solution of glucose (VWR Chemicals, catalog number 101174Y). Interference from peroxidase activity was tested by spiking samples with a concentrated solution of ascorbic acid (Sigma-Aldrich, catalog number 33034).

GA and Alb concentrations were measured at basal and increased concentrations of interfering substances. GA, Alb, or GA% bias values >10% were considered affected by the interferent.

**Method comparison**

A cohort of 99 anonymized patient serum samples, collected at San Raffaele Hospital laboratory, were assayed for GA% on ILab Taurus, ILab Aries, and ADVIA 2400. Sample selection was made upon HbA1c values (obtained using Sebia Capillarys 2 Flex Piercing) ranging from 18 to 137 mmol/mol (3.8%–14.7%).

**Plasma-serum matrix comparison**

Paired patient samples (n = 76), collected for both serum and K3-EDTA plasma matrix, were analyzed on the ILab Taurus. GA% was determined and results were compared with a pairwise Student t-test where P < 0.05 was considered significant.

**Statistical analysis**

Statistical analyses were performed using MedCalc for Windows, version 15.6 (MedCalc Software) (linearity, method comparison, and plasma-serum matrix comparison), and Microsoft Excel 14.0.7153.5000 (LoB, LoD, LoQ, precision, accuracy, and interference). Data were analyzed with SPSS/Win program (version 19.0; SPSS). Values were expressed as mean (SD), CVs, or median when not normally distributed. To assess the significance of the differences, a paired t-test was used. Linear re-
gression analysis was performed by the least squares method. \( P \) values <0.05 were considered statistically significant.

RESULTS

Performance results data shown here were mainly run on the ILab Taurus system; comparable analytical performance was observed for ILab Aries system, for which data are not shown except where noted.

LoB, LoD, and LoQ

LoB, LoD, and LoQ were determined both for Alb and for GA using a scalar dilution with saline; values obtained were 0.1, 0.3, and 2.0 g/L and 0.26, 0.36, and 1.15 g/L, respectively.

Precision

The CV% for repeatability ranged from 0.58% to 0.80% for Alb, 0.68% to 1.25% for GA, and 0.96% to 1.26% for GA%. The CV% for within-device precision ranged from 0.78% to 1.61% for Alb, 0.95% to 2.11% for GA, and 1.17% to 1.56% for GA%. Data results are reported in Table 1.

Linearity

The method is linear within a range from 5.5% to 92.2% for GA% \((y = 1.006 \times -1.792; R^2 = 0.9992)\). The highest deviation from linearity was <11% and recovery was higher than 90%. The method was also linear from 1.2 to 36.8 g/L for GA \((y = 1.02 \times -0.30; R^2 = 0.9997)\). Alb linearity was from 2.0 to 66.8 g/L \((y = 0.96 \times +1.5; R^2 = 0.999)\). No influence of Alb on GA quantification was previously demonstrated (14). The linear range for GA% assay permits evaluation of the expected reference interval (13.5% ± 1.5%) (18, 19) and expected GA% values in diabetic patients of >16.5% (20).

Accuracy vs the JCCRM 611

JCCRM 611 is the certified reference material for GA; it is provided as a 3-level material with as-

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signed values of GA%. Table 2 shows the recovery and percentage differences of GA% assay on the ILab systems. The absolute percentage difference between experimental data and certified data are between 0.0% and 3.1%.

### Analytical interferences

Classical interfering substances such as hemoglobin, bilirubin, triglycerides, ascorbic acid, and glucose were assayed for GA. GA% was not influenced by any of the interfering substances at the following concentration values (accepted bias < ±10%): hemoglobin <2.48 g/L, bilirubin <0.156 g/L, triglycerides <8.08 g/L, ascorbic acid <1.1 g/L, and glucose <19.3 g/L. Moreover, turbidity lower than 1490 FTU (formazin turbidity unit) did not affect the GA% assay.

### Method comparison

A total of 99 samples were analyzed on ILab Taurus, ILab Aries, and ADVIA 2400 instruments. Fig. 2 shows the correlation of GA% measurement on the 3 analytical platforms; slopes and intercepts are also reported. Correlation coefficients were ≥0.983. No significant difference was found among them.

### Plasma-serum matrix comparison

A total of 76 patient samples, collected for both serum and K3-EDTA plasma matrix, were analyzed on ILab Taurus, and no difference was found in GA% results (P = 0.1477). The regression equation was $GA_{\text{plasma}} = 0.999 \times GA_{\text{serum}} + 0.001$ ($r = 0.985$).

### DISCUSSION

Glycation of human serum protein is a biological nonenzymatic reaction that can be observed upon glucose exposure of serum proteins. Glycation usually occurs at few amino acidic sites that are characteristic for each protein (12, 18). This phenomenon is directly proportional to physiological glucose concentration and has several biological effects documented in many studies investigating diabetic patients (19). In most of the clinical settings, HbA₁c is currently used as a marker for diabetes diagnosis, staging of diabetic patients, and follow-up both for antidiabetic drug therapy and diet profiles. Moreover, HbA₁c level has a high correlation with diabetes prognosis and its complications (19, 20).

The downside of HbA₁c is that its diagnostic and clinical performance are strictly dependent on the hemoglobin molecule structure and erythrocyte life span (20). Genetic abnormalities can produce abnormal hemoglobin proteins with different glycation sites and consequently different affinity for sugars. These pathologies can also be characterized by a reduced erythrocyte life cycle. Other factors such kidney disease can influence Hb life span. The effect of diabetes on vascular tissues can be one of the most important triggering factors for kidney disease, being at the same time a limiting factor for HbA₁c.
clinical performance and reliability. Pregnancy can also represent a clinical setting with low reliability for HbA1c (21–23).

Serum proteins have been considered good glycation markers for the past 10 years. Several reports studied their usefulness in the diagnosis and follow-up of diabetes with different or opposite conclusions (24). Fructosamine, the first assay available on the market, is nowadays considered poorly reliable, although the test is still used for glycation assessment (25, 26).

We have evaluated a new enzymatic method coupled to a colorimetric output for the quantification of GA by ketoamine oxidase and an Alb-specific protease, developed by the Asahi Kasei Pharma Corporation, that is able to measure GA and total Alb, thus giving GA% value. Analytical performance studies conducted on the ILab Taurus clinical chemistry analyzer showed high analytical reliability with this method. LoB, LoD, and LoQ data were reliable considering that very low values were detected. The assay demonstrated reliable analytical performance for precision (repeatability and/or within-device) <2.11% for GA, <1.61% for Alb, and <1.56% for GA%.

Alb concentration in the pools were in the low-normal or low range. This fact might have negatively affected Alb (g/L) and GA (g/L) precision performance in the high pool.

Linearity was excellent for the 2 quantified (GA and Alb) and 1 calculated (GA%) tests with the latter having a linear range from 5.5% to 90.0%. The principle analytical interferences were studied and the 3 tests were found not to be susceptible to abnormal values of hemoglobin, icterus, ascorbic acid, glucose, triglycerides, and turbidity. Moreover, these data met the manufacturer’s claims for interference that are provided only for GA%. No difference was found in serum-plasma matrix comparison.

Accuracy was confirmed using a certified reference material (JCCRM 611) (17). Using this material recovery was never lower than 99% on the
ILab Taurus and 97% on ILab Aries. Results correlating GA% on the 3 analytical platforms in 99 fresh samples showed good agreement as indicated by the correlation coefficients and slopes on ILab Taurus, ILab Aries, and ADVIA 2400, whose performance was previously correlated with the HPLC GA method (14, 27).

Results presented here show the reliability of GA enzymatic quantification with the ILab Taurus and ILab Aries systems and confirm previous data obtained from different analyzers (28). GA% quantification does not require any preanalytical procedures, thus resulting in a better ease-of-use when compared to other laboratory methods.

Diabetes is managed by clinicians with few laboratory medicine tools. GA is an analytically robust and reliable assay, that is easy to run, and has no sample pretreatment.

Thanks to its ability to evaluate midterm glycation (last 3 weeks) and its independence from Hb abnormalities, GA% can be trusted to be a good glycation indicator and used in conjunction with HbA1c measurements may be compromised.

Critical patients, such as those affected by kidney disease, may have advantage from GA evaluation paired with HbA1c in naïve diabetic patient diagnosis, GA% could represent a way to assess glycation exposure in an earlier time-frame. GA has been also proposed as a useful marker for assessing postprandial hyperglycemia (25, 29). This is also due to the faster glycation rate of Alb compared to Hb (25, 30, 31). GA may also be able to contribute to assessment of prediabetes, for which average glucose concentration is increasing, but below the diabetes cutoff value.

Our study represents the analytical performance of the GA method on an automated clinical chemistry analyzer. Now there is the need to further explore the clinical value of the test in different patient subsets. Determination of reference intervals in a selected population according to American Diabetes Association (ADA) criteria is next to be investigated to pair Caucasian population values with those previously published (32–35). Furthermore, GA% vs HbA1c comparisons will be investigated.

The use of this assay could give to laboratory medicine a chance to provide further information to clinicians (diabetologists, nephrologists, etc.), allowing earlier identification and better management of some classes of diabetic patients.

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PERFORMANCE OF THE GLYCATED ALBUMIN ASSAY


