Interlaboratory Variation of GHb Assays in Victoria, Australia

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OBJECTIVE — To determine the extent of interlaboratory variation and accuracy in the measurement of glycated hemoglobin (GHb).

RESEARCH DESIGN AND METHODS — All laboratories that measure glycated hemoglobin in the State of Victoria, Australia, were invited to participate, and positive responses were received from 27 of 30 laboratories. An aliquot of blood drawn from three patients with diabetes and varied glycemia and from one nondiabetic subject was sent to each participating laboratory. Distribution of results was analyzed according to the reported results and their variance from an assigned reference value and were expressed as differing from this latter value as percentage bias and in absolute terms. A bias ≥10% or an absolute difference of ≥1% HbA_{1c} from the reference value was considered significant.

RESULTS — Reported results for the same blood sample ranged from 4.1 to 5.8%, 5.1 to 8.2%, 6.7 to 9.3%, and 10.1 to 14.7% for the specimens from the nondiabetic subject and the diabetic patients with good, moderate, and poor glycemic control, respectively. The proportion of laboratories with results that differed by ≥10% bias from the reference value were 39% (12 of 30), 29% (9 of 30), 16% (5 of 30), and 32% (10 of 30), and the proportion reporting results that differed by ≥1% HbA_{1c} in absolute terms from the reference values were 3% (1 of 30), 6% (2 of 30), 16% (5 of 30), and 23% (7 of 30) for the specimens from the nondiabetic subject and the diabetic patients with good, moderate, and poor glycemic control, respectively.

CONCLUSIONS — A substantial degree of interlaboratory variation for GHb measurement exists in Victoria, Australia. This may lead to difficulties in interpretation when GHb is assayed by different laboratories in the same patient over time. Interlaboratory standardization may be achievable by calibration to a standard assigned by a reference laboratory and distributed to all laboratories measuring GHb.

The Diabetes Control and Complications Trial (DCCT) has provided compelling evidence that the likelihood of developing long-term complications of diabetes may be reduced by improving long-term glycemic control (1). The assessment of long-term glycemic control is based on the measurement of glycated hemoglobin (GHb). In IDDM, isolated blood glucose measurements yield little information regarding long-term glycemia, and even the mean of random blood glucose measurements over an extended period correlates poorly with GHb (2). While the relationship between GHb and either fasting or postprandial blood glucose measurements is better in NIDDM (3), the measurement of GHb is still useful in IDDM patients. Current American Diabetes Association (4) and European Consensus Guidelines (5,6) advocate the regular measurement of GHb in patients with IDDM and NIDDM.

GHb is formed by the nonenzymatic glycation of hemoglobin A (HbA). In this process, glucose is adducted to certain amino acids in the hemoglobin chains and then undergoes a chemical (Amadori) rearrangement to form a stable (ketoamine) product referred to as GHb. The Amadori process occurs continuously and essentially irreversibly such that the proportion of HbA that becomes glycated reflects the integrated (time averaged) blood glucose concentration during the life of the erythrocyte. Thus, GHb reflects the level of glycemia over the preceding 2–3 months.

Current assay methods distinguish glycated from nonglycated hemoglobin on the basis of charge (cation exchange chromatography, electrophoresis), chemical reactivity (thiobarbituric acid method, affinity chromatography), and antigenicity (immunoassay). Charge based methods such as ion exchange chromatography and electrophoresis use the fact that modification of hemoglobin at the NH_2-terminal valine of the β-chain alters hemoglobin charge causing it to migrate more rapidly in a polarized electrical field. This fast hemoglobin is referred to as HbA_1, which consists of three components, HbA_{1a}, HbA_{1b}, and HbA_{1c}, that may be detected by high performance liquid chromatography (HPLC). Only HbA_{1c} reflects modification by glucose, while HbA_{1a} and HbA_{1b} arise from adduction of other substrates such as glucose 6-phosphate and fructose 1,6-diphosphate. Structural methods such as affinity chromatography measure total GHb, i.e., hemoglobin that has been glycated at other sites in addition to the NH_2-terminal valine of the β-chain. These other sites that constitute ~10% of GHb in a normoglycemic milieu include the NH_2-terminal valine of the α-chain and the ε-amino groups of lysine residues throughout the hemoglobin chains. In nondiabetic subjects, ~4–6% of hemoglobin is HbA_{1c}, 5–8% is HbA_1, and 4–7% is total GHb (7).

Because different methods measure different GHb fractions, results obtained may not be directly comparable. The diversity of methods that may be used and the lack of an available standard render the interpretation of results obtained from different laboratories difficult. The

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CV, coefficient of variation; DCCT, Diabetes Control and Complications Trial; HPLC, high performance liquid chromatography; LPC, low pressure chromatography.

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Table 1—*Methods of GHb assay*

Method	Laboratories	CV (%)	Manufacturers
Cation exchange chromatography			
HPLC	7	2.8 (1.6–6.4)	Diamat (Bio-Rad Laboratories, Hercules, CA; n = 3), MDMS (Bio-Rad, n = 1), ICI (GBC Scientific, Dandenong, Victoria, Australia; n = 1), Waters (Waters Corporation, Milford, MA; n = 1), in house (n = 1)
Microcolumn	6	5.9 (3.8–11.6)	Microcolumn (Bio-Rad; n = 6)
LPC	1	5.4	765 Glycomat (Ciba-Corning Diagnostic, Medfield, MA; n = 1)
Immunoassay	9	4.5 (2.2–7.6)	DCA 2000 (Bayer Diagnostics, Elkhart, IN; n = 7), Tinaquant (Boehringer-Mannheim, Mannheim, Germany; n = 2)
Affinity chromatography	6	7.6 (1.5–8.6)	IMX (Abbott Laboratories, Chicago, IL; n = 3), Endocrine Sciences (Calabasas Hills, CA; n = 2), Columnate (Helena Laboratories, Beaumont, TX; n = 1)
Electrophoresis	1	3.6	Diatrac (Beckman Instruments, Fullerton, CA)

CV refers to the in-house quality control procedures of all laboratories using each method expressed as median (range).

present study was undertaken to assess the interlaboratory variation and the manner in which measured GHb components (total GHb, HbA_1, HbA_{1c}) were reported in Victoria.

RESEARCH DESIGN AND METHODS

Recruitment

A list of laboratories that assay GHb was obtained from the Pathology Services Accreditation Board of Victoria. All laboratories were invited to participate, and positive responses were received from 27 of 30 laboratories.

Specimens

Three subjects with diabetes and varying degrees of long-term glycemic control (good, moderate, poor) and one nondiabetic subject were recruited from the diabetes clinic at the Austin Hospital. Blood from each subject was collected by venipuncture into EDTA-evacuated tubes and kept at 4°C before and during transport to other laboratories. An aliquot from each of the four specimens was sent to the 27 participating laboratories and arrived at their destination within 12 h of specimen collection. At each laboratory, the four specimens were measured in a single assay.

Standardization

HPLC was chosen as the reference method because of its excellent long-term precision, the specificity of the various hemoglobin fractions measured, the ease with which quality-control procedures may be preformed, and its use in the DCCT (8). Results obtained by Diamat HPLC (Bio-Rad Laboratories, Hercules, CA) at the Biochemistry Laboratory of the Austin Hospital were chosen as reference standards because of their excellent long-term performance in European (9) and College of American Pathologists (10) external quality assurance programs as well as their use in longitudinal studies of glycemic control and the development of long-term diabetic complications (11).

Results obtained as total GHb (n = 6) were expressed as HbA_{1c} after conversion by empirically derived equations for each method using the manufacturers guidelines and/or from a series of samples assayed using the method of interest compared with those obtained at the Austin Hospital by HPLC (r = 0.96–0.98, n = 54–59, $P < 0.0001$).

A previous interlaboratory quality control survey in Victoria (I.G., unpublished observation) demonstrated that results for HbA_{1c} obtained by immunoassay and microcolumn methods were linear over the entire measurement range when compared with HPLC. The linear regression equations are as follows: $y_1 = 0.992x - 0.304$ ($r^2 = 0.999$, $P < 0.001$) and $y_2 = 0.937x - 0.092$ ($r^2 = 0.97$, $P < 0.01$), where x indicates HPLC, y_1 indicates immunoassay, and y_2 indicates assay by microcolumn ion exchange.

Accuracy

The distribution of results was analyzed according to their variance from the reference value. Results were expressed as differing from this value in absolute terms. However, because analysis in absolute terms alone will diminish differences found in specimens obtained from nondiabetic subjects and those with good glycemic control when compared with those derived from patients with moderate and poor glycemic control, results were also expressed as percentage bias, where bias = $(X_1 - X_2)/X_1$ in which X_1 is the reference value and X_2 is the measured result of interest. An absolute difference of ≥1% HbA_{1c} from the reference value or a bias ≥10% was considered significant.

The selection of a bias of >10% and an absolute difference of >1% as separating satisfactory from unsatisfactory was based on the knowledge that the within laboratory coefficient of variation (CV) for GHb is at best ~3%. Therefore, two standard deviations would be ±0.6% for a true HbA_{1c} of 10%. Hence, by choosing a deviation in HbA_{1c} of 1% rather than, say, 0.5%, significant deviations was more likely to reflect assay bias rather than imprecision.

Imprecision

All laboratories participating in the study assayed GHb in singlicate in both their routine clinical practice and for the study samples. Imprecision was ascertained by the in-house interassay CV of each participating laboratory and expressed as median (range) according to the assay method used (Table 1).

Between-method comparisons

Results were analyzed according to percentage bias for each of the four major assay methods: HPLC, immunoassay, microcolumn ion exchange chromatography, and affinity chromatography. Be-

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Table 2—*Results of assays for HbA_{1c} according to method used*

HbA_{1c} (%)	Glycemic control			
	Nondiabetic	Good	Moderate	Poor
Reference HPLC	5.3	6.7	8.5	11.4
Median and range				
For all laboratories	5.1 (4.1–5.8)	6.7 (5.1–8.2)	8.3 (6.7–9.3)	11.3 (10.1–14.7)
By HPLC	5.2 (4.5–5.8)	6.7 (6.5–7.2)	8.7 (8.3–9.3)	11.8 (11.1–12.4)
By microcolumn chromatography	5.3 (5.0–5.4)	6.3 (5.1–6.6)*	7.7 (6.7–8.6)*	11.4 (10.3–11.9)
By immunoassay	5.0 (4.1–5.5)	6.7 (5.9–6.9)	8.2 (7.8–9.3)	11.0 (10.1–14.7)
By affinity chromatography	5.0 (4.7–5.6)	6.7 (6.3–7.1)	8.1 (7.7–9.2)	10.9 (10.1–12.7)
LPC	5.7	6.9	8.7	11.4
Electrophoresis	5.5	8.2	9.3	12.2

The median and range refer to the results obtained for each sample using the same method of analysis in different laboratories. Results for affinity chromatography are expressed as HbA_{1c} equivalents after conversion from total GHb using the manufacturer's guidelines or empirically derived conversion formula (see RESULTS). *$P < 0.05$ compared with HPLC.

cause results obtained by affinity chromatography were derived from conversion formulas based on the reference method, these were excluded from between-method comparison analysis.

Statistics

All analyses were performed using the Statview SE+ Graphics package (Abacus Concepts, Berkeley, CA) on an Apple Macintosh Quadra 605 (Apple Computer, Cupertino, CA). The percentage bias of results obtained by each method was compared by analysis of variance. A P value of <0.05 was considered statistically significant.

RESULTS

Of 30 laboratories measuring GHb in Victoria, 27 participated in the study. At the time of the study, three laboratories were assaying GHb by two different methods, and both were used in the subsequent analysis. Methods of GHb measurement included HPLC, immunoassay, ion exchange chromatography, affinity chromatography, electrophoresis, and low pressure chromatography (LPC) (Table 1). All laboratories included in the study either removed the labile fraction as a routine part of their assays (HPLC, LPC, microcolumn ion exchange chromatography, electrophoresis) or used assays that measure only the ketoamine and not the aldimine fraction of GHb (affinity chromatography, immunoassay).

Reporting of results

The range of results reported for each sample assayed by each method was wide (Table 2). Of the six laboratories using affinity chromatography and thus measuring total GHb, three converted their results to HbA_{1c} using the manufacturers guidelines before issuing reports, while the other three reported their results as total GHb. Of these three, one laboratory issued results with a table in which total GHb and approximate HbA_{1c} results were compared. Another laboratory routinely assayed both total GHb and HbA_{1c} allowing direct comparisons, and the third reported total GHb results and equivalent HbA_{1c} values based on a conversion formula obtained by comparing their affinity chromatography method with HPLC.

Accuracy

The proportion of laboratories with results that differed by ≥10% bias from the reference value were 39% (12 of 30), 29% (9 of 30), 16% (5 of 30), and 32% (10 of 30) for specimens from the nondiabetic subject and the diabetic patients with good, moderate, and poor glycemic control, respectively. The proportion reporting results that differed by ≥1% HbA_{1c} in absolute terms from the reference values were 3% (1 of 30), 6% (2 of 30), 16% (5 of 30), and 23% (7 of 30), respectively.

Between-method comparisons

The percentage bias for HPLC, immunoassay, and microcolumn ion exchange chromatography are detailed in Table 2 and Fig. 1. The degree of bias was least for HPLC. Results obtained by immunoassay and microcolumn ion exchange chromatography revealed bias that was signifi-

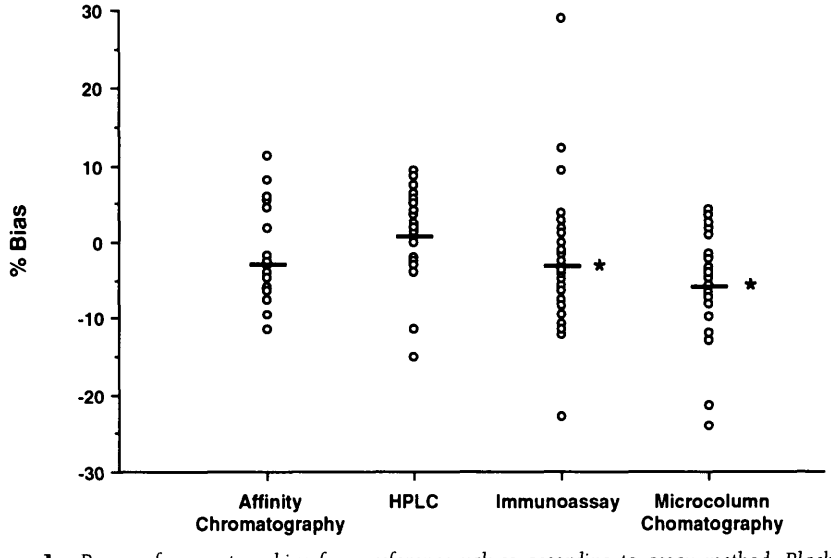

Figure 1—*Range of percentage bias from reference values according to assay method. Black line indicates mean percentage bias. *$P < 0.05$ compared with HPLC.*

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cantly lower than that of HPLC, suggesting that these methods would tend to give lower results for HbA_{1c} when compared with HPLC. The degree of dispersion was greatest for immunoassay (Table 2).

CONCLUSIONS— The present study demonstrates that in our study area a variety of different methods were used to measure GHb and that there was considerable variation in the measurements among laboratories. Patients with diabetes frequently obtain their care in different clinical settings and may thus have their GHb measured in different laboratories using different methods that may not be directly comparable. Indeed, in the present study, the range of GHb results obtained for each method varied widely despite similar median values. Confusion may ensue when, despite apparently similar levels of glycemia, different GHb results are obtained. For instance, in the present study, results obtained from the patient with moderate glycemic control varied by 2.6% from 6.7 to 9.3%. These results span those obtained for the conventionally and intensively treated groups in the DCCT in which a 2% decrease in HbA_{1c} over a 9-year period was associated with ~50% reduction in the risk of developing long-term complications such as retinopathy, nephropathy, and neuropathy (12). Since the publication of the DCCT findings, it is possible to estimate the risk of microvascular complications in patients with diabetes indicating a need to relate the results of GHb obtained in an individual patient to those of the DCCT.

The results obtained in the present study are similar to those found in other surveys of GHb (9,10,13). A survey of the American College of Pathologists in 1990 reported interlaboratory CV that ranged from 3.4% for HPLC to 17.2% for one type of HbA_1 assay by ion exchange chromatography (10). Similarly, in the U.K. interlaboratory CV for GHb varied between 11.2–20.1% (13). The acknowledged differences in results obtained by different methods and in different laboratories using the same method have led to the adoption of various strategies to address this problem. While education of clinicians and patients about different assay methods is important, it does not address the underlying need for standardization (15). Approaches such as the use of a single centralized laboratory (16) or a single type of assay system are unlikely to receive universal approval. Indeed, each assay method has its own advantages and disadvantages, which include cost, speed, convenience of automation, and interference with hemoglobin variants, fetal and carbamylated hemoglobin (17).

In its recently published consensus guidelines on targets for metabolic control, the European IDDM Policy Group addressed the problem of interlaboratory variation by expressing recommendations for glycemic control in terms of the mean and standard deviation (SD) of the assay used as well as HbA_1 and HbA_{1c} (18). For instance, in IDDM, good glycemic control is defined by the Policy Group as HbA_{1c} <6.5%, HbA_1 <8.0%, or <3 SD above the nondiabetic mean. This follows a similar European consensus view for NIDDM in which good glycemic control is defined as <2 SD above the nondiabetic mean (19). Unfortunately, this approach may still be associated with problems of interpretation when different assay methods are used. For instance, in two separate studies conducted in the U.K. (20,21), the proportion of patients with NIDDM and good control (GHb < mean + 2 SD) was 17–20% when HbA_1 was measured by electrophoresis. However, when simultaneously drawn specimens were also assayed for HbA_{1c} by immunological methods, the proportion of patients with good glycemic control decreased to 6–8% when interpreted according to the advocated SD approach.

In the U.S., Bodor and colleagues (22) have approached the problem of interlaboratory standardization by the use of calibrators. With this method, hemolysates are obtained from the blood of patients with varying glycemic control. Hemoglobin A_{1c} values are determined by HPLC, expressed as a percentage of total Hb, then distributed to participating laboratories that use them to calibrate their own assays. Despite the theoretical nonlinear relationship between HbA_{1c} and total GHb, this use of calibrators resulted in low long-term imprecision and comparability between different assay methods. Using this approach, similar improvements have also been demonstrated in Europe by Weykamp and colleagues (9,24) and the feasibility of this method has recently been shown in Australia (25).

Recently, a Glycohemoglobin Standardization Subcommittee was formed under the auspices of the American Association for Clinical Chemistry, which includes representatives from other key organizations such as the International Federation of Clinical Chemistry, the American Diabetes Association, the College of American Pathologists, and the Center for Disease Control (26). The preparation of primary and secondary calibration material and the quantitation of such material is the ultimate aim of this group. These would be available worldwide and should greatly reduce the interlaboratory variation and imprecision for all GHb assays. As an interim measure, the International Federation of Clinical Chemistry has suggested calibration and standardization using freeze-dried hemolysate available from the European external quality control laboratory with GHb values assigned by the DCCT reference laboratory (9,27). The outcome of such endeavors is keenly awaited. However, while health professionals should be aware of the imperfections in assaying GHb (28), its measurement remains a valuable clinical tool in the management of patients with diabetes.

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