HELENA GLYCO-Tek AFFINITY COLUMN METHOD

Cat. No. 5351

Helena Laboratories

The Helena GLYCO-Tek Affinity Column Method is an affinity microchromatographic methodology for the quantitation of glycated hemoglobin (GHb) in whole blood to indicate a 120-day time-averaged blood glucose level. An algorithm is provided for calculating the % HbA_{1c} from the % GHb.

SUMMARY

Accurate assessment of chronic (time averaged) glucose control is one of the major difficulties in managing diabetic patients. Even patients with mild disease may show large fluctuations in blood glucose, and single glucose determinations may correlate poorly with mean blood glucose levels. Measurement of glycated hemoglobin (GHb) has gained acceptance as a good assessment of diabetic control.[1-7] Glycated hemoglobins are all hemoglobins binding a glucose or other carbohydrate at any one of many sites on the hemoglobin molecule.[8] The term "glycated hemoglobin" has been applied in the past to certain hemoglobin fractions (HbA_1) separated on the basis of molecular charge, but these fractions do not wholly represent the glycated hemoglobins.

Glycation does not occur during biosynthesis but is a non-enzymatic, two-stage condensation of glucose with various amino groups of the hemoglobin molecule. Glycation occurs slowly throughout the life span of the mature red blood cell and is dependent on the circulating level of blood glucose. Therefore, it represents the time-averaged (chronic) blood glucose level. Two advantages are evident in measuring glycated hemoglobins: (1) a single determination can replace multiple glucose determinations performed at timed intervals and (2) glycated hemoglobin levels, determined by affinity chromatography, are not affected by the labile fraction or physical exercise.[9]

Affinity chromatography has several characteristics that increase the accuracy, specificity and linearity of glycated hemoglobin determinations.[9] The method detects all glycated hemoglobins, not just HbA_1,[8, 9] and it is not affected by moderate fluctuations in temperature and pH,[8, 9] or by the presence of abnormal hemoglobins.[10] A significant advantage of affinity chromatography is the lack of interference by "labile" glycated hemoglobin[9] and carbamylated hemoglobin (in uremic patients).[11,12] The labile GHb is a transition state in the formation of glycated hemoglobin that changes in response to acute changes in blood glucose and increases HbA, quantitations determined by cation-exchange chromatography or electrophoresis. In uremic patients there is condensation of urea-derived cyanate with the N-terminal amino groups on the beta chains of HbA. This urea-bound hemoglobin elutes with HbA_1 on cation-exchange columns producing falsely elevated results.

The Helena GLYCO-Tek Affinity Column Method minimizes limiting factors associated with other methodologies and provides a fast, simple, and accurate assessment of glycated hemoglobins.

PRINCIPLE

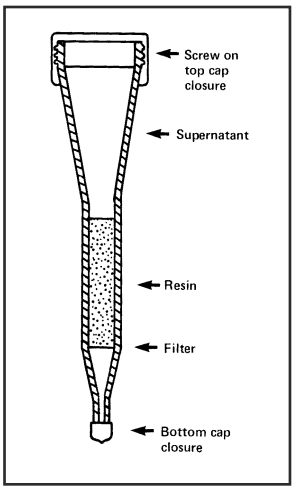
The Helena GLYCO-Tek Affinity Column Method employs an affinity column utilizing a dihydroxyboryl group bound to an insoluble cellulose resin. The unique properties of the dihydroxyboryl group include an affinity to the cis-diol groups present in many simple sugars including glucose, thus allowing separation of glycated hemoglobins from non-glycated hemoglobins.

Elution with a slightly basic developer removes non-glycated hemoglobins, carbamylated hemoglobin and the labile form of HbA_1, while retaining the glycated form while the glycated hemo-globins are eluted with a sorbitol buffer. The glycated hemoglobin value is determined by comparison of the two solutions utilizing a spectrophotometer operating at 415 nm.

The algorithm for calculating the HbA1c is a linear regression equation derived from a standardization with the reference lab for the Diabetes Control and Complications Trial (DCCT).[13]

REAGENTS

NOTE: The components provided in this kit are lot specific and cannot be interchanged with components of another kit.



1. **GLYCO-Tek Affinity Columns**
   Ingredients: Each column contains cellulose resin covalently bonded to dihydroxyboryl groups, in a low ionic strength preservative solution containing 0.1% sodium azide.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE. Refer to the Sodium Azide Warning.
   Preparation for Use: Store the columns in the dark at room temperature. Instructions for preparation of the columns for testing are provided in the Step-by-Step Method.
   Storage and Stability: The columns should be stored in the dark at 15 to 30°C and are stable until the expiration date indicated on the box.
   Signs of Deterioration: The columns should contain a dense off-white resin with a clear supernate.
2. **GLYCO-Tek Hemolysate Reagent**
   Ingredients: The reagent contains 0.05 M magnesium chloride, 2% Triton X-100, 0.1 M glycine and sodium azide as a preservative.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE ONLY. DO NOT INGEST. Refer to the Sodium Azide Warning.
   Preparation for Use: The reagent is ready for use as pack- aged. Allow to equilibrate overnight to room temperature prior to use. A water bath or running tap water may be used to equilibrate the reagent for a shorter length of time. Proper temperature should be maintained for one hour before use.
   Storage and Stability: The reagent should be stored at 2 to 6°C and is stable until the expiration date on the label.
   Signs of Deterioration: Discard the reagent if any signs of fungal or bacterial growth are present.
3. **GLYCO-Tek Developer A**
   Ingredients: The reagent contains 0.05 M magnesium chloride, 0.1 M glycine, and sodium azide as a preservative; pH 8.1-8.6.
   WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST. DO NOT PIPETTE BY MOUTH. Refer to the Sodium Azide Warning.
   Preparation for Use: Allow the reagent to equilibrate over night to room temperature before using. A water bath or running tap water may be used to equilibrate the reagent

for a shorter length of time. Proper temperature should be maintained for one hour before use.

Storage and Stability: The developer should be stored in the dark at 2 to 6°C and is stable until the expiration date indicated on the label.

Signs of Deterioration: Do not use developer if discolored or if fungal or bacterial growth is present.

4. **GLYCO-Tek Developer B**

Ingredients: The reagent contains sorbitol, buffer and 0.1% sodium azide as a preservative; pH 6.0.

WARNING: FOR IN-VITRO DIAGNOSTIC USE. DO NOT INGEST. DO NOT PIPETTE BY MOUTH. Refer to the Sodium Azide Warning.

Preparation for Use: Allow the reagent to equilibrate over night to room temperature prior to use. A water bath or running tap water may be used to equilibrate the reagent for a shorter length of time. Proper temperature should be maintained for one hour before use.

Storage and Stability: The reagent should be stored in the dark at 2 to 6°C and is stable until the expiration date indicated on the label.

Signs of Deterioration: Do not use developer if discolored, or if fungal or bacterial growth is present.

SODIUM AZIDE WARNING

To prevent the formation of toxic vapors, sodium azide should not be mixed with acidic solutions. When discarding reagents containing sodium azide, always flush sink with copious quantities of water. This will prevent the formation of metallic azides which, when highly concentrated in metal plumbing, are highly explosive. In addition to purging pipes with water, plumbing should occasionally be decontaminated with 10% NaOH.

INSTRUMENTS

A spectrophotometer capable of reading absorbance accurately at 415 nm with a range of 0.0-2.0 absorbance (Abs) must be used. The HemeSpec® Plus (Cat. No. 1103) is recommended for reading and automatically determining the % GHb.

SPECIMEN COLLECTION AND HANDLING

Specimen: Fresh, whole blood, aseptically collected into sterile venipuncture tubes containing EDTA, heparin or citrate as an anticoagulant, or packed cells are the specimens of choice. Do not use specimens collected with fluoride as an anticoagulant.

Packed Cell Preparation: Centrifuge whole blood specimen at 2000 g for 5-10 minutes. Carefully remove the stopper and aspirate all plasma and buffy coat from the cells. Thoroughly mix the red blood cells before use. Make hemolysates according to instructions in the STEP-BY-STEP section.

Interfering Substances: Hemolyzed, lipemic or icteric specimens are not recommended for use in this procedure.

Specimen Storage: The use of fresh blood samples is recommended. If necessary, specimens may be stored up to 15 days at 2 to 6°C. Storage at room temperature for more than 12 hours will cause erratic results, as will prolonged storage at 2-6°C.

Specimen Preparation: Detailed instructions for specimen preparation are included in the STEP-BY-STEP METHOD.

PROCEDURE

Materials Provided: The following materials needed for the method are provided in the GLYCO-Tek Affinity Column Kit (Cat. No. 5351).

- GLYCO-Tek Affinity Columns (50)
- GLYCO-Tek Developer A (2 x 250 mL)
- GLYCO-Tek Developer B (1 x 200 mL)
- GLYCO-Tek Hemolysate Reagent (1 x 50 mL)

Additional materials provided by Helena Laboratories but not contained in the kit:

- GLYCO-Tek Normal Control (Cat. No. 5352)
- GLYCO-Tek Abnormal Control (Cat. No. 5353)
- Quik Column® Equipment Kit (Cat. No. 5336)
  - 10 Small Collection Tubes (3 mL)
  - 10 Large Collection Tubes (15 mL)
  - Ouik Column® Rack
- HemeSpec® Plus (Cat. No. 1103)

Materials Needed but not provided:

- Deionized Water
- Disposable Pasteur pipettes
- Disposable serological pipettes (5 mL)
- Disposable glass test tubes

STEP BY STEP METHOD

IMPORTANT: Allow the GLYCO-Tek Affinity reagents to equilibrate overnight to room temperature (15 to 30°C) before testing. If the reagents are used with incomplete temperature equilibration, slow reagent flow will be observed. Perform all steps of the procedure at this temperature.

1. Obtain a Small Collection Tube and a Large Collection Tube and a GLYCO-Tek Affinity Column for each patient and control to be tested.
2. Preparation of the Patient Sample(s)
   a. Place 50 µL of packed cells or whole blood in a small disposable glass test tube.
   b. Add 400 µL (0.4 mL) GLYCO-Tek Hemolysate Reagent to the test tube.
   c. Shake or vortex the tube to ensure complete hemolysis of the sample. Excessive foaming should be avoided. Complete lysis is essential for accurate results. Samples that do not readily hemolyze should be subjected to freeze-thaw technique.
   d. Allow the sample(s) to stand at least 5 minutes but not more than 45 minutes prior to use.
3. Prepare the GLYCO-Tek Affinity Column
   a. Up-end each column twice to remove resin adhering to the top cap closure. Place the column in the Quik Column Rack.
   b. Remove the top cap closure and resuspend the resin completely using a disposable Pasteur pipette. While squeezing the bulb of the pipette, position the tip slightly above the top of the resin bed. Suck the resin in and out of the pipette as you move it down to the top of the filter in the column. Be careful not to let any of the slurry spill from the top of the column. Do not introduce air bubbles into the resin bed at this point as it may slow the flow of the eluent.
   c. Place the columns over the sink or a container and remove the bottom tip closure. Allow solution to elute and the resin to pack.
   d. When the resin has settled to a level of 1.1 cm, or the level of supernatant has drained to below the shoulder of the column, remove the liquid to the top of the resin bed with a transfer pipette.
   e. Add 3 mL Developer A to each column, and allow complete elution of the developer into the sink or a container. Complete elution of Developer A from the column in this step is critical. Failure to elute the developer completely will result in falsely low patient GHb values.

   NOTE: The technique used to add the developers to the columns is critical to accurate, reproducible results. Using a disposable serological pipette, allow about five drops of developer to drip down the wall of the column, providing a shield to prevent the disturbance of the top of the resin bed. Allow remainder of the developer to drain from the pipette down the wall and into the column.
4. After the developer has drained from the column, place the column over a Large Collection Tube (non-GHb). Do not allow the column to set dry for longer than 10 minutes.
5. Apply 50 µL of sample hemolysate prepared from packed cells, or 100 µL of hemolysate prepared form whole blood

to the top of the resin bed.

6. Allow the sample to set on the resin for 8 minutes (acceptable range 6-10 minutes). Begin the timing of the sample incubation after the addition of sample to the first column.
7. After 8 minutes incubation, add 0.5 mL GLYCO-Tek Developer A to each column, washing any hemolysate adhering to the sides of the column into the resin. Allow to elute.
8. Apply an additional 4 mL GLYCO-Tek Developer A to the column carefully avoiding any disturbance of the resin bed. Allow complete buffer elution (requires approximately 5-10 minutes). The eluent in the Large Collection Tube contains the non-glycated hemoglobins.
   Excess GLYCO-Tek Developer A applied to the column results in falsely low values. An insufficient amount of this developer results in falsely high values because the nonglycosylated hemoglobins will not be entirely eluted.
9. Adjust the volume in the Large Collection Tube to 15 mL with deionized water.
10. Place the column over a Small Collection Tube and carefully add 3.0 mL GLYCO-Tek Developer B to the column avoiding any disturbance of the resin bed.
11. Allow complete buffer elution into the Small Collection Tube (GHb). The eluent in the small tube contains all the glycated hemoglobins.
12. Invert the Large and Small Collection Tubes in such a manner that the air bubble travels completely from top to bottom of the tube and back to the top. Repeat two times for a total of three inversions.
13. Transfer collected fraction to cuvette and read cuvette immediately after last inversion.
14. Determine GHb% in each sample:
    a. Using the HemeSpec® Plus Spectrophotometer
       1) Turn the HemeSpec Plus on and allow it to warm up for at least 30 minutes prior to use.
       2) Place the HemeSpec Plus in the GHb % MODE.
       3) Zero the HemeSpec Plus by placing a cuvette filled with distilled water into the cuvette holder, and pressing the BLANK key.
       4) Fill a cuvette with the GHb fraction eluate, place it into the cuvette holder, and press the STORE key.
       5) Fill a cuvette with liquid from the non-GHb fraction tube, place it into the cuvette holder, and press the STORE key.
       6) The HemeSpec Plus automatically performs all calculations and displays the GHb percentage.
    b. Using a standard spectrophotometer
       1) Adjust wavelength to 415 nm.
       2) Zero instrument with water.
       3) Read and record the absorbance (Abs) of both the GHb and non-GHb solutions.
       4) Determine the GHb% as per instructions in the RESULTS Section.

Stability of End Product: The final test solutions are stable for 2 hours at 15-30°C.

Calibration: No calibration curve is necessary.

Quality Control: The GLYCO-Tek Normal (Cat. No. 5352) and Abnormal Controls (Cat. No. 5353) are recommended for use with each run of the GLYCO-Tek system. Refer to the package insert provided with each control for assay values. If results do not perform as expected, results should be considered invalid.

RESULTS

Calculation of the % GHb:

Following the completion of Step 13 in STEP-BY-STEP METHOD using a standard laboratory spectrophotometer, GHb results are calculated by the following formula:

$$\frac{\text{Abs. of GHb Tube} \times 100\%}{(\text{Abs. of GHb Tube}) + 5.0\ (\text{Abs. of non-GHb Tube})} = \%\ \text{GHb}$$

%GHb = percentage of glycated hemoglobins in the sample
Abs.of GHb tube = absorbance of the contents of the Small Collection Tube at a wavelength of 415 nn
Abs. of non-GHb tube = absorbance of the contents of the Large Collection Tube at a wavelength of 415 nm.

5.0 = dilution factor (15 mL or non-GHb tube/3 mL of GHb tube)
100 = percentage conversion factor

EXAMPLE: Abs of GHb tube = 0.140;
Abs of non-GHb tube = 0.411

$$\frac{0.140}{0.140 + 5.0\ (0.411)} \times 100\% = 6.4\%$$

Calculation of the % HbA_{1c}[13]:

After calculation of the % GHb, the following equation can be used to determine the % HbA_{1c}.

$$HbA_{1c} = 0.6846 \times \%\ GHb + 0.973258$$

LIMITATIONS

The results of the GLYCO-Tek Affinity Column Method may be affected by the following conditions:

1. **Hemolytic Anemia:** Erythrocytes of patients who have hemolytic anemia have a shortened life span. Because the erythrocytes are being destroyed prematurely, normal or low values for GHb may be obtained, although the time averaged blood glucose level may be elevated. Glycated hemoglobin levels can still be used to monitor the hemolytic anemia patient, but this type of patient must be monitored against himself - not against published normal or abnormal values.
2. **Temperature:** For optimum results, kit components must be equilibrated to room temperature (15-30°C) prior to use. Lower temperatures cause slower flow times. Unlike the ion exchange column method, the affinity column methodology is not significantly affected by variations in room temperature. The following chart shows actual % GHb variation with temperature variation.

Donor	15°	23°	30°
Abnormal Control	16.7%	16.6%	16.6%
Abnormal Patient	15.4%	15.1%	15.1%
Normal Control	6.1%	6.4%	6.3%
Normal Patient	6.7%	6.9%	6.7%

3. **Sample Absorption:** Failure to allow the sample to sit on the column the appropriate length of time will result in erroneous values.
4. **Drug Therapy:** At present, no data exist to indicate that drug therapy interferes with glycated hemoglobin measurements using the GLYCO-Tek Affinity Column methodology.
5. **Abnormal Specimens:** Lipemic specimens and those with elevated bilirubins can be used with this method. It is suggested that packed cells be used instead of whole blood since the nature of the specimen will cause retarded flow rates.

Polycythemic specimens may also be used without interfering with the accuracy of the test.

REFERENCE RANGE

A typical normal range obtained using the Helena methodology-for quantitating glycated hemoglobin (GHb%) was determined by Helena Laboratories. Fresh whole blood samples from 44 normal adults 18 years of age or older were quantitated according to the procedure.

Reference GHb Range = 4.3 - 7.7%

Calculated HbA_{1c} Range = 3.9 - 6.2%

Each laboratory should establish its own normal range using this Helena methodology.

INTERPRETATION OF RESULTS

In the normal individual the glycated hemoglobin comprises approximately 5 to 9% of the total hemoglobin.[3, 6] This level may more than double in the diabetic patient. Because the glucose attachment to the hemoglobin molecules occurs slowly and depends on the circulating level of blood glucose, the glycated hemoglobin level represents a time-averaged blood glucose level. There is a time lag of approximately three to four weeks before changes occurring in the blood glucose level are reflected in the glycated hemoglobin levels.

Known insulin-dependent diabetic patients usually have elevated HbA_1 levels.[2] HbA_1 quantitations on these patients may be in the 9-17% range depending on the degree of hyperglycemia. How ever, diabetic patients in good control may have HbA_1 values in the normal range. To date, there is no specific HbA_1 range that is accepted as “good” control or “poor” control. When using GHb to monitor the diabetic patient, results must be interpreted on an individual basis; that is, the patient should be monitored against himself.

PERFORMANCE CHARACTERISTICS

Comparison of % GHb

Results obtained on samples from 44 non-diabetic and 11 diabetic donors were compared to HbA_1 results obtained on Helena HbA_{1c} Automated Analyzer (HPLC). The derived correlation coefficient was 0.943.

Comparison of % HbA_{1c}

A study was performed on 47 samples (13 normal and 34 abnormal) comparing the % HbA_{1c} automatically calculated by the ColumnMate® and the manually calculated % HbA_{1c} derived from GLYCO-Tek Affinity column results. The following linear regression equation and correlation coefficient was obtained.

Y = 0.969X + 0.169	X = ColumnMate
r = 0.997	Y = GLYCO-Tek

Precision:

Precision studies were conducted to assess within-run and run-to-run, reproducibility of the GLYCO-Tek Affinity Column Methodology.

Within-Run

Within-run precision of the method was determined using a total of 4 different donor samples (two from normal donors and two from diabetic donors). Each donor sample was tested with 10 different GLYCO-Tek Columns. The coefficients of variation obtained were:

Normal samples:	N = 10,	C.V. = 2.0%
	N = 9,	C.V. = 4.7%
Diabetic samples:	N = 10,	C.V. = 1.1%
	N = 10,	C.V. = 1.7%

Run-to-Run

Run-to-run precision was determined using four different donor samples (two from a normal donor and two from diabetic donors). The coefficients of variation obtained were:

Normal Sample I: N = 30, C.V. = 3.9%
Normal Sample II: N = 14, C.V. = 4.0%
Diabetic Sample I: N = 35, C.V. = 1.5%
Diabetic Sample II: N = 45, C.V. = 2.4%

Effect of Temperature:

Samples from a normal and a diabetic donor were run at temperatures varying from 15 to 30°C. No significant differences in values were noted within this range.

Linearity:

The method is linear from GHb values of 1.5% to 33.3%.

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GLYCO-Tek Affinity Column Kit

Cat. No. 5351
GLYCO-Tek Affinity Columns (50)
GLYCO-Tek Developer A (2 x 250 mL)
GLYCO-Tek Developer B (1 x 200 mL)
GLYCO-Tek Hemolysate Reagent (1 x 50 mL)

Other Supplies and Equipment

The following items, needed for performance of the GLYCO-Tek Affinity Column Kit, must be ordered individually.

	Cat. No.
GLYCO-Tek Normal Control	5352
GLYCO-Tek Abnormal Control	5353
Quik Column® Equipment Kit (10 small and 10 large collection tubes, Quik Column® Rack)	5336
HemeSpec® Plus	1103

For Sales, Technical and Order Information, and Service Assistance, call 800-231-5663 toll free.