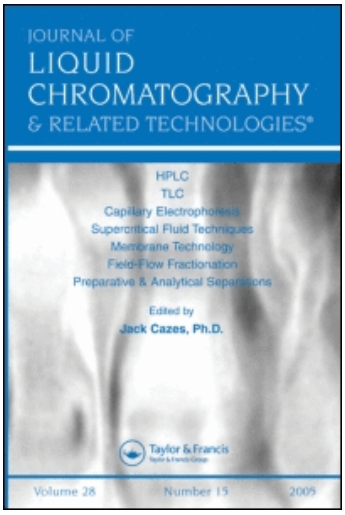

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Glycosylated Hemoglobin Determinations by Isocratic High-Performance Liquid Chromatography

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GLYCOSYLATED HEMOGLOBIN DETERMINATIONS BY ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Glycosylated hemoglobins were separated isocratically by high-performance liquid chromatography (HPLC) on a cation-exchanger (CM-300). Both the stable and the labile fractions eluted together. The labile fraction was eliminated by incubating the red blood cells at 37°C for 20 min in a acidic buffer before injecting the sample on the column.

The column plate number was found to be dependent on the amount of sample injected. The capacity factor was dependent on the type of buffer, pH and ionic strength. Controls were preserved by preparing the hemolysates in 5% ethylene glycol. The method compared favorably with a commercial disposable minicolumn method.

INTRODUCTION

The aldehyde group of glucose reacts non-enzymatically and rapidly with the amino groups of hemoglobin (Hb) to form a reversible adduct (aldamine) which rearranges slowly to form a more stable ketoamine compound, HbA_{1c} (1). Other carbohydrates give similar reactions with Hb. These Hb derivatives are

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generally known as glycosylated Hb (2). Their level is greatly increased in diabetes. Measuring the level of glycosylated Hb is very helpful in monitoring the long-term therapy in the diabetic patient (3).

Several methods including high-performance liquid chromatography have been used for separating and determining the glycosylated Hb based on charge differences of the molecules. The weak cation-exchanger Bio-Rex 70 was used previously in HPLC methods for this determination (4,5). This ion-exchanger was not designed for HPLC work. Because the particles are large (>40 μm) the column did not yield a very high plate number. The Hb peak was therefore broad and overlapped with the non-glycosylated Hb peak (5). A buffer gradient in addition to double wavelength monitoring was necessary for the test. This complex instrumentation is not quite suitable for routine assays.

Recently, cation-exchangers designed specifically for protein separation by HPLC became available from different manufacturers. These ion-exchangers have small particle size with narrow particle distribution and wide pores yielding columns with high plate number. Here, we describe the isocratic separation of glycosylated Hb on such a column without the need for complex instrumentation. Factors which affect the separation, e.g., plate number, capacity factor, and temperature are studied. We also describe the preparation of stable controls.

MATERALS AND METHODS

Instrumentation:

The liquid chromatograph consisted of a Model 970A variable-wavelength detector (Tracor Instruments, Austin, TX, U.S.A.), a Constrametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.) and a 20 µl loop injector, Model 2120 (Rheodyne, Berkeley, CA U.S.A.). The detector was set at 405 nm and 0.010 A. The flow- rate was 1.0 ml/min.

A 250 mm x 4.6 (i.d.) column was packed with a CM-300 ion-exchanger, (Synchrom, Linden, IN) 6.5 µm average particle size, in the mobile phase by the slurry packing technique.

Reagents:

1. Mobile phase: Ammonium phosphate 28 mmol/l, pH 6.85. Initially the column may require higher concentration of buffer for elution.

2. Hemolysates: A 5 µl aliquot of blood collected in EDTA tubes was hemolyzed in 4 ml ammonium phosphate-buffer, pH 4.5, 14 mmol/l. The hemolysates were incubated for 20 min at 37°C before injecting on the column to eliminate the unstable fraction of the glycosylated Hb.

3. Controls: A large aliquot of a patient's hemolysate (twice concentrated) is mixed with ethylene glycol (20:1) and stored frozen in small aliquots. It is diluted 1:1 with hemolysate buffer before use.

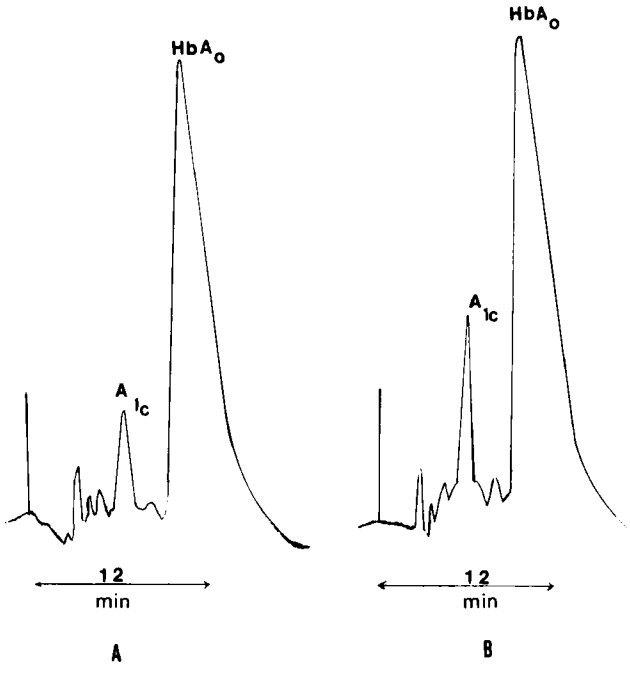

Figure 1:

Separation of the glycosylated Hb on the CM-300 at flow rate 0.7 ml/min: (A) without incubation of the red blood cells with glucose; (B) after glucose at (2000 mg/dl) is incubated with red blood cells for 1 hr. The hemolysates were injected immidiately on the column.

Procedure:

Inject 20 µl of the hemolysate on the column. Elute isocratically with the mobile phase.

Calculation:

The areas under the peaks were cut and weighed on a balance

and the ratio of the sum of all the glycosylates to the total hemoblobins were calculated.

RESULTS AND DISCUSSION

The isocratic separation of glycosylated Hb on the CM-300 column reveals several peaks, Fig. 1-A. This separation is better than the one obtained previously on the Bio-Rex 70 column with solvent gradient (5). Samples can be injected without the need for column equilibration. The need for double wavelength monitoring which is associated with the solvent gradient is eliminated.

When the red blood cells are incubated at 37°C with glucose even for short periods of time - e.g., 1 hr - a rapid increase in the height of several peaks on the chromatogram, especially HbA_{1c}, Fig. 1-B is noted. The degree of this increase depends on time of incubation, Fig. 2A. In contrast, if cell hemolysates are left for 20 min at 37°C instead of being injected directly on the column there is a gradual decline of the peaks, Fig. 2. These findings are consistent with the idea that glucose binds rapidly to hemoglobin altering the number of positive charges on the molecule; and this reaction is reversible in the acidic buffer used for hemolyzing the red blood cells (6). The fraction of the glycosylated Hb which reacts rapidly and reversibly with glucose represents the unstable fraction of aldamine. This fraction is not clinically useful. It arises from sample handling and from rapid fluctuations of glucose in the patient's

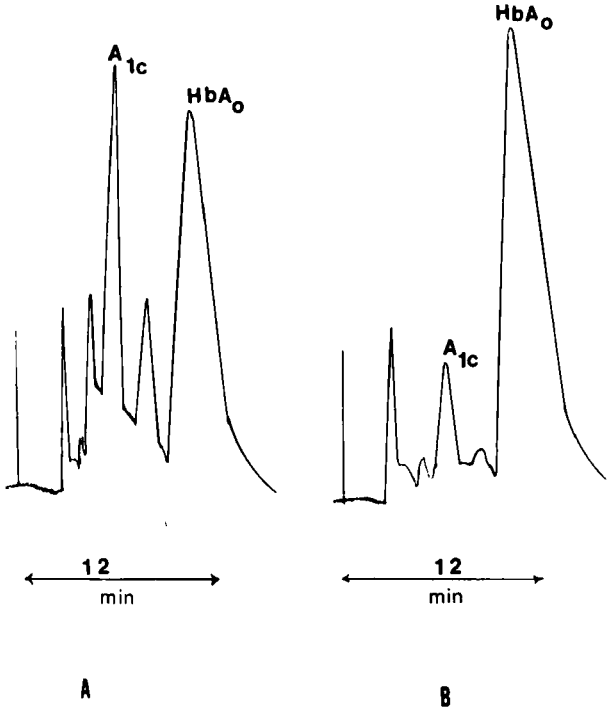

Figure 2:

A - Glycosylated Hb separation as in Fig. 1-B except the cells were incubated 24 hr. B - Same as in A except the hemolysates were kept for 30 min at 37°C before injection on the column.

blood. Unfortunately, the aldamine co-elutes with the stable fraction of the Hb (8). Thus it is necessary to incubate the patient's hemolysates in an acidic buffer for at least 20 min to eliminate this fraction (6).

The plate number (N), for the CM-300 column based on HbA_{1c} peak, is quite dependent on the amount of sample injected,

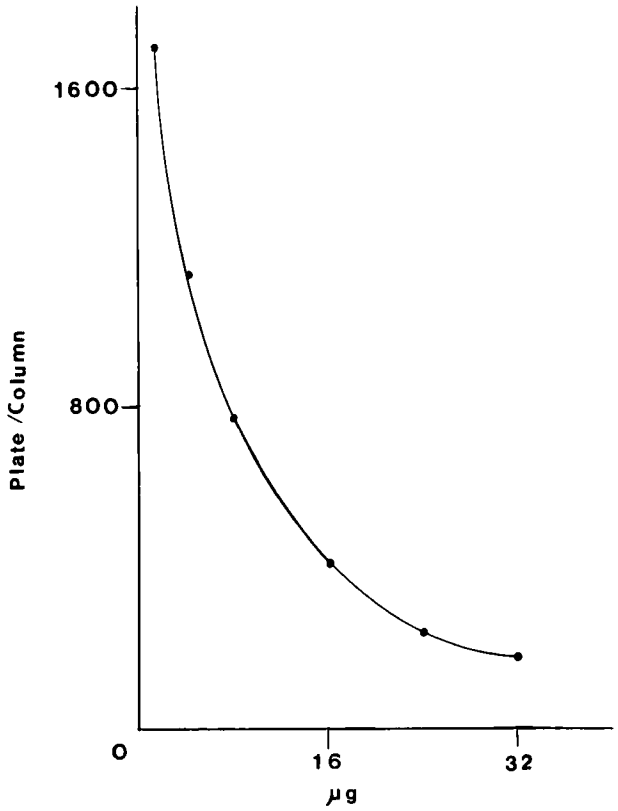

Figure 3:

Relationship of plate number of HbA_{1c} peak and the amount of Hb applied on the column as determined.

Fig. 3. Thus, in order to obtain a good separation it is necessary to inject a minimum amount of sample while operating the detector at maximum sensitivity. Injection of small amounts of sample increases the column lifetime. The present column yields about 10 times the plate number of the Bio-Rex column

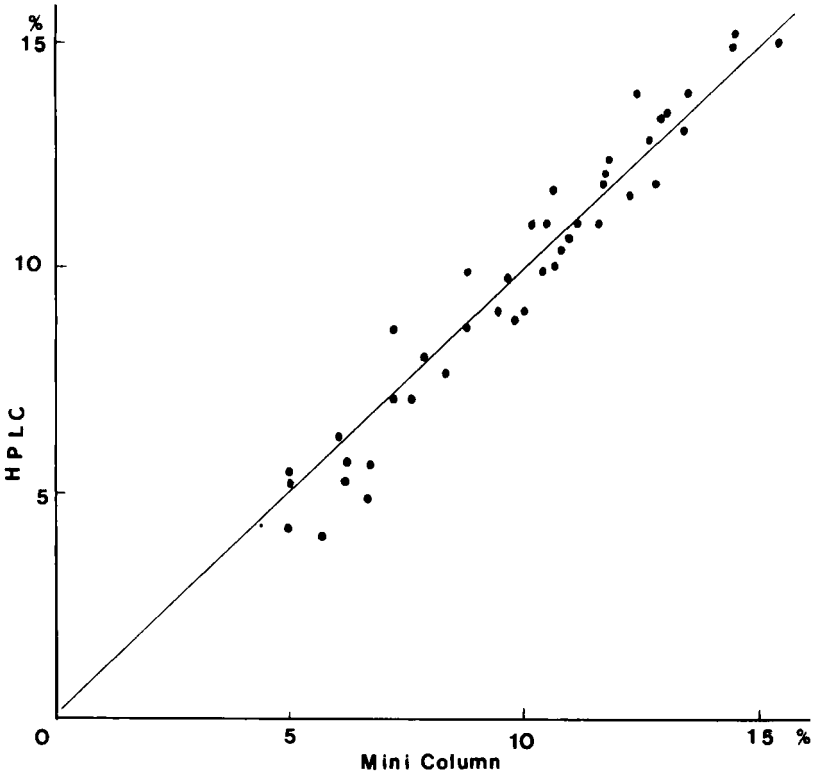

Figure 4:

The correlation of the results between CM-300 column by HPLC and the mini-column of Isolab for the glycosylated Hb. ($y = 1.26\ x + 2.8$; $r = 0.962$)

previously used (5). Flow-rates between 0.5 to 1.5 ml/min did not affect the plate number.

The within-run CV for 20 replicate determinations at a mean of 9.8% Hb is 3%. The correlation of the results of this method with that of the disposable commercial column (Isolab, Inc., Akron, OH) is 0.90, Fig. 4.

The capacity factor of HbA_{1c} decreased with increasing the ionic strength or increasing pH between 6 to 7.5. The potassium salts of the phosphate buffer gave the same capacity factor as the ammonium salts; however, the sodium salts decreased the capacity factor.

Column temperature between 25 and 40°C did not affect the percentage of glycosylated Hb but did affect the separation speed. For routine assays the separation was performed at room temperature.

Hemoglobin variants other than HbA elute at different capacity factors. The monozygotes will be recognized by the absence of HbA_o while the heterozygotes will have reduced HbA_o (about half of the concentration) relative to the other patients on the chromatograms.

Stable controls are very important in every assay. Hemoglobins are known to be unstable (8). They undergo continuous denaturation even when frozen. Several agents were added to the hemolysates in an attempt to preserve the glycosylated Hb as well as the total Hb. In the absence of any preservative, total Hb denatured and did not go back into solution giving low peak areas, (Fig. 5) and erratic values for the glycosylated Hb. However, we found that when ethylene glycol was added at 5% to the hemolysate, both the glycosylates and the total fractions were more stable. At this time, we have not studied the effect of different concentration of ethylene glycol or the effects of long-term storage.

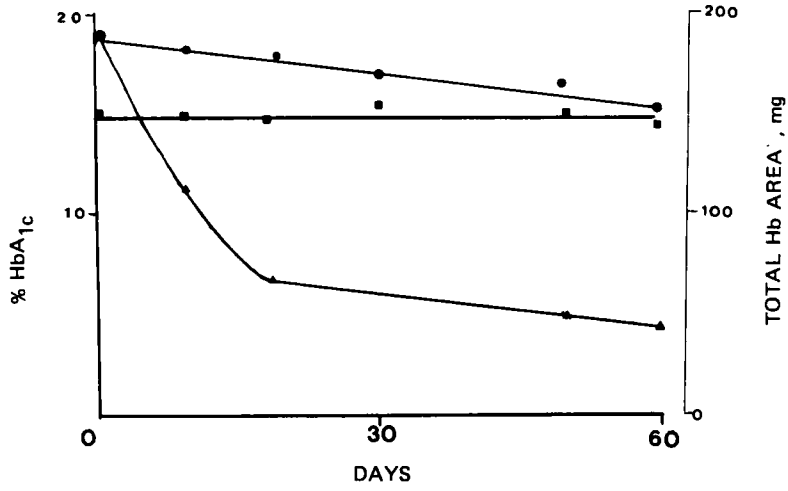

Figure 5:

The effect of ethylene glycol on % HbA_{1c} and total HbA_o after storage at -20°C:%HBA_{1c} (■——■) and total HBA_o (●——●) preserved with 5% ethylene glycol. Total HBA_o in absence of ethylene glycol (▲——▲).

Determination of glycosylated Hb on the CM-300 column is rapid (less than 10 min at flow-rates of 1.0 ml/min) and eliminates the need for solvent gradient and column equilibration after each run. In addition the column can be used for determination of other proteins.

ACKNOWLEDGMENT

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