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CATION-EXCHANGE, HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF HEMOGLOBIN A_{1c}

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SUMMARY

We describe a rapid, simple, and sensitive high-performance liquid chromatographic method for the determination of hemoglobin A_{1c} with a bonded-phase cation-exchange column. About five measurements are obtained per hour on small quantities of whole blood. Sample preparation requires no centrifugation or washing of cells. The results correlate well ($r = 0.918$, $n = 51$) with a commercially available, disposable mini-column kit. The results are insensitive to small changes in pH and temperature. Variants arising from hemoglobinopathies display different chromatographic profiles.

INTRODUCTION

The determination of the percentage of hemoglobin (Hb) A_{1c} or glycosylated Hb (A_1 or $A_{1a} + A_{1b} + A_{1c}$) has recently assumed considerable interest because the level of glycosylated Hb is proportional to the integrated, long range (approximately 12 weeks) level of blood glucose. It is, therefore, a measure of diabetic control and therapy. The normal range for glycosylated Hb is from 5 to 8% and results can be two to three times higher for diabetics. The normal range varies with the method.

HbA_1, sometimes called fast hemoglobin, has been determined by electrophoresis[1], by isoelectric focusing[2], by spectrophotometry[3], by column chromatography[4] (a classical reference procedure), recently by mini-column, cation-exchange methods, *e.g.*, ref. 5 and very recently by affinity chromatography[6]. Many of these procedures were compared very recently[7].

Davis *et al.*[8] improved on the method of Trivelli *et al.*[4] which used a macroscale ion-exchange column, by adapting the procedure to high-performance liquid chromatography (HPLC). They used a cation-exchange resin and elution with a step-gradient. This procedure greatly reduced analysis time; however, the cation-exchange resin used could not sustain high back-pressures, and the larger particles used adversely affected resolution. Recently, intact hemoglobin proteins were separated with good resolution on 5–10 μm, bonded-phase, anion-exchange columns[9]; but the elution order on anion-exchange packings does not permit isolation of HbA_1 which is eluted last.

With the recent commercial availability of microparticulate, bonded-phase,

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cation-exchangers, we have developed a rapid, simple and precise procedure for the separation and quantitation of HbA_{1c}.

EXPERIMENTAL

Apparatus

All liquid chromatography was performed on a Spectra-Physics Model 8700 ternary gradient solvent delivery system (Spectra-Physics, Houston, TX, U.S.A.) equipped with a 50-μl Rheodyne Model 7125 injection loop.

The chromatographic column, 180 × 4.1 mm, was made from precision-bore stainless-steel tubing (Alltech, Houston, TX, U.S.A.) and was slurry-packed in isopropanol with Synchropak 5–10 μm cation exchanger, CM-300 (SynChrom, Linden, IN, U.S.A.), using a Micromeritics Model 705 slurry-packer (Micromeritics, Norcross, GA, U.S.A.).

Absorbance was measured with a Model 440 dual channel detector equipped with 405-nm filters and phosphors (Waters Assoc., Milford, MA, U.S.A.). Detectors 1 and 2 were set at attenuations of 0.5 and 1.0 a.u.f.s., respectively. The response from each detector was acquired with the analogue-to-digital converter of a DEC LAB 11/V03 computer (Digital Equipment, Maynard, MA, U.S.A.). These data were reduced and plotted with the DEC GT-46 system previously described[10].

Reagents

The weak buffer was 0.01 *M* $NaH_2PO_4 \cdot H_2O$, 0.004 *M* Na_2HPO_4, pH 6.4 (buffer 1) and the strong buffer (buffer 2) was 0.03 *M* $NaH_2PO_4 \cdot H_2O$, 0.012 *M* Na_2HPO_4, 1 *M* NaCl adjusted to pH 6.4.

Sample preparation

Samples of whole blood (EDTA anticoagulant) obtained from the Clinical Laboratory at the University of South Alabama Medical Center were refrigerated until analyzed (within 2 days). These samples were hemolyzed (1:10) with 0.0016 *M* KCN to which 1.0 g Triton X per liter had been added. Further dilution, normally 1:50 (total dilution 1:500), with glass-distilled water provided suitable concentrations for the absorbance detection. No additional sample preparation was necessary.

Identical samples were analyzed by the Clinical Laboratories at the University of South Alabama Medical Center by the Sigma procedure[5] for the quantitative determination of glycohemoglobin (HbA_1) at 415 nm to establish a correlation with the HPLC method.

Procedure

A 5%/min gradient, beginning with 100% buffer 1 at a flow-rate of 2.0 ml/min, was passed through the column for 7 min and then, by returning to 100% buffer 1 for at least 5 min, the column was equilibrated. Immediately after the injection of 50 μl of each hemolyzed and diluted sample at ambient temperature (except in the temperature study), the 5% gradient was started. Responses generated by the absorbance detectors were treated as previously described[10].

RESULTS AND DISCUSSION

A typical chromatogram obtained from the hemolyzate of a normal individual is shown in Fig. 1. Chromatograms of samples from diabetic patients in most cases are similar, but with a larger percentage of the total area shown under the HbA_{1c} peak. The upper tracing of Fig. 1 is the response observed with detector 1 (0.5 a.u.f.s.), and the lower tracing is the response from detector 2 (1.0 a.u.f.s.). Two methods were used to calculate the percentage of HbA_{1c}; in both cases the total area was measured with detector 2. The most reproducible results came from the integration of the HbA_{1c} area from detector 2 response, even though it would appear that the precision of the relative HbA_{1c} area should improve with higher sensitivity. In several trials the sensitivity of detector 1 was increased even further with no noted improvement. The coefficient of variation, C.V., for $\%HbA_{1c}$ in "normal-range" samples is 2.7% (within analysis, $n = 20$) when the data from detector 2 were used, but 5.4% ($n = 20$) when detector 1 was used. For samples from diabetic patients the C.V. values were 3.0% ($n = 20$) and 0.7% ($n = 20$), respectively. The accuracy of the method is difficult to assess because standards are not available; however, the results agree with secondary control pools as prepared by the Clinical Laboratories; their C.V. is about 6% overall.

Fig. 2 is a scattergram of the HbA_{1c} results obtained with this HPLC method, all areas totally determined from detector 2 response, compared to the column method for routine assay of total HbA_1 (glycohemoglobin) in the Clinical Labora-

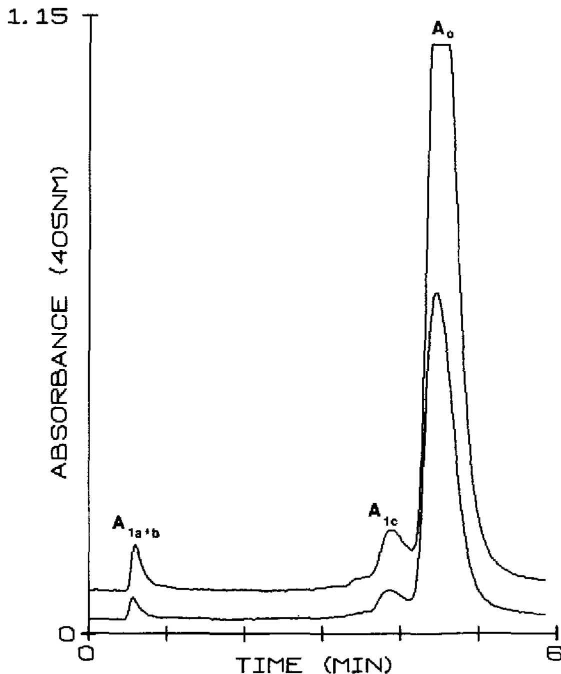

Fig. 1. Typical chromatogram of a hemolyzate from a normal individual (6.4% HbA_{1c}). The upper tracing was measured at detector 1 (0.5 a.u.f.s.) and the lower tracing was measured at detector 2 (1.0 a.u.f.s.). The traces are offset for clarity. The calculation method is described in the text.

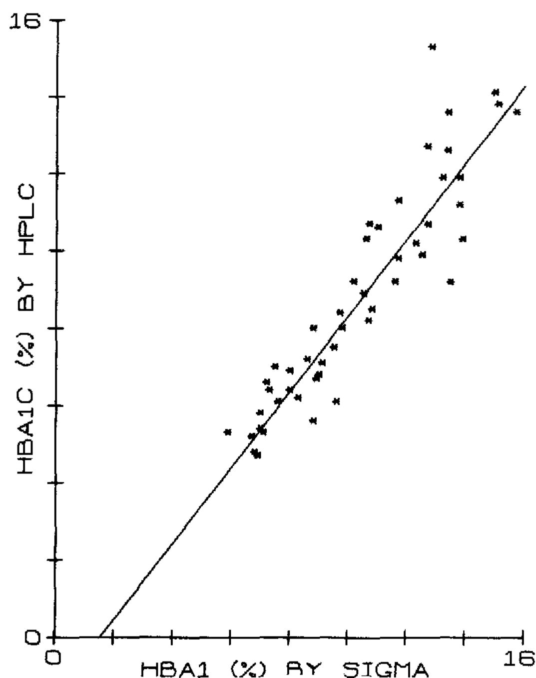

Fig. 2. Scattergram and regression line of the results of our method compared with the Sigma kit method[5]. The regression line equation is described in the text.

tories. The regression line has the equation $y = -(1.50 \pm 0.65) + 0.988x$. The correlation coefficient is 0.9182 with a standard error of the estimate of 1.11 for $n = 51$. The regression equation obtained when using the HbA_{1c} area from detector 1 is $y = -(1.08 \pm 0.76) + 1.006x$. The correlation coefficient is 0.9025 with a standard error of the estimate of 1.19 for $n = 44$. The negative intercept results because the mini-column method measures total HbA_1 (the percentage of HbA_{1a} + HbA_{1b} is typically 2.4%[11]). Other small sources of difference may be attributed to the 405-nm phosphor and filter used with the Model 440 detector; 415 nm is not available. Because both methods of calculation correlate well with the Sigma method (and with each other) ($r = 0.98$ for $n = 52$), it is not necessary to use a dual-channel detector or a single-channel detector at different attenuations[8].

Mini-column, ion-exchange methods for glycosylated hemoglobin are notorious for a large dependence on temperature and buffer pH. To investigate these variables, the column temperature and eluent pH were varied. As the column temperature was varied from 40 to 20°C (ambient ≈25°C), the percentage of HbA_{1c} increased only from 9.9 to 10.0% ($n = 3$) when integrated detector 1 data were used and from 8.9 and 9.1% ($n = 2$) for detector 2 data. As the pH was increased from 6.3 to 6.5 (normally 6.40), the percentage of HbA_{1c} decreased from 10.7 to 10.2% ($n = 3$) using detector 1 and decreased from 9.5 to 9.2% ($n = 2$) using detector 2 data. (The samples are 10.2 and 9.2%, respectively at pH = 6.5.) These data indicate that the HPLC method is relatively insensitive to large changes in temperature and pH; therefore, changes due to typical laboratory variations of temperature and pH should fall well within the C.V. of this method.

Another common problem occurring in ion-exchange determinations of HbA_{1c} or HbA_1 is the interference by the labile, aldimine form or pre-HbA_{1c}[12] and HbF, both of which are eluted together with HbA_{1c}. We have been unable to investigate the effect of HbF at present; however, we have investigated the effect of the labile pre-A_{1c}. We incubated normal hemolyzates with 10 g/l glucose for 4 h and noted no difference (within the C.V. of the method) between unincubated and incubated hemolyzates. We believe that the pre-A_{1c} may be eluted together with HbA_{1c} (note the very small peak immediately before A_{1c} in Fig. 1), but its effect is minimized by the integration algorithm that in effect strips off the A_{1c} area. More elaborate procedures to remove or study the effects of pre-A_{1c} such as treatment with semicarbazide and aniline[13], or dialysis[14] from washed red cells were not used in order to keep sample treatment simple.

Hemolyzates of samples from patients with variant hemoglobinopathies were chromatographed by this method; an HbSF variant is shown in Fig. 3. Presently we have not examined sufficient samples to characterize the different profiles for normal and other hemoglobinopathies. We shall report on these findings after more data are obtained.

Fig. 1 illustrates that under the chromatographic conditions described above, a sample may be chromatographed in about 7 min. With an equilibration time of 5 min, it should be possible to process five samples per hour, especially when a whole blood specimen can be prepared during the 5-min equilibration period. Although we have used an elaborate detection and data reduction system, a single detector that

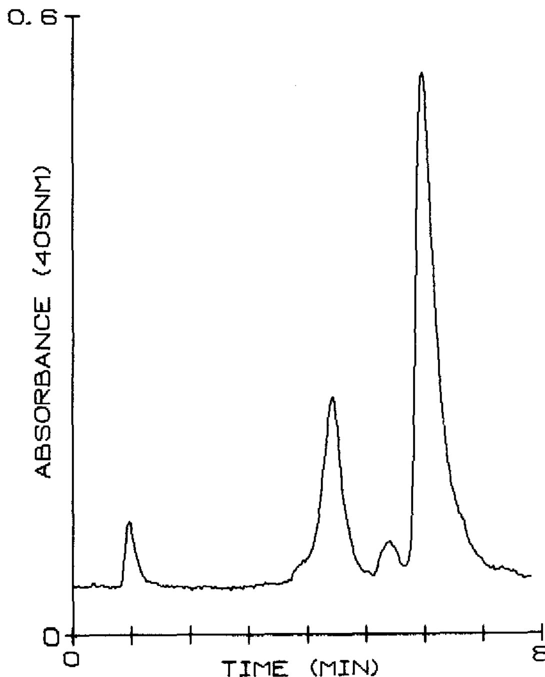

Fig. 3. Chromatogram of a hemolysate from a patient with a hemoglobin SF variant. Note that the pattern differs from Fig. 1. Peak assignments have not been made.

will operate in the 400–425 nm range coupled with a simple integrating recorder or a recorder and planimeter[7] should provide comparable results.

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