Quantitation of Hemoglobin A\textsubscript{1a+b} and Hemoglobin A\textsubscript{1c} by Automated “High-Performance” Liquid Chromatography

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The glycosylated hemoglobin A\textsubscript{1a+b} and A\textsubscript{1c} have been rapidly and precisely quantitated in 5-µL samples of human blood hemolysate (~240 µg of hemoglobin) by cation-exchange column chromatography. Total chromatographic time is 22.0 min. Proportions of Hb A\textsubscript{1c} range from 3.85 to 6.71% in normal individuals and from 4.23 to 19.90% in diabetic subjects. Within-day variation was 1.58 and 1.10% for mean Hb A\textsubscript{1c} proportions of 4.92 and 10.32%, respectively. Hb A\textsubscript{1c} and Hb A\textsubscript{1} are stable in hemolysates stored at 4 °C for as long as seven days, and indefinitely under liquid nitrogen.

The concentration of the glycosylated hemoglobin (Hb A\textsubscript{1a+b}, Hb A\textsubscript{1c}) reflects the integrated state of carbohydrate metabolism of an individual during the preceding four to six weeks. Quantitation of Hb A\textsubscript{1c} is suggested (1–4), not only for the diagnosis of diabetes, but also as a means of monitoring glucose metabolism in the diabetic with the expectation that establishment of effective metabolic control as monitored by this assay would reduce the incidence of secondary sequelae associated with the diabetic state.

Previous methods are either too time-consuming to support clinical protocols or do not permit the Hb A\textsubscript{1c} fraction to be separately quantitated.

Materials and Methods

Reagents


Phosphate buffer, pH 6.42, and 50 mmol/L cyanide-phosphate buffer, pH 6.70, are prepared according to Travelli (5). The resin is equilibrated in the cyanide-phosphate buffer before column packing (5).

Apparatus

Automatic Sample Injector, equipped with a 5-µL loop (The Upjohn Co., Kalamazoo, MI 49001).


Model 970A Variable Wavelength Detector (Tracor, Inc., Austin, TX 78766).

CSI-3 Programmable Computing Integrator (Columbia Scientific Ind., Austin, TX 78706).

Water-jacketed glass column, 0.6 × 15-cm (Glenco Scientific, Houston, TX 77007).

Procedure

Whole-blood specimens (treated with ethylenediaminetetraacetic acid anticoagulant) are kept at 4°C up to 24 h. The specimen is processed into stroma-free hemolysate and dialyzed according to Drabkin (5). A 5-µL sample of hemolysate equivalent to ~240 µg of hemoglobin (range, 125–280 µg) is injected and chromatographed on the cation-exchange resin packed to a column height of 11.0 cm. Hb A\textsubscript{1a+b} and Hb A\textsubscript{1c} are eluted sequentially within 6.4 min with the cyanide-phosphate buffer, at a constant flow of 3.0 mL/min. Hb A\textsubscript{1} is eluted with the phosphate buffer, pH 6.42. The eluents are monitored at 410 nm, 0.08 A full scale, and the area under each curve is integrated and expressed as a percentage of total hemoglobin eluted. A solenoid valve activated by a programmable time-event sequence of the computing integrator makes the appropriate buffer changes at 4.2 and 15.6 min. The retention times for Hb A\textsubscript{1a+b}, Hb A\textsubscript{1c}, and Hb A\textsubscript{1} are 0.9, 2.5, and 9.7 min, respectively. Total chromatographic time is 22.0 min. The precision of this assay is enhanced by maintaining room and column temperature at 22 °C.

Subjects

Our “normal” subjects were diabetes-free volunteers whose values for serum glucose during fasting ranged from 700 to 1200 mg/L and who had no familial history of diabetes. The data on diabetic patients were obtained for samples from previously diagnosed diabetic subjects who were either currently being treated or were enrolled in clinical protocols designed to test the efficacy of various drugs as metabolic-control agents.

Results

Precision. Within-day variation was 1.58 and 1.10% for Hb A\textsubscript{1c} values of \( \overline{x} = 4.921 \pm 0.0778 \) (SD), \( n = 10 \), and \( \overline{x} = 10.3210 \pm 0.1133 \) (SD), \( n = 10 \), respectively. Between-day variation for a normal subject, assayed in triplicate each day during the previous 24 months, was 5.7% for Hb A\textsubscript{1c} \( \overline{x} = 4.525 \pm 0.2621 \), \( n > 1000 \). Duplicate analysis of 100 diabetic samples indicated no statistically significant variation (Student’s paired t-test) from the original analysis.

Stability. Hb A\textsubscript{1c} and Hb A\textsubscript{1} (Hb A\textsubscript{2a+b+c}) are stable for 24 h in whole blood if kept at 4 °C (\( p > 0.05 \)), but the concentration of Hb A\textsubscript{1a+b} significantly increases (\( p < 0.01 \)). A delay of 48 h or more in processing the whole-blood specimen signifi-
Clinical studies have shown a relationship of Hb A1c to carbohydrate control in diabetic subjects (1-5), in part because of tightly controlled assay conditions and sample preparation. The variation inherent in many of the "fast" systems, coupled with sample instability, particularly of the Hb A1a + b fraction, may obscure that relationship. The more precise quantitation of Hb A1c by the method we describe fills the requirements for the uses mentioned in the introduction.

Added in proof: While this paper was in revision, a similar methodology was reported by Dunn et al. (11).

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References