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Note

Enhanced high-performance liquid chromatographic resolution of hemoglobin A_{1c} at low temperatures

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Hemoglobin A_{1c} is a glycosylated hemoglobin which shows elevated levels in the hemolysates of uncontrolled diabetics [1,2], and provides a means for monitoring patient compliance.

Schnek and Schroeder [3] devised a liquid chromatographic method which was further developed by Trivelli et al. [4] and Schwartz et al. [5]. These methods had the disadvantage of being time-consuming. Faster high-performance liquid chromatographic methods (HPLC) were developed by Davis et al. [6], Cole et al. [7] and Dunn et al. [8]. While these methods are fast and provide adequate separation, they suffer from a relatively short column life; they employ minus 400 mesh Bio-Rex 70 packed in glass columns and operate at room temperature. On repeated use, column operating pressure would rise and cause either destruction of the resin or bursting of the columns. Use of coarser Bio-Rex 70 in HPLC columns at room temperature results in poorer resolution.

Our laboratory has modified the HPLC method of Davis et al. by utilizing an optimum of 200—400 mesh Bio-Rex 70 resin at column temperatures approaching 0°C. The coarse resin has prolonged the lifetime of the columns without any significant rise in the operating pressure. The low column temperature provided an excellent resolution of hemoglobin A_{1c} with a coarse resin which would otherwise give poor performance at elevated temperatures.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatograph consisted of two Model 110 A

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pumps, a Model 421 controller, an Hitachi 100-40 spectrophotometer with an Altex flow cell (Beckman, Fullerton, CA, U.S.A.). The output was displayed on an Altex Model C-RIA.

Temperature control of the water-jacketed glass column was achieved using a thermostatically controlled circulation pump, Polytemp Model 80 (Polyscience, Niles, IL, U.S.A.).

Reagents

Reagent grade $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, and KCN (Fisher Scientific, Springfield, NJ, U.S.A.) were used to prepare low ionic strength buffer: 4.55 g of $NaH_2PO_4 \cdot H_2O$, 1.18 of g Na_2HPO_4 and 0.64 g of KCN were dissolved in distilled water and brought up to a total volume of 1 liter. The pH was then adjusted to 6.7 at 22°C.

The high ionic strength buffer of Trivelli et al. [4] was prepared by dissolving 14.35 g of $NaH_2PO_4 \cdot H_2O$ and 6.52 g of Na_2HPO_4 in water and diluting to 1 liter. The pH was then adjusted to 6.4.

Column

A jacketed 1-cm I.D. Altex glass column (Beckman) was packed with 200—400 mesh Bio-Rex 70 (Bio-Rad Labs., Richmond, CA, U.S.A.) replacing the minus 400 mesh resin used by Davis et al. Resin height was between 8 and 9 cm. The columns were conditioned by eluting a sample of hemolyzed blood through the column and storing the column overnight, filled with high ionic strength buffer. Reconditioning of the column was carried out on each day of use.

Preparation of sample

Red blood cells were separated from plasma, washed twice in saline, and hemolyzed with 20 vol of distilled water. The hemolyzate was centrifuged for 20 min at 5000 *g* and the supernate analyzed by HPLC.

Assay

After conditioning the column, low ionic strength buffer was pumped through the column at 2 ml/min for 10 min prior to sample injection and continued until hemoglobin A_{Ic} had been eluted. This was followed by pumping high ionic strength buffer at the same rate to elute unglycosylated hemoglobin. Since the retention time for hemoglobin A_{Ic} varied with temperature, the shift from low to high ionic strength buffer was conducted at varying times. Experiments were performed on the day after fresh columns were packed. Since retention times and theoretical plates varied slightly from day to day, smooth curves could not be obtained from observations taken several days apart.

RESULTS AND DISCUSSION

The use of 200—400 mesh Bio-Rex 70 eliminated pressure buildup and column rupture experienced with minus 400 mesh resin. Our column operates consistently at 100—200 p.s.i. Lowering the column temperature from 37.5°C to 0°C also brought about partial separation of hemoglobins A_{1a} and A_{1b} which became more pronounced at lower temperatures (Fig. 1A and B) and effected

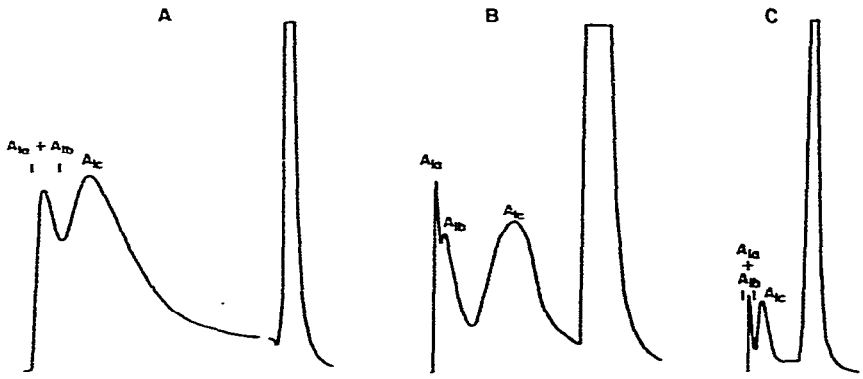

Fig. 1. Separation of hemoglobins A_{1a}, A_{1b} and A_{1c} at: (A) 37.5°C, utilizing column packed with 200—400 mesh Bio-Rex 70; (B) 0°C, utilizing column packed with 200—400 mesh Bio-Rex 70; (C) room temperature, utilizing column packed with minus 400 mesh Bio-Rex 70.

resolution of hemoglobin A_{1c} comparable to that obtained with another column packed with minus 400 mesh and used at room temperature (Fig. 1C).

Lowering the temperature of the column packed with 200—400 mesh resin also increased the retention time for hemoglobin A_{1c} from 3 to 28 min (Fig. 2). Theoretical plate numbers were calculated by measuring peak half-widths for hemoglobin A_{1c}. It was found that plate number increased sharply as the jacket temperature approached 0°C (Fig. 3). Although peak widths increased at low temperatures, longer retention times offset this negative effect as the temperature dropped. A minimum number of theoretical plates is observed at approximately 20°C in this study; two earlier studies with freshly packed columns also yielded minima in this region.

Preliminary results indicated normals average 5.65 + 0.7% of hemoglobin

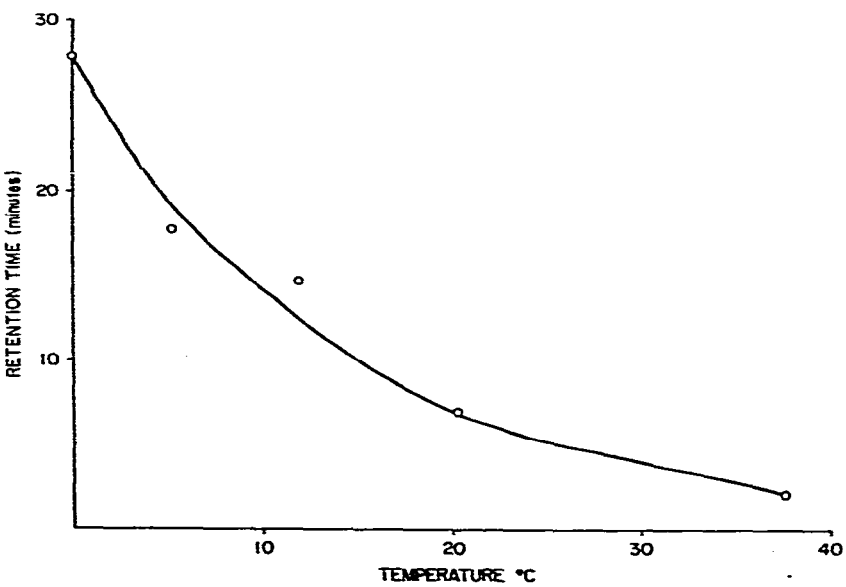

Fig. 2. Retention time of hemoglobin A_{1c} versus temperature.

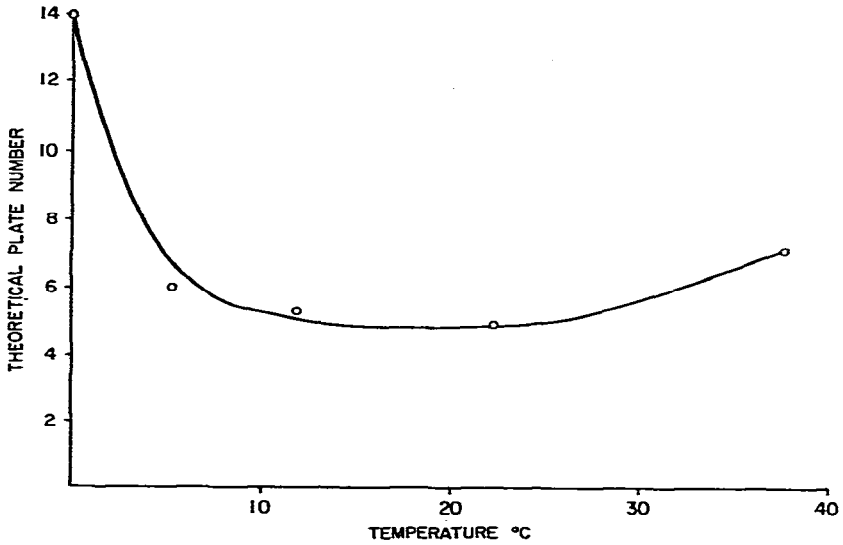

Fig. 3. Theoretical plate number versus temperature. Peak half-widths of hemoglobin A_{1c} were used to calculate plate numbers.

A_{1c} and poorly controlled diabetics average 12.4 + 0.9%. This column provides good resolution of hemoglobin A_{1c} while avoiding the high pressures associated with minus 400 mesh resin which may burst the glass column.

On the basis of our present results we make the following recommendations for the assay of hemoglobin A_{1c}: (1) In order to maintain a reliable long-lived column, we recommend substitution of 200—400 mesh Bio-Rex 70 for the minus 400 mesh resin used by Davis et al. [6]. (2) We recommend a low column temperature of 0°C in order to obtain adequate separation of hemoglobin A_{1c} with this column. This temperature may be achieved easily by immersing the column in a mixture of water and crushed ice.

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