In this liquid-chromatographic assay for hemoglobin A₂ (Hb A₂) in human blood, the blood samples are hemolyzed by dilution and the hemolyzates are chromatographed on a weak cation-exchanger with a solvent gradient of a buffer with two different ionic strengths. The absorbance of the effluent is monitored at 405 nm. The retention time of Hb A₂ is less than 10 min. The mean within-assay CV was 1.6%, the between-assay CV 4.9%. I describe the effects of various column dimensions and sample preparation methods on the separation of Hb A₂. The normal reference interval (for 54 healthy adults) was 1.3 to 3.0%. A comparison between results by a minicolumn method and by the present method is performed. Rapid and reproducible, this method is suitable for use in routine determination of Hb A₂.

Additional Keyphrases: reference intervals, erythrocytes, screening for β-thalassemia trait, anemia, chromatography, cation-exchange

In human erythrocytes hemoglobin A₂ (Hb A₂) accounts for 1.4 to 3.2% of the total hemoglobin (I). Quantification of Hb A₂ in blood is a useful diagnostic tool in the genetic disorder of β-thalassemia, which represents an imbalance in α and β chain synthetic ratios. In β-thalassemia, β chain production is decreased, resulting in anemia (2). The detection of an abnormally high proportion of Hb A₂ is widely regarded as diagnostic for carriers of the trait, and quantification of Hb A₂ is important for accurate diagnosis of anemias caused by this disorder.

The method most widely utilized for Hb A₂ assay has been electrophoresis on cellulose acetate at an alkaline pH (3). Later, ion-exchange micro-chromatographic procedures became increasingly important, and the use of an anion-exchanger for this purpose was presented (4). This method was modified (5) and the improvements were reflected in commercially available kits. Cation-exchange column microchro...
Here I describe a liquid-chromatographic method involving a weak cation-exchanger, PolyCAT A, for determination of human Hb A₂. The present assay is based on the method of Ou et al. (10) for human hemoglobins, but I have especially modified it for the fast determination of Hb A₂. The practicality of the resulting procedure makes it suitable as a routine method for screening for β-thalassemia trait.

Materials and Methods

Apparatus. The chromatographic system consisted of a Model 5500 liquid chromatograph combined with a Vista 402 chromatographic data system (Varian Associates, Walnut Creek, CA). I used 100 × 4.6 mm, 70 × 4.6 mm, and 50 × 4.6 mm columns of PolyCAT A, 5-μm-diameter particle size. The column packing material was from PolyLC, Columbia, MD, and the columns were packed by Kaukomarkkinat, Espoo, Finland. Disposable filter units, Milllex-HV 0.45-μm (av pore size), were from Millipore, Yonezawa, Japan. "Sickle-thal quick column™" kits (cat. no. 5334) were from Helena Laboratories, Beaumont, TX.

Reagents. 2-[(2-hydroxyethyl)amino]-1-(hydroxy-methyl)-1,3-propanediol ("Bistris") was from Sigma Chemical Co., St. Louis, MO. Hemoglobin controls (cat. no. 5328) containing hemoglobins A₂ and A₂ were from Helena Laboratories. Triton X-100 detergent was from Koch-Light Laboratories Ltd., Colnbrook, Berks., U.K. Buffer A (pH 6.8) contained 40 mmol of Bistris and 4 mmol of KCN per liter. Buffer B (also pH 6.8) contained the same plus 200 mmol of sodium acetate per liter. These buffers were filtered through a 0.22-μm (av pore size) filter before use. All other chemicals were of analytical grade.

Sample treatment. Collect blood by venipuncture, using EDTA as anticoagulant. I used four different sample preparation methods: (a) Mix the blood sample and pipet 1 mL of blood into 5 mL of isotonic saline (0.15 mol/L NaCl), mix, and incubate for 18 h at 37 °C. Wash the erythrocytes twice with isotonic saline, then centrifuge the suspensions at 4000 × g for 10 min. Hemolyze the cells by adding 500 μL of 32 mmol/L KCN to 400 μL of cells, mix, and to 800 μL of the hemolyzate add 200 μL of CCl₄, mix for 2 min, and centrifuge (6000 × g, 15 min). To 50 μL of the supernate add 950 μL of buffer A and filter through a disposable 0.45-μm pore-size filter unit. (b) As in method a, but with no incubation. (c) To 40 μL of mixed blood sample add 1 mL of a 0.5 mL/L solution of Triton X-100 in buffer A; mix well. Centrifuge at 7000 × g for 2 min and filter through a disposable 0.45-μm pore-size filter unit. (d) As in method c, but use only 5 μL of blood.

Chromatographic conditions. Inject 15 μL of the filtered hemolysate into the chromatographic system. Use a linear gradient consisting of buffers A and B, at a flow rate of 1 mL/min at room temperature (Table 1). The detection wave-length is 405 nm, the detector range 0.2 A full-scale, and the chart speed 0.5 cm/min. With the Vista 402 data system the attenuator setting was 64, corresponding to 0.128 A full-scale.

Results

Choice of column. I optimized the solvent gradients for the three above-mentioned columns and found that the gradients detailed in Table I gave the best separations. The separation was very similar with each column (Figure 1). In all cases Hb A₂ was well resolved from the other peaks. The smallest column is the most preferable for use in routine determinations of Hb A₂ because of its good separating properties and short analysis times. Using the 50-mm column, I analyzed a control (2.6% Hb A₂), a normal (2.5%), and a high-concentration (4.6%) Hb A₂ sample. The control sample was included to confirm the identity of the Hb A₂ peak. The peaks for Hb A₂ and Hb A₀ were well resolved in all three types of sample, eluting at 8.5 and 5.8–6.3 min, respectively.

Sample preparation. I evaluated the effect of the labile glycated hemoglobin fraction on the determination of Hb A₂. In sample-preparation method a, the labile fraction of glycated hemoglobins was removed by incubating the sample overnight at 37 °C. In method b it was not removed. In methods c and d the sample was hemolyzed only by dilution. When a sample with a high concentration of Hb A₂ was assayed by each of the four sample-preparation techniques, no major differences were found, the mean Hb A₂ percent-ages for three different determinations being 4.4, 4.5, 4.3, and 3.9%, respectively, by methods a–d. Evidently sample-preparation methods a–c are equally applicable; method d gives somewhat lower values than the other methods.

Precision. I evaluated the precision of the method at two concentrations of Hb A₂. The within-assay CVs were 0.92% (n = 10, ̅x = 2.2%, SD = 0.021%) and 2.2% (n = 10, ̅x = 4.0%, SD = 0.090%); the mean CV for the two was 1.6%. The between-day CV for 1.4% Hb A₂ was 5.3% (n = 5, SD = 0.072%) and for 4.5% Hb A₂ it was 4.4% (n = 5, SD = 0.19%); the mean CV for the two was 4.9%.

Reference values. I determined the proportion of Hb A₂ in normal blood by using samples from 54 apparently healthy hospital personnel. Values ranged from 1.3 to 3.1% (mean 2.15%, SD 0.43%). The normal reference interval for Hb A₂, mean ± 2 SD, was 1.3–3.0%.

Comparison with a minicolumn method. I determined the correlation between results by a minicolumn procedure (x) for Hb A₂ ("Sickle-thal quick column™" method) and those by the present assay (y) for seven patients' samples. The linear correlation was described by the equation y = 0.886x − 0.328 (r = 0.985).

Discussion

The purpose of the present study was to develop a rapid, practical method for determining Hb A₂ in human blood by HPLC. PolyCAT A, a weak cation-exchanger that contains a carboxylic acid function in the form of poly(aspartic acid) linked to silica, has been developed by Alpert (12). Ou et al. (10) used this support in resolving hemoglobin variants. In these systems the retention time of Hb A₂ was about 20 or 30 min, depending on the buffer gradient used. Even with hemoglobins S or C present, the PolyCAT A column completely resolves Hb A₂ and provides for its accurate quantification (10).
I developed the present assay by using short columns and simplifying the sample-preparation technique. Because the amount of Hb A₂ is calculated as a fraction of total hemoglobin, the absolute amount of hemolysate injected into the system is not critical. Therefore, the sample processing should be the shortest possible. In my method the most practical sample-preparation technique found to give reliable results was to dilute 40 μL of blood with 1 mL of buffer A containing 0.5 μL of Triton X-100, filter the mixture, and inject 15 μL into the column. The use of 50-mm columns instead of conventional 200-mm columns improves laboratory efficiency by decreasing the analysis time.

Because this assay is designed to measure Hb A₂, the gradients have been optimized to give a fast and good separation of Hb A₂ from the main hemoglobin peak, Hb A₀. With the 50-mm column this is achieved in 8.4 min. If both glycated hemoglobins and Hb A₂ are to be determined during the same run, the analysis time is increased somewhat because a shallower gradient is required at the beginning of the run.

The reference interval for Hb A₂, 1.3–3.0%, is in general agreement with results by other HPLC methods: Huisman et al. (7) report a reference interval of 1.7–2.8% for assays with a Synchropak CM 300 column, and Stenman et al. (9), using a Mono S column, report somewhat higher values: 3.0–4.2%.

The present assay is precise but fine tuning of the peak integrator is essential to obtain similarly integrated peaks from run to run. After about 200 runs the column performance gradually deteriorates and the peaks become broader and start to tail. When this occurs, the integrator does not resolve the Hb A₂ peak from the main hemoglobin peak, resulting in falsely increased Hb A₂ values. However, the peak shapes can usually be restored by making minor adjustments to the buffer gradient.

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Liquid-Chromatographic Determination of 4-Hydroxyproline in Urine

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In this method for 4-hydroxyproline in urine, hydroxyproline is derivatized with 4-chloro-7-nitrobenzofurazan, with subsequent estimation by reversed-phase “high-performance” liquid chromatography. The ranges for excretion of free and total hydroxyproline while the subjects were ingesting unrestricted diets were 2–29 and 122–374 μmol/L h (n = 21), respectively, with no significant sex-related difference. A comparison with results by colorimetry indicated no significant differences: mean (n = 18) concentrations (μmol/L) of hydroxyproline in urine were 180 (SD 149) by the present method, 163 (SD 166) by colorimetry. For protein hydrolyzate the respective values were 5.9 (SD 2.7) and 6.7 (SD 2.9).

Additional Keyphrases: chromatography, reversed-phase · amino acids · colorimetry compared · monitoring therapy of (e.g.) Paget’s disease, hyperparathyroidism.

Degradation of bone collagen releases free 4-hydroxyproline and peptides containing 4-hydroxyproline into the plasma. Both ordinarily are excreted in the urine (1). The increased concentration of total hydroxyproline in urine during conditions of increased bone resorption—e.g., in Paget’s disease and hyperparathyroid bone disease—can be used to monitor the response of such disorders to treatment. Estimation of the concentration of hydroxyproline in urine has generally been based on its oxidation to various pyrrole derivatives and their subsequent reaction with 4-dimethylaminobenzaldehyde (Ehrlich’s reagent). Colored products based on the reaction with ninhydrin and isatin have also formed the basis of quantitative techniques (2).

Several variations of this technique have been described, some involving partial purification of the specimen before analysis (e.g. 3, 4). Other oxidizing agents have also been used (5). Roth (6) described fluorometric detection of free hydroxyproline and proline in plasma, after ion-exchange chromatography; subsequently, Bohlen and Mellet (7) described an automated fluorometric assay of amino acids that was applicable to proline and hydroxyproline. However, these procedures either lack specificity or are time consuming.

Recently, several methods for estimating hydroxyproline by “high-performance” liquid chromatography (HPLC) have been published (8–10), but none of them has specifically addressed its estimation in urine.

Materials and Methods

Materials. We used a chromatograph equipped with a Model 441 ultraviolet detector (both from Waters Associates, Milford, MA) and a Kratos Model 970LC fluorescent detector (Analytical Instruments, Ramsey, NJ). The 25 cm × 4.6 mm (i.d.) column was packed with 5-μm (av. particle size) octadecylsilyle-treated silica (Lichrosorb RP18; BDH Chemicals, Vancouver, B.C.). All solvents used were “HPLC” grade, and the buffer salts were “Analar” grade. The heptanesulfonic acid was purchased from Sigma Chemical Co., St. Louis, MO, the NBD chloride from Aldrich Chemical Co., Milwaukee, WI. "NBD" is 4-chloro-7-nitrobenzofurazan, a fluorogenic reagent for amino groups.

Derivatization. The derivatization procedure was essentially that described by Ahnoff et al. (11), which briefly is as follows. Add 1.0 mL of a methanolic solution of 250 mmol/L NBD chloride to 2.0 mL of 19.1 μmol/L hydroxyproline in a saturated solution of sodium tetraborate (approximately 0.17 mol/L). Heat at 60 °C in a capped tube wrapped in aluminum foil for 3 min, excluding light. Add 100 μL of HCl (5.0 mol/L) to stop the reaction. Cool the sample in ice-water, then inject 25 μL of the reaction mixture into the chromatograph.

Chromatographic conditions. The eluting solvents were: (A) 0.2 mol of KH₂PO₄ and 5 mmol of heptanesulfonic acid per liter, adjusted to pH 2.4, and (B) 600 mL of 0.33 mol of KH₂PO₄ and 8.33 mmol of heptanesulfonic acid per liter, adjusted to pH 2.4, plus 400 mL of acetonitrile.

Operate the chromatograph isocratically for 22 min with 75% A, 25% B, at a flow rate of 0.7 mL/min. Change to gradient elution to 100% B at 1 mL/min over 3 min, then run it isocratically for 10 min to elute other derivatized amino acids from the column. Take 3 min to re-establish the initial column conditions (75% A, 25% B), then allow the column to equilibrate for 12 min before injecting the next sample. Measure the absorbance of the effluent at 340 nm and its fluorescence at 340 nm excitation, >470 nm emission.

Procedures. The urine samples used in comparing the described method with the colorimetric method were all-