

## Analysis of Hemoglobins and Globin Chains by High-Performance Liquid Chromatography

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### 1. Introduction

In recent years, high-performance liquid chromatography (HPLC) has become a reference method for the study of hemoglobin (Hb) abnormalities. This technique is used in two distinct approaches. The first is quantitative analysis of the various Hb fractions by ion-exchange HPLC, which is now done in routine hospital laboratories mostly by using fully automated systems. The second is reverse-phase (RP)-HPLC, which is of interest for more specialized studies (*see Note 1*).

### 2. Materials and Methods

#### 2.1. Ion-Exchange HPLC Separation of Hbs

Cation-exchange HPLC is the method of choice to quantify normal and abnormal Hb fractions (*1–4*). This is the method of reference for measuring glycosylated Hb for monitoring diabetes mellitus. It is also generally used for measuring of the levels of HbA<sub>2</sub>, HbF, and several abnormal Hbs.

According to some researchers, this method could even replace electrophoretic techniques for primary screening of Hbs of clinical significance (*3,5–7*) or, at least, should be an additional tool for the identification of Hb variants (*8*). Automated apparatuses have been developed for large series measurement. I describe the Bio-Rad Variant Hemoglobin Testing System (Bio-Rad, Hercules, CA), using the  $\beta$  Thalassemia Short program as an example of this type of equipment.

### 2.1.1. Bio-Rad Variant Hb Testing System

The Bio-Rad apparatus is a fully automated HPLC system, using double wavelength detection (415 and 690 nm). The  $\beta$  Thalassemia Short program is the most widely used system for HbA<sub>2</sub> and HbF measurements, but other elution methods, including specific columns, buffers, and software, are available from the manufacturer according to the test to perform. This program has been designed to separate and determine, in 5 to 6 min, area percentage for HbA<sub>2</sub> and HbF and to provide qualitative determinations of a few abnormal Hbs. Windows of retention time have been established for presumptive identification of the most commonly occurring Hb variants. The  $\beta$  Thalassemia Short program uses a  $3.0 \times 0.46$  cm nonporous cation-exchange column that is eluted at  $32 \pm 1^\circ\text{C}$ , with a flow rate of 2 mL/min, by a gradient of pH and an ionic strength made of two phosphate buffers provided by the manufacturer. This material and procedure have been used worldwide in many laboratories over the last several years. Since recommendations for experimental procedure are fully detailed by the manufacturer, I describe only a few additional notes of practical import.

1. Blood is collected on adenine citrate dextrose (ACD).
2. Samples for analysis (about 0.2% Hb) are obtained by hemolysis of 20  $\mu\text{L}$  of blood in 1 mL of a buffer containing 5 g/L of potassium hydrogenophthalate, 0.5 g/L of potassium cyanide, 2 mL of a 1% solution of saponine, and distilled water. This procedure for sample preparation, which is currently used for HPLC determination of HbA<sub>1c</sub>, avoids some of the Hb components present in low amounts (about 1%) eluted together with HbF in the HbF retention time window (8).
3. Twenty microliters of hemolysate is applied onto the column for analysis.

Under these experimental conditions an excellent agreement is found between chromatographic measurement of HbF, down to 0.2%, and resistance to alkali denaturation, up to 15% (9). Presumptive identification of the most commonly occurring variants (Hb S, HbC, HbE, and HbD Punjab) is made using the retention time windows named S-Window, D-Window, A<sub>2</sub>-Window, and C-Window, which have been specified by the manufacturer. Aged Hb specimens display some degraded products that are eluted in the P2 and P3 windows (e.g., glutathione-Hb) (Table 1).

Slight differences in the elution time of the various Hb components are observed from column to column and from one reagent batch to another, which should be taken into account by a program supplied by the manufacturer. The elution time of an Hb component varies also slightly according to its concentration in the sample. For a given column, a more accurate calibration than that proposed by the manufacturer could be obtained using HbA<sub>2</sub> as reference. The concentration of this Hb, which varies between narrow limits, prevents significant modification of its elution time.

**Table 1**  
**Analyte Identification Window<sup>a</sup>**

Analyte name	Retention time (min)	Band (min)	Window (min)
F	1.15	0.15	1.00 -1.30
P2	1.45	0.15	1.30-1.60
P3	1.75	0.15	1.50-1.90
A0	2.60	0.40	2.20-3.30
A2	3.83	0.15	3.68-3.98
D-window	4.05	0.07	3.98-4.12
S-window	4.27	0.15	4.12-4.42
C-window	5.03	0.15	4.88-5.18

<sup>a</sup> Example provided by manufacturer.

Two methods are available for comparing data when the elution time of HbA<sub>2</sub> differs between two runs done with a different column or reagent batch. The first consists of slightly modifying the experimental procedure (temperature or pH) to reproduce exactly the elution times of the previous runs. The second method consists of establishing a normalized retention scale taking as references two Hbs eluted within a linear part of the gradient.

The elution patterns of more than 100 variants have been published, but, in my opinion, these data should be used as a confirmatory test for characterization of a variant after a careful multiparameter electrophoretic study (8) rather than as a primary identification method.

### 2.1.2. Alternative Methods

When a dedicated machine is not available for Hb analysis, or when the chromatographic separation is done for “preparative” purposes, alternative techniques have to be used. These procedures are suitable for conventional HPLC equipment. Several anion-exchange and cation-exchange HPLC columns may be used for Hb separation; some are silica based and others are synthetic polymers. These methods have been well standardized for several years (10,11).

PolyCat A (Poly LC, Columbia, MD) is one of the more popular phases for Hb separations (6). It consists of 5- $\mu$ m porous (100-nm) spherical particles of silica coated with polyaspartic acid. For analytical purposes, a 5.0  $\times$  0.40 cm column is used; elution is obtained at 25°C with a flow rate of 1 mL/min, by developing in 20 min at pH 6.58 a linear gradient of ionic strength from 0.03 to 0.06 *M* NaCl in a 50 *mM* Bis-Tris, 5 *mM* KCN buffer. The presence of KCN is necessary to convert methemoglobin into cyanmethemoglobin, which displays ion-exchange chromatographic properties similar to those of oxyhemoglobin (see Note 2).

## 2.2. HPLC Analysis of Globin Chains

### 2.2.1. Analysis of HbF Composition (see Note 3)

The solvent system, acetonitrile–trifluoroacetic acid (TFA), which is used for RP-HPLC, dissociates the Hb molecule into its subunits and removes the heme group. This method is therefore used to analyze or separate the globin chains. This kind of study may be useful in the investigation of many human Hb disorders. For instance, the determination of HbF composition ( $G\gamma:A\gamma$  ratio) is of interest in several genetic and acquired disorders.

A good separation is obtained between the  $G\gamma$  and  $A\gamma^I$ , with most of the RP columns by using a very flat acetonitrile gradient. By contrast, it is often much more difficult to separate  $G\gamma$  from  $A\gamma^T$ , a frequent allele of  $A\gamma^I$ . Among the procedures that have been successfully proposed for this analysis, one of the most popular is the RP-HPLC method described by Shelton et al. (12). They used a Vydac C4 column (The Separation Group, Hesperia, CA) eluted at a flow rate of 1 mL/min by developing in 1 h a linear gradient from 38 to 42% acetonitrile in 0.1% TFA with detection at 214 nm. Under these conditions, the chains were eluted in the following order:  $\beta$ ,  $\alpha$ ,  $A\gamma^T$ ,  $G\gamma$ , and  $A\gamma^I$ . In recent years, a modification introduced in the manufacturing process of this type of column (13) made necessary the use the higher acetonitrile concentrations to elute the  $\gamma$ -chains. Unfortunately, it also resulted in the low resolution of  $A\gamma^T$ .

#### 2.2.1.1. RP PERFUSION CHROMATOGRAPHY

Perfusion chromatography involves a high-velocity flow of the mobile phase through a porous chromatographic particle (14–16). The Poros R1<sup>®</sup> media (Applied Biosystems, Foster City, CA) used in this technique consists of 10- $\mu$ m-diameter particles. These particles are made by interadhering under a fractal geometry poly(styrene-divinylbenzene) leading to throughpores of 6000- to 8000-Å-diameter microspheres with short, diffusive 500- to 1000-Å-diameter pores connected to them. As a result, relatively low pressures are obtained under high flow rates. The Poros R1<sup>®</sup> beads may be considered a fimbriated stationary phase having retention properties somewhat similar to those of a classic C4 support (15). The column (10  $\times$  0.46 cm) is packed on a conventional HPLC machine at a flow rate of 8 mL/min using the Poros self-pack technology<sup>®</sup> according to the manufacturer's protocol. More than a thousand runs may be performed without alteration of the resolution.

##### 2.2.1.1.1. Sample Preparation

1. Samples containing about 0.1 mg of Hb/mL are obtained by lysis, in 1 mL water (or 5 mM KCN), of 2–5  $\mu$ L of washed red blood cells (RBCs).
2. Membranes are removed by centrifuging at 6000g for 10 min.

3. According to the HbF level, 20–100  $\mu\text{L}$  of these hemolysates are applied onto the column. To avoid additional chromatographic peaks owing to glutathione adducts, 10  $\mu\text{L}$  of a 50 mM solution of dithiothreitol in water is added per 100  $\mu\text{L}$  of sample. An in-line stainless steel filter (0.5- $\mu\text{m}$  porosity) needs to be used to protect the column.

**2.2.1.1.2. Equipment.** Any conventional HPLC machine can be used. In the method described here, the analyses were performed on a Shimadzu LC-6 HPLC machine equipped with an SCL-6B system controller, an SIL-6B autoinjector, and a C-R5A integrator (Shimadzu, Kyoto, Japan). A flow rate of 3.0–4.5 mL/min was convenient for synchronization of injection, integration, and column equilibration.

**2.2.1.1.3. Experimental Procedure (see Note 4).** Using a flow rate of 3 mL/min, the various  $\gamma$ -chains are isolated by developing in 9 min a linear gradient from 37 to 42% acetonitrile in a 0.1% solution of TFA in water. In practice, this is done by using two solvents (A: 35% acetonitrile, 0.1% TFA in water; B: 50% acetonitrile, 0.1% TFA in water) and a linear gradient from 15 to 45% B. Before injection, the column is equilibrated by a 10 column volume wash with the starting solvent, thus allowing completion of a cycle of analysis every 14 min. Elution is followed at 214 nm (wavelength at which double bonds absorb), and the recorder is set to 0.08 AUFS. Higher flow rates may be used, but the slope of the gradient will need to be increased in proportion. Keeping the same initial and final acetonitrile concentrations as above, elution is achieved in 6 min at a flow rate of 4.5 mL/min and in 4 min at a flow rate of 6.0 mL/min.

## **2.2.2. RP-HPLC Analysis of Globin Chains (see Note 5)**

Globin chain analysis is also important as an additional test that allows discrimination between Hb variants for the identification of structural abnormalities. Several RP-HPLC procedures have been proposed (**10,14,17,18**).

On a conventional HPLC apparatus, a  $20 \times 0.46$  cm column packed with Lichrospher 100 RP8 (Merck, Darmstadt, Germany) is used. Samples are prepared as described in **Subheading 2.2.1.1.1**. Elution is obtained at 45°C with a flow rate of 0.7 mL/min using a 90-minute linear gradient of acetonitrile, methanol, and NaCl made by a mixture of two solvents (**18**). Solvent A contains acetonitrile, methanol, and 0.143 M NaCl, pH 2.7 (adjusted by a few drops of 1 N HCl), in the proportion of 24, 38, and 36 L/L, respectively. Solvent B is made from the same reagents but in the proportion of 55, 6, and 39 L/L, respectively. The gradient starts with 10% B and ends with 70% B. The design of the gradient may be modified according to the machine, the geometry of the column, and the separation to be achieved. Elution can be followed at 214 or 280 nm. Globin chains are eluted in the same order as on the Vydac C4 column.

A kit for globin chain analysis with similar performance is also commercially available from Bio-Rad (ref. 270.0301).

### 2.2.3. Scaled Up Methods for Chain Separation

For biosynthetic or structural studies, milligram amounts of globin chains need to be separated. This can be achieved either by scaling up the RP-HPLC procedure using semipreparative size columns or by cation-exchange -HPLC done in the presence of dissociating concentrations of urea.

#### 2.2.3.1. SEMIPREPARATIVE SIZE RP-HPLC COLUMNS

**2.2.3.1.1. Samples.** Globin solution rather than Hb solution is used. Globin is prepared from a 1% Hb solution obtained by hemolysing washed RBCs in distilled water. Stromas are removed by centrifuging at 6000g for 30 min, and the globin is precipitated by the acid acetone method. Usually, the sample is made from 1 to 2 mg of globin dissolved in 250  $\mu$ L of 0.1% TFA, which requires the use of a 500- $\mu$ L injection loop.

**2.2.3.1.2. Chromatographic Procedure.** A 240  $\times$  10 mm Vydac C4 column (ref. 214TP510) is used. Elution is obtained by a gradient of acetonitrile in 0.1% TFA made by two solvents (solvent A contains 35% acetonitrile and solvent B 45%). A typical elution program, using a flow rate of 1.2 mL/min, consists of a 10-min equilibration at 35% B, 70 min of a linear gradient from 35 to 55% B, 30 min of a linear gradient from 55 to 90% B, and 5 min of an isocratic step at 90% B for cleaning the column. Elution of the column is followed at 280 nm with a full scale of 0.16 absorbance units (AU).

#### 2.2.3.2. CATION-EXCHANGE HPLC IN PRESENCE OF 6 M UREA USING A POLYCAT COLUMN

Procedures that are modified from the classic CM cellulose chromatography described by Clegg et al. (19) may be transposed to the HPLC technology (20). The retention capacity of this type of column is higher than that of RP supports, allowing the handling of larger samples. I describe here a method using a PolyCat 300-Å, 10- $\mu$ m particle column (150  $\times$  4 mm).

**2.2.3.2.1. Reagents and Buffers.** Two buffers are used. Buffer A consists of 6 M urea, 0.1 M sodium acetate, and 0.4%  $\beta$ -mercaptoethanol, with the pH adjusted to 5.8 by acetic acid. Buffer B consists of 6 M urea, 0.25 M sodium acetate, and 0.35%  $\beta$ -mercaptoethanol, with the pH adjusted to 5.8 by acetic acid. Both buffers need to be filtered through a membrane with 0.45- $\mu$ m porosity before being used. In addition, an in-line stainless steel filter (0.5- $\mu$ m porosity) is needed to protect the column.

**2.2.3.2.2. Samples.** Up to 5–10 mg of globin, prepared by the acid acetone method, is dissolved in 200–600 mL of buffer A.

**2.2.3.2.3. Chromatographic Procedure.** Elution is obtained by a gradient of ionic strength developed with the two buffers. A typical elution program, using a flow rate of 1.0 mL/min, consists of a 10-min equilibration at 0% B, 5 min of a linear gradient from 0 to 25% B, 50 min of a linear gradient from 25 to 100% B, and 5 min of an isocratic step at 100% B for cleaning the column. Elution of the column is followed at 280 nm with a full scale of 0.32 UA.

### 3. Notes

1. Why should one method be preferred over another? The choice of a separation method between RP or ion-exchange chromatography depends on the purpose of the separation. Ion-exchange is the only chromatographic method that allows preparation of native Hb fractions. The presence of cyanide ions in the buffers (or during sample preparation) will nevertheless hinder any further oxygen-binding study. If the aim of the separation is to obtain Hbs suitable for functional studies, the technique will have to be modified accordingly by removing cyanide from all the steps. It may be of interest in some cases to work with carbonmonoxy-hemoglobin, since Hb is very stable under this form and procedures are available to return to the oxyform. For several applications, salts in excess also need to be removed. RP separation methods always lead to denatured proteins that cannot be used for functional studies. Techniques involving an ionic strength gradient can only be used for analytical purposes. By contrast, using fully volatile buffers, such as the acetonitrile-TFA system, the isolated globin fractions can be vacuum dried and readily used for further structural studies such as mass spectrometry measurements.
2. To isolate amounts of Hb in the milligram range, larger columns ( $15.0 \times 0.46$  cm) may be used. According to the separation to be achieved, the dimensions of the column, and the apparatus used, slightly different experimental conditions may have to be designed. Elution is followed at 415 nm for analytical purposes or at 540 nm in preparative runs. This buffer system is not suitable for ultraviolet (UV) detection. The use of an in-line stainless steel filter (0.5- $\mu$ m porosity) is recommended to increase the column life expectancy. Reproducibility requires careful preparation of the buffers and temperature control. Since in these chromatographic methods the elution is recorded at one of the wavelengths of absorption of the heme, any factor modifying the absorption spectrum of the Hb molecule will hinder accurate quantitative measurement. For instance, unstable Hb variants, which lose their heme groups or lead to hemichrome formation, will be underestimated. HbMs, which are hardly converted into cyanmethemoglobin, display a much higher extinction coefficient than oxyhemoglobin at 415 nm and a lower one at 540 nm. As a consequence, HbMs will be overestimated when measured at the first wavelength, and underestimated at the second one. A modified experimental procedure allowing for a simultaneous measurement of HbF, glycated Hb, and several other Hb adducts has been proposed by using a combination of pH and ionic gradients (11).



3. In my laboratory, for routine determination of the  $\gamma$ -chain composition, we replaced this procedure with an RP perfusion chromatography using a Poros R1<sup>®</sup> column (Applied Biosystems) (14).
4. To obtain good reproducibility, we recommend using the same glassware for preparing the solvents. Solvents may be kept refrigerated at 4°C for a few days. Accurate balance of the TFA between both solvents is important to avoid baseline drift. Acetonitrile must be of HPLC grade with low UV absorbancy in the 210-nm region. With this Poros R1 column, the  $\alpha$ -chain is eluted before the  $\beta$ -chain. Resolution may be improved by modifying the geometry of the column or the design of the gradient. A 10  $\times$  0.2 cm column may be used to improve separation between the various  $\gamma$ - or adult chains. In this case, with a flow rate of 1 mL/min, after 5 min of equilibration at 5% B, the column is eluted using a 15-min linear gradient between 5 and 25% B of the described solvents. This is followed by a 2-min isocratic elution at 25% B.
5. Several columns may be used, but I have found that a method adapted from that described in **ref. 17** leads to a good resolution. Other columns or techniques may nevertheless be more appropriate for some specific separations. When chromatographic methods are used for globin chain quantification, it is important to consider the absorption coefficient of the various chains at the wavelength of detection. In some cases, it may be identical, such as when comparing the various  $\gamma$ -chains. In other cases, the absorption may differ considerably; for example, at 280 nm,  $\gamma$ -chains, because of their 3 Trp residues, have a higher  $\epsilon$  coefficient than  $\beta$ -chains (2Trp) and  $\alpha$ -chains (1 Trp). Abnormal Hbs containing a number of aromatic residues different from the normal may also display modified absorption coefficient.

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