DIONEX

Application Note 126

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Determination of Hemoglobin Variants by Cation-Exchange Chromatography

INTRODUCTION

Clinical laboratories frequently separate and quantify the levels of hemoglobin variants in blood samples. Two types of hemoglobin most commonly measured are glycosylated hemoglobins and hemoglobin sequence variants.[1,2] For the physician, the assay of glycated (nonenzymatically glycosylated) hemoglobin levels in the blood of a diabetic patient serves as an excellent indication of the average level of blood glucose during the preceding one to two months. In addition, the determination of hemoglobin variants helps to identify a variety of hemoglobinopathies, including sickle cell, hemoglobin C, and Bart's Disease. Moreover, these assays are extremely important in the diagnosis, treatment, and counseling of afflicted children.[3]

Typically, isoelectric focusing (IEF) gel electrophoresis is used for the analysis of hemoglobin variants, including HbS, HbC, HbF, HbA, and HbA_2. However, two IEF procedures are necessary: cellulose acetate electrophoresis at alkaline pH, followed by citrate agar electrophoresis at acidic pH for confirmation. In comparsion, the ProPac™ SCX-10 column successfully resolves these hemoglobin species in a single run within 20 min. This column uses a unique, hydrophilic-coated, polymeric resin with sulfonate functional groups on grafted linker arms. The physicochemical characteristics of this strong cation exchange support afford minimal band spreading and very high selectivity.[4]

This Application Note describes two examples that illustrate the use of the Dionex ProPac SCX-10 strong cation exchange column for the rapid, high-resolution separation of hemoglobin variants.

EQUIPMENT

DX-500 BioLC® Liquid Chromatograph
- GP50 Gradient Pump
- AD20 Variable Wavelength Absorbance Detector
- LC25 Chromatography Enclosure
- AS50 Autosampler (with a 50-µL sample loop)

PeakNet Chromatography Workstation

REAGENTS AND STANDARDS

High-purity deionized water prepared with a Milli-Q system (Millipore, Bedford, MA, USA)

Sodium phosphate, monobasic and dibasic, analytical-reagent grade (J. T. Baker, Phillipsburg, NJ, USA)

Sodium chloride, analytical-reagent grade (Fluka, Ronkonkoma, NY, USA)

Potassium cyanide, analytical-reagent grade (Sigma, St. Louis, MO, USA)

Hemoglobin variants (Helena Labs, Beaumont, TX, USA)

PREPARATION OF SOLUTIONS AND REAGENTS

Two eluents are used for this chromatography: 50 mM sodium phosphate with 2 mM potassium cyanide (KCN) (pH 6.0) and 50 mM sodium phosphate (pH 6.0) with 500 mM sodium chloride (NaCl) and 2 mM potassium cyanide (KCN). The sodium phosphate buffer system was prepared by diluting appropriate quantities of the monobasic and dibasic sodium phosphate concentrate solutions with water to attain the desired pH 6.0. The following procedure is a recommended starting point for obtaining

the desired eluents, but some deviation from this formula may be necessary after checking the pH when using reagents in other labs. If the pH is not 6.0, then adjust the proportions of monobasic and dibasic solutions added. The combined total volume of monobasic and dibasic solutions should remain at 500 mL to produce 50 mM sodium phosphate for 2 L of eluent.

1M Sodium Chloride

Dissolve 116.90 g sodium chloride in water, and fill to a final volume of 2.0 L. Filter through a 0.45-µm filter.

200 mM Potassium Cyanide

Dissolve 13.02 g anhydrous potassium cyanide in 1000 mL of water. Filter through a 0.45-µm filter. *Caution: exercise necessary care and precautions when handling concentrated potassium cyanide.*

200 mM Sodium Phosphate, Dibasic

Dissolve 28.38 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in 1000 mL of water Filter through a 0.45-µm filter. Store frozen until needed.

200 mM Sodium Phosphate, Monobasic

Dissolve 27.60 g monohydrate monobasic sodium phosphate ($NaH_2PO_4 \bullet H_2O$) in 1000 mL of water. Filter through a 0.45-µm filter. Store frozen until needed.

50 mM Sodium Phosphate, 2 mM Potassium Cyanide, pH 6.0

Combine 70 mL of 200 mM dibasic sodium phosphate, 430 mL of 200 mM monobasic sodium phosphate, 20.0 mL of 200 mM potassium cyanide, and 1480 mL water.

50 mM Sodium Phosphate with 0.5 M Sodium Chloride, 2 mM Potassium Cyanide, pH 6.0

Combine 70 mL of 200 mM dibasic sodium phosphate, 430 mL of 200 mM monobasic sodium phosphate, 1000 mL 1 M sodium chloride, 20.0 mL of 200 mM potassium cyanide, and 480 mL water.

CONDITIONS

Column: ProPac SCX-10, 4 x 250 mm

Flow Rate: 1 mL/min

Detection: Absorbance, 220 nm

Mobile Phase: A: 50 mM sodium phosphate, 2 mM potassium cyanide, pH 6.0
B: 50 mM sodium phosphate, 2 mM potassium cyanide, 0.5 M sodium chloride, pH 6.0

Gradient: Method 1: 0 min, 3% to 12% B in 20 min to 40% B at 30 min
Method 2: 0–50% B in 30 min

Method

Method 1

Separation of glycated hemoglobin variants (Figure 1)

Time	A (%)	B (%)	Comments
Initial	97.00	3.00	Equilibration
0.00	97.00	3.00	Injection
20.00	88.00	12.00	
30.00	60.00	40.00	End Gradient
30.10	97.00	3.00	Re-equilibration
40.10	97.00	3.00	

Method 2

Separation of hemoglobin sequence variants (Figure 2)

Time	A (%)	B (%)	Comments
Initial	100.00	0.00	Equilibration
0.00	100.00	0.00	Injection
30.00	50.00	50.00	End Gradient
30.10	100.00	0.00	Re-equilibration
40.00	100.00	0.00	

SAMPLE PREPARATION

Protein samples were prepared by dissolving hemoglobin variants in Mobile Phase A at a concentration of 0.5 mg/mL.

RESULTS AND DISCUSSION

Figure 1 shows the separation of hemoglobin variants on the ProPac SCX-10 column. This strong cation-exchange resin column produced a rapid, high resolution separation of hemoglobin variants found in a sample known to contain elevated levels of glycated hemoglobin. About 10% of the total hemoglobin was glycated. The peaks are labeled in accordance with established conventions.[2,5] Figure 1 reveals the presence of numerous glycated

forms of hemoglobin. Hemoglobin glycation is a modification that occurs nonenzymatically between hemoglobin and sugars in the blood. In principle, nonenzymatic glycation can occur with any free amino group in the hemoglobin protein (e.g., at the N-terminus of the protein chains or on the side chains of lysine residues). The major glycated component is formed when the N-terminal residues of the protein chains react with glucose to produce HbA_{1c}, although other forms have been identified and described elsewhere.[6]

The separation of several hemoglobin sequence variants, including sickle cell hemoglobin, fetal hemoglobin, and hemoglobin C, is shown in Figure 2. The high rate of mass transfer associated with the pellicular resin resulted in narrow, efficient peaks and, consequently, very high levels of resolution. The high peak efficiency observed in this separation and the relatively steep nature of the gradient profile indicate that the ProPac SCX-10 column has the potential to resolve a large number of hemoglobin variants. Two peaks (1 and 4) are tentatively identified in the chromatogram as HbA_{1c} and HbA_2.

CONCLUSION

These results demonstrate that the ProPac SCX-10 column has a high degree of selectivity for hemoglobin variants. The ability to separate these variants makes the ProPac SCX-10 column a powerful tool for the analysis of hemoglobin in clinical and research laboratories.

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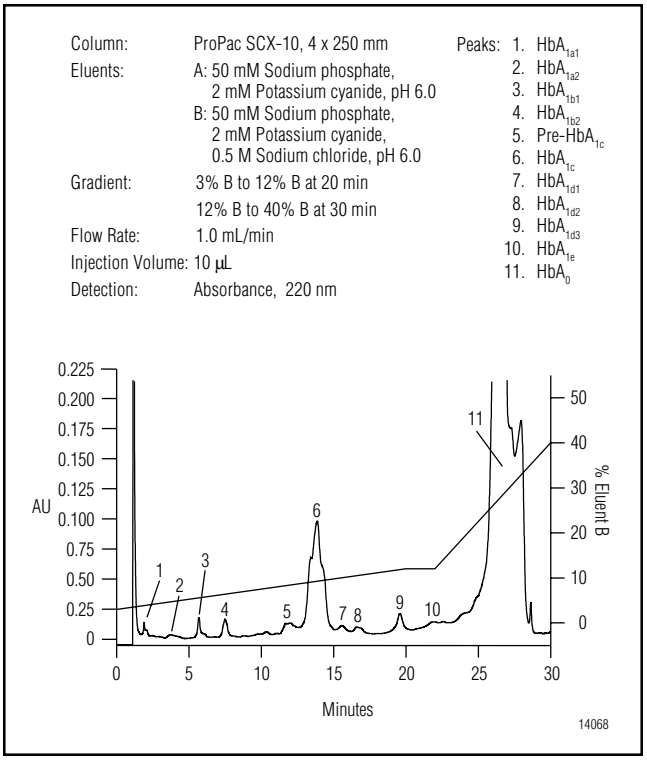

Figure 1 *Separation of hemoglobin variants in a sample with elevated levels of glycated hemoglobins. Sample concentration is 0.5 mg/mL.*

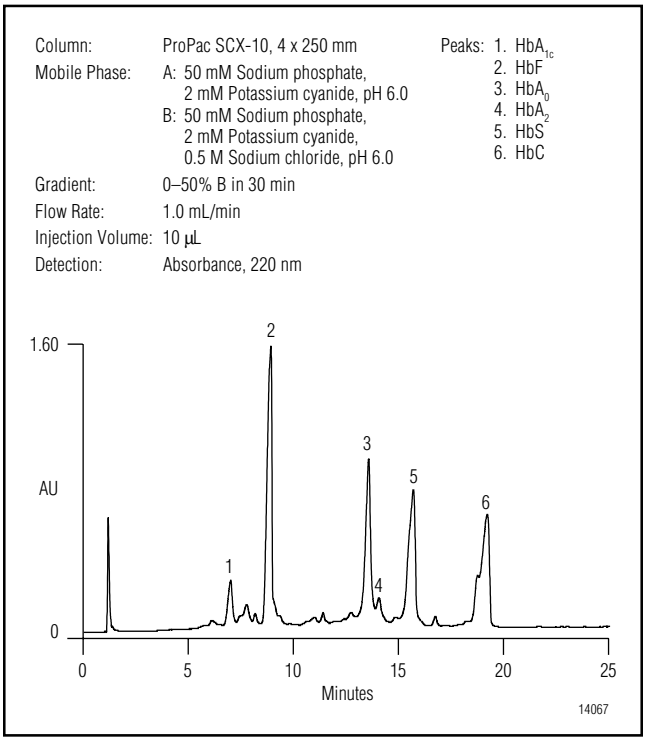

Figure 2 *Separation of hemoglobin variants, including fetal (HbF), sickle cell (HbF), normal (HbA), and C-type (HbC) hemoglobins. Sample concentration is 0.5 mg/mL.*

LIST OF SUPPLIERS

Millipore Corporation, 80 Ashby Road, Bedford, MA 01730, USA. Tel: (800) 645-5476

J. T. Baker Incorporated, 222 Red School Lane, Phillipsburg, NJ 08865, USA. Tel: (800) 582-2537

Fluka Chemika-BioChemika, Fluka Chemie AG, Industriestrasse 25, CH-9471 Buchs, Switzerland. Tel: +81 755 25 11

Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178, USA. Tel: (800) 325-3010

Helena Labs, 1530 Lindbergh Dr., Beaumont, TX 77707, USA. Tel: (409) 842-3714

Dionex Corporation
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

North America
U.S. (847) 295-7500
Canada (905) 844-9650

South America
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Asia Pacific
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