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High-performance liquid chromatographic evaluation of pyridoxyl 5′-phosphate hemoglobin derivatives produced by different reduction procedures[a]

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ABSTRACT

Pyridoxylated hemoglobin derivatives have been studied by many investigators In this study hemoglobin A_0 rather than stroma-free hemoglobin was used as a starting material in order to reduce the number of proteins to A_0 and A_{1c} Derivatives were characterized using a Synchropak Q300 strong anion-exchange column, a PolyCAT A weak cation-exchange column and a VYDAC reversed-phase high-performance liquid chromatographic column Resulting peak profiles of these two ion-exchange separations demonstrated enhanced resolution and showed the presence of pyridoxylated hemoglobin products not previously described. We compared products from the reduction of these Schiff base derivatives using either sodium borohydride or sodium cyanoborohydride reduction procedures The sodium cyanoborohydride reduction method produced a lower percentage of products with multiple-site pyridoxylation modifications than the sodium borohydride reduction process

INTRODUCTION

Stroma-free hemoglobin (SFHb) has been investigated as a potential blood substitute [1,2]. When used *in vivo*, SFHb binds oxygen very tightly, which may reduce the oxygen delivery to tissues. Benesch *et al.* [3] showed that when pyridoxal phosphate (2.5 mol/mol tetramer) is bound to hemoglobin (Hb) at the 2,3-diphosphoglyceric acid (DPG) binding site, the high oxygen affinity is reduced to within a desirable range (between 23 and 26 Torr) [2,3]. These pyridoxylated hemoglobin products (Schiff bases) can be stabilized without losing these reduced oxygen-binding properties by reduction with sodium borohydride ($NaBH_4$), thereby forming secondary amines [4]. An alternative reduction process using sodium cyanoborohydride ($NaCNBH_3$) was also studied since it is a gentler process and has been shown in our laboratory to produce much less methemoglobin than the $NaBH_4$ reduction.

[a] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official nor do they reflect the views of the Department of the Army or the Department of Defense (AR360-5).

Anion-exchange [5–7] and cation-exchange [5,8] high-performance liquid chromatography (HPLC) have been used to characterize pyridoxylated products. Because sample preparation and chromatographic procedures differ, the number of observed pyridoxylated fractions have ranged from four [6] to six [5,7]. The fractions described by Benesch *et al.* [6] and McGarrity *et al.* [5] are included in Table I. The main modification sites have been reported at the N-terminal valine of the α- and β-chains and at β-82-lysine [8], both within the β-cleft and part of the DPG binding site.

Human hemoglobin A_0 (HbA_0) was used instead of SFHb in order to reduce the number of starting protein species to two (A_0 and A_{1c}). The A_0 preparation removes all other forms of hemoglobin, such as A_2, and all the non-heme red cell proteins. Thus the use of HbA_0 reduces the number of pyridoxal phosphate (PLP) reaction products and simplifies product characterization.

During these studies improved HPLC methods using a Synchropak Q300 strong anion-exchange column and a PolyCAT A weak cation-exchange column were developed. This paper details these new HPLC procedures and evaluates the

TABLE I

COMPARISON OF PYRIDOXAL PHOSPHATE DERIVATIVES

Method	This paper[a]	McGarrity *et al.* [5][b]	Benesch *et al.* [6][c]	Area percent[d]	dpm per peak area[e]	mol P per dimer
$NaBH_4$	Peak 1	Peak I	Po	13.8	0	0
$CNBH_4$	Peak 1	Peak I	Po	46.0	0	0
$NaBH_4$	Peak 2	Peak II	PLP 2	8.1	0 21	1
$CNBH_4$	Peak 2	Peak II	PLP 2	10.5	0 16	1
$NaBH_4$	Peak 3			5.3	0.22	1
$CNBH_4$	Peak 3			9.0	0.16	1
$NaBH_4$	Peak 4	Peak III	PLP 2	10.4	0 23	1
$CNBH_4$	Peak 4	Peak III	PLP 2	7.6	0 15	1
$CNBH_3$	Peak 4B			3.2	0.11	1
$CNBH_3$	Peak 4C			6.9	0 14	1
$NaBH_4$	Peak 5	Peak IV		14.0	0 35	2
$CNBH_4$	Peak 5	Peak IV		3.7	0 26	2
$NaBH_4$	Peak 6	Peak V	PLP 4	18.4	0 41	2
$CNBH_4$	Peak 6	Peak V	PLP 4	2.0	0 26	2
$NaBH_4$	Peak 7	Peak VI		15.2	0 54	3
$CNBH_4$	Peak 7	Peak VI		< 1 0	–[f]	–[f]
$NaBH_4$	Peak 8			3.3	0.84	4 +

[a] Peaks 1–8 from Fig. 1 reduced with $NaBH_4$ and $NaCNBH_3$

[b] McGarrity's corresponding peak identification.

[c] Benesch's peak identification according to McGarrity *et al.* [5].

[d] Peak area expressed as a percentage of the total area

[e] Peak area is expressed in mV/s.

[f] Peak too small to quantitate accurately

differences in products formed from the two different methods of Schiff base reduction.

EXPERIMENTAL

Reagents and chemicals

All buffer salts were obtained from Sigma (St. Louis, MO, U.S.A.). Hemoglobin samples were deionized by passing them through a mixed-bed ion-exchange column (Bio-Rad, Bio-Rex, RG 501-X8, Richmond, CA, U.S.A.); the conductivity was always less than 10 μmhos. [^{14}C]PLP was purchased from Amersham (Arlington Heights, IL, U.S.A.) as a custom synthesis. The $NaCNBH_3$ and $NaBH_4$ were purchased from Aldrich (Milwaukee, WI, U.S.A.)

Oxygen dissociation curve

The hemox analyzer (Southhampton, PA, U.S.A.) was used to determine the P_{50} or oxygen dissociation curve. The samples were run at 37°C in hemox pH 7.4 buffer.

Preparation of PLP-HbA_0

SFHb was isolated from out-dated human red blood cells, and chromatographically purified HbA_0 was made using procedures of Christensen *et al.* [9]. This HbA_0 contained only A_0 and A_{1c} hemoglobin and had a methemoglobin level of 3.1% The PLP-HbA_0 samples were prepared at 22°C in a Radiometer (Copenhagen, Denmark) TTA 80 titration assembly with a Radiometer ABU autoburette using a 1:1 molar ratio of PLP to heme. Before pyridoxylation, the sample that was to be reduced with $NaCNBH_3$ (PLP-$CNBH_3$-HbA_0) was blanketed with air saturated with water. The sample to be reduced with $NaBH_4$ (PLP-BH_4-HbA_0) was blanketed with nitrogen and constantly stirred. The deoxygenation process was considered complete when no additional 0.1 *M* potassium hydroxide titrant was added by the autoburette for a period of 0.5 h [7], *i.e.* the pH change of 0.13 between oxy- and deoxy-Hb was complete. The PLP was added in a powder form, and the mixture was adjusted with 0.1 *M* potassium hydroxide to pH 6.8 with the autoburette [7]. The reduction procedure was completed in 1 h. An aliquot of oxy-PLP-HbA_0 was reduced with $NaCNBH_3$ (3 mol/mol per heme) and then passed through the mixed-bed column. The deoxy-PLP-HbA_0 sample was reduced with $NaBH_4$ (3 mol/mol per heme) under nitrogen with constant stirring to prevent excessive foaming and appreciable methemoglobin formation. The PLP-BH_4-HbA_0 samples were then passed through the mixed-bed resin.

HPLC analytical procedures

HPLC separations were carried out with a Waters (Waters Assoc., Milford, MA, U.S.A.) system consisting of a WISP autosampler, two 510 pumps, a 490

detector and an 820 controller. All samples were filtered through a 0.45-μm filter (Schleicher and Schuell, Keene, NH, U.S.A.) prior to injection.

Anion-exchange analyses were carried out on a Synchropak (SynChrom, Lafayette, IN, U.S.A.) Q300 column (6 5 μm, 250 mm × 4.6 mm I.D.). Buffer A was 1.5 m*M* potassium cyanide (Fischer, Pittsburgh, PA, U.S.A.) and 15 m*M* Tris (Sigma) at pH 8.0; buffer B was 1.5 m*M* potassium cyanide, 15 m*M* Tris at pH 8 0 and 400 m*M* sodium acetate (J. T. Baker, Phillipsburg, NJ, U.S.A.). Samples (0.5–1 mg per 25–200 μl) were applied to the column. The samples were then eluted at a flow-rate of 1.0 ml/min with a linear gradient of 22 to 50% buffer B over 30 min, then 50 to 100% buffer B in 25 min and then 100% buffer B for 5 min. Peaks were collected to measure the labeled products. Radioactivity was measured by liquid scintillation counting (Packard Instrument, Downers Grove, IL, U.S.A.) for 10 min after adding 1 ml of fraction to 9 ml of Opti-Fluor (Packard). Two drops of 30% hydrogen peroxide were added to decolorize the samples.

Cation-exchange analyses were done on a PolyCAT A (PolyLC, Columbia, MD, U.S.A.) column (5 μm, 200 mm × 4.6 mm I.D.) [10] Buffer A was 1.5 m*M* potassium cyanide and 30 m*M* Bis–Tris as pH 5.8; buffer B was 1.5 m*M* potassium cyanide and 30 m*M* Bis–Tris at pH 5.8 and 200 m*M* sodium acetate. The samples were run at the same concentration as the anion-exchange procedure and were eluted from the column at 1.0 ml/min with a linear gradient of 5 to 30% buffer B over a period of 50 min and then to 100% B over the next 60 min. To distinguish heme from non-heme protein the effluent was monitored at 280 and 540 nm.

Reversed-phase analyses were done on a VYDAC (Separation Group, Hesperia, CA, U.S A.) C_4 column (5 μm, 200 mm × 4.6 mm I.D.) [11]. Solvent A was 20% acetonitrile in water and 0.1% trifluoracetic acid (TFA); solvent B was 60% acetonitrile and 0.1% TFA. The samples were eluted from the column at 1.0 ml/min with a linear gradient of 47 to 50% solvent B over a period of 40 min and then to 70% solvent B over the next 20 min. The effluent was monitored at 220 nm. The samples were run at the same concentration as the anion-exchange procedure and were eluted from the column at 1.0 ml/min with a linear gradient of 5 to 30% solvent B over a period of 50 min and then to 100% solvent B over the next 60 min. The effluent was monitored at 280 and 540 nm to distinguish heme from non-heme protein.

RESULTS

Fig. 1 shows the separation of [^{14}C]PLP-BH_4-HbA_0 and [^{14}C]PLP-$CNBH_3$-HbA_0 on a strong anion-exchange Synchropak Q300 column. The PLP-BH_4-HbA_0 mixture was resolved into eight major peaks and several minor peaks of various sizes. The PLP-$CNBH_3$-HbA_0 mixture was resolved into nine major peaks and a few minor ones. A fraction containing each peak was collected.

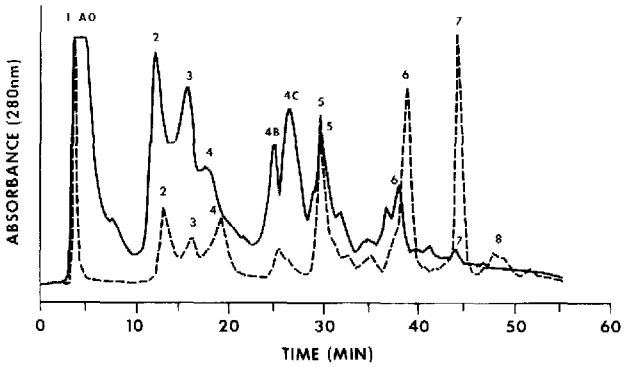

Fig 1 Chromatograms of PLP-BH_4-HbA_0 (---) and PLP-$CHBH_3$-HbA_0 (——). Samples were run on a Synchrom Q300 strong anion-exchange column and monitored at 280 nm. Peak 1 = A_0 The remaining numbered peaks correspond to the values given in Table I

Potassium cyanide was added to the HPLC buffers to convert ferrihemoglobin to ferrihemoglobin cyanide which has the same chromatographic behavior as oxyhemoglobin [12,13]. The comparison in Fig. 1 shows a close correspondence in the identity of the products from the two reduction procedures. However, in the $NaBH_4$ reduction procedure only 13.8% of A_0 (Table I) remained unreacted while 46% of the A_0 remained unreacted in the $NaCNBH_3$ reduction method. In the $NaCNBH_3$ method, the peaks eluting between peaks 4 and 5 are relatively larger than the corresponding peaks produced by the $NaBH_4$ reduction method. The fractional distribution of the main pyridoxylated products is shown in Table I along with the phosphate/dimer molar ratios. The counts ranged from 600 to 4200 dpm per peak for the $NaBH_4$-reduced derivatives and from 129 to 614 dpm per peak for the $NaCNBH_3$-reduced derivatives. The correlation between the dpm per peak area in the $NaCNBH_3$-reduced PLP-HbA_0 and the $NaBH_4$-reduced PLP-HbA_0 is reasonable considering there was less than complete baseline resolution in the chromatogram (Fig. 1). The phosphate/dimer molar ratios of 2 or higher comprised approximately 51% of the reaction products in the $NaBH_4$ method whereas such a ratio comprised only about 6% in the $NaCNBH_3$ procedure. The chromatograms monitored at 540 and 280 nm were identical, but the absorbance at 540 nm was lower.

A weak cation-exchange separation using both reduction procedures was done on a PolyCAT A column (Fig. 2). At least twenty peaks can be seen in the PLP-BH_4-HbA_0 chromatogram. The $NaCNBH_3$ reduction method produced a relatively higher percent area of peaks with a less negative charge as shown in the anion-exchange chromatogram (Fig. 1). The relative percent area of the peaks eluted within 64 min in the PLP-BH_4-HbA_0 chromatogram (Fig. 2) was 51% of the total area, whereas in the PLP-$CNBH_3$-HbA_0 chromatogram (Fig. 2) the peaks of the first 64 min comprised approximately 9% of the total area. This finding correlated well with anion-exchange data which showed the phosphate/

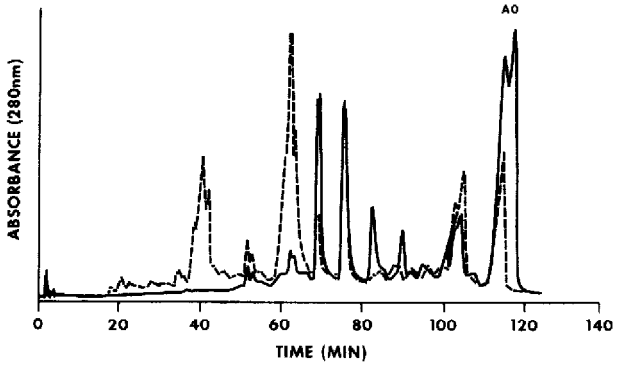

Fig 2 Chromatograms of PLP-BH_4-HbA_0 (---) and PLP-$CNBH_3$-HbA_0 (——). Samples were run on a PolyCAT A weak cation-exchange column and monitored at 280 nm The A_0 peak has a retention time of approximately 120 min.

dimer molar ratios of 2 or more in 51% of the $NaBH_4$-reduced products and 6% of $NaCNBH_3$-reduced products. Fig. 3 shows a comparison between the chromatograms of unreduced PLP-HbA_0 and that of the same compound reduced with $NaCNBH_3$. The main peaks eluting at 69 and 120 min (A_0) appeared to be the same in both chromatograms, but the PLP-$CNBH_3$-HbA_0 chromatogram contained a few additional although less prominent peaks.

The hemoglobin chains were studied by reversed-phase chromatography. Fig. 4 shows an overlay comparison between A_0 control and PLP-BH_4-HbA_0. In the modified Hb an additional peak could be seen prior to the β-chain (labeled β mod), and the retention time of the peak was shifted from 36 to 33 min compared to A_0. Radioactivity was detected in the β-modified chain (59%), β-chain (14%) and α-chain (27%) fractions. In Fig. 5 the $NaCNBH_3$-reduced samples were compared to A_0. A small additional peak prior to the β-peak (20 min) was seen,

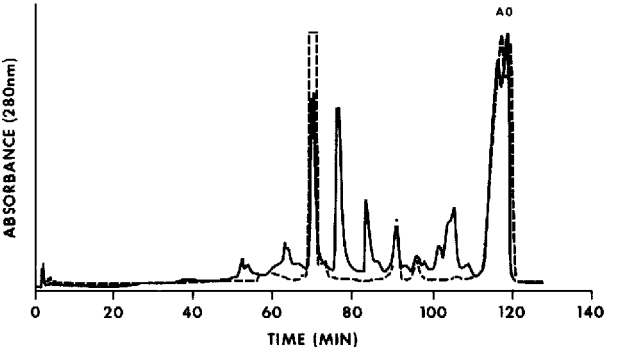

Fig. 3. Chromatograms of PLP-$CNBH_3$-HbA_0 (---) and unreduced PLP-A_0 (——) Samples were run on a PolyCAT A weak cation-exchange column and monitored at 280 nm.

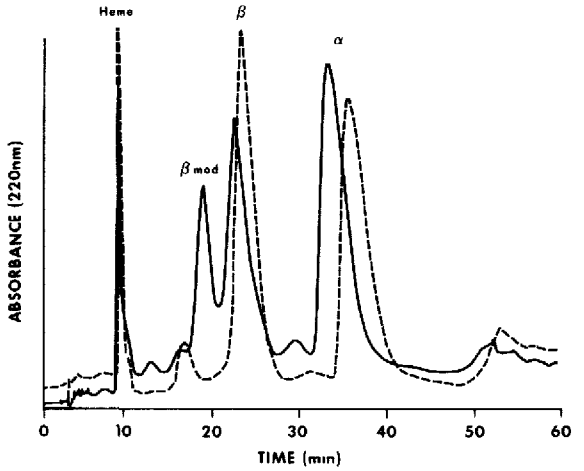

Fig 4 Chromatograms of A_0 (---) and PLP-BH_4-HbA_0 (——) chains Samples were run on a VYDAC C_4 reversed-phase column and monitored at 220 nm

but the retention time of the main β-peak stayed about the same. The α-peak shifted by 1 min to a shorter retention time. Radioactivity was seen in the small peak at 20 min (24%), the β-peak (63%) and the α-peak (13%).

The P_{50} value of the PLP-BH_4-HbA_0 and PLP-$CNBH_3$-HbA_0 solutions were 23.5 and 18.5, respectively. The P_{50} value of the A_0 control was 12.0.

The anion- and cation-exchange chromatographic results of this pyridoxylation procedure were compared to the results obtained from a sample prepared by

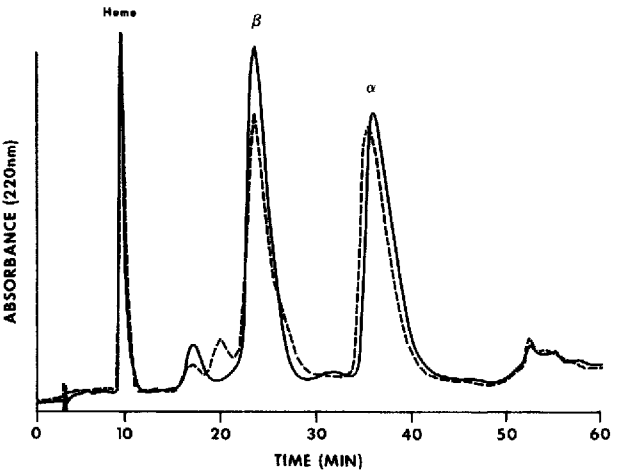

Fig. 5 Chromatograms of A_0 (——) and PLP-$CNBH_3$-HbA_0 (---) chains. Samples were run on a VYDAC C_4 reversed-phase column and monitored at 220 nm

the method of DeVenuto *et al.* [1] and McGarrity *et al.* [5]. In comparison, Tris buffer and caprylic alcohol were eliminated in our pyridoxylation process, and nitrogen was blanketed over the reaction mixture rather than bubbled through it. These differences in the sample preparation procedure could account for at least some of the differences in peak profiles between our sample and those of other investigators. Benesch *et al.* [6] have shown that the use of Tris and deoxy conditions favors PLP reactions with the β-chains, while residual oxy-Hb reacts with PLP at α-chains.

DISCUSSION

In previous studies by Benesch *et al.* [6], McGarrity *et al.* [5] and DeVenuto and Zegna [4] SFHb was used as the starting material for the preparation of pyridoxylated hemoglobin. In the present study we started with a more purified form of hemoglobin consisting mainly of A_0 (95%). The remaining 4–5% eluted in the same position on cation-exchange and isoelectric focusing as HbA_{1c} and within the same percentage range as normal HbA_{1c} (4–6%) [9,14]. McGarrity *et al.* [5] showed that the composition of their pyridoxylated mixtures was more complex than that reported by Benesch *et al.* [6]. Using a Mono Q strong anion exchanger, they were able to distinguish six major peaks and correlate them with Benesch's fractions (Table I). Peak I was designated as HbA, peaks II and III each contained 1 mol of phosphate per dimer and were modified at the β-1-valine site. The peaks were shown to be an equilibrium mixture dependent on sample concentration. Peak IV contained 2 mol of phosphate per dimer and represented hemoglobin modified at the β-1-valine and the α-1-valine sites; peak V contained 2 mol of phosphate per dimer and was modified at the β-1-valine and the β-82-lysine sites, peak 6 had a phosphate/dimer ratio of 3 and was modified at the β-1-valine, β-82-lysine and α-1-valine sites. Based on phosphate/dimer molar ratios and the similarity of the strong anion-exchange chromatographic profile (see Fig. 1), we were able to correlate six of our peaks with the six major peaks McGarrity *et al.* [5] described in their chromatogram (see Table I) Our PLP-BH_4-HbA_0 anion- and cation-exchange chromatograms contained more peaks despite the fact that we started with HbA_0 rather than with SFH. The anion-exchange chromatogram also showed at least one hemoglobin species with a phosphate/dimer molar ratio of 4. As expected, the peaks with the higher phosphate/dimer molar ratio had longer retention times on the anion-exchange column due to the phosphate contributing negative charges to the molecule.

The $NaCNBH_3$-reduced PLP-HbA_0 had a peak profile similar to the $NaBH_4$-reduced PLP-HbA_0, but there were marked differences in the relative quantities of each of the PLP-HbA_0 products. As shown in Table I, the $NaCNBH_3$ reduction process produced a much lower percentage of multiple-site pyridoxylation on the globin chain than the $NaBH_4$ reduction process. A small amount of radioactivity was detected in the α-chain in reversed-phase chromatography. The pyri-

doxylation varied slightly from results in the literature [9] which showed no radioactivity on the α-chain. This discrepancy could be due to slight variations in the sample preparation process, and we are now investigating this possibility.

When the sample PLP-BH_4-HbA_0 was run on a weak cation exchanger (PolyCAT A), more than twenty peaks could be observed in the chromatogram. We supported these results from the weak cation exchanger with isoelectric focusing gels and observed at least twenty bands [7].

A comparison of our two reduction methods on weak cation-exchange chromatography confirmed the anion-exchange data showing that the chromatographic profiles were very similar, but this $NaBH_4$ reduction method resulted in the production of a greater amount (percent area) of components with higher negative charges and consequently more pyridoxylation McGarrity *et al.* [5] showed that the higher P_{50} values occurred in fractions with a phosphate/dimer molar ratio of 2 and 3. Therefore, the P_{50} value of $NaCNBH_3$-reduced PLP-HbA_0 would be predictably less than that of $NaBH_4$-reduced PLP-HbA_0. Our P_{50} data confirmed this prediction PLP-$CNBH_3$-HbA_0 showed a very close resemblance to non-reduced PLP-A_0 (Fig. 3). The difference between the two chromatograms is the addition of one large peak at 75 min and additional smaller peaks between 52 and 69 min and 82 and 110 min in the PLP-$CNBH_3$-HbA_0 chromatogram. However, unreduced PLP-A_0 has been reported to be unstable [9].

Ion-exchange separations have been shown to be slow relative to the hemoglobin dimer dissociation and re-association process [6]. Benesch *et al.* [6] found that when electrophoresis was carried out under aerobic conditions, the final major reaction products were symmetrical due to a dimer exchange equilibrium reaction. The initial presence of asymmetric products was shown by anaerobic electrophoresis [6]. Therefore, these HPLC profiles do not represent the initial reaction products, but were a result of dimer exchange during the equilibrium reaction favoring the formation of symmetrical tetramers.

In the formulation of blood substitute solutions, the ability to resolve adequately the components of a solution is highly desirable for molecule characterization and quality control purposes. By using state-of-the art high-resolution cation- and anion-exchange HPLC columns, complex mixtures of pyridoxylated hemoglobin derivatives have been described and compared using both $NaCNBH_3$ and $NaBH_4$ reduction procedures. When our pyridoxylation procedure was used, $NaBH_4$-reduced PLP-HbA_0 was shown to have a higher degree of pyridoxylation than the $NaCNBH_3$-reduced mixture. High-resolution cation-exchange chromatography using a PolyCAT A column has been especially effective in showing the complexity of these pyridoxylated derivatives.

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