Journal of Chromatography, 417 (1987) 397–402
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3632

Note

Haemoglobin pyridoxylation: optimization and observation of an additional new pyridoxal phosphate–haemoglobin species by high-performance liquid chromatographic resolution

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(First received October 17th, 1986; revised manuscript received January 28th, 1987)

Human haemoglobin cannot be used in blood transfusion: its oxygen affinity is too high, thus it does not readily release oxygen to the tissues. Furthermore, its vascular persistence is too short, because it passes into capillaries and urine. Various chemical modifications of haemoglobin have been tried in attempts to overcome these difficulties. Benesch et al. [1] showed that the affinity of haemoglobin for oxygen could be reduced by coupling it to pyridoxal phosphate (PLP), the active form of vitamin B_6, which reacts with the amine functions of the globin to form Schiff bases that can be stabilized by reduction with agents such as sodium borohydride.

We have found no reports of attempts at standardization and quality control of the products obtained. Definite criteria are needed for the quality and properties of pyridoxylated haemoglobin, so that it can be easily and quickly checked, just like other injectable blood products. The objective of this work was to develop a simple, reproducible method of coupling, and a fast, automatic procedure for quality control by high-performance liquid chromatography (HPLC). Our chromatographic results also shed new light on the phenomenon of haemoglobin pyridoxylation, since a hitherto undescribed species was detected.

EXPERIMENTAL

The haemoglobin solution was prepared from outdated blood as previously described [2] and was sterilized by filtration (Swinnex, 0.22 μm pore diameter, Millipore, Molsheim, France).

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The technique used to couple haemoglobin with PLP was an adaptation of the combined techniques of Benesch et al. [3] and Sehgal et al. [4]. Haemoglobin was diluted to 70 g/l in a 1 *M* Tris–HCl buffer (E. Merck, Darmstadt, F.R.G.), pH 7.7. The resulting solution was kept at 4°C and was reduced to deoxyhaemoglobin by a current of nitrogen overnight. A deoxygenated solution of PLP (4 mol per mol haemoglobin) (Sigma, St. Louis, MO, U.S.A.), diluted in a 200 m*M* Tris–HCl buffer, was added and the mixture allowed to stand for 3 h. The imine formed was reduced in the absence of an antifoaming agent by sodium borohydride (Merck) in 1 m*M* sodium hydroxide, at a ratio of 20 mol per mol of haemoglobin. The coupling lasted 20 h for 0.5 l.

The deoxyhaemoglobin solution was stirred under oxygen for 1 h, centrifuged (20 000 *g* for 15 min) and dialysed against distilled water and sterilized. The degree of coupling was estimated by HPLC (Varian LC 5020 equipped with a UV 100 detector coupled to a Vista 401 integrator; Les Ulis, France). The sample, 10 μl of 15 g/l haemoglobin, was injected on a mono S cation-exchange column Pharmacia HR 5/5 (50×5 mm I.D. containing spherical particles of diameter 10 μm; Pharmacia, Uppsala, Sweden). The flow-rate of the mobile phase was 1 ml/min. The mobile phase was 10 m*M* sodium malonate buffer (Merck), pH 5.7, and the elution was a stepwise gradient from 10 to 300 m*M* lithium chloride buffer (Merck), pH 5.7. The eluent was monitored at 415 nm (only haemoglobin is detected at this wavelength, which is not the case at 280 nm [5]). The HPLC analysis time was 15 min. The oxygen dissociation curve of haemoglobin was traced by a Hem-O-Scan apparatus (Aminco, Silver Spring, MD, U.S.A.) at 37°C and P_{CO_2} 5.32 kPa, and the P_{50} was measured [6]. Methaemoglobin was estimated by the method of Evelyn and Malloy [7].

RESULTS AND DISCUSSION

The chromatogram of Fig. 1 shows that under our conditions of chromatography the pyridoxylated haemoglobin solution was separated into four and often five major fractions, not counting the Hb A1c fraction. The overall percentages of the various F fractions obtained are given in Table I. Non-pyridoxylated haemoglobin (called P_0 by Benesch et al. [8]) did not exceed 20% in our most recent experiments, this result being consequent on a greater agitation efficiency. The other three or four fractions presumably correspond to P_1, P_2 and P_4 (see schematic representation 1 given by Benesch et al. [8]). The penultimate one (i.e. F_3) may be P_3 (see Scheme 2), a pyridoxylated species that Benesch et al. did not describe. Peak P_4 (F_4) was not always detected, and the chromatogram then showed only four peaks: F_1, F_2, F_3 and F_0.

We tried to show graphically the percentages of the various fractions, F, as a function of the relative retention time t_R (Fig. 2). The best distribution was obtained with F_1 used as a reference. In this case, clusters of weakly scattered points are observed, indicating that each population is relatively homogeneous and distinct (Fig. 2). Only the F_4 population, whose relative t_R was shortest, is more scattered, to the point of being differentiated into two subgroups. One is presumably F_4 ($7.2 \pm 4.4\%$, $n=8$) and the other, with a shorter relative t_R, may

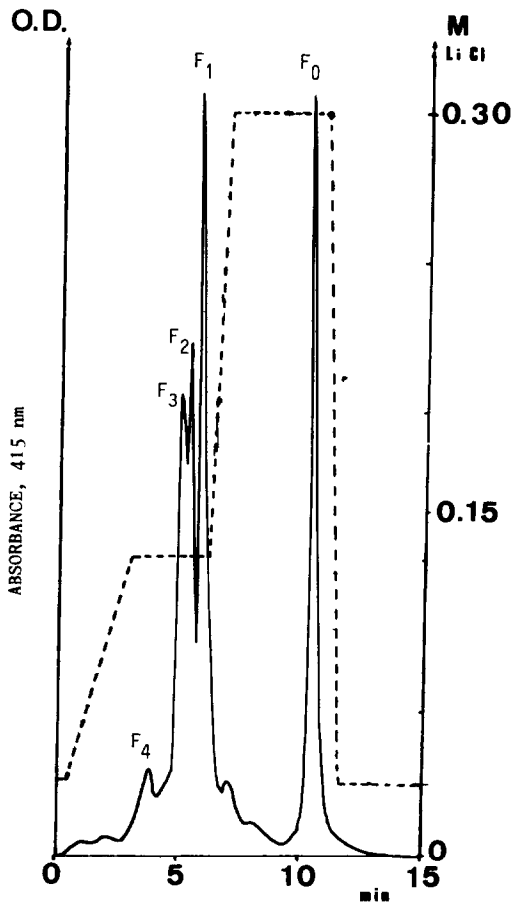

Fig. 1. HPLC of Hb–PLP eluted from a HR 5/5 Mono S cation-exchange column (Pharmacia). The dashed line shows the lithium chloride non-linear gradient in 10 m*M* sodium malonate buffer (pH 5.7). The flow-rate was 1 ml/min.

TABLE I

PERCENTAGES OF THE VARIOUS F FRACTIONS OF PYRIDOXYLATED HAEMOGLOBIN OBTAINED BY CATION-EXCHANGE CHROMATOGRAPHY

The subscripts to F indicate the number of PLP per mole of tetrameric haemoglobin molecule (see text); n = number of measurements.

Fraction	n	Percentage (mean ± S.D.)
F_0	22	33.3 ± 6.6
F_1	22	23.6 ± 6.0
F_2	22	20.4 ± 6.5
F_3	22	12.1 ± 4.4
F_4	16	7.6 ± 4.2

$$\alpha\beta^{1\ \text{Val PLP}} + \alpha\beta^{1\ \text{Val PLP}} \rightleftharpoons \alpha\beta^{1\ \text{Val PLP}} + \alpha\beta$$
$$\alpha\beta \qquad\qquad \alpha\beta \qquad\qquad \alpha\beta^{1\ \text{Val PLP}} \qquad\qquad \alpha\beta$$
$$F_1 \qquad\qquad F_1 \qquad\qquad F_2 \qquad\qquad F_0$$

$$\alpha\beta^{1\ \text{Val PLP}}_{82\ \text{Lys PLP}} + \alpha\beta^{1\ \text{Val PLP}}_{82\ \text{Lys PLP}} \rightleftharpoons \alpha\beta^{1\ \text{Val PLP}}_{82\ \text{Lys PLP}} + \alpha\beta$$
$$\alpha\beta \qquad\qquad \alpha\beta \qquad\qquad \alpha\beta^{1\text{Val PLP}}_{82\ \text{Lys PLP}} \qquad\qquad \alpha\beta$$
$$F_2 \qquad\qquad F_2 \qquad\qquad F_4 \qquad\qquad F_0$$

Scheme 1.

$$\alpha\beta^{1\ \text{Val PLP}} + \alpha\beta^{1\ \text{Val PLP}}_{82\ \text{Lys PLP}} \rightleftharpoons \alpha\beta^{1\ \text{Val PLP}}_{82\ \text{Lys PLP}} + \alpha\beta$$
$$\alpha\beta \qquad\qquad \alpha\beta \qquad\qquad \alpha\beta^{1\ \text{Val PLP}} \qquad\qquad \alpha\beta$$
$$F_1 \qquad\qquad F_2 \qquad\qquad F_3 \qquad\qquad F_0$$

Scheme 2.

be a more highly pyridoxylated form such as P_6 or P_8 ($8.0 \pm 4.3\%$, $n=8$).

According to this hypothesis, it appears that the separations between the mean t_R values for F_1, F_2 and F_3 are relatively constant. The same separation is also seen with the first subgroup of the cluster, supporting the argument that these are F_4 subunits. The second subgroup, in contrast, has a mean t_R whose separation is twice that of the previous ones, suggesting that there may be an F_8 subunit in which four PLPs may be bound (?) outside the pocket.

In order to increase haemoglobin pyridoxylation, we performed a second pyridoxylation on a haemoglobin solution that had already reacted once. About a quarter of subunit F_0 was pyridoxylated; this fraction thus fell from 23 to 17% of the total and increased the concentrations of the other four. If pyridoxylation were quasi-total, it would have the advantage of avoiding the purification step, as carried out by Sehgal et al. [4] on DEAE Sephadex. To our minds, this may result in dilution and denaturation of the haemoglobin, but it does not seem profitable in this case, in view of the small change in the percentage of F_0.

In conclusion, our method of pyridoxylation is rapid (5 h for the chemical reactions, the deoxygenation being conducted overnight) and reproducible; the coupling obtained is consistent (70–75%) and results in a strong increase of the P_{50}, which rises from 2.53 to 4.0 kPa, or a 58% increase. The low methaemoglobin content (less than 3%), high saturation (96% HbO_2) and non-use of an anti-foaming agent, which is poorly tolerated in vivo, all convince us that the procedure does not greatly damage the haemoglobin.

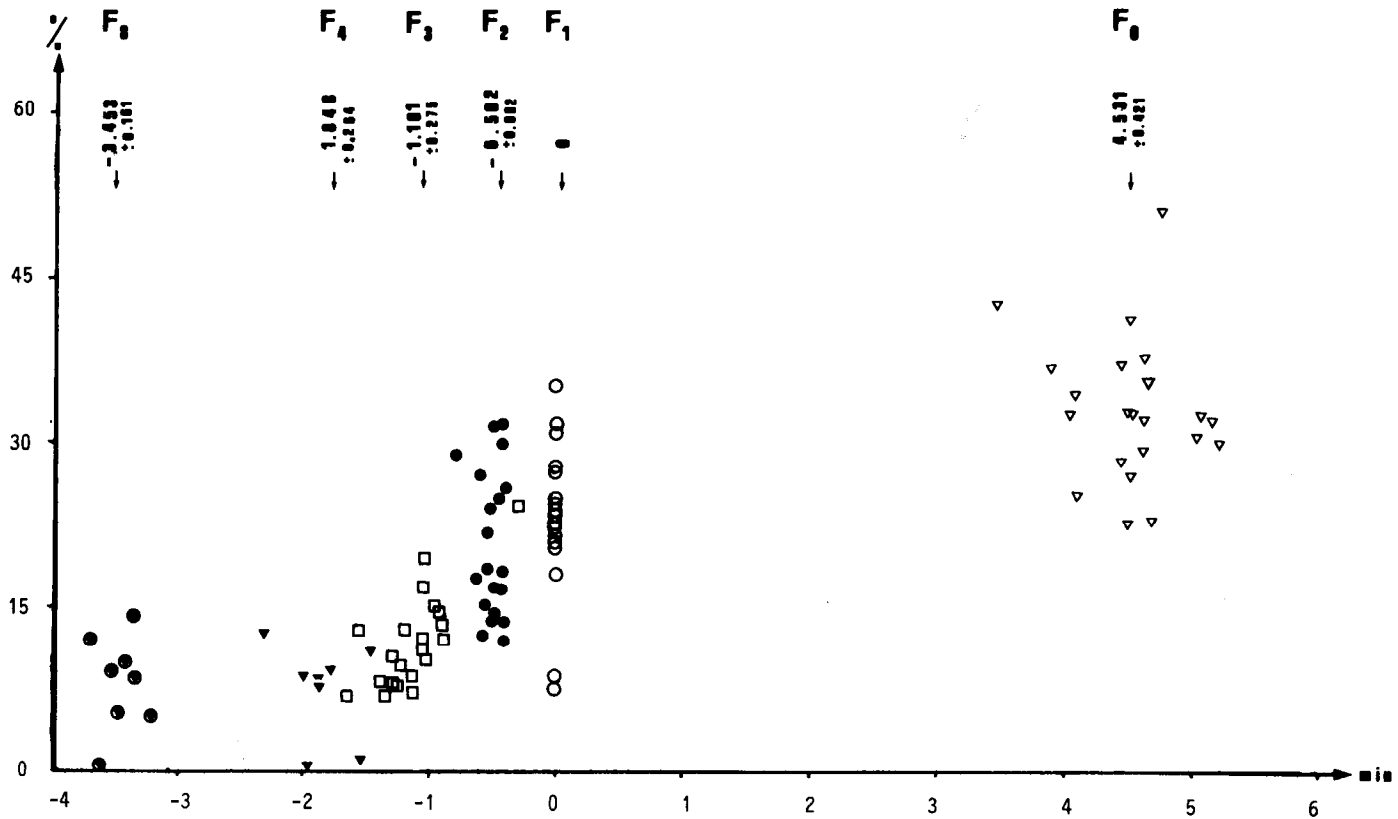

Fig. 2. Percentages of pyridoxylation as a function of retention time. Data points: ▽ = non-pyridoxylated haemoglobin; ○ = F_1 fractions; ● = F_2 fractions; □ = F_3 fractions; ▼ = F_4 fractions; ⊛ = F_8 fractions. Mean ± S.D. of relative retention time for each fraction is indicated at the top of the figure.

HPLC permits rapid and accurate evaluation of the overall level of coupling. Use of this HPLC separation resulted in resolution of P_3 and P_4. Benesch et al. [3] reported 3–4 mol per tetramer of deoxyhaemoglobin for pool I in 1972. Finally, this improvement of pyridoxylation — i.e. a better yield, a compromise between a large volume and a short reaction time, which favours protection of the active site of the haemoglobin (preventing other compounds from entering the DPG binding site), molecular cohesion (with better intravascular retention and lesser urinary elimination, as described by Greenburg et al. [9] and also found by ourselves [10]) and the release of oxygen — makes it possible to proceed later to polymerization or to coupling of macromolecules without significant risk of reducing the oxygen-carrying properties of the conjugates.

REFERENCES

1 R.E. Benesch, R. Benesch, A. Bank, R. Renthal and B.A. Bray, in T. Arends, G. Bemski and R.L. Nagel (Editors), Proceedings First Inter-American Symposium on Hemoglobins, Caracas, 1969, Karger, Basel, 1971, p. 134.
2 P. Menu, P. Voisin, P. Labrude and C. Vigneron, Int. J. Artif. Organs, 8 (1985) 341.
3 R.E. Benesch, R. Benesch, R.D. Renthal and N. Maeda, Biochemistry, 11 (1972) 3576.
4 L.R. Sehgal, A.L. Rosen, G. Noud, H.L. Sehgal, S.A. Gould, R. DeWoskin, C.L. Rice and G.S. Moss, J. Surg. Res., 30 (1981) 14.
5 T.I. Přistoupil, M. Kramlová, J. Kraml and S. Ulrych, J. Chromatogr., 219 (1981) 436.
6 Hem-O-Scan™, Oxygen Dissociation Analyser, Operator's manual, Aminco, Silver Spring, MD, 1978, pp. 5–9.
7 K.A. Evelyn and H.I. Malloy, J. Biol. Chem., 126 (1938) 655.
8 R. Benesch, R.E. Benesch and S. Kwong, J. Biol. Chem., 257 (1982) 1320.
9 A.G. Greenburg, G.W. Peskin, D.B. Hoyt and W.Y. Moores, Crit. Care Med., 10 (1982) 266.
10 P. Menu, F. Pauly, P. Labrude and C. Vigneron, unpublished results.