Journal of Chromatography, 355 (1986) 438–441
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 391

Note

Gel chromatography of pyridoxalated and glutaraldehyde-treated human haemoglobin on Superose™ 12

M. KRAMLOVÁ*, T. I. PŘISTOUPIL and V. FRIČOVÁ
Institute of Haematology and Blood Transfusion, Prague (Czechoslovakia)
and
J. KRAML
1st Department of Medical Chemistry, Charles University, Prague (Czechoslovakia)
(Received December 4th, 1985)

The characterization and batch monitoring of chemically modified haemoglobins (Hb), under investigation as possible oxygen-carrying and offloading infusable fluids, needs sensitive methods of analytical fractionation. Rapid micromethods are usually preferred[1–3].

In the present work we used a recently developed variant of gel chromatography on monodisperse microbeads of Superose™ 12 (Pharmacia) with a fast protein liquid chromatography (FPLC) apparatus (Pharmacia)[4]. The results are compared with those achieved by gel chromatography on Sepharose 6B. Irregularities and discrepancies observed during gel chromatography of the labile haemoglobin preparations are discussed as well as certain implications concerning the retention times of haemoglobins in the bloodstream.

MATERIALS AND METHODS

Human stroma-free haemoglobin (SFH) and its derivatives (PHIR-PG) modified with pyridoxal-5'-phosphate, borohydride and glutaraldehyde were prepared as before[2]. The samples, usually containing 60 g Hb/l, were filtered through a 0.22-μm sterile Millipore filter prior to application to an HR 10/30 chromatographic column prepacked with Superose™ 12 (ref. 4). A 0.05 *M* phosphate buffer pH 7.0 containing 0.15 *M* sodium chloride was degassed, filtered through a sterile 0.22-μm filter and used for equilibration of the column and elution of the SFH samples. The chromatographic column was connected to the Pharmacia FPLC system and separations were achieved according to the instruction manual[4]. A gel-filtration calibration kit of standard proteins for molecular weight determination (Pharmacia) was used. Standard gel chromatography was performed on columns of Sepharose 6B (55 × 1.7 cm) and Sephacryl S-200 (70.5 × 1.7 cm) at a flow-rate of 0.13 ml/min.

RESULTS AND DISCUSSION

Fig. 1 shows a typical elution pattern of a mixture of standard native proteins

0021-9673/86/$03.50

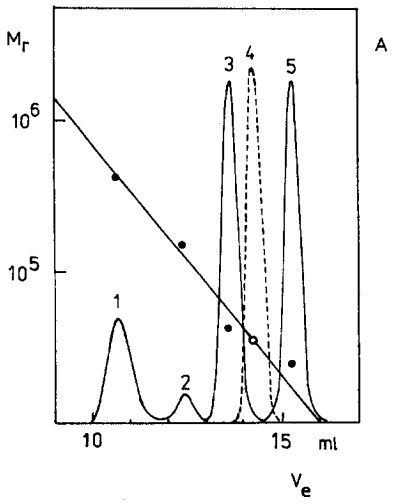

Fig. 1. Elution profiles of proteins and calibration curve for M_r estimation on Superose™ 12. Curves: 1, ferritin; 2, aldolase; 3, ovalbumin; 4, native human haemoglobin (separate experiment); 5, chymotrypsinogen. Buffer: 0.15 *M* sodium chloride, 0.05 *M* sodium phosphate pH 7.0. Flow-rate : 0.4 ml/min. *A* = Absorbance at 280 nm.

on Superose™ 12. The haemoglobin samples were chromatographed separately under the same conditions. Repeated experiments led to very sharp and reproducible separations. The plots of V_e (elution volume) *versus* log M_r (relative molecular mass) at the two flow-rates used, 0.4 and 0.9 ml/min, were identical. The separation on Superose™ 12 usually required about 25 min in contrast to 13–15 h on Sepharose 6B. Moreover, the peaks were markedly more distinct on Superose™ 12. The V_e/log M_r relationship was not exactly linear with the given calibration kit, evidently due to the effects of factors other than M_r, *e.g.*, molecular size, shape and various interactions with the gel matrix which influenced the V_e values. The haemoglobin tetramer Hb_4 (M_r 64 500) is known to dissociate in aqueous solution into subunits of lower M_r. The statistical ratio of dissociated to undissociated haemoglobin molecules at equilibrium in a given buffer solution depends on the Hb concentration[5]. Therefore, gel chromatography of haemoglobin on either Sephadex G-100 (ref. 5), Sepharose 6B (ref. 2) or Superose™ 12 led always to higher V_e values than expected[5]. However, when a standard amount of native haemoglobin (either freshly prepared or stored dry with sucrose[2]), *e.g.*, 1 mg in 0.1 ml, was repeatedly applied to a given column and chromatographed under standard conditions, reproducible V_e values were achieved for the haemoglobin moiety, corresponding to a mean M_r of 37 000. In the above sense, haemoglobin can be used as a coloured substance for M_r calibration.

An irregularity was observed repeatedly during gel chromatography of native haemoglobin on columns of Sephacryl S-200. In parallel experiments, where haemoglobin was chromatographed on Sepharose 6B and Superose™ 12 columns without any problems, on Sephacryl S-200 the haemoglobin was denatured spontaneously after about 1 h, flocculated in the column and the whole chromatographic procedure deteriorated. In contrast to native haemoglobin, the modified samples (PHIR-PG)

were chromatographed without problems under the same conditions on the same Sephacryl S-200 column. The reason might be either the stabilization of Hb molecules of PHIR-PG by glutaraldehyde, *cf.*, Fig. 3, or hydrophobic interactions of the native haemoglobin with the cell matrix of Sephacryl S-200, leading to denaturation.

The elution curve 1 in Fig. 2 indicates the presence of three main molecular populations in modified haemoglobin of the type PHIR-PG. The peaks correspond to M_r 240 000 (22%), 120 000 (23%) and 43 000 (55%) which indicates roughly the prevalence of polycondensates Hb_{16}, Hb_8 and of subunits Hb_2 + Hb_3 (in ratio *ca.* 2:1). Intramolecularly stabilized tetramers Hb_4 (Fig. 3) which are also present in modified haemoglobins, according to other analytical methods[1,6–8], are believed to be hidden between the two higher peaks.

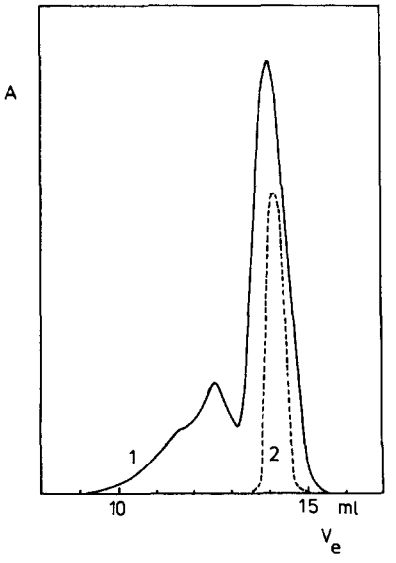

Fig. 2. Gel chromatography of haemoglobin samples on Superose™ 12. Curves: 1, human haemoglobin treated with pyridoxal-5'-phosphate, glutaraldehyde and borohydride (PHIR-PG); 2, native human stroma-free haemoglobin (SFH). Other conditions as in Fig. 1. *A* = Absorbance at 280 nm.

The fact that the biggest peak (55%) of PHIR-PG and the peak of native haemoglobin had very similar elution volumes was felt to be a discrepancy since in biological experiments[7] (*cf.*, ref. 6) PHIR-PG and native haemoglobin differed markedly in their half retention times ($T/2$) in the bloodstream of rats. After infusion of haemoglobin solutions, PHIR-PG showed a $T/2$ of 11–18 h while native haemoglobin showed $T/2$ = 1.5–2 h due to dissociation of Hb_4 into subunits and their rapid renal filtration. The prolonged $T/2$ of modified polycondensed haemoglobin (PHIR-PG) was not surprising. More puzzling was the high amount of Hb_2 and Hb_3 in PHIR-PG during gel chromatography as well as of Hb_1 in other experiments, where sodium dodecyl sulphate (SDS)-treated PHIR-PG was analyzed by gel permeation techniques[1,6,8].

An explanation could be found in terms of the very simple scheme shown in

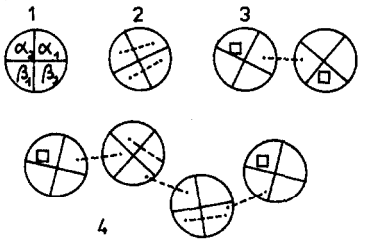

Fig. 3. A simplified scheme of modified haemoglobin molecules assumed to be present in PHIR-PG samples. Circles: haemoglobin tetramers Hb_4. Squares: bound pyridoxalphosphate. Dashed lines: glutaraldehyde cross-links. 1, Native tetramer Hb_4 with subunits; 2, intramolecularly cross-linked Hb_4 (after full dissociation, two "true" dimers would appear); 3, pyridoxalated Hb_8 with one intermolecular cross-link (after dissociation, two pyridoxalated and four intact monomers as well as one cross-linked "untrue" dimer would appear); 4, pyridoxalated cross-linked Hb_{16}, assumed to form after full dissociation of two pyridoxalated and five intact monomers, one "untrue" trimer, two "untrue" and one "true" dimers.

Fig. 3. Hypothetically, some of the Hb molecules and subunits, which have been altered by the reaction with pyridoxal-5'-phosphate and covalently bound by glutaraldehyde, do not dissociate, while those remaining intact are able to dissociate (partly in water and fully in SDS-containing media) and can enter the gel cavities or pass through the gel network during gel permeation. Moreover, about 10–20% of haemoglobin molecules in modified Hb are known to be unaffected by the chemicals added. A different treatment of chemically modified haemoglobin molecules and subunits, even if they are of the same size as the native ones, in the kidney must be assumed, too.

In conclusion, gel chromatography of proteins on Superose™ 12 proved most useful in shortening significantly the analysis time and in sharpening the elution peaks in comparison with gel chromatography on, *e.g.*, Sepharose 6B. Gel chromatography on Superose™ 12 together with isoelectric focusing remain the most suitable methods for a direct characterization of the molecular heterogeneity of intact haemoglobin products, unchanged by a supplementary addition of denaturing agents, *e.g.*, SDS[2,6–8] or 4-hydroxymercuribenzoate[3].

REFERENCES

1 T. I. Přistoupil, M. Kramlová, V. Fričová, S. Ulrych, J. Kraml and K. Jojková, *J. Chromatogr.*, 213 (1981) 183.
2 T. I. Přistoupil, M. Kramlová, H. Fořtová, V. Fričová and L. Kadlecová, *J. Chromatogr.*, 288 (1984) 469.
3 T. I. Přistoupil, M. Kramlová, H. Fořtová and V. Fričová, *J. Chromatogr.*, 346 (1985) 401.
4 *Pharmacia instruction manual 52-1768-00-01 (1985).*
5 P. Andrews, *Biochem. J.*, 91 (1964) 222.
6 L. R. Sehgal, A. L. Rosen, S. A. Gould, H. L. Sehgal, G. S. Moss, in R. B. Bolin, R. P. Geyer and G. J. Nemo (Editors), *Advances in Blood Substitute Research*, Alan. R. Liss, New York, 1983, pp. 19–28.
7 V. Fričová, E. Paluska, T. I. Přistoupil and M. Kramlová, *Biologia*, in press.
8 H. Fořtová, J. Suttnar, M. Kramlová and T. I. Přistoupil, *J. Chromatogr.*, 357 (1986) in press.