ELSEVIER

Journal of Chromatography B, 656 (1994) 119–122

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short Communication

EDMA 2000 as a matrix for high-performance liquid chromatography of human haemoglobin chains

Jiří Suttnar*, Hana Fořtová, Václav Brabec

Department of Biochemistry, Institute of Hematology and Blood Transfusion, U nemocnice 1, 128 20 Prague 2, Czech Republic

Abstract

An ethylenedimethacrylate polymer-based matrix (EDMA 2000) is described that shortens the time for separation of haemoglobin (Hb) chains to 30 min, compared with the use of C_4 large-pore Vydac columns, commonly used for reversed-phase HPLC of Hb variants. EDMA 2000 was successfully used for the isolation of an abnormal β-chain of the unstable Hb recently characterized as Hb Nottingham.

1. Introduction

Human haemoglobin (Hb) abnormalities have been studied for many years since 1949 when HbS was discovered in sickle cell anaemia [1]. The structural analyses of these proteins, the clinical evaluation of haemoglobinopathies and direct DNA analyses have provided data that are invaluable for a better understanding of the basis of various haematological abnormalities [2,3].

Most haemoglobinopathies, connected with the presence of abnormal Hb, are caused by point mutation in one of the five types of Hb chains (α, β, γ, δ, ϵ) normally found in healthy persons in various stages of development. Thalassaemias, special part of haemoglobinopathies, result from the underproduction of α (α-thalassaemia) or β (β-thalassaemia) Hb chains. An imbalanced α/non-α chain synthesis is the major factor in determining the severity of the disease. Information about the relative amounts of the different individual human haemoglobin chains in blood are therefore valuable in the diagnosis of haemoglobinopathies.

The development of accurate and rapid chromatographic procedures using reversed-phase high-performance liquid chromatography (RP-HPLC) has allowed the separation and determination of many types of haemoglobin chains [4–7]. The time for separation of haemoglobin chains was thus shortened from several hours (on CM cellulose with 8 *M* urea [8]) to several tens of minutes. Separation of Hb chains in a few minutes has also been performed by capillary zone electrophoresis [9].

We describe in this paper a relatively fast HPLC technique for the separation of haemoglobin chains in an artificial mixture of haemoglobins and in some erythrocyte lysates, and further the separation of Hb chains of an unstable Hb (characterized as Hb Nottingham [10])

* Corresponding author.

0378-4347/94/$07.00
SSDI 0378-4347(94)00077-I

on an ethylenedimethacrylate (EDMA) polymer-based matrix.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and methanol (Lichrosolv grade) were supplied by Merck (Darmstadt, Germany). Water was doubly glass distilled and deionized on a Milli-Q50 system (Millipore, Vienna, Austria). Trifluoroacetic acid (TFA) for protein sequential analysis was obtained from Fluka (Buchs, Switzerland).

2.2. Sample preparation

Peripheral blood samples were obtained from normal subjects, from a patient with unstable Hb, from a case of homozygous β-thalassaemia and from heterozygotes for HbS, HbE. Cord blood samples from normal newborns were collected at delivery. Red blood cells were washed in saline and lysed with a double volume of distilled water. The Hb concentration in the haemolysate was adjusted to 1 g/dl with distilled water. Different haemoglobins, HbA ($\alpha_2\beta_2$), HbA_2 ($\alpha_2\delta_2$), HbS ($\alpha_2\beta_2^S$), HbF ($\alpha_2\gamma_2$) and HbE ($\alpha_2\beta_2^E$), were obtained by chromatography on MonoQ (Pharmacia, Uppsala, Sweden) performed in 50 m*M* Tris–HCl buffer with 100 mg/l KCN with a pH gradient from pH 8.5 to 6.5 [11]. Samples were concentrated, if necessary, by ultrafiltration and stored at −50°C if not assayed immediately.

2.3. Apparatus and chromatographic conditions

Separations were carried with an LKB low-pressure gradient liquid chromatograph fitted with a Rheodyne Model 7125 sample injector, an LKB 2510 Uvicord SD fixed-wavelength detector operating at 226 nm, a membrane degasser (LDC Analytical, Riviera Beach, FL, USA) and a data station (Watrex, Prague, Czech Republic). The column (150 × 3 mm I.D.) contained EDMA 2000 (7 μm) (Tessek, Prague, Czech Republic). Temperature control was achieved by using a water jacket and an U1 thermostat (MLW, Freital, Germany).

Elution was performed with the following linear segment gradient: 0 min, 0% B; 8 min, 14% B; 25 min, 30% B; 30 min, 30% B. The flow-rate was 0.4 ml/min and the temperature was maintained at 44°C. Solvent A was 60% methanol–0.3% TFA, solvent B was 75% methanol–0.3% TFA.

Erythrocyte lysates, isolated haemoglobins or their mixtures were centrifuged at 15 000 *g* and then injected directly in various volumes (1–10 μl) corresponding to about 10–100 μg of total haemoglobin. Peaks were identified by comparing their retention times with those of peaks obtained with standard haemoglobin solutions.

3. Results and discussion

Low-pH eluents used for HPLC promote the dissociation of Hb into the prosthetic group haem and the two pairs of polypeptide chains. Separation of Hb chains resulting from an artificial mixture of haemoglobins A, A_2, E and F, in approximately equimolar proportions, is illustrated in Fig. 1. The β-chain of HbS co-eluted with the β-chain of HbA (data not shown). γ-Chains are produced by two non-allelic genes coding for either Gly ($^G\gamma$) or Ala ($^A\gamma$) at position 136. Fig. 1 shows that peaks of $^G\gamma$ and $^A\gamma$ are well separated under the conditions described above. The quantitative ratio $^G\gamma/^A\gamma$ is different in various haemoglobinopathies, especially in thalassaemia and in cases of elevated HbF in adults, and can serve as a potential prognostic factor in patients with myelodysplastic syndrome [12].

When comparing the results achieved with commonly used columns for the separation of Hb chains (mostly Vydac C_4), EDMA 2000 shows shorter retention times and different orders of eluted peaks. The peaks of Hb chains and haem were eluted on EDMA 2000 under conditions described above in the following order: α, haem, β^E, β, δ, $^G\gamma$ and $^A\gamma$. The

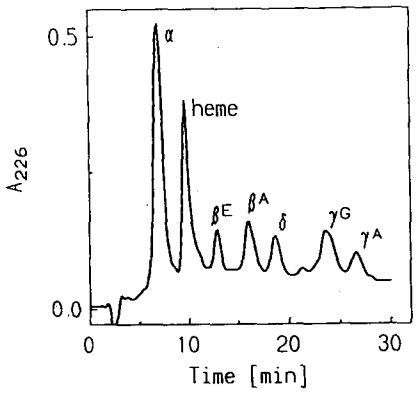

Fig. 1. Chromatogram of an artificial mixture of haemoglobins A, A_2, E and F in approximately equimolar proportions (5 μl of a mixture of chromatographically pure haemoglobins). Hb chains generated by dissociation of Hbs under chromatographic conditions are indicated by Greek letters. Conditions: eluent A, 60% methanol–0.3% TFA; eluent B, 75% methanol–0.3% TFA; linear segment gradient as in text; flow-rate, 0.4 ml/min; column temperature, 44°C; detection wavelength, 226 nm.

resolution of peaks with eluents containing 0.3% or 0.1% TFA was the same. We preferred the higher concentration of TFA to facilitate complete dissociation of haemoglobins to globin chains and haem groups. Elevated temperature reduced the column pressure and broadening of peaks without any effect on the column lifetime. As many as 350 separations have been performed to date on each column without a significant decrease in reproducibility.

The mean retention times of the main Hb chains and haem are shown in Table 1. The reproducibility was assessed from 30 separations carried out on the same column within 3 days and with different Hb samples. However, some differences in selectivity were noted between columns under the same operating and ageing conditions. A correct and reproducible eluent preparation is critical if reproducible retention times are to be obtained. The lower limit of detection of Hb chains at 226 nm was *ca.* 1 μg of haemoglobin. An attempt to improve the sensitivity using UV detection at 206 nm failed owing to a high background and sloping of the baseline. In preliminary experiments we used a moderate gradient of acetonitrile in water containing 0.3% TFA, which only slightly resolved β^A- and δ-chains and did not resolve β^A- and β^S-chains; the haem was eluted as the last peak (Fig. 2).

Table 1
Reproducibility of globin chains and haem separations

Chain or haem	Retention time (min)	Coefficient of variation (%)
α-Chain	6.45 ± 0.19	2.95
Haem	9.80 ± 0.15	1.53
β-Chain	16.11 ± 0.37	2.30
δ-Chain	18.25 ± 0.25	1.37

Values are the means ± standard deviations of 30 determinations within 3 days. Experimental conditions as in Fig. 1.

EDMA 2000 was successfully used for the separation of Hb chains of the unstable Hb in the blood of an 8-year-old girl from the Czech Republic. The presence of the unstable Hb was revealed by a blood stability test [13]. HPLC of normal HbA and of abnormal Hb (HbX) is shown in Fig. 3. The slight difference in the elution volume of the β^X-chain in comparison with that of the β^A-chain, and the same elution

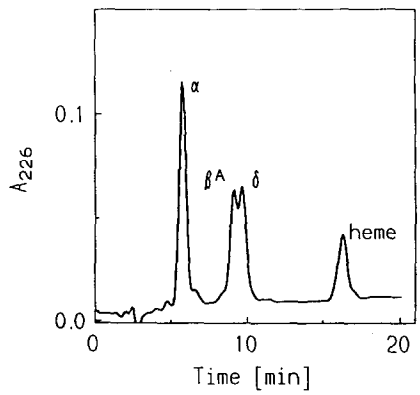

Fig. 2. Chromatogram of an artificial mixture of haemoglobins A and A_2 in approximately equimolar proportions (1 μl of a mixture of chromatographically pure haemoglobins). Hb chains generated by dissociation of Hbs under chromatographic conditions are indicated by Greek letters. Conditions: eluent A, 35% acetonitrile–0.3% TFA; eluent B; 50% acetonitrile–0.3% TFA; linear segment gradient, 0 min, 0% B; 16 min, 60% B; 20 min, 60% B; flow-rate, 0.4 ml/min; column temperature, 41°C; detection wavelength, 226 nm.

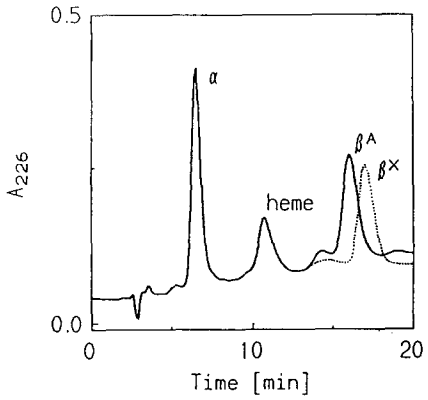

Fig. 3. Chromatogram of Hb chains from erythrocyte lysate from a normal blood donor (solid line) and the abnormal Hb from a patient with unstable HbX/β^X (dotted line). Conditions as in Fig. 1.

volume of the α^X- and α^A-chains, indicated the abnormality in the β-chain. Isolated β^X- and β^A-chains were aminoethylated and digested with trypsin. The resulting peptides were separated by RP-HPLC on a CGC C_{18} column (Tessek) with a linear gradient of acetonitrile in 50 m*M* H_3PO_4 (pH 2.9). Peptides from the β^X- and β^A-chains differing in elution volumes were rechromatographed on the same column with a linear gradient of acetonitrile in 30 m*M* phosphate buffer (pH 5.5) and subjected to sequential analysis. The abnormal Hb was thus identified as Hb Nottingham with amino acid substitution Val→Gly in position 98 [10].

The EDMA 2000 column allows the separation of globin chains in 30 min with satisfactory sensitivity and reproducibility. The method can be applied in studies of physiological and clinical problems concerning haemoglobins.

4. Acknowledgement

This work was supported by a grant from IGA MZ 3723 (Prague, Czech Republic).

5. References

[1] L. Pauling, H.A. Itano, S.J. Singer and I.C. Wells, *Science*, 110 (1949) 543.
[2] H. Lehmann and R.G. Huntsman, *Man's Haemoglobins*, North-Holland, Amsterdam, 1974, p. 237.
[3] J.G. Adams, III, and M.B. Coleman, *Sem. Hematol.*, 27 (1990) 229.
[4] J.O. Jeppsson, I. Källman, G. Lindgren and L.G. Fägerstam, *J. Chromatogr.*, 297 (1984) 31.
[5] J.B. Shelton, J.R. Shelton and W.A. Schroeder, *J. Liq. Chromatogr.*, 7 (1984) 1969.
[6] L. Leone, M. Monteleone, V. Gabutti and C. Amione, *J. Chromatogr.*, 321 (1985) 407.
[7] F. Kutlar, H. Moscoso, C.R. Kiefer, F.A. Garver, S. Beksac, L. Önderoglu, A. Gurgey, C. Altay and T.H.J. Huisman, *J. Chromatogr.*, 567 (1991) 359.
[8] J.B. Clegg, M.A. Naughton and D.J. Wetherall, *J. Mol. Biol.*, 19 (1966) 91.
[9] P. Ferranti, A. Malorni, P. Pucci, S. Fanali, A. Nardi and L. Ossicini, *Anal. Biochem.*, 194 (1991) 1.
[10] V. Brabec, K. Indrák, J. Zeman, H. Fořtová, J. Suttnar, B. Blažek, J. Čermák, V. Divoký, L. Vepřeková and T.H.J. Huisman, *Ann. Hematol.*, in press.
[11] F. Winston, W.F. Moo-Penn and D.L. Jue, *J. Chromatogr.*, 318 (1985) 325.
[12] K.L. Bourantas, I. Georgiou and K. Seferiadis, *Haematologica*, 76 (1991) 337.
[13] J.V. Dacie, A.J. Grimes, A. Meisler, L. Steingold, E.H. Hemsted, G.H. Beaven and J.C. White, *Br. J. Haematol.*, 10 (1964) 388.