ELSEVIER

Journal of Chromatography A, 718 (1995) 397–404

JOURNAL OF CHROMATOGRAPHY A

Separation of hemoglobin variants in single human erythrocytes by capillary electrophoresis with laser-induced native fluorescence detection

Sheri J. Lillard[a], Edward S. Yeung[a,*], Roy M.A. Lautamo[b], David T. Mao[b]

[a] *Department of Chemistry and Ames Laboratory-USDOE, Iowa State University, Gilman Hall, Ames, IA 50011, USA*

[b] *J&W Scientific, 91 Blue Ravine Road, Folsom, CA, USA*

First received 4 April 1995; revised manuscript received 15 June 1995; accepted 21 June 1995

Abstract

Single human red blood cells, in which the hemoglobin (Hb) molecules exist in their native, tetrameric states, were analyzed. Upon injection and lysis of a cell, the tetramers were dissociated on-column into their respective polypeptide chains, separated, and detected by laser-induced native fluorescence detection with 275-nm excitation. This technique was applied to the determination of hemoglobin variants as found in adult (normal and elevated Hb A_1) and fetal erythrocytes. Normal adult cells contained 9.6% and 4.8% glycated β- and α-chains, respectively. Cells with elevated Hb A_1 gave 30% and 12%, respectively. The amounts of glycated Hb and total Hb in a given cell were found to be uncorrelated.

1. Introduction

The separation of hemoglobin (Hb) variants by capillary electrophoresis (CE) represents a special challenge due to the similarities among the various hemoglobin molecules which are present in a human erythrocyte. The hemoglobin molecule exists as a tetramer of four polypeptide chains to each of which a heme group is attached. Human hemoglobin has an approximate molecular mass of 65 500 [1]. The main adult component, Hb A_0, consists of two $\alpha\beta$ dimers ($\alpha_2\beta_2$) and comprises about 90% of the total hemoglobin content [2]. While genetic variants involve one or more differing amino acids in the protein sequence of one or both types of globin chains, other variants, such as Hb A_{1c}, arise from a post-translational modification [2]. In fact, Hb A_{1c}, which constitutes roughly 5% of the total hemoglobin in normal erythrocytes, only differs from Hb A_0 by one glucose molecule attached to the N-terminal valine of each β-chain [2].

With more than 500 variants known to human hemoglobin [3], there are many hemoglobinopathies for which diagnosis is based on the detection of variants. Examples include the presence of the abnormal variant Hb S in sickle cell anemia [4] or the increased amount of Hb A_2 in β-thalassemia [5]. Hb A_{1c} is known to be elevated two- to three-fold in untreated diabetes mellitus, and its monitoring serves to give a measure of long-term blood glucose control [6].

Additionally, Hb A_{1c} may also be used to assess relative cell age. The life span of a

* Corresponding author.

0021-9673/95/$09.50 © 1995 Elsevier Science B.V. All rights reserved
SSDI 0021-9673(95)00702-4

circulating human erythrocyte is about 120 days [7,8]. Most studies, in which a comparison between a cellular constituent (or property) and cell age is made, utilize a density-gradient centrifugation method, such as that of Murphy [9]. In such a physical separation of cells, the more dense cells are presumed to be older, while the less dense cells correspond to the younger population. Fitzgibbons et al. [10] conducted a study in which Hb A_{1a+b} and A_{1c} were determined as a function of cell density, and a positive correlation of the amounts was seen. Glycohemoglobins were also determined by Elseweidy et al. [11], in which a dextran 40 density-gradient centrifugation method was utilized. Glycohemoglobin amounts in both adult and fetal red blood cells were shown to increase with increasing cell density. Some investigators still question whether density centrifugation can truly separate cells on the basis of age, hence, the validity of correlations of density with cell age have remained somewhat ambiguous [8]. However, the mechanism by which glycohemoglobin is formed [2] makes this class of compounds a good candidate for the determination of relative cell age in a population of cells, independent of density-based fractionations.

The total hemoglobin content in human erythrocytes is about 450 amol [12], of which Hb A_{1c} is about 5% in normal adults, and 2–3 times higher in diabetics. This amount is sufficient to allow determination at the level of a single cell by employing a sensitive detection scheme such as laser-induced native fluorescence [13]. Determinations of single erythrocytes, when compared to a sample of lysed cells in which the average is assessed, allow subtle intercellular differences to be seen, which in turn may give further insights into the aging process.

Although CE has been utilized to efficiently separate hemoglobin variants and/or their corresponding globin chains, the absorption detection methods used with most of these studies would not permit low enough detection limits for single-cell analysis [14–19]. Separations of globin chains under denaturing CE conditions, as well as intact genetic variants by capillary isoelectric focusing (cIEF), were demonstrated by Zhu and co-workers [14,15]. Ferranti et al. [16] also separated normal globin chains by free-zone CE, utilizing a coated capillary. The separation of Hbs A, F, S and C by free-zone electrophoresis was demonstrated by Huang et al. [17], in which a neutral, hydrophilic coated capillary was used. Using a dynamic cIEF technique, Molteni et al. [18] have separated Hb A_{1c} from A_0 as well as from other variants. Hempe and Craver [19] used a similar cIEF approach to separate hemoglobins, including Hb A_{1c} in blood samples. However, they injected only 40-nl sample volumes, instead of filling 25–100% of the capillary length as in normal or dynamic cIEF [15,18].

In this work, we demonstrate a separation scheme based on free-zone electrophoresis for the separation of hemoglobin variants in single human erythrocytes. To our knowledge, this is the first report in which Hb variants were determined in single cells. Previous work involved the separation of Hb A_0 from carbonic anhydrase and methemoglobin in single human erythrocytes [13]. In the surfactant-based system described here, the Hb molecules are denatured on-column into their corresponding polypeptide chains. Hemoglobin variants from single human adults (normal and elevated Hb A_1) and fetal red blood cells are separated and identified. Capillary lifetime, as well as the effects of the cell suspension buffer, are also discussed.

2. Experimental

The experimental setup used in this work has been described elsewhere [13]. Briefly, 20 μm I.D., 360 μm O.D. fluorocarbon (FC)-coated experimental capillaries were used (J&W Scientific, Folsom, CA, USA). The total length is 75 cm (65 cm to the detector). A high-voltage power supply (Glassman High Voltage, Whitehorse Station, NJ, USA; EH Series; 0–40 kV) was used for electrophoresis, with an applied voltage of +25 kV at the injection side. The capillary was rinsed with running buffer for ca. 1 h, then equilibrated at +25 kV for an equivalent amount of time before use. The standards were injected hydrodynamically by

raising the sample vial to a height of 11 cm relative to the detection end for 30 s. Electropherograms were recorded via a 24-bit A/D interface (ChromPerfect Direct, Justice Innovation, Palo Alto, CA, USA) and stored on a computer. Further peak analysis was performed by Peakfit (Jandel Scientific Software, San Rafael, CA, USA).

An argon-ion laser (Spectra Physics, Mountain View, CA, USA; Model 2045) was used as the excitation source, from which the 275.4-nm line was isolated with a prism. The laser was focused with a 1-cm focal length quartz lens onto the detection window. Two UG-1 color filters (Schott Glass Technologies, Duryea, PA, USA) were used to reject scattered light and placed directly in front of the photomultiplier tube.

All chemicals were obtained from Fisher Scientific (Fair Lawn, NJ, USA), unless otherwise noted. The running buffer for electrophoresis was 50 m*M* H_3PO_4, and 0.05% (w/v) fluorocarbon surfactant (FC, J&W Scientific, Folsom, CA, USA), adjusted to pH 2.7 ± 0.1 with NaOH. Deionized water was used from a Waters Purification System (Millipore, Milford, MA, USA). The buffer was filtered before use with a 0.22-μm cutoff cellulose acetate filter (Costar, Cambridge, MA, USA). Hemoglobin A_0 was purchased from Sigma Chemical (St. Louis, MO, USA).

Normal adult erythrocytes were obtained from a presumably healthy female volunteer. Fetal erythrocytes (cord blood sample), and elevated Hb A_1 samples were drawn into EDTA-tubes and obtained from Mercy Hospital (Des Moines, IA, USA). Phosphate buffered saline (PBS) was comprised of 135 m*M* NaCl and 20 m*M* $NaH_2PO_4 \cdot H_2O$; pH 7.4. All cells were treated the same way prior to injection: 20 μl of whole blood was washed with PBS, centrifuged and the supernatant discarded. This procedure was repeated five times, after which the cells were resuspended in 8% (w/v) D-glucose. The cell injection procedure has been described previously [13,20]. Briefly, the injection end of the capillary was aligned by micropositioners with the cell of interest, as confirmed visually in the field of view of a 100 × microscope. An air-tight syringe connected to the distal end of the capillary served to draw the cell into the capillary. Once injected, the capillary was placed in the buffer vials to initiate electrophoresis. The osmotic pressure inside the cell causes lysis when the buffer solution was drawn over the cell. If the same cell solution was to be used the following day, the glucose solution was washed away and the cells were suspended in PBS for refrigerated storage overnight.

3. Results

3.1. Electrophoretic separation

In this work, a favorable environment for hemoglobin separation is created by an approach which is different from, but as effective as, focusing techniques. In Fig. 1, an electropherogram of hemoglobin in single human erythrocytes (normal adult) is shown. The mechanism and important features of the separation are explained as follows. Firstly, a hydrophobic fluorocarbon coating is used, in which protein adsorption to the capillary wall is minimized. This is evident by the extremely narrow peak widths. The half-widths of the β- and α-chains are 0.09 and 0.06 min, respectively.

Secondly, a fluorocarbon surfactant (FC) is also added to the acidic (pH 2.7 ± 0.1) running buffer. When a cell is introduced into the capil-

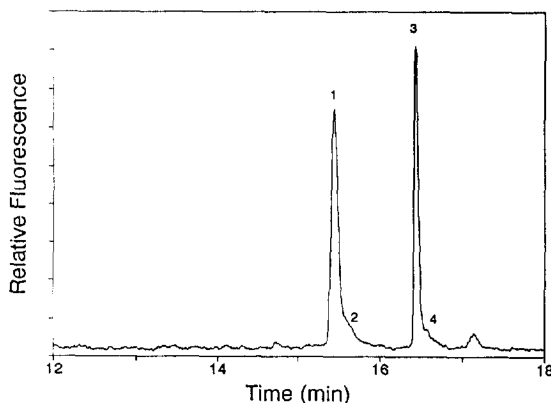

Fig. 1. Single red blood cell (normal adult). Peaks: $1 = \beta$, $2 = \beta$-glycated-, $3 = \alpha$-, and $4 = \alpha$-glycated-chains.

lary, it is initially suspended in an isotonic solution of 8% glucose, hence, the hemoglobin molecules contained within are in their native, tetrameric state. Upon contact with the lower ionic strength running buffer, in which a surfactant is also present, the cell is easily lysed, and the hemoglobin molecules released. When these molecules are then exposed to the acidic, surfactant-containing running buffer, the protein is denatured (i.e., the tetramers are dissociated) and their constituent polypeptide chains are separated.

Thirdly, the running buffer serves to oxidize the heme groups from the ferrous to the ferric state. This was demonstrated by adding Hb A_0 (ferrous) to a solution of running buffer. The color changed from red to brown immediately upon contact; this brown color is evidence that oxidation has taken place. Subsequent determinations of A_0 versus methemoglobin revealed no differences in migration time (data not shown). Our previous results [13] showed that A_0 and methemoglobin have different electrophoretic mobilities. The simplicity of the electropherograms here confirms that only one form (the latter) exists in this buffer. Although we will continue to use the familiar Hb notation, it is clear that all variants are altered similarly. When the α- and β-chains of A_0 were detected at 415 nm, a signal decrease of approximately two orders of magnitude was observed, versus 210-nm detection. It is apparent that with our conditions, essentially all heme groups dissociated from the polypeptide chains; the small signal at 415 nm being due to a relatively small number of chains which still contain the heme. We therefore conclude that the compounds which are separated and detected in this work are globin chains without the heme groups.

Although Hb A_{1c} ($\alpha_2(\beta\text{-glucose})_2$) is the major glycated hemoglobin in human red blood cells, glycation may occur at the N-terminus of the α- or β-chain, or at the ϵ-NH_2 groups of specific lysine residues [2]. Like Hb A_{1c}, these glycohemoglobins are also elevated in diabetics. In most cases, four peaks are evident in the adult erythrocytes studied. The first and third peaks correspond to the β- and α-chains, respectively, while we can assign the second and fourth peaks to the β- and α-glycated chains, respectively. Because any glycated polypeptide chain only differs from its respective unglycated form by one sugar molecule, it is expected that the glycated and unglycated chains will migrate very close to one another. Because Hb A_{1c} is known to be the major glycated component, with glycation on the β-chain, this implies that peak 2 corresponds to the β-glycated chains, and peak 4 to the α-glycated ones. The average amounts of β- and α-glycated chains are 9.6% and 4.8%, respectively, of the total Hb.

Fig. 2 shows the separation of Hb in a single adult erythrocyte containing an elevated amount of fast Hb ($A_1 = A_{1a} + A_{1b} + A_{1c}$, all of which are glycated at the β-N-terminus). This blood sample was independently assayed by the hospital, and was found to contain 16.2% fast Hb (i.e., Hb A_1). The fact that peaks 2 and 4 are substantially larger in Fig. 2 compared to Fig. 1 is positive confirmation of our peak assignments. From our single-cell determinations, the relative β- and α-glycated fractions were found to be 30% and 12%, respectively. Differences between the two types of assays may be explained as follows. Firstly, the fast-Hb assay constitutes an average, whereas values from individual cells will display the heterogeneity of the cell population. Thus, the effects of glucose (or sugar) exposure can be seen, probably reflecting the cell age. Secondly, there can be additional glycohemog-

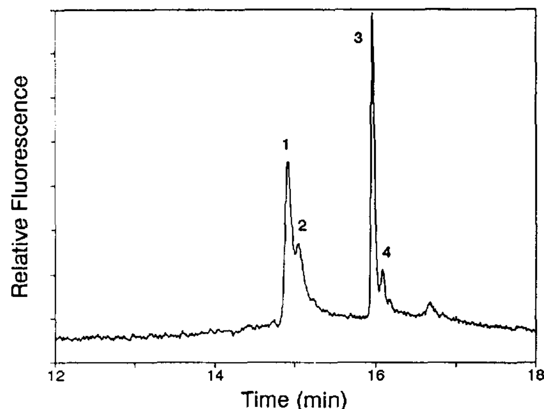

Fig. 2. Single red blood cell (diabetic adult; i.e., elevated Hb A_1). Peak identification same as Fig. 1.

lobin chains present in peak 2, other than those of the fast Hbs. This would also account for a higher observed amount over the assayed value.

Structural differences in the hemoglobin variants give rise to unique information from the electropherograms. Approximately 80% of the total hemoglobin in the fetal erythrocyte is hemoglobin F ($\alpha_2\gamma_2$), with about 10% of Hb F existing as the acetylated metabolite, F_1 ($\alpha_2(\gamma$-acetyl$)_2$) [21]. It is also known that there are two structural genes for the γ-chain, as residue 136 may either be glycine (G_γ) or alanine (A_γ). At birth, these are present in the ratio of 3 G_γ:1 A_γ [21]. Fig. 3 shows the separation of hemoglobins from a single fetal erythrocyte. The first peak corresponds to the γ-chains while the second one is probably a variant of Hb F, since it appears in neither the A_0 standard sample nor the adult cells. The total Hb F content (peaks 1 + 2) is 78%, of which peak 2 is 9%. Therefore, we have identified peak 2 tentatively as Hb F_1, which agrees with the literature value, rather than a genetic variant. As with the glycated chains, the acetylated chain migrates very close to the unacetylated form. Peak 3 corresponds to the β-chain, and is present at about 22%. Because the β and both of the γ-peaks only correspond to one variant each, the percentages of the globin chains are considered equal to the percentage of the intact variant. Finally, the fourth peak corresponds to the α-chain, as in all other electropherograms.

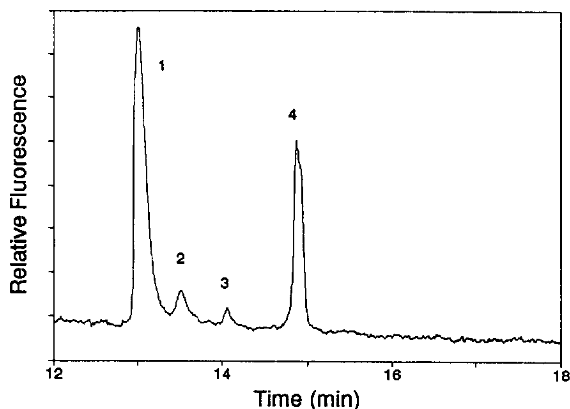

Fig. 3. Single fetal red blood cell. Peaks: 1 = γ-, 2 = γ-acetyl-, 3 = β-, and 4 = α-chains.

The peak identities in Fig. 3 were confirmed by coinjections of hemoglobin standards (A, A_2, S, F), as compared to clinical samples with known Hb C and G variants (200-nm absorption detection; data not shown). The assignments given to the peaks from single cells are consistent with those of the standards. It is interesting to note that the migration order of the globin chains in this work is reversed compared to other reports [14,16], under seemingly similar conditions. The elution order appears to depend on the specific composition of the buffer components. When a sample of Helena Hb standard was pretreated by (a) dilution in water, (b) acidified acetone to remove the heme groups with dilution in water, or (c) dissolution in 7 *M* urea and 0.2% dithiothreitol, no changes in the electropherograms were observed. When a polyacrylamide (PA)-coated capillary was used in conjunction with a pH 3.0 phosphate buffer, the elution order was again identical, even though the resolution becomes poorer. However, when 7 *M* urea was added to our running buffer (either PA- or FC-coated columns), the elution order was reversed (i.e. became identical to that in Refs. [14] and [16]). We further found that adding urea changed the pH of the buffer from 3.0 to 4.15 (apparent value). Since the pK_a of the carboxyl groups is around 4.5, the reversed elution order is not unexpected. Finally, the results of Ref. [16] (without urea) are probably due to a higher than expected pH in the running buffer.

Because all the hemoglobin subfractions in the fetal cell presumably contain the normal α-chain, it is expected that its peak area should be the highest. This is what is seen for lysed (cord blood) erythrocytes using 210-nm absorption detection (data not shown). With native laser-induced fluorescence (LIF) detection, the fluorescence efficiency of the polypeptide chains depends on the aromatic amino acid residues, primarily tryptophan. The number of tryptophan, tyrosine, and phenylalanine residues are, respectively, 3, 4, and 7 for the γ-chain, 2, 3, and 7 for the β-chain, and 1, 3, and 6 for the α-chain [23]. This is the primary reason the peak corresponding to γ (Fig. 3) is larger than that of α.

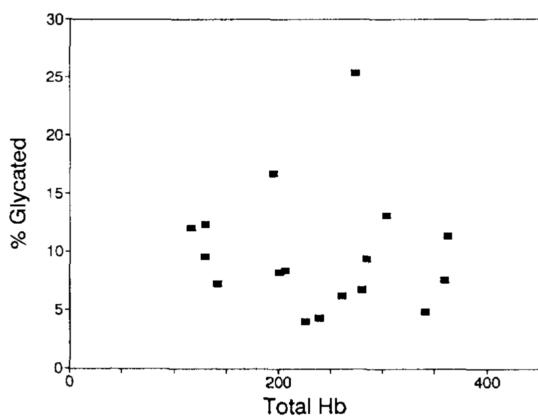

Fig. 4. Correlation between the fraction of glycated β-chain and the total Hb in different red blood cells in a normal adult.

In Fig. 4, the amount of glycated Hb (measured as glycated β-chain amount) is plotted against total Hb, for the adult cells. These amounts are shown to be uncorrelated with each other. As a cell ages, the amount of glycated hemoglobin is increased; however, the amount of total hemoglobin is not decreased. This means that total hemoglobin content is not a function of cell age, and as a cell circulates, loss of hemoglobin occurs by another mechanism. We note that there is a large variation in the amounts of total hemoglobin from cell to cell. This is consistent with our earlier study [13], and confirms that the intercellular variations, in this study and in the earlier study, are not due to artifacts of either experiment.

3.2. Effect of cell suspension solution

The primary concern, when choosing a cell-suspension solution, is that it is isotonic, such that the integrity of the cells is maintained during storage. The PBS solution in which the cells are washed normally serves this purpose well. However, with our system, the high salt concentration (135 mM NaCl) caused severe broadening and distortions in peak shape in the electropherograms. In the separations of Hb A_0 chains as a function of solvent, the β-chain peak half-width increased from 0.087 ± 0.005 to 0.181 ± 0.007 min for 8% glucose and PBS, respectively; the α-chain peak half-width increased from 0.055 ± 0.004 to 0.202 ± 0.011 min. This effect is likely due to localized heating (due to the high salt concentration) or disturbance of the capillary coating–surfactant equilibrium by the same matrix. Another possibility is that a reverse stacking effect is occurring; the more slowly traveling molecules speed up when the running buffer is contacted, thus broadening the zones. Satow et al. [22] also reported a similar effect of severe peak broadening for samples containing high salt concentrations.

3.3. Capillary life

The fluorocarbon capillary coating has been shown to be extremely rugged. One capillary can be used for several months, with regeneration possible should the separation efficiency start to degrade. Electroosmotic flow does change from one capillary to another, and changes gradually as a function of use, as evident in the migration times in Figs. 1–3. Normalization of the migration times based on standards is a solution, but is not implemented here because of the simplicity of the electropherograms. Several things become apparent when the capillary starts to break down. Normally, the α- and β-chain peaks of Hb A_0 (a standard) are approximately equal in peak height. When the coating begins to degrade, the β peak becomes very short and broadens, while the α peak always remains sharp and at a constant height.

When this phenomenon occurs, the column can be regenerated in one of two ways. Firstly, the capillary can be flushed with a higher percentage surfactant buffer (e.g., 1% FC) for more than one hour, as recommended by the manufacturer for 50-μm capillaries. However, in our experience, this did not seem to be effective for small I.D. capillaries. Alternatively, we have found that filling the capillary with the running buffer (50 mM phosphate, pH 2.7, 0.05% FC-surfactant) and leaving that inside (without flow)

for several hours to several days seems to regenerate the capillary. When poor resolution developed in one capillary, precluding its further use, it was stored for more than one month filled with the running buffer. After this length of time, with static regeneration, it was put to use again, with acceptable performance.

With this combination of coating and buffer, it seems that an important factor towards good performance is letting the surfactant molecules diffuse to coat the capillary wall on their own–without the influence of pressure or potential, as is typically used to equilibrate capillaries. Naturally, this results in a much slower equilibration, but its effectiveness seems to outweigh the time it takes to regenerate the column. It does not take weeks, however, to regenerate a capillary. In most cases, several hours, or overnight, is sufficient time to allow the performance to return to normal.

4. Conclusions

This separation system, in which hemoglobin molecules are denatured on-column to their respective polypeptide chains has shown to be an effective technique for the separation of hemoglobin variants in single human erythrocytes. Fetal, normal adult, and elevated Hb A_1 adult red blood cells were analyzed, with the corresponding (oxidized) polypeptide chains determined at the attomole level. Glycated Hb fractions were identified, and confirmation was made by noting the overall increase in diabetic cells versus normal ones. In a given cell, the amount of glycated chains was found to be uncorrelated with total hemoglobin amount. It is concluded that loss of hemoglobin from a cell is independent of cell age, since glycated Hb is known to increase with the length of time the cell is in circulation. The 20-μm capillaries that were used in this study are still in the development stages, thus one would expect even better performance once reproducible manufacturing protocols are implemented.

Acknowledgements

The authors wish to thank Ms. Carol Deal Eilers at Mercy Hospital in Des Moines, IA, USA, for donation of and assistance with the fetal and diabetic blood samples. In addition, we would like to acknowledge Phillips Petroleum for a graduate fellowship. The Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under Contract No. W-7405-Eng-82. This work was supported by the Director of Energy Research, Office of Basic Energy Sciences, Division of Chemical Sciences.

References

[1] R.E. Dickerson and I. Geis, Hemoglobin, Benjamin/Cummings, Menlo Park, CA, 1983, p. 21.
[2] H.F. Bunn, in D.F.H. Wallach (Editor), The Function of Red Blood Cells: Erythrocyte Pathobiology, 1981, Alan R. Liss, New York, pp. 223–239.
[3] H.G. Bunn and B.G. Forget, Hemoglobin: Molecular, Clinical and Genetic Aspects, W.B. Saunders, Philadelphia, PA, 1986, pp. 381–451.
[4] H.F. Bunn, B.G. Forget and H.M. Ranney, Hemoglobinopathies, W.B. Saunders, Philadelphia, PA, 1977, p. 131.
[5] H.F. Bunn, B.G. Forget and H.M. Ranney, Hemoglobinopathies, W.B. Saunders, Philadelphia, PA, 1977, p. 2.
[6] H.F. Bunn, K.H. Gabbay and P.M. Gallop, Science, 200 (1978) 21–27.
[7] P.M. Ness and J.M. Stengle, in D.M. Surgenor (Editor), The Red Blood Cell, Vol. 1, Academic Press, New York, 1974, pp. 22–24.
[8] M.R. Clark, Physiol. Rev., 68 (1988) 503–554.
[9] J. Murphy, J. Lab. Clin. Med., 82 (1973) 334–341.
[10] J.F. Fitzgibbons, R.D. Koler and R.T. Jones, J. Clin. Invest., 58 (1976) 820–824.
[11] M.M. Elseweidy, M. Stallings and E.C. Abraham, J. Lab. Clin. Med., 102 (1983) 628–636.
[12] R.B. Pennell, in D.M. Surgenor (Editor), The Red Blood Cell, Vol. 1, Academic Press, New York, 1974, pp. 100–101.
[13] T.T. Lee and E.S. Yeung, Anal. Chem., 64 (1992) 3045–3051.
[14] M. Zhu, R. Rodriguez, T. Wehr and C. Siebert, J. Chromatogr., 608 (1992) 225–237.
[15] M. Zhu, T. Wehr, V. Levi, R. Rodriguez, K. Shiffer and Z.A. Cao, J. Chromatogr. A, 652 (1993) 119–129.
[16] P. Ferranti, A. Malorni, P. Pucci, S. Fanali, A. Nardi and L. Ossicini, Anal. Biochem., 194 (1991) 1–8.

[17] T.L. Huang, P.C.H. Shieh and N. Cooke, J. High Resolut. Chromatogr., 17 (1994) 676–678.

[18] S. Molteni, H. Frischknecht and W. Thormann, Electrophoresis, 15 (1994) 22–30.

[19] J.M. Hempe and R.D. Craver, Clin. Chem., 40 (1994) 2288–2295.

[20] B.L. Hogan and E.S. Yeung, Anal. Chem., 64 (1992) 2841–2845.

[21] H. Lehmann and R.G. Huntsman, Man's Haemoglobins, North-Holland, Oxford, 1974, p. 61.

[22] T. Satow, A. Machida, K. Funakushi and R. Palmieri, J. High Resolut. Chromatogr., 14 (1991) 276–279.

[23] H. Lehmann and P.A.M. Kynoch, Human Haemoglobin Variants and Their Characteristics, North-Holland, Amsterdam, 1976, pp. 13–19.