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Review

Chromatographic and electrophoretic methods for modified hemoglobins

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Abstract

The discovery of the clinically important glycohemoglobin adducts and their relation to diabetes mellitus have greatly stimulated the study of other minor post-translational modifications of hemoglobin. Chromatographic and electrophoretic procedures have played an important role in these studies. Today several hemoglobin adducts are known and the formation of adducts with glucose, phosphorylated carbohydrates, urea/cyanate, aspirin, vitamins, acetaldehyde, penicillin and acetyl CoA have been described. Furthermore, new adducts, such as those observed using hemoglobin as a biochemical marker monitoring environmental, occupational and lifestyle exposures to reactive toxic chemicals are constantly being reported. This review deals with chromatographic and electrophoretic separation methods available for the study of non-enzymatic post-translational modifications of hemoglobin. Suitability, perspectives and biomedical applications are discussed. © 1997 Elsevier Science B.V.

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Contents

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1. Introduction

Heterogeneity of hemoglobin (Hb) has been reported by several investigations isolating minor fractions from red cell hemolysates [1–3]. Additionally more than 600 mutant variants have so far been reported.

From a clinical point of view the most important post-translational non-enzymatically modified Hb is without doubt the carbohydrate adducts termed glycated hemoglobins. The study of the minor HbA1 fraction, composed of HbA1a, HbA1b and HbA1c [1], produced by post-translational attachment of glucose and phosphorylated carbohydrates to the peptide chain of HbA0, have been greatly stimulated by the fact that their proportion was found to increase in diabetes mellitus [4]. During their study, several new minor fractions of Hb have been discovered, both new genetic variants and various chemically modified Hbs. Different post-translational modifications of Hb are now well known to occur and Hb adducts of glucose, urea/cyanate, aspirin, vitamins, acetaldehyde, penicillin and acetyl CoA have been described. Additionally, new and up to now not yet fully characterised Hb adducts, such as those found using Hb as a biochemical marker for environmental exposure, are constantly being reported.

Chromatographic and electrophoretic procedures have played an important role in the assessment of Hb variants, both genetic and post-translational. This review discusses available separation methods, focusing on methods used in the study of post-translational modifications of Hb. Methods devoted to the study of genetic variants will not be discussed unless the procedures and separation techniques presented are of general interest and importance in the study of adducted Hb.

2. Structure and synthesis of modified hemoglobins

Several of the residues in the polypeptide chain of hemoglobin, i.e., amino and sulfhydryl groups, are reactive to form adducts with many substances. Most of these adducts are unstable and therefore of minor clinical interest, yet others, such as glycated hemoglobin (GHb), are in routine clinical use.

2.1. Glycated Hb

The carbonyl group of the open chain structure of glucose reacts with free aminogroups of Hb to form an aldimine via nucleophilic addition. The labile Schiff base formed undergoes Amadori rearrangement resulting in a stable non-reversible ketoamine adduct. The formation of GHb is dependent upon the surrounding blood glucose concentration and the time of incubation, and takes place throughout the life span of the erythrocytes. Correspondingly, GHb disappears according to the half-life of the erythrocytes. GHb has become widely accepted as an objective and quantitative index of long term blood glucose levels and is today in routine use in monitoring diabetes [5]. The glycation process also includes the reaction of hexose phosphates in a similar process resulting in the adducts HbA1a1 and

HbA1a2 [6], however, neither of these are elevated in diabetes.

Clegg and Schroeder [2] demonstrated by chromatography the presence of five minor Hbs and named them HbA1a–e according to their elution order. Further improvements resolved the HbA1 fraction into nine components [7]. The HbA1c fraction constituting the major portion of HbA1 have been identified as the glycated variant of importance for the assessment of diabetes treatment. It has also been given most of the attention in terms of structural characterisation and has been identified as the ketoamine adduct of glucose at the N-terminal valine of the β-chain of Hb [8]. The presence of this adduct in a ring-stabilised fructopyranose form have been substantiated by several reports [9–12]. However, glycation of Hb is known to occur not only at the N-terminal residues but also at several other amine sites on the protein chains of the Hb tetramer [13]. Therefore, the term GHb, i.e., total glycated hemoglobins, is used to designate all glycated species of Hb irrespective of glycation site. The labile Schiff base referred to as pre-GHb or HbA1d (when addressing the labile N-terminal modification of the β-chain valine) [14] is not clinically important, yet of considerable analytical importance since it interferes in the separation of HbA1c when using separation methods based on charge difference. For further details regarding biosynthesis, nomenclature and naming of the different glycated species, several reviews dedicated to glycated Hb are available [5,15,16].

2.2. Acetaldehyde Hb adducts

Hemoglobin as well as other proteins are known to form adducts with acetaldehyde, the primary oxidative metabolite of ethanol [17–20]. The interest in acetaldehyde adducts is due to their potential of working as a biochemical and clinical marker of alcohol consumption. Used in a manner analogous to GHb monitoring diabetes, acetaldehyde adducts could serve as alcohol abuse markers similar to carbohydrate deficient transferrins [21]. Although comprehensive experimental data on the formation and structure of these adducts so far are lacking, modifications of Hb have been identified at multiple sites, among others at the N-terminal valine of both the α- and β-chain [22]. Similar to glycation the adduct formation is depending on the concentration of acetaldehyde itself, however other reaction conditions that may vary significantly between in vivo and in vitro reactions are also of importance [23].

2.3. Carbamylated Hb

Non-enzymatically carbamylation of Hb occurs in patients with uremia and is a result of the spontaneous dissociation of urea to form cyanate and ammonium ions. The reactive isocyanic acid then forms irreversible carbamoyl adducts with amino-groups in Hb [24]. Carbamylated Hb has been found in patients with elevated levels of urea and in renal failure [25,26]. Combined with the interference on the analysis of HbA1c, hyperuremic individuals often exhibit decreased red cell survival and glucose intolerance, making accurate assessment of glycation problematic in this group of patients.

2.4. Acetylated Hb

Hb adducts formed by acetylation of Hb, either by the action of an acetyltransferase catalysing the transfer from acetyl CoA to the amino-terminal ends of Hb [27] or by acetylsalicylic acid [28–32] is another often investigated adduct of Hb. These adducts are found in human cord blood, in alcoholics and in pregnant women, and both HbA0 and HbF are known to be acetylated [28,33]. Chemical blocking of amino-terminal valine residues, both by carbamylation and acetylation, have been the subject of studies related to HbS for ameliorating the severity of sickle cell anaemia [34,35].

2.5. Xenobiotic-Hb adducts

The determination of environmental compounds or their metabolites excreted in the urine of humans is a possible method of biomonitoring. However, detection of Hb adducts have shown to be a more suitable way of measuring exposures over a longer period of time and for providing information of the genotoxic potency of the exposure [36,37]. The use of chemically stable Hb adducts as biomarkers of occupational and lifestyle exposures is, similar to the use of GHb, governed by the availability and long bio-

logical life time of Hb and a dose-dependent covalent binding.

In addition to amine residues, the sulfhydryl groups in proteins are prone to attachment by reactive compounds. One of the principal sites of adduct formation with xenobiotics in human Hb is the sulfhydryl group of β93 cysteine, where reactions with compounds like halogenated hydrocarbons [38,39] and nitroso compounds [40,41] have been described. Aromatic nitroso compounds are found to be active intermediates in the metabolism of aromatic nitro, amino and azo compounds binding covalently to thiol residues of compounds in the body fluids to give sulfinamides [42].

2.6. Other Hb adducts

Glutathione-Hb adducts (HbSSG) are observed during storage of blood samples [15,16]. Thiol-disulfide interchange taking place during oxidation of the thiol group of cysteine β-93 is a possible mechanism for the formation of this adduct. Depending upon the redox potential of thiols present, i.e., cysteine, glutathione and CoA [43], various disulfides including glutathione-Hb can potentially be formed with the thiol groups of Hb [44]. The reactivity of thiol reagents with Hb is of interest since blocking of the thiol group has been shown to inhibit red cell sicling [45].

Another adduct worth mentioning, although conflicting results are available, is ascorbinated Hb formed between vitamin C and Hb.

Some Hb adducts are presented in the reaction schemes of Fig. 1, illustrating the formation of glycated, carbamylated and acetylated Hb, and finally an acrylamide and a nitroso adduct of Hb. The last two representing adducts found after occupational exposure to hazardous chemicals.

3. Separation methods used in the study of post-translationally modified hemoglobins

In addition to the more fundamental choices when selecting a separation method, the evaluation of available methods is often done with emphasis on separation ability, practicability and for the routine clinical laboratory; applicability and aspects related to quality assurance. However, these elements are sometimes diverging parameters not always possible to combine and optimise in one single method.

The following is a brief description of separation techniques used in the analysis of adducted Hb, focusing on separation ability, benefits and limitations.

3.1. Ion-exchange chromatography

Hb modified at the N-terminal valine of the β-chain, either by glycation or other post-translational modifications, results in distinct charge differences compared to unmodified HbA0 and ε-lysine modified Hb. The reduced charge of some forms of modified Hbs are the fundamental basis for their separation using charge separating techniques. Cation-exchange chromatography and electrophoresis are therefore frequently used in the analysis of Hb. Using cation exchangers at neutral pH allows separation of the glycated variant HbA1c from HbA0 and also partial separation of the glycated N-terminal end of the α-chain [46].

3.1.1. Mini-columns

The charge difference between HbA0 and the glycated HbA1 fraction is the basis for the classical method for rapid determination of HbA1a, HbA1b and HbA1c introduced by Trivelli et al. [4]. Using the weak cation exchanger Biorex 70 (Bio-Rad) they were able to separate HbA1c from HbA0 and also partly separate it from other HbA1 fractions.

Modifications of this method focusing on speed and simplicity have resulted in disposable mini-columns with higher flow-rates based on weak cation exchangers. Such mini-columns dedicated to the separation and quantification of the HbA1 fraction have been available for several years (Bio-Rad, Helena, Isolab). Although in widespread use because the separation is easy, rapid to perform and does not require specialised equipment, they have several drawbacks. In manual systems, variations in assay conditions are difficult to avoid and even small variations in temperature, pH and sample load affects the elution characteristics of the columns [47,48].

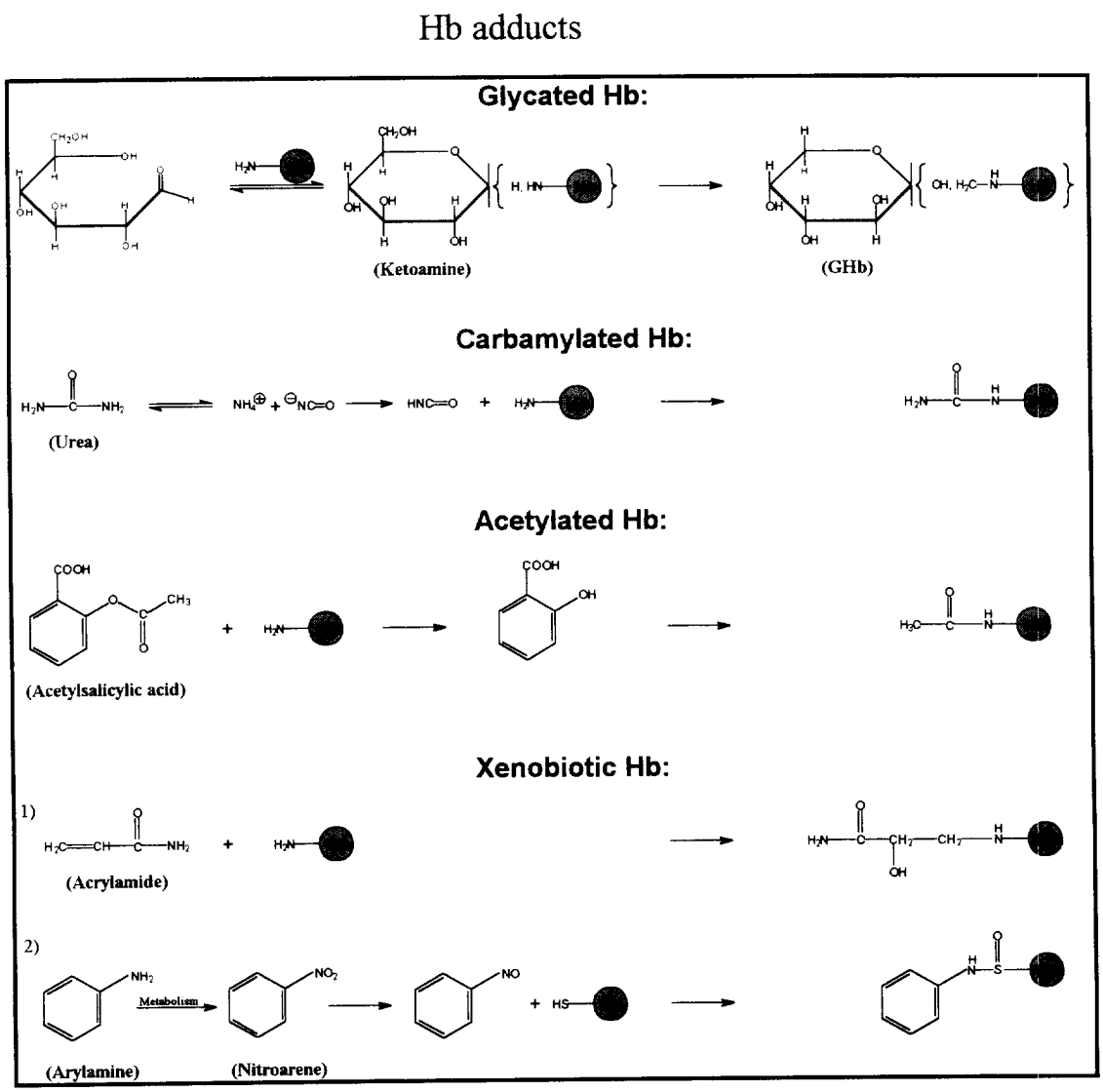

Fig. 1. Reaction scheme illustrating the formation of different Hb adducts described in the literature, i.e., glycated, carbamylated and acetylated Hb. The illustrated Hb adducts formed with acrylamide (1) and aromatic amines (2) (metabolised to nitroso compounds) represent typical adducts seen using Hb as a biochemical marker studying environmental exposure to toxic chemicals.

3.1.2. HPLC

The inherent problems of the mini-columns led to the development and use of several new high-performance ion-exchange matrices in the chromatographic study and separation of Hb. This has resulted in HPLC columns from several suppliers dedicated to the analysis of HbA1c. Examples are the strong ion-exchanger Mono S [49] (Pharmacia Biotech, Uppsala, Sollentuna, Sweden) based on sulphonated highly monosized polymer particles and the polymeric Chrompack 8 column (Chrompack, Middelburg, Netherlands). Correspondingly, dedicated HPLC systems, like the Diamat/Variant system from Bio-Rad (Hercules, CA, USA), using Glycopak resins, and the Daiichi HA8110/21 analysers (Kyoto, Japan), using the Micropearl SF-W-A1c

resin, are in routine use in clinical laboratories. A comparison of elution profiles using high-performance columns and automated ion-exchange systems is shown in Fig. 2. Although they all manage to separate HbA1c from the HbA0 fraction it is obvious that the quality of separation differs among the matrices shown, particularly studying the minor forms. As seen in the figure, HbA1a and HbA1b are not resolved by the Daiichi analyser [50,51]. Several publications have been devoted to the feasibility of

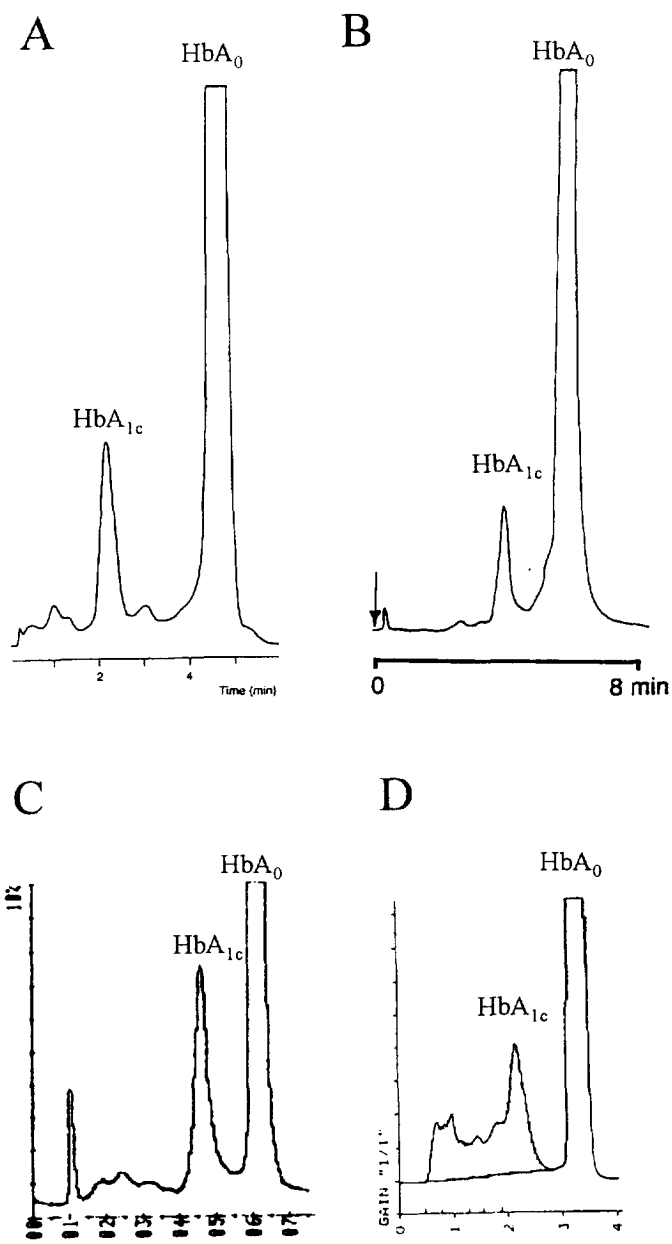

Fig. 2. Comparison of elution profiles using high-performance columns and automated ion-exchange systems. Mono S-column (Pharmacia) (A), Chrompack 8-column (Chrompack) (B), the Diamat-system (Bio-Rad) (C) and the Daiich HA8121-system (Kyoto) (D).

available ion-exchange systems for the analysis of HbA1c [49,50,52–55]. Although not as vulnerable as mini-columns, some HPLC-HbA1c systems still have problems with interference from HbF [53,54].

Since all post-translational chemical modification of N-terminal amine residues of HbA0 result in charge modifications similar to glycation and the resulting HbA1 fraction, acetylated, carbamylated and acetaldehyde Hb adducts are often detected and observed as interfering, coeluting or scarcely resolved peaks during routine ion-exchange analysis of HbA1c [51,56,57]. Similarly, new genetic variants have also been discovered this way [58,59].

3.2. Electrophoretic methods

3.2.1. Cellulose acetate/citrate agar/agarose gel

One of the most widely used method for the analysis of hemoglobin in order to detect genetic variants is cellulose acetate electrophoresis at alkaline pH [60]. Still, some variants (HbA2, HbC, HbO and HbE) [61] are unresolved and citrate agar electrophoresis at acidic pH is needed to detect these [62]. Although HbA0, HbA1a+b and HbA1c exhibit distinct mobility on agarose gel electrophoresis [60,63,64], this method has been shown to be ineffective in determining HbA1c in the presence of elevated amounts of HbF [16,33]. Under such conditions foetal Hb (both glycated and nonglycated) co-migrates with the HbA1c fraction [53,65]. The same problems are seen when carbamylated Hb [57,66,67] or acetylated Hb [57,67] are present in the sample. Although the interferences experienced during agar gel electrophoresis are similar to those seen using ion-exchange methods, HPLC normally gives more reliable and reproducible results than agar gel electrophoresis [53,68].

3.2.2. Isoelectric focusing (IEF)

Since charge differences resulting from post-translational modifications of the β-N-terminal valine, or the variability in the primary structure of Hb like the one seen in HbA0, HbA2 and HbF, alters the isoelectric point (p*I*) of the protein, separation can be performed using isoelectric focusing. Although the p*I* difference between HbA0 and the HbA1c fraction is only 0.01 pH unit of magnitude, IEF manage to separate them. For this reason IEF is used as a routine method for the analysis of HbA1c [69]. Fig. 3 illustrates graphically the focusing pattern achieved when separating human adult Hb by this method.

3.2.3. Capillary electrophoresis (CE)

With the introduction of CE approximately ten years ago, new analytical possibilities have arisen. As the utility have become apparent to those familiar with more traditional techniques of liquid–gas chro-

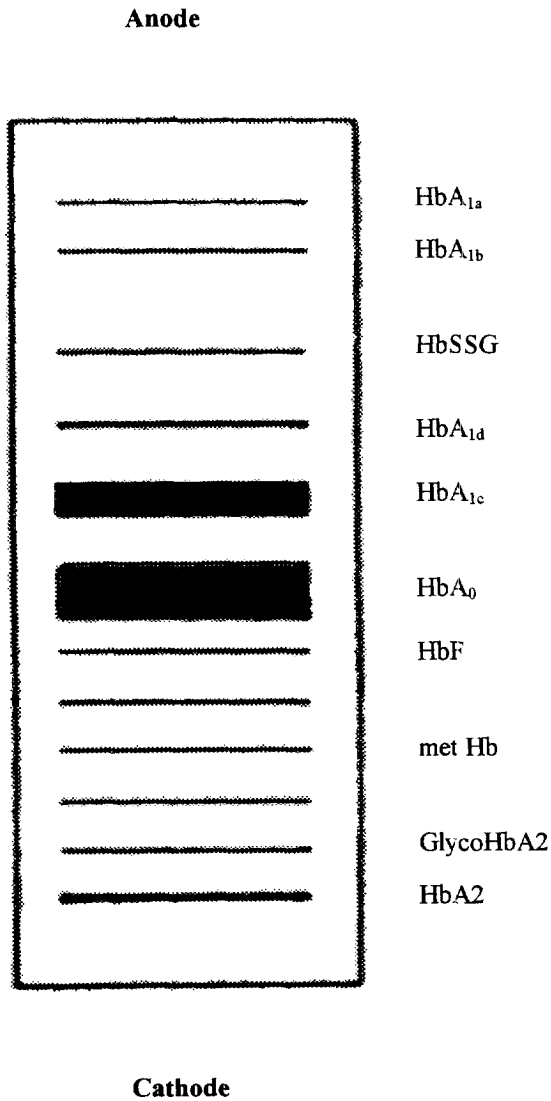

Fig. 3. Graphical representation of IEF pattern of human Hb.

matography, several applications have been published separating various compounds including Hb [70–72]. A number of excellent reviews covering methods and applications have been published [73,74], and only a brief introduction to the area will be given.

As in electrophoresis and LC, CE can resolve analytes on the basis of charge, size, hydrophobicity, absorption, affinity etc. However, it has the ability to handle sample volumes in the picoliter (10^{-10}) range and have detection limits in the attomol (10^{-18}) level. Some of the CE techniques are comparable or equivalent to LC techniques, while others are unique. Most capillaries used are made of silica with an external rugged coating of polyamide making them less fragile and susceptible to fracture. The internal surface with exposed chargeable silanol groups can be left uncoated to allow them to interact with an annulus of cations, secondly producing the electroendosmotic flow through the capillary as the current is turned on. This flow is normally greater than the electrophoretic mobility of the different charged species in the injected sample, making it possible to separate both anions and cations at the same time. Capillary zone electrophoresis (CZE) in uncoated capillaries is the most commonly used technique in CE. However, coating the capillary makes it possible to control or even eliminate the electroendosmotic flow and is the basis of techniques like IEF, isotachophoresis (ITP) and gel electrophoresis (GE). CE requires specialised equipment, but is faster and much less labour intensive than traditional electrophoresis and the direct detection of the capillary format out-passes the quantitative information achieved with traditional staining. A problem with the use of traditional IEF ampholytes in cIEF is that they are not optimised for CE and show varying degree of transparency, thus affecting the detection limit. Similarly, detection of the focused zones inside the capillary represents a technical problem [75].

3.3. Boronate affinity

The ability of boronic acids to form boronate esters with compounds containing coplanar *cis*-diol groups have been used in the detection of glycated amino acids, peptides and proteins [76–78]. Mallia et al. [78] was the first to present the method for the separation of GHb. The method relied on the binding of an immobilised boronated ligand, usually *m*-aminophenyl boronic acid, to the *cis*-diol configuration of the glycated residues of GHb irrespective of glycation site. The binding of the glycated protein is carried out in alkaline solution to facilitate esterification and is usually being performed on commercially available mini-columns (Pierce, Hellena, Isolab). However, elution is achieved using acidic non-binding conditions, or more preferably by competitive displacement using diol compounds like sorbitol. The method is frequently being used in the determination of GHb due to its benefits compared to the ion-exchange methods. These are first of all related to low sensitivity towards variation in pH and temperature, analytical parameters known to interfere in several of the alternative methods. Additionally, and equally important, the method is insensitive towards the presence of interfering Hb species like the labile pre-glycated aldimine hemoglobin variant (HbA1d), HbF and various modified Hbs [79,80].

3.4. Immunological methods

Due to the massive interest in glycated Hb as a diagnostic tool in diabetes mellitus, the development of non-chromatographic analytical methods for the clinical laboratory have lead to immunological methods and the availability of mono- and polyclonal antibodies. Polyclonal antibodies against glycated Hb became available by Javid et al. in 1978 [81], however, monoclonals directed against HbA1c rapidly evolved [82]. Today monoclonal antibodies directed against deoxyfructosyl residues in Hb [83] and polyclonals/monoclonals against Hb-acetaldehyde adducts [84,85] are also available.

Antibodies directed against glycated Hb and acetaldehyde-Hb adducts have primarily been raised for the development of ELISA [86] and immunoturbidimetric assays [87] as alternatives to chromatographic assays. Although their use is limited outside these systems, the specificity of the antibodies have the potential of being utilised in different affinity studies of the Hb derivative in question. Such studies can be performed either on the native tetrameric protein or on single chains, using immobilised or non-immobilised antibody.

3.5. Reversed-phase gas chromatography

The detection, isolation and characterisation of in vivo formed minor Hb variants are difficult because they are present in very low amounts; for this reason HPLC methods sometimes fail [88]. In addition to the separation methods described above separating Hb in its native tetrameric stage, three other methods are often used in the analysis and characterisation of Hb adducts; (1) chemical release of the adduct from Hb (e.g., hydrolysis), followed by chromatographic analysis of the derivatised adduct [89–92], (2) complete hydrolysis of the protein followed by derivatisation and separation [93,94] and (3) analysis of N-terminal valine adducts by the modified Edman degradation method [95,96]. The analytical methods most often used following approaches 1 and 2 are reversed-phase (RPC)-/ gas chromatography (GC), often coupled with mass spectrometry (MS) as one of the most powerful techniques for structural characterisation.

New developments now makes it possible to obtain information on the adducting moiety in intact protein subunits by the combination of electrospray ionisation–mass spectrometry (ESI–MS) and HPLC [94,97,98] or cIEF [99].

Several separation and processing steps are involved during the structural characterisation of Hb adducts, both preparative and analytical. Following the preparative isolation of the minor Hb in study, further isolation of single globin subunits is necessary. The most simple and rapid method is the combination of chain dissociation and RPC using C_2–C_4 columns [100]. Further splitting of the chains to identify specific sites of modification is performed using trypsin, followed by separation on C_{18} RPC media.

4. Chromatographic and electrophoretic methods in the study of hemoglobin adducts – evaluation, perspectives and biomedical applications

4.1. Ion-exchange methods

4.1.1. Glycated Hb

Glycation is by far the most studied modification of Hb and more than ten different analytical methods have been described [5,16]. Some of these methods, like the fructosamine [101] and the TBA [102] assays, are chemical methods not discussed here. Cation-exchange chromatography and electrophoresis were the first methods available for the analysis of glycated Hb and have dominated the methods used for this clinically important analyte. When the importance of glycated hemoglobin was established, much effort was dedicated to the improvement of the separation methods. The method of Trivelli et al. [4], although improved compared to other methods at that time, needed 4 h for separation of "fast" moving Hbs (HbA1a–c). This rapidly led to the development of the so called mini-columns in the determination of HbA1c; columns still available. Although suppliers claim that HbA1a and HbA1b can be separated from HbA1c and HbA0 by isocratic elution using the buffers supplied, the separation ability is not satisfactory. In our experience the fraction designated to be HbA1c contains both HbA1a and HbA1b. In addition, others have shown that a substantial portion of HbA1c in an applied sample does not elute in this fraction at all [48]. Although providing a number to the physician that might be useful to assess long-term diabetic control, there is a question whether these assays for HbA1c actually measures what they say they do. Additionally HbF, co-eluting with the HbA1c fraction, is often experienced to be a problem [54]. Unless automated equipment are available for thorough control of the separation conditions using mini-columns, HPLC media is definitely a better choice for the analysis of HbA1c. This is also illustrated by the separation ability of different ion-exchange media presented in Fig. 2.

Despite the frequent use and importance of HbA1c in diabetes monitoring there has not been possible to fully agree on reference methods, although ion-exchange HPLC has been recommended because of its high level of precision. This has further been complicated by the fact that there is no single standard available for glycated Hb that can be used in all methods (chromatographic as well as non-chromatographic) [103]. Still, regarding standardisation there are also problems associated with the chromatographic methods. This can be exemplified by the fact that the Diamat analyser, although often used as a reference method in correlation studies and used as one of the methods in the landmark study by the

Diabetes Control and Complications Research Group [68], report higher reference values for HbA1c than alternative HPLC-media, for instance using the Mono S column [49,50]. Lower resolving power and specificity of the Diamat system, combined with aspects related to signal integration/data treatment might be an explanation [49,52].

With the recent advances in ion-exchange HPLC methodologies, Hb can quickly be separated in more than ten minor Hb fractions [7] with even better separation using longer separation time. The high resolution Mono S column has been widely used in the analysis of HbA1c, and found favourable in comparison with several other HPLC-columns [49]. Optimisation of important variables using this column for the separation of HbA1c has been addressed by several groups [52,104,105]. It shows very good long term stability and more than a thousand injections should be expected if recommendations regarding sample load are followed [52]. Other matrices/columns frequently used in the study of Hb variants are the TSK-gel SP/CM-3SW columns [49,106] (Tosoh, Tokyo, Japan), the Poly-Cat A column [61,107,108] (Poly LC, Columbia, MD, USA), Spherogel TSK SP-5PW [49] (Beckman, San Ramon, CA, USA) and ProteinPak 8 HR SP [48,49,56,83] (Waters, Milford, MA, USA). The performance of some of these columns in separating HbA1c and other Hb variants is very good. This can be illustrated by the work of Nakatani et al. [106] using the non-porous TSK gel SP-NPR (Tosoh) and the even more impressive separations obtained using the PolyCat A column, illustrated by the work of Itälä et al. [108] and Bisse and coworkers [109,110]; the last illustrated in Fig. 4.

Since commercially available ion-exchange HbA1c systems are optimised to separate human Hb, their direct utilisation in the analysis of glycated Hb variants in other species are limited and result in fused, scarcely resolved peaks [111] not directly comparable to the separation of human Hb. To obtain satisfying separation of Hb variants of non human origin, new analytical conditions must be established. An example is the work of Hasegawa et al. [112] presenting separation conditions enabling the separation of canine glycated Hbs on an cation exchanger.

Two adducts reported to interfere in the determination of HbA1c are the glutathione-Hb adduct (HbSSG) [16], and a possible vitamin C-Hb adduct [113]. The HbSSG adduct is formed during prolonged blood sample storage and can easily be avoided using by fresh samples [114]. Similarly, vitamin C has been reported to affect the determination of GHb due to adduct formation with Hb resulting in ascorbinated Hb [113]. However, these findings were not confirmed by Weykamp et al. [115] using electrophoresis, HPLC, affinity chromatography and immunoassay.

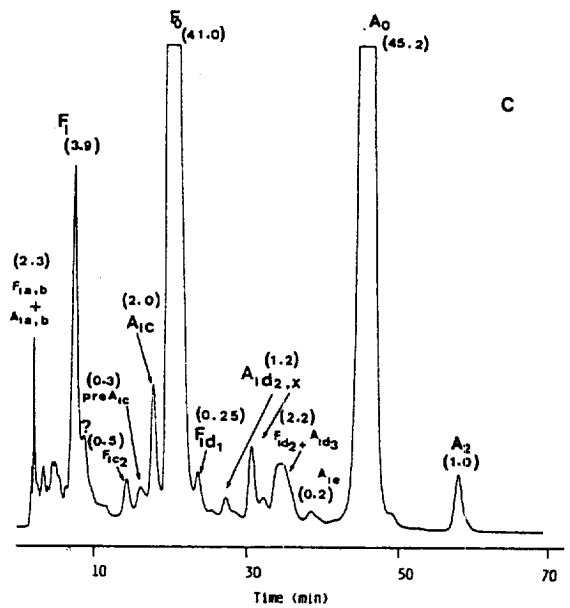

Fig. 4. Separation of Hb components in human adult red cell hemolysate using the weak cation exchanger Poly Cat A (Poly LC). Number in parentheses are percentages. From Bisse and Wieland [110].

As a closing remark regarding ion-exchange methods measuring HbA1c, the interference from the preglycated Schiff base form of HbA1c (HbA1d) [14] has to be addressed. Coeluting with HbA1c it has to be removed prior to separation. Since this interference is well known several sample pre-treatment methods are available [15], among them elimination using tetrapolyphosphate at pH 6 [116], incubation in borate buffer pH 5.9 [49] or incubation in saline at 37°C [15]. Unless removed, the preglycated Hb adduct also interferes in gel electrophoretic determinations of HbA1c.

4.1.2. Acetaldehyde Hb

Apart from glycated Hb, the acetaldehyde adducts of Hb have been given much attention. Due to their potential clinical usefulness as a marker of alcohol consumption, several chromatographic as well as immunological methods have been presented. Although frequently studied, structural evidences together with comprehensive experimental data on the formation and stability of these adducts have not yet been presented. A major problem in their study is the very low amounts found in vivo. This has led to experimental set-ups studying acetaldehyde adducts using elevated non-physiological concentrations of acetaldehyde in order to generate adducts in vitro or in vivo [88]. This results in an increase of minor Hb components which can be separated from HbA0 by ion-exchange or electrophoretic methods [117,118], however, some studies have found these modified Hbs in samples from alcoholics [117], while others have not [88]. Sillanaukee and Koivula [119] have presented a method based on the Mono S column (Pharmacia) and identified an Hb fraction, HbA1ach. This fraction was found to be dependent upon the formation of an acetaldehyde-Hb adduct, however the measurement was disturbed by glycation. Recently Itälä et al. [108], using a Poly Cat A column, reported a procedure able to separate three different acetaldehyde Hb adducts, termed HbA1ach1–3. This method benefits from being quite fast (25 min), resulting in the separation of the specific acetaldehyde-Hb adduct, HbA1ach3, free of interference from varying amounts of acetylated and glycated Hb adducts. The separation obtained is illustrated in Fig. 5.

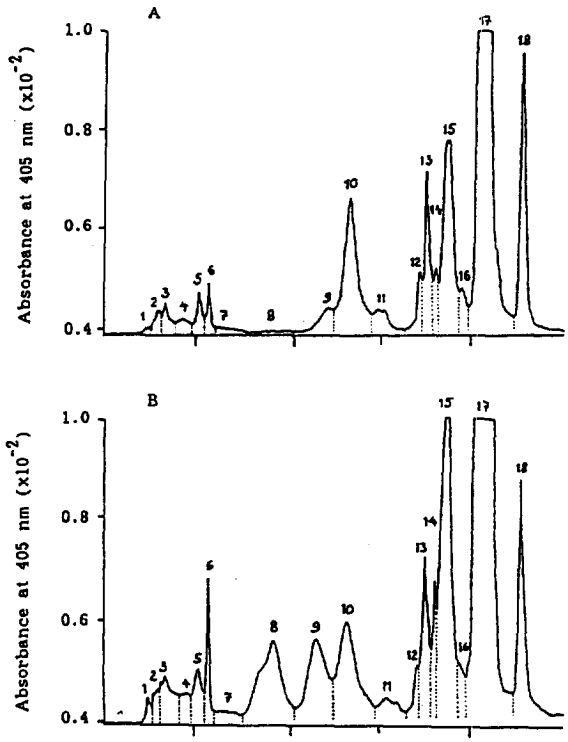

Fig. 5. Separation of acetaldehyde (ach) adducts using the Poly Cat A column (Poly LC). Chromatogram of normal hemolysate (A) and after incubation with 1 m*M* acetaldehyde (B). Peaks are 1=HbA1a1, 2=HbA1a2, 3=HbA1b1, 4=HbA1b2, 5=HbA1b3, 6=HbA1ach1, 7=HbA1ach2, 8=HbA1ach3, 9=HbA1prec, 10=HbA1c, 11=HbF, 12=HbA1d1, 13=HbA1d2, 14=HbA1d1x, 15=HbA1d3, 16=HbA1e, 17=HbA0, 18=HbA2. Peak 8 (HbA1ach3) was identified as the specific acetaldehyde fraction. From Itälä et al. [108].

4.1.3. Carbamylated and acetylated Hb

Acetylated and carbamylated Hb adducts have both been observed as Hb adducts interfering in the determination of HbA1c using analytical methods separating based on charge differences. Several reports have described this interference and also evaluated the sensitivity of different analytical HbA1c methods against these adducts [33,57]. Interfering in the determination of glycation using ion-exchange and gel electrophoretic methods, boronic acid affinity chromatography [56,57] or immunoassays [66] are very good alternatives when these adducts are found or expected. However, ion-exchange methods have been presented both for the measurement of HbA1c in the presence of acetylated or carbamylated Hb and for the specific separation and assaying of these adducts. Brunnekreeft and Eidhof [56] separated HbA1a, HbA1b, HbA1c, HbA0 and HbC/HbS with carbamylated and acetylated Hb indicated as extra peaks. However, although useful for screening purposes, quantification of HbA1c was affected by their presence. Bissè et al. [109] managed to separate carbamylated Hb from HbA1c, but acetylated Hb was not resolved from the HbA1c-peak. To avoid the effect of glycation on the determination of acetylated Hb, Turpeinen et al. [30] used a combination of boronic acid affinity and ion-exchange chromatography. Addition-

ally, the method presented by Itälä et al. [108] previously described for the separation of acetaldehyde adducts, should also be mentioned due to the separation of acetylated Hb adducts from the specific acetaldehyde adduct which they studied.

Before closing this section presenting ion-exchange methods, an illustration of another approach for the measurement of adducted Hb present in small amounts is the use of GC analysis after extracting the adduct in study from the Hb chains. This principle was used by Manning et al. [120] when measuring carbamylated Hb as valine hydantoin released by acid hydrolysis of globin extracts. However, since the method is tedious and has a very low sample throughput (40 samples a week), it is hardly an alternative to the ion-exchange HPLC methods described. Still, it can be mentioned that Kwan et al. [121] managed to increase the throughput to approximately 20 samples per day by measuring the released valine hydantoin by HPLC.

Since no single chromatographic method has managed to separate all Hb adducts simultaneously, the choice of ion-exchanger and method is not obvious. Depending on whether the method should be used as a routine analysis or as a method for research purposes, this choice will often be a balance between speed and resolution. For the routine analysis of HbA1c, columns from several suppliers have shown good performance [122]. Similarly, for the research laboratory the PolyCat A column with porous silica coated with polyaspartic acid (Poly LC), is a valuable tool shown able to separate all adducts previously discussed [61,107–109].

4.2. Boronic acid affinity chromatography

The use of boronic acid affinity chromatography is a unique solution to the problem of separating glycated Hb variants, irrespective of glycation site. As mentioned before, non-enzymatic glycation is not unique to the N-terminal amine of the β-chain of Hb [13]. However, glucose attached to ε-lysine residues in the primary structure of Hb does not alter the p*I* enough to be separated from HbA0 based on charge differences. These glycated variants elute on cation-exchange HPLC in the first part of the HbA0 fraction [46] and HPLC and gel electrophoresis are therefore not applicable. These adducts can efficiently be separated using the boronate *cis*-diol affinity interaction and numerous reports have described its use in the analysis of glycated Hb [57,83,123–125]. The advantage of the method is related to its low sensitivity towards variation in analytical parameters and lack of interference from HbF/acetylated HbF [33] carbamylated [57,126] and acetylated Hb [57].

Boronic acid affinity chromatography is, contrary to the use of ion-exchange methods, applicable for the analysis of GHb in non-human blood without the need to change or optimise the chromatographic conditions to achieve separation. This has been utilised in the determination of rat GHb [111] to overcome problems with the heterogeneity of Hb in this species [127], making determination of GHb difficult and unreliable using ion-exchange methods.

However, since boronic acid affinity chromatography often is being performed using commercial mini-columns, the assay is tedious and has low throughput. To account for these problems automated systems have now become available (e.g., Primus, Cansas, MO, USA; Drew Scientific, Cumbria, UK) and their suitability for the analysis of glycated Hb have been evaluated [124,125].

Commercially available boronate affinity columns are based on 3-aminophenyl boronic acid immobilised on agarose. Although possible to regenerate several times, the total life time of the matrix is limited. The need for new high-performance boronated media have resulted in several reports dealing with the preparation of stable high-performance media with immobilised phenyl boronic acids [128–133] using both porous and non-porous media. Although boronic acid affinity chromatography has proved efficient in separating different *cis*-diol containing analytes other than GHb, both the nature of the solid matrix, the boronic acid derivative used as well as the coupling chemistry applied has proved to be of importance [76,134,135]. In an attempt to make a hydrophilic boronic acid affinity media, Frantzen et al. [128] used protein–3-aminophenyl boronic acid conjugates immobilised on non-porous polymer particles. The resulting column showed good flow and separation characteristics, however, the total capacity of the matrix was low and though easily overloaded. The use of porous media for the immobilisation seems to be more applicable [132,133]. Fig. 6 shows the correlation between GHb

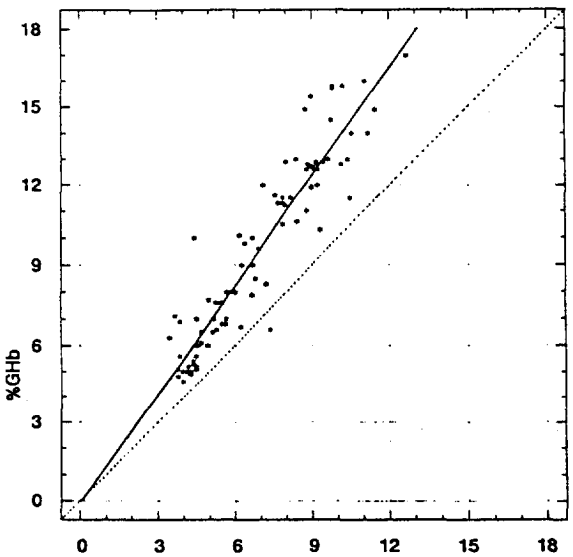

Fig. 6. Correlation between glycated Hb measured by boronic acid affinity chromatography based on an Sephacryl–3-aminophenyl boronic acid matrix (y-axis) and HPLC HbA1c-values (x-axis). From Bisse and Wieland [132].

obtained using the Sephacryl–3-aminophenyl boronic acid matrix described by Bisse and Wieland [132] and ion-exchange HbA1c values. The figure illustrates the positive deviation between results obtained using boronate affinity chromatography, measuring total glycated Hb and HbA1c results obtained using ion-exchange HPLC. Although 3-aminophenyl boronic acid is used most often, basically because of its availability and simple conjugation chemistry, it is the experience of the author that 4-carboxyphenyl boronic acid is more suited than 3-aminophenyl boronic acid. This because of greater chemical stability of the conjugates and lower pKa of the boronic acid residue, both of importance for the separation and use of the resulting media. Both ligands are utilised in the synthesis of soluble coloured boronic acid conjugates developed for the determination of GHb based on a completely new non-chromatographic principle presented by Frantzen et al. [136].

Another separation method relaying on the boronate–*cis*-diol interaction seldom used after the boronate affinity matrices became available, is to separate glycated Hb (or other *cis*-diol containing analytes) on an anion-exchange column using borate containing buffers. This method utilise the extra negative charge induced by borate after diol esterification with the glycated residues of Hb and was used by Shapiro et al. [13] in their elucidation of specific glycation sites in Hb.

4.3. Immunoaffinity chromatography

Similar to the boronate affinity system specific for glycated Hb, affinity media can be prepared using immobilised antibodies specific to glycated Hb or other adducts. This approach is illustrated by the work of Wu et al. [83] using an immobilised antibody directed against glycated ϵ-lysine epitopes in an immunoaffinity chromatographic separation of HbA0 and HbA1c. Chromatographic separation of purified HbA1c showed that this Hb fraction, in addition to its N-terminal glycation, may also contain some additional glycation at ϵ-lysine sites.

4.4. RPC-GC methods

4.4.1. Xenobiotic-Hb adducts

Environmental and occupational exposure to toxic chemicals is of great concern to the public. Methods to identify biological effects and to quantitate the risk associated with such exposure are therefore required. Different biochemical markers have been used for biomonitoring the human population. Similar to other Hb adducts discussed in this paper, adducts formed with carcinogens and other xenobiotics are based on the reactivity of the amino-groups or the β93 Cys sulfhydyl group. However, stable xenobiotic-Hb adducts are present in very low amounts and ion-exchange methods are seldom applicable unless the adducts are generated in vitro using high concentrations [137]. A number of studies describing different xenobiotic Hb adducts have used GC or HPLC methods to measure and quantitate the released Hb-adduct after derivatisation. In biomonitoring, the total amount of the released adduct is most often reported [89,90,92,96], however, some reports on structural characterisation and localisation of the adducts in the subunits of Hb are also available [91,95,97].

4.4.2. Characterisation of Hb-adducts

Standard procedures are developed for the structural characterisation of proteins. Regarding Hb, RPC is often used both for the analysis and isolation of Hb chains after dissociating the chains of tetrameric Hb. Updated procedures are presented by Jones [138] illustrating an approach to the structural characterisation of modified Hbs using distinct successive steps. However, with the introduction of new MS techniques like ESI–MS, mass spectra can be obtained for intact globin chains. Combined with globin chain separation and tryptic digests, this technique provides a new tool for the determination of both the nature and extent of Hb modifications. A few publications describing this technique have appeared on the characterisation of Hb variants [93,94,98] and Hb adducts [91,97,139].

4.5. Electrophoresis

Although HPLC systems show better performance in the separation of HbA1c, gel electrophoresis is frequently used. Several commercial systems are available and their benefit is first of all related to their suitability for routine laboratory use. Generally, modification of N-terminal valine of Hb, like acetylation after high doses of aspirin, lead to increases in a fast moving Hb affecting electrophoretic separations [31]. Additionally, carbamylation, pre-glycated HbA1c, HbF and genetic Hb variants also affects the quantification of HbA1c [57].

IEF on the other hand shows several benefits over traditional electroendosmosis and the analysis of HbA1c can be used to illustrate its resolving power. Using IEF HbA1a1, HbA1a2, HbA1b, normal levels of HbF and even the preglycated form of HbA1c (HbA1d) can be resolved from HbA1c [139]. High levels of HbF and acetylated HbF bands may still fuse and complicate the interpretation of the results [33,69,140], however, acetylated HbF will only be a problem at high HbF levels.

For the study of non-carbohydrate modified Hbs it is worth mentioning that IEF has the potential of separating several of these adducts. The glutathione-Hb adduct focuses anodally from pre-HbA1c and acetylated Hb can be resolved from HbA1c, but still not from the HbA1b fraction. Correspondingly, HbA2 focuses near the cathode and glycation of this minor Hb have been detected using IEF [31].

CE opens new possibilities in the use of electrophoretic methods for the analysis of Hb adducts and variants. The information provided by CZE is comparable to that obtained by traditional electrophoresis of native Hb on cellulose acetate or cation HPLC. Generally cIEF shows higher resolution than HPLC but lower speed, still, high resolution separations of Hb variants (HbA, HbF, HbS, HbC) can be performed within 2 min [75]. Most of the reports so far using CE for the analysis of Hb have been dealing with the analysis of genetic variants, both in humans and in other species [71,141–143,145], however, some studies have demonstrated the separation of HbA1c from HbA0 [144,145]. Yet, as illustrated in Fig. 7 the separation of the HbA0 and HbA1c fraction is still a bit behind what is obtained using traditional IEF and HPLC methods. The resolution obtained is at the 0.04 pH unit level, not enough to completely resolve N-terminally glycated Hb from the main non-glycated HbA0 fraction. To fully

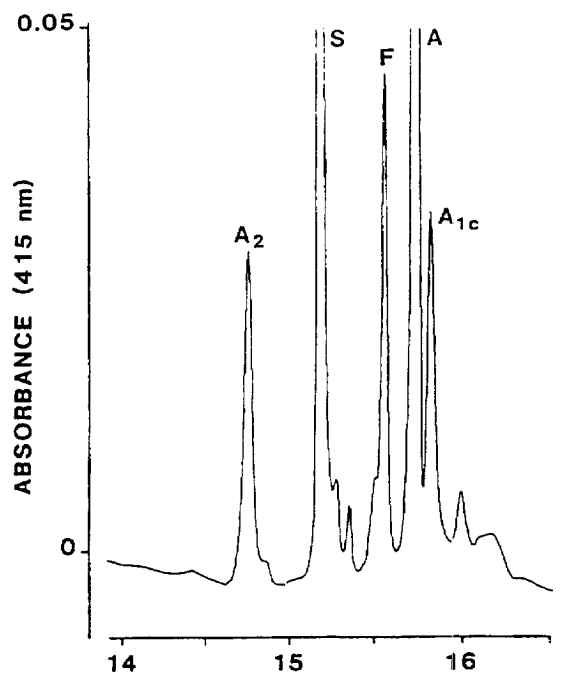

Fig. 7. Capillary IEF of Hb variants with a resolution at the 0.03 pH unit level. Blood from a person heterozygote for HbS. From Molteni et al. [145].

utilise the potentials of CIEF, further work with optimisation of the separations and development of automated procedures are required. Still, exciting new analytical possibilities arise with CE, exemplified by the work of Lillard et al. [72] separating Hb variants in single human erythrocytes. Cells introduced into the capillary were lysed by an detergent containing buffer, then secondly denatured, i.e., dissociated, before separated as globin chains by free-zone electrophoresis. Using red cells from normal and diabetic adults, they were able to detect the α- and β-chains and their glycated counterparts, as illustrated in Fig. 8 [72]. Similarly, using foetal erythrocytes they were able to tentatively detect acetylated γ-chains. Correspondingly, Tang et al. [99] have demonstrated the integration of electrospray ionisation mass spectrometry (ESI-MS) with CIEF, enabling a two dimensional separation/detection system based on p*I* and mass determination.

Using CE in the separation of tetrameric Hb with intact quaternary structure requires mild conditions to avoid denaturation or chain dissociation. However, globin chain analysis [146] offers better versatility than that available for tetrameric Hb and is an excellent alternative to analytical RPC in the characterisation of Hb adducts.

Although there is a need for further optimisation using CE in Hb analysis, we will certainly experience improvements and new applications for the separation of minor Hbs in the future.

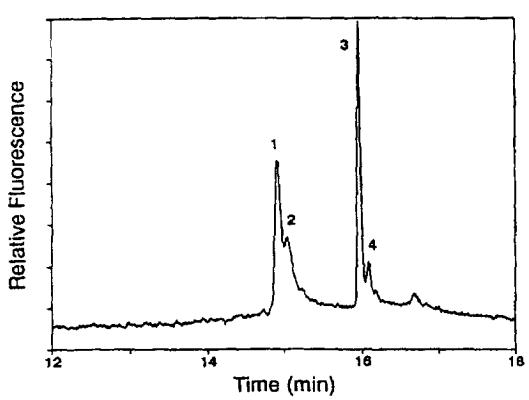

Fig. 8. Separation of Hb chains by CE. The sample used is in a single red blood cell injected into the capillary. Peaks are: 1=β-chain, 2=glycated β-chain, 3=α-chain, 4=glycated α-chain. From Lillard et al. [72].

5. Concluding remarks

In addition to more basic requirements regarding separation ability the choice of separation method for the study or quantitative determination of hemoglobin adducts very much depends on whether the method should be used for research purposes or established as a routine analysis. For the determination of clinically important Hb adducts, first of all glycated hemoglobin, several high-performance chromatographic media are available. Cation exchangers are dominating, however, boronic acid based media showing less interference due to variation in separation parameters like temperature and pH, are also frequently being used in the assessment of glycation. In some particular situations, i.e., dealing with hemoglobinopathies and studying glycation in non-human species, boronic acid media is often the best choice since separations can take place without prior optimisation related to the Hb variant in study.

In the routine clinical laboratory automated HPLC and electrophoretic systems dedicated to HbA1c are frequently being used. Although the interferences experienced using agar gel electrophoresis are similar to those seen using high-performance ion-exchange methods, HPLC normally gives the most reliable results. Using standard electrophoretic methods for the determination of minor Hbs, IEF is often to be preferred due to high resolving power and the limited number of interferents affecting this technique.

Although no single chromatographic media or procedure is able to separate the most studied Hb adducts in one single run, high-performance cation-exchangers capable of resolving an impressing number of minor hemoglobins are available.

The availability of poly- and monoclonal antibodies directed against some Hb adducts have opened the possibility of immunoaffinity studies, however, this is a technique not frequently used.

With the minute concentrations present of some of the Hb adducts being studied, detection problems are often experienced using traditional HPLC methods separating tetrameric Hb. RPC, GC or chemical determination of Hb chains, chain digests or derivatized adducts released after hydrolysis are alternative technique. Yet, new developments, first of all in the

area of capillary electrophoresis have shown promising results regarding the separation of both tetrameric Hb and Hb chains. Although some technical optimisations still remains, the possibility of easily varying the separation principle, high resolving power, speed and sensitivity makes this a very attractive technique for the further study of minor hemoglobins.

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