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REVIEW

GLYCATED HAEMOGLOBINS

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CONTENTS

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LIST OF ABBREVIATIONS

C.V. Coefficient of variation
Hb Haemoglobin
HPLC High-performance liquid chromatography
IEC Ion-exchange chromatography
IEF Isoelectric focusing
TBA Thiobarbituric acid

1. INTRODUCTION

Glycated haemoglobins (Hb) arise from the non-enzymatic attachment of glucose, glucose-6-phosphate or fructose-1,6-diphosphate to haemoglobin. Of clinical interest are the glucose adducts. They are formed and accumulate in the erythrocyte in proportion to the prevailing blood glucose concentration. Therefore, glycated haemoglobins have become an important parameter for the objective assessment of averaged long-term glycaemia in patients with diabetes mellitus [1].

Measurement of glycated haemoglobins is especially useful in insulin-dependent diabetic patients where blood glucose concentrations fluctuate widely and where the fasting blood glucose does not reflect averaged glycaemia, as is often the case in non-insulin-dependent patients. With regard to detecting glucose intolerance, glycated haemoglobin levels are less sensitive than results of oral glucose tolerance tests consistent with a conservative approach to the diagnosis of diabetes [2].

An unaltered life span of the erythrocyte [3] and the absence of significant concentrations of the reactive galactose in blood [4], are prerequisites for glycated haemoglobins to yield reliable information concerning averaged long-term glycaemia. Conversely, it has been proposed to use glycated haemoglobin levels in haemolytic anaemia or galactosaemia to follow the effect of therapeutic intervention.

2. GLYCATED HAEMOGLOBINS AND PRINCIPLES OF ESTIMATION

Human haemoglobin is quite heterogeneous. This heterogeneity is mainly a consequence of its glycation (Table 1)*.

Haemoglobin gets glycated at several sites: the amino termini of both its α- and β-chains, as well as at certain ϵ-amino groups [5]. Modification, such as glycation of the amino terminus of the β-chains imparts enough negative charge to the haemoglobin molecule to allow separation of the respective haemoglobins by charge-dependent techniques. Haemoglobins glycated at the amino termini of the β-chains elute from a cation-exchange resin in the HbA1 fraction well in front of the main haemoglobin peak. They are referred to in order of their elution as HbA1a, HbA1b and HbA1c.

Glucose is the carbohydrate in the major glycated haemoglobin, HbA1c, and glucose-6-phosphate and fructose-1,6-diphosphate in the two components of HbA1a. These phosphorylated carbohydrates get attached to the amino terminus

*For a more general review see T.H.J. Huisman, J. Chromatogr., 418 (1987) 277.

TABLE 1

NOMENCLATURE AND STRUCTURE OF RELEVANT HAEMOGLOBINS

Haemoglobin/glycohaemoglobin	Structure
Haemoglobin	
A	$\alpha_2\beta_2$
F	$\alpha_2\gamma_2$
A2	$\alpha_2\delta_2$
*Glycated forms of HbA (glycohaemoglobin)**	
A1a1	$\alpha_2(\beta\text{-N-FDP})_2$
A1a2	$\alpha_2(\beta\text{-N-G6P})_2$
A1b	$\alpha_2(\beta\text{-N-CHO})_2$
A1c (labile)	$\alpha_2(\beta\text{-N=Glc})_2$
A1c (stable)	$\alpha_2(\beta\text{-N-Glc})_2$
A-Glc	$\alpha_2(\beta\text{-LysN-Glc})_2$ $(\alpha\text{-LysN-Glc})_2\beta_2$ $(\alpha\text{-N-Glc})_2\beta_2$

*FDP = fructose-1,6-diphosphate; G6P = glucose-6-phosphate; Glc = glucose; CHO = unidentified carbohydrate.

TABLE 2

METHODOLOGY FOR ESTIMATION OF GLYCATED HAEMOGLOBINS

Irrespective of the methodology used, glycated haemoglobins do not allow assessment of averaged glycaemia in galactosaemia or if the life-span of the erythrocyte is shortened.

Charge-dependent methods	Glycation-specific methods
Cation-exchange chromatography	TBA method
IEF	Boronate affinity chromatography
Mobile affinity chromatography	
Parameter measured:	
β-Chain amino terminal modifications (HbA1a+b + HbA1c = HbA1)	Total glycated haemoglobins: β-chain amino terminal glycation, α-chain amino terminal glycation, glycation at ϵ-amino groups (glycohaemoglobin)
Analytical interferences:	
Fetal haemoglobin (HbF)	
Variant haemoglobins (HbS, C, D, etc.)	
Modifications other than glycation (carbamylation, acetylation, etc.)	
Hemichromes formed during storage	

of the β-chains only. This site is located in the positively charged 2,3-diphosphoglycerate binding cleft, which affinity directs phosphorylated carbohydrates to this site [6]. HbA1b is also glycated, but its structure has not been established with certainty.

Haemoglobins glycated at sites other than the β-chain amino terminus elute in the leading edge of the main haemoglobin peak [7]. This portion of glycated haemoglobin represents ca. 50% of total glycated haemoglobin and is detected by

glycation-specific methods, such as the thiobarbituric acid (TBA) method or boronate affinity chromatography [8,9] (Table 2).

Glycation at specific sites can be detected with high specificity by immunological assays. Antibodies were raised against HbA1c so that glycation of the β-chain amino terminus is measured with existing immunological assays [10–13]. So far, such assays have not been available for use in the routine clinical laboratory.

Two other methods that have not found wide use in the routine clinical laboratory rely on the altered spectral and functional properties of haemoglobin modified at the β-chain amino termini. The phytic acid assay relies on the high affinity of this ligand for the 2,3-diphosphoglycerate binding site and the altered spectral properties of this complex compared with free haemoglobin [14]. The other assay relies on the fact that the complexes of HbA1 and HbA with haptoglobin differ in stability and hence have differing peroxidase activity [15]. Both these assays have attractive features, the phytic acid assay because it is a purely spectral assay that does not require separation and the HbA1/haptoglobin method because it can be installed on the autoanalyser. However, their evaluation for use in the clinical routine laboratory was not entirely satisfactory [16,17].

3. SAMPLE PREPARATION AND STORAGE

3.1. Sample storage

Sample storage and preparation are important aspects in the determination of glycated haemoglobins. In the past, de novo formation of HbA1c in the intact erythrocyte and preferential lysis of older HbA1c-rich erythrocytes were considered important and hence erythrocytes were lysed as soon as possible after blood withdrawal. Haemolysates were stored in the cold until analysis. In view of more recent experience this practice should be discontinued. De novo formation of glycated haemoglobin does not occur because glycation is a slow process and old erythrocytes do not seem to get preferentially lysed.

3.2. Labile HbA1c

With the advent of fast chromatographic techniques it was recognized that glycated haemoglobin fluctuates acutely with blood glucose and that these fluctuations are caused by the labile glucose adduct of haemoglobin, which co-chromatographs with stable HbA1c [18,19]. This labile HbA1c represents the initial Schiff's base adduct or aldimine, which has not yet undergone the Amadori rearrangement to form the more stable ketoamine (1-amino-1-deoxy-2-keto compound). It has also been referred to as pre-HbA1c [20] or HbA1d [21].

The strategies for the elimination of labile HbA1c comprise incubation of erythrocytes with saline (5 h, 37°C) [22], dialysis of haemolysates [23], diluting and reconcentrating haemolysates by ultrafiltration [24], use of haemolysis reagents containing the eliminators semicarbazide–aniline [25–27] or borate [28] or simply haemolysis at pH 5 [29,30]. The eliminators are supposed to trap the liberated glucose and thereby accelerate the dissociation. We could not confirm

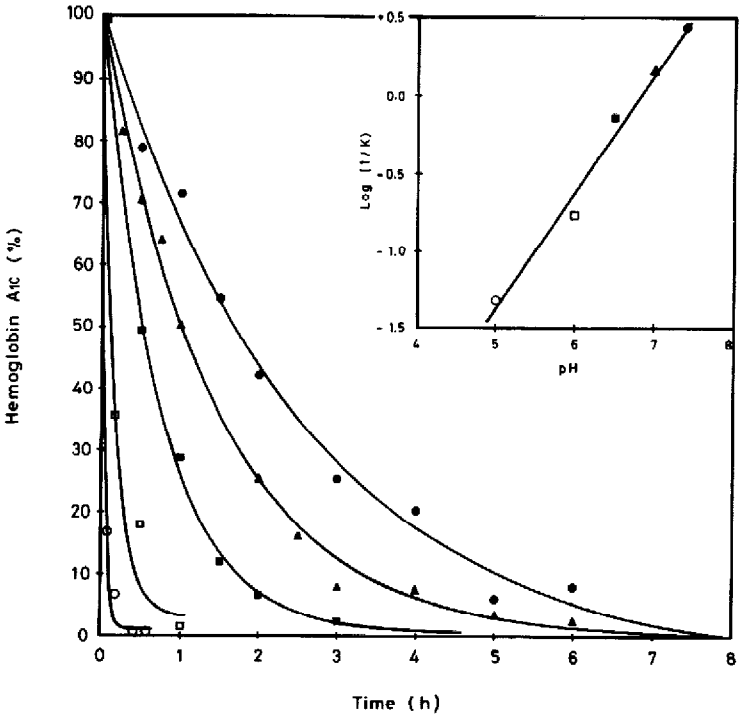

Fig. 1. pH dependence of dissociation of labile HbA1c. The inset illustrates the linear dependence of dissociation on pH (experimental procedure as in ref. 29).

this acceleration by semicarbazide–aniline [29] and found that the rate of dissociation depends linearly on the proton concentration (Fig. 1). The pH values of both the semicarbazide and borate eliminator reagents are acidic; under these conditions dissociation proceeds rapidly and borate complexes are not stable. Haemolysis at an acidic pH is therefore sufficient to promote efficient dissociation of labile HbA1c.

Labile HbA1c can be resolved from stable HbA1c by isoelectric focusing (IEF) or by ion-exchange chromatography (IEC) on Synchropak CM 300 [31].

4. SEPARATION METHODS

4.1. Ion-exchange chromatography

The classical method for the rapid determination of HbA1a–c by chromatography on the weak cation-exchange resin Biorex 70 was introduced by Trivelli et al. in 1971 [32]. Several approaches, including use of minicolumns or automated equipment for high-performance liquid chromatography (HPLC) were used to make IEC acceptable for the routine clinical laboratory. These methods measure HbA1 or HbA1c, usually including HbF.

4.1.1. Minicolumns

Commercial minicolumns have found widespread use for HbA1(c) determinations because chromatography on such columns is simple to perform and does not require specialized equipment. This approach is, however, not without problems. Separations on minicolumns are quite sensitive to variations in assay conditions, such as pH, ionic strength and temperature. Originally, use of temperature nomograms was advocated [33], then strict temperature control [34] or use of calibrators. Precision is better if the temperature is controlled than if corrections based on calibrators are made [35]. Also, conditions were sought where separations are least temperature-sensitive [36].

Commercial minicolumn kits have repeatedly been compared [37–39] and differences of the normal range and precision noted. All systems tested were found to be of a high standard. One comparison includes a detailed description and discussion of the practical advantages and disadvantages of the various test-kits [37].

In minicolumn systems, variations in assay conditions cannot be avoided and, therefore, the eluted haemoglobin fractions are not always quantitatively collected. This inherent feature sets limits to the performance of minicolumns. Monitoring of the effluent and use of an integrator, as is the case when automated HPLC equipment is used, overcomes this problem. When the elution profile changes, peak integration is still adequate. Results are no longer temperature-dependent [40].

4.1.2. High-performance liquid chromatography

Though good resolution can be obtained with Biorex 70 (Fig. 2A) this resin does not tolerate pressures above 20 bar [41] and column life is rather short. This drawback stimulated synthesis and use of several ion-exchange resins for HPLC, such as non-commercial CM-polyamide resin [42], Synchropak CM 300 (SynChrom, Linden, IN, U.S.A.) [43], polyCAT (Custom LC, Houston, TX, U.S.A.) [42], MonoS HR 5/5 (Pharmacia) [45–48], as well as Micropearl SF-W-A1c (composition proprietary, Daiichi analyser) [49] and the Glykopak resin (composition proprietary) of the Diamat analyser (Biorad).

Resolution on the two commercial HbA1c analysers, the Daiichi analyser (Haemoglobin A1c analyser, Model HA-8110, manufactured by Kyoto Daiichi, Kagaku, Japan) and the Diamat analyser (manufactured by Toya-Soda, Tokyo, Japan; distributed by Biorad) is compared in Fig. 2 with that obtainable on Biorex 70 using automated HPLC equipment. On Biorex-70 HbF is not separated from HbA1c, barely so on the resin used with the Daiichi analyser and with baseline resolution on the Diamat.

Furthermore, on the resin used with the Diamat analyser, the percentage of HbA1c+b is less than on other resins. This is of importance when HbA1c and HbA1c values are compared. There is a high correlation between HbA1c and both HbA1c+b [50] and HbA1 [51]. Taking this constant relation into account, HbA1 and HbA1c can both be used for the estimation of haemoglobin glycation, at least in fresh samples.

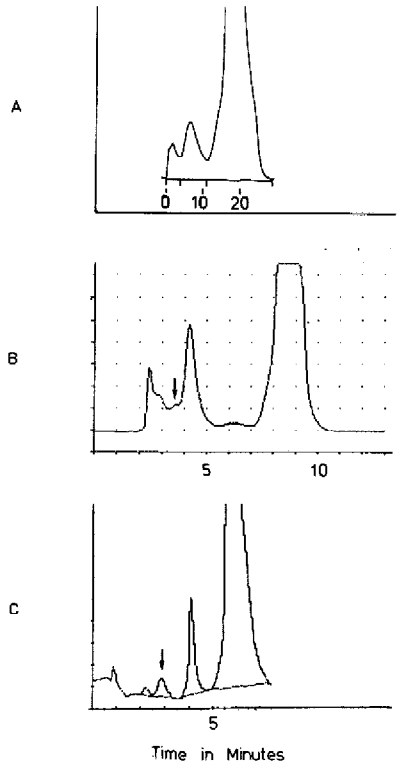

Fig. 2. Chromatograms of haemolysate analysed by (A) HPLC on Biorex 70, (B) the Daiichi analyser and (C) the Diamat analyser. The arrow indicates the elution position of HbF.

The normal range for stable HbA1c determined by IEC is 4–6%. Intra-assay precision (coefficient of variation, C.V.) is in the range 2–6% [37, 49].

Published data on the stability of samples at the various stages of preparation are not consistent (for a review see ref. 52). Fig. 3 shows data on the stability of HbA1 and HbA1c in whole blood. During storage at room temperature HbA1c remains unchanged. Blood samples can, therefore, be mailed to the analytical laboratory unrefrigerated [53] and may then be stored at 4 °C for up to four weeks without increases in HbA1 or HbA1c [54]. If blood samples are exposed to room temperature for more than two to three days HbA1a+b levels tend to increase, especially in the presence of high concentrations of glucose. Capillary blood specimens may, therefore, be mailed to the analytical laboratory by the patient before attending the physician.

Both the minicolumns and the automated HPLC systems have attractive features for use in the routine clinical laboratory. The minicolumns are easy to use and do not require capital investment. Capital investment is high for automated HPLC systems but they offer high precision.

4.2. Isoelectric focusing

IEF separates proteins according to their isoelectric points. The isoelectric points of the glycated haemoglobins HbA1a (pI=6.88), HbA1b (pI=6.92) and

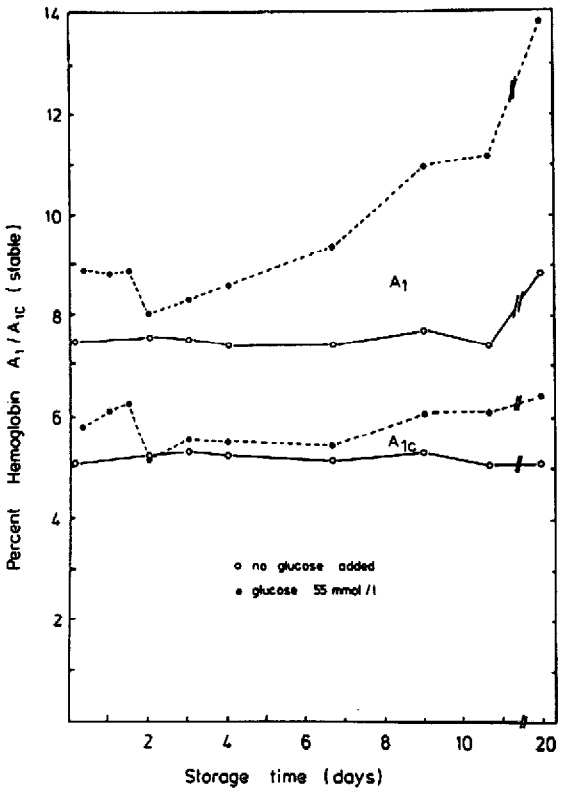

Fig. 3. Behaviour of HbA1 and HbA1c in whole blood stored at room temperature as analysed by HPLC on Biorex 70.

HbA1c (pI=6.94) are lower than the pI of HbA (6.95) [55] and can be resolved by this technique (Fig. 4). Satisfactory resolution is achieved in polyacrylamide gels with a simple pH 6–8 gradient [56]. HbA1a+b focus anodally from the glutathione adduct of haemoglobin (HbASSG; HbA3) and HbF cathodally from HbA, so that HbA1c is specifically determined.

Resolution is improved by addition of separators or pH gradient modifiers, such as β-alanine, which flatten the gradient in the proximity of the pI of the separator [57]. An even better resolution is obtained with ready-made narrow-range (pH 6.8–7.3) polyacrylamide gel plates, commercially available from LKB (Bromma, Sweden). The separation between HbA and HbA1c is ca. 2 mm using β-alanine and 3 mm in the ready-to-use narrow-range plates. HbA1c can be quantitated with equal sensitivity and reproducibility by either spectrophotometry or densitometry [58].

In the routine clinical laboratory ready-to-use plates are most often used for the determination of HbA1c. Handling of the hazardous polyacrylamide and the time-consuming gel casting which can give rise to reproducibility problems, are thus avoided. The intra-assay and day-to-day precision with the narrow pH-range plates is ca. 4% (C.V.) [58].

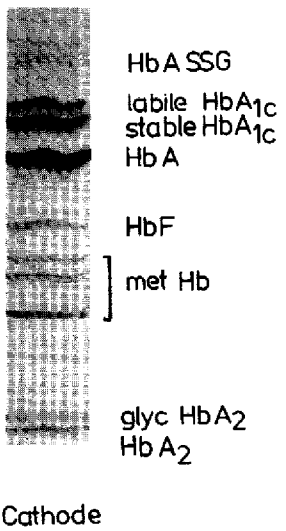

Fig. 4. IEF pattern of normal haemolysate. HbASSG and methaemoglobin are increased in old haemolysates.

Resolution by IEF is so high that labile HbA1c is resolved from stable HbA1c [59, 60]. Resolution between labile and stable HbA1c is, however, insufficient for quantitation by microdensitometry, so that for routine HbA1c determinations elimination of labile HbA1c is necessary. Elimination should be performed by incubation of erythrocytes with normal saline rather than with a pH 5 step, because the latter gives rise to significant and variable methaemoglobin formation and spuriously low HbA1c [61].

The reference level in control persons for stable HbA1c is $4.95 \pm 0.51\%$ [62]. The different values of published reference intervals are likely to be in part a consequence of lack of removal of labile HbA1c.

Whole blood was found to be stable for five days when kept at 4°C [58], and for seven days at room temperature [63, 64]. Analysis of outdated samples will lead to falsely decreased HbA1c values because of methaemoglobin formation [61].

IEF has a high capacity: 60 samples can be analysed daily but this requires special equipment and skilled personnel.

4.3. Mobile affinity electrophoresis

Mobile affinity electrophoresis exploits the affinity of dextran sulphate for haemoglobin not glycated at the β-chain N-termini. Inclusion of this active ligand in the electrophoresis buffer increases the mobility of HbA relative to that of the glycated haemoglobins of the HbA1 fraction. Electrophoresis may be performed on agarose [65], cellulose acetate [66], or agar gel membranes [67]. Ready-to-

use agar gel cassettes are available from Corning (Sullivan Park, NY, U.S.A.). Use of such plates avoids variability of results arising from differences in agar batches [68].

HbF and labile HbA1 are not resolved from HbA1 with this technique [67, 69]. In agar gel electrophoresis, temperature variations ranging from 4 to 30°C do not affect results [70]; the only difference between gels run at different temperatures is the increased migration of HbA and HbA1 at higher temperatures [65]. The normal range is 6.0–7.1% HbA1, and values correlate closely with results of HPLC determinations [70, 71].

Precision studies show an overall C.V. of 9%, with a C.V. for repeat scanning of the gels of less than 1.5% [72], indicating that most of the imprecision arises during specimen processing, electrophoresis and drying of the gels. Haemolysates can be stored at 4°C for one week without significant changes in HbA1 [72].

The commercial agar gel electrophoresis is simple to perform and not critically influenced by assay variables, but does require special equipment.

4.4. Boronate affinity chromatography

Boronate affinity chromatography relies on the complex formation in alkaline solution between an immobilized boronic acid and the coplanar *cis*-diol groups of the carbohydrate moiety in glycated molecules. It should, therefore, have very high specificity for glycated products and allow their absolute quantitation with little effect of assay conditions on results.

These characteristics apply for the measurement of amino acids and peptides [73]. Binding is weak with glycated proteins, and secondary interactions have to be eliminated with a carefully optimized buffer [74]. Quantitative binding occurs only if the residence time on the gel is sufficiently long. Under such optimized conditions the glycohaemoglobin content of haemolysates is twice that of HbA1c [9] (Fig. 5).

In routine clinical practice, boronate affinity chromatography is usually performed on 1 ml of the phenylboronic acid-agarose, Glycogel B (Pierce) [75]. This leads to underestimation of the glycohaemoglobin content [9] and dependence of results on pH and assay temperature [76–78]. The normal range in this 1-ml system is 5–8% and values in diabetics are 1.2–1.4 times that of their HbA1c [75]. Both precision within a run (C.V. 3%) and from day to day (C.V. 3.4–5.3%) are satisfactory [79]. While some authors have detected binding of labile HbA1c [9, 80], others have not [76, 81].

Attractive features of boronate affinity chromatography for the routine clinical laboratory are reusability of columns (if kept in the dark the affinity gel can be reused more than ten times) [82], lack of interference from HbF, variant haemoglobins [83] and sample stability during storage. Whole blood can be stored at room temperature for three weeks [83] or for up to two months at 4°C [81] with no effect on results, but glycohaemoglobin was found to be increased in haemolysates after one month's storage at 4°C [84].

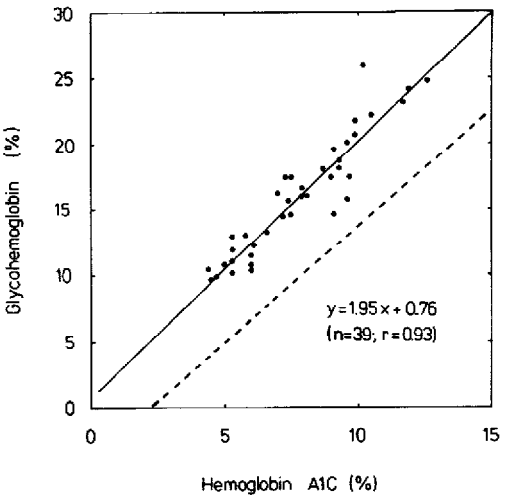

Fig. 5. Comparison of affinity chromatography on Glycogel B performed under optimal conditions, in Pasteur pipettes. The broken line indicates a regression obtained in the commercial 1-ml columns [76].

TABLE 3

CHARACTERISTICS OF METHODS FOR ESTIMATING GLYCATED HAEMOGLOBINS IN THE ROUTINE CLINICAL LABORATORY

Method	Specificity	Normal range (%)	Precision (C.V.) (%)
IEC with minicolumns	HbA1/HbA1c+HbF	4–6	9
HPLC	HbA1c±HbF	4–6	2
IEF	HbA1c	4.5–6.1	4
Mobile affinity chromatography	HbA1+HbF	6–7.1	9
Boronate affinity chromatography	GlycoHb*		
Commercial		5–8	5
Optimized		7–12	5

*The only parameter not affected by variant and non-glucose modified haemoglobins.

5. ADVANTAGES AND LIMITATIONS OF CHROMATOGRAPHIC METHODS

The relationship between averaged glycaemia and glycated haemoglobin defines the precision requirements for the quantitation of glycated haemoglobins. One percentage point of HbA1c corresponds to a difference in averaged glycaemia of 30 mg/dl. In order to obtain quantitative rather than merely qualitative information from such determinations, precision should be maintained at the highest possible level.

The presence of variant haemoglobins [85] or haemoglobin modified by other reactive molecules such as cyanate [86], aspirin [87], penicillin [88] or metabolites arising in alcoholism [87] interfere in all chromatographic methods with

the exception of the specific boronate affinity chromatography, in such a way that interpretation of results requires special attention.

A chemical procedure that specifically detects glycation, the TBA method, is not affected by the above modifications of haemoglobin [90]. Performance of the TBA reaction, even in its semi-automated setup [91], is more laborious than the chromatographic techniques and hence it is less often used.

The immunological assays that have not yet become commercially available have the potential of overcoming the methodological difficulties of glycated haemoglobin analyses.

Some important characteristics of chromatographic methods for estimating haemoglobin glycation in the routine clinical laboratory are summarized in Table 3. Precision and specificity are the requirements imposed by the nature of the parameter and cost-effectiveness or automatability by the clinical laboratory. The recent introduction of a high-pressure boronate affinity resin, Fractogel TSK AF-phenyl boronate, may allow these opposing aspects to be reconciled.

6. SUMMARY

The association between elevated levels of glycated haemoglobins and diabetes mellitus has been known for twenty years [92]. Since then the determination of glycated haemoglobins has become a valuable tool for the objective assessment of long-term glycaemia in diabetic patients.

The marked clinical interest in reliable measurements of glycated haemoglobins has stimulated the development and perfection of the necessary methodology. Limitations of the techniques have led to investigation of the underlying causes. Some of them led to the recognition of processes that were not known to occur in vivo before, such as glycation at sites other than the amino terminus of the β-chains, modification of haemoglobin by reactants other than glucose or the existence of labile haemoglobin adducts. With ideal methodology these features would have gone unnoticed. Furthermore, the determination of glycated haemoglobin in large populations of diabetic patients has lead to the discovery of new, clinically silent mutant haemoglobins. Today, the routine determination of glycated haemoglobins in diabetic patients probably represents the broadest screening for mutant haemoglobins.

The experience with glycated haemoglobins shows that overcoming difficulties in their determination, and progress in biomedical research, are closely intertwined.

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