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Approved IFCC Reference Method for the Measurement of HbA_{1c} in Human Blood

International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)[1)2)]

Scientific Division
Working Group on HbA_{1c} Standardisation[3)] and
Network of Reference Laboratories for HbA_{1c}[4)]

Prepared for publication[5),6)] by

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HbA_{1c} is the stable glucose adduct to the N-terminal group of the β-chain of HbA_0. The measurement of HbA_{1c} in human blood is most important for the long-term control of the glycaemic state in diabetic patients. Because there was no internationally agreed reference method the IFCC Working Group on HbA_{1c} Standardization developed a reference method which is here described. In a first step haemoglobin is cleaved into peptides by the enzyme endoproteinase Glu-C, and in a second step the glycated and non-glycated N-terminal hexapeptides of the β-chain obtained are separated and quantified by HPLC and electrospray ionisation mass spectrometry or in a two-dimensional approach using HPLC and capillary electrophoresis with UV-detection. Both principles give identical results. HbA_{1c} is measured as ratio between the glycated and non-glycated hexapeptides. Calibrators consisting of mixtures of highly purified HbA_{1c} and HbA_0 are used. The analytical performance of the reference method has been evaluated by an international network of reference laboratories comprising laboratories from Europe, Japan and the USA. The intercomparison studies

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of the network showed excellent results with intra-laboratory CVs of 0.5 to 2% and inter-laboratory CVs of 1.4 to 2.3%. Possible interferences have been carefully investigated. Due to the higher specificity of the reference method the results are lower than those generated with most of the present commercial methods which currently are calibrated with unspecific designated comparison methods. The new reference method has been approved by the member societies of the International Federation of Clinical Chemistry and Laboratory Medicine and will be the basis for the future uniform standardization of HbA_{1c} routine assays worldwide. Clin Chem Lab Med 2002; 40(1):78–89

Key words: HbA_{1c}; Glycohaemoglobin; Diabetes; International Standardization; Reference method; Electrospray ionisation mass spectrometry; Capillary electrophoresis.

Abbreviations: amu, atomic mass unit; HPLC-CE, HPLC/capillary electrophoresis; HPLC-ESI/MS, HPLC/electrospray mass spectrometry; KCN, potassium cyanide; MES, β-Morpholino-ethanesulfonic acid; SOP, standard operation procedure; TFA, trifluoroacetic acid.

Contents

1. Introduction

The measurement of HbA_{1c}/glycohaemoglobin in blood has become the gold standard for the long-term control of the glycaemic state of diabetic patients as presented in the DCCT and UKPDS studies (1, 2). The optimal therapy of diabetic patients requires carefully validated, method-independent therapeutic target values for the glycohaemoglobin levels of diabetic patients in order to reduce the long-term risk of the late complications such as retinopathy, nephropathy and neuropathy as well as the short-term risk of life-threatening hypoglycaemia. More than 15 different analytical methods are currently used by the clinical laboratories, which are based on ion exchange or affinity chromatography, electrophoresis and immunological principles (3). There is no internationally agreed reference method or reference material to which routine assays could be traced back (4). However, there are a several national initiatives for harmonisation and certification such as the National Glycohaemoglobin Standardisation Program (NGSP) in the USA based on the DCCT HbA_{1c} work (5–7) and standardisation schemes in Japan (8) and Sweden (9) which are based on different designated comparison methods. As a consequence of the lack of international standardisation, values vary considerably between methods, as many surveys have shown (10–12).

HPLC methods often used as "reference method" for the standardisation of routine tests provide good precision and long-term stability but they are rather unspecific. Different values for HbA_{1c} can be obtained when the same blood samples are measured, depending on the chromatographic system, *e.g.* the kind of resin, lot-to-lot variation of resins, column size, buffer composition and elution times. The peak considered to be HbA_{1c} may contain variable proportion of substances which have the same elution behaviour as HbA_{1c} but are not HbA_{1c} according to definition, since they lack immunological activity or do not bind during affinity chromatography. The differences between some methods can be rather large. The comparison between the Bio-Rex 70 HPLC method used as designated comparison method in the DCCT study and in the NGSP programme in the USA, and the Mono S HPLC method recommended for standardisation in Sweden, shows about 20% differences in values at the cut-off level in spite of the fact that both HPLC methods claim to measure HbA_{1c}. It is obvious that none of the methods meet the requirements of a reference method in a metrological sense.

There are a few glycohaemoglobin species present in human blood due to several potential glycation sites of the haemoglobin molecule (N-terminal ends of the β- and α- chains and lysine residues) (13–16). HbA_{1c} is the major form of all glycohaemoglobin species in human blood and is defined as the stable adduct of glucose and the N-terminal amino group of the β-chain of haemoglo-

bin A_0 [N-(1-deoxyfructosyl)haemoglobin]. Most of the commercial tests already claim to measure this form of glycohaemoglobin and some assays, which measure the sum of glycohaemoglobin species in blood, are already internally standardised against a HbA_{1c} designated comparison method. This is possible since the total glycohaemoglobin and HbA_{1c} values correlate very well (17).

To overcome the problem of poor standardisation the IFCC has created a working group to establish a uniform, scientifically well founded international standardisation. The group decided to develop a reference system to which routine methods can be traced (3, 18–20). A major component of the reference system is the reference method, which specifically measures HbA_{1c} and is necessary for assigning values to reference materials. Due to the fact that all currently used designated comparison methods are rather unspecific, a new reference method which specifically measures glycated N-terminal residue of the β-chain of haemoglobin had to be developed. Searching for an appropriate analytical technique, two analytical approaches have been proven to be adequately suited – namely HPLC /electrospray mass spectrometry (HPLC-ESI/MS) and HPLC/capillary electrophoresis (HPLC-CE). Both principles work very well and generate identical results. Since flexibility with regard to equipment in the reference laboratories is an advantage both principles have been developed (Figure 1).

2. Principle (Figure 2)

In the first step haemoglobin is cleaved into peptides by the proteolytic enzyme endoproteinase Glu-C. Thereafter the resulting glycated and non-glycated N-terminal hexapeptides of the β-chains are separated from the crude peptide mixture and quantified by HPLC and electrospray mass spectrometry or by HPLC followed by capillary electrophoresis with UV detection. The percentage of HbA_{1c} is determined as a ratio of the glycated to non-glycated β-N-terminal hexapeptides of haemoglobin. There are three steps:

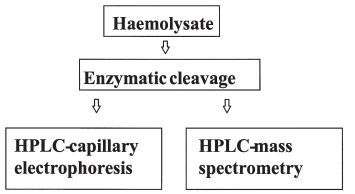

Figure 1 Flow chart of the reference method procedure.

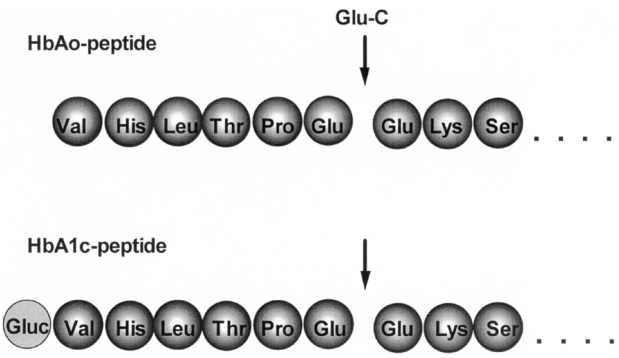

Figure 2 Principle of the proteolytic digestion of haemoglobin chains.

2.1 Isolation of erythrocytes and haemolysis

Human erythrocytes are separated, washed and haemolysed in water and kept in a storage buffer at pH 6.2.

2.2 Enzymatic cleavage of the haemoglobin

The haemoglobin solution is treated with endoproteinase Glu-C in order to hydrolyse the protein into several peptides, among them the glycated (HbA_{1c}) and the non-glycated (HbA_0) N-terminal hexapeptides of the β-chains.

2.3 Analysis and calibration

The peptide mixture is analysed to measure the ratio of glycated to non-glycated hexapeptide. These analyses are done either by HPLC-ESI/MS (approach A) or HPLC-CE (approach B). In both cases the areas containing the N-terminal hexapeptides of haemoglobin are measured and ratios of the glycated and non-glycated peptides are calculated. Calibrators consisting of the mixture of pure HbA_{1c} and HbA_0 are used for calibration.

3. Instrumentation and Equipment

3.1 HPLC-ESI/MS approach

An analytical HPLC system suitable for microbore columns is necessary. A slow gradient of water-trifluoroacetic acid (TFA)/acetonitrile-TFA is used on the equipment with a switching valve between the HPLC-column and the mass spectrometric detector, together with a temperature-controlled autosampler and autoinjector.

The HPLC column consists of cyanopropyl stationary phase. The electrospray ionisation mass spectrometer is directly coupled to the microbore HPLC system (HPLC-ESI/MS).

3.2 HPLC-CE approach

An analytical HPLC system with a C_{18} column is used in the first dimension along with a temperature controlled fraction collector. An analytical capillary electrophoresis system with 214 nm photometric detector (or DAD) and a temperature controlled fused-silica capillary is used for the final separation in the second dimension.

3.3 Data processing

For both approaches chromatography data systems are necessary which are capable of precise automatic or manual integration

4. Reagents

Acetic acid
Acetonitrile
Ammonium acetate, CH_3COONH_4

β-Morpholino-ethanesulfonic acid, MES
Endoproteinase Glu-C: sequencing grade
Ethylenediamine-tetraacetic acid disodium salt, Na_2-EDTA
HbA_{1c}: human haemoglobin with glycated N-terminal β-chain, purity ≥ 95% assigned by standard addition approach.
HbA_0: human haemoglobin with non-glycated N-terminal β-chain, purity ≥ 99%
Water, HPLC grade
Ortho-phosphoric acid
Potassium cyanide, KCN
Sodium chloride, NaCl
Sodium hydroxide, NaOH
Trifluoroacetic acid, TFA

The purity of chemicals having no specified purity is of analytical grade.

5. Preparation of Solutions

5.1 Buffer solutions

Incubation solution: saline, NaCl 0.15 mol /l
Digestion buffer: NH_4-acetate, 50 mmol/l, pH 4.3
Storage buffer: MES, 50 mmol/l, KCN, 10 mmol/l, Na_2EDTA, 1 mmol/l, pH 6.2
The enzyme endoproteinase Glu-C is freshly dissolved in HPLC grade water, 200 µg/ml, and stored at 4 °C. The solution should be used within 8 hours.

5.2 Calibrator solutions

Calibrator solutions are prepared by mixing pure HbA_0 and HbA_{1c} solutions (19). A set of calibrators with six levels of HbA_{1c} (0, 3, 6, 9, 12 and 15%) is prepared.

HbA_0 solution in storage buffers approximately 120 mg/ml and HbA_{1c} solution approximately 20 mg/ml in the same buffer is used. The exact haemoglobin content of both solutions is determined by the ICSH reference method for haemoglobin. For the HbA_{1c} solution the content of residual HbA_0 is determined as specified in appendix B. Both solutions are mixed by weighing the volumes from calibrated pipettes. For the calculation of volumes to be mixed, the haemoglobin concentrations of both solutions and the amount of HbA_0 contribution by the HbA_{1c} sample are considered. The pipetting volumes are adjusted, to get a haemoglobin content of 1 mg for each vial of calibrator within a total volume of 30 µl, by filling up with storage buffer. In order to reduce pipetting errors, for each calibrator level a bulk solution of several hundred millilitres is prepared, which is split into 30 µl aliquots. The calibrator solutions are stable for at least 8 hours at room temperature and can be stored at –20 °C or at –80 °C for at least 1 year.

6. Specimen Procurement

6.1 Sample preparation

6.1.1 Patient samples

Fresh blood is collected in the presence of EDTA. The cells are washed and incubated with saline solution for 4 hours at 37 °C to remove the pre-HbA_{1c} (Schiff base). Haemolysates are prepared by mixing cells with water and the storage solution. The clear haemolysates are stored at –20 °C and are stable for at least 1 year.

6.1.2 Calibrators

One vial of each concentration (1 mg total Hb in 30µl storage buffer) is used.
Enzyme solution and digestion buffer are added as for patient samples.

6.1.3 Controls

Previous haemolysates of patient pools stored at – 70 °C, with HbA_{1c} values established by the Network, were used as controls in each experiment.

6.2 Enzymatic cleavage

For the measurement of patient samples and calibrators an aliquot containing approximately 1 mg of total Hb is taken, 50 µl enzyme solution is added and the mixture is made up with digestion buffer to a total volume of 500 µl.

The vials are carefully closed with crimp caps and incubated under gentle shaking or rotating at 37 °C (± 2 °C) for 18 hours. The digestion is stopped by freezing the material at –20 °C. The digests of calibrators and samples are stable and may be stored for at least 3 months at –70 °C until analysis. After thawing the digests can be stored up to three days at 4 °C – 8° C.

7. Measurement

7.1 The measuring sequence for samples and calibrators

Measurements are done in following sequence: calibrator set- controls- sample 1 to sample n –controls- calibrator set (n= up to maximum 20–30 samples). Calibrators and samples are taken from the same digest and the same lot of enzyme. For the HPLC-CE system two to three injections per vial are suitable; for the HPLC-ESI/MS system two to four injections per vial may be needed, depending on the stability of the ion source

7.2 HPLC–ESI/MS

An analytical reversed phase HPLC column with cyanopropyl stationary phase, 2.1 mm in diameter, directly connected to an electrospray ionisation mass spectrometer is used. Flow rate is set to 300 µl/min and column temperature to 50 °C. Injection volume is 10 – 25 µl of the digest. A gradient elution is performed with eluent A (0.025% TFA acid in water) and eluent B (0.023% TFA in acetonitrile). A column-switching valve is positioned after the HPLC column and, to avoid contamination of the electrospray ion source, only the fraction between 0 min and 12 min elution time is allowed to enter the detection system.

The mass spectrometer is tuned and calibrated according to the instructions of the manufacturer; resolu-

tion is set to 0.7 amu peak half width. The acquisition mode is set at centroid and multiple ion detection at m/z 348.2 and 429.2 for the double protonated ions of non-glycated and glycated N-terminal hexapeptides of the haemoglobin β-chain.

7.3 HPLC-CE

7.3.1 HPLC-UV

An analytical HPLC system with a reversed phase C_{18} column is used together with a fraction collector able to collect 0.5 min fractions at 4 °C – 8 °C. The flow rate is 0.8 ml/min at a temperature of 20–25 °C; 200 µl of the digest solution is injected after centrifugation at 14000 *g* for 2 min. The gradient consists of a mobile phase A: 0.1% TFA in water and phase B: 0.1% TFA in acetonitrile. The major peak around 15–18 minutes, depending on chromatography system, is collected and freeze-dried. The freeze-dried material from the C_{18} fraction is dissolved in 50 µl 0.01% TFA/water immediately before CE analysis.

7.3.2 Capillary electrophoresis – UV

A capillary electrophoresis system containing an efficient cooling system and a fused capillary 77 cm × 75 µm ID is used. The absorbance is monitored at 214 nm, the temperature is 25 °C and the separation is performed in a H_3PO_4/NaH_2PO_4 buffer 0.1 mmol/l, pH 2.50 at 18 kV during 43 min.

8. Calibration and Calculation

The peaks for the hexapeptides β 1–6 and glc-β 1–6 are carefully integrated. The calculation algorithms are identical for HPLC-CE and HPLC-ESI/MS. From the peak area raw data the ratio glc-β 1–6 to β 1–6 is calculated. The ratio data of repetitive injections (two to four) are averaged.

The following notation is used:

x:= $[HbA_{1c}]$ = concentration of HbA1c in the sample
y:= $[HbA_0]$ = concentration of HbA0 in the sample
Hb_{tot} := total concentration $[HbA_{1c}] + [HbA_0]$
z_{conc} := percent HbA_{1c} in the sample

$$z_{conc} = \frac{100 \cdot x}{x + y} = \frac{100 \cdot x}{Hb_{tot}},\ Hb_{tot} = x + y.$$

S_x := signal obtained for $[HbA_{1c}]$ = *area (glc ß1–6)*
S_y := signal obtained for $[HbA_0]$ = *area (ß1–6)*
a := response coefficient of HbA_{1c} of the measurement devices
b := response coefficient of HbA_0 of the measurement devices
r_{sig} := ratio of signals

$$r_{sig} = \frac{Sx}{Sy} = \frac{area(glc\beta 1-6)}{area(\beta 1-6)}$$

$$S_x = \alpha \cdot x,\ S_y = \beta \cdot y$$

For calibration of the method we simply plot the ratio of the signals $r_{sig} = S_x/S_y$ *versus* the ratio of the concentrations $r_{conc} = x/y$ of the calibrators prepared by mixing pure HbA_{1c} and HbA_0.

$$r_{sig} = \frac{\alpha}{\beta} \cdot r_{conc}$$

Since the calibration function is always linear, irrespective of the response coefficients, a linear regression r_{sig} on r_{conc} is performed. From this calibration function r_{conc} data for unknown patient samples are obtained. To obtain the wanted quantity z_{conc} (% HbA_{1c}) an additional calculation is needed.

$$z_{conc} = \frac{x}{Hb_{tot}} = \frac{100 \cdot r_{sig}}{\frac{\alpha}{\beta} + r_{sig}} = \frac{100 \cdot r_{conc}}{1 + r_{conc}}$$

9. Analytical Variability

The transferability, robustness and the analytical performance of the reference method have been evaluated several times by a network of reference laboratories in Europe, the USA and Japan (publication in preparation). All reference laboratories were able to implement the candidate reference method according to the standard operation procedure (SOP) based on an earlier publication (18). Through the collaboration of the reference laboratories the SOP has been optimised and finally fixed. Excellent calibration curves were achieved for both approaches. In order to check the intra-laboratory and the inter-laboratory analytical performance intercomparison studies were organised. The results of the 4th Comparison Study, in which 11 reference laboratories participated, are listed in Table 1. The intra-laboraotry CV varied from 0.47 to 2.07% and the inter-laboratory CV from 1.35 to 2.27%. The criteria for acceptance were intra-laboratory CV<3% and the deviation from the mean <2%.

Table 1 Mean value, inter-laboratory CV and mean intra-laboratory CV in the 4th Comparison Study of the Network of HbA_{1c} Reference Laboratories (1999). Participants: 11 reference laboratories from Europe, USA and Japan.

Sample	Mean value (% HbA_{1c})	Inter-laboratory CV (%)	Mean intra-laboratory CV (%)
1	3.94	1.71	1.23
2	4.62	2.27	0.65
3	5.26	1.46	2.07
4	6.41	1.87	0.58
5	7.24	1.70	1.54
6	7.83	1.45	0.66
7	8.57	2.01	0.47
8	9.08	1.35	1.34
9	9.96	1.80	0.90
10	11.08	2.00	0.82
Mean CV		1.76	1.05

10. The Uncertainty of Measurements

The uncertainty of target values assigned with the reference methods to secondary reference materials, calibrators or trueness control materials depends on the uncertainty of the target value of the primary calibrator used for the calibration of the reference methods, and the uncertainty which results from the value assignment procedure. The uncertainty of the primary calibrator is the combined standard uncertainty (u_{cal}) of the uncertainties which are related to the purity of the HbA_0 and HbA_{1c} preparations, the uncertainties of the target values for the haemoglobin concentration of these two materials, and the uncertainty resulting from mixing these materials in order to get calibrators with a defined concentration of HbA_{1c}. The combined standard uncertainty u_{cal} for the target values of the calibrators calculated according to the GUM (21) is 0.63%. In the comparison studies of the Network of HbA_{1c} Reference Laboratories the average inter-laboratory CVs were about 2.0% (each sample was measured in duplicate on two occasions by each reference laboratory). If four reference laboratories participate in a value assignment exercise the uncertainty of the value assignment procedure u_{VA} is 1.0% (for 10 laboratories u_{VA} = 0.63%), and a combined standard uncertainty u_{total} (including the uncertainty of the primary calibrator) of about 1.2% can be attained for the target value assigned to a secondary reference material, calibrator or control material.

11. Interference

We have investigated the possible interference of N-terminal haemoglobin adducts, genetic variants and some of the added chemicals.

11.1 Carbamylated and acetylated haemoglobin

Carbamylation and acetylation can also modify the N-terminal valine that is the major glycation site. Clinically, carbamylation occurs in normal individuals but to a much higher degree in patients with reduced kidney function and elevated serum urea levels. These compounds interfere with many ion exchange chromatography methods (14, 22). A normal sample was carbamylated *in vitro* by potassium cyanate (1 mmol/l, for 60 min at 37 °C). HbA_{1c} increased from 5.2 to 5.9 measured by Mono S ion exchange chromatography but the HPLC-CE procedure gave identical results.

Some patients on high dose of aspirin show small amount of acetylated haemoglobin. Normal HbA_{1c} samples were therefore incubated with acetylsalicylic acid, 5 mmol/l, for 30 min at 37 °C. The HbA_{1c} increased from 5.2 to 5.5%. The HPLC-CE results were the same before and after acetylation. Thus no interference was shown.

11.2 Haemoglobin variants

More than 750 different haemoglobin variants exist. The most common world-wide are Hb S and Hb C with the amino acid substitutions of glutamic acid to valine and glutamic acid to lysine, respectively at position 6 from the N-terminal end. Hb S and C were purified by standard chromatography on a DEAE Sepharose A50 column and treated as normal haemoglobin in both procedures. No signal at the position of the N-terminal hexapeptide was obtained in the HPLC-MS and in the HPLC-CE method.

Corresponding procedure for purified Hb_{A2} (2.0–2.8% of total Hb) gave a normal signal for glycated and non-glycated hexapeptide since the first part of N-terminal amino acid sequence is identical to Hb_0. The experiment with the Hb S and C also demonstrate the specificity of the proteolytic enzyme used in the reference method.

11.3 Interference of reagents

Possible interference from potassium cyanide and sodium azide was evaluated by running sets with and without these chemicals. Potassium cyanide is originally added to avoid methaemoglobin formation during the purification and sodium azide as a preservative during storage of calibrators (0.02%,w/v). There was no interference of potassium cyanide but sodium azide gave an unexpected interference. Sodium azide has therefore been eliminated from the mixed calibrators.

12. Reference Ranges

A preliminary reference range study has been carried out by utilising EDTA-washed red cells collected from a Danish population study (DiaRisk, Steno Diabetes Centre, Copenhagen, Denmark). one hundred and twenty non-diabetic subjects (aged 35–55 years) were selected according to the current WHO-ADA criteria (fasting plasma glucose <7.0 mmol/l and 2 h glucose during a standard OGTT <11.1 mmol/l). The preliminary reference range for HbA_{1c} as measured in this study using the reference methods was 3.33 $\pm$ 0.48% (mean $\pm$ 2 SD). There was no influence of sex. As expected, the reference range for the new reference method is lower and narrower than the ranges for many routine methods, which vary considerably. The preliminary 97.5 percentile is 3.8% compared to 5.0% or 6.2% for many routine methods.

Appendix A: Description of Pertinent Factors for Optimal Conditions for the Measurement

1. Introduction

In order to obtain reliable results, the following factors had to be optimised:

- the cleavage of haemoglobin into peptides by a proteolytic enzyme generating representative N-terminal peptides of the β-chain (glycated and non-glycated), so that the ratio of the glycated and non-glycated peptides is representative for the HbA_{1c} content of the haemoglobin sample and can reliably be quantified.

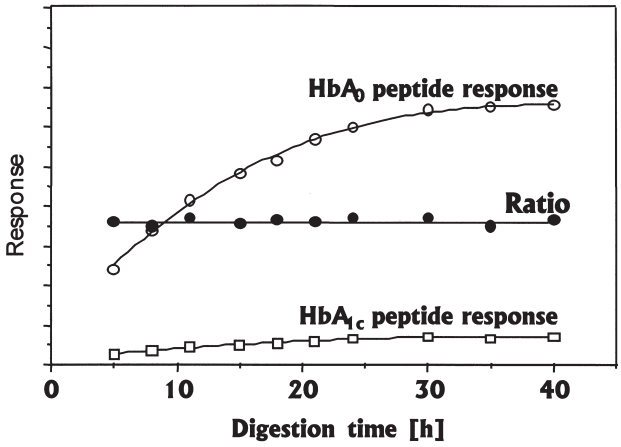

Figure 3 Time dependency of hexapeptide release during digestion of HbA_{1c} and HbA_0 by endoproteinase Glu-C.

HPLC-ESI/MS approach

- the optimal separation of the β-chain N-terminal peptides by an appropriate HPLC system,
- the detection and quantification of the β-chain N-terminal peptides by electrospray mass spectrometry.

HPLC-CE approach

- the optimal separation of the two β-chain N-terminal peptides from other peptides and the quantitative co-elution of this peptide fraction by an appropriate HPLC system (HPLC-CE method),
- the separation of the two β-chain N-terminal peptides in an appropriate capillary electrophoresis (CE) system and the quantitative detection of the peptides in the chosen system.

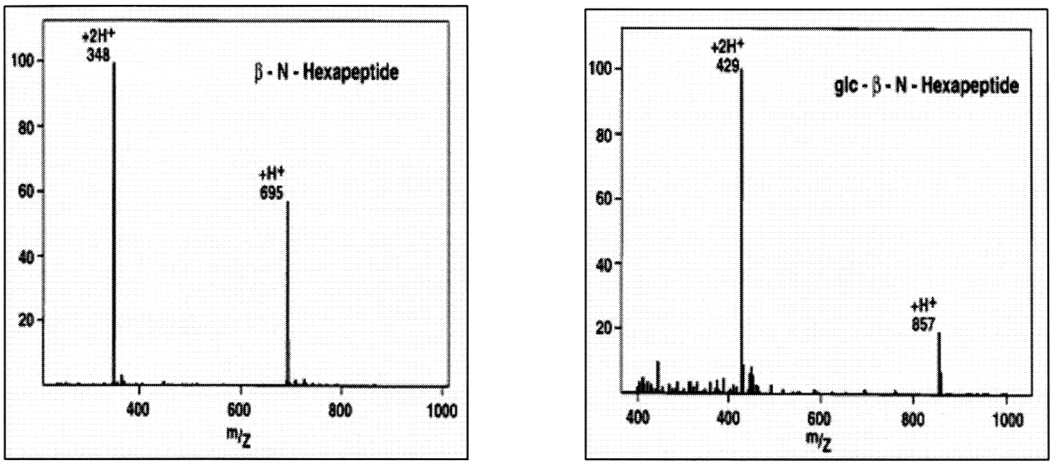

Figure 4 Electrospray mass spectra of synthetic N-terminal peptides of the β-chain of haemoglobin.

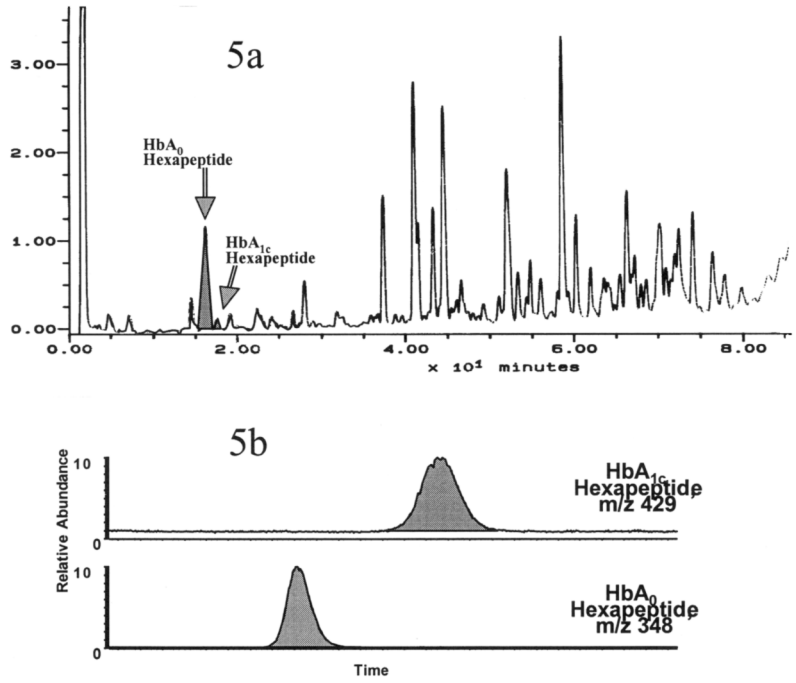

Figure 5 (a) Non-specific scan mode detection of all peptides. (b) Highly specific detection of the N-terminal hexapeptides of HbA_{1c} and HbA_0.

2. Enzymatic cleavage of haemoglobin

The enzyme endoproteinase Glu-C cleaves the haemoglobin molecule so that the N-terminal hexapeptides $C_4H_9O_4$-CO-CH_2-NH-Val-His-Leu-Thr-Pro-Glu-COOH from HbA_{1c} and NH_2-Val-His-Leu-Thr-Pro-Glu-COOH from HbA_0 are released from the two β-chains. We selected the Glu-C enzyme instead of the more common trypsin, which cleaves at lysine and arginine residues. The first lysine residue is in position 8 and an octapeptide could easily be obtained after trypsin digestion but this lysine residue is a potential glycation site which could interfere in samples with a higher extent of haemoglobin glycation.

The digestion conditions were optimised to get a high reproducibility and equal digestion kinetics for both HbA_0 and HbA_{1c} β- chains. To study the digestion kinetics a patient sample was digested over a period of 4 to 40 hours. Aliquots were taken every 4 hours. The reaction was stopped by freezing. The ratio of released glycated to non-glycated hexapeptides was constant over a period of 40 hours (Figure 3) under the chosen digestion conditions. For the final methods we used 18 hours digestion at 37 °C.

3. HPLC separation and on-line ESI-MS detection

For the HPLC-ESI/MS system the HPLC separation has been optimised to get good resolution between glycated and non-glycated β-N-terminal hexapeptides and a good separation from all other peptide fragments. Mass spectra of synthetic glycated and non-glycated β-N-terminal hexapeptide standards are shown in Figure 4. As it is typical for ESI-MS spectra of small peptides, single and doubly protonated ions are produced by the ionisation process and no fragmentation is observed. For quantitative measurements the doubly protonated ions were chosen, because of their better response. Resolution of the mass analyser was set to 0.7 amu peak half width. The very high specificity of the mass spectrometric detection is shown in Figure 5.

A chromatogram recorded in a scan mode (Figure

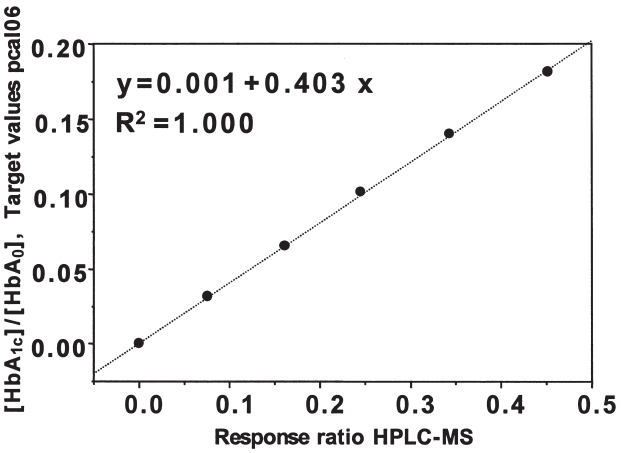

Figure 6 Calibration function for HPLC-ESI/MS.

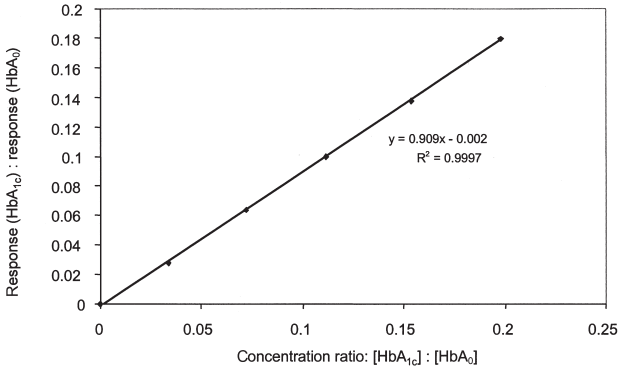

Figure 7 Calibration function for the HPLC-CE approach.

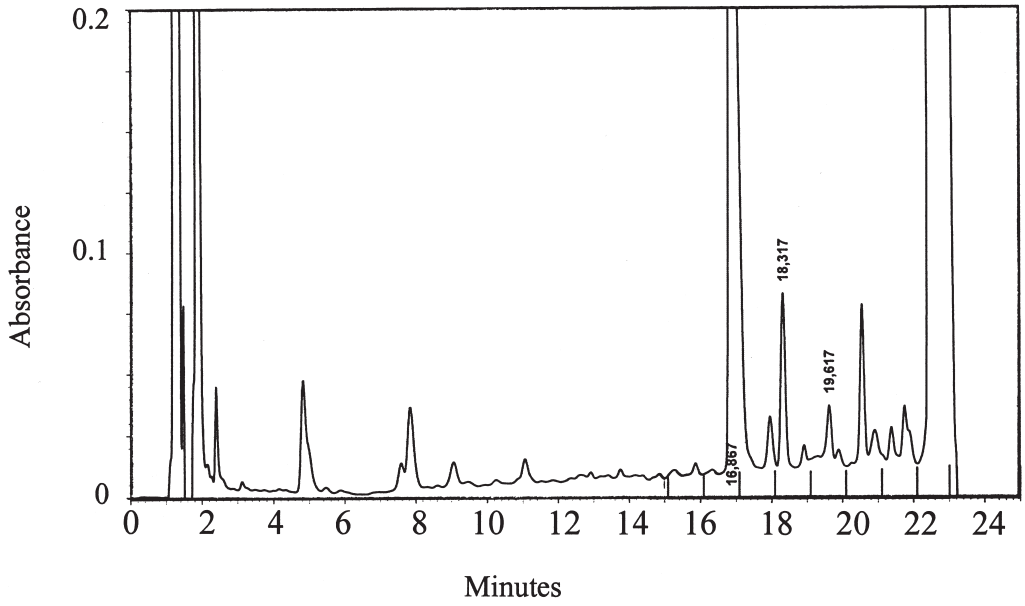

Figure 8 HPLC-C_{18} chromatogram of a mixture of peptides from proteolytic digestion of haemoglobin chains. Fractions 16–18 min are collected for capillary electrophoresis.

5a), which is similar to a photometric detection at 215 nm, is compared to the multiple ion detection mode for the doubly protonated ions at m/z 348.2 and 429.2 (Figure 5b), which represent the hexapeptides released from HbA_{1c} and HbA_0. This comparison clearly shows the superior specificity of the MS detection. The sensitivity of the analytical system at multiple ion detection modes is sufficient to get superior signal-to-noise ratios. By simple least-squares regression a linear calibration function was generated (Figure 6). Linearity has been proven by measuring a set of six primary calibrators covering the range from 0 to 15% HbA_{1c}.

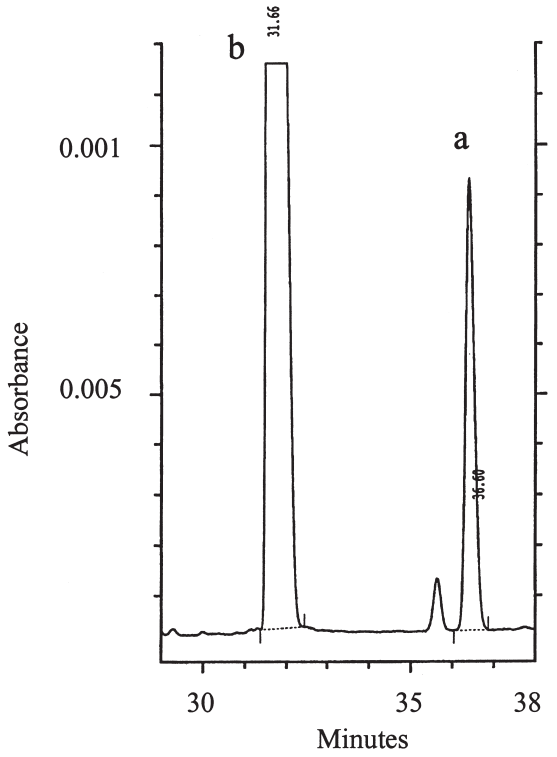

Figure 9 Electropherogram of the collected C_{18} fraction. Peak (a) represents the glycated hexapeptide and (b) the non-glycated hexapeptide.

4. HPLC separation and capillary electrophoresis

We have also employed a second independent two-dimensional approach to quantify the ratio of glycated to non-glycated β-N-terminal hexapeptides. This was done by reverse-phase HPLC combined with off line capillary electrophoresis and photometric detection. The electrophoresis system has been optimised to obtain sufficient separation and to avoid interfering contamination. The cleaning between runs has been carefully worked out to yield reproducible results over longer periods.

Glycated and non-glycated β-N-terminal hexapeptides co-eluted together on the C_{18} column used. This step was used for an enrichment of these peptides. The response ratio at 214 nm absorbance for capillary electrophoresis is linear over the whole range of calibrators (Figure 7) but different compared to the MS detection. The C_{18} peak is collected carefully to harvest all glycated peptide in the tail of the non-glycated fraction (Figure 8). This has to be confirmed in each individual HPLC setting. In a second step, capillary electrophoresis was introduced which separated the C_{18} fraction into two major separated peaks and some minor impurities (Figure 9). The system is calibrated in the same way as in the ESI-MS approach, using the same calibrators. The HPLC-CE approach generates identical results when the mean values from reference laboratories which use the HPLC-CE method are compared with those of reference laboratories which use the HPLC-MS method (Figure 10).

Appendix B: Reagent and Equipment Specifications

1. Introduction

Mixtures of pure HbA_{1c} and pure HbA_0 are needed for the calibration of the reference method. HbA_{1c} and

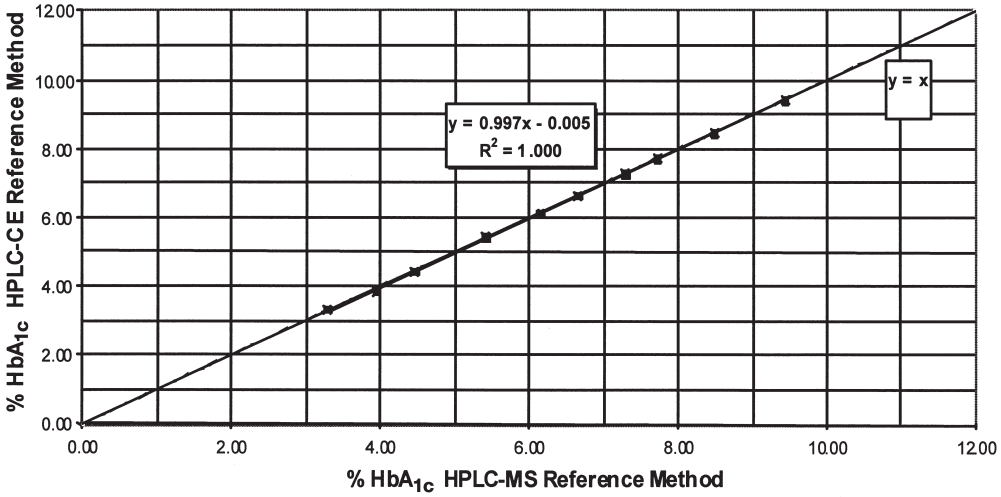

Figure 10 The measurement of HbA_{1c}: a comparison of four HPLC-MS and six HPLC-CE methods reference laboratories 1999.

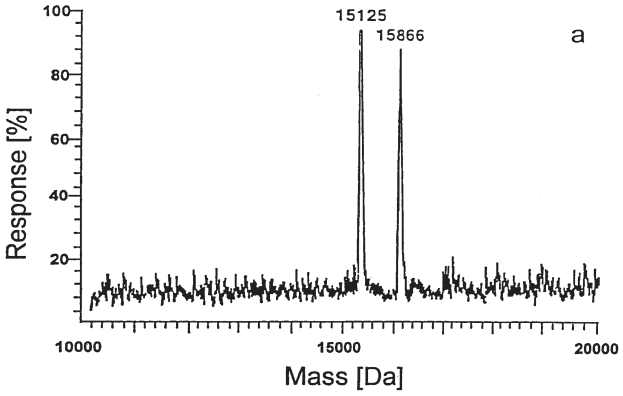

Figure 11 ESI/MS of purified HbA_0. 15125 Da non-glycated α-chain, 15866 Da non-glycated β-chain.

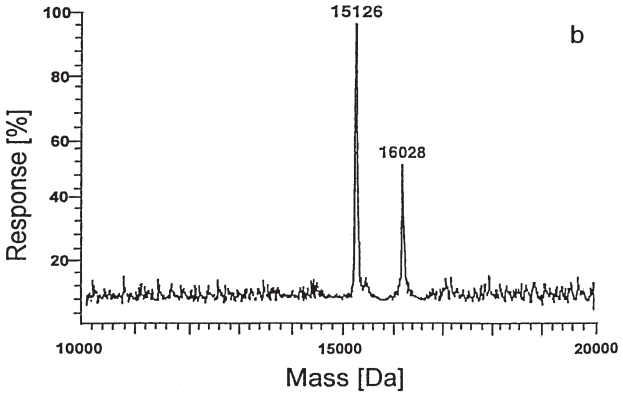

Figure 12 ESI/MS of purified HbA_{1c}. 15126 Da non-glycated α-chain, 16028 Da mono-glycated β-chain.

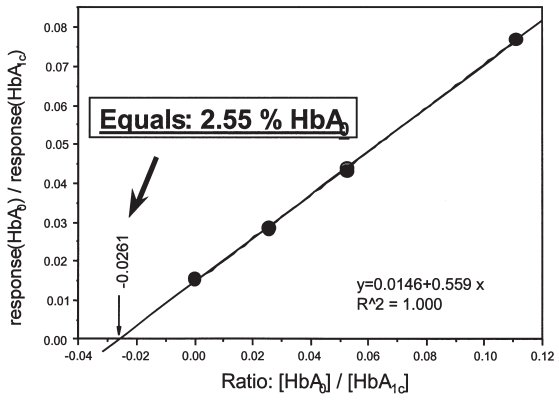

Figure 13 Determination of the amount of HbA_{1c} preparation by standard addition procedure.

HbA_0 were isolated, purified to homogeneity, and characterised (19). The techniques used for the characterisation were cation exchange and affinity chromatography for the purification as well as HPLC, capillary isoelectric focusing, electrospray ionization mass spectrometry (Figures 11 and 12) and peptide mapping. With these methods haemoglobin from healthy, non-diabetic volunteers was obtained with a purity determined by ion exchange chromatography (Mono S) of > 99.5% HbA_0 and > 98.5% HbA_{1c}.

However, results from peptide mapping indicate that the HbA_{1c} preparations still contain about 5% non-β-N-terminally glycated haemoglobin co-eluting with HbA_{1c} on Mono S. The exact amount of this fraction was determined by a procedure consisting of standard addition, enzymatic cleavage and quantification of the resulting β-N-terminal peptides (Figure 13). The total haemoglobin concentration in the HbA_{1c} and HbA_0 preparations was determined by the ICSH haemoglobin reference method. Mixtures of both components could be successfully used to calibrate the reference methods.

2. Sample preparation

Fresh blood is collected in EDTA-containing tubes (Becton Dickinson, Rutherford, NJ); 1.5 ml blood is centrifuged (10 min at 3000 *g* at +8 °C), the plasma is removed and the sedimented cells are washed twice with a 10 ml saline solution. The cells are incubated in 10 ml saline solution at +37 °C for 4 hours in order to eliminate pre-HbA_{1c}. The supernatant is discarded and hemolysate is prepared by mixing the cells with 1.0 ml water. The concentration of total haemoglobin is determined. The haemolysates are diluted to 50 mg/ml total haemoglobin by mixing with equal amounts of storage solution MES 50 mmol/l, EDTA 1 mmol/l, and potassium cyanide 10 mmol/l. The pH is adjusted to 6.2 by adding sodium hydroxide, 4 mol/l. Cell debris is removed by centrifugation for 20 min at 3000 *g*. Total haemoglobin concentration is determined in an aliquot. The haemolysates are stable at –70 °C for at least 3 years.

3. Equipment for the HPLC-ESI/MS

A combined HPLC-ESI-MS system suitable for microbore columns is used. Essential is the possibility to run a slow gradient of water-TFA/acetonitrile-TFA on equipment with a switching valve between HPLC-column and the detector. A temperature-controlled autosampler and autoinjector are mandatory. The electrospray mass spectrometric equipment coupled to the microbore HPLC system (HPLC-ESI/MS) must give stable and sensitive signals with good reproducibility, in order to allow quantitative data evaluation. The HPLC column is of cyanopropyl stationary phase.

An example of the appropriate equipment is as follows: The HPLC system consisting of an HP 1090 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) with a DR 5 solvent delivery system, a thermostat-equipped autosampler and an autoinjector. A column-switching valve Rheodyne No. 7010P (ERC, Regensburg, Germany), a T-piece 0.159 cm Swagelok No. SS-100-3 (B.E.S.T., Munich, Germany), a relay box for control of pneumatic valves (Festo, Murnau, Germany). Programmable Absorbance Detector Spectroflow 783, Kratos (GmbH Bioanalytische Instrumente, Bensheim, Germany) with 2.4 µl cell volume. Analytical HPLC column ZORBAX SB-CN, 5 µ, 2.1 mm * 150 mm, No.AS-RT-1245; P/N:883700.905 (Axel Semrau, Sprockhövel, Germany).

The mass spectrometric system is an SSQ700 single-stage quadrupole mass spectrometer with an electrospray ion source (Finnigan MAT, Bremen, Germany). The HPLC system is connected on-line with the photometric detector and the electrospray ion source by 0.12 mm ID steel capillaries. The electrospray ion source is supplied with 60 psi nitrogen sheath gas and nitrogen auxiliary gas at a HPLC flow rate of 300 µl/min. Spray voltage is 4.5 kV, transfer capillary temperature 200 °C. The mass spectrometer is tuned and calibrated with MRFA, myoglobin mixture; resolution is set to 0.7 amu peak half width and the electron multiplier set to 13 kV. Acquisition mode is set to centroid, multiple ion detection at m/z 348.2 and 429.2 for the double protonated ions of non-glycated and glycated N-terminal hexapeptides of the haemoglobin β-chain.

Gradient:

Time (min)	0	3	9	13.5	13.6	17	17.1	23
% B	0	0	5	5	100	100	0	stop run

Between-run time 14.5 to 18.0 min the ion source is switched out of the HPLC flow. Expected retention time for hexapeptides is 8–12 min.

4. Equipment for the HPLC-CE

Analytical HPLC system with a 214 nm photometric detector can be used together with a fraction collector with a collection device and a C18 Supelco TPR-100 column (Cat no. 59154, Supelco, Bellafonte, PA, USA) together with a guard column, Super guard TPR-100 (Cat no. 5-9570). As an alternative a µRPC C2/C18 ST 4.6/100 column (Cat no. 17-5057-01, Amersham Pharmacia Biotech, Uppsala, Sweden) can also be used. The dead volume in the system between UV-detector and collector must be taken into consideration. The third dominating peak with retention time at approximately 17 min using the Pharmacia Biotech column (Figure 8) is collected and freeze-dried and later dissolved in 50 µl 0.01% TFA/water immediately before capillary electrophoresis.

A typical gradient is as follows:

Time (min)	0	3	19	19.1	23.0	23.1	30
% B	7	7	18.5	100	100	7	7

Analytical Capillary Electrophoresis System with 214 nm photometric detector was used with fused-silica capillary, 75 µm ID, 77 cm in length (Beckman-Coulter Inc., Fullerton, USA, No: 33 84 72). The temperature was 25 °C and samples were injected during 10 s using low hydrodynamic flow. The separation was optimised at 18 kV during 43 min. The instruments operate for 24 hours. The rinsing of the capillary between runs is important to obtain reproducible results over longer periods. The following 1 min scheme per solvent is recommended: HCl, 100 mmol/l; water; NaOH, 100 mmol/l; H_3PO_4/H_2NaPO_4 buffer, 1.0 mol/l, pH 2.5.

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