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Preparation of a Candidate Primary Reference Material for the International Standardisation of HbA1c Determinations

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We prepared a candidate primary reference material for the forthcoming international standardisation of **β**-N-terminal glycated hemoglobin A measurements. It consists of well-defined mixtures of purified **β**-N-terminal glycated hemoglobin A and non-glycated hemoglobin A.

First, **β**-N-terminal glycated hemoglobin A and non-glycated hemoglobin A were isolated, purified to homogeneity, and characterised. The techniques used were cation exchange and affinity chromatography for the purification, and high performance liquid chromatography, capillary isoelectric focusing, electrospray ionisation mass spectrometry, and peptide mapping for the characterisation. Hemoglobins from blood of healthy, non-diabetic volunteers were obtained with a purity of >99.5% for non-glycated hemoglobin A and of >98.5% for **β**-N-terminal glycated hemoglobin A. However, results from peptide mapping indicate that the **β**-N-terminal glycated hemoglobin A preparations still contain some non-**β**-N-terminal glycated hemoglobins, co-eluting with **β**-N-terminal glycated hemoglobin A. The exact content of **β**-N-terminal glycated hemoglobin A in these preparations could be determined by a procedure consisting of standard addition, enzymatic cleavage and quantification of the resulting **β**-N-terminal peptides to be in the range from 95 – 97.5%.

Since the **β**-N-terminal glycated hemoglobin A and non-glycated hemoglobin A content could be exactly determined in the materials prepared, mixtures of both components could be successfully used to calibrate the candidate reference methods.

Key words: β-N-terminal glycated hemoglobin A (HbA1c); Non-glycated hemoglobin A (HbA0); Hemoglobin; Glycohemoglobin; Reference material; Peptides; Electrospray ionisation mass spectrometry; Reference method; International standardisation.

Introduction

The measurement of β-N-terminal glycated hemoglobin A (HbA1c) in blood is the parameter of choice for the long-term control of the glycemic state of diabetic patients. A wide variety of different analytical methods is currently used by clinical laboratories. These are based on physical, chemical or immunological principles. The methods are standardised either internally or against arbitrarily chosen HPLC comparison methods since there is no internationally accepted reference system available. As a result, HbA1c values differ considerably with different methods. (1-5). On the other hand, the studies have clearly shown that a sufficient degree of comparability between different methods can be achieved by uniform calibration. Therefore, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has established a working group on HbA1c standardisation to develop a reference system for the all routine assays. The system consists of a primary reference material, a reference method, and a set of human-matrix-based secondary reference materials with target values assigned by the reference method (6-10).

The IFCC working group decided to base the reference system on HbA1c, as HbA1c is the major component of glycohemoglobins in human blood and is biochemically well characterised as a stable adduct of glucose to one or both N-terminal amino groups of the β-chains of hemoglobin A (10-12).

There was an earlier attempt to establish a reference material on the basis of a lyophilised human hemolysate. However, it was impossible to determine the HbA1c content in this material and the program failed (13). To overcome the problems associated with using hemolysate, defined mixtures of the pure components of a primary reference material, HbA1c and HbA0, can be used.

As a glucose modification at an N-terminal α-amino group of the hemoglobin (Hb) molecule shifts significantly the isoelectric point, cation exchange matrices such as Bio-Rex 70 (14,15), PolyCAT A (16) or MonoS (17-19) are widely used for preparative or analytical separation of HbA1c from HbA0 and other minor hemoglobins.

Aminophenylboronate matrices can be used for affinity chromatography to separate HbA1c from co-eluting non-glycated species, such as carbamylated or acetylated hemoglobin (20-22), and to separate the non-glycated HbA0 from the co-eluting species glycated at ε-amino groups of lysine residues (21). The aminophenylboronate ligand binds to cis-diols on glycohemoglobins forming a five-membered ring complex. This complex can be dissociated by using sorbitol or by lowering the pH (23-25).

For the analysis of hemoglobins, cation exchange chromatography and isoelectric focusing are the clas-

sical tools. To obtain more structural information about certain hemoglobins, methods such as capillary isoelectric focusing (26), mass spectrometry (27) and peptide mapping (9,10,28,29) have been established.

The purpose of this investigation was to purify and characterise HbA1c and HbA0 and to use these proteins for the preparation of a candidate primary reference material suitable for the calibration of the IFCC candidate reference methods (9,10).

Materials and Methods

Materials

Endoproteinase Glu-C (EC 3.4.21.19), synthetic non-glycated and glycated β-N-terminal hexapeptides of hemoglobin A, 4-morpholine-ethanesulfonic acid (MES), 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), Test-Combination Hemoglobin for the determination of the hemoglobin concentration, and the molecular weight standard for gel filtration experiments were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Control sera Lyphocheck® Diabetes Control Level 1 and 2 were obtained from BioRad (Munich, Germany).

SP Sepharose High Performance and MonoS HR 5/5 were obtained from Pharmacia (Freiburg, Germany), TSK G2000 SW from Tosoh (Stuttgart, Germany), ZORBAX SB-CN HPLC column (5 µm, 2.1 x 150 mm, No.AS-RT-1245, P/N:883700.905) from Axel Semrau (Sprockhoevel, Germany) and GLYCO-GEL™ II Boronate Affinity Gel from Pierce (Bender & Hobein, Munich, Germany).

Preparative methods

All steps were performed at 4 °C in a cold room with the exception of the chromatographic steps which were carried out at room temperature due to the cyanide content of the buffers which were used. To stabilise the hemoglobins, potassium cyanide and, in some steps, EDTA were used during the whole process. To isolate HbA0 and HbA1c, approximately one week was needed for each protein.

Preparation of hemolysates

Human blood was obtained from healthy, non-diabetic volunteers using EDTA as anticoagulant. Each sample was screened for HIV and hepatitis. To isolate hemoglobins, erythrocytes were sedimented by centrifugation, washed three times with equal volumes of 150 mmol/l sodium chloride and finally hemolysed with 2 volumes of 20 mmol/l EDTA pH 7.0. Cell debris was removed by centrifugation. The hemoglobins were stabilised in 10 mmol/l potassium cyanide.

Preparative chromatography

Preparative chromatography was performed at room temperature under a hood on an liquid chromatography system equipped with an Econo Pump (BioRad), a Single Path Monitor UV-1 (Pharmacia) and a 2210 2-Channel Recorder (Pharmacia). Absorbance was monitored at 405 nm. Fractions were collected with a 2211 SuperFrac fraction collector (Pharmacia) and analysed on MonoS. Fractions of suitable purity were pooled.

Cation exchange chromatography / liquid chromatography

To improve the performance of the following affinity chromatography step, a preliminary cation exchange chromatography was used for a crude separation of HbA1c and HbA0. Hemolysate was dialysed against 50 mmol/l MES pH 6.2, containing 10 mmol/l potassium cyanide. A column (5 x 46 cm) was packed with SP Sepharose High Performance and equilibrated with the same buffer. After application of the hemolysate to the column (approximately 10 mg Hb/ml column volume at a concentration of 40 mg/ml), different hemoglobin subtypes were eluted with a linear salt gradient (0–200 mmol/l lithium chloride) within 10 column volumes of buffer at a linear flow rate of 60 cm/h. Fractions of approximately 40 ml were collected.

For the final purification of HbA1c and HbA0 we used a similar system but with smaller columns (1.5 x 24 cm for HbA1c and 3 x 52 cm for HbA0). Approximately 7 mg hemoglobin were applied per ml column volume at a concentration of 15 – 20 mg/ml to obtain optimal separation. HbA1c was eluted with a linear salt gradient (0–120 mmol/l lithium chloride) within 15 column volumes at a linear flow rate of 50 cm/h and, HbA0 with a linear salt gradient (0–300 mmol/l lithium chloride) within 10 column volumes at a linear flow rate of 70 cm/h.

Affinity chromatography / liquid chromatography

To separate glycohemoglobins from non-glycohemoglobins, Glyco-Gel™ II Boronate Affinity Gel, an aminophenylboronate agarose, was used. The fractions from the first cation exchange chromatography step were dialysed against 100 mmol/l HEPES pH 8.0, containing 50 mmol/l magnesium chloride and 10 mmol/l potassium cyanide. After equilibration of the gel with the same buffer, samples were applied to the column (3 x 78 cm); 1 mg crude HbA1c (25 mg/ml) or 5.5 mg crude HbA0 (90 mg/ml) per ml column volume were applied. The non-glycated hemoglobins were washed from the column with the HEPES buffer. Glycohemoglobins were eluted in a first step with the above-described MES buffer and in a second step with MES buffer containing 100 mmol/l sorbitol. Affinity chromatography was run at a linear flow rate of 80 cm/h.

Analytical methods

Determination of hemoglobin concentration

The determination of the hemoglobin concentration was performed according to the International Committee for Standardisation in Haematology (ICSH) cyanide reference method (30).

Analytical chromatography

Analyses were performed at room temperature on an LKB/Pharmacia HPLC system equipped with a 2141 Variable Wavelength Monitor, a 2249 Gradient Pump, a 2210 2-Channel Recorder, and a Perkin Elmer Nelson Series 900 Interface (Cupertino, CA, USA) for data collection. Absorbance was monitored at 405 nm.

Cation exchange chromatography / HPLC

For routine analysis of hemoglobins on cation exchange resin, MonoS 5/5 HR (5 x 50 mm) was used according to Jeppsson *et al.* (17). To increase the stability of the buffer, the malonic acid concentration was increased to 20 mmol/l. As a control for the identification of HbA0 and HbA1c, Lyphocheck® Diabetes Control was used prior to every set of MonoS runs.

Gel filtration / HPLC

Gel filtration experiments were performed at a flow rate of 1 ml/min using a TSK G2000 SW column (7.5 x 600 mm) with different buffers as indicated in the text. A molecular weight standard was used to calibrate the system before running hemoglobin samples. Both standard and sample were dialysed against the appropriate buffer prior to injection of approximately 50 µl at a protein concentration of 1 mg/ml.

Reversed-phase chromatography / HPLC

The HPLC system consisted of a HP 1090 liquid chromatograph (Hewlett-Packard, Waldbronn, Germany) with a DR 5 solvent-delivery system, a thermostat-equipped autosampler and an autoinjector. Analyses were run on a Zorbax SB-CN HPLC column (5 µm, 2.1 x 150 mm).

Flow rate was set to 300 µl/min and the column temperature to 50 °C. Fifty µl of the enzymatic digest of the respective hemoglobin was injected. A gradient elution (0 min, 0 % B / 30 min, 15 % B / 31 min, 100 % B / 34 min, 100 % B / 35 min, 0 % B/ 39 min, stop run) was performed with 0.025 % trifluoroacetic acid in water (eluent A) and 0.023 % trifluoroacetic acid in acetonitrile (eluent B). Absorbance was monitored at 214 nm or eluting peptides were directly characterised by electrospray ionisation mass spectrometry (ESI-MS) coupled on-line to the HPLC system (see below).

Capillary isoelectric focusing

Capillary isoelectric focusing was performed on a P/ACE instrument (Beckman Inc., Fullerton, CA, USA). A neutral-coated capillary (Beckman P/N 477445) was used according to the manufacturer's instructions. The gradient was pH 3.5 – 10. Anodal mobilisation was used and absorbance monitored at 415 nm.

Electrospray ionisation mass spectrometry

The mass spectrometric system was a SSQ700 single stage quadrupole mass spectrometer with an electrospray ion source (Finnigan MAT, Bremen, Germany). Spray voltage was set to 4.5 kV, nitrogen sheath gas to 60 psi, and transfer capillary temperature to 200 °C. It was tuned and calibrated with a mixture of an MRFA-tetrapeptide and myoglobin. Resolution was set to 0.7 amu peak half width.

For the determination of molecular weights, a solution of the sample (5 to 20 pmol/µl) in 0.5 % acetic acid in methanol/water (1:1) was infused at a flow rate of 5 µl/min. Data acquisition was set to "profile mode", scan range was 700 to 2000 m/z and up to 32 scans were averaged. De-convolution of the raw data was carried out with ICIS™ software from Finnigan MAT.

Enzymatic cleavage

An aliquot of 1 mg hemoglobin was transferred to a 1 ml crimp cap glass vial, mixed with 20 µl of a solution of 50 µg Glu-C in 250 µl water, and diluted with 25 mmol/l ammonium acetate buffer, pH 4.0, to a total volume of 500 µl. Vials were carefully closed with crimp caps and incubated by gentle shaking at 37 °C for 18 hours. Digestion was stopped by freezing the sample at –20 °C.

Under these conditions β-N-terminal hexapeptides of the β-chain of human hemoglobin, that is $C_4H_9O_4$-CO-CH_2-NH-Val-His-Leu-Thr-Pro-Glu-COOH for HbA1c and NH_2-Val-His-Leu-Thr-Pro-Glu-COOH for HbA0, as well as other peptides are obtained.

Quantification of glycated and non-glycated β-N-terminal hexapeptides

For the quantification of glycated and non-glycated β-N-terminal hexapeptides, released by enzymatic cleavage of HbA1c and HbA0, respectively, the reversed-phase HPLC system was connected on-line to the above-described ESI-MS instrument by 0.12 mm inner diameter steel capillaries. To avoid contamination of the electrospray ion source, only the fraction with a retention time between 5 and 20 min, containing the β-N-terminal hexapeptides, was allowed to enter the detection system using a column switching valve.

Data acquisition mode was set to "centroid" and "multiple ion detection" to m/z 348.2 and 429.2 for the double protonated ions of the non-glycated and glycated N-terminal hexapeptides of the hemoglobin β-chain.

To characterise the purity of a HbA1c preparation more precisely, the amount of hemoglobins not glycated at their β-N-termini was determined by standard addition. The total hemoglobin concentration of the HbA1c and HbA0 preparation was determined using the reference method (30). HbA1c was subsequently spiked with HbA0 to obtain samples containing 0, 2.5, 5.0 and 10.0 % HbA0. Assuming the HbA1c to be pure, the calculated concentration ratios [HbA0]/[HbA1c] for those samples were 0.00000, 0.02564, 0.05263 and 0.11111, respectively. The hemoglobins were then digested by endoprotease Glu-C and the resulting β-N-terminal hexapeptides were quantified by HPLC – ESI-MS. The response (peak area) ratios of non-glycated to glycated hexapeptides correspond to the true concentration ratios of HbA0 plus other hemoglobins not modified at their β-N-termini to HbA1c. These ratios were plotted against the calculated concentration ratios of the spiked samples and a linear regression analysis was performed. By extrapolation to a response ratio equal to zero, a concentration ratio for the unspiked HbA1c was obtained. The amount of impurities in this HbA1c sample could be calculated according to the formula

$$\% \text{ impurity} = \text{ratio} * 100 / (1 + \text{ratio})$$

Preparation of calibrators

For the calibration of the candidate reference methods developed by the IFCC working group on HbA1c standardisation (9,10), a set of six calibrators covering the range from 0 to 15 % HbA1c was prepared. To do this, weighed amounts of HbA1c and HbA0 were mixed. Target values for HbA1c could be assigned to these calibrators according to the total hemoglobin concentration, determined in an accredited laboratory, and the degree of purity of both components. The calibrators were stored at –20 °C.

Results

Purification

As the candidate reference methods for the standardisation of HbA1c determinations are based on the quantification of glycated and non-glycated β-N-terminal hexapeptides after enzymatic digest of the hemoglobins (6,9,10), it was a prerequisite not to use synthetic peptides as calibrators but to isolate the respective hemoglobins in a form in which they are indistinguishable from native hemoglobins.

In order to be able to use the isolated HbA1c for the preparation of a candidate primary reference material, firstly, it had to be determined whether only one or both β-N-termini of HbA1c are glycated (8).

Possible changes of the quaternary structure of the tetrameric hemoglobin A molecule ($\alpha_2\beta_2$) (31) during the purification process were therefore analysed by gel filtration on TSK G2000 SW. The chromatograms given in Figure 1 (data for pH 8.0 and 5.7 not shown) indicate that at pH ≥ 6.8 hemoglobins elute from a gel filtration column as tetramers, and at pH ≤ 6.2 as dimers ($\alpha\beta$).

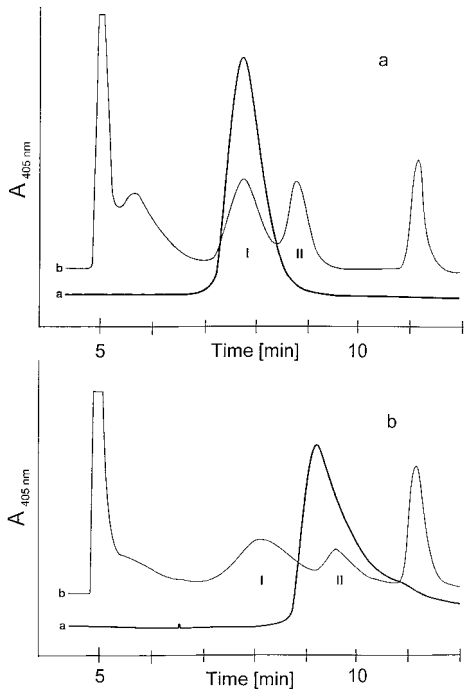

Fig. 1 Gel filtration on TSK G2000 SW. Elution pattern of hemoglobin (a) and a molecular weight standard (b) with Fab (I – 50 KDa) and myoglobin (II – 17 KDa). Fig. 1a: pH 6.8; Fig. 1b: pH 6.2.

These results implicate that using a combination of cation exchange chromatography and boronate affinity chromatography where glycohemoglobins are retarded on the gel at pH > 8.0, only hemoglobins with both β-N-termini glycated or non-glycated should be obtained.

The purification steps employed to obtain pure HbA1c and pure HbA0 from human blood are shown schematically in Figure 2.

With the first chromatographic step using SP Sepharose High Performance (Fig. 3), crude HbA1c could be separated from HbA1a and HbA1b. Crude HbA0 was isolated in the same run. Total recovery from this first step was about 90 % with approximately 5.4 % crude HbA1c and 82 % HbA0. Recovery from this first step was about 90 %. According to the data from separation on MonoS, the purity of these fractions was approximately 90 % and 98 % respectively (data not shown).

Affinity chromatography on a boronate matrix was used to eliminate co-eluting, non-glycated hemoglobins from the HbA1c preparation, and glycated hemoglobins from HbA0.

In initial experiments with crude HbA1c the conditions for the affinity chromatography were evaluated. No difference was observed whether the well established ammonium acetate buffer (23-25) or HEPES buffer (32) was used. For stability reasons the HEPES buffer system was chosen for further experiments.

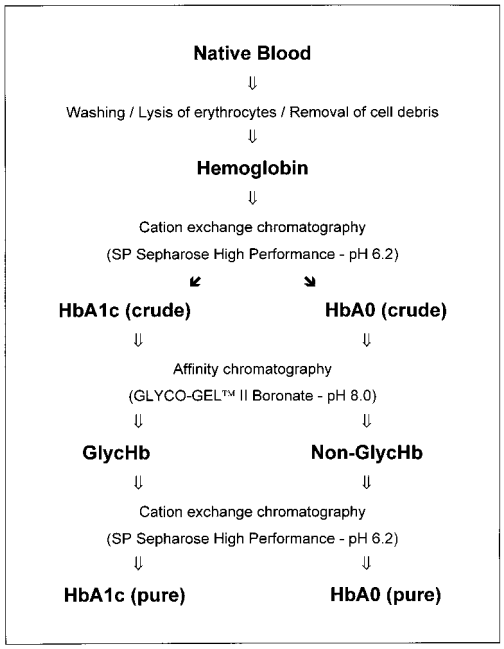

Fig. 2 Purification scheme for the isolation of HbA1c and HbA0.

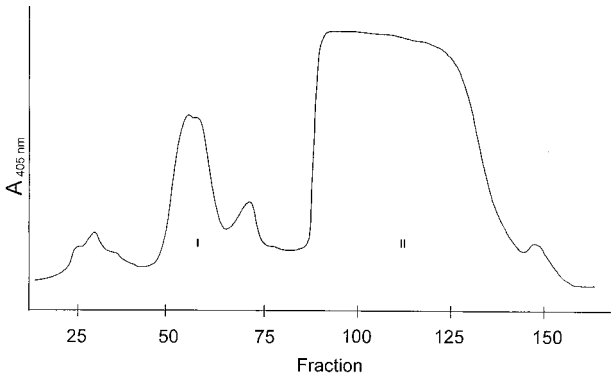

Fig. 3 Preparative cation exchange chromatography on SP Sepharose High Performance. Elution pattern of a human erythrocyte hemolysate. (I – HbA1c, II – HbA0). Sensitivity of the detector was lowered before HbA0 was eluted from the column.

There was also no difference in recovery whether 0.5 or 1.5 mg crude HbA1c were applied per ml column volume. In all cases recovery was between 90 and 95 %. About 40 % of the applied material passed the column without any retardation and approximately 50 % was eluted either by lowering the pH of the eluent or by addition of sorbitol. Similar results have been reported previously (32,33).

According to these initial experiments, crude HbA1c and HbA0 were further purified on a preparative scale.

Approximately 1 mg crude HbA1c was applied per ml column volume at a hemoglobin concentration of about 25 mg/ml. On the preparative scale, almost exactly the same results as described above were obtained. For the final step, only the material eluted by lowering the pH was used.

During the purification of crude HbA0, about 90 % of

the applied hemoglobin was found in the void volume, and only 3.2 % was eluted by lowering the pH and addition of sorbitol to the elution buffer.

Affinity-purified HbA1c and HbA0 were applied to a second SP Sepharose High Performance column to obtain material suitable to prepare calibrators for a candidate reference method (10). HbA1c was obtained with a yield of 71.4 % , and HbA0 with a yield of 71.9 % in this step.

Characterisation of HbA1c and HbA0

For further characterisation of HbA1c and HbA0, and comparison to native hemoglobins, cation exchange chromatography (HPLC / MonoS), capillary isoelectric focusing, ESI-MS and peptide mapping were used. A summary of the analytical results for HbA1c and HbA0 preparations is given in Table 1.

Tab. 1 Characterisation of HbA1c and HbA0.

	HbA1c	HbA0
Capillary isoelectric focusing	Native	Native
HPLC/MonoS	> 98.5 %	> 99.5 %
Electrospray ionisation mass sprectrometry	15 126 Da α-chain	15 125 Da α-chain
	(15 290 Da glycated α-chain)	
	16 028 Da glycated β-chain	15 866 Da β-chain
Peptide mapping		
Glycated β-N-terminus	> 95 %	< 0.1 %
Non-glycated β-N-terminus	< 5 %	> 99.9 %

Cation exchange chromatography on MonoS

Hemoglobins obtained during the purification process and the hemolysate were analysed on MonoS. The same technique was used to determine the purity of the isolated HbA1c and HbA0. As can be seen from Figure 4, HbA1c was obtained with a purity of more than 98.5 %, and HbA0 with a purity of more than 99.5 %. The HbA0 preparation contained less than 0.5 % of a substance that elutes at a position similar to HbA1d and less than 0.1 % HbA1c. HbA1c preparations contained approximately 0.5 – 1 % HbA0.

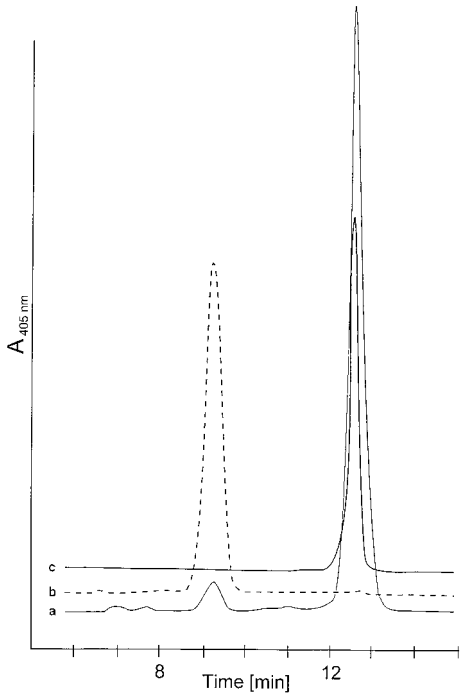

Fig. 4 Analytical cation exchange chromatography on MonoS HR 5/5. Elution pattern of a human erythrocyte hemolysate (a) and preparations of HbA1c (b) and HbA0 (c).

Capillary isoelectric focusing

Capillary isoelectric focusing (Fig. 5) of both purified HbA1c and HbA0 showed patterns very similar to native hemolysate and agreed with the results from MonoS chromatography. Beside the fully reduced hemoglobins (Fe^{2+}), intermediate forms (Fe^{2+}/Fe^{3+}) and a small amount of fully oxidised hemoglobin (Fe^{3+}) in the case of HbA1c are detectable in the same magnitude as in fresh hemolysate. Thus the addition of potassium cyanide to all buffer systems is important to avoid the appearance of oxidised and denatured forms of hemoglobin.

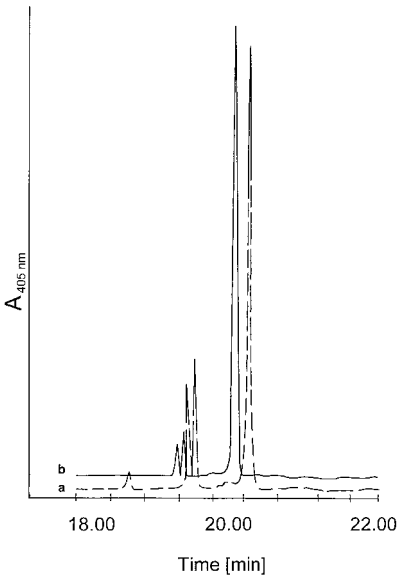

Fig. 5 Capillary isoelectric focusing of preparations of HbA1c (a) and HbA0 (b).

Electrospray ionisation mass spectrometry

Electrospray ionisation mass spectra of purified HbA1c and HbA0 are given in Figure 6. The spectrum of HbA0 contains two distinct polypeptides with molecular masses of 15125, and 15866 daltons (Da) corresponding to the non-glycated α- and β-chain respectively. From the amino acid sequence, the theoretical molecular masses for the non-glycated polypeptide chains are 15126 and 15867 Da.

For HbA1c a similar spectrum was obtained: 15126 Da for the non-glycated α-chain but 16028 Da for the mono-glycated β-chain. In this spectrum, a minor peak (5-10 %) containing a polypeptide with a molecular mass of 15288 Da, which corresponds to mono-glycated α-chain, could be detected. The obtained data agree well with literature values (27) and demonstrate the integrity of the purified material.

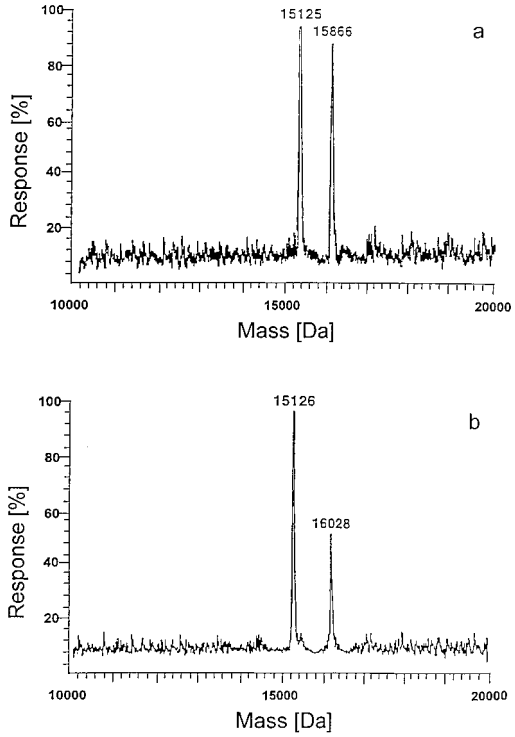

Fig. 6 Electrospray ionisation mass spectrometry of HbA0 (a) and HbA1c (b).
Fig. 6a: 15125 Da – non-glycated α-chain / 15866 Da – non-glycated β-chain;
Fig. 6b: 15126 Da – non-glycated α-chain / 16028 Da – mono-glycated β-chain.

Quantification of β-N-terminal hexapeptides by peptide mapping

As the only structural difference between HbA0 and HbA1c is in the β-N-terminus, it was useful to examine this part of the molecule more closely to obtain more information about the purity of the isolated hemoglobins. Therefore, HbA0 and HbA1c were fragmented by endoproteinase Glu-C digests. The peptide mixture containing β-N-terminal hexapeptides was then analysed by both reversed-phase chromatography with photometric detection at 214 nm and ESI-MS to obtain ratios between glycated and non-glycated β-N-termini. After optimisation of both the digest and the chromatography conditions, a differentiation between the glycated and the non-glycated β-N-terminal hexapeptide, and thus a quantification, could be achieved (9,10). As a control, the kinetics of the digestion of HbA0 and HbA1c over a period of 40 hours and the stability of synthetic glycated and non-glycated β-N-terminal hexapeptides were assessed. Stability during the process was demonstrated.

While the peptide maps of different HbA0 lots showed no impurities (data not shown), HbA1c lots reproducibly contained 2.5 – 5 % hemoglobins which are not glycated at their β-N-termini and thus had non-glycated β-N-terminal hexapeptides (Fig. 7).

To determine the purity of a HbA1c preparation more precisely, the amount of hemoglobins not glycated at their β-N-termini was determined by standard addition and quantification of the peptides by highly specific ESI-MS as described in Materials and Methods.

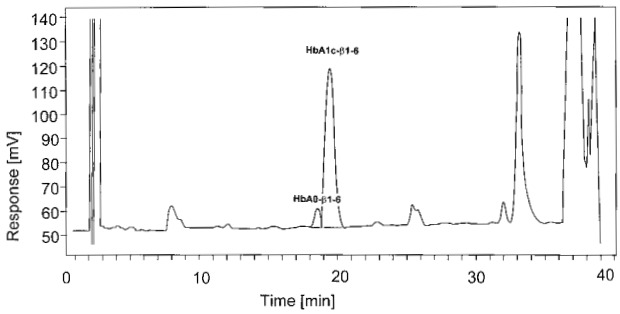

Fig. 7 Analytical reverse phase chromatography on Zorbax SB-CN. Elution pattern of β-N-terminal hexapeptides after enzymatic digestion of HbA1c.
HbA1c-β 1-6: glycated hexapeptide;
HbA0-β 1-6: non-glycated hexapeptide.

For HbA1c lot #16 the concentration ratio [HbA0]/[HbA1c] was determined to be 0.02614 (Fig. 8). This concentration ratio is equivalent to 2.55 % impurity within the HbA1c preparation.

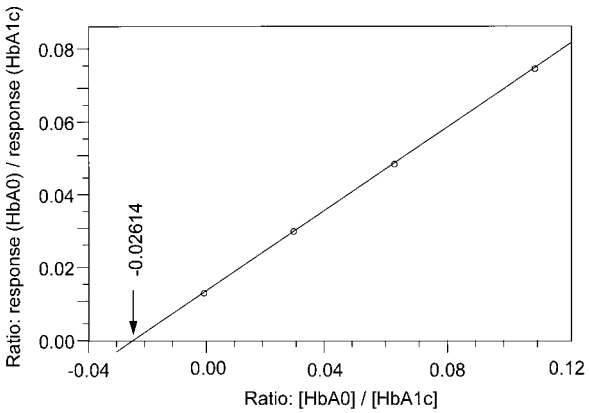

Fig. 8 Standard addition experiment with HPLC-ESI detection (Response: peak areas (mV x min)) to determine the amount of non-β-N-terminal-glycated hemoglobins in HbA1c lot#16. A ratio [HbA0]/[HbA1c] of 0.02614 corresponds to 2.55 % impurity.
y = 0.01462 + 0.55924x; R^2 = 0.9908

Primary calibrators

For the preparation of six calibrators in the range between 0 and approximately 15 % HbA1c, used during evaluation of the candidate reference methods, HbA1c lot #16 (see above) and HbA0 lot #9 were used. It could be clearly demonstrated that it is possible to calibrate both candidate reference methods with these primary calibrators (Fig. 9).

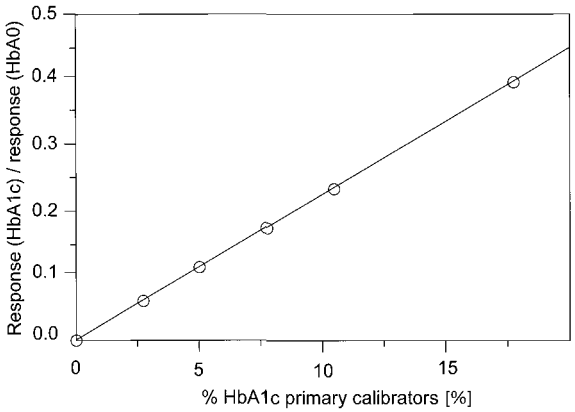

Fig. 9 Calibration curve for the candidate reference method (Response: peak area (mV x min)) for the standardisation of HbA1c assays obtained with the candidate reference material. y = –0.004 + 0.0268x; R = 0.99988; N = 6

Discussion

The quantification of HbA1c in blood has become a major tool in the management of diabetes mellitus. More than 20 different routine methods are currently used by clinical laboratories to measure HbA1c. As there is no internationally accepted reference system, values differ widely between methods and laboratories (1-5). This situation is not acceptable because the therapy of diabetes requires carefully validated method-independent clinical target values for glycohemoglobin levels in order to minimise long-term risk of microvascular complications as well as the short-term risk of life-threatening hypoglycemia (34-36). To overcome this problem, the IFCC has established a working group to develop a system for the uniform international standardisation of all HbA1c/glycohemoglobin assays. The group decided to develop an HbA1c reference system consisting of a primary reference material, a reference method which specifically measures HbA1c and secondary reference materials with HbA1c values assigned by the reference method (6). The standardisation of all routine glycohemoglobin assays on the basis of an HbA1c reference system is acceptable from a scientific as well as from a practical point of view, because most of the routine tests such as HPLC methods and immunological assays claim to measure HbA1c (11,12). Tests, which measure the sum of glycohemoglobin species in blood such as affinity chromatography and related methods, are already internally standardised against HbA1c comparison methods since HbA1c and total glycohemoglobin values correlate very well.

As it is well known that the HbA1c peak on cation exchange systems reflects a complex mixture of singly and multiply glycated hemoglobins which co-elute together with carbamylated and acetylated hemoglobin, and different amounts of other substances (depending on the system used), these methods overestimate the true value for HbA1c. Due to this lack of specificity, it was not possible to adopt one of the HPLC consensus methods used in existing national standardisation programs (NGSP) (e.g. USA (National Glycohemoglobin Standardization Program – NGSP), Japan, Sweden) as the reference method for the IFCC reference system. A specific method had to be identified. Therefore, two independent candidate reference methods, both measuring specifically the total amount of N-terminal glycated β-chains, were developed by the IFCC group and published recently (10). As the reference system has to be method-independent and highly specific, all routine assays for the determination of HbA1c can be calibrated to this method. Due to the higher specificity of the proposed reference methods the clinically relevant reference ranges will obviously be lower.

The main goal of the work presented here was to develop the candidate primary reference material for the calibration of the candidate reference methods.

The use of synthetic β-N-terminal peptides was not possible because calibrators and samples must behave in the same way during the enzymatic cleavage of hemoglobin, an essential step within the candidate reference methods. Since digestion is not complete for a native sample (although the ratio of released glycated to non-glycated hexapeptides is constant) (10), the status of peptide-based calibrators and native sample would be different.

Consequently, the primary reference material should consist of well-defined mixtures of HbA1c and HbA0. The native components had to be isolated and characterised. With the demand for a pure HbA1c, it was important to establish whether only one or both β-N-termini of hemoglobin A become glycated. *In vitro* experiments of Weykamp and Penders (37) demonstrated that, with increasing glucose concentration and time, a small amount of the double Schiff base was formed, but the stable double Amadori rearrangement product was not observed. Thus, the major glycation product is mono-glycated hemoglobin and tetramers glycated on both β-N-termini constitute only a minor fraction in human blood. The IFCC group has defined HbA1c as hemoglobin which is irreversibly glycated at one or both N-terminal valines of the β-chains. Since double glycation is a sign of higher glucose concentration in blood, double-glycated hemoglobin should also give double signals in the reference method.

To analyse the integrity of the hemoglobin molecule during the purification process we examined the quaternary structure of isolated hemoglobins. There seems to be a dissociation into dimers when lowering the pH of a hemoglobin solution from pH 6.8 to 6.2 (Fig. 1). From the mass spectrometry data it can be concluded that $\alpha\beta$ dimers are formed. This seems to be important for several reasons.

First, analytical methods for the determination of HbA1c based on cation exchange chromatography with buffer systems at pH 6.2 and below quantify dimers. Differences in the photometric quantification of hemoglobins with different cation exchange chromatography methods such as MonoS, PolyCAT A etc. might be at least partially related to the extent of dissociation. It might be speculated whether methods with combined salt and pH-gradient, for example PolyCAT A chromatography with a pH range between 6.5 and 7.0 (16,38), can resolve dimers as well as tetramers.

Second, by using preparative cation exchange chromatography, it is possible to separate N-terminal glycated from non-N-terminal glycated $\alpha\beta$ dimers, because a modification at an N-terminal α-amino group of the hemoglobin molecule shifts the isoelectric point significantly. Nevertheless, with a combination of cation exchange and affinity chromatography it is possible to obtain HbA1c that is indistinguishable from native hemoglobins during isoelectric focusing.

The most efficient purification procedure started with a cation exchanger at a pH of 6.2 to separate HbA1c and HbA0. This reduced the amount of hemoglobin to be applied to the affinity matrix. Due to inconvenience in handling the classical Bio-Rex 70 cation exchange resin, we used SP Sepharose High Performance. This matrix proved to be a very good material for the fast preparative separation of hemoglobin subclasses with good resolution.

Although the obtained crude HbA1c seemed to be quite pure on MonoS, about 40 % of the hemoglobin applied to the boronate matrix passed through the gel. Mass spectrometry data obtained with this material indicate that these hemoglobins were indeed not glycated (data not shown). These findings confirm the data of Abraham *et al.* and Garlick *et al.* (32,33). To reduce the amount of minor contaminants in the HbA1c preparation, a second cation exchange chromatography proved to be necessary.

When applying the crude HbA0 to the boronate affinity gel, approximately 3.5 % of the material was glycated as indicated by retardation on the gel. To improve the purity of the non-glycated HbA0, a second cation exchange step was employed.

Our data indicate that it is possible to isolate native HbA0 with a purity of approximately 99.5 % which is suitable for the preparation of a candidate primary reference material. However, HbA1c preparations, which were obtained with a purity of >98.5 % according to MonoS data, contain small amounts of hemoglobin species that are retarded on a boronate affinity matrix similarly to HbA1c, co-elute with HbA1c on MonoS and are not glycated on their β-N-termini. The latter could be shown by peptide mapping. According to electrospray mass spectrometry data, all β-chains are glycated (Fig. 6). These β-chains must be glycated on an ε-amino-group of a lysine residue. Small amounts of glycated α-chain are detectable in ESI-MS, so the shift in the retention time on MonoS of these hemoglobins might be due to an α-N-terminal glycation. On the other hand, Mayer and Freedman (39) describe that the α-N-terminal modified glycohemoglobin elutes from a cation exchanger on the leading edge of the HbA0 peak.

By standard addition, a procedure allowing to determine the degree of purity of a substance precisely, it could be demonstrated that our HbA1c preparations were up to 97.5 % pure. The HbA1c and HbA0 lots could be used reproducibly to prepare sets of calibrators containing HbA1c and HbA0 in proportions which cover the clinically relevant concentration range of HbA1c. These calibrators were suitable for the calibration of the candidate reference methods developed by the IFCC working group on HbA1c standardisation (10). This was confirmed in a first evaluation of the candidate reference methods by an international network of reference laboratories.

Nevertheless, the work is ongoing to improve the purity of HbA1c. Since a polyclonal antibody specific for the non-glycated β-N-terminus of human hemoglobin is available, it should be also possible to remove the remaining amounts of non-β-N-terminal glycated hemoglobins from HbA1c preparations by negative immunosorption. Initial experiments have been started to achieve this. Independently of the outcome of this additional effort, the materials currently available are suitable for preparing the primary reference material, and can be used for HbA1c reference system so that the subsequent stages in the international standardisation of HbA1c/glycohemoglobin can be carried out.

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