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Candidate reference methods for hemoglobin A_{1c} based on peptide mapping

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A reference method that specifically measures hemoglobin (Hb) A_{1c} is an essential part of the reference system for the international standardization of Hb A_{1c}/glycohemoglobin. We have developed a new method for quantification, based on the specific N-terminal residue of the hemoglobin β-chains. Enzymatic cleavage of the intact hemoglobin molecule with endoproteinase Glu-C has been optimized to obtain the β-N-terminal hexapeptides of Hb A_{1c} and Hb A_0. These peptides have been separated by reversed-phase HPLC and quantitated by electrospray ionization-mass spectrometry (method A) or by capillary electrophoresis (method B). With these peptides and hyphenated separation techniques, it has been possible to overcome the insufficient resolution of currently used protein separation systems for Hb A_{1c}.

Hemoglobin (Hb)[4] A_{1c} is the major glycohemoglobin species in human blood *[1, 2]* and has been used for ~20 years for long-term assessment of glycemic control in diabetic patients. The comprehensive Diabetes Control and Complications Trial (DCCT) has provided ample evidence that microvascular complications such as retinopathy, nephropathy, and neuropathy are directly related to the degree of hyperglycemia in patients with insulin-dependent diabetes and that measurement of Hb A_{1c} in blood is an excellent tool for long-term monitoring of the glycemic state of diabetic patients *[3–6]*. The DCCT study has also clearly demonstrated the need for reliable and reproducible measurements of Hb A_{1c} because only a relatively small difference exists between the average Hb A_{1c} concentrations in patients at low and high risk of developing the dreaded late complications.

Many different routine methods (>20) claiming to measure Hb A_{1c} are currently used by the clinical laboratories. The methods either exploit the charge differences existing between Hb A_{1c} and Hb A_0 (ion-exchange chromatography, electrophoresis, isoelectric focusing) or are immunological methods that use poly- or monoclonal antibodies directed towards the glycated N-terminal group of the β-chain of Hb. At present, no internationally accepted reference system, to which the routine assays could be adjusted *[7]*, exists nor do any internationally approved primary or secondary reference materials or a reference method. As a result, values differ in a clinically unacceptable magnitude between methods and laboratories as has been demonstrated in several trials *[8–12]*. To overcome this problem, national standardization activities have been initiated in some countries to achieve standardization on a national scale, but these activities are independent of each other and tend to use different approaches.

To achieve a uniform international standardization, the IFCC has established a working group on Hb A_{1c} standardization that coordinates activities worldwide. The working group is developing a reference system that will be the basis for the international standardization *[13]*. The first step was a clear definition of the analyte Hb A_{1c} on the basis of its molecular structure. Historically Hb A_{1c} was defined as a certain peak in an HPLC system, but this is no longer scientifically acceptable. To overcome this problem, one suggested definition for Hb A_{1c} was Hb that is glycated at the N-terminal of the β-chain [β-(*N*-deoxyfructosyl)hemoglobin] because this is the major compound in the HPLC peaks and the major form of all glycohemoglobins; however, the issue of potential double glycation at both β-chains or an additional glycation at any lysine residue was not discussed explicitly *[2]*. To make this clear, the IFCC group has now defined Hb A_{1c} as Hb that is irreversibly glycated at one or both N-terminal valines of the β-chains. This also covers Hb that

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[4] Nonstandard abbreviations: Hb, hemoglobin; DCCT, Diabetes Control and Complications Trial; ESI-MS, electrospray ionization-mass spectrometry; CE, capillary electrophoresis.
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is additionally glycated at any lysine residue in the β-chain. Hb that is solely glycated at a lysine residue is not considered to be Hb A_{1c}.

The second step of the standardization process is the development of a reference method that can measure Hb A_{1c} specifically according to this molecular definition. At this time, such a method is not yet available. HPLC methods, which have often been used as reference methods for standardization of routine tests, are not really reference methods in the scientific sense. They provide precision and long-term stability but lack specificity. Different values for Hb A_{1c} in the same blood are obtained depending on the conditions used, because the Hb A_{1c} peaks contain different kinds and amounts of substances that are not Hb A_{1c} *[14–19]*. The differences between certain methods can be rather large. Between the Bio-Rex 70 HPLC method used as a designated comparison method for the internal standardization of the DCCT study *[2]* and the Mono S ion-exchange chromatography method *[20, 21]*, differences as large as 20% were found at the reference range concentrations, despite both methods' claiming to measure Hb A_{1c} *[22]*. Although the Mono S system is more specific than the Bio-Rex 70 method, it cannot be accepted as a reference method because carbamylated Hb and dimers of glycated α- and nonglycated β-chains coelute with Hb A_{1c} in this system *[19, 23]*. Because none of the currently available methods for Hb A_{1c} determination shows sufficient specificity to be used as a reference method, we have developed one that distinguishes the smaller N-terminal parts of the β-chains of Hb A_{1c} from those of the Hb A_0 molecules, thus avoiding the heterogeneity created by modifications of other glycation sites of the Hb molecule.

The most specific Reference Methods in clinical chemistry available today are based on mass spectrometric detection (gas chromatography–mass spectrometry with isotopic dilution). Most were developed in the 1980s, e.g., for cholesterol, progesterone, testosterone, cortisol, estradiol, or thyroxine *[24]*. We have applied electrospray ionization (ESI-MS), which is a relatively new mass spectrometric technique suited for the analysis of polar biomolecules of low and high molecular mass *[25]*. The process of ESI is characterized by formation of polyprotonated or polydeprotonated ions of intact analyte molecules that are produced from a fine spray of an aqueous solution of the analyte, assisted by a strong electrical field at atmospheric pressure. The ions have low internal energy and are not prone to fragmentation. The measurement of mass-to-charge values of these multiple charged ions gives the inherent molecular mass of the analyte molecules. The ion currents produced by ESI are dependent on the analyte concentration. The ESI technique has improved dramatically within the last 5 years, and detection limits in the lower picomole to upper femtomole range are now available. ESI-MS is ideally suited for an on-line coupling with HPLC, and the combination of these techniques might become a powerful instrument for the development of new Reference Methods for a variety of analytes.

Considering the high cost of ESI-MS equipment, we concurrently investigated the possibility of developing and providing another method based on capillary electrophoresis (CE), a technique that is more generally available in clinical laboratories.

Materials and Methods

PREPARATION OF BLOOD SAMPLES

Fresh blood samples collected in EDTA-containing tubes (Becton Dickinson) were used. Mixed blood (1.5 mL) was washed twice with 10 mL of saline and centrifuged for 10 min at 3000*g*. The sedimented cells were incubated in 10 mL of saline at 37 °C for 4 h to eliminate pre-Hb A_{1c}. Hemolysate was prepared by mixing the cells with 1.5 mL of water, followed by centrifugation for 20 min at 3000*g*. Supernatants were mixed with equal parts of buffer, 50 mmol/L 2-(*N*-morpholino)propanesulfonic acid), 1 mmol/L EDTA, 10 mmol/L potassium cyanide, and 0.2 g/L sodium azide. The pH was adjusted to 6.2 with 2 mol/L hydrochloric acid. Cell debris was removed by centrifugation before storage of a clear hemolysate at −70 °C.

ENZYMATIC CLEAVAGE OF HEMOGLOBIN

Endoproteinase Glu-C sequencing grade (Boehringer Mannheim; cat. no. 1047817) was used for cleavage of Hb. An aliquot of 1 mg of Hb from the patients' samples or the primary calibrators was transferred to a 1-mL crimp cap glass vial, mixed with 20 μL of a solution of 50 μg of Glu-C in 250 μL of water and diluted with 25 mmol/L ammonium acetate buffer (pH 4.0) up to a total volume of 500 μL. Vials were carefully closed with crimp caps and incubated with gently shaking at 37 °C for 18 h. Digestion was stopped by freezing at −20 °C. For the cleavage experiments with trypsin an Hb solution consisting of 2 mg of Hb in 300 μL of distilled water was used. After 3 min of boiling, 200 μL of ammonium bicarbonate (0.5 mol/L, pH 8.2) was added. The solution was incubated for 2 h at 37 °C with 20 μL of *N*-tosyl-L-phenylalanyl chloromethyl ketone-treated trypsin (Sigma, 5 mg/mL in 1 mmol/L HCl and 12 mmol/L $CaCl_2$). The digestion was stopped by adding 2 drops of 6 mol/L HCl.

CALIBRATORS

Human blood from healthy, nondiabetic volunteers was used to isolate Hb A_0 and Hb A_{1c} for the preparation of primary calibrators. Erythrocytes were sedimented by centrifugation, washed with sodium chloride, hemolyzed with water, and stabilized in an EDTA/KCN solution to isolate hemoglobins. Purification was achieved by three chromatographic steps: cation-exchange chromatography on SP-Sepharose HP, affinity chromatography on GLYCO-GEL® II Boronate Affinity Gel, and cation-exchange chromatography on SP-Sepharose HP again. Purified Hb A_0 and Hb A_{1c} were stored stabilized by KCN in

an EDTA/2-(*N*-morpholino)propanesulfonic acid buffer system. Purified Hb A_{1c} and Hb A_0 were analytically characterized by Mono S cation-exchange chromatography, capillary isoelectric focusing, ESI-MS, and quantification of β-N-terminal peptides by enzymatic cleavage and reversed-phase HPLC. Total Hb was determined by the Hb cyanide method *[26]*. Details of the preparation and characterization are described elsewhere *[23]*. Calibrators were prepared by mixing Hb A_{1c} and Hb A_0 primary materials. Mixing of calibrators was done with calibrated pipettes, on the basis of weight according to the total Hb concentration and the degree of Hb A_0 impurity in the Hb A_{1c} preparation. For calibration of the analytical systems described below, a set of 6 calibrators covering the range of 0–15% Hb A_{1c} (relative to total Hb) was used.

METHOD A. HPLC SEPARATION AND ON-LINE ESI-MS DETECTION

Samples prepared as described above were analyzed in a combined HPLC-ESI-MS system. The HPLC system consisted of an HP 1090 liquid chromatograph (Hewlett-Packard) with a DR 5 solvent-delivery system, a thermostat-equipped autosampler, an autoinjector, a Rheodyne no. 7010P (ERC) column-switching valve, a 0.159-cm Swagelok no. SS-100-3 (B.E.S.T.) T-piece, a relay box for control of pneumatic valves (Festo), a Kratos Programmable Absorbance Detector Spectroflow 783 (Bioanalytische Instrumente) with 2.4-μL cell volume, and a 2.1 × 150 mm analytical HPLC column (ZORBAX SB-CN, 5 μm, no. AS-RT-1245; P/N:883700.905; Axel Semrau).

Flow rate was set to 300 μL/min, column temperature to 50 °C. Injection volume was 50 μL of digest. A gradient elution was performed with eluent A (0.25 mL/L trifluoroacetic acid in water) and eluent B (0.23 mL/L trifluoroacetic acid in acetonitrile): 0 min, 0% B; 30 min, 15% B; 31 min, 100% B; 34 min, 100% B; 35 min, 0% B; 39 min, stop run). For quantitative determinations a column switching valve was positioned after the HPLC column, and only the fraction between the 5-min and 20-min elution times was allowed to enter the detection system to avoid contamination of the electrospray ion source. The photometric detector was set to 214 nm and was used for control purposes only.

The mass spectrometric system was an SSQ700 single-stage quadrupole mass spectrometer with an electrospray ion source (Finnigan MAT). The HPLC system was connected on-line with the photometric detector and the electrospray ion source by 0.12-mm (i.d.) steel capillaries. The electrospray ion source was run with 414 kPa nitrogen sheath gas and nitrogen auxiliary gas at an HPLC flow rate of 300 μL/min. Spray voltage was 4.5 kV, transfer capillary temperature 200 °C. The mass spectrometer was tuned and calibrated with tetrapeptide MRFA, myoglobin mixture; resolution was set to 0.7 amu peak half-width and the electron multiplier to 13 kV. Acquisition mode was set to centroid, multiple ion detection at *m/z* 348.2 and 429.2 for the double-protonated ions of nonglycated and glycated N-terminal hexapeptides of the hemoglobin β-chain.

For measurements a sequence was set up for calibration bracketing: primary calibrators–samples–primary calibrators. Four injections were made for each vial. The ion chromatograms for *m/z* 348.2 and 429.2 were recorded, the peak areas integrated, and the ratios of area *m/z* 429.2 vs *m/z* 348.2 calculated. The mean values of four injections at a time per vial were calculated. The ratio of peak areas was a linear function of the percent Hb A_{1c} for a set of primary calibrators. With the data set of primary calibrators a calibration function was calculated by linear regression of the measured ratio against the default values of percent Hb A_{1c}. This calibration function was used to determine the percent Hb A_{1c} values of the measured patient samples.

METHOD B: HPLC AND CE

Samples prepared as described above were separated by reversed-phase chromatography on a C_{18} column (Pep-RPC HR 5/5; Pharmacia). HPLC separation was done as described earlier *[27]* with the following modifications: an HPLC system, including two P-500 pumps, a GP-250 gradient programmer, and a UV detector 2141 (LKB-Pharmacia); flow rate was 1 mL/min, gradient elution was performed with eluent A (1 mL/L trifluoroacetic acid in water) and eluent B (1 mL/L trifluoroacetic acid in acetonitrile): 0 min, 0% B; 40 min, 18% B; 41 min, 100% B; 43 min, 100% B; 44 min, 0% B; 52 min, stop run. Absorbance was monitored at 214 and 280 nm. The actual fraction, the first dominating peak, was identified by amino acid analysis, collected, and lyophilized. The material was dissolved in 40 μL of 0.1 mL/L trifluoroacetic acid in water immediately before electrophoresis. In a second step the mixture of glycated and nonglycated β-N-terminal hexapeptides was separated by CE performed on a Beckman P/ACE system 5000 equipped with System Gold™ version 8.1 software. Fused-silica capillaries, 50 μm (i.d) × 67 cm (no. 338472; Beckman Instruments), were used. New capillaries were conditioned by rinsing with 0.1 mol/L NaOH for 10 min and buffer for another 10 min. A typical run would be 100 mmol/L phosphate buffer, pH 2.5, 15-s pressure injection, 25-kV voltage, temperature at 20 °C, and absorbance monitored at 214 nm. Calibration was carried out as described in method A.

HPLC COMPARISON METHOD

Mono S ion-exchange chromatography, a reliable and well-established routine method, was chosen for a comparison with the newly developed methods. In this method Hb A_{1c} is separated from other hemoglobins Hb A_{1a}, Hb A_{1b}, Hb F, acetylated Hb, α-chain dimers, the glutathione adduct Hb A_3, and Hb A_0 by a salt gradient in malonate buffer at pH 5.7 on a Mono S™ HR 5/5 column (Pharmacia) as described earlier *[20, 21]*. The pre-Hb A_{1c}

is eliminated by incubation of samples in a citrate/phosphate buffer, pH 5.4, for 30 min at 37 °C *[21]*.

METHOD COMPARISON STUDIES

Two sets of patient samples were measured on different occasions with the HPLC-MS procedure, the HPLC-CE procedure, and Mono S ion-exchange chromatography. The results were statistically evaluated.

Results

ENZYMATIC CLEAVAGE OF HEMOGLOBIN

The enzyme endoproteinase Glu-C cleaves the hemoglobin molecule so that the N-terminal hexapeptides $C_4H_9O_4$-CO-CH_2-NH-Val-His-Leu-Thr-Pro-Glu-COOH from Hb A_{1c} and NH_2-Val-His-Leu-Thr-Pro-Glu-COOH from Hb A_0 were released from the β-chains. The digestion conditions were optimized to get high reproducibility and equal digestion kinetics for both Hb A_0 and Hb A_{1c} β-chains. A patient's sample was digested over 5–40 h to study the digestion kinetics, and aliquots were taken every 4 h. The reaction was stopped by freezing. Under the chosen digestion conditions the ratio of released glycated and nonglycated hexapeptides was constant over a period of 40 h (Fig. 1). For the final methods we used 18-h digestion at 37 °C.

METHOD A: HPLC SEPARATION AND ON-LINE ESI-MS DETECTION

The HPLC separation has been optimized for the HPLC-ESI-MS system to achieve good resolution between glycated and nonglycated β-N-terminal hexapeptides and good separation from all other peptide fragments. Mass spectra from synthetic glycated and nonglycated β-N-terminal hexapeptide calibrators are shown in Fig. 2. The spectra show both single- and double-protonated ions, and no fragmentation is observed. For quantitative measurements the double-protonated ions were chosen because of their better response behavior. Resolution of the mass analyzer was set to 1 Da. The very high specificity of the mass spectrometric detection is shown in Fig. 3. The chromatogram recorded in a scan mode, which is similar to a photometric detection at 215 nm, is compared with the multiple-ion detection mode for the doubly protonated ions at *m/z* 348.2 and 429.2, which represent the hexapeptides released from Hb A_{1c} and Hb A_0. This comparison clearly shows the superior specificity of the MS detection. The detection limit of the analytical system at multiple-ion detection mode is sufficient to get superior signal-to-noise ratios. By simple least-squares regression a linear calibration function was generated (Fig. 4). The range over which the response (ratio of glycated hexapeptide to nonglycated hexapeptide) is linearly proportional to the percent Hb A_{1c} was evaluated by measuring a set of six primary calibrators covering the range 0–15% Hb A_{1c}. The stability and reproducibility of the total analytical system were proven by repeated measurement of three patients' samples (~4%, 5%, and 7.5% Hb A_{1c}) within a period of 4 weeks (duplicates on 4 different days). The three samples were run together with a larger set of patients' samples. Including all calibration and test assays, >250 injections were done during this period, in

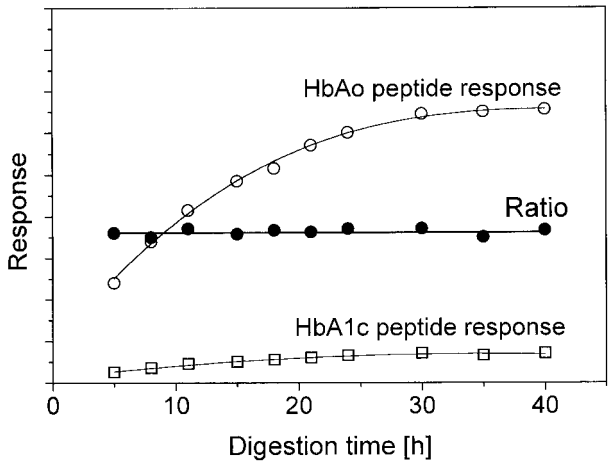

Fig. 1. Digestion of hemolysates by endoproteinase Glu-C: formation of glycated and nonglycated β-N-terminal hexapeptides of Hb and their ratios.

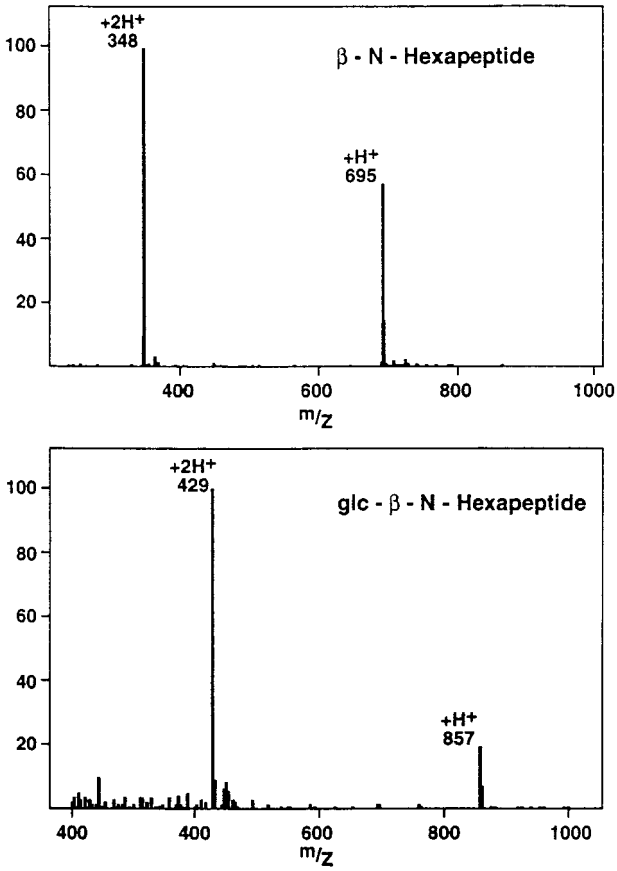

Fig. 2. Electrospray mass spectra of glycated and nonglycated β-N-terminal hexapeptides.

$+H^+$, singly protonated peptide; $+2H^+$, doubly protonated peptide.

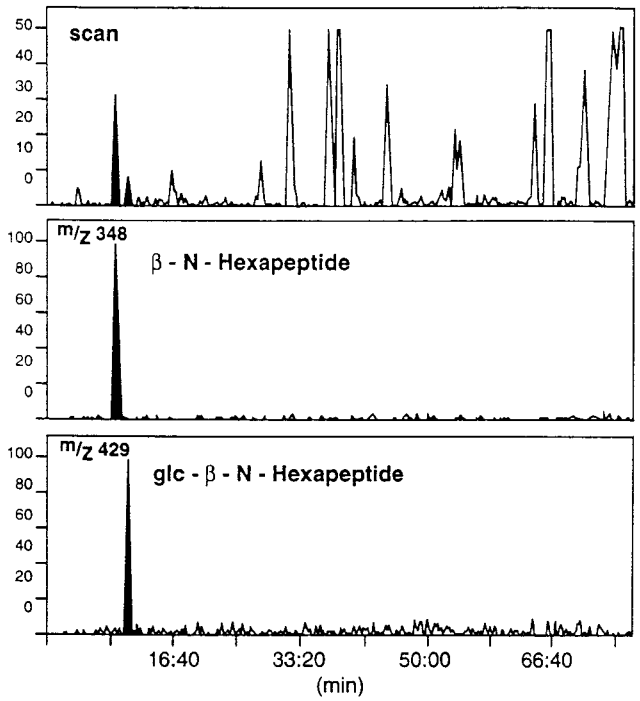

Fig. 3. Scan mode HPLC-ESI-MS and multiple ion detection chromatograms.

Scan mode and selected ion monitoring on doubly protonated ions: *m/z* 348, *β*-N-terminal hexapeptide; *m/z* 429, Glc-*β*-N-terminal hexapeptide.

addition to the routine use of the mass spectrometer for qualitative peptide and protein analysis. The overall CVs for Hb A_{1c} amounts of 4.09%, 5.37%, and 7.91% were 2.0%, 1.2%, and 2.5%, respectively. This demonstrates a very good reproducibility of the system. The overall procedure of the complete method is shown in the flow chart in Fig. 5.

METHOD B: HPLC SEPARATION AND CE

We also studied a second independent multidimensional approach to quantify the ratio of glycated to nonglycated *β*-N-terminal hexapeptides. This was done by reversed-phase HPLC combined with off-line CE and photometric detection. Glycated and nonglycated *β*-N-terminal hexapeptides coeluted together on the C_{18} column used. This step was used for an enrichment of these peptides. In a second step, CE, which separated the C_{18} fraction into two peaks, was introduced (Fig. 6). By comparing synthetic glycated and nonglycated *β*-N-terminal hexapeptides, it was obvious that the peptides showed different absorbance values at 214 nm. The system was therefore calibrated in the same way as the ESI-MS method with the same calibrators (area % used for calculations). The within-day CVs of the HPLC-CE system for Hb A_{1c} at 3.86%, 6.36%, and 12.0% were 1.92%, 1.58%, and 1.62%, respec-

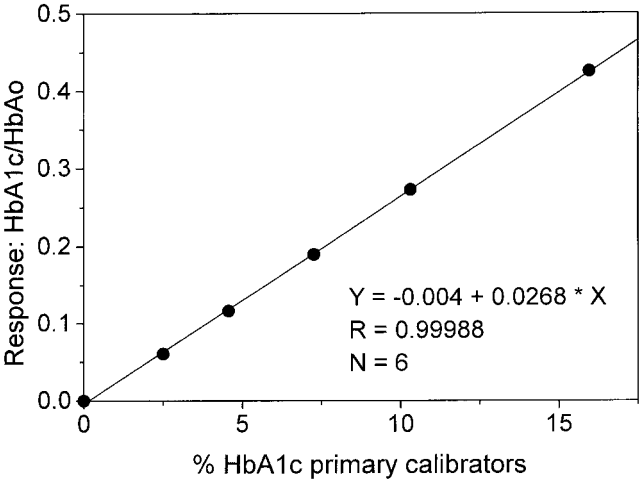

Fig. 4. Calibration curve for HPLC-ESI-MS with primary calibrators.

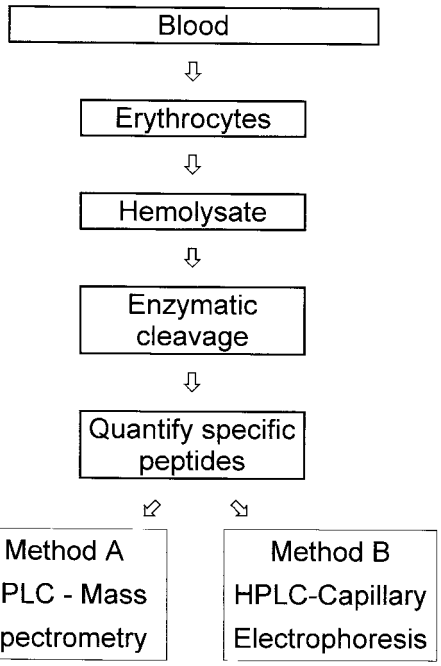

Fig. 5. Flow chart of methods A and B.

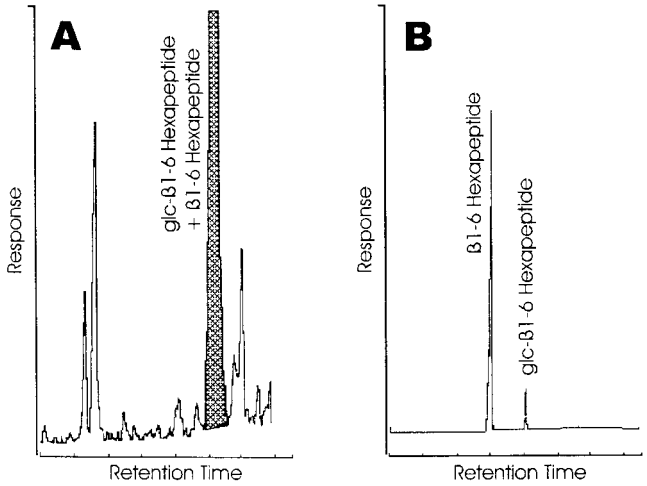

Fig. 6. HPLC and off-line capillary electrophoretic separation.

A, HPLC chromatogram for separation on C_{18} column; *B*, chromatogram for CE separation.

tively. The overall procedure of the complete method is shown in the flow chart in Fig. 5.

METHOD COMPARISON STUDIES

These studies were carried out to compare the two newly developed methods and also to compare the new methods with a reliable well-established routine HPLC method, the Mono S method. The comparison between the HPLC-CE and HPLC-MS methods (Fig. 7) shows an excellent agreement. The comparisons of the Mono S method against HPLC-MS also demonstrate good correlations (Fig. 8). Comparison of Mono S vs HPLC-CE is similar ($y = 0.836\,x + 1.35$, $r = 0.994$). The remarkable slope is expected because of the theoretical considerations that Mono S gives higher Hb A_{1c} values in the lower concentration range, because of impurities that coelute with the Hb A_{1c} peak, and gives lower values in the upper concentration range, because of the additional glycation of ϵ-amino groups of lysine that will not coelute with the original Hb A_{1c} peak, thus resulting in a reduction of the Hb A_{1c} signal. Because all ion-exchange chromatographic systems are traceable to each other *[9, 10]*, there will be traceability between currently used ion-exchange systems and the described candidate Reference Methods.

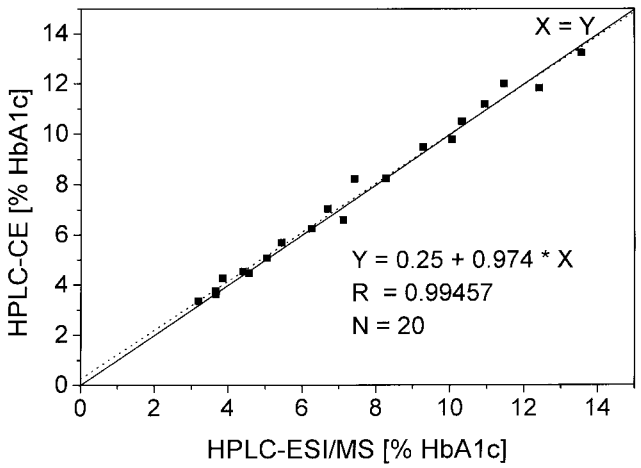

Fig. 7. HPLC-CE vs HPLC-ESI-MS.

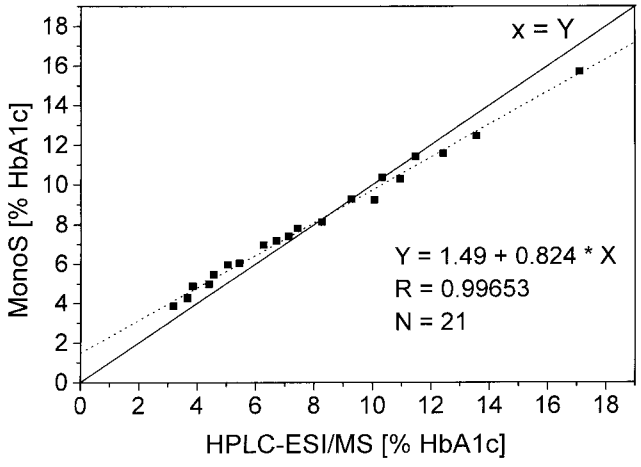

Fig. 8. Mono S vs HPLC-ESI-MS.

Discussion

Because the currently available methods for the measurement of Hb A_{1c} in human blood do not fulfill the high requirements necessary for a Reference Method, we searched for a new principle that could result in a more specific method. Many analytical problems lie in the complexity of the Hb molecule and the multitude of glycohemoglobin species and other Hb adducts that exist in human blood. Because Hb A_{1c} is defined as an Hb that is glycated at the N-terminal of the β-chain *[3]*, we carried out studies to see whether it is possible to change from the determination of the total molecule to the measurement of the glycated and nonglycated β-N-terminal peptides of Hb. Therefore we studied the possibilities for an appropriate enzymatic cleavage of the Hb molecule.

Trypsin, which cleaves peptide bonds with lysine and arginine at the C-terminal side, is a commonly used enzyme for peptide mapping in hemoglobinopathies. We were able to show that the lysine residues at position 8 in the β-chain are glycated in samples with increased Hb A_{1c} concentrations but not in those with normal concentrations. Thus the use of trypsin to release N-terminal octapeptides for quantification purposes would include the risk of getting doubly or singly glycated octapeptides at the Lys-8 position. Therefore trypsin cleavage was not usable. Endoproteinase Glu-C cleaves the N-terminal part of the β-chain between the two glutamic acid residues at positions 6 and 7. The resulting fragments contain only a single glycation site at the N-terminal valine and can thus be used to separate Hb A_{1c}. The actual cleaving site is easily exposed to the enzyme under mild denaturing conditions at pH 4.0. Complete denaturation before digestion exposes additional substrates to the enzyme and yields a more complex peptide mixture. With the modern multidimensional analytical techniques of on-line HPLC and ESI-MS or the off-line system of HPLC and CE, the two β-N-terminal hexapeptides of Hb A_{1c} and Hb A_0 could be separated and quantified with the necessary analytical performance. By analyzing the mixture of peptide fragments resulting from the endoproteinase Glu-C digestion of whole-blood samples, we obtained high specificity and a low detection limit. The approach excludes the interference of carbamylated and acetylated N-terminal species as well as other N-terminal adducts or nonglycated β-chains from the dimer of glycated α-chain–nonglycated β-chain (which coelute for example with Hb A_{1c} in the Mono S system).

We have developed two quantitative analytical methods that specifically measure the ratio of the N-terminal hexapeptides of Hb A_{1c} and Hb A_0. The calibration of both methods was done with primary calibrators that are mixtures of Hb A_{1c} and Hb A_0 primary calibrators. The CE method is a simple and robust technique but requires an off-line preparation of samples. The ESI-MS method

requires advanced equipment but permits highly specific detection like the classical Reference Methods in clinical chemistry and has the practical advantage of on-line coupling without time-consuming sample processing. In our method, MS has been used for the first time in a Reference Method for proteins or peptides. The method is a good example of how to adapt the well-known concept of Reference Methods for low-M_r analytes to high-M_r proteins. From a theoretical point of view HPLC-ESI-MS offers higher specificity than HPLC-CE. This motivates its position as the most advanced candidate for the forthcoming Hb A_{1c} Reference Method. However, with the observed analytical performance, the HPLC-CE method could also serve as a Reference Method. According to the NCCLS definition of analytical methods, the HPLC-CE system could be classified as a Reference Method while the HPLC-ESI-MS method has the potential to be a Definitive Method. Both methods will now be evaluated further in an international network of reference laboratories organized by the IFCC Working Group on Hb A_{1c} Standardization.

Clearly, however, the Hb A_{1c} values found with the new Reference Methods will be considerably lower than those found with many current routine methods, especially when they are calibrated against the DCCT method *[3]*. The designated comparison method in that study was a cation-exchange HPLC method with Bio-Rex 70 as resin. Although the method was very stable during the 9-year period of the DCCT study, it has become evident that the Hb A_{1c} result by this method is only 60% specific, due to β-*N*-deoxyfructosyl Hb *[17]*. More sophisticated assays with other resins, e.g., Mono S, yield lower Hb A_{1c} results, but still show a perfect correlation with the Bio-Rex 70 method *[19, 20]*. The new Reference Methods will solve this lack of specificity. For this reason lower values of Hb A_{1c} will be expected in nondiabetic persons as well as in diabetic patients. Therefore, reference ranges from nondiabetics and recommended values for optimal therapy in diabetic patients will have to be revised and adjusted to the new accuracy. Preliminary experiments with more specific methods, e.g., the Mono S method *[18]*, have already shown that translation of the Hb A_{1c} values found in the DCCT study into values based on these more specific methods is feasible. The translation of Hb A_{1c} values found in the DCCT and other important clinical trials into values that are based on the new reference system will be a task of the IFCC Working Group on Hb A_{1c} Standardization *[13]* because the previous clinical experience must be maintained.

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