

What is hemoglobin A_{1c}? An analysis of glycosylated hemoglobins by electrospray ionization mass spectrometry

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Hemoglobin A_{1c} (HbA_{1c}) is a stable minor Hb variant formed *in vivo* by posttranslational modification by glucose, originally identified by using cation exchange chromatography, and containing primarily glycosylated N-terminal β -chains. However, the structure(s) of the quantified species has not been elucidated, and the available methods lack a reference standard. We used electrospray ionization mass spectrometry to determine the extent of glycosylation of samples separated by boronate affinity and/or cation exchange chromatography. Analyses of clinical samples were consistent with the curvilinear relationship of patient glucose and HbA_{1c}. As glycosylation increased, the ratio of β -chain to α -chain glycosylation increased, and the number of glycosylation sites on the β -chain increased, although these were relatively minor components. We found several glycosylated species that cochromatographed with HbA_{1c} on cation exchange, including species with both glycosylated α - and β -chains, nonglycosylated α - and glycosylated β -chains, and multiply glycosylated β -chains. The combined use of affinity and cation exchange chromatography with structural confirmation by electrospray ionization mass spectrometry was found to be useful in producing samples of sufficient purity for the standardization of glycosylated hemoglobin clinical assays.

Glycosylated hemoglobin is formed *in vivo* by the nonenzymatic attachment of glucose to hemoglobin (Hb).⁵ HbA_{1c} is a stable minor hemoglobin variant separated by charge that is composed primarily but variably of glycosylated hemoglobin. A clinical relationship between HbA_{1c} and fasting plasma glucose, peak plasma glucose on the glucose tolerance test, the area under the curve of the glucose tolerance test, and mean glucose concentrations over the preceding several weeks was elucidated in the mid-1970s (1, 2).

Allen et al. (3) in 1958 suggested that chromatographic hemoglobin fractions as separated on cation exchange resin be given the designations HbA₀, HbA_{1a}, HbA_{1b}, and HbA_{1c}, pertaining to their order of elution. Their roman numerals were later replaced with Arabic numerals. Subsequently, as other minor components of hemoglobin were identified, they were given names such as HbA_{1d}, HbA₂, and HbA₃. The nomenclature for these fractions has changed somewhat over the years, generating increasing confusion as to the meaning of the fraction names, because the order of elution can vary with temperature, pH, and conditions of elution.

Glycemic control over the previous 3–4 months can be monitored by the determination of the ratio of glycosylated to nonglycosylated hemoglobin, as reflected *inter alia* by the HbA_{1c} chromatographic fraction. As early as 1984, a National Diabetes Data Group expert committee on glycosylated hemoglobin published an editorial outlining the lack of understanding of the glycosylation process and calling for standardization and characterization of the glycosylated hemoglobins that were quantified by current assays (4). Over 12 years later, the glycosylated species still has not been completely characterized, and the assay methods have not been standardized. Currently available methods give different values, thus limiting the usefulness and clinical

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⁵ Nonstandard abbreviations: Hb, hemoglobin; ESI, electrospray ionization; and MS, mass spectrometry.

credibility of the test. A recent editorial (5) highlighted the need for standardization of the analytes quantified by available assay methods worldwide, as well as the potential contributions of analytical mass spectrometry (MS) methods to the quantification of glycohemoglobins.

Early structural studies of glycohemoglobins and in particular the A_{1c} fraction as separated on cation exchange chromatography qualitatively demonstrated glycation of the N terminus of the β -chain (6–8). The techniques available for these types of structural studies have vastly improved in recent years, allowing for more sensitive and nondestructive analyses to be performed on macromolecules (9). Recent advances in MS such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization have resulted in the capability to analyze biological macromolecules including hemoglobin (9). ESI is a mild ionization technique that does not cause the fragmentation of intact protein molecules when operated under appropriate experimental conditions. In addition to total ion mass information, ESI can be used to probe noncovalent interactions (10, 11), to accomplish protein and peptide sequencing (12–14) and DNA sequencing (15), to study protein folding (16), and most importantly to probe posttranslational modifications of proteins (17–24). The versatility and accuracy of ESI-MS make it especially valuable for characterization, and the technique has been applied to the quantification (25) of glycohemoglobins.

We studied hemolysate samples from patients with low, medium, and high glycohemoglobin concentrations as quantified by a clinical assay corresponding to HbA_{1c} as studied by the Diabetes Control and Complications Trial (26). The HbA_{1c} values were 5–8%, 8–10%, and >10%, respectively. We compared the spectra obtained from the clinical samples with those obtained from a pooled sample prepared by a combination of cation exchange and affinity chromatography to further characterize the species that cochromatograph with HbA_{1c}. In addition, we performed ESI-MS on various chromatographic fractions obtained using both affinity and ion exchange chromatography.

Materials and Methods

PREPARATION OF INDIVIDUAL PATIENT SAMPLES FOR DIRECT INJECTION ONTO ESI-MS

All studies were approved by appropriate institutional committees. Hemolysate was prepared from fresh human whole blood. After centrifugation of the sample at 2000g for 10 min to remove plasma, erythrocytes were incubated in an equal volume of water for 10 min. The mixture was centrifuged at 2000g for 10 min. This process was repeated two times, followed by centrifugation at 14 000 g for 1 min.

PREPARATION OF POOLED PATIENT HEMOLYSATE SAMPLES

Hemolysate was prepared from fresh human whole blood. First the sample was centrifuged at 2000g for 10

min to remove plasma. Erythrocytes were then incubated with phosphate-buffered saline equal to the volume of removed plasma for 30 min at 37 °C, followed by centrifugation at 2000g for 10 min to remove labile glucose adducts. An equal volume of H₂O and 0.4 volume of CCl₄ was added and mixed on a rotator for 15 min to remove lipids and lipid-soluble material. To remove cell debris, the mixture was centrifuged for 15 min at 2000g. Hemolysate was removed and stored at –70 °C before use.

CHROMATOGRAPHIC PURIFICATION OF POOLED PATIENT HEMOLYSATE SAMPLES

Fresh human whole blood from persons with diabetes and HbA_{1c} values of 10% or greater as obtained on cation exchange (Bio-Rad) was collected in EDTA-containing tubes, pooled, and frozen as defined above.

The affinity protocol used a Pierce Glycogel II boronate affinity (Pierce) column with ammonium acetate (0.25 mol/L), magnesium chloride (0.05 mol/L), and phenoxyethanol (4.5 mL/L) in the equilibration buffer (pH 8.5) and sorbitol (0.2 mol/L), Tris (0.1 mol/L), and phenoxyethanol (4.5 mL/L) in the elution buffer. Hemolysate (5 g) was applied to the column (20-cm long; 4.4-cm diameter). Both the nonbound fraction and the bound fraction were eluted and collected in glass bottles and concentrated using ultrafiltration (Amicon) before storage at –70 °C. The affinity protocol was repeated on both fractions to further purify them.

The cation exchange protocol used Bio-Rex 70-400 mesh cation exchange resin sized to ~40 mm and a column of 15 cm in length and 2 cm in diameter. Buffer A consisted of sodium phosphate (monobasic; 0.033 mol/L), sodium phosphate (dibasic; 0.08 mol/L), and potassium cyanide (0.01 mol/L); adjusted to pH 6.75. Buffer B consisted of sodium phosphate (monobasic; 0.104 mol/L) and sodium phosphate (dibasic; 0.045 mol/L) adjusted to pH 6.4, with a conductivity between 7.5 and 8.2 g/L total dissolved solids. The column was eluted by using a linear gradient at a flow rate of 1.4 mL/min after an initial equilibration for 45 min using Buffer A. Approximately 500 mg of hemoglobin was applied.

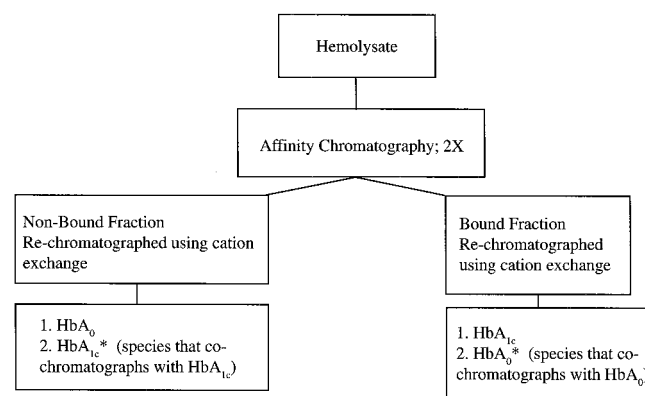


Fig. 1. Flow diagram of the chromatographic separation protocol of hemolysate samples to generate HbA₀, HbA₀*, HbA_{1c}, and HbA_{1c}*.

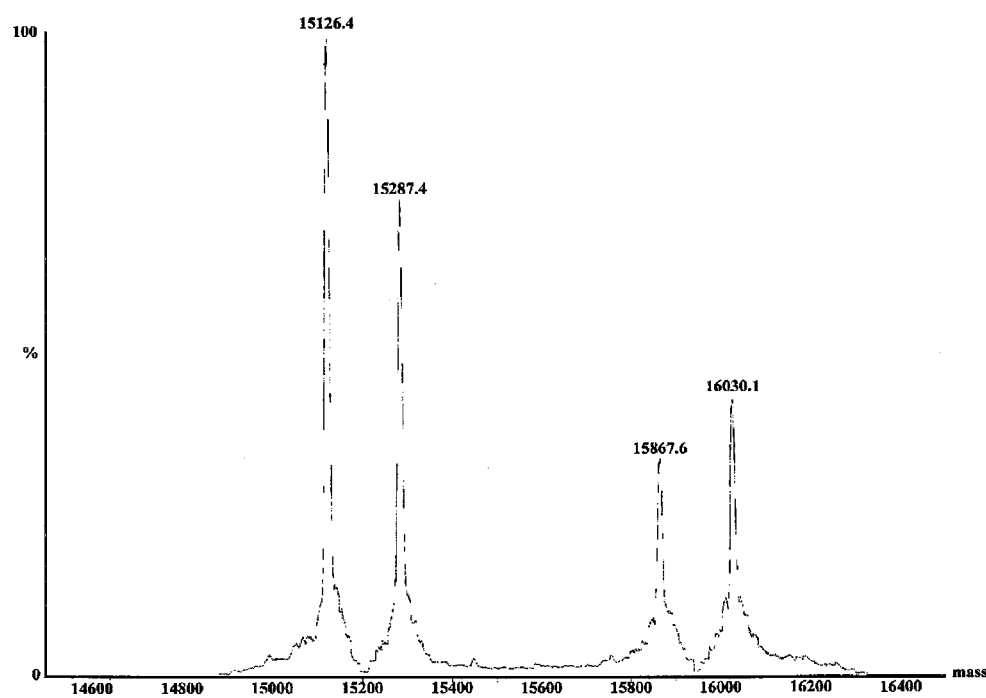
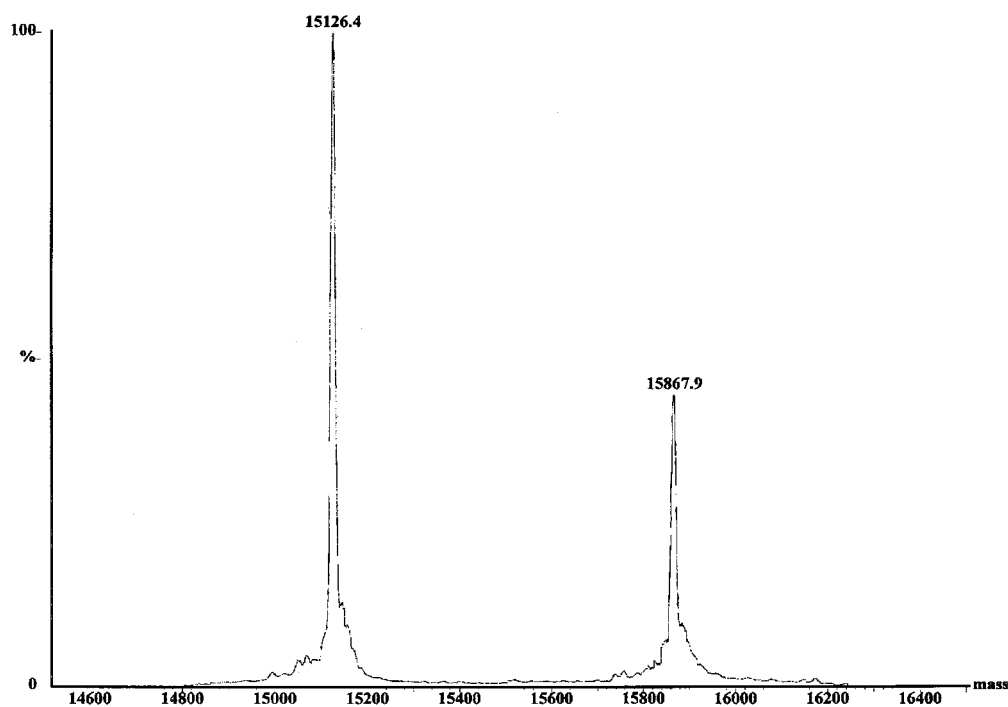


Fig. 2. Deconvoluted ESI spectra of unmodified (A_0 , upper panel) and glycated (lower panel) hemoglobin.

After affinity chromatography, the nonbound and bound effluents were collected, concentrated (10 000 M_r cutoff; Amicon), and chromatographed on Bio-Rex 70 cation exchange resin. The species not binding to affinity that cochromatographed with HbA_0 and HbA_{1c} on cation

exchange were collected and labeled HbA_0^* and HbA_{1c}^* , respectively (Fig. 1). The species that bound to affinity and cochromatographed on cation exchange with HbA_0 and HbA_{1c} were labeled HbA_0 and HbA_{1c} , respectively (Fig. 1).

MASS SPECTROMETRY

Each sample was analyzed by ESI-MS operating in the positive ion mode on a VG Fisons Platform II single quadrupole mass spectrometer with ESI source. Data were acquired over a mass range of 600-2000 m/z and analyzed with VG MassLynx operational software (including maximum entropy deconvolution algorithm) on a DEC pc XL560. The α -globin chain was used as an internal reference for mass calibrations.

Before infusion, each sample was diluted 1:1000 with deionized water. Sample was introduced to the instrument via infusion at a flow rate of 20 $\mu\text{L}/\text{min}$. Source temperature was set at 90 $^{\circ}\text{C}$, and all samples contained 1 mL/L formic acid to aid ionization.

Results

Fig. 2 documents that two passes on boronate affinity chromatography separated glycated from nonglycated hemoglobin (Fig. 1, step 1). The deconvoluted spectra in Fig. 2 correspond to the calculated masses for each globin chain, obtained on concentrated eluent that bound (lower panel) and did not bind (upper panel) to boronate affinity. Both the α - and β -globin chains were modified in the bound sample (lower panel), as indicated by the additional mass peaks at 15288.0 Da (singly glycated α -chain) and 16029.0 Da (singly glycated β -chain). No glycation is detected on the sample that did not bind to affinity.

The spectra in Fig. 3 are from three whole hemolysate

samples drawn from clinic patients with various concentrations of glycohemoglobin (see *Materials and Methods*). Fig. 4 shows the charge envelopes for the sample in the bottom panel of Fig. 3. The inset of Fig. 4 is an expansion of one region of the spectrum, showing the identities of each species in the sample.

Each sample in Fig. 3 is representative of a different range of clinical values indicative of different concentrations of glycemic control. The top panel is a "low" sample (HbA_{1c} 4.2%) representing normoglycemia. The middle panel is a "medium" range sample (HbA_{1c} 8.0%) representing marginal glucose control. The bottom panel is a "high" range sample (HbA_{1c} 9.9%) representing poor glucose control. In the top panel, a small peak indicative of glycated α -chain (15287.0 Da) is observed. The corresponding peak for glycated β -chain is found at 16020.0 Da. The α -chain with intact heme attached corresponds to the peak at 15740.0 Da. In the middle panel, both glycated α (15286.0 Da) and glycated β (16025.0 Da) are observed. In the lower panel, both glycated species are present in greater abundance.

Fig. 5 shows the deconvoluted mass spectrum of a high range clinical sample, obtained after cation exchange and subsequent affinity chromatography. This sample shows multiple glycation of the β -chain (16029.5 Da, 16191.0 Da, and 16351.5 Da) as well as single glycation of the α -chain (15287.5 Da). The peak at 16642.0 Da corresponds to glycated β -chain plus intact heme. No nonglycated β -chains are observed. Although this sample elutes as one

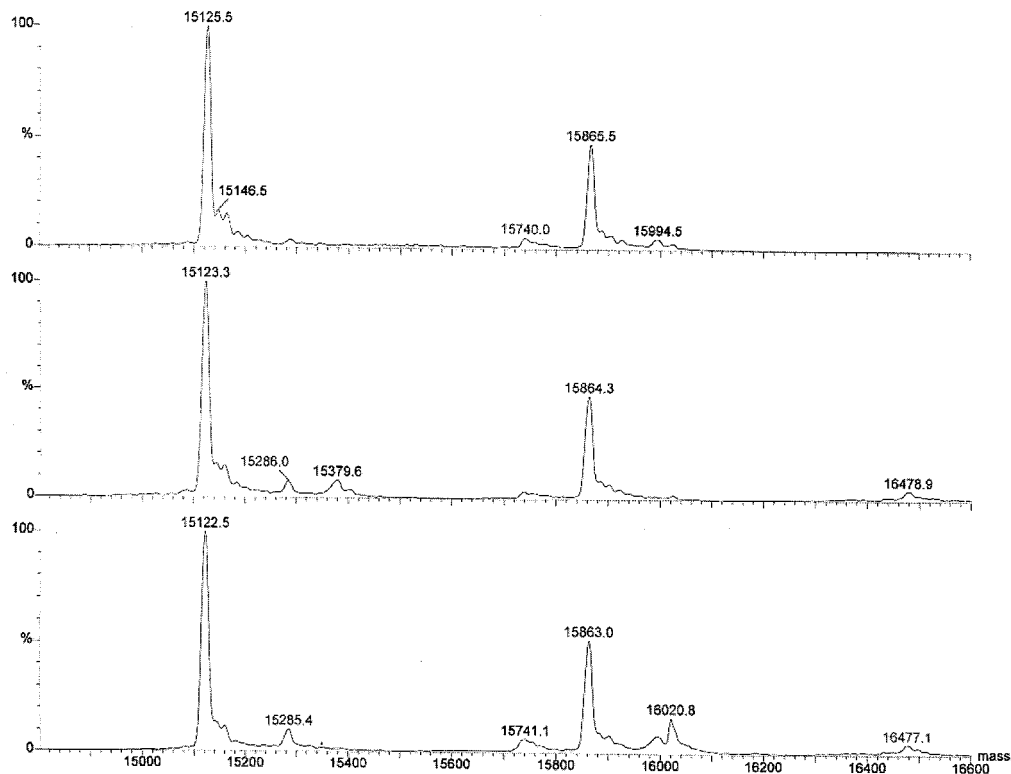


Fig. 3. ESI-MS deconvoluted spectra of patient hemolysate samples.

The top panel is a "low" sample (HbA_{1c} 4.2%) representing normoglycemia; the middle panel is a "medium" range sample (HbA_{1c} 8.0%) representing marginal glucose control; and the bottom panel is a "high" range sample (HbA_{1c} 9.9%) representing poor glucose control.

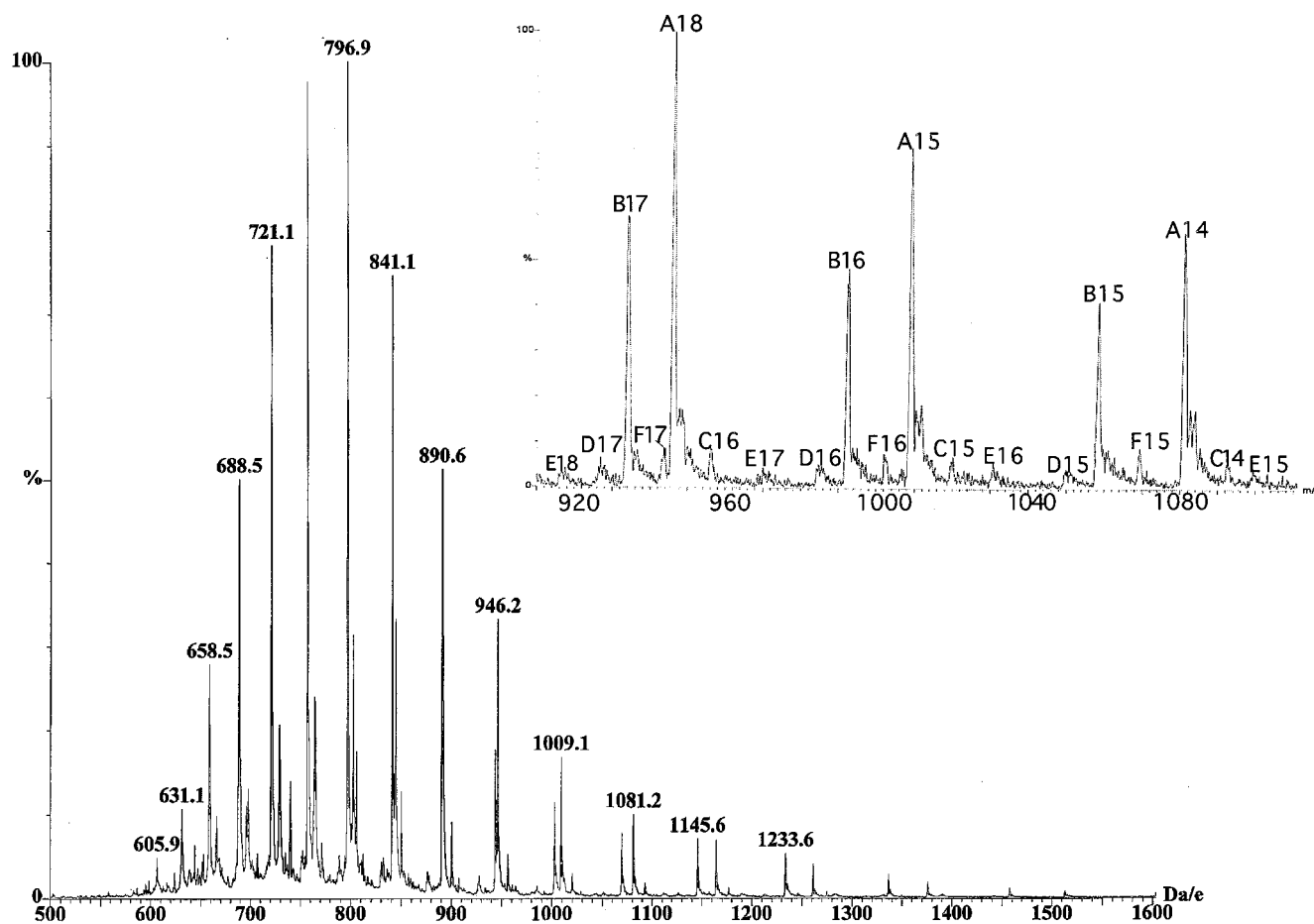


Fig. 4. Charge envelopes of hemolysate of the "high" patient sample (shown deconvoluted in Fig. 3C).

The inset shows a portion of the charge envelope for each species present in the sample. The peaks labeled A correspond to the charge envelope for nonglycated α -chain; those labeled B correspond to nonglycated β -chain; C corresponds to glycated α -chain; D corresponds to nonglycated α -chain attached to heme; E corresponds to nonglycated β attached to heme; and F corresponds to glycated β -chain.

peak on ion exchange chromatography, it contains multiple sites of glycation. Multiple glycation of the β -chain has been observed in some other patient samples as well.

Fig. 6 shows the deconvoluted mass spectra after affinity and cation exchange chromatography as described in *Materials and Methods* (see Fig. 1). The sample in panel A is the chromatographic fraction that cochromatographed with HbA₀. No observable glycated α - or β -chains are present. Panel B contains the spectrum of the species that cochromatographed with HbA₀ on cation exchange chromatography but bound to affinity (HbA₀*). This material contains glycated α -chains (15289.0 Da) as well as glycated β -chains (16029.0 Da).

The sample in panel C cochromatographed with HbA_{1c} on cation exchange chromatography and bound to affinity gel. This material contains glycated α -chains (15288.0 Da), and all β -chains were glycated (16029.0 Da). The material in panel D (HbA_{1c}*) cochromatographed with HbA_{1c} on cation exchange but did not bind to affinity gel. It contains no glycated α -chains, and all β -chains were glycated (16030.0 Da).

Discussion

The clinical use of glycohemoglobin measurements as an indicator of long-term glycemic control continues to grow. The quantification of glycohemoglobins, notably the HbA_{1c} chromatographic fraction, is currently in the process of standardization. However, the degree of homogeneity of the quantified species may differ from sample to sample and from method to method, and a universally accepted "gold standard" does not exist. It has been suggested that "HbA_{1c}" be used as a reference. The present work documents how difficult it is to define HbA_{1c}.

The chemistry of nonenzymatic glycation has been studied for many years (6–8). However, to our knowledge, the extent of glycation and the relative involvement of the α - and β -chains remain unclear, in part because of the lack of nondestructive instrumentation to observe the modified hemoglobin molecules without first altering their structure in an unpredictable way. For example, potential points of modification have been deduced after the incubation of radiolabeled glucose with purified HbA₀

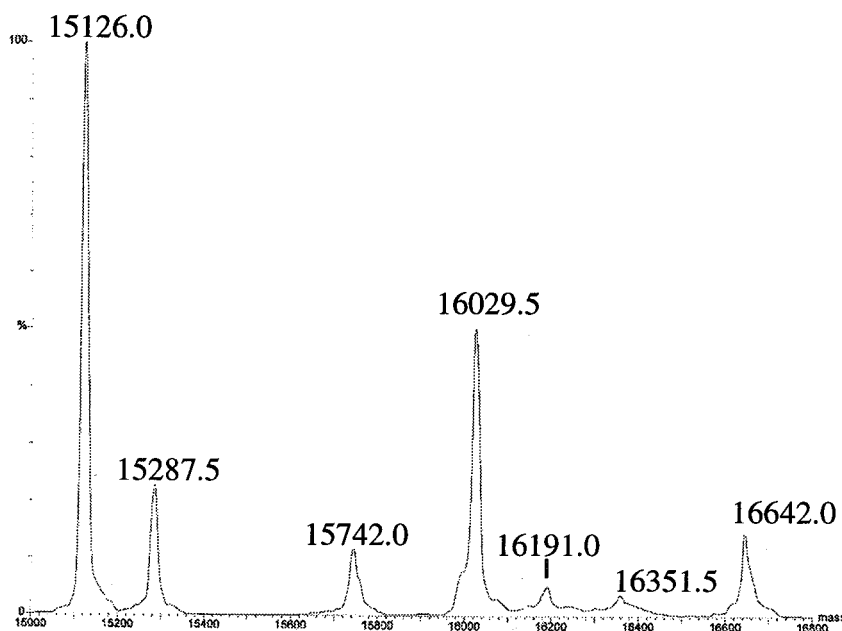


Fig. 5. ESI-MS deconvoluted spectrum of a high-range clinical hemoglobin sample, demonstrating the presence of multiple glycation of the β -chain (16029.5 Da, 16191.0 Da, and 16351.5 Da) as well as single glycation of the α -chain (15287.5 Da).

The peak at 16642.0 Da corresponds to glycated β -chain plus intact heme.

and digestion with a protease such as trypsin (27). The modified globin residues in HbA_{1c} were deduced by reduction with tritiated borohydride, followed by chromatographic separation (27). The borohydride reduction will identify some, but not necessarily all, of the modifications on the globin chains, due in part to varying accessibility of the reagent to different sites on a large molecule. Because the glycation process is a slow and continuous one that is known to occur over days to months in vivo (7, 28, 29) and to produce many structurally different adducts (e.g., browning products), the above approaches can only provide a rough estimate of the extent of glycation in a sample. Other adducts may be unstable to the various in vitro processes involved in the preparation and analysis of the sample and may have not been observed. Furthermore, the harsh conditions used may have fostered low yield as well as intra- and inter-chain exchange of adducts.

It has been known for some time, based on both in vitro and in vivo studies (7, 28, 29), that the N-terminal valine residue of the β -globin chain can be glycated. The chromatographic fraction measured in clinical assays as HbA_{1c} has been assumed to be this species. However, our analysis of the HbA₀ and HbA_{1c} fractions obtained from ion exchange chromatography (Fig. 6) indicates that this is not necessarily the case. Panel C shows the material clinically defined as HbA_{1c}, and panel D shows a material that cochromatographs with the material in panel C. By using the definition of HbA_{1c} as Hb with a single glycation on the β -globin chain, the component in panel D is likely to be HbA_{1c}. However, the clinical assay will quantify all other species that cochromatograph. Our observations of higher β to α glycation with increasing values in the lower glucose ranges and that multiple β -chain sites are glycated at high glucose concentrations

may explain the curvilinear relationship of glucose to HbA_{1c} seen in clinical studies (26).

We confirm that MS can be used to determine the extent of glycation of hemoglobin. This analysis requires no additional pretreatment of the sample than is used for conventional chromatographic methods of HbA_{1c} determination. Because of the high sensitivity of the mass spectrometric method and the separation of species in the sample by mass to charge rather than by charge, even minor components in a sample can be determined accurately and reproducibly.

Our analysis of patient samples (Fig. 3) demonstrates that the judicious use of MS is helpful for determining the extent of glycation and which globin chain(s) are glycated. The increase in β -chain glycation with increasing clinical glycohemoglobin value was not unexpected. Unexpected was that α -chain glycation also increases with increasing glycohemoglobin value, although apparently not at the same rate as with the β -chain. If the cation exchange chromatography-based clinical assay of HbA_{1c} is used, several glycated species will cochromatograph with HbA_{1c}, one with α - and β -chain glycation and others with one or more β -chain glycation sites. In our experience to date with patient samples, every sample with observable β -chain glycation also had α -chain glycation. The purified reference material (Fig. 6, panel C), which has been determined to be homogeneous by several analytical methods, contains both α - and β -chain glycation. These observations document the importance and difficulty in standardizing the different methods of glycohemoglobin measurement, because the site(s) as well as the quantity of glycation change with deterioration in glycemic control.

The quantitation of glycohemoglobin in patient samples has been reported recently (25). Although this appears to be the first attempt at quantitation of proteins by

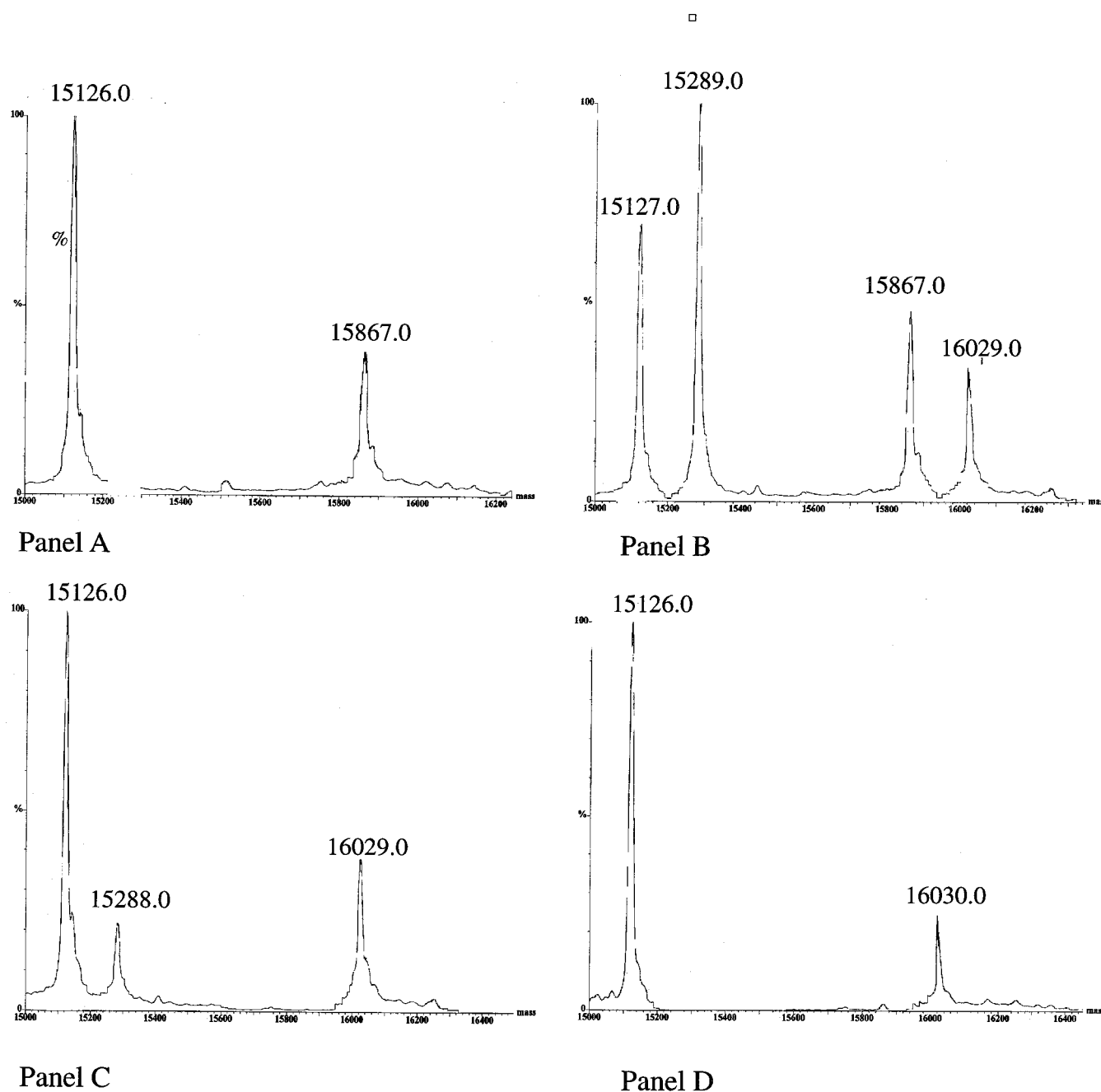


Fig. 6. ESI-MS of chromatographic fractions after affinity and cation exchange chromatographic separation of pooled hemolysate from diabetic patients.

ESI-MS in the literature, this use of the ESI technique raises some concerns. As noted by Bunk and Welch (30), good accuracy can be obtained when ESI is coupled to an efficient separation method such as liquid chromatography to separate the analyte from other species in the matrix and when internal standards are used.

Analysis using other state-of-the-art techniques such as matrix-assisted laser desorption/ionization and tandem (MS/MS) methods may further assist in the determination of the precise positions of glycation on both α - and

β -chains. By using these additional methods, analysis of patient samples from persons with various degrees of glycemic control should aid in the determination of preferred binding sites and the relative sequence of adduct formation with increasing glycemia. Thus, MS can play an important role in the chemical definitions of glycohemoglobins, HbA_{1c}, and the references and standards used in their measurement. Our studies confirm the heterogeneous nature of glycohemoglobins, as well as the potential for effective standardization of the HbA_{1c} assay and

characterization of the clinical analyte. Further analyses with other mass spectrometric techniques should improve our understanding of the glycation process and its clinical relevance for patients with diabetes and the clinicians who care for them.

References

- Koenig RJ, Peterson CM, Jones RL, Saude C, Lehman M, Cerami A. Correlation of glucose regulation and hemoglobin A_{1c} in diabetes mellitus. *N Engl J Med* 1976;295:417–2.
- Koenig RJ, Peterson CM, Kilo C, Cerami A, Williamson JR. Hemoglobin A_{1c} as an indicator of the degree of glucose intolerance in diabetes. *Diabetes* 1976;25:230–2.
- Allen DW, Shroeder WA, Balog J. Observations on the chromatographic heterogeneity of normal adult and fetal human hemoglobin: a study of the effects of crystallization and chromatography on the heterogeneity and isoleucine content. *J Am Chem Soc* 1958; 80:1628–34.
- Baynes JW, Bunn HF, Goldstein D, Harris M, Martin DB, Peterson CM, Winterhalter K. National diabetes data group: report of the expert committee on glycosylated hemoglobin. *Diabetes Care* 1984;7:602–6.
- Miedema K. Electrospray mass spectrometry for measurement of glycohemoglobin [Editorial]. *Clin Chem* 1997;43:705–7.
- Holmquist WR, Schroeder WA. A new N-terminal blocking group involving a Schiff base in hemoglobin A_{1c}. *Biochemistry* 1966;5: 2489–503.
- Bookchin RM, Gallop PM. Structure of hemoglobin A_{1c}: nature of the N-terminal β -chain blocking group. *Biochem Biophys Res Commun* 1968;32:86–93.
- Bunn HF, Haney DN, Gabbay KH, Gallop PM. Further identification of the nature and linkage of the carbohydrate in hemoglobin A_{1c}. *Biochem Biophys Res Commun* 1975;67:103–9.
- Siuzdak G. The emergence of mass spectrometry in biochemical research. *Proc Natl Acad Sci U S A* 1994;91:11290–7.
- Chait BT, Want R, Beavis RC, Kent SB. Protein ladder sequencing. *Science* 1993;262:89–92.
- Cox AL, Skipper J, Chederson RA, Darrow TL, Shabanowitz J, Engelhard VH, et al. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science* 1994; 264:716–9.
- Chowdhury SK, Eshraghi J, Wolfe H, Forde D, Hlavac AG, Johnston D. Mass spectrometric identification of amino acid transformations during oxidation of peptides and proteins: modifications of methionine and tyrosine. *Anal Chem* 1995;67:390–8.
- Musatov A, Robinson NC. Subunit analysis of bovine cytochrome bc₁ by reverse-phase HPLC and determination of the subunit molecular masses by electrospray ionization mass spectrometry. *Biochemistry* 1994;33:10561–7.
- Crowther J, Adusumalli V, Mukherjee T, Jordan K, Abuaf P, Corkum N, et al. Determination of nanogram levels of peptide drug in rabbit and human plasma using high-performance liquid chromatography coupled with electrospray ionization mass spectrometry. *Anal Chem* 1994;66:2356–61.
- Pieles U, Zurcher W, Schar M, Moser HE. Matrix-assisted laser desorption time-of-flight mass spectrometry: a powerful tool for the mass and sequence analysis of natural and modified oligonucleotides. *Nucleic Acids Res* 1993;21:3191–6.
- Katta V, Chait BT. Conformational changes in proteins probed by hydrogen-exchange electrospray-ionization mass spectrometry. *Rapid Commun Mass Spectrom* 1991;5:214–7.
- Wagner DS, Melton LG, Yan Y, Erickson BW, Anderegg RJ. Deuterium exchange of α -helices and β -sheets as monitored by electrospray ionization mass spectrometry. *Protein Sci* 1994;3: 1305–14.
- Springer DL, Bull RJ, Goheen SC, Sylvester DM, Edmonds CG. Electrospray ionization mass spectrometric characterization of acrylamide adducts to hemoglobin. *J Toxicol Environ Health* 1993; 40:161–76.
- Resing KA, Mansour SJ, Hermann AS, Johnson RS, Candia JM, Fukasawa K, et al. Determination of v-Mos-catalyzed phosphorylation sites and autophosphorylation sites on MAP kinase by ESI/MS. *Biochemistry* 1995;34:2610–20.
- Packman LC, Berry A. A reactive, surface cysteine residue of the class-II fructose-1,6-bisphosphate aldolase of *Escherichia coli* revealed by electrospray ionisation mass spectrometry. *Eur J Biochem* 1995;227:510–5.
- Chen H-M, Scott BK, Braun KP, Peterson CM. Validated fluorimetric HPLC analysis of acetaldehyde in hemoglobin fractions separated by cation exchange chromatography: three new peaks associated with acetaldehyde. *Alcohol Clin Exp Res* 1995;19: 939–44.
- Shackleton CHL, Witkowska HE. Mass spectrometry in the characterization of variant hemoglobins. In: Desiderio DM, ed. *Mass spectrometry: clinical and biomedical applications*, Vol. 2. New York: Plenum Publishing Corp., 1994:135–99.
- Braun KP, Cody R, Jones DJ, Peterson CM. A structural assignment for a stable acetaldehyde-lysine adduct. *J Biol Chem* 1995; 270:11263–66.
- Braun KP, Pavlovich JG, Jones DR, Peterson CM. Stable acetaldehyde adducts: structural characterization of acetaldehyde adducts of human hemoglobin N-terminal β globin chain peptides. *Alcohol Clin Exp Res* 1997;21:40–3.
- Roberts NB, Green BN, Morris M. Potential of electrospray mass spectrometry for quantifying glycohemoglobin. *Clin Chem* 1997; 43:771–8.
- Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 1993;329:977–86.
- Shapiro R, McManus MJ, Zalut C, Bunn HF. Sites of nonenzymatic glycosylation of human hemoglobin A. *J Biol Chem* 1980;255: 3120–7.
- Bunn HF, Shapiro R, McManus M, Garrick L, McDonald MJ, Gallop PM, Gabbay KH. Structural heterogeneity of human hemoglobin A due to nonenzymatic glycosylation. *J Biol Chem* 1979;254: 3892–8.
- Mohammad A, Fraenkel-Conrat H, Olcott HS. The “browning” reaction of protein with glucose. *Arch Biochem Biophys* 1949;24: 157–78.
- Bunk DA, Welch MJ. Electrospray ionization mass spectrometry for the quantitation of albumin in human serum. *J Am Soc Mass Spectrom* 1997;8:1247–54.