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Potential of electrospray mass spectrometry for quantifying glycohemoglobin

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An electrospray ionization–mass spectrometric procedure has been developed for determining glycohemoglobin. Whole-blood samples from 78 diabetic and 50 nondiabetic subjects (glycation range 3–15%, as determined by electrospray mass spectrometry) were diluted 500-fold in an acidic denaturing solvent and introduced directly into a mass spectrometer. The resulting mass spectra were then processed to estimate the percentage of glycohemoglobin present in the sample. Total analysis time, including plotting the spectra and computing the percentage of glycation, was ~3 min. The imprecision (CV) of the method was <5.1% for inter- and intrabatch analyses for total glycohemoglobin in the range 3.6–14%. Comparison of the mass spectrometric results with those from established affinity chromatographic procedures showed good overall agreement. The relative glycation of the α- and β-chains was determined directly and was shown to be constant (0.64:1) over the glycation range measured. Only single glucose attachment to both the α- and β-chains was observed.

INDEXING TERMS: diabetes • method comparison

The measurement of glycohemoglobin (GHb) is widely accepted as a valuable indicator for long-term diabetic control *[1, 2]*. Development of diabetic complications is substantially decreased when the GHb proportions in blood from diabetics are maintained as close as possible to those in nondiabetics *[3]*. Several procedures and numerous commercial instruments, mainly based on chromatographic separation methods, have been developed for measuring this indicator, but these can give quite different results *[4–6]* unless carefully calibrated against a reference "standard." Interferences, e.g., uremia, hemoglobinopathies, therapy with acetylsalicylate, and sample degradation under adverse storage conditions, can also affect the results *[7, 8]*. The need to rationalize this situation has been made a major priority of the US National Diabetes Data Group *[9]*. However, as has been highlighted elsewhere, methods of GHb determination may also lack long-term assay reproducibility *[9]*.

GHb is a general term referring to the products of glucose binding slowly and nonenzymatically to hemoglobin with a ketoamine structure *[10]*. Included in these products are those resulting from the condensation of glucose with the N-terminal valines of one or both of the β-chains (originally specified as Hb A_{1c}) *[11]* with, to a lesser extent, glycation of the N-terminal valine(s) of the α-chain(s) *[12]*. In addition, several of the ε-amino groups of the globin lysine residues may also be glycated *[12]*, and evidence indicates that the site of modification can vary, depending on whether the hemoglobin glycation occurs in vitro or in vivo *[12, 13]*.

A more detailed analysis with enzymatic digestion of the glycated proteins and subsequent separation and analysis has been used to uniquely identify the sites of glycation on the α- and β-chains *[14]*. Miedema, Kobold, and Jeppsson demonstrated the utility of using enzymatic digestion coupled with chromatographic or electrophoretic separation and mass spectrometric detection to determine the absolute amount of Hb A_{1c} present in a blood sample and proposed that method as a primary reference method for measuring Hb A_{1c}. Participants at that symposium *[14]* also highlighted the lack of an available primary standard for Hb A_{1c} determination.

For several years, electrospray mass spectrometry (ES-MS) has been recognized as one of the tools available for characterization of variant hemoglobins *[15]*. In this technique, the hemoglobin is introduced into the mass spectrometer in a denaturing solution, so that all of the proteins (including posttranslationally modified proteins) in the native material are analyzed as covalently bound entities. In effect, the mass spectrometer separates these components according to their molecular weights (masses); thus, the α- and β-chains, their glycoforms, other chains, and posttranslationally modified chains are detected separately, provided their masses differ sufficiently

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from one another. However, using mass spectrometry this way cannot differentiate between different sites of glycation because all of the glycated species that exhibit single glycation on a given chain have the same mass.

In essence, the electrospray ion source of the mass spectrometer generates a series of multiply protonated molecules for each protein present in the sample solution. These ions are separated by the mass spectrometer according to their mass-to-charge ratio (*m/z*), producing a spectrum on an *m/z* scale. Software used to transform this *m/z* spectrum represents each protein as a single peak on a true molecular mass scale. The relative intensities of the peaks so displayed are proportional to the concentrations of the proteins in the sample solution, but proteins that differ significantly in their sequence, and thus their solution and gas-phase chemistries, do not necessarily produce the same response. Previous ES-MS studies of hemoglobin indicate that the relative signals observed for α- and β-chains are ~4:3 *[15]*. Because both glycated α- and β-chains differ from their nonglycated forms by a similar posttranslational modification, the responses of the glycated forms and their nonglycated forms would be expected to be similar. If the response of the glycated forms differed greatly from that of the nonglycated forms, the calculation of percentage of glycation from the mass spectrometric data would be unrealistic and would not correlate to the other methods of GHb determination.

In the case of hemoglobin, the resolution (the ability of ES-MS to separately analyze the various components according to their molecular masses) is ~1300 (12 Da at 16 kDa), and the accuracy and precision of measuring their molecular masses is ±0.005%, or about ±0.8 Da for the components in the relevant mass range of 15–16.5 kDa. Given that the average molecular masses calculated from the sequences of the α- and β-chains in normal (nonvariant) human hemoglobin are 15126.4 and 15867.2 Da, respectively—162.1 Da higher for their glycated forms (15288.5 and 16029.4 Da, respectively)—these components are well separated on the mass scale and may be unambiguously identified from their measured masses. ES-MS, therefore, should be well suited to determinations of the percentage of glycation of the separate globin chains. The high resolution and high mass accuracy provided by ES-MS may also reveal the presence of components in the hemolysate that might interfere with other analytical techniques, and identify them, in many cases, simply from their molecular masses.

Here we describe an investigation into the potential of ES-MS for quantifying GHb. Analyses were carried out on blood from healthy volunteers and diabetic patients and the results compared with those from various established procedures such as ion-exchange chromatography and affinity-based techniques. We also illustrate that GHb can be determined in the presence of aberrant chains (e.g., a heterozygote β-chain variant) in a suspected diabetic blood sample and, moreover, can provide some information about the nature of the aberrancy.

Materials and Methods

GENERAL

Blood specimens were taken from 50 fasting healthy volunteers and 78 diabetic patients attending the Royal Liverpool University Hospital outpatient clinic. All the fasting volunteers had plasma glucose concentrations <6.0 mmol/L. Samples were collected in fluoride-oxalate tubes (Sarstedt, Leicester, UK) and stored for later subdivision for separate analyses. The routine (affinity chromatography) GHb analyses were carried out with Abbott Vision and IMx-based methods (Abbott Diagnostics, Maidenhead, UK) and with an HPLC column technique (Primus Corp., Kansas City, MO).

The collection of samples and analysis was part of the routine assessment of the patients' glycemic control, and complied with the ethical standards of the Royal Liverpool University Hospital. The additional analyses were performed anonymously, in accordance with the ethical guidelines of the Hospital.

The samples were stored at 4 °C between collection and analysis by non-mass-spectrometric methods (within 2–3 days of collection). They were further stored at −20 °C until analysis by mass spectrometry, generally within 1 week.

SAMPLE PREPARATION FOR MASS SPECTROMETRY

Initially, 10 μL of the whole-blood samples was diluted 50-fold with 490 μL of deionized water (Elga, High Wycombe, UK). Portions (20 μL) of these solutions were then diluted another 10-fold with 40 μL of 10 mL/L formic acid, 40 μL of deionized water, and 100 μL of HPLC-grade acetonitrile (Fisons, Loughborough, UK) to give working solutions for mass spectrometric analysis.

To assess the effect of salt present in the samples on the analysis, we desalted some samples, using 10-kDa-cutoff microconcentrators (Amicon, Stonehouse, UK). A 100-μL aliquot of the 50-fold-diluted solutions was further diluted with 200 μL of 10 mL/L formic acid and concentrated with a microconcentrator almost to dryness. The concentrate was then washed with 300 μL of deionized water and reconcentrated, after which the hemoglobin residue was taken up in 100 μL of deionized water. From this solution, 20 μL was then diluted with 40 μL of 10 mL/L formic acid, 40 μL of water, and 100 μL of acetonitrile to give the mass spectrometer working solutions.

A working solvent of equal volumes of water and acetonitrile acidified with 2 mL/L formic acid was generally used for preparing samples for the ES-MS analysis of peptides and proteins and serves to denature the protein. The acid also assists protonation of the molecules in solution before the analysis.

MASS SPECTROMETRY

The working solutions prepared above were introduced as 10-μL aliquots at 5 μL/min into the electrospray source of a Quattro II mass spectrometer (Micromass, Altrincham, Cheshire, UK) in a flowing liquid phase of equal

amounts of water and acetonitrile with no additives. The instrument, operated in the positive ion mode, summed for each sample 10 × 8-s scans over an *m/z* range of 980–1300 in 1.5 min to obtain the final spectrum. After baseline subtraction, these data were transformed to a scale for true molecular mass by use of software routines supplied with the instrument. The transformed spectra were then smoothed and the heights of the peaks used as a measure of their intensities. We used peak heights rather than peak areas because the heights discriminate more efficiently against potential interferences from partially resolved peaks. Initially, the data were manually processed, but the original *m/z* spectra from later batches of samples were processed automatically to carry out the transformation, identify the appropriate peaks from their measured masses, and calculate the %GHb from the peak intensities. Mass-scale calibration was performed by using the multiply protonated molecules from the normal α-chain (15126.4 Da) present in the samples.

CALCULATION OF % GLYCATION

A typical electrospray mass spectrum from diluted human blood contains the following species:

1) α-, β-, glycated α-, and glycated β-chains.

2) Several covalently modified chains, mainly associated with the β-chain. Their concentrations vary, apparently depending on the age of the sample, e.g., putative cysteinylated and glutathionylated β-chain and their corresponding glycated species.

3) Minor non-α-chains, e.g., δ and γ.

4) Non-covalently-bound adducts associated with all the species already listed, e.g., heme adducts and alkali metal adducts, predominantly sodium and potassium.

In the absence of direct measurements of the sensitivities (mass spectrometer response for a given molar concentration in the analyte) of many of these species, we made the following assumptions to derive an expression for calculating the percentage of glycation from the experimental data:

1) All α-chain species, including noncovalent adducts, have the same sensitivity. We believe this is a reasonable assumption because these species all have the same protein chain with relatively minor modifications.

2) All β-chain species, including noncovalent adducts, have the same sensitivity.

3) The sensitivities of the α- and non-α-chain species differ and are related by:

$$\text{sensitivity of } \alpha\text{-chain species} = K \times \text{sensitivity of non-}\alpha\text{-chain species}$$

where K is constant for a given set of operating conditions.

4) The relative sensitivities of adducted glycated α-chain to glycated α-chain species are the same as those of adducted α-chain to α-chain species. Similar assumptions are made for the corresponding β-chain species.

5) Minor non-α-chain species may be ignored.

6) There is no contribution to the glycated species by multiple alkali metal adducts of the nonglycated species (see comparison of results after desalting samples, Fig. 2).

On the basis of these assumptions and the knowledge that the ES-MS analyte originates from whole-blood hemoglobin that contains equal molar amounts of α- and non-α-chain species, it can be rigorously shown that

$$\%\text{GHb} = 50\{[\alpha_g/(\alpha + \alpha_g)] + [\beta_g/(\beta + \beta_g)]\}$$

where α and β represent the intensities of the α- and β-chains, and α_g and β_g represent the intensities of the glycated α- and β-chains.

This remarkably simple expression means that only four peaks from the transformed spectrum need to be measured; however, many of the assumptions on which it is based are difficult to prove empirically. Nevertheless, the close correlation between the ES-MS results and the results obtained with other techniques imply that the assumptions are not widely divergent from reality.

The ratio of glycated α to glycated β was calculated as $\alpha_g(\beta + \beta_g)/\beta_g(\alpha + \alpha_g)$.

Results

Figure 1a shows a typical *m/z* spectrum (after baseline subtraction) for a blood sample with ~11% glycation; Fig. 1b shows the same data after transformation to a true molecular-mass scale. The prominent peaks observed in Fig. 1a are attributed to the series arising from the multiply protonated α- and β-chains, the degree of protonation observed under these experimental conditions varying from 12 to 15 for the α-chain (e.g., A12 = α-chain + 12 protons) and from 13 to 16 for the β-chain. The region in the lower *m/z* region (*m/z* 600–980)—not shown and not used in the analysis—contains multiply protonated molecules extending up to the 20-protonated α-chain and the 18-protonated β-chain. The presence of free heme (*m/z* 616.2) is also observed.[3] The transformed spectrum in Fig. 1b shows peaks from the α- (15126.4 Da) and β- (15867.2 Da) chains, in addition to the glycated α- (15288.5 Da) and glycated β- (16029.4 Da) chains. Also observed are peaks for α + heme (15742.9 Da) and β + heme (16483.7 Da). Peaks observed at a slightly higher mass than the α- and β-chains are attributable to the adduction of sodium and potassium; they were considered a possible source of interference in measuring glycated globins if multiple adduction of alkali metals formed species that had the same mass as the glycated forms.

Figure 2a shows a typical transformed spectrum from a sample with ~11% glycation. In addition to peaks corre-

[3] The monoisotopic mass (^{12}C) for a free heme is 616.2 Da. For calculating the mass of the protein, the resolution of the technique is insufficient to resolve the separate isotopes, and the *average* mass is determined. For reporting the mass of the protein + heme, the average mass of the heme (616.5 Da) is used.

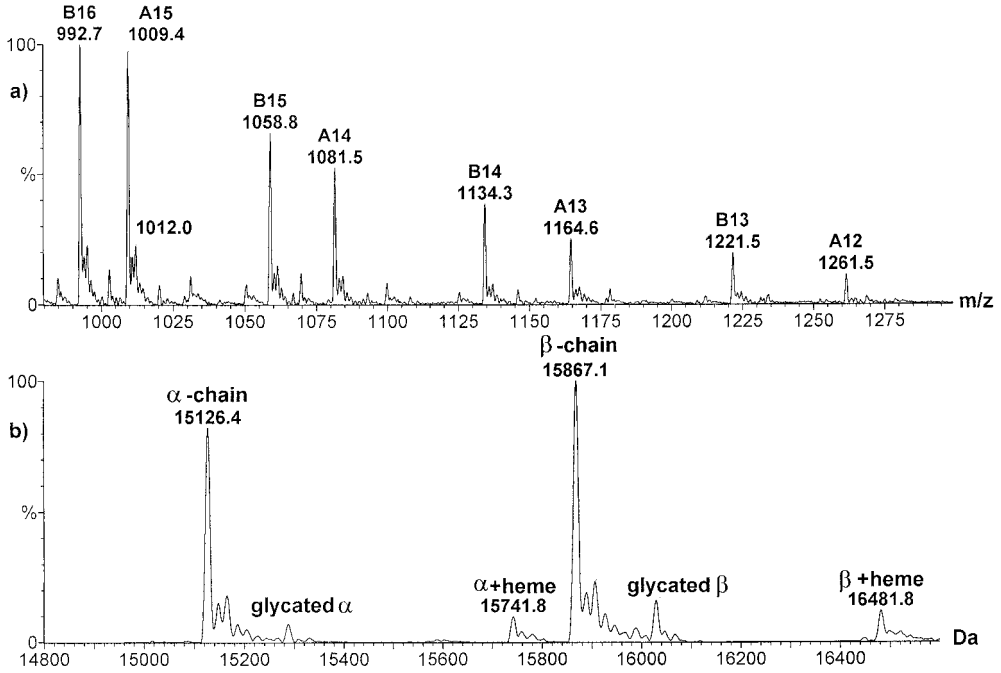

Fig. 1. Electrospray mass spectrum of 500-fold-diluted normal (nonvariant) hemoglobin from whole blood (~11% glycated): (*a*) raw data plotted on a mass/charge scale and (*b*) transformed data presented on a true mass scale.

(*a*) The two series annotated are those arising from multiply protonated α-chains (e.g., A15 = α-chain + 15 protons) and β-chains (e.g., B16 = β-chain + 16 protons).

sponding to the α- and β-chains and their corresponding glycated forms (+162.1 Da), peaks are present corresponding to the addition of heme and sodium and potassium single adducts. These alkali metal adducts, although not directly a problem, indicate the possibility of multiple adducts occurring at the same masses as the glycated chains, which would thus increase the measured quantities of the latter. To assess this possibility, we remeasured the percentage of glycation after desalting selected samples. The transformed spectrum of a desalted sample is shown in Fig. 2b. The measured proportion of glycation for this sample was 11.1% both before and after desalting, despite the reduction in adducting observed after desalting (Fig. 2b). This result indicates that using diluted whole blood without further purification to remove alkali metal salts is adequate for determining GHb by ES-MS. It further suggests that the assumptions made regarding alkali metal adducts in calculating %GHb are reasonable. All the GHb results presented in this report are from samples that were simply diluted from whole blood and analyzed without desalting.

Data from one batch of samples showed evidence of

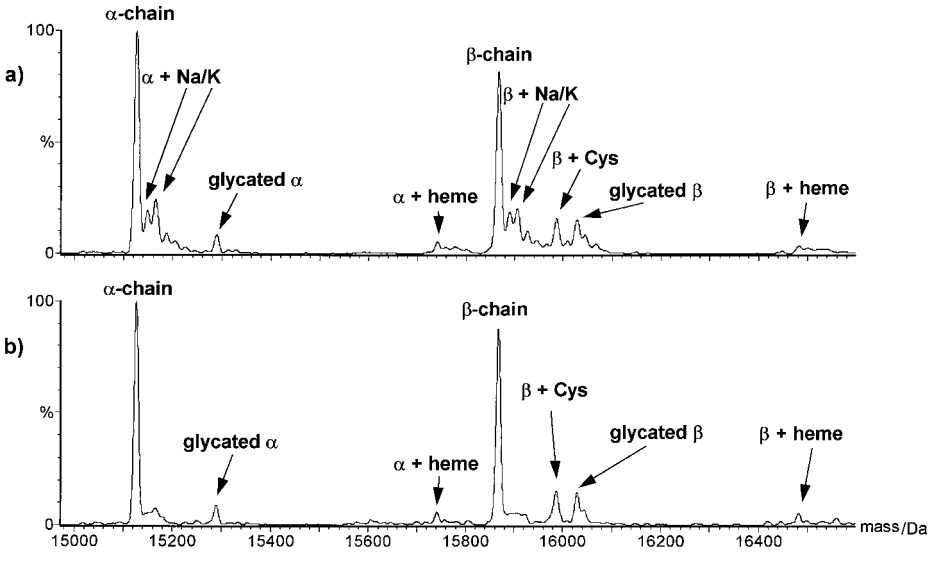

Fig. 2. A comparison of the mass spectra of a single blood sample after (*a*) standard dilution and (*b*) desalting to remove alkali metal salts.

Note that the glycated globin peaks are unchanged in relative intensity. The peaks labeled β+Cys are believed to result from cysteinylation of the β-chains in the samples during storage.

sample degradation by exhibiting additional peaks in their mass spectra. The spectra in Fig. 2 are from a sample from this batch. The additional peaks, at masses 119.1 and 176.2 Da higher than the β-chain peak, may be due to cysteinylation and the covalent addition of Cys-Gly, respectively. Peaks for these adducts reached significant heights for whole-blood samples that had been stored at 4 °C for several weeks. These types of adducts were not observed when analyzing fresh samples, and thus are not considered a problem.

The transformed mass spectra for three blood samples are shown in Fig. 3, demonstrating glycation of 5%, 7%, and 12% of the total hemoglobin. As can be clearly seen in these spectra, the peaks attributed to the glycated α- and β-chains proportionately increase as the degree of glycation increases. In these experiments, only single glycation of both the α- and β-chains was observed; i.e., no signals were seen 324.2 Da greater than the molecular masses of the free chains, which would have corresponded to the addition of two glucose units. No evidence for multiple glycation was observed even at the highest value analyzed for GHb (15%, as determined by ES-MS).

The ratio of α-chain glycation to β-chain glycation was 0.64 ± 0.07 (SD) and was independent of the degree of glycation. Both α-chain glycation (r^2 = 0.9669) and β-chain glycation (r^2 = 0.9838) increased linearly with the increase in total measured GHb (Fig. 4).

The imprecision (CV) of the ES-MS measurements was determined by performing 10 replicate analyses on five separate samples. During a single day the CV was 2.0% (n = 10); it was <5.1% (n = 10) over a period of several days. For the various proportions of GHb determined, the CVs were: 5.1% at 3.6% GHb, 2.8% at 4.9% GHb, 3.1% at 6.5% GHb, 3.0% at 9.7% GHb, and 2.3% at 14% GHb.

A comparison of GHb determined by ES-MS with GHb determinations by other established methods is presented graphically in Fig. 5. All the established methods demonstrated a reasonably linear correlation with the ES-MS method, although the latter generally reported a lower %GHb than the separation methods. Whether this lower response is a function of the ionization method used or the result of chemical noise artificially increasing the results in the other techniques is unclear. The HPLC affinity method showed excellent correlation (r^2 = 0.9840; Fig. 5a) with ES-MS, whereas the Abbott Vision method showed more scatter around a well-correlated trend (r^2 = 0.9754; Fig. 5b). The worst correlation of the ES-MS method, that with the IMx affinity method (r^2 = 0.8640; Fig. 5c), still showed an overall similarity in results by the two methods. Comparison between the HPLC affinity method and the IMx affinity method (Fig. 5d) showed the poorest correlation (r^2 = 0.8554) of all the methods compared.

We also compared the HPLC affinity determinations with those obtained by ion-exchange chromatography. The resulting correlation (r^2 = 0.9278) was comparable with that observed between ES-MS and HPLC affinity measurements (N.B. Roberts, B.N. Green, and M. Morris, unpublished results). This implies that the correlation between ES-MS and ion-exchange chromatography should also be good.

An additional, incidental, result of the ES-MS quantification of GHb is the detection of hemoglobin variants. Mass differences as little as 12 Da can be detected for heterozygous variants in α- and β-globin chains *[16]*; using additional processing based on maximum entropy algorithms can resolve species differing by only 6 Da at 16 kDa. An example of a sample containing a hemoglobin

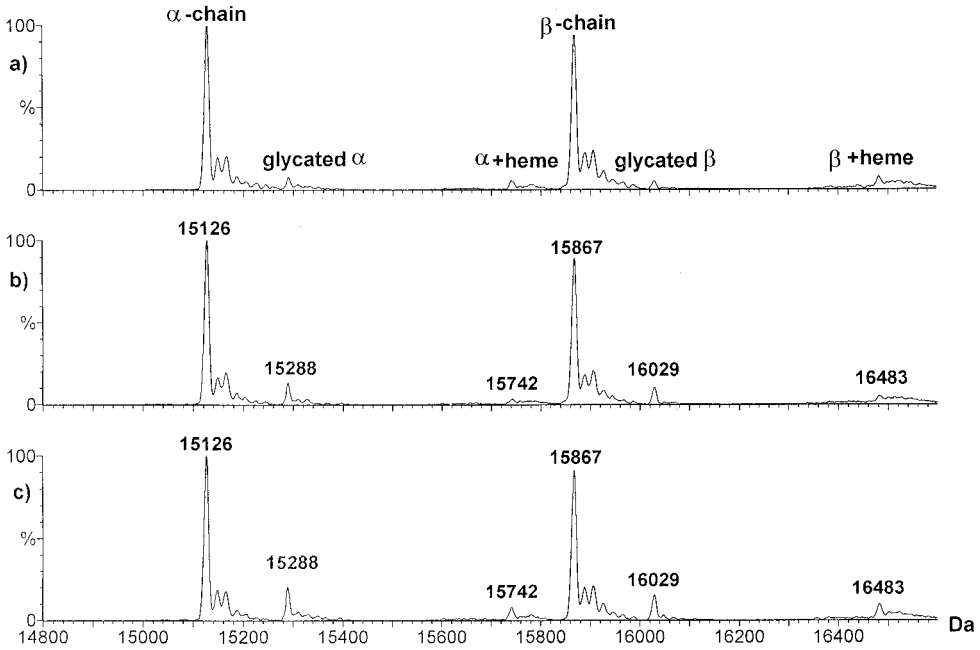

Fig. 3. Typical mass spectra from blood samples taken from diabetic patients and showing three proportions of glycation of the hemoglobin: (*a*) 5% glycation, (*b*) 7% glycation, and (*c*) 12% glycation.

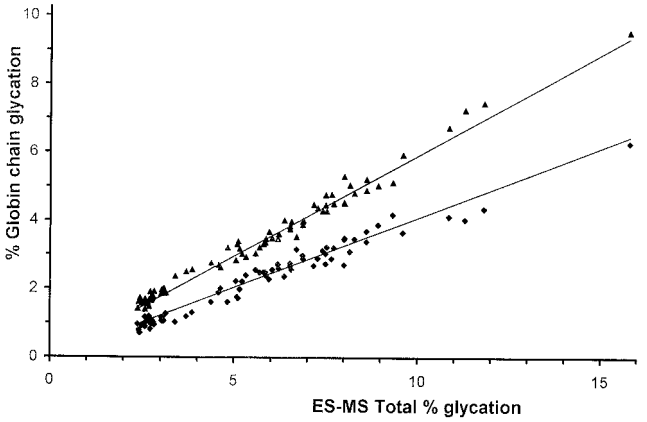

Fig. 4. Individual chain glycation plotted against total glycohemoglobin (x) as determined by ES-MS.

(♦, *lower curve*), α-chain glycation = $0.41x - 0.06$ ($r^2 = 0.9669$); (▲, *upper curve*), β-chain glycation = $0.59x + 0.06$ ($r^2 = 0.9838$).

variant is shown in Fig. 6. The blood, analyzed because it was thought to have increased proportions of GHb, was found to contain a heterozygous β-chain variant at 15 881 Da, 14 Da more than the normal β-chain. The hemoglobin was also determined to be 3.1% glycated. A mass difference of +14 Da in β-globin corresponds to a single-point mutation that may occur at one of 49 of the 146 residues in the β-chain (M. Morris, B.N. Green, and N.B. Roberts, manuscript in preparation). Further investigation showed the variant to be Hb-Raleigh (β_1 Val→Ac-Ala) *[17]*. Homozygous variants may be analyzed more easily, and one can determine by mass spectrometry a homozygous variant differing by 1 Da from the normal form *[15]*.

Discussion

This ES-MS method for the quantitative analysis of GHb is robust, simple, and rapid, with an intersample analysis time of <3 min. Minimal sample preparation is required: Whole-blood samples are simply diluted 500-fold in an acidic denaturing solvent. This method is suitable for development to automated sample preparation, analysis, and reporting. Moreover, the data were obtained without any calibration except for the mass scale. However, the technique measures either α-chain glycation, β-chain glycation, or total glycation, but cannot analyze specifically for Hb A_{1c}. At its present stage of development, the technique is more applicable to the research environment than to a routine clinical laboratory and would obviously need further validation before use in the latter.

The ES-MS results demonstrate that the degree of glycation of the α-chain is 0.64 ± 0.07 that of the β-chain, in reasonable agreement with a previously reported ratio of 0.53 *[18]*. Because the amounts of the two chains in the solution being analyzed are the same, i.e., $\alpha_2\beta_2$ *[19]*, any difference in relative glycation between the two chains is likely to be structurally determined.

Previous studies showed that glycation at the N-terminus of the β-chain (Hb A_{1c}) and at other sites

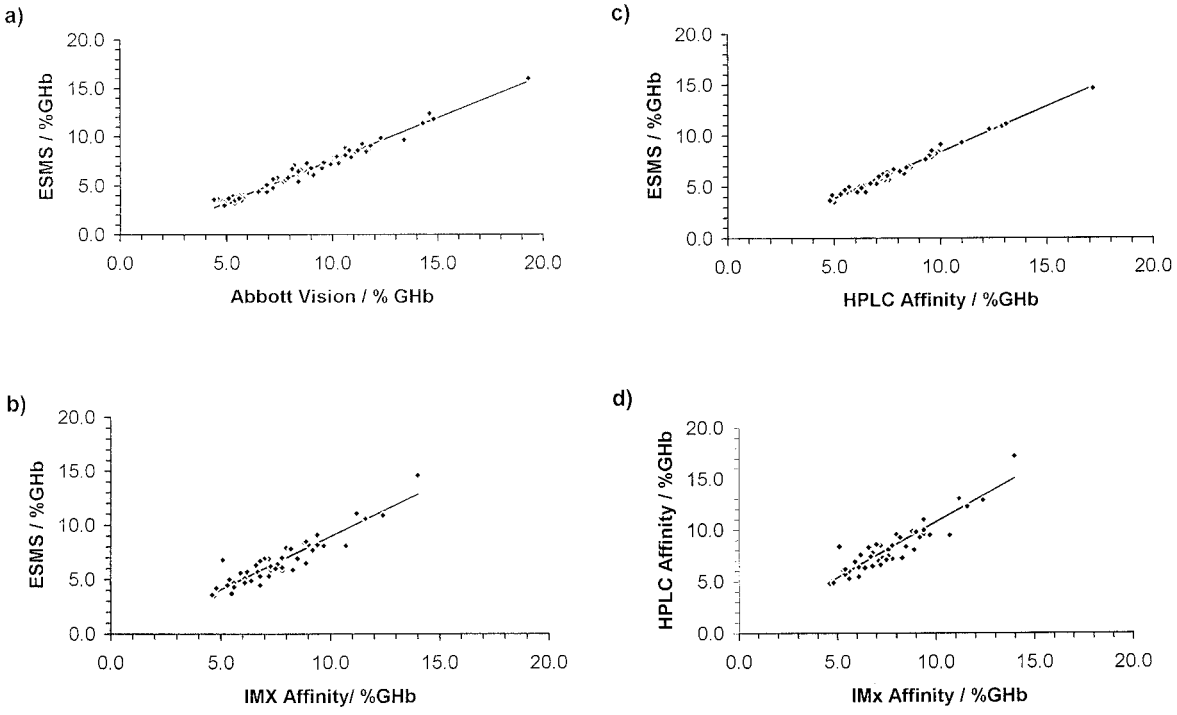

Fig. 5. Comparison of glycohemoglobin data from diabetic patients: (*a*) ES-MS vs Abbott Vision, $y = 0.8665x - 1.0487$ ($r^2 = 0.9754$); *(b)* ES-MS vs IMx affinity, $y = 0.9780x - 0.8224$ ($r^2 = 0.8640$); (*c*) ES-MS vs HPLC affinity, $y = 0.8954x - 0.5847$ ($r^2 = 0.9840$); and (*d*) HPLC affinity vs IMx affinity, $y = 1.0781x - 0.1589$ ($r^2 = 0.8554$).

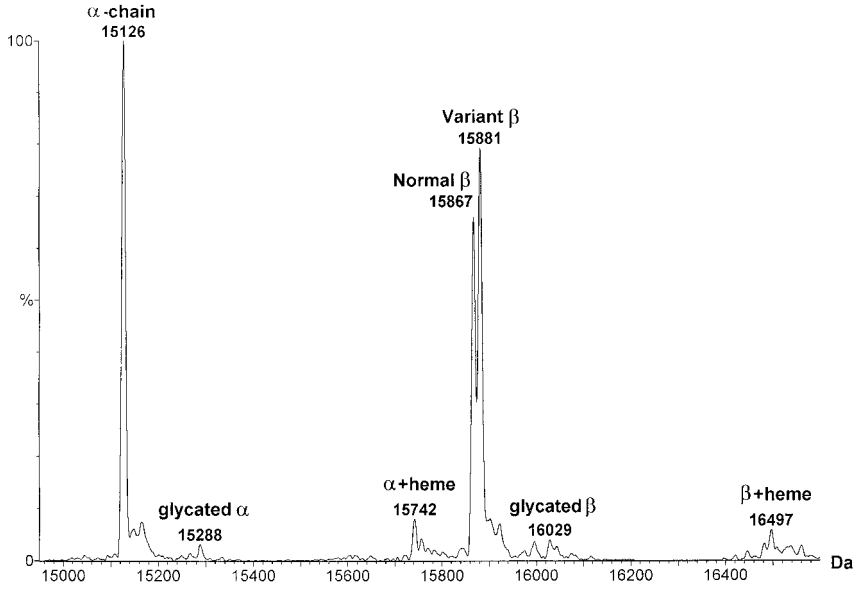

Fig. 6. Electrospray mass spectrum of hemoglobin variant Hb-Raleigh (β_1 Val→Ac-Ala, Δm = +14 Da).

(N-terminus of the α-chain, α-chain lysines, and β-chain lysines) accounts for ~8% *[12]* of Hb A_0 in nondiabetic subjects. The data from these ES-MS analyses averaged ~2.7% ± 0.5%, somewhat lower than the values previously determined for normal samples *[13, 18]*. The higher-resolution PolyCAT A cation-exchange method described by Turpeinen et al. *[5]* reports lower values for (specifically) Hb A_{1c}, primarily because the more efficient chromatographic separation resolves the peak measured as Hb A_{1c} from interferences that coelute in other separation techniques. Turpeinen et al. reported a mean glycation percentage (Hb A_{1c}) of 3.5% ± 0.5% (95% confidence interval) for nondiabetic patients, fairly close to the slightly lower total GHb value by ES-MS of 2.7% ± 0.5%.

To test whether ES-MS analysis of the samples in acid solution could have caused some hydrolysis of the glucose adducts and hence led to lower GHb values in our results, we also analyzed two blood samples having ~14% GHb, as follows. Two solutions were prepared from each sample in the aqueous acetonitrile solvent plus 2 mL/L formic acid, as described above. One solution was kept at room temperature (~20 °C); the other was kept at −20 °C except when brought to room temperature for analysis. The four solutions were each analyzed as a group 14 times over 4 days. The time between analyzing each room temperature solution and its corresponding −20 °C solution was <15 min. The solutions kept at −20 °C gave mean ± SD %GHb values of 13.52% ± 0.31% and 14.48% ± 0.21% and showed no trend in variation of %GHb with time. Calculating the %GHb ratio for each pair of measurements (i.e., the room temperature solution and its corresponding −20 °C solution) showed a barely detectable rate of decrease of %GHb in the room temperature solutions, averaging 0.12% and 0.16% per 24 h for the 13.5% and 14.5% GHb samples, respectively. Thus we conclude that no significant reduction occurred in the %GHb values reported here as a result of the samples being prepared and analyzed in acid solution, given that the time between preparing and analyzing the solutions was <3 h.

Overall, our observations may help explain some of the intermethod variations observed in GHb determinations. In particular, we demonstrated the presence of strongly attached alkali metal ion adducts, which may affect both electrophoretic and ion-exchange separations. None of the other postulated complexing agents (urea, acetylsalicylate) were observed in the analyses reported here, probably because the relative amount of these complexes normally detected (<1.0% of total hemoglobin for an individual component) *[7]* would be too low to detect by ES-MS under the nonseparatory conditions used. Even if such complexes were observed, the mass differences between the complexes and the glycated analytes of interest would not generally interfere in the assay.

Decomposition of the samples on storage, with the formation of Cys and Cys-Gly addition products of the β-chain observed in the mass spectra, may also affect separations that are charge-dependent. However, these species do not occur at the same mass as the glycated chains and are not considered to be a possible source of interference in the mass spectrometric assay. The putative Cys and Cys-Gly addition products were not observed in samples that had not undergone prolonged storage.

Despite the existence of multiple glycosylation sites in both α- and β-chains *[13]*, only single glucose addition was observed by ES-MS. Careful comparison of the spectra from two samples having 13.5% and 14.5% GHb with those from two samples having ~5% GHb failed to positively detect diglycosylated species at values noticeably greater than background (~1%). We estimate that in these two highly glycated samples, the diglycosylated

species are probably <1% and are certainly <2% of the nonglycated α- and β-chains.

This result is perhaps not unexpected. Previous work *[13]* showed that each chain has several sites capable of being glycated with single glucose units in addition to the N-terminus of the β-chain. However, the N-terminus of the β-chain has the greatest probability of being glycated, the other sites having lower probabilities. For the two samples with high GHb, the ratio of monoglycated (G_1) to nonglycated (G_0) β-chain species was ~0.18 (by ES-MS). Simple probability theory for this ratio predicts that the ratio of diglycated (G_2) to G_0 species is at most 0.007. This calculation assumes that two glycation sites in the β-chain have equal probability of being occupied, which would give the maximum probability for the G_2 species. If one site has a lower probability than the other for adding glucose, then the G_2/G_0 ratio must be <0.007. For the less likely situations that three and four sites in the β-chain would have equal probabilities of being occupied by glucose, the maximum ratio of G_2/G_0 would be 0.011 and 0.012, respectively. For lower proportions of the G_1 species, the predicted amounts of the G_2 species rapidly decrease; e.g., for the case with two glycation sites having equal probability of being occupied and $G_1/G_0 = 0.12$ and 0.06, the predicted G_2/G_0 ratios would be 0.003 and 0.0008, respectively. For a given % GHb, the predicted G_2/G_0 ratios for the α-chain are about half those for the β-chain.

We suggest that proportions of G_2 species occurring at GHb values up to ~15% can be neglected. At higher values for %GHb, the G_2 species should become detectable, but this may be of only academic interest because higher glycation percentages are uncommon in our experience.

The analytical comparisons make clear that the chromatography-based procedures are in good agreement with both themselves and the ES-MS. From this we conclude that the analyte actually being measured is probably the same in all these instances, i.e., glycated hemoglobin. However, all the procedures have some problems in resolving potential interference signals, especially as observed in the nondiabetic patient group, whose GHb is in the 2.7% ± 0.5% range as measured by ES-MS. Several possibilities have been proposed to explain why these interferences are less prevalent in the diabetic population *[9]*.

Mass analysis of the globin chains may also be used to identify the presence of some hemoglobin variants in the same experiment, a procedure well documented in the literature *[15]* and demonstrated by the example in Fig. 6. ES-MS thus offers the potential for the simultaneous automated analysis of glycated and variant hemoglobins.

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