Clinical Chemistry 47:2
316–321 (2001)

Endocrinology and Metabolism

Long-Term Evaluation of Electrospray Ionization Mass Spectrometric Analysis of Glycated Hemoglobin

Norman B. Roberts,[1*] Alieu B. Amara,[1] Michael Morris,[2] and Brian N. Green[2]

Background: **Electrospray ionization mass spectrometry (ESIMS) has been successfully applied to the identification of hemoglobin (Hb) variants and the presence of glucose adducts (mass difference of 162 Da) on the separate Hb α and β chains. To establish the potential of ESIMS as a routine and/or a reference method for the quantification of glycohemoglobin (HbA1c), we carried out a detailed evaluation over a 4-month period in a routine laboratory environment.**

Methods: **We optimized a procedure using ESIMS suitable for the routine quantitative analysis of HbA1c. We determined reliability and reproducibility over 4 months and assessed the potential for automated sample injection. We then compared values of 1022 blood samples from diabetic patients with a routine HPLC-based ion-exchange procedure (HA-8140; Menarini).**

Results: **Results of HbA1c measurement by ESIMS were available within 3 min. The analytical imprecision (CV) was 1.6–5.0% for both manual and automated injections. Data collection over the m/z 980-1400 range confirmed lower glycation of the α chain relative to the β chain (0.66:1). Only one glycation was observed per globin chain. The overall glycohemoglobin (i.e., the average of α- and β-chain glycations) measured by ESIMS (x) on 1022 blood samples was lower than by HPLC (y): $y = 1.0432x + 0.4815$. However, the β-chain glycation measured by ESIMS was up to 20% higher than the value measured by ion-exchange HPLC and showed a close conformity, particularly at 5–10% HbA1c, with the ion-exchange Diabetes Control and Complications Trial (DCCT)-corrected and the United Kingdom National External Quality Assessment Scheme DCCT mean return values.**

Conclusions: **ESIMS provides a precise measurement of HbA1c and, in particular, glycation of the β chain. The method is robust and could be proposed as a procedure to substantiate HbA1c measurement and/or calibration.**

There are several different ways of measuring glycohemoglobin (HbA1c);[3] the longer term (months) glucose control marker *(1)* uses ion-exchange and affinity chromatography, electrophoresis, and/or immunologically based assays. Unfortunately, these separate procedures produce different analytical values *(2, 3)*. It is therefore widely recognized that both a reference method and a reference standard are required. To date, most assays report values in Diabetes Control and Complications Trial (DCCT) "equivalents" based on an ion-exchange procedure, the designated comparison method *(2)*. Although actual measurement of the glycation of the N-terminal valine of the β chain of hemoglobin (the fraction defined as HbA1c) is possible, this is not yet available as a routine procedure *(4)*.

Electrospray ionization mass spectrometry (ESIMS) has been successfully applied to the identification of hemoglobin (Hb) variants *(5)* and the measurement of glucose adducts on the α and β chains. A mass increase of 162 Da is observed incremental to the native protein chain and is assigned as the addition of glucose (mass, 180 Da) through elimination of water (−18 Da). ESIMS has been successfully applied to the quantification of this glucose addition to Hb as a measure of HbA1c *(6)*. However, establishing the potential of ESIMS as a routine and/or a reference method for HbA1c requires a more detailed

[1] Department of Clinical Chemistry, Royal Liverpool University Hospital, Liverpool L7 8XP, United Kingdom.
[2] Micromass (UK) Ltd., Wythenshawe, Manchester M239LZ, United Kingdom.
*Author for correspondence. Fax 44-0151-706-5813; e-mail n.b.roberts@liverpool.ac.uk.
Received October 5, 2000; accepted November 30, 2000.

[3] Nonstandard abbreviations: HbA1c, glycohemoglobin; DCCT, Diabetes Control and Complications Trial; ESIMS, electrospray ionization mass spectrometry; Hb, hemoglobin; and UKNEQAS, United Kingdom National External Quality Assessment Scheme.

evaluation over a longer period in a routine laboratory environment *(7)*.

We therefore assessed the reliability and precision of the measurement of HbA1c by ESIMS, in particular its potential for total automation, in a study that lasted several months.

Materials and Methods

CHEMICALS

All of the chemicals used were Analar grade (Sigma). Acetonitrile was HPLC grade (BDH Laboratory Supplies), and water was deionized grade (Elga Ltd). Quality-control samples used were Lyphocheck Whole Blood Diabetic Controls 1 and 2 with stated HbA1c values (ion-exchange chromatography) of 4.8% ± 1.0% and 8.8% ± 1.2% (mean ± SD), obtained from Bio-Rad Laboratories Ltd.

Blood samples were obtained from patients (n = 1022) with diabetes mellitus attending the Royal Liverpool University and Broadgreen Hospitals over a 4-month period. The routine analysis of these samples for HbA1c was part of the assessment of the patients' glycemic control and was carried out using a HI-AUTO, HA-8140 automated analyzer (Menarini). To correct ion-exchange values (x) to DCCT equivalents (y), a correction factor, $y = 1.02x + 0.87$ (supplied by Menarini using a certified method comparison), was used. Blood analysis by ESIMS was carried out anonymously and in accordance with the ethical regulations of the Royal Liverpool University Hospital.

MASS SPECTROMETRIC ANALYSIS

Whole blood, always less than 3 days old, was diluted 10-fold in deionized water and a further 50-fold in aqueous acetonitrile to yield a final aqueous solution containing 500 mL/L acetonitrile and 2 mL/L formic acid to form the working solutions. The working solutions were then introduced into a single quadrupole Platform LC mass spectrometer (Micromass) at flow rates of ~20 μL/min in 500 mL/L aqueous acetonitrile mobile phase. The instrument was run in the positive ion electrospray (ES+) mode, and a 3.5 kV potential was applied to the spraying capillary tip. The spray was desolvated using nitrogen gas at a source temperature of 80 °C. Eleven 5-s scans were summed over a *m/z* range of 980-1400 with an interscan time of 0.1 s and a total analysis time of 1 min. The mass scale of the instrument was externally calibrated using horse heart myoglobin (mass, 16 951.1 Da), and the acquired mass spectra were individually internally calibrated using human α chain (15126.4 Da) typically present in the samples, as described previously *(6)*. Automated injection of the diluted samples was also carried out using a Waters Alliance 2690 autosampler module (Waters UK Ltd). The flow program of the solvent management system allowed for an interinjection time of ~4 min, with an observed carryover from sample to sample of <1%.

The raw data were processed using the MassLynx data processing algorithms provided with the instrument. The signal-to-noise ratio observed for the raw data was typically in excess of 50 for the base peak in the spectrum [at *m/z* 1009.4 for $(\alpha+15H)^{15+}$ or *m/z* 992.7 for $(\beta+16H)^{16+}$]; the noise was measured at a point where no sample-related signals were observed (e.g., *m/z* 1280–1310). The algorithm used to transform the data was the MassLynx Transform algorithm. Although the routines are not explicitly named (Background, Subtract, Centroid, and Transform), their use is implicit in the text. Thus, after baseline subtraction, the raw data were transformed to a true molecular-weight scale and smoothed, and the heights of the peaks were used as a measure of their intensities. The ion intensities observed under electrospray ionization conditions are concentration dependent. Relative concentrations may therefore be measured by comparing the ion signals observed for the compounds of interest. However, the relative sensitivities of the α and non-α species might be expected to be slightly different because of the different compositions of the proteins. This was countered by tuning the conditions as outlined such that the signal intensities of the α-chains [$(\alpha+15H)^{15+}$, *m/z* 1009.4] and β-chains [$(\beta+16H)^{16+}$, *m/z* 992.7] were within 10% of each other. Any analyses outside these limits were discarded and the samples reanalyzed. This was checked by analyzing quality-control samples at the beginning, middle, and end of the daily run and to provide three daily sets of control values.

CALCULATION OF PERCENTAGE OF GLYCATION

The principles of the calculation of the percentage of glycation have been described previously *(6)*. In the absence of direct measurements of the sensitivities of many of the species apparent in the electrospray mass spectrum, the following assumptions were made to derive an expression to allow the percentage of glycation to be calculated from the experimental data:

1. All α-chain species, including noncovalent adducts, have the same sensitivity (same mass spectrometer response for a given molar concentration in the analyte). We believe that this is a reasonable assumption to make because these species are 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 are different (set to within 10%) and are related by: sensitivity of α-chain species = $K \times$ sensitivity of non-α-chain species (where K is constant for a given set of operating conditions).
4. The relative intensities 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. The most prevalent other non-α species present in apparently

healthy adults is the δ chain, which is ~2% of the β chain. However, at this concentration, glycation of the δ chain would be undetectable. On this basis, we chose to ignore any contribution from minor non-α species.

6. There is no contribution to the glycated species by multiple alkali metal adducts of the nonglycated species; desalting the sample does not affect the overall measurement *(6)*.

On the basis of these assumptions and the knowledge that the ESIMS analyte originates from whole-blood Hb, in which there are 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 GHb is glycohemoglobin, 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 simple expression means that only four peaks need to be measured from the transformed spectrum, but it is based on the assumptions outlined, many of which are difficult to prove empirically. Nevertheless, the close correlation between the ESIMS results and the results obtained with other techniques imply that the assumptions are not unreasonable.

Data comparisons were carried out using standard regression confirmed by Passing and Bablok analysis and Bland-Altman comparative analysis.

Results

A typical transformed mass spectrum is shown in Fig. 1, indicating the α and β chains and the glycated fractions. Only one glucose addition was seen at the respective chain mass + 162 Da, i.e., normal α chain at 15 288 Da and normal β chain at 16 029 Da, with no peak at mass + 324 Da (equivalent to two glucose molecules) at any HbA1c value in this study. A comparison of the α- and β-chain glycation with increasing HbA1c values confirmed the increased glycation of the β chain relative to the α chain ($\beta_g = 1.43\alpha_g + 0.87$; Fig. 2A). The difference between the two values increased consistently as glycation increased (Fig. 2B), with α-glycation always ~66% of the β-glycation.

The imprecision analysis in Table 1 shows good analytical performance during a typical working day (intraassay variation) and over the 4-month study period (interassay variation), with values of 1.6–5.0% for both manual and automated sample processing.

Comparison of the overall glycation determined by ESIMS and an ion-exchange chromatographic procedure showed good agreement (Fig. 3). The graphical statistical analysis indicated that values for HbA1c were on average 0.7% lower with ESIMS. Comparison of ESIMS β-glycation with DCCT-corrected ion-exchange values gave good agreement, particularly for 5–10% HbA1c (Fig. 4) with ESIMS showing an overall lower value of mean 0.4%.

The β-glycation values determined by ESIMS (y) show good agreement (Fig. 5) with the mean [United Kingdom National External Quality Assessment Scheme (UKNEQAS)] DCCT-aligned values (x) of 5.8%–12.0%. The correlation r^2 was 0.96 ($P < 0.001$) and the equation for the line was: $y = 0.7825x + 1.4761$ (n = 23). The ESIMS values were consistently higher at glycation values >10%.

Discussion

The data presented on the measurement of HbA1c confirm the overall reliability and reproducibility of ESIMS,

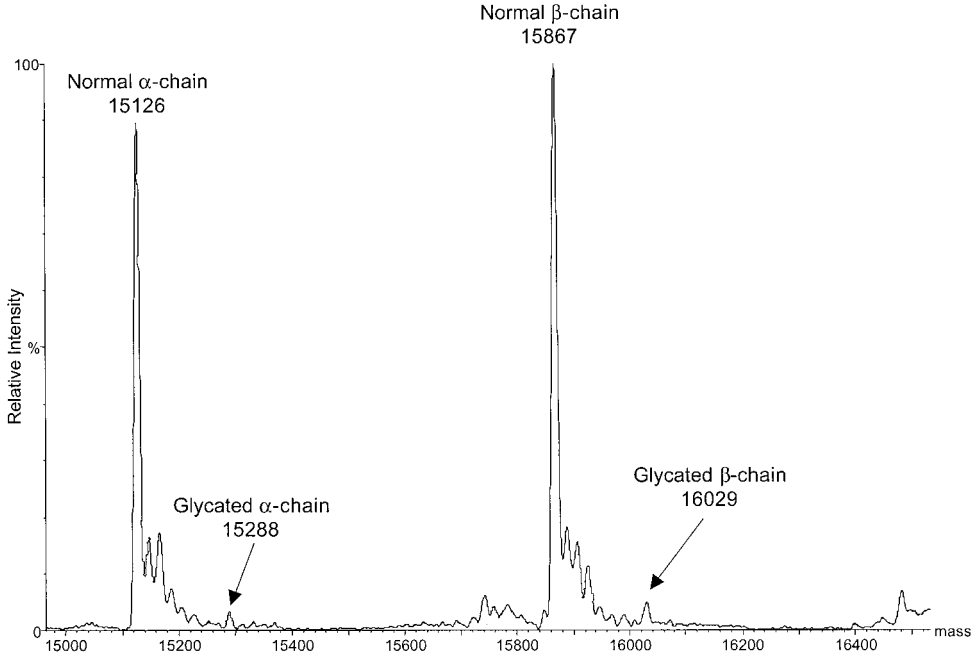

Fig. 1. Typical transformed spectrum showing α and β chains and the corresponding glycated fractions.

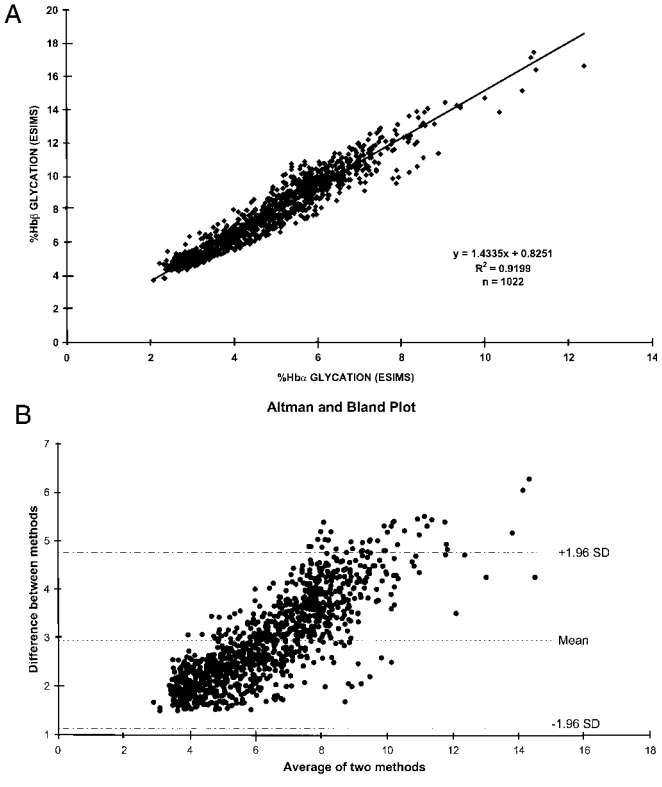

Fig. 2. Comparison between percentages of Hbα (*%Hb*α) and Hbβ (*%Hb*β) glycation, as measured by ESIMS (*A*) and Bland-Altman plot showing the differences between the methods (*B*).

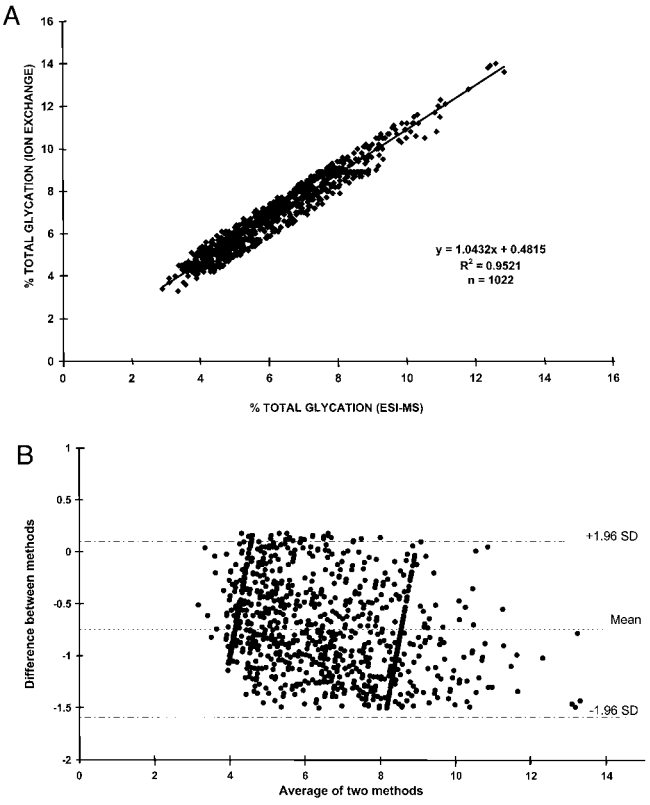

Fig. 3. Comparison of percentage of total glycation as measured by ESIMS vs ion-exchange chromatography (*A*) and Bland-Altman plot showing the differences between the methods (*B*).

thus satisfying the various criteria suggested *(7)* to substantiate the possibility of using this technique as a routine procedure. Indeed, the technique is well established as routine in the pharmaceutical industry and is now recommended for the complex analysis of amino acid abnormalities in the newborn *(8)*. However, for reliable glycated Hb analysis, the standard operating procedures we have outlined *(6)* must be strictly adhered to. Studies *(9)* where mass spectra were acquired over the *m/z* 600-1400 range showed substantial reduction in the signal intensity for β chain (particularly with the higher *m/z* ratios). In these reports, ESIMS was used to identify components obtained during chromatography, i.e., in a qualitative mode. Accordingly, if the intensities of the α and β chains are not within 10% of each other, then the measured β glycation will be grossly overestimated and an inappropriate value obtained. We have also confirmed that α-chain glycation is consistently less than β-chain glycation. The explanation for this needs further study to establish whether these differences are related to site-specific glycation, i.e., whether the N-terminal NH_2 of the α chain is the only site of glycation and, therefore, whether the other possible sites on the α chain are less accessible than those on the β chain.

Interestingly, the percentage of glycation of the β chain showed much closer agreement with the DCCT-corrected ion-exchange values, in particular, at 5–10% HbA1c. This would infer that the DCCT procedures are measuring the glycated fraction of the β chain. A similarity between an ESIMS-based assay for β chain glycation and ion-exchange chromatography for HbA1c values, in particular for concentrations between 6% and 10%, has also been observed *(4)*. These authors argued that the poor agree-

Table 1. Assessment of the analytical imprecision of ESIMS using manual and automated procedures.

Samples	IE[a]	Mean ± SD	CV, %
Interassay precision:[b] daily quality-control data over 4 months			
LI[c] (n = 25)	4.80	3.74 ± 0.16	4.3
L2 (n = 25)	8.80	7.85 ± 0.17	2.1
Intraassay precision: manual method			
Low[d] (n = 10)	4.3	3.96 ± 0.14	3.5
Medium (n = 10)	7.3	6.64 ± 0.11	1.6
High (n = 10)	12.9	10.91 ± 0.20	1.6
Intraassay precision: automated method			
Low (n = 10)	3.4	3.74 ± 0.19	5.0
Medium (n = 10)	8.4	7.41 ± 0.23	3.1
High (n = 10)	13.9	11.71 ± 0.20	1.7

[a] IE, ion-exchange values.
[b] Manual only.
[c] L1 and L2, Bio-Rad Diabetic Controls.
[d] Low, Medium, and High are from three different patients analyzed 10 times in 1 day.

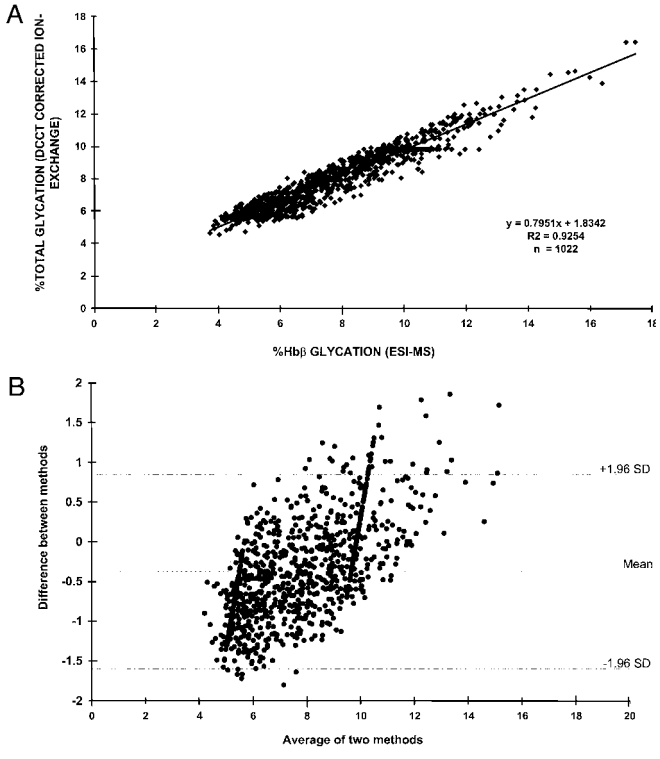

Fig. 4. Comparison of percentage of Hbβ glycation, as measured by ESIMS, with total glycation, as calculated using DCCT-corrected ion exchange results (*A*) and Bland-Altman plot showing the differences between the methods (*B*).

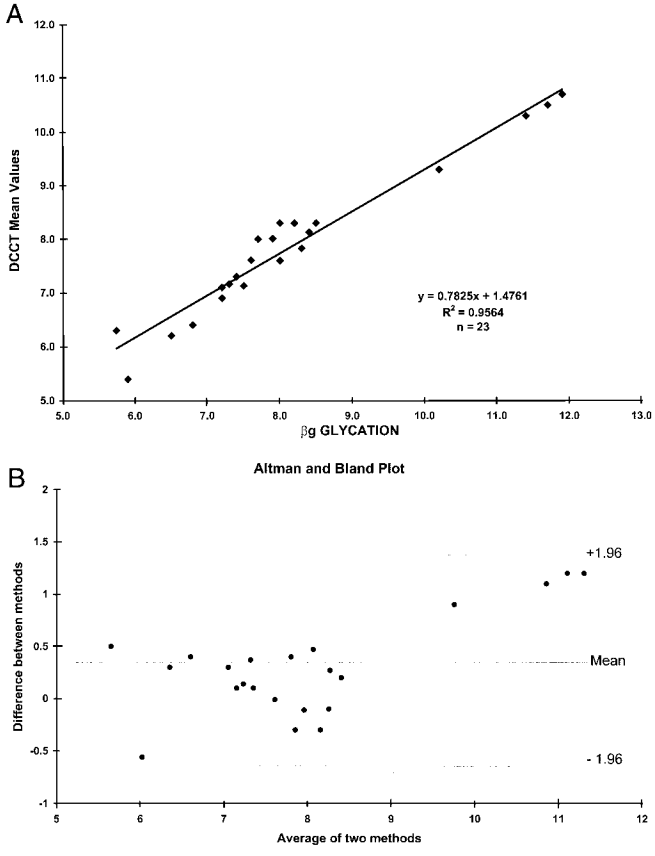

Fig. 5. Comparison of percentage of Hbβ glycation, as measured by ESIMS, and the mean DCCT-aligned HbA1c values from UKNEQAS (*A*) and Bland-Altman plot showing the differences between the two methods (*B*).

ment obtained at <5% HbA1c was probably attributable to impurities in the chromatographic peak causing higher values in high-performance ion-exchange chromatography, whereas the lower values obtained at >12% HbA1c suggested additional glycation of the ϵ-amine groups of lysine. This latter explanation, however, seems unlikely because this would be associated with a doubly glycated β chain, which we have never observed even at a high percentage of glycation (18%). It may be that higher glycation values somehow affect column separation efficiency, causing more HbA1c to co-elute with HbAo. The possibility that more than one glycation occurs at a detectable concentration was again not substantiated by our findings. Indeed, applying the following argument suggests that double glycation will be always low. If we assume that there two sites for glycation, then the possibility of seeing both sites occupied on one molecule can be expressed by the following binomial distribution. Suppose the two glycation sites, e.g., on the β chain, can randomly attach a glucose moiety with probabilities $p1$ on site 1 and $p2$ on site 2. The proportions of no glycation (0G) to one glycation (1G) to two glycations (2G) are $(1 - p1)(1 - p2)$ to $p1(1 - p2) + p2(1 - p1)$ to $p1p2$. The sum of these proportions is 1. The highest proportion of two glycations should occur when two sites have equal probability of being occupied, i.e., when $p1 = p2$, simplified as p. In this case, the probabilities of obtaining 0G:1G:2G are $(1 - p)^2$:$2p(1 - p)$:p^2. The highest proportion of one glycation, 1G/(0G + 1G), observed to date by ESIMS was 0.18, and in the above equation, the value of p that gives 0.18 on the β chain is 0.0989. Thus, p^2, the predicted proportion of double glycation, is 0.0098, or ~1.0%. If the second site has a lower probability of being occupied than the first, then the fraction of double glycations on the β chain will be reduced. Thus, even in the most favored conditions for two glycations occurring simultaneously, only 1% of the molecules will be doubly glycated even at the high single glycation of 18% on the β chain. To date, we have not been able to reliably detect double glycations at this percentage.

The analysis we have outlined measures the overall percentage of glycation of the β chain and not the percentage of glycation of the N-terminal valine. This is unlike the method proposed as a reference procedure for HbA1c, where the actual terminal glycated cleavage product from the β chain (as confirmed by ESIMS) is measured *(4)*. It is nevertheless interesting that there is such close agreement between our procedure for whole-blood analysis of the percentage of β-chain glycation by ESIMS and the DCCT-aligned values obtained by high-performance ion-exchange chromatography. This would imply that

validation of DCCT alignment could be confirmed by direct analysis of whole blood using ESIMS. If further analytical validation was required, comparative analysis with washed cells or even partially purified hemoglobin from cells could be carried out and a certified reference material produced. The question then arises as to how accurate is measurement by mass spectrometry. Clearly, instrumental conditions for the mass spectrometer must be properly defined to ensure that the signal intensities of the α and β chains are similar or the analysis will be only qualitative *(9)*. Quantitative agreement between whole-blood analysis of β-chain glycation by ESIMS and a DCCT-aligned chromatographic procedure for HbA1c was confirmed using data collected over the *m/z* 980-1300 range *(10)*. In addition, independent observations *(11)* using similar conditions have confirmed the reliability and robustness of ESIMS for measurement of HbA1c, whereas poorer agreement was observed using data collection over a larger range, *m/z* 700-1400 *(12)*.

The notion that the ESIMS technique is too sophisticated and/or expensive for most routine clinical laboratories may be an erroneous supposition. Indeed, although the overall capital cost is high, the actual running cost of reagents is very low (a few pence/cents), which means that the overall cost per test would be little different from current methodologies. Certainly the speed of analysis is not an issue because with automated analysis and calculation of data, the result can be available within 3 min. Measures are currently being investigated to reduce the cycle time, and analysis times approaching 2 min have been demonstrated after manual injection. However, the conditions for automation would need to be optimized further because initial data on analytical precision were relatively worse (although just acceptable) after automated compared with manual injection. Other benefits of ESIMS include the identification of Hb variants, although the classification of the amino acid alteration requires further detailed analysis *(13)*.

In conclusion, we have confirmed that accurate and reliable quantification of HbA1c is possible by ESIMS in a routine environment. The glycation of the α and β chains enables an overall HbA1c index to be measured. The separate β-chain glycation values obtained by ESIMS show good agreement with DCCT-aligned ion-exchange chromatography values, inferring that these different techniques are measuring a common analyte. Thus, the mass spectrometric technique, operated under the appropriate conditions, may be suitable as a reference method for β glycation and thus for DCCT alignment, particularly if previously used methods become unavailable.

References

1. DCCT 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;329:977–98.
2. John WG. Glycated haemoglobin analysis. Ann Clin Biochem 1997;34:17–31.
3. Weykamp CW, Penders TJ, Muskiet FAJ, Van der Slik W. Effect of calibration on dispersion of glycohemoglobin values determined by 111 laboratories using 21 methods. Clin Chem 1994;40:138–44.
4. Kobold U, Jeppsson J-O, Dulffer T, Fuike A, Hoelzel W, Miedema K. Candidate reference methods for hemoglobin A1c based on peptide mapping. Clin Chem 1997;43:1944–51.
5. 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, 1994:135–99.
6. Roberts NB, Green BN, Morris M. Potential of electrospray mass spectrometry for quantifying glycohemoglobin. Clin Chem 1997; 43:771–8.
7. Miedema K. Electrospray mass spectrometry for measurement of glycohemoglobin. Clin Chem 1997;43:705–7.
8. Rashed MS, Bucknall MP, Little D, Awad A, Jacob M, Alamandi M, et al. Screening blood samples for inborn errors of metabolism by electrospray tandem mass spectrometry with a microplate batch process and a computer algorithm for automated flagging of abnormal profiles. Clin Chem 1997;43:1129–41.
9. Peterson KP, Pavlovich JG, Goldstein D, Little R, England J, Peterson CM. What is hemoglobin A1c? An analysis of glycated hemoglobin by electrospray ionization mass spectrometry. Clin Chem 1998;44:1951–8.
10. Rocks BF, Morris M, Cooper D, Iversen SA. Automated measurement of glycated hemoglobin by mass spectrometry. In: Proc ACB National Meeting. London: Association of Clinical Biochemists, 1999:112.
11. Nakanishi T, Mizaki A, Kishikawa M, Yasuda M, Tokuchi Y, Kanada Y, Shimizu A. Quantification of glycated hemoglobin by electrospray ionization mass spectrometry. J Mass Spectrom 1997;32: 773–8.
12. Lapolla A, Fedele D, Plebani M, Garbeglio M, Seraglia R, D'Alpioso M, et al. Direct evaluation of glycated evaluation of glycated and glyco-oxidized globins by matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom 1999; 13:8–14.
13. Witkowska HE, Bitsch F, Shackleton CH. Expediting rare variant hemoglobin characterization by combined HPLC/electrospray mass spectrometry. Hemoglobin 1993;17:227–42.