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Development of an isotope dilution mass spectrometry assay for HbA1c based on enzyme-cleaved peptide analysis

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Abstract

HbA1c is an index of control in diabetes patients. We report a highly reproducible measurement method for HbA1c based on analysis of the enzyme-cleaved peptide by electrospray ionization mass spectrometry using deuterium-labeled synthetic peptides as internal standards. Intra- and inter-assay coefficients of variation for the novel method ranged from 1.23 to 1.99% for samples with high and low HbA1c. Using this method, we clarified the extent of discrepancies among the indices of diabetes measured by conventional methods and the ESI method for clinical samples including those from patients with Hb variants. High-performance liquid chromatography (HPLC) methods for most samples with variants underestimate the true HbA1c value, although a few variants give a positive error for HbA1c. Immunoassays may also underestimate the values, if the reactivity of the antibody is low against the glycated N-terminal of the variant β-chains by conformational change. The method proposed here is an important step to establish a candidate definitive method, and is also useful in assessing specific HbA1c test systems using samples containing Hb variants.

Keywords: Enzyme-cleaved peptide analysis; Hemoglobins

1. Introduction

Stable isotope dilution mass spectrometry (ID-MS) incorporates a precise method that has proved successful for the measurement of small molecules, including cholesterol [1], cortisol [2], and dioxin [3]. However, its use for macromolecules such as proteins is less developed due to the difficulty in ionization. Recently, owing to the progress in soft ionization MS, the study of macromolecules has been simplified and quantification of peptides and proteins has also been performed by ID-MS [4–6]. An excellent quantitative analysis method for C-peptide, which is generated from proinsulin by enzyme cleavage in vivo and serves as an important index of insulin secretion during insulin therapy for diabetic patients, has been established by electrospray ionization-isotope dilution-liquid chromatography mass spectrometry (ESI-ID-LC-MS) [6]. This method could provide a basis for a reference measurement procedure for C-peptide analysis.

HbA1c, originally designated as a fraction separated by Amberlite IRC-50 cation-exchange resin [7], is now defined as hemoglobin (Hb) that is irreversibly glycated at the N-terminal valine of the β-chain [8], and serves as a useful index in the monitoring of glucose control in patients with diabetes [9]. Various analytical methods for measuring glycated-hemoglobin in human blood are currently being used by clinical laboratories, based on affinity chromatography, cation-exchange chromatography, or immunoassay. Each method is calibrated using reference materials, which are adjusted to an international reference material. Unfortunately, considerable discrepancies in the measured values of HbA1c have been observed among methods and among laboratories [10]. The International Federation of Clinical Chemistry (IFCC) proposed the approved reference method for measurement of HbA1c, applying either capillary zone electrophoresis or the LC-ESIMS [11]. The LC-ESIMS assay was devised by Kobold et al. [12], based on LC-ESIMS analysis of the glycated and non-glycated N-terminal hexapeptide of the Hb β-chains, which are released by enzymatic cleavage of the intact Hb molecule with endoproteinase Glu-C. We have used the LC-ESIMS method, however, assay values in repeated tests of the same solution

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fluctuated greatly, depending on the condition of the mass spectrometer. To improve the reproducibility of measurement, we propose a method for HbA1c measurement based on peptide analysis by ESIMS using deuterium-labeled synthetic peptides as internal standards. The HbA1c values measured by high-performance liquid chromatography (HPLC), immunoassay, and glycated albumin values measured by enzyme assay [13] were compared with the values obtained by the method proposed in the present paper for clinical samples including those from patients with various Hb variants. Part of this work was preliminarily published as a technical briefs [14].

2. Materials and methods

2.1. Materials

We purchased four kinds of chemically synthesized peptide, consisting of six amino acids of the N-terminus hemoglobin β-chain, from Peptide Institute Inc. (Osaka, Japan): VHLTPG (Lot No. 749-901201), 1-deoxyfructosyl-VHLTPG (480709), VHL*TPG (740-102051), and 1-deoxyfructosyl-VHL*TPG (740-101295). L*: is deuterium (D)-labeled with isopropyl-D_7:leucine, $(CD_3)_2$–CD–CH_2 –$CH(NH_2)$COOH. The homogeneity of the peptides was ascertained by HPLC: 99.1, 99.3, 99.4 and 99.5%, respectively. Endoproteinase Glu-C (Lot No. PBIO 160-016, an enzyme that specifically cleaves the carboxyl side of glutamic acid residue), was purchased from PE Biosystems Co. (Foster City, CA, USA). Other reagents were purchased from Nakalai Tesque (spectrophotometric grade, Kyoto, Japan) and used without further purification.

Clinical samples from 52 subjects, including both normal persons and diabetic patients, were analyzed, of which 22 were homozygous for HbA, and 30 were heterozygous for various Hb variants (see Table 1). The structures of most Hb variants were determined by MS and by DNA analysis in our laboratory, primarily to elucidate the cause of the unexpected values of HbA1c measured by HPLC. Some of the studies on the structure of Hb variants were published [15–18].

2.2. Measurement of the percentage of HbA1c by mass spectrometry by using deuterium labeled internal standard

Approximately 3 mg of each peptide was weighed and dissolved in distilled water to adjust the concentration to

Table 1
Amino acid substitution of samples with variant hemoglobins

Case no.	Variant hemoglobin		Amino acid substitution		HbA1c (%)			Glycated albumin (%)
					HA8150	DCA2000	ID-MS	
1	I-Interlaken		α15	G → D	2.9	4.5	5.3	
2	Le Lamentin	1	α20	H → Q	2.8	4.8	4.2	14.9
3		2	α20	H → Q	3.9	5.0	5.0	
4	Shaare Zedek		α56	K → E	1.1	4.9	3.9	
5	J-Meerut		α120	A → E	4.7	5.2	4.9	15.9
6	Okayama		β2	H → Q	21.9	5.5	5.4	
7	G-Coushatta		β22	E → A	2.7	4.7	4.5	14.1
8	Hoshida	1	β43	E → Q	2.2	5.2	5.0	14.3
9		2	β43	E → Q	1.4	5.1	4.0	14.5
10		3	β43	E → Q	1.4	5.4	4.6	8.8
11	Hokusetsu		β52	D → G	5.8	11.0	9.3	
12	Hamadan	1	β56	G → R	1.4	4.7	4.3	16.5
13		2	β56	G → R	2.1	4.9	5.0	13.3
14		3	β56	G → R	2.4	5.2	5.3	14.4
15		4	β56	G → R	1.4	4.4	4.2	14.3
16	Hikari		β61	K → N	3.2	4.8	4.4	16.4
17	G-Szuhu		β80	N → K	2.7	4.5	4.3	15.4
18	Moriguchi		β97	H → Y	1.1	4.6	4.8	
19	Yoshizuka		β108	N → D	2.8	5.0	4.3	13.5
20	Shizuoka		β108	N → H	4.9	5.6	5.3	13.5
21	Peterborough		β111	V → F	2.3	3.8	4.4	22.3
22	Stanmore		β111	V → A	3.7	4.1	4.0	13.9
23	Masuda		β114/119	L → M, C → D	2.5	4.8	4.1	20.7
24	Riyadh	1	β120	K → N	6.8	11.5	10.9	23.6
25		2	β120	K → N	3.1	4.8	4.5	20.2
26		3	β120	K → N	1.4	6.3	6.5	17.4
27	Yamagata		β132	K → N	35.3	3.6	2.7	
28	K-Woolwish		β132	K → Q	33.2	5.7	5.9	
29	Sagami		β139	N → T	0.4	1.1	3.6	15.4
30	Mito		β144	K → E	39.9	12.1	10.6	

10 pmol/μl, and the peptide solutions were mixed in the desired molar ratios. As internal standards, we added a mixture of labeled glycated and labeled non-glycated peptides to test samples, that is 10 μl containing 10 pmol labeled-glycated and 100 pmol of labeled-non-glycated peptide. To measure the calibration curve, the internal standard was added to the solution containing various ratios of synthetic non-labeled glycated and non-labeled non-glycated peptides. For calibration with synthetic peptides, 100 μl solutions containing 200 pmol non-glycated hexapeptide and 0, 5.0, 9.5, 14.0 and 17.5 pmol glycated peptide (both were non-labeled with isotope) were measured. For calibrators, Kobold et al. used Hb solution prepared by mixing isolated HbA1c and HbA_0 [12,19]. To compare the calibrators, we also measured reference materials kindly donated by the IFCC Working Group for HbA1c intercomparison study by our ID-MS method (see "Acknowledgments"). Standard hemoglobin purchased from Kokusai Reagent Co. Ltd. (Kobe, Japan) with high (noted value 11.1%) and low (5.5%) values of HbA1c were analyzed. This sample was standardized to the certified reference material. Samples from controls, patients with diabetes mellitus, and those with Hb variants were also analyzed.

The digestion of Hb by Glu-C was performed according to the method of Kobold et al. [12]. Briefly, Glu-C (ca. 50 μg) dissolved in 25 mmol/l ammonium acetate buffer was added to the hemoglobin solution (ca. 1 mg), and incubated for 18 h at 37 °C. For measurement of HbA1c with Hb solution, the internal standard containing labeled glycated and non-glycated peptides (1:10 molar ratio, 10 μl above mentioned solution) was added to the Hb solution after digestion with endoproteinase, Glu-C.

ESI-MS was carried out in positive ionization mode using a TSQ7000 triple stage quadruple mass spectrometer with a conventional ESI source (ThermoQuest, San Jose, CA, USA). The HPLC system was an Ultrafast Microprotein Analyzer (Michrom BioResources Inc., Pleasanton, CA, USA) with a reversed phase microcolumn (ZORBAX-SB-CN, 0.5 mm × 150 mm, 5 μm). The ES ion source was run with 0.4 MPa nitrogen. Sheath gas and nitrogen auxiliary gas were used at an HPLC flow rate of 40 μl/min. Spray voltage was 4.5 kV, and transfer capillary temperature was 200 °C. The mass spectrometer was tuned and calibrated with Met-Arg-Phe-Ala and horse muscle apomyoglobin mixture. To compare the signal intensities of the deuterium-labeled and unlabeled peptides, mixtures of equal moles of each labeled and unlabeled peptide were analyzed. Univalent ions for 4 peptides were selected for ion monitoring, *m*/*z* 695.4 for non-glycated non-labeled hexapeptide (HD0), 702.4 for non-glycated-labeled hexapeptide (HD7), 857.4 for glycated non-labeled hexapeptide (GD0), and 864.4 for glycated-labeled hexapeptide (GD7). The peak area of selected ion monitoring of each elution profile was measured, and the ratio of glycated to non-glycated peptides was calculated by the following equations:

$$\text{the ratio of glycated/non-glycated hexapeptide} = \frac{1}{10} \times \frac{\text{GD0/GD7}}{\text{HD0/HD7}}$$

$$\text{the percentage of glycated peptide} = \left\{ \frac{\text{ratio}}{1 + \text{ratio}} \right\} \times 100.$$

2.3. Measurement of HbA1c and glycated albumin by conventional methods

For immunoassay we used the DCA2000 kit from Bayer Diagnostics (Pittsburgh, PA), which measures the intact glycated β-chain of hemoglobin without proteolytic digestion, according to the manufacturer's instructions. For HPLC to measure HbA1c, a Hi-AUTOA1c HA-8150 HbA1c analyzer (Arkray, Kyoto, Japan) was used and operated according to the manufacturer's instructions. Glycated albumin was measured by the method using albumin-specific proteinase and ketoamine oxidase, developed by Asahi Kasei Corporation (Shizuoka, Japan) [13].

3. Results

3.1. ESI spectra of the standard peptide mixture, and selected ion chromatogram for measurement of samples

An equal-mole mixture of labeled and unlabeled non-glycated peptides and those of the glycated peptides were analyzed by LC-ESIMS. A wide range of LC peak scans was summed for calculation. Fig. 1 shows the ESI spectra of non-glycated (Fig. 1a), and glycated (Fig. 1b) peptides. The signal intensities of the same deuterium-labeled and unlabeled peptides in the mixture were almost equivalent. Fig. 2 shows selected ion mass chromatograms of a case (clinical sample) to measure HbA1c by ESI-ID-LC-MS. Clear symmetric peaks without any overlapping signals are seen, suggesting the accuracy of this measurement method. The elution positions of the four peptides are slightly different, which suggests the peak area of the ion chromatogram is suitable for calculation, rather than peak height.

3.2. Calibration curves using calibrators of peptides and hemoglobin standard solution

Calibration curves by the measurement of both peptide calibrators (Fig. 3, solid line) and by hemoglobin standard solution of IFCC (Fig. 3, dotted line) were highly linear over a wide range of glycated components. Both were superimposed. The observed values (*y*-axis) were calculated by the equation described above, and all data points are means of five experiments ± S.E. (bars) for peptides calibrator and means of two experiments for hemoglobin calibrator. Parameters for calibration lines are shown in the figure legend.

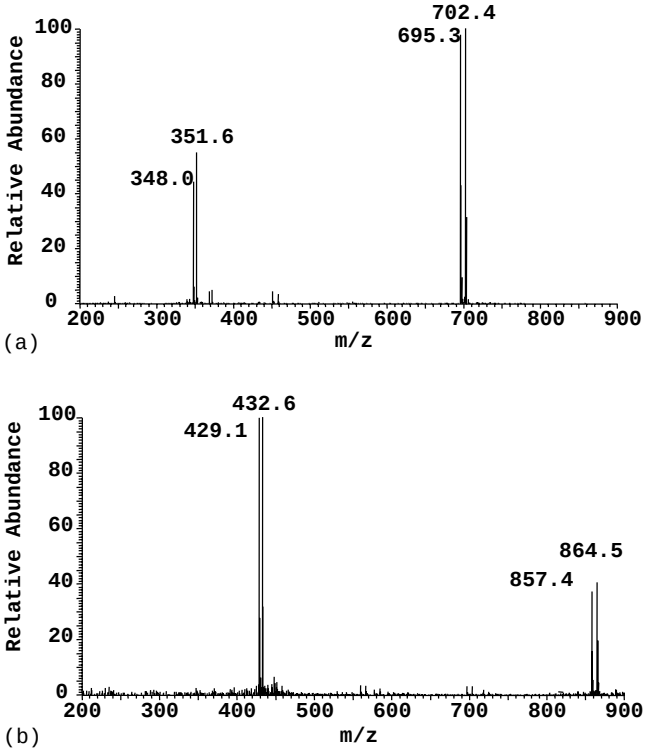

Fig. 1. Comparison of the signal intensities of the deuterium-labeled and unlabeled peptides with the same sequence. Mixture of equal moles of the non-glycated labeled and non-labeled peptides (a), and mixture of equal moles of glycated labeled and non-labeled peptides (b). Each mixture was analyzed by LC-ESIMS.

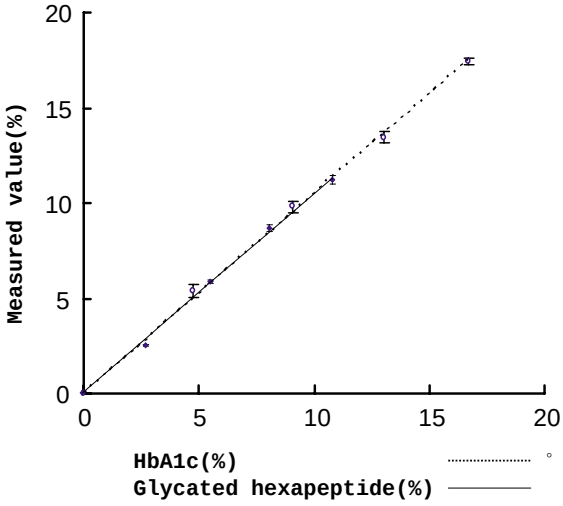

Fig. 3. Calibration curves of the percentages of the observed peak area of the glycated peptide with the molar percentages of the glycated peptides prepared by mixing synthetic glycated and non-glycated peptides (solid line), and with HbA1c values of Hb standard solution provided by IFCC working group for HbA1c standardization (dotted line). The peak intensity (%) of glycated peptide was measured by the present ID-MS method using deuterium-labeled internal standards. The percentage was calculated by the equation described in the text; *x*-axis: the % of glycated peptide/total peptides (solid line), the HbA1c (%) of the target values certified by IFCC working group (dotted line); *y*-axis: the peak intensity (%) of the glycated peptide measured by ID-MS method using labeled internal standards. All data points are means of five experiments ± S.E. (bars) (peptide calibrator); all data points are means of two experiments (hemoglobin calibrator). The regression equations for the calibration line are: peptide calibrator $y = 0.975x + 0.11\%$ ($R^2 = 0.988$), hemoglobin calibrator $y = 1.049x + 0.09\%$ ($R^2 = 0.998$).

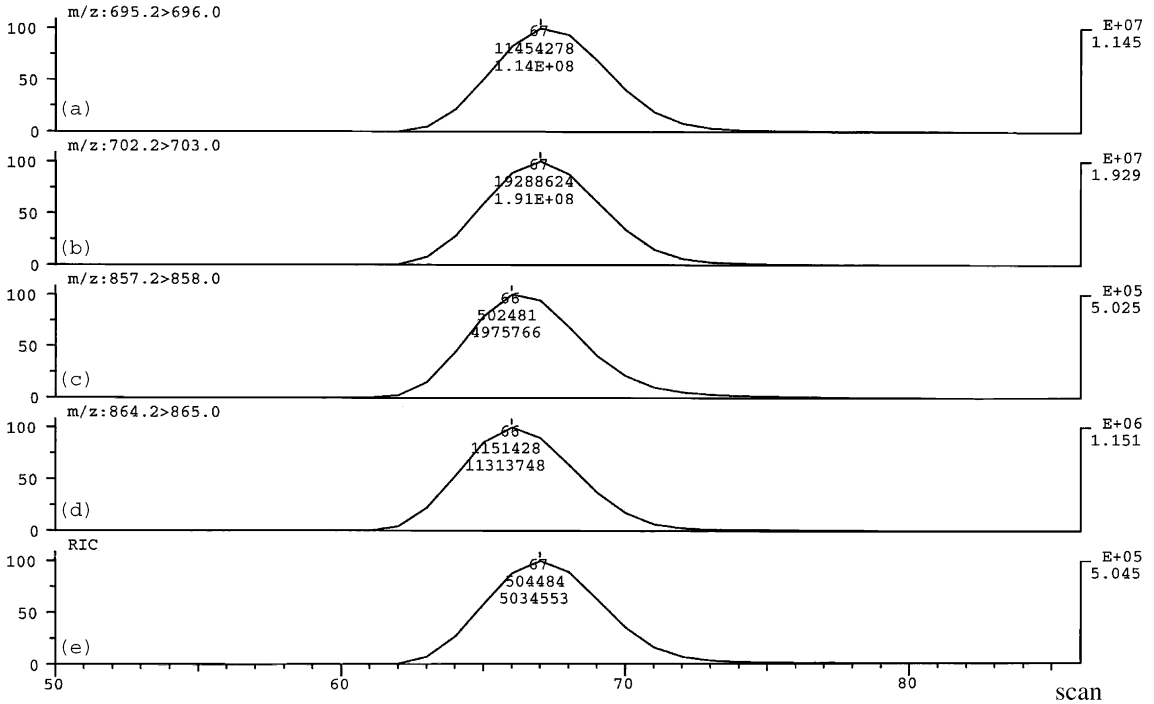

Fig. 2. Selected ion mass chromatogram of a case (clinical sample), HbA1c measured by ESI-ID-LC-MS.

The observed values calculated by the former calibration curve and those by the latter calibration curve were almost the same. For measurement of clinical samples, HbA1c was calculated by the calibration curves obtained by peptide calibrators.

3.3. Intra- and inter-assay coefficient of variation (CV)

The measurement of standard solution (Kokusai, Kobe, Japan) showed an intra-assay CV of 1.99% for low HbA1c control sample ($N = 10$, mean HbA1c = 4.38%) and 1.23% for high HbA1c control sample ($N = 10$, mean HbA1c = 8.73%). The inter-assay CVs were 1.82 and 1.42% ($N = 5$, mean HbA1c = 4.34 and 8.81%, respectively).

3.4. Comparative study of clinical samples including those with Hb variants

The percentage obtained by the ESI-ID-LC-MS analysis and the values obtained by a conventional HPLC method and immunoassay were compared (Fig. 4a and b). The correlation coefficient of both conventional methods for samples without variant Hb was good, and the slope was close to 1. We applied ESI-MS to identify hemoglobin variants, and to assess the effect of the variants on routine measurements of HbA1c.

Comparative studies of a variety of HbA1c test systems on samples containing hemoglobin variants revealed discrepancies of varying degrees. In most samples containing variant Hb, HPLC divides glycated Hb into two fractions, glycated HbA and glycated variant Hb, resulting in underestimation of HbA1c. For Hb Okayama, Hb Yamagata, Hb K-Woolwish, and Hb Mito, the HbA1c value obtained by HPLC was much higher than that by ID-MS because the variant components comigrated with the HbA1c fraction of HPLC.

Some variants also yielded considerably different values by immunoassay (DCA2000) from those obtained by ID-MS. For example, as we suggested previously [21], in Hb Sagami [β139Asn → Thr] (see Fig. 4b, no. 29), the value (1.1) obtained by repeated immunoassay (DCA2000) was significantly lower than that obtained by ID-MS, 3.6. It is possible that the reactivity of the antibody is relatively low against the glycated N-terminal of the variant β-chains by

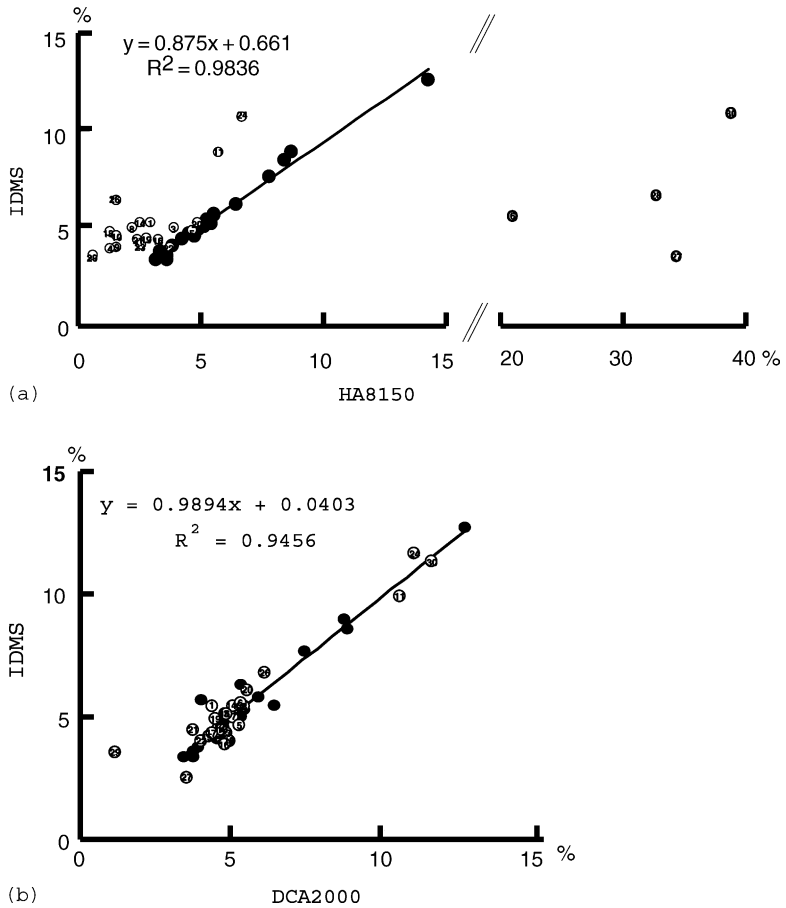

Fig. 4. Correlation between the percentage obtained by conventional methods and the value obtained by the method using ID-MS. Deming regression analysis of results for homozygous HbA samples is shown in the figures: (●) results for homozygous HbA; (○) samples heterozygous for Hb variant and HbA. (a) Comparison between the percentage obtained by ID-MS and that by an HPLC method (HA-8150). (b) Comparison between the percentage obtained by MS and that obtained by the immunoassay, DCA2000. Numbers in circles correspond to that of no shown in Table 1.

conformational change.Glycated albumin was also measure for clinical samples, and the values are shown in Table 1. The correlation between the values of glycated albumin and HbA1c by ID-MS was low (see Section 4).

4. Discussion

4.1. Calibration line

We previously reported [20–22] on the method utilizing monitoring with both univalent and divalent ions, although Kobold et al. [12] used only divalent ion and we used only univalent ion in the present paper. We chose univalent ions for monitoring because noise signals were much lower by monitoring with univalent ions than with divalent ions.

The measured values assayed on different days using the same solution by the method using isotope-labeled internal standards were constant, but those not using isotope varied widely in CV from 5 to 100%. The measured values scattered depending on the condition of the equipment. The use of isotope-labled internal standards is indispensable to establish reference method for HbA1c measurement.

The enzyme cleavage of HbA and HbA1c is not controlled via internal standardization. We estimated that the cleavage is equal for both forms of Hb. However, the standard Hb and HbA1c, which are labeled with stable isotope at the N-terminal part, are prerequisites for a measurement procedure at this level of the hierarchy. When the cleavage is proved to be always equal to Hb and HbA1c, the method based on peptide analysis by ESI-MS using deuterium-labeled synthetic peptides as internal standards may offer a reference method to assess HbA1c measurement.

4.2. HbA1c and abnormal Hb

Many variant Hbs have been found during the clinical test of HbA1c by the HPLC procedure, which is widely applied, especially in Japan. Without considering the possibility of error by Hb variant, attending physicians might choose the wrong index for their patients. We previously reported [21] the comparison among routine methods and MS method for samples containing Hb variants by the method without isotope-labeled internal standards. However, we occasionally obtained erroneous values by the previous method. By the method proposed in the present paper, we can obtain reliable data irrespective of the conditions of the mass spectrometer. Assessment of HbA1c using ESI-ID-LC-MS might help physicians correctly select the measurement method.

4.3. Glycated albumin and HbA1c measured by ESI-ID-LC-MS method

These clinical samples were also analyzed for the percentage of glycated albumin. We expected that the values of subjects with variant Hbs measured by the methods proposed here would correlate with glycated albumin percentage, and that the correlation would be better than those between HbA1c values obtained by conventional methods and the ID-MS method. However, the correlations between the values of glycated albumin and HbA1c by ID-MS were weaker than those between HbA1c values obtained by HPLC or immunoassay and by ID-MS, which was similar for samples with and without Hb variants. The possible causes of this unexpected data include: (1) difference of the half life of Hb and albumin, (2) errors in glycated albumin values for sera containing Hb by hemolysis. We cannot tell at the present time whether glycated albumin measurement is suitable as an index for control diabetes patients who have Hb variants.

5. Conclusion

The method proposed here for HbA1c measurement based on peptide analysis by ESI-MS using deuterium-labeled synthetic peptides as internal standards, is an important step to establish a candidate reference method for HbA1c. Labeled peptides are stable, can be stored for years, and are easily distributed anywhere in the world. We are ready to provide labeled peptides to laboratories with a strong interest in improving the standardization of HbA1c measurement. Secondly, by ESI-ID-LC-MS measurement of HbA1c in patients who have Hb variants, attending physicians can choose the proper control index for their patients.

Acknowledgements

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