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Determination of ionization efficiency of glycated and non-glycated peptides from the N-terminal of hemoglobin β-chain by electrospray ionization mass spectrometry

Toyofumi Nakanishi, Akira Shimizu*

Department of Clinical Pathology, Osaka Medical College, 2–7 Daigakumachi, Takatsuki, Osaka 569-8686, Japan

Abstract

We compared the ionization efficiency of glycated and non-glycated peptides for the HbA1c measurement method developed by Kobold et al. [Clin. Chem., 43 (1997) 1944] based on LC–ESI-MS analysis of the N-terminal peptides of the β-chains released by cleavage of the hemoglobin with endoproteinase Glu-C. Taking half the peak area of the doubly charged ion and adding it to the area of the singly charged ion, we determined that the slope of the resulting calibration curve was nearly equal to 1, and the reproducibility of the added values was better than the values calculated by the doubly or the singly charged ion alone.

Keywords: Peptides; Hemoglobin β-chain

1. Introduction

HbA1c, which is defined as hemoglobin (Hb), that is irreversibly glycated at the N-terminal valine of the β-chain, is an important index in the monitoring of glucose control in patients with diabetes [1]. Various analytical methods for measuring glycohemoglobin in human blood are currently being used by clinical laboratories, methods based on affinity chromatography, cation-exchange chromatography, or immunoassay. Unfortunately, considerable discrepancies among methods have been observed [2]. To standardize these assays, Kobold et al. [3] reported a high-level reference method for measuring HbA1c, based on liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) 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. There are two functional groups with proton accepting ability in both peptides. One is N in 1-deoxyfructosyl-NH-Val of the glycated peptide and NH_2-Val of the non-glycated peptide, and the other is N in the imidazole ring of both peptides. Kobold et al. [3] calculated the ratio of glycated to non-glycated peptides by monitoring the peak areas of doubly charged ions of both peptides. The ratio of the singly to doubly charged ions was considerably different between the glycated and non-glycated peptides, and varied slightly even among analyses of the same peptides. In the present paper, we examined the calculation method of the peak ratio of glycated to non-glycated peptides using both singly and doubly charged ions instead of the calculation of the intensities of the singly or doubly

*Corresponding author. Tel.: +81-726-846-448; fax: +81-726-846-548.

E-mail address: shimizu@poh.osaka-med.ac.jp (A. Shimizu).

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charged ions alone. We used synthetic glycated and non-glycated hexapeptides for this experiment.

The intensity of the singly and doubly charged ions of the N-terminal heptapeptides released from β-chains of an abnormal Hb (Hb Niigata, β1Val→Leu with complete retention of the initiator Met and 24% acetylation of the amino terminal Met) [4] was compared to examine the proton affinity of N-terminal free, glycated and acetylated amines. In addition, the method proposed in the present paper was applied to assess a conventional high-performance liquid chromatography (HPLC) method.

2. Experimental

2.1. Materials

Non-glycated and glycated hexapeptides, Val–His–Leu–Thr–Pro–Glu, and 1-deoxyfructosyl–Val–His–Leu–Thr–Pro–Glu (Lot Nos. 749-901201 and 480709, respectively), were chemically synthesized by Peptide Institute (Osaka, Japan). The homogeneity of the peptides was ascertained by HPLC. This was 99.1% and 99.3%, respectively, and both contained trifluoroacetic acid, the content of which was 15.9% and 24.8%, respectively. Approximately 3 mg of each peptide was weighed and dissolved in distilled water to adjust the concentration to 90 pmol/μl. Both peptides were mixed in the desired molar ratios.

Immobilized endoproteinase Glu-C, Poroszyme (Lot No. PBIO 160-016, an enzyme that specifically cleaves the carboxyl side of glutamic acid residue), was purchased from PE Biosystems (Foster City, CA, USA). Other reagents, which were purchased from Nakalai Tesque (Kyoto, Japan), were spectrophotometric grade and used without further purification.

2.2. LC–ESI-MS

The MS system was a TSQ7000 triple stage quadrupole mass spectrometer with a conventional electrospray ion source (Finnigan MAT, San Jose, CA, USA). The HPLC system was Ultrafast Microprotein Analyzer (Michrom BioResources, Pleasanton, CA, USA) with a reversed-phase microcolumn (Vydac C_{18}, 150 mm×0.5 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. The acquisition mode was set to full scan to confirm the homogeneity of the synthetic peptides and to determine the ratio of singly and doubly charged ions. For determination of the ratio of glycated to non-glycated peptides, selected ion monitoring at m/z 348–349 and 429–430 for the divalent, and m/z 695–696 and 857–858 for the univalent ions of non-glycated and glycated peptides were performed. The various methods for calculation of the ratio of peak intensity of ion chromatogram of both peptides were compared. These were (1) peak height of singly charged ion, (2) peak area of singly charged ion, (3) peak height of doubly charged ion, (4) peak area of doubly charged ion, (5) to assess both ions, the following equation was used, 0.5× peak area of doubly charged ion+1×peak area of singly charged ion. The detector gain of TSQ 7000 was adjusted to obtain peak intensities proportional to the molar quantity of ions [5]. Therefore, the peak intensity of doubly charged ion of a peptide was theoretically twice that of the singly charged ion of the same amount of the same peptide. The total mole of molecules protonated on either one or two sites was expected to be measured by the above equation. The molar ratios of both peptides for given mixtures were plotted against the ratio of the peak intensity calculated by these methods.

2.3. Preparation of peptides from blood with an abnormal hemoglobin, Hb Niigata and clinical samples

Hemolysate and globin were prepared by the method previously reported [6]. Globin (ca. 1 mg) was dissolved in 1 ml of 50 m*M* ammonium acetate buffer (pH, 4.0), and 50 μl of the solution was mixed with roughly 375 μg of Porozyme and incubated for 4 h at 42°C. An aliquot (5 μl) of the supernatant of the mixture was applied onto the LC–ESI-MS system. Peak intensity of the ion chro-

matogram was calculated by the equation described above, and HbA1c percentage was calculated as follows: [Gpi]/([Gpi]+[nGpi]), where [Gpi] and [nGpi] represent the peak intensity of glycated and non-glycated peptides, respectively. Clinical samples, including normal and diabetic samples, were analyzed by the MS method, and by conventional HPLC. A Hi-AUTOA1c HA-8121 HbA1c analyzer (Kyoto Daiichi, Kyoto, Japan) was used for HPLC, and operated according to the manufacturer's instructions.

The structure of β-N-terminal peptides released by the enzymatic digestion were: Val–His–Leu–Thr–Pro–Glu, and 1-deoxyfructosyl–Val–His–Leu–Thr–Pro–Glu from normal Hb. In addition to these peptides, Met–Leu–His–Leu–Thr–Pro–Glu, acetyl–Met–Leu–His–Leu–Thr–Pro–Glu and 1-deoxyfructosyl–Met–Leu–His–Leu–Thr–Pro–Glu were released from Hb obtained from a heterozygote with Hb Niigata [4].

3. Results

3.1. Full scan ESI spectra and selected ion chromatogram of the synthetic non-glycated and glycated peptides

Full scan ESI mass spectra of the synthetic glycated and non-glycated hexapeptides are shown in Fig. 1 insets. The spectra showed that the specimens were almost homogeneous. The observed mass at m/z 348.4 and 695.4 (inset top) corresponded to doubly and singly charged ions of non-glycated hexapeptide, respectively. The observed mass at m/z 429.3 and 857.4 (inset bottom) corresponded to doubly and singly charged ions of glycated hexapeptide, respectively. The peak height ratio of doubly to singly charged ion of non-glycated peptide was ca. 1.7:1, but that of glycated peptide was ca. 5:1. Ion chromatograms using selected ion monitoring mode of the mixture of equal moles of synthetic non-glycated and glycated hexapeptides are shown in Fig. 1. For non-glycated peptide, doubly charged ion, m/z 348–349, and singly charged ion, m/z 695–696, were monitored. For glycated peptide, doubly charged ion, m/z 429–430, and singly charged ion, m/z 857–858, were monitored. The ratio of peak area of doubly to singly charged ion of both peptides was similar to the ratio of peak height of full scan spectra (insets). These data showed that the ionizing efficiency of both peptides was considerably different.

3.2. Calibration curves for the ratio of the peak intensity with the molar ratio of peptides

Fig. 2 shows calibration curves for the ratio of the peak intensity of the selected ion chromatogram of both peptides with the molar ratio of peptides prepared by mixing synthetic peptides. The values calculated by the peak area (solid line) and that calculated by the peak height (dotted line) of the same ion chromatogram were compared. The ratio of the peak height of singly charged ion and that of doubly charged ion are shown in Fig. 2a and b, respectively. All data points are means of five experiments±standard error (SE). The reproducibility calculated by the peak area was better than that calculated by the peak height. The average standard deviation (SD) over the entire curve for the peak area of singly charged ion was 1.7%, that for the peak height of singly charged ion was 2.8%, that for the peak area of doubly charged ion was 1.8%, and that for the peak height of doubly charged ion was 2.5%. The slope of the calibration curve using doubly charged ion was ca. 2.0 (both by area and by height), and that using singly charged ion was ca. 0.5. The value of the slope may reflect the difference in ionization efficiency between the peptides.

Fig. 3 shows the calibration curve for the ratio of the ion peak area calculated by the equation described above with the molar ratio of the peptides prepared by mixing synthetic glycated and non-glycated hexapeptides. The reproducibility of each point determined by the calculation with the equation was better than those determined by singly or doubly charged ions alone. The average SD over the entire curve for the peak area ratio calculated by the equation was 1.3%. The slope of the calibration line calculated by the equation was nearly 1, meaning that the ratio of the observed ion intensity was almost identical to the mixing molar ratio of both peptides.

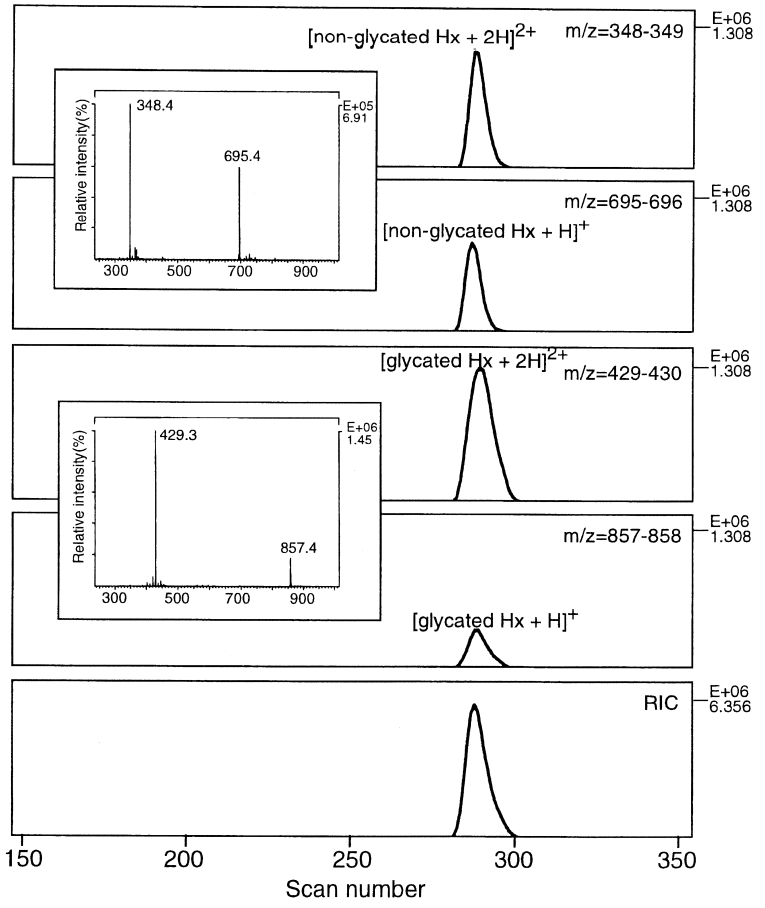

Fig. 1. Ion chromatogram using selected ion monitoring mode of the mixture of equal moles of synthetic non-glycated and glycated hexapeptides. For non-glycated peptide, doubly charged ion, m/z 348–349, and singly charged ion, m/z 695–696, were monitored. For glycated peptide, doubly charged ion, m/z 429–430, and singly charged ion, m/z 857–858, were monitored. Full scan ESI spectra of synthetic non-glycated (top), and glycated (bottom) peptides are shown in insets. m/z 348.4: Doubly charged ion, m/z 695.4: singly charged ion of non-glycated hexapeptide and m/z 429.3: doubly charged ion, m/z 857.4: singly charged ion of glycated hexapeptide.

3.3. Measurement of Hba1c from clinical samples

The percentage obtained by the LC–ESI-MS analysis ($n=45$) and the value obtained by a conventional HPLC method were compared (Fig. 4). The correlation coefficient was good (0.99), and the slope was 0.92. The intercept of the y axis was -0.85.

3.4. Ionizing efficiency of heptapeptides from Hb Niigata

The intensity of the singly and doubly charged ions of the N-terminal heptapeptides cleaved from β-chains of Hb Niigata was examined. The observed mass at m/z 420.9 and 840.7 corresponded to doubly and singly charged ions of non-glycated heptapeptide, respectively. The observed mass at m/z 501.9 and 1002.5 corresponded to doubly and singly charged ions of glycated heptapeptide, respectively. The observed mass at m/z 441.9 and 882.8 corresponded to doubly and singly charged ions of acetylated heptapeptide, respectively. The ratio of peak intensity of doubly to singly charged ions of non-modified heptapeptide was ca. 3:1, that of glycated peptide was ca. 4:1, and that of acetylated peptide was ca. 0.4:1.

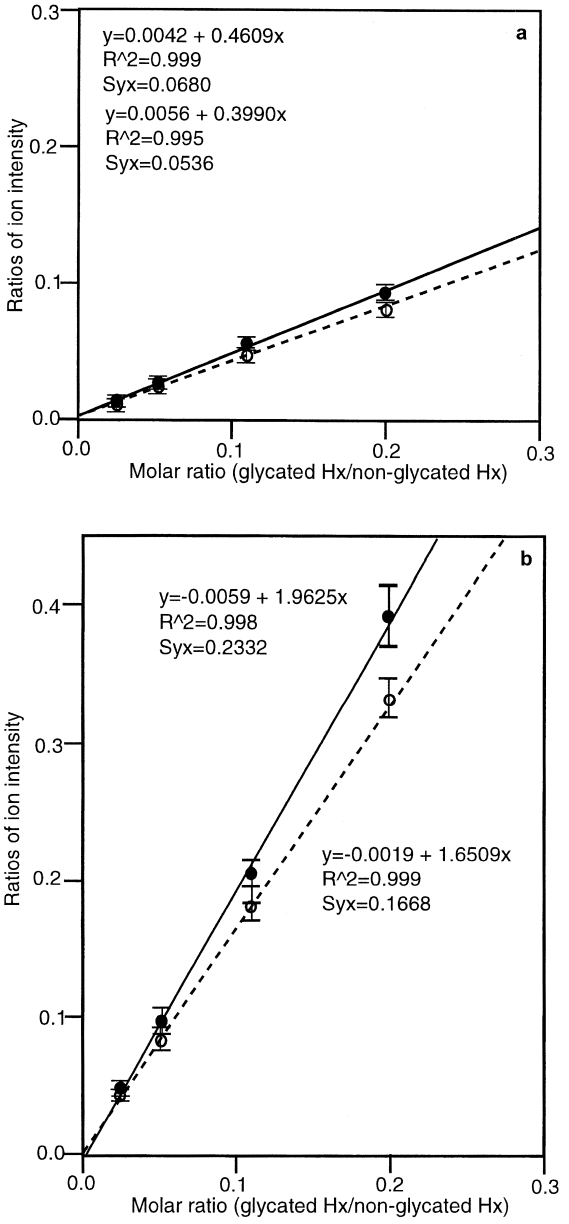

Fig. 2. Calibration lines for the observed peak intensity ratio with the molar ratio of peptides prepared by mixing synthetic glycated and non-glycated hexapeptides. Hx: Hexapeptide. Glycated/non-glycated Hx ion peak area (solid line) and peak height (dotted line) of the singly charged ion (a), and the doubly charged ion (b) are shown. All data points are means of five experiments±SE (bars). Parameters for calibration lines are shown in the figure.

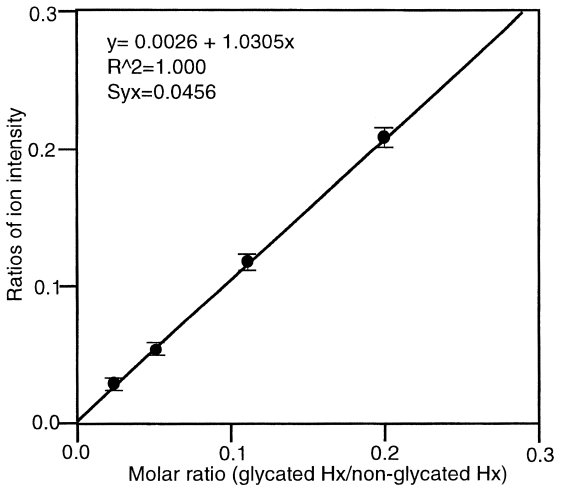

Fig. 3. Calibration lines for the ratio of peak intensity with the molar ratio of peptides prepared by mixing synthetic glycated and non-glycated hexapeptides. Hx: Hexapeptide. Glycated/non-glycated Hx ion peak area was calculated by the equation, 0.5× peak area of divalent ion+1×peak area of monovalent ion. All data points are means of five experiments±SE (bars). Parameters for calibration lines are shown in the figure.

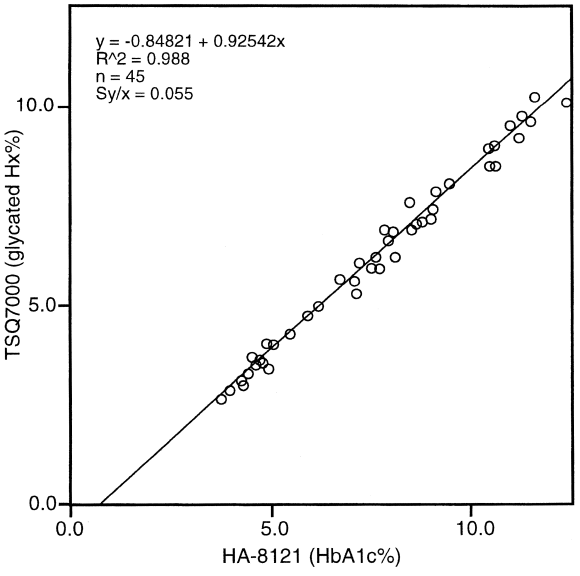

Fig. 4. Correlation between the percentage obtained by conventional HPLC method and the value obtained by the method using LC–ESI-MS with Glu-C enzyme digests and calculated by the proposed equation with singly and doubly charged ions.

4. Discussion

4.1. Calibration line

The ratio of the observed peak area of glycated to non-glycated peptides calculated by adding half intensity of doubly charged ion to full intensity of singly charged ion gave good values. The reproducibility of the ratio calculated by this method was better than those calculated either with the peak area or the peak height of only doubly charged ion or only singly charged ion. The ratio obtained by this method almost coincided with the molar ratio of each mixture of both peptides. Therefore, under the MS conditions applied, the factor (ratio of peak intensity of both peptides in a given mixture)/(the molar ratio of both peptides in the mixture) was almost 1.

4.2. Comparison of the intensity of the doubly and the singly charged ions

The ratio of peak intensity of the doubly to the singly charged ions showed a rather large difference between glycated and non-glycated peptides. The doubly charged ion was ca. five-times of the singly charged ion in the ion chromatogram of the glycated hexapeptide. In contrast, it was ca. 1.7-times for the non-glycated hexapeptide. The difference of the ratio may have been caused by the different ionization efficiency of the N-terminal amine. If so, the ionization efficiency of N-terminal amine was in the order, glycated>non-modified. The spectra of heptapeptides from Hb Niigata showed that the order of the relative intensity of doubly charged ion of the glycated and non-modified heptapeptide coincided with those of hexapeptides from normal Hb. However, the relative intensity of the doubly charged ion of the acetylated heptapeptide (0.4:1) was much less than the glycated (4:1) or non-modified (3:1) forms. The ionization efficiency of N-terminal amine was in the order, glycated>non-modified>>acetylated forms. It may be commonly accepted that NH_2- is more easily protonated than R-NH– in ESI, as in the solution [7]. Thus the order should be non-modified>acetylated, glycated, which was different from the observed result. Glucose, possibly the hydroxy group of the molecule, may promote the protonation of the neighboring N.

4.3. Comparison of the measurement methods

The regression line of the percentage of HbA1c obtained by the proposed calculation versus HbA1c measured by routine HPLC analyzer showed good correlation (0.994), and good slope value (0.925). The intercept of the *y* axis was rather large (−0.85), and HbA1c values measured by the conventional HPLC method were ca. 20% (10–30%, depending on the HbA1c level) higher than those measured by MS. This was probably due to substances co-eluting in the HbA1c peak during routine HPLC.

Although more experiments under different analytical conditions, or using other MS instruments, are needed, we expect that the slope of calibration line is always 1 under standard MS conditions. The calculation method we propose in the present paper may offer a unique reference method for Hb A1c. Furthermore, this method may be widely applied for accurate measurement of the ratio of a peptide and its modified form in various biological specimens. A part of this work was reported as a rapid communication in a Japanese journal [8].

5. Conclusion

The glycated peptide formed doubly charged ions preferentially, unlike the non-glycated molecule. Under the MS conditions applied, the ratio of the sum of half of the doubly charged peak area and the full singly charged peak area of the glycated to non-glycated peptides was almost equal to the molar ratio of the peptide mixture. The reproducibility of the ratio was better than that calculated with the singly or doubly charged ions alone. The calculation method proposed here may offer a more accurate and precise measurement of HbA1c by LC–ESI-MS.

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