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Optimization of Microwave-Assisted Method for Accelerated Glycated Hemoglobin Quantification from Amino Acids to Proteins

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Abstract : Glycated hemoglobin (HbA_{1c}) has been commonly used to screen and diagnose for patients with diabetes mellitus. Here the accelerated procedure of microwave-assisted sample treatment from acid hydrolysis to enzyme digestion followed by isotope dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) was optimized and applied to measure HbA_{1c} in an effort to speed up analysis time. First, two signature peptides of HbA_{1c} and hemoglobin A_0 were certified with amino acid analysis by setting optimized acid hydrolysis conditions to 150°C, 1.5 h and 10 μM sample concentration in 8 M hydrochloric acid. Consequently, the accurate certified peptides above were used as calibration standards to implement the proteolytic procedure with endoproteinase Glu-C at 37°C, 700 W for 6 h. Compared to the traditional method, the microwave heating not only shortened dramatically sample preparation time, but also afforded comparable recovery yields. The optimized protocol and analytical conditions in this study are suitable for a primary reference method of HbA_{1c} quantification with full SI-traceability and other similar proteins in complex biological samples.

Keywords : glycated hemoglobin, microwave-assisted methodology, isotope-dilution mass spectrometry, acid hydrolysis, enzyme digestion, diabetes mellitus

Abbreviations : SI, International System of Units; CRM, certified reference material

Introduction

Glycated hemoglobin (HbA_{1c}) describes a stable minor hemoglobin component formed by the non-enzymatic interaction of glucose with the N-terminal amino acid (AA) valine in the β-chain of hemoglobin.[1,2] The use of HbA_{1c} as an indication to measure chronic glycaemia rather than instantaneous blood glucose levels is a promising alternative test for monitoring diabetic patients.[3]

Throughout continuous efforts and modifications from the establishment of the International Federation of Clinical Chemistry (IFCC) Working Group[4] to the development of an isotope dilution-mass spectrometry (ID-MS) method,[5] a HbA_{1c} measurement procedure of highest metrological order has been achieved. This measurement was based on ratio calculation of glycated to non-glycated β-N-terminal of hemoglobin cleaved by enzyme endoproteinase Glu-C.[4] Both proteolytic hexapeptides were analyzed by isotope dilution-ultra performance liquid chromatography–tandem mass spectrometry (ID-LC-MS/MS) with synthetic hexapeptides and their isotopic analogues as reference materials, and internal standards, respectively. Prior to using of those peptides, the concentration of the peptide standard solutions was, in turn, certified with amino acid analysis (AAA) via acid hydrolysis using AAs as primary reference materials, and isotope labeled AAs as internal standards as well.

Generally, sample preparation is recognized as a bottleneck in the entire analytical procedure. Not only for the two-stage SI-traceable quantification method for HbA_{1c}, consequently, this step should be more straightforward to accustom to the fast advancements. The introduction of microwave radiation energy offers several benefits over the conventional thermal reaction method in remarkable reduction of reaction time from hours to minutes, minimization of sample contamination, safe, and

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automation.[6-8] Microwave reactions occur under the effect of electromagnetic field of high frequency on a molecular level in contrast with convection currents and heat transfer used in conventional oven.[9] This fundamental mechanism of microwave amounts to boost up the molecular collisions and hence faster interactions. The outstanding applications of microwave irradiation as a heating method to biologically relevant processes have been recognized.[10]

In this study, rapid microwave-assisted methods for both acid hydrolysis and proteolytic digestion are investigated to perform full traceability of glycated hemoglobin measurement using ID-LC-MS/MS. Each parameter in both stages was optimized in the manners of reaction efficacy and stability. The reliability of proposed procedure was confirmed by result equivalency with the traditional oven method.

Experimental

Chemicals and analytes

Formic acid (FA) and methanol (MeOH) were from Merck & Co. (Kenilworth, NJ, USA) and Honeywell B&J (Muskegon, MI, USA), respectively. Hydrochloric acid (HCl) was purchased from Junsei (Tokyo, Japan). Ammonium acetate (AmAc), trifluoroacetic acid (TFA), sodium chloride (NaCl) were all purchased from Sigma-Aldrich (St.Louis, MO, USA), and endoproteinase Glu-C (Glu-C) was obtained from Roche Diagnostics (Rotkreuz, Switzerland). All other chemicals and reagents used were of high analytical grade and obtained from commercial sources. The 3 AAs produced by National Metrology Institute of Japan (Tsukuba, Japan) with purity over 99% were used as reference materials: L-proline (Pro), L-valine (Val), and L-leucine (Leu). The following isotope-labeled AAs, L-proline (U-$^{13}C_5$, 98%; ^{15}N, 98%; Pro*), L-valine (U-$^{13}C_5$, 98%; ^{15}N, 98%; Val*), and L-leucine (U-$^{13}C_6$, 97-99%; ^{15}N, 97-99%; Leu*) were used as internal standards and purchased from Cambridge Isotopes Laboratory (Andover, MA, USA). The water purified from a standard in-house Millipore Alpha-Q water purification system (Millipore, Billerica, MA, USA) was used to prepare all aqueous solutions, and solvents were filtered through a disposable membrane filter (0.2 μm pore size) under vacuum.

Both synthetic hexapeptides VHLTPE (Hexa) and Glc-VHLTPE (G-hexa), and corresponding stable isotope-labeled hexapeptides VH[$^{13}C_6$, ^{15}N]LTPE (Hexa*) and G-VH[$^{13}C_6$, ^{15}N]LTPE (G-hexa*), with claimed purity over 95%, were synthesized by Peptron (Daejeon, South Korea).

The two hemolysate CRMs with different levels of HbA_{1c} (111-01-013, 111-01-014; Korea Research Institute of Standards and Science (KRISS), Daejeon, South Korea) were used to confirm reliability of proposed procedure. Moreover, real clinical sample of fresh whole blood was kindly provided by Chungnam National University hospital (Daejeon, South Korea) to investigate commutability of proposed procedure. The sample was collected with EDTA anticoagulant.

Peptide hydrolysis

The microwave hydrolysis system (CEM corp., Matthews, NC) is designed to perform acid hydrolysis of samples under finely controlled energy. A mixture of 200 μL of internal standard and working sample solutions was placed into microvials (part No. 908265, CEM corp.) and dried under vacuum prior to putting into the microwave system. In operation, approximately 10 mL of 8-M HCl was placed in the bottom of a Teflon® PFA vessel. The microvials were inserted into a micro vial tray setting inside the vessel. The vessel was flushed with nitrogen, tightly sealed, and connected with proper fiber optic thermometer; then, hydrolysis was conducted for 1.5 h at 150°C. Following completion of a hydrolysis cycle, the samples were dried again under vacuum for evaporation of HCl. The residues were reconstituted in 200 μL of distilled water, filtered through disposable syringe filters, and finally injected into LC-MS system for analysis. All preparation steps for pre-operating microwave oven were carefully manipulated.

Enzyme digestion

The frozen blood samples were equilibrated to room temperature for 1 h. Followed by a ten-fold dilution with distilled water, 20 μL of working sample were transferred to a 0.5 mL vial and spiked with 60 μL of 100 mM AmAc buffer (pH 4.0) enriched with isotope-labeled internal standards. Total volume of 100 μL solution was then made after adding 0.2 mg/mL endoproteinase Glu-C. The digestion was carried out using the Rapid Enzyme Digestion System (Hudson Surface technology, NJ, USA) at 37°C for 6 h with microwave power of 700 W. In order to stop the digestion, the solutions were frozen at -70°C for 2 more hours. The samples were thawed again and injected into LC-MS system. Cleaning steps were added to discard cell debris in whole blood prior to enzyme digestion, if necessary. Although the above optimized protocol has also been proposed for increased digestion rate upon classic method,[11] it is clear that the time required for cleavage of peptide linkages using microwave heating was considerably shortened by 67%.

LC-MS conditions

An ACQUITY series UPLC system connected in-line to a Xevo TQ-S electrospray ionization triple quadrupole-mass spectrometer (Waters, Massachusettes, USA) was used to perform chromatographic separation. Data acquisition and system control were computerized by analytical software MassLynx™ (Version 4.1). For identification and quantification, positive ion multiple reaction monitoring (MRM) mode was used and all

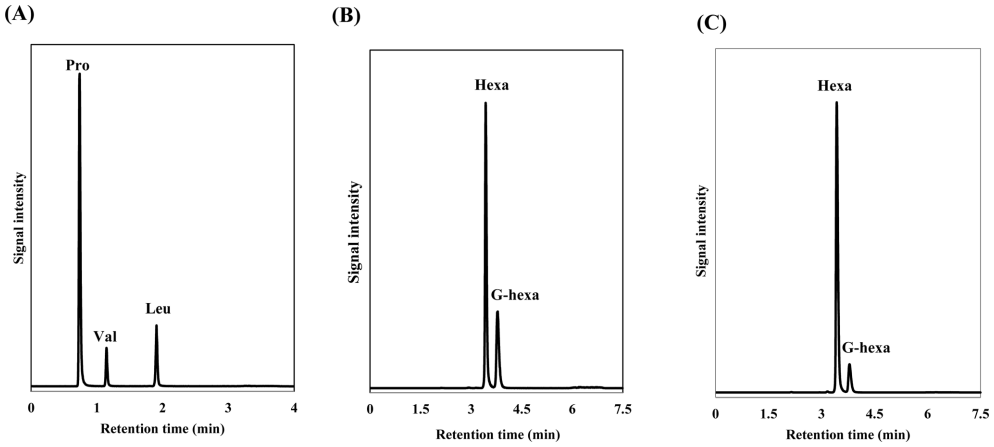

Figure 1. Typical total ion chromatograms (TICs) of (A) three AAs at 20 μmol/kg, (B) hexapeptides in the standard solutions at 40 μmol/kg, and (C) hexapeptides in the whole blood sample treated by proposed procedure.

operating conditions were well tuned and optimized prior to sample analysis.

To achieve the chromatographic separation of individual AAs, 1 μL injections were made onto a ACQUITY UPLC C_{18} column (1.7 μm, 100 × 2.1 mm i.d., Waters, Massachusettes, USA) coupled with a guard column (security Ultra, Phenomenex) at 40°C. Solvents A (0.3% (v/v) TFA in 10 mM AmAc) and B (0.3% (v/v) TFA in 50% (v/v) ACN) were used as mobile phases. The separation was obtained at a flow rate of 400 μL/min with a gradient program that allowed for 2.5 min at 95% mobile phase A followed by a 1 min step that reduced mobile phase A to 70%. The column was then washed with 20% of mobile phase A for 1 min before returning to initial conditions and re-equilibrating for 1 min. The precursor-to-product ion transitions were monitored for Pro (116.03 > 70.04), Pro* (122.06 > 75.05), Val (118.07 > 72.05), Val* (124.08 > 77.06), Leu (132.08 > 86.07), and Leu* (139.10 > 92.12).

For the determination of Hexa and G-hexa, 3 μL of analytes were injected into a KINETEX C_{18} column (2.6 μm, 50 × 2.1 mm i.d., Phenomenex, Torrance, CA, USA) and eluted at a flow rate of 400 μL/min at 40°C. Mobile phase A and B consisted of 0.1% (v/v) FA in both water and MeOH, respectively. The separation was obtained with a linear gradient elution from 95:5 (A:B) to 80:20 (A:B) for 5.5 min followed by a 1 min cleaning step with 20:80 (A:B) and re-equilibration for 1 min. The ion transitions *(m/z)* were as follows: Hexa (695.35 > 350.00 and 450.99), G-hexa (857.59 > 558.83 and 803.05), Hexa* (702.29 > 356.99 and 458.35), and G-hexa* (864.57 > 565.87 and 810.03).

Results and discussions

Separation of AAs and peptides

Figure 1A shows the typical total ion chromatogram (TIC) for all three selected AAs (Pro, Val, and Leu) using the optimized conditions described above. All the analytes were satisfactorily eluted without any interference within a gradient time of 4 min. A cleaning step in the gradient program for each injection and a blank sample after 20 sample injections were taken to eliminate possible contaminants. All standard curves varied linearly over the concentration range from 5 to 100 μmol/kg. Good peak shape, stable retention time, and resolution led to confidence that the method is robust and reproducible.

High peak intensities of both isotope-labeled and unlabeled hexapeptides without any overlapping signals are observed in the Figure 1B, C. Hexa was eluted at 3.43 min with G-hexa separated at 3.73 min of run time. The requirements of stable peak ratios and linear calibration curves were fulfilled for the accurate and precise outcomes. Effects which refer to reduced detector response such as ion suppression was carefully examined (data not shown).

Optimization of the hydrolysis conditions

Microwave-assisted vapor phase acid hydrolysis represents a strategy for potentially decreasing reaction time to less than a few hours from days. Figure 2 shows measured values of three AAs (Pro, Val, and Leu) with respect to the applied parameter changes of temperature, time, acid concentration, and sample concentration in both Hexa and G-hexa. At least 3 independent experiments in each condition were progressed to check precision of hydrolysis. The *y*-axis represents ratio of measured value-to-certified value by using oven.

As presented in Figure 2A, the first factor concerned was temperature. Elevated temperature has been considered as a critical parameter to increase the hydrolysis rate.[12] Nevertheless, the unwanted effects of temperature such as heat loss or excessive temperature might ruin several heat-labile AAs during the microwave radiation period. Good

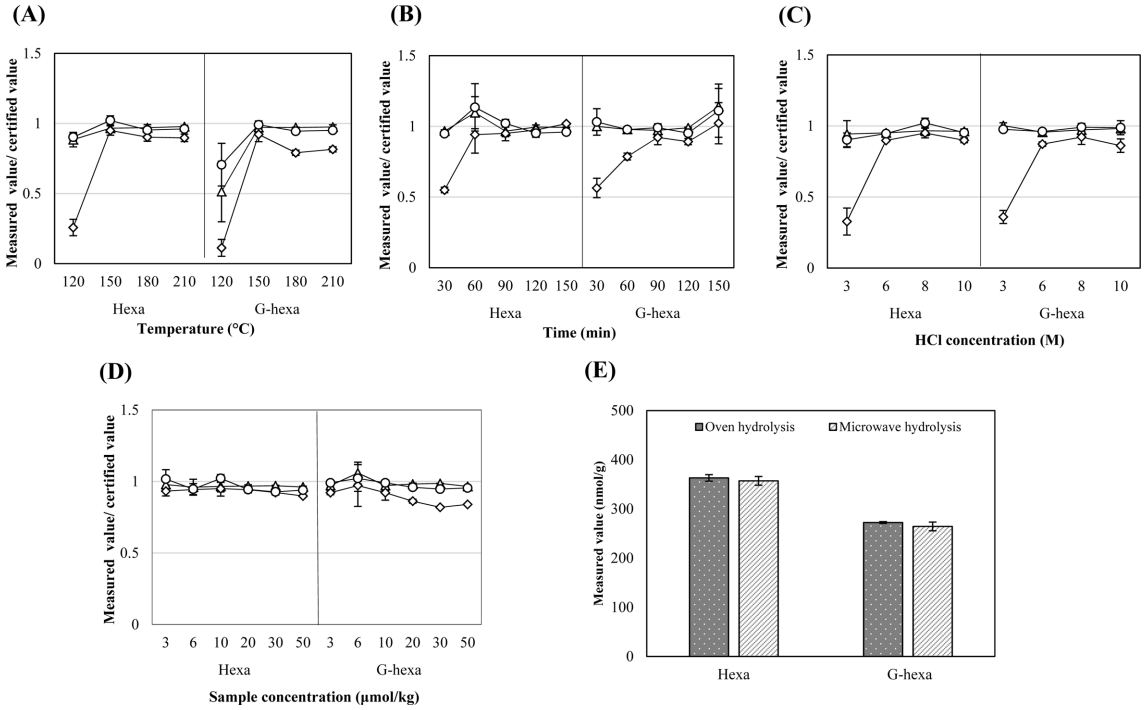

Figure 2. The effect of hydrolysis conditions for the microwave-assisted acid hydrolysis. (A) Temperature, (B) time, (C) HCl concentration, and (D) protein concentration on the release of AAs. Symbols: △, Pro; ◇, Val; ○, Leu. (E) Comparison of microwave and conventional hydrolysis method for both Hexa and G-hexa. The *y*-axis represents ratio of measured value-to-certified value by traditional method.

reproducibility of residues in proper temperature, therefore, is much more crucial. According to the recommended temperature (150°C) by the manufacturer, the highest recovery yields were obtained. The drop from 150°C to 120°C accounted for the fall in recovery of all residues in both peptides, notably a drastic reduction in Val. Release of the AAs was apparently complete during microwave hydrolysis at high temperature (over 150°C), with the exception of Val in G-hexa due to incomplete hydrolysis by linkage of glucose. Additionally, increasing the temperature enhanced the variability between the samples for each analyte (< 2%).

Comparing with conventional hydrolysis method, the benefit of shorter reaction time performed in microwave-assisted hydrolysis was investigated with 30 min increments from 30 to 150 min (Figure 2B). The yield rates of all AAs were markedly lowest at the 30 min time points, while extending time profile from 90 to 120 min afforded the greater improved recoveries and agreement of residues. This time point range seems to be optimal as it can cleave most of peptide bonds and show less loss.

As illustrated in Figure 2C, acid concentrations did not significantly affect the release of Pro and Leu but there was a trend for Val concentration to be maximized with high concentrated HCl. Particularly, the peptide bonds of aliphatic amino acids such as Val and Leu might be difficult to completely hydrolyze these bulky side chains in weak acid condition. In our study, 6 M HCl (the standard acid concentration for acid hydrolysis)[7] was the minimum concentration with the aim of obtaining the maximum AAs using the microwave-promoted hydrolysis. Increasing acid concentration up to 10 M still remained the stability in measurements of all residues. As a result, the quantification can be performed with high confidence and low uncertainty.

Figure 2D illustrates the recoverable concentration of AAs as a function of sample concentration. The range of sample concentration between 3 and 50 μmol/kg provided a similar recovery in both Hexa and G-hexa. It indicates that potential dipeptides and larger peptides in high concentrated solutions were successfully liberated during the hydrolysis period of 90 min. Our assay allows the complete hydrolysis of the peptides as well as a quantitative recovery of the residues in the hydrolysate to use up to 50 μmol/kg of starting material. This quantity could be lowered to 10 μmol/kg and used as an optimum concentration for further experiments.

The suitability of microwave radiation for peptide hydrolysis can be well acknowledged.[13,14] Experiments comparing the merits of the liquid- and gas-phase modes showed the beneficial time saving of the latter mode. In our study, it is found that the results derived from

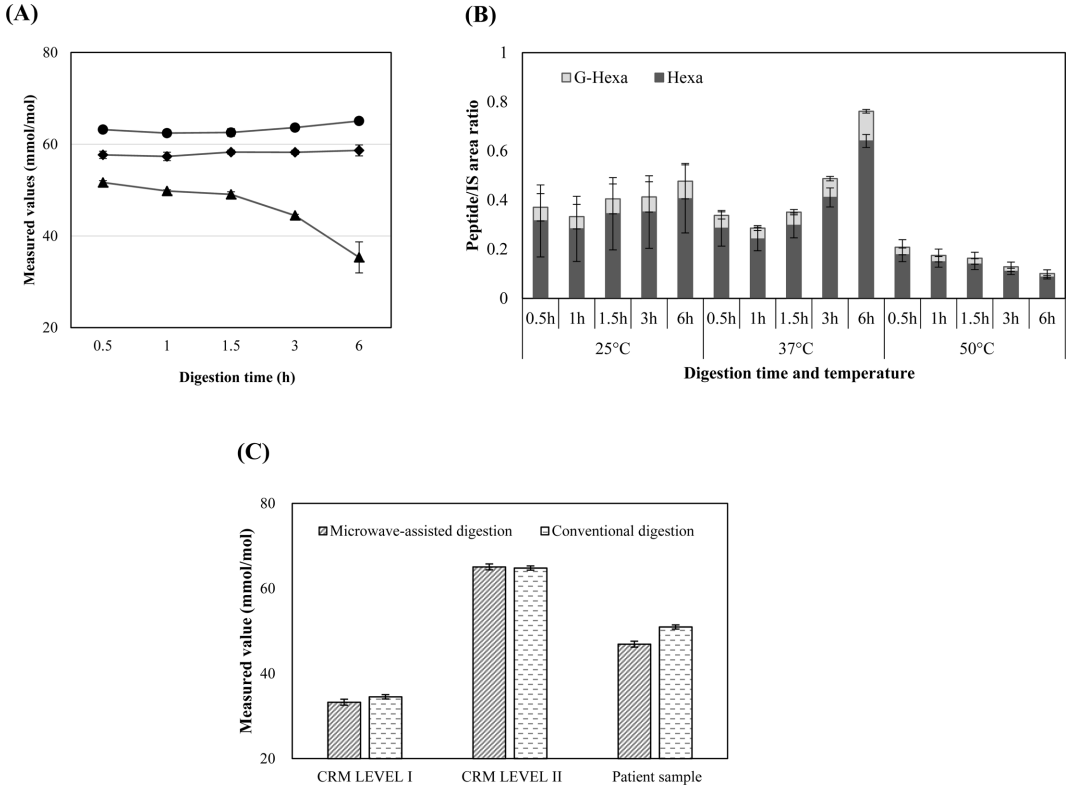

Figure 3. Optimization of enzyme digestion for the microwave-assisted digestion. (A) Absolute measured concentration of HbA_{1c} with different digestion times and temperatures. Symbols: ◆, 25°C; ●, 37°C; ▲, 50°C. (B) Area ratio of peptide and IS peptide, and (C) comparison of microwave and conventional enzyme digestion method.

conventional and microwave radiation hydrolysis were equally reliable and comparative (Figure 2E). The protocol using microwave radiation energy for peptides hydrolysis is basically similar to the traditional decomposition, except for fast hydrolysis time after spiking with internal standard solution.

Optimization of the enzyme digestion conditions

Microwave-assisted digestion experiments were carried out at instrumental settings of 700 W; digestion times and temperatures were compared in parallel at 0.5, 1, 1.5, 3, 6 h and 25, 37, 50°C, respectively. These results are plotted in Figure 3A. An evaluation of absolute HbA_{1c} concentrations revealed that at 25 (enzyme manufacturer's recommendation to protect autolysis) and 50°C the concentrations were markedly lower compared to the digestion conducted at 37°C over the time periods tested. At high temperature, the digestion efficiency considerably reduced because of heat-induced inactivation of the enzyme. Similar to classic digestion method, the digestion at 37°C was found to be over 90% complete from early stage. At either low or high temperature, increasing the digestion time enhanced the reproducibility of the repeated measurements, for instance, the variability between the measurements for each sample decreased with increased digestion time from coefficient of variation (CV) of over 25% for 0.5 h to less than 5% CV for 6 h at 37°C (Figure 3B). This indicates that the activity of Glu-C has gradually been destroyed and the target peptides are completely cleaved after 6 h. As opposed to high CV within measurements of each sample, the between-sample variations showed acceptable results (< 1%) during the period of 6 h. In this study, the optimum digestion conditions of 37°C, 700 W, and 6 h under controlled microwave irradiation is highly recommended.

For the comparison of two digestion methods, 3 different samples were examined for each method; all results are an average of values obtained from triplicate analyses. The final HbA_{1c} concentrations were found to strongly correlate between traditional and microwave heating (discrepancy < 5%) in Figure 3C. This, once again, claims that microwave-assisted enzymatic digestion not only accelerates digestion time but also provides reliable results. These results can be beneficial for qualitative and quantitative analysis of peptides.

Conclusions

This study described the merit of microwave irradiation

as a versatile tool to determine HbA_{1c} in complete SI-traceability without sacrificing analytical quality. The demand for standard peptides traceable to SI units with ID-LC-MS/MS was also successfully achieved via AAA. The time-consuming for whole process has been saved 90% when using microwave technique over traditional oven method. An agreement with previous oven results was used to demonstrate the reliability of the optimized protocol. The method described here satisfactorily served as reference measurement procedure for absolute quantification of clinically relevant proteins.

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