



Diagnosis of hemoglobinopathy and β -thalassemia by 21-Tesla Fourier transform ion cyclotron resonance mass spectrometry

Jae-Seok Kim¹, Hyun Sik Kim²

¹Department of Laboratory Medicine, Kangdong Sacred Heart Hospital, Hallym University College of Medicine, Seoul, Korea; ²Division of Mass Spectrometry & Advanced Instrumentation Research Group, Korea Basic Science Institute, Cheongju, Chungbuk, Korea

Correspondence to: Hyun Sik Kim, PhD. Division of Mass Spectrometry & Advanced Instrumentation Research Group, Korea Basic Science Institute, 161 Yeongu danji-ro, Ochang, Cheongju, Chungbuk 28119, Korea. Email: fticr@kbsi.re.kr.

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Hemoglobin is the oxygen-transport protein in red blood cells consisting of four globulins. Human adult hemoglobin A (HbA), containing two identical α -chains (141 amino acids, 15,126.4 Da) and two identical β -chains (146 amino acids, 15,867.2 Da), accounts for around 97% of the total hemoglobin in a normal adult, whereas HbA₂, with two α -chains and two delta-chains, accounts for 1.5–3.5% of the total hemoglobin in normal adults. Moreover, the proportions of HbA, HbA₂, and HbF can fluctuate during human development from the fetal to adult stage.

Hemoglobinopathy and thalassemia are two major inherited disorders of hemoglobin that cause hemolysis. Hemoglobinopathies involve structural variants of hemoglobin proteins (i.e., HbS, HbC, HbE, HbD), which can be detected by DNA sequencing or by protein mass changes, whereas thalassemias are characterized by the abnormal production of each globin chain, requiring their quantitative measurement for diagnosis.

Although many hemoglobin variants do not induce clinical symptoms or disease, some hemoglobinopathies and thalassemias can cause a variety of clinical problems. For example, an imbalance in α -non- α -globin chains is the basis of β -thalassemia, and the accumulation and precipitation of α -globulin tetramers in red blood cell precursors cause oxidative membrane damage and extensive premature destruction in the bone marrow (1). Sick cell hemoglobin (HbS) is the most predominant hemoglobin in patients with

sickle cell disease. The HbSS homozygous form of sickle cell disease is a life-threatening genetic disorder associated with many acute and chronic complications that require immediate medical attention, such as serious chronic hemolysis, vaso-occlusive crisis, and increased risks of several types of bacterial infections (2).

Numerous laboratory tests to screen for hemoglobin variants have been developed to date; however, Hb pattern analysis using automated and high-precision systems such as high-performance liquid chromatography and capillary electrophoresis are now the most prevalent diagnostic testing methods (3) for initial screening of Hb variants and determination of the percentages of HbA, HbA₂, and other Hbs. Molecular tests such as Sanger sequencing can be used for a definitive diagnosis of hemoglobinopathies, but this process is time-consuming, requiring several days to obtain results.

Recent advances in protein characterization using mass spectrometry (MS) have enabled identifying Hb variants based on mass differences caused by the associated amino acid substitutions or deletions. Here, He *et al.* report the use of 21-Tesla Fourier-transform ion cyclotron resonance (FT-ICR) MS to analyze the peptide sequences of Hb subunits with top-down approaches. This system can ionize and fragment complete Hb chains, and quantify the intact α , β , and delta subunits of Hb with minimal sample preparation.

The FT-ICR mass spectrometer acquires information

of molecular weight by measuring the frequency of the circulating ion motion induced by the Lorentz force in the uniform magnetic field. Thus, the performance of FT-ICR MS is influenced by the strength of the magnetic field. The accuracy, resolving power, intensity, LC-MS scan speed, and trapping ion number of the FT-ICR MS all increase with increasing magnetic field strength (4,5). The 21-Tesla magnet is the highest field superconducting magnet applied for FT-ICR to date, and features advantages of high spatial homogeneity and temporal stability (6). The 21-Tesla FT-ICR mass spectrometers at the National High Magnetic Field Laboratory (NHMFL) showed the highest resolving power and mass accuracy employing the highest magnetic field superconducting magnet. This high performance of FT-ICR MS can provide extremely accurate analysis of complex mixtures, including not only proteins (7,8) but also natural metabolites in animals (9), plants (10,11), crude oils (12,13), and even environmental microdust (14).

In electrospray ionization-mode FT-ICR MS and MS/MS, the Hb peptide chains are multiply charged, and thus capable of resolving long-chain peptides with a minor mass difference that are typically not clearly separated along the m/z axis due to the broad ^{13}C isotopic distribution and low S/N of the multiply charged peptides. The peak intensities of the high-mass peptides of Hb chains decrease by being distributed across multiply charged peak envelopes, resulting in decreased S/N and resolution compared with those of singly charged low mass peptides. Moreover, the fragment peptide ions are still sufficiently large to allow for accurate measurements in the sequence determination of the variants of the Hb long chains by top-down methods. In this study, the highest field of FT-ICR MS was sufficient to provide good mass accuracy and S/N for successfully resolving the variants from a mixture of peptides and large fragment ions.

The amino acid sequences of normal Hb subunits were analyzed by electron transfer dissociation (ETD) (7) and collision-induced dissociation (CID). The ETD technique enables the immediate fragmentation of multiply protonated proteins, which are commonly observed with electrospray ionization, via radical reactions. ETD generates c-type and z-type ions by the cleavage of almost all amide bonds. By contrast, a slow heating-based activation method such as CID generates mostly b-type and y-type fragment ions, but may not fragment every amino acid residue at definite sites [see Figure 2C,2F in He *et al.*'s report (15)].

For the diagnosis of hemoglobinopathies, differentiating HbS (HBB p.E6V), one of the most clinically important variants, can be easily achieved by observing the Hb β -chain

mass shift of 'calculated' 29.97418 Da, which is calculated by the mass differences of the amino acid residue of glutamic acid (E; residue formula, $\text{C}_5\text{H}_7\text{NO}_3$; monoisotopic mass, 129.04259 Da) to valine (V; residue formula, $\text{C}_5\text{H}_9\text{NO}$; monoisotopic mass, 99.06841 Da). Although another "heterozygotic" peak with a 28.006-Da difference from β chain ($\text{M}+19\text{H}$)⁺¹⁹ was observed, *de novo* sequencing by ETD only showed an abnormality at the position after the c_{38} ion. In this case, the dipeptide at the N-terminal 39–40 position was not fragmented, and QK and KK were suspected by *de novo* sequencing using accurate mass calculation within a 10-ppm window. Hb unknown heterozygotes were estimated based on the 28.00615-Da mass differences of the monoisotopic mass of arginine (R; 156.10111 Da) and lysine (K; 128.09496 Da).

Differentiation among Hb variants such as HbA and HbD could be achieved by calculating the mass difference of the β -chain ($\text{M}+17\text{H}$)¹⁷⁺. The overlap of the isotopic distributions of HbA and HbD hinder this ability due to the very minor difference of 0.01935 Da with the closest isotopic peak (one additional ^{13}C vs. ^{12}C); however, HbA and HbD heterozygotes could be much more easily determined by analyzing the z_{26}^{2+} and z_{26}^{3+} fragments generated by ETD due to the higher mass resolving power of the smaller peptide fragment ions (7).

In this report, He *et al.* also demonstrated the feasibility of diagnosing β -thalassemia by directly comparing the amount of β -chains and delta-chains at the precise position of the β -chain ($\text{M}+\text{Na}+\text{K}+15\text{H}$)¹⁷⁺. Importantly, this did not require any removal step of the β -chain using methanol/chloroform/water precipitation.

Overall, this report supports that FT-ICR MS is an extremely effective instrument given the ultra-high resolution that can achieve spectrum interpretation, especially for highly charged ions of target molecules (7). Moreover, this top-down approach by analyzing the protein sequence with ETD and CID MS/MS could be further applied for the diagnosis of hemoglobinopathies and thalassemia with great ease and confidence. A general robust FT-ICR MS diagnostic method was established and tested successfully against unknown Hb variants to detect hemoglobinopathies such as HbA, C, D, E, S, J-Baltimore, Hope, Athens-GA, Himeji, Park Ridge, and β -thalassemia. In addition, this ultrahigh-mass resolving power 21 T FT-ICR MS provides reference information for expanding other FT-ICR MS or Orbitrap MS applications in routine clinical laboratories to identify these common hematologic disorders.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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