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Electrospray mass spectrometric characterization of hemoglobin Q (Hb Q-India) and a double mutant hemoglobin S/D in clinical samples

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Abstract

Objectives: The clinical analysis of hemoglobin by ion exchange chromatography can result in ambiguities in identification of the nature of the globin chain present in patient samples. LC/ESI-MS provides rapid and precise determination of globin chain masses.

Design and methods: Hemolysate of hemoglobin Q-India and hemoglobin S/D/F have been analyzed using ESI-MS. Tandem-MS has been used to establish mutation in α chain of hemoglobin Q.

Results: The identification of hemoglobin Q-India is readily achieved by LC/ESI-MS, which establishes the presence of a mutant α chain differing in mass from normal α chain by 22 Da. The site of mutation has been identified by tandem-MS analysis of a tryptic fragment encompassing residues αV62-K90. LC/ESI-MS screening has also provide an example of simultaneous occurrence of mutant globin chains containing β6E→V (Hb S, sickle) and β121E→Q (Hb D) variant. Expression of γ_G globin chain is also demonstrated in this sample.

Conclusions: The site of mutation in hemoglobin Q-India is identified as α64D→H which differs from mutations α74D→H in Hb Q-Thailand and α75D→H in Hb Q-Iran. Mass spectrometric analysis of hemoglobins from a patient and her parents suggests inheritance of mutant β globin genes from both parents.

Keywords: Hemoglobin Q; Hemoglobin variants; Electrospray ionization mass spectrometry; Tandem mass spectrometry; Mutant hemoglobin; Hemoglobin clinical analysis

Introduction

Hemoglobin (Hb) analysis in clinical laboratories is routinely accomplished using electrophoretic methods or ion exchange HPLC procedures [1–5]. The detection of mutant polypeptide chains as well as chemically modified hemoglobins containing glycated or glutathionylated polypeptides can be achieved by HPLC procedures [6,7]. In specific instances, co-elution of different hemoglobins can result in ambiguities of interpretation. Liquid chromatography electrospray ionization mass spectrometry (LC/ESI-MS) procedures which have been widely used in the last few years to identify hemoglobin variants are rapidly emerging as the method of choice for high resolution characterization of the nature of mutations/modifications in hemoglobin, obtained from patients presenting specific clinical conditions [8]. In this report, we compare LC/ESI-MS procedures with ion exchange HPLC and demonstrated the characterization of hemoglobin Q (Hb Q-India). In addition, we describe the characterization of a relatively rare Hb S/D occurrence in a patient inheriting mutant β-globin genes from both parents.

Materials and methods

Patients

The study group consisted of a number of patients clinically diagnosed as mild anemic, thalassemic and diabetic, referred to the Manipal Hospital, Bangalore, for hemoglobin analysis.

Abbreviations: LC/ESI MS, Liquid chromatography electrospray ionization mass spectrometry; MALDI MS, matrix-assisted laser desorption ionization mass spectrometry; Hb S, sickle hemoglobin; Hb F, fetal hemoglobin; Hb Q, hemoglobin Q; Hb D, hemoglobin D.

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Samples for analysis were also obtained from patients undergoing general health checkup. Patients were recruited after obtaining written consent. The study was approved by the Ethics Committee of the Manipal Hospital.

Hemoglobin analysis

Hemoglobin variants and HbA1c were estimated by standard high performance liquid chromatography (HPLC) methods using an automated D-10 HPLC system (Bio-Rad, USA).

Sample preparation

Venus blood anticoagulated with EDTA was used. Plasma and buffy coat was aspirated off after centrifugation at 805×*g* for 10 min at 25 °C. The packed cells were washed with 0.9% NaCl thrice before lysis with eight volumes of ice-cold distilled water. The hemolysate was centrifuged at 12880×*g* for 10 min to remove the erythrocyte membranes. The clear supernatant was diluted 100 times with distilled water prior to mass spectrometric analysis.

Enzyme digestion

Enzyme digestion of hemoglobin of both normal and patient samples was performed using trypsin (TPCK treated) in 50 mM NH_4HCO_3 buffer pH 8.0. Hemoglobin concentration in clinical samples was determined by usual procedures in clinical laboratories. Enzyme to hemoglobin ratio was maintained at 1:50 and the reaction mixture was incubated at 37 °C for 12 h. After first 4 h of incubation, enzyme was added to the reaction mixture once again. The digested samples were fractionated through a C18 reverse phase column (Vydac, 4.6×250 mm, 5 μm). Different peptides obtained from hemoglobin digestion were eluted using a linear gradient of acetonitrile (2%/min) containing 0.1% trifluroacetic acid. Fractions were collected and concentrated in a Speed Vac system and spotted on to a MALDI plate for MS analysis.

Mass spectrometry

Electrospray ionization mass spectra were acquired in positive ion mode using a single quadrupole mass spectrometer (Hewlett Packard HP 1100 MSD series). Ten microliters of sample was injected through a C18 reverse phase column (Zorbax, 4.6 mm×150 mm, 5 μm). Different polypeptide chains of hemoglobin were eluted using a linear gradient of acetonitrile (2% raise/min; 20% acetonitrile/water–90% acetonitrile/water) containing 0.1% acetic acid with a flow rate of 0.2 mL/min. Spectra were acquired over the mass range 200–3000 *m*/*z*. Mass scale calibration was done using ES tuning mix supplied by the manufacturer. Deconvolution of the multiply charged species was performed using the Chemstation software. Mass determination by deconvolution of the charge states in ESI-MS spectra under our condition have the reliability of ±1 Da. Most single site mutants result in ΔM values much greater than the estimated uncertainty.

MALDI mass spectra were obtained on trypsin digested samples using an Utraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Germany) in the reflectron mode, using 200-ns time delay and a 25-kV accelerating voltage in the positive ion mode. The system utilizes a 50-Hz pulsed nitrogen LASER, emitting at 337 nm. α-Cyano-4-hydroxy cinnamic acid was used as a matrix.

Tandem mass spectra (tandem-MS) were acquired by selecting the precursor ion with a 10-Da window, and fragments were generated in post source decay (PSD) mode. A single acquisition run was the sum of at least 50 series with 600 total added shots to generate the MS/MS spectra. Mass spectra were analyzed using Flex-analysis software, provided by the manufacturer. *De novo* sequencing for different peptide was done by manual assignment of daughter ions in MS/MS spectra.

Results and discussion

In a clinical laboratory setting, routine analysis of hemoglobin samples is carried out by separation of various hemoglobin components by ion exchange chromatography using an HPLC system. In general, the use of the BioRAD D-10 HPLC system has proved widely applicable to a diverse range of problems encountered in a clinical setting. Ion exchange chromatography separates hemoglobin as $\alpha_2\beta_2$ tetramers and is highly sensitive to the overall charge in hemoglobins. On occasion, samples in clinical laboratories do yield intense bands on ion exchange chromatography, which are not easily assigned, resulting in difficulties in the preparation of definitive analytical reports. Under our laboratory conditions 30 to 40 samples are routinely analyzed per day, with unambiguous analysis being possible in most cases. However, over a period of time unusual blood samples accumulate which merit analysis by complementary techniques. While molecular analysis using PCR and DNA sequencing are proving popular in characterizing hemoglobin mutations, we have turned to exploring the use of reverse phase HPLC in conjunction with electrospray ionization mass spectrometry as a measure of definitively establishing the chemical constitution of hemoglobin polypeptide chains present in unusual clinical samples. This methodology has the advantage of providing molecular information on the individual polypeptide chains α and β in hemoglobin, and also allows the rapid detection of variant chains. In this paper we exemplify our approach by presenting an analysis of seven hemoglobin samples, four of which has turned out to be Hb Q and one in each group of Hb S, Hb D, Hb S/D.

Fig. 1 compares the ion exchange HPLC profiles of a normal hemoglobin (Panel A) sample with that of a patient possessing a variant chain (Panel B). The intense peak at 4.41 min is assigned as an unknown, clearly differentiating it from a number of commonly occurring hemoglobin variants like Hb S (β6E→V) and Hb D (β121E→Q), which are readily identified. Fig. 2 shows an ESI-MS spectrum of the unassigned Hb sample. Inspection of the reverse phase HPLC profile (Inset A) and the charge state distribution clearly reveals the presence of a mutant α-globin chain. Deconvolution yields a mass of 15147 Da for the mutant alpha chain which is 22 Da greater than a normal alpha chain (15125 Da) (Inset B). A mass change of 22 Da can,

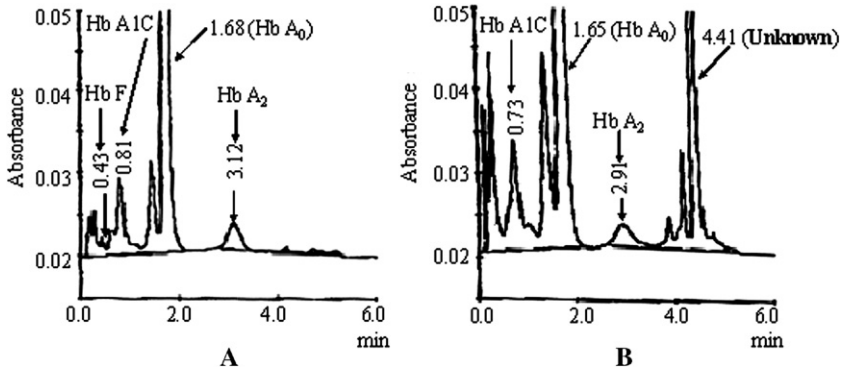

Fig. 1. Ion exchange HPLC profile of a normal subject (A) and a Hb Q patient (B) using an automated D-10 HPLC system (BioRad, USA).

in principle, be ascribed to the formation of an Na^+ adduct. However, under conditions of LC-MS using reverse phase columns and 0.1% acetic acid in the eluant, cationised species are largely eliminated. The mass difference may therefore be assigned to a mutation of an aspartic acid (Asp, D) residue to a histidine (His, H) residue, identifying the hemoglobin sample as an Hb Q variant [9–16]. Thus far, three distinct Hb Q forms have been characterized. Hb Q Thailand, which is a form observed in Far East populations with an α74D→H mutation (10). Hb Q India has been identified as α64D→H mutation (11) while Hb Q Iran has been identified as α75D→H mutation (10). These assignments had been carried out by sequencing of the relevant regions of the mutant alpha genes (10–11). In an interesting report, ESI-MS-MS has been used to reassign an Hb Hasharon variant as Hb Q-Iran (9). We have applied MALDI-MS/MS following trypsin digestion in order to directly identify the site of mutation. Fig. 3 compares the MALDI-MS tryptic peptide finger prints of normal (Panel A) and variant hemoglobin (Hb Q) (Panel B). It is seen that while the normal Hb sample contains a tryptic fragment with an *m/z* value 2997.3, the variant sample also exhibits an additional peak at *m/z* 3019.3, which corresponds to a 22-Da increase in mass (inset). This fragment was therefore chosen for subsequent sequencing. An attempt to directly sequence the fragment by MALDI-MS/MS procedures without further purification did not yield adequately intense fragment ions. We therefore fractionated the tryptic fragments on reverse phase HPLC and separated two peptides corresponding to $[M+H]^+$ masses of 3019 and 2997 Da

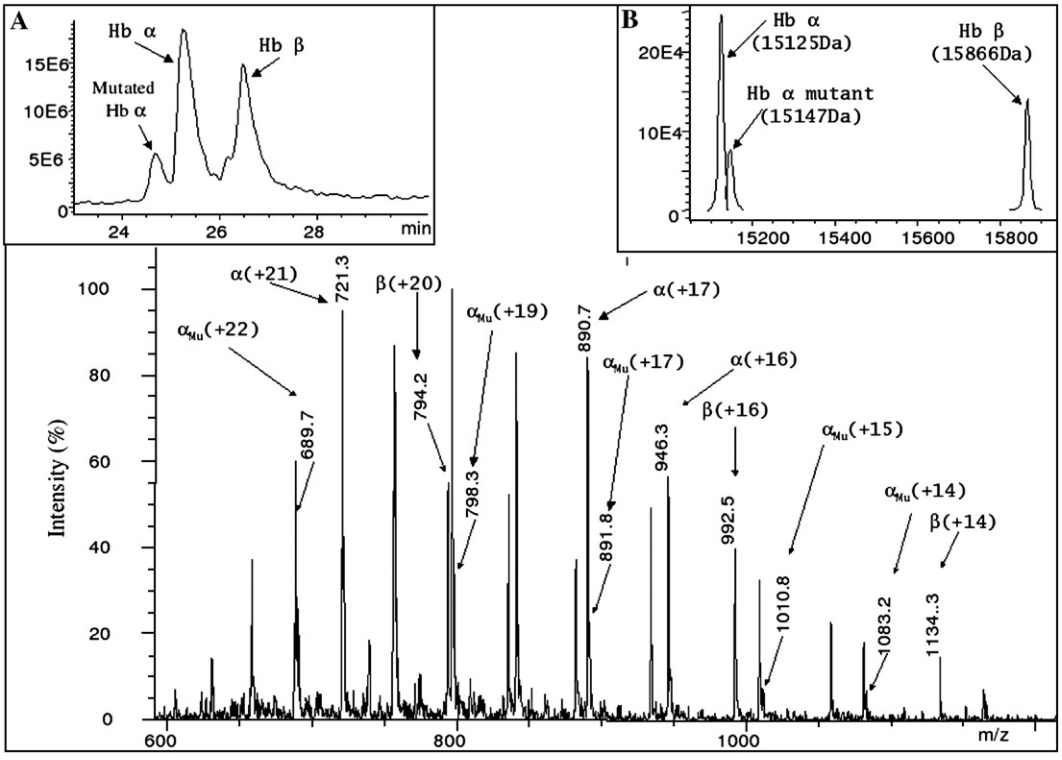

Fig. 2. LC-ESI mass spectrum of hemoglobin of a Hb Q sample. Inset: (A) Total ion chromatogram of the hemoglobin sample in LC/ESI-MS, (B) deconvoluted masses of Hb α (15125 Da), mutated Hb α (15147 Da) and Hb β(15866 Da). [Note: The total ion chromatogram has been integrated over the entire window (24.5 min to 27.5 min) resulting in the simultaneous observations of the mass spectra of all three chains.]

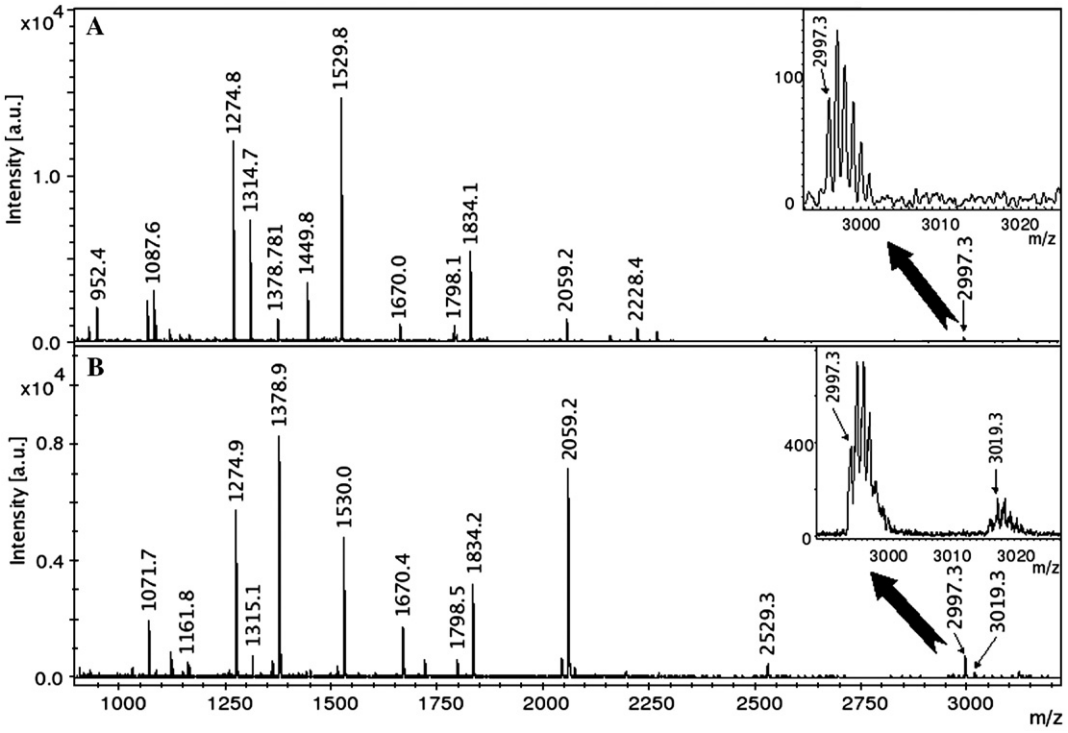

Fig. 3. MALDI-MS of a peptide fragment of human hemoglobin obtained after trypsin digestion from a normal subject (A) and an Hb Q patient (B).

(Fig. 4). Figs. 5 and 6 show the MALDI-MS/MS spectrum obtained from the two purified peptide fragments. The assignment of the relevant fragment ions is indicated. The observed tryptic peptide corresponds to the V62-K90 fragment of the hemoglobin alpha chain. Interestingly, this stretch of polypeptide contains as many as four Asp (D) residues. Comparison of the mass spectral fragmentation patterns in Figs. 5 and 6 readily permits identification of the site of mutation as residue 64 (D→H). It may be noted that the observation of the b_4 ion at *m*/*z* 357.2 in the normal chain and the corresponding ion at *m*/*z* 379.1 in the mutant chain confirms the site of mutation. This is also supported by the observation of the y_{27} ion at *m*/*z* 2826.3 in the normal chain and the corresponding ion at *m*/*z* 2848.9 in the mutant. The coincidence of *m*/*z* values of the lower y_n ions precludes mutation at the other Asp residues. In the course of our study, we examined a total of four clinical

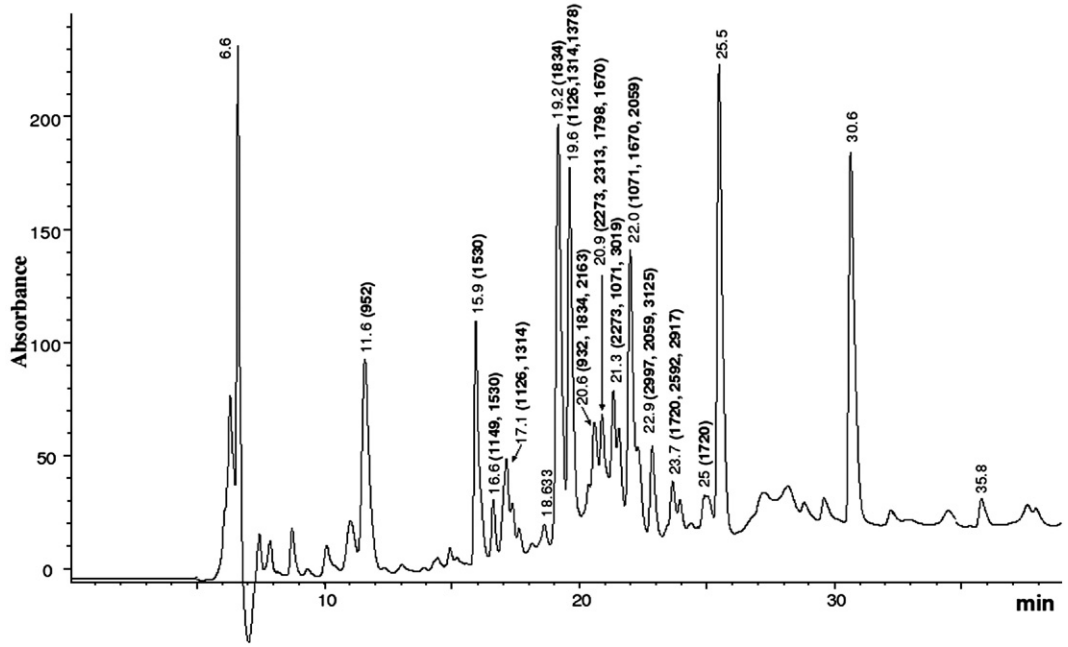

Fig. 4. Reverse phase HPLC profile of trypsin digested hemoglobin Q sample. Masses of different peptides eluting at specific retention time are shown in parentheses.

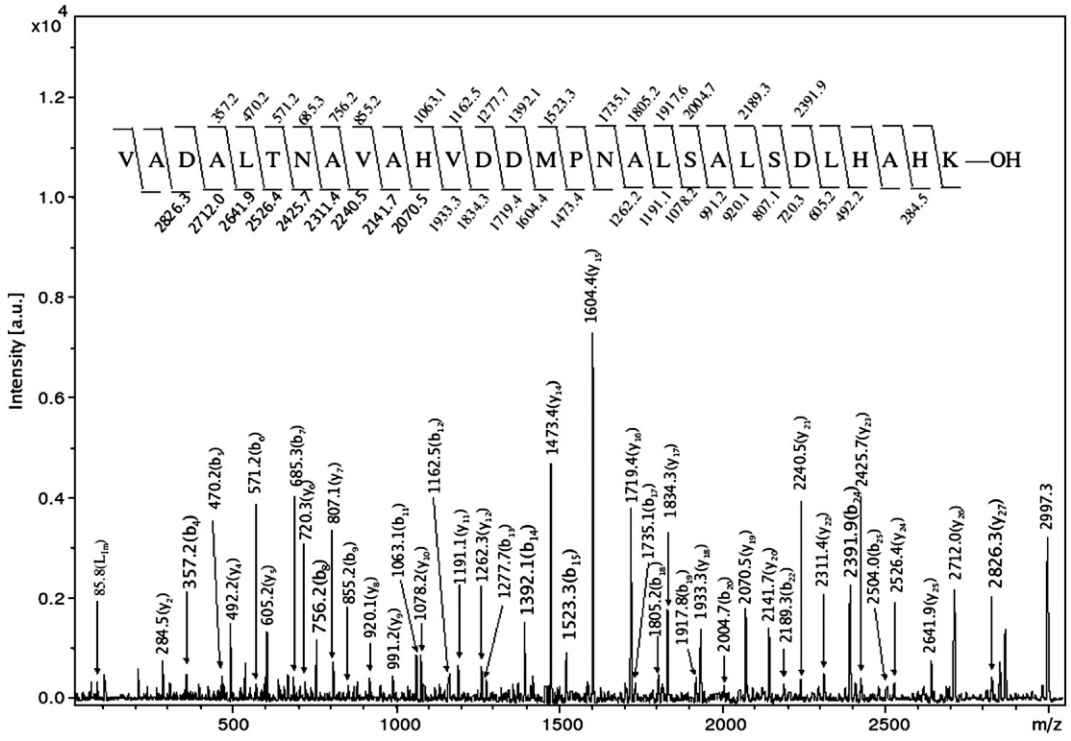

Fig. 5. MALDI MS/MS spectrum of a tryptic peptide with $[M+H]^+$ at *m/z* 2997.3. Series of 'b', 'y' ions and molecular ions obtained by neutral losses are labeled. Inset shows the sequence of the precursor peptide ion.

samples which were tentatively identified as unknown hemoglobin variants by ion exchange HPLC. In all the cases, ESI-MS procedures permitted a rapid classification of these samples as Hb Q. For all four samples, we also sequenced the 62–90 tryptic fragment and established the α64D→H mutation.

Analysis of Hb S/D

Ion exchange HPLC provides a ready identification of Hb S (β6E→V) and Hb D (β121E→Q) mutations. The relatively frequent observation of these mutations in clinical laboratories

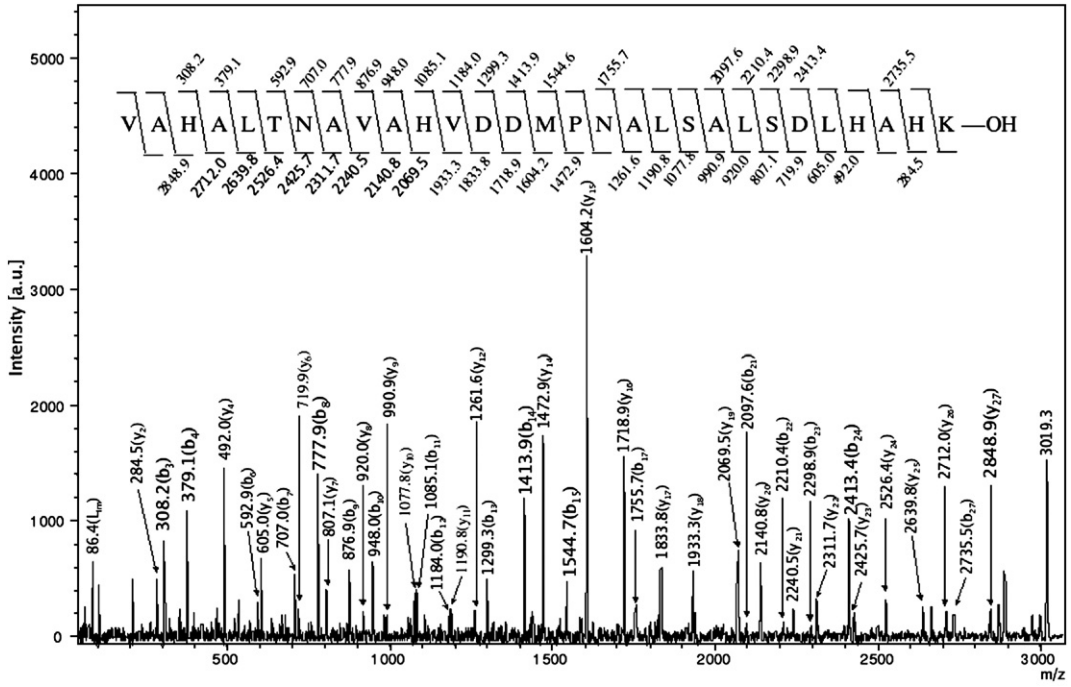

Fig. 6. MALDI MS/MS spectrum of a tryptic peptide a with molecular ion at *m/z* 3019.3. Series of 'b', 'y' ions and molecular ions obtained by neutral losses are labeled. Inset shows the sequence of the precursor peptide ion, determined *de novo*.

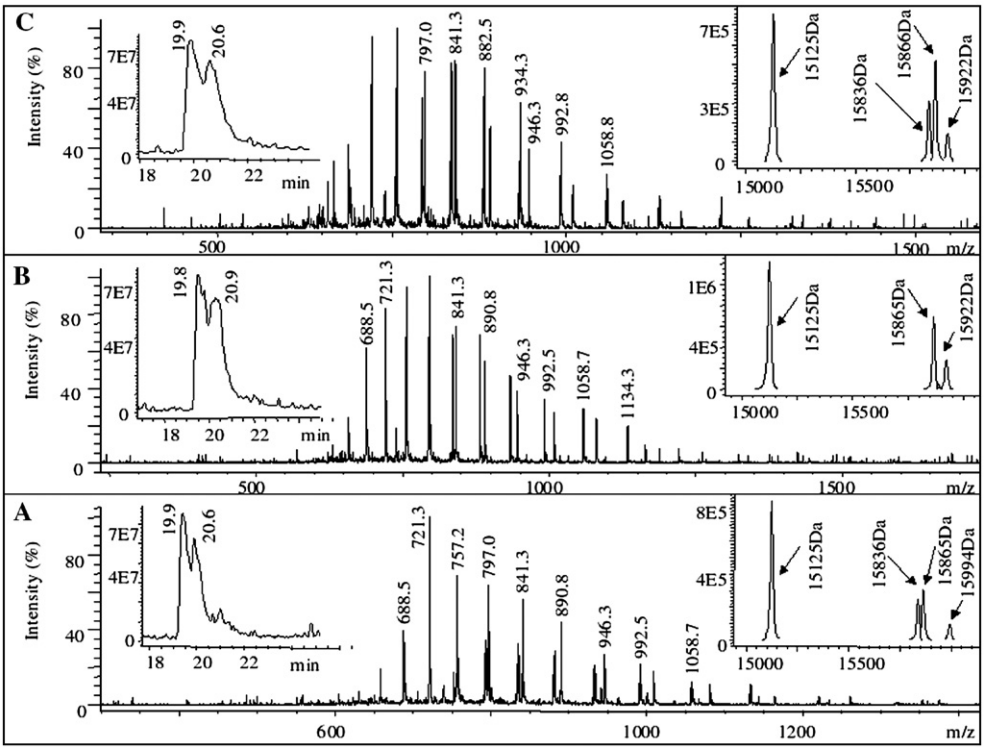

Fig. 7. ESI mass spectra of unusual hemoglobin samples (A) Hb FSD, (B) Hb D and (C) Hb S. (Inset) Deconvoluted masses of individual Hb chains: α 15125 Da, β 15866 Da, β S 15836 Da, β D 15865 Da, δ 15922 Da, γ 15994 Da. [Note: The total ion chromatogram has been integrated over the entire window (19.5 min to 23 min) resulting in the simultaneous observations of the mass spectra of all three chains.]

has resulted in quick identification by ion exchange HPLC procedures, in which separation is facilitated by charge differences in the mutants.

During the course of routine screening of clinical samples, we came across an example of an unusual ion exchange HPLC profile, which appeared to suggest the simultaneous presence of Hb S, Hb D and Hb F. Fig. 7 (Panel A—daughter) shows an ESI-MS spectrum of the sample (female patient aged 2 years), which reveals the presence of four polypeptide chains with masses 15125 Da (α normal), 15836 Da (β sickle), 15865 Da (β) and 15994 Da (γ_G). The polypeptide chain with mass 15865 Da may be assigned to either beta normal or beta D. The Hb D mutation is characterized as β121E→Q, which results in a mass change of only 1 Da [17]. Under our mass spectral conditions, there is an ambiguity in assignment of this polypeptide chain. The ion exchange HPLC profile reveals an extremely low amount of normal hemoglobin (Hb A_0), pointing to the absence of the normal $\alpha_2\beta_2$ tetramer. These observations suggests that the patient sample corresponds to a situation where two mutant beta globin genes (Hb S, Hb D) have been inherited from parents, along with elevated expression of fetal hemoglobin. Fig. 7 also shows the electrospray ionization mass spectra obtained from hemoglobin isolated from both parents (Panel B—mother, C—father). In the case of the mother, a beta chain with mass 15865 Da which could be assigned to either β normal (15866 Da) or β D (15865 Da). In the hemoglobin obtained from the father, the presence of the beta sickle (β S) chain with a mass of 15836 Da is clearly identified, in addition to a normal β chain at 15866 Da. In the case of both parents, Hb A_2 expression is appreciable as evidenced by the presence of a δ globin chain at 15922 Da, but for daughter expression of the δ chain is very low. The ESI MS data taken together with the ion exchange HPLC analysis indicates an almost complete absence of normal hemoglobin (Hb A_0) in the case of the patient. This observation suggests that both parents harbor a mutant β globin genes, β S in the case of the father and β D in the case of the mother. Inheritance of the mutant gene results in a complete absence of normal β globin chain in the case of the daughter. Interestingly, there is also a relatively high level of the fetal chain (γ_G) [18] in the case of the patient.

Conclusion

Electrospray ionization mass spectrometry provides a rapid measurement of the masses of the individual polypeptide chains in hemoglobin. Under normal condition of electrospray ionization, subunit dissociation is facile, permitting highly accurate mass characterization of individual chains. Identification of hemoglobin Q variants by conventional ion exchange HPLC procedures is still not routine in the clinical laboratory. LC/ESI-MS offers a rapid and unambiguous characterization of Hb Q. In the present study, MALDI MS/MS sequencing of the 62–90 alpha globin tryptic peptide fragment permits identification of the site of mutation as α64D→H. An example of characterization of an unusual clinical sample containing Hb S/D/F is reported. Mass spectrometric analysis of hemoglobins from the patient and her parents suggests inheritance of mutant β globin genes from both parents. A direct analysis of the

expressed polypeptide chains of hemoglobin genes can provide insights into relatively rare clinical conditions. Future analyses which focus on quantitation of hemoglobin tetramer in the gas phase may provide valuable insights into the relationship between clinical presentation and the constitution of hetero oligomers.

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References

[1] Riou J, Godart C, Hurtrel D, Mathis M, Bimet C, Bardakdjian-Michau J, et al. Cation-exchange HPLC evaluated for presumptive identification of hemoglobin variants. Clin Chem 1997;43:34–9.
[2] Wilson JB, Headlee ME, Huisman THJ. A new high-performance liquid chromatographic procedure for the separation and quantitation of various hemoglobin variants in adults and newborn babies. J Lab Clin Med 1983;102:174–85.
[3] Kutlar A, Kutlar F, Wilson JB, Headlee ME, Huisman THJ. Quantitation of hemoglobin components by high-performance cation exchange liquid chromatography: its use in diagnosis and in the assessment of cellular distribution of hemoglobin variants. Am J Hematol 1984;17:39–53.
[4] Rogers BB, Wessels RA, Ou CN, Buffone GJ. High-performance liquid chromatography in the diagnosis of hemoglobinopathies and thalassemias. Am J Clin Pathol 1985;84:671–4.
[5] Joutovsky A, Hadzi-Nesie J, Nardi MA. HPLC retention time as a diagnostic tool for hemoglobin variants and hemoglobinopathies: a study of 60000 samples in a clinical diagnostic laboratory. Clin Chem 2004;50:1736–47.
[6] Khuu HM, Robinson CA, Goolsby K, Hardy RW, Konrad RJ. Evaluation of a fully automated high-performance liquid chromatography assay for hemoglobin A1c. Arch Pathol Lab Med 1999;123:763–7.
[7] Pastore A, Francesca Mozzi A, Tozzi G, Maria Gaeta L, Federici G, Bertini E, et al. Determination of glutathionyl–hemoglobin in human erythrocytes by cation-exchange high-performance liquid chromatography. Anal Biochem 2003;312:85–90.
[8] Wild BJ, Green BN, Cooper EK, Lalloz MRA, Erten S, Stephens AD, et al. Rapid identification of hemoglobin variants by electrospray ionization mass spectrometry. Blood Cells Mol Diseases 2001;27:691–704.
[9] Rai DP, Alvelius G, Landin B, Griffiths WJ. Electrospray tandem mass spectrometry in the rapid identification of α-chain haemoglobin variants. Rapid Commun Mass Spectrom 2000;14:1184–94.
[10] Lorkin PA, Charlesworth D, Lahman H, Rahbar S, Tuchinda S, Eng LI. Two haemoglobins Q, alpha-74 [EF3] and alpha-75 [EF4] aspartic acid to histidine. Br J Haematol 1970;19:117–25.
[11] Sukumaran PK, Merchant SM, Desai MP, Wiltshire BG, Lahmann H. Hemoglobin Q India (alpha 64(E13) aspartic acid to histidine) associated with beta-thalassemia observed in three Sindhi families. J Med Genet 1972;9:436–42.
[12] Desai DV, Dhanani H, Kapoor AK, Yeluri SV. HbQ-India in a Sindhi family: an uncommon hemoglobin variant. Lab Hematol 2004; 10:212–4.
[13] Sanchaisuriya K, Chunpanich S, Fucharoen S, Fucharoen G, Sanchaisuriya P, Changtrakun Y. Association of Hb Q-Thailand with homozygous Hb E and heterozygous Hb constant spring in pregnancy. Eur J Haematol 2005;74:221–7.
[14] Viprakasit V, Chinchang W, Pung-Amritt P, Tanphaichitr VS. Identification of Hb Q-India (alpha64 Asp → His) in Thailand. Hematology 2004;9: 151–5.
[15] Abraham R, Thomas M, Britt R, Fisher C, Old J. Hb Q-India: an uncommon variant diagnosed in three Punjabi patients with diabetes is identified by a novel DNA analysis test. J Clin Pathol 2003;56:296–9.
[16] Zeng FY, Fucharoen S, Huang SZ, Rodgers GP. Hb Q-Thailand [alpha 74 (EF3)Asp → His]: gene organization, molecular structure, and DNA diagnosis. Hemoglobin 1992;16:481–91.
[17] Zeng YT, Huang SZ, Li HJ. Identification of Hb D-Punjab gene: amplification in the study of abnormal hemoglobins. Am J Hum Genet 1989;44:886–9.
[18] Jones RW, Old JM, Trent RJ, Clegg JB, Weatherall DJ. Major rearrangement in the human beta-globin gene cluster. Nature 1981;291:39–44.