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# Hot Electron Capture Dissociation Distinguishes Leucine from Isoleucine in a Novel Hemoglobin Variant, Hb Askew, $\beta 54(\text{D5})\text{Val}\rightarrow\text{Ile}$

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Population migration has led to the global dispersion of human hemoglobinopathies and has precipitated a need for their identification. An effective mass spectrometry-based procedure involves analysis of the intact  $\alpha$ - and  $\beta$ -globin chains to determine their mass, followed by location of the variant amino acid residue by direct analysis of the enzymatically digested chains and low-energy collision induced dissociation of the variant peptide. Using this procedure, a variant was identified as either  $\beta 54\text{Val}\rightarrow\text{Leu}$  or  $\beta 54\text{Val}\rightarrow\text{Ile}$ , since the amino acids leucine and isoleucine cannot be distinguished using low-energy collisions. Here, we describe how hot electron capture dissociation on a Fourier transform-ion cyclotron resonance mass spectrometer was used to distinguish isoleucine from leucine and identify the mutation as  $\beta 54(\text{D5})\text{Val}\rightarrow\text{Ile}$ . This is a novel variant, and we have named it Hb Askew. (J Am Soc Mass Spectrom 2009, 20, 1707–1713) © 2009 American Society for Mass Spectrometry

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**H**emoglobin (Hb) exists in the blood cells of vertebrates as a noncovalently assembled tetramer of  $\alpha$ - and  $\beta$ -chains ( $\alpha_2\beta_2$ ), in which each chain is associated with a heme group. Its primary function is to supply oxygen to the organs of the body. Abnormalities of human hemoglobin (Hb) are the most common autosomal recessive inherited disorders in man. These disorders can be both quantitative, due to impaired synthesis of the globin chains (thalassemia syndromes), or qualitative (structural variants). Many hemoglobinopathies do not cause clinical problems, but some are responsible for morbidity and mortality. Hb variants initially tended to be located with high-frequency in tropical and subtropical regions such as Africa, the Caribbean, the Mediterranean, Asia, and the Far East. The large degree of population migration has led to the worldwide spread of Hb disorders.

Hb variants are often detected as part of a routine glycohemoglobin screen during diabetic monitoring or antenatal and neonatal screening healthcare programs. Hemoglobinopathy diagnosis in clinical laboratories is

routinely accomplished using isoelectric focusing (IEF) or cation exchange high-performance liquid chromatography (ce-HPLC) procedures. These methods presumptively identify variants but cannot positively identify any variant. Precise variant identification requires protein sequencing or DNA analysis. The majority of Hb variants listed in the Globin Gene Server [1] database result from a single point mutation in either the  $\alpha$ - or  $\beta$ -globin gene that leads to the production of a single amino acid substitution in either the  $\alpha$ - or  $\beta$ -chain of the Hb protein. Although many of these variants may be innocuous, once detected in a first-line hospital screen, it is sensible to precisely identify the mutation.

A procedure for identifying Hb variants by electrospray ionization mass spectrometry (ESI-MS) has been described previously [2, 3]. There are three steps in this procedure. The first step involves analyzing blood diluted in a denaturing solvent to determine the molecular weight of the variant chain and assign the variant to the  $\alpha$ - or  $\beta$ -chain. In the second step, diluted blood is digested with trypsin and the resulting mixture of peptides analyzed directly by ESI-MS. The third step involves sequencing the variant tryptic peptide by tandem mass spectrometry if required. No chromatographic separation of the tryptic peptides before or during analysis is required. The accurate average mass

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measurement involved in step one allows for the detection of variants in heterozygotes that differ by as little as 1 Da from normal, providing the variant is present at >10% relative abundance [3, 4]. Using this procedure and applying the  $\alpha$ -chain mass for internal calibration, the normal  $\beta$ -chain mass can be determined with a precision of  $\leq 0.03$  Da standard deviation [4]. Important minor Hb fractions, such as HbA<sub>1c</sub> (assessment of long-term glycemic control in diabetics) [5–7] and HbA<sub>2</sub> (biomarker for  $\beta^0$ -thalassemia trait) [8], are also detected and quantified using these procedures following calibration with standards.

The above procedure involves sequencing peptides via low-energy collision-induced dissociation (CID) in a triple quadrupole instrument, and >95% of the variants encountered in practice can be identified. However, there are a number of variants that involve amino acid exchanges governed by single mutations in the nucleotide codon to either leucine (Leu) or isoleucine (Ile), which cannot be differentiated using this procedure. Leu and Ile are isomeric species. Therefore, low-energy CID of variant peptide precursor ions, which contain either Leu or Ile, generates sequence ions [9] of the same  $m/z$  and renders unequivocal assignment of these species impossible.

Here, we describe a mass spectrometric approach, which allows distinction between Leu and Ile when characterizing human hemoglobinopathies. This approach utilizes hot electron capture dissociation (HECD) [10] on a Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. In conventional (low-energy) ECD [11, 12], peptide backbone cleavage occurs at the N-C $\alpha$  bond to produce  $c'$  and  $z\bullet$  ions. These fragments also result from HECD but are accompanied by extensive secondary fragmentation due to the excess energy. Secondary fragmentation of amino acid side chains of  $z\bullet$  ions result in the formation of  $w$  ions. The loss of  $\bullet\text{CH}(\text{CH}_3)_2$  (–43 Da) or  $\bullet\text{CH}_2\text{CH}_3$  (–29 Da) from  $z\bullet$  ions containing N-terminal Leu or Ile, respectively,

enables the two amino acids to be distinguished, see Scheme 1. This method has been demonstrated on synthetic peptides [10] and the bovine milk protein PP3 [13]. In the present example, a blood sample was found to contain three variants, one of which involved a mutation to either Leu or Ile. Here we show how HECD was used to resolve the ambiguity and identify a novel  $\beta$ -chain variant. To confirm the assignment, HECD of Leu- and Ile-containing synthetic analogues of the tryptic peptide containing the variant was also performed. This work demonstrates the significant potential role of HECD in the characterization of these types of Hb mutation, and also constitutes the first true clinical application of the HECD technique.

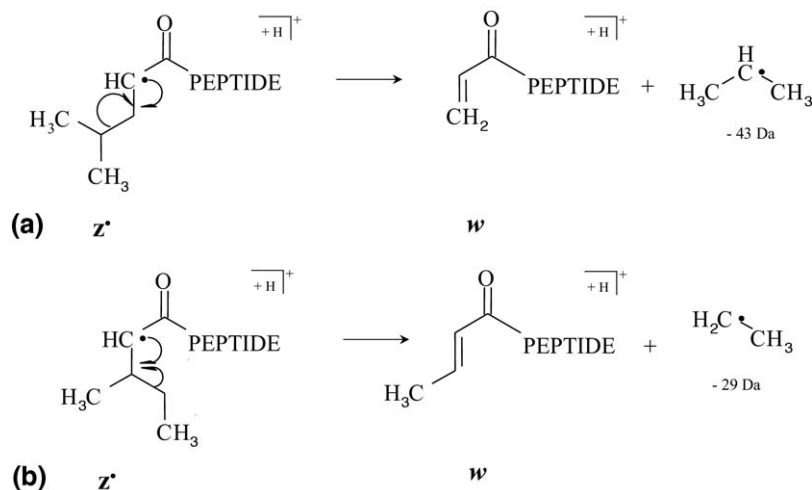
## Methods

### Cation-Exchange HPLC

Cation-exchange HPLC with UV detection was performed on a VARIANT HPLC system using the Variant  $\beta$ -thalassemia Short program (Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK).

### Sample Preparation

The procedures for identifying variants in blood samples by mass spectrometry have been previously described in detail elsewhere [2, 3]. Briefly, 10  $\mu\text{L}$  of the blood sample (in ethylene diamine tetra-acetic acid (EDTA) anti-coagulant) was diluted 50-fold with 490  $\mu\text{L}$  of water to give a stock solution. Then, 20  $\mu\text{L}$  of the stock solution was diluted a further 10-fold with 180  $\mu\text{L}$  of 5:4 acetonitrile:water containing 0.2% formic acid (Solution A). Tryptic digests were prepared as follows: first, 100  $\mu\text{L}$  of the stock solution was denatured by mixing with 20  $\mu\text{L}$  of 50% aqueous acetonitrile containing 0.5% formic acid. Then, 6  $\mu\text{L}$  of 1 M ammonium bicarbonate solution was added, followed by 5  $\mu\text{L}$  of a



**Scheme 1.** Secondary fragmentation of a  $z\bullet$  ion to produce the  $w$  ion from a peptide containing (a) a leucine residue and (b) an isoleucine residue.

5 mg/mL solution of trypsin (T1426, Sigma-Aldrich Corp., St. Louis, MO, USA). The resulting solution was then incubated at 37 °C for 30 min, after which aliquots were diluted 10-fold with Solution A.

The peptides FFESFGDLSTPDAIMGNP and FFESFGDLSTPDALMGNP were synthesized by AltaBioscience, University of Birmingham, UK, and used without further purification. The peptides were diluted to 2 pmol/ $\mu$ L in methanol (Fisher Scientific, Leicestershire, UK):water (J. T. Baker, Deventer, The Netherlands) (75:25) with 1% formic acid (Fisher Scientific).

### Mass Spectrometry

ESI-MS was performed on two instruments. Initial experiments were undertaken on a triple quadrupole mass spectrometer (Quattro Ultima; Waters MS Technologies, Manchester, UK) equipped with the standard Z-spray electrospray ion source and operated at a source and desolvation temperature of 110 °C. Sample solutions were introduced into the source region of the instrument at a rate of 5  $\mu$ L/min. The instrument was operated in positive mode of ionization, with a capillary voltage of 3 kV, a cone voltage of 80 V, and hexapole 1 ion guide set to 60 V. Tandem mass spectrometry (MS/MS) was carried out using argon collision gas at a pressure of  $2.5 \times 10^{-3}$  mbar within the RF-only hexapole collision cell. Fragmentation of the precursor tryptic peptide ions was achieved with a collision energy of 30 eV.

FT-ICR analysis was performed on a Thermo Finnigan LTQ FT mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Samples were injected by use of an Advion Biosciences Triversa electrospray source (Advion Biosciences, Ithaca, NY, USA) at a flow rate of  $\sim$ 200 nL/min. For all experiments, scans were acquired in the ICR cell with a resolution of 100,000 at  $m/z$  400. ECD and HECD of tryptic peptide: precursor ions were isolated in the linear ion trap and transferred to the ICR cell for (H)ECD. AGC target was  $1 \times 10^6$ . Isolation width was 6 Th. The electrons for (H)ECD were produced by an indirectly heated barium-tungsten cylindrical dispenser cathode (5.1 mm diameter, 154 mm from the cell, 1 mm off axis) (Heat-Wave Labs, Watsonville, CA, USA). The current across the electrode was  $\sim$ 1.1 A. Ions were irradiated for 70 ms at 5% energy (ECD) (corresponding to ECD cathode potential  $-4.35$  V) or 15% energy (HECD) (corresponding to ECD cathode potential  $-14.35$  V). Each (H)ECD scan comprised four co-added microscans. Mass spectra shown comprise fifty averaged scans. HECD of synthetic peptides: instrumental parameters were as above, except that the current across the electrode was  $\sim$ 1.0 A. Ions were irradiated for 70 ms at 17.5% energy (corresponding to ECD cathode potential  $-16.18$  V). Each HECD scan comprised 20 co-added microscans. Mass spectra shown comprise one scan.

### Results

A blood sample was submitted for investigation by mass spectrometry because abnormalities had been detected during a routine antenatal screen in a hospital hematology laboratory using ce-HPLC. The ce-HPLC chromatogram (Figure 1) showed two abnormal peaks, which eluted after the time for normal adult Hb (A0, 2.44 min). The elution time of the earlier peak (4.50 min) corresponds to that of the clinically significant Sickle variant, but the peak itself is atypical in that there appear to be shoulders on both its leading and trailing edges. A sickle cell solubility test was positive. Apart from the unusual ce-HPLC chromatogram, no other hematological abnormalities were observed. To clarify these anomalies, the sample was submitted for analysis by ESI-MS.

Initially, mass spectrometric analysis was performed on a triple quadrupole instrument. Analysis of the intact Hb chains revealed the presence of one  $\alpha$ -chain variant and, surprisingly, two  $\beta$ -chain variants (Figure 2). The  $\alpha$ -chain variant was 14 Da heavier than normal and its proportion of total  $\alpha$ -chains was 28.3%. It was identified from a 30-min tryptic digest as Hb Stanleyville II ( $\alpha$ 78[EF7]Asn $\rightarrow$ Lys). The lighter  $\beta$ -chain variant (normal  $\beta$   $-30$  Da and 42.2% of total  $\beta$ -chains) was confirmed from the same digest as Hb Sickle ( $\beta$ 6[A3]Glu $\rightarrow$ Val). The unexpected third variant was 14 Da heavier than the normal  $\beta$ -chain and its mutant amino acid was shown from the spectrum of the tryptic digest to occur in the  $\beta$ T5 peptide (Figure 3). There are nine potential mutations in this peptide that could give a 14 Da mass increase by a single base change in the nucleotide codon. To distinguish these possibilities, the normal and variant  $\beta$ T5 $^{2+}$  ions were sequenced by tandem mass spectrometry using low-energy CID (Figure 4). The 14 Da mass increase at  $y''_6$  between the spectra from (a) the normal peptide and (b) the variant peptide placed the mutation at  $\beta$ 54, but did not allow  $\beta$ 54Val $\rightarrow$ Leu to be distinguished from  $\beta$ 54Val $\rightarrow$ Ile.

To precisely characterize the mutation and resolve the ambiguity between these two isomeric amino acids, the variant  $\beta$ T5 $^{2+}$  ion was subjected to hot ECD. The ECD mass spectrum obtained under standard operating

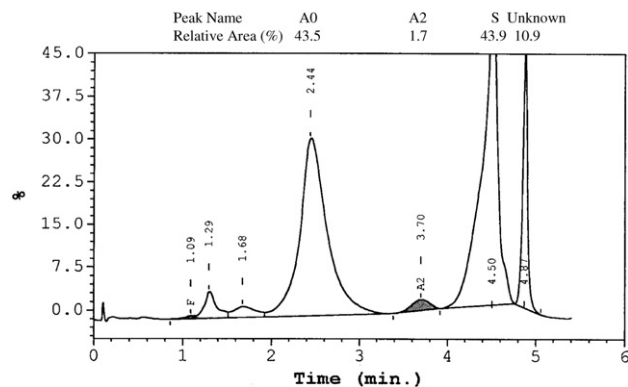
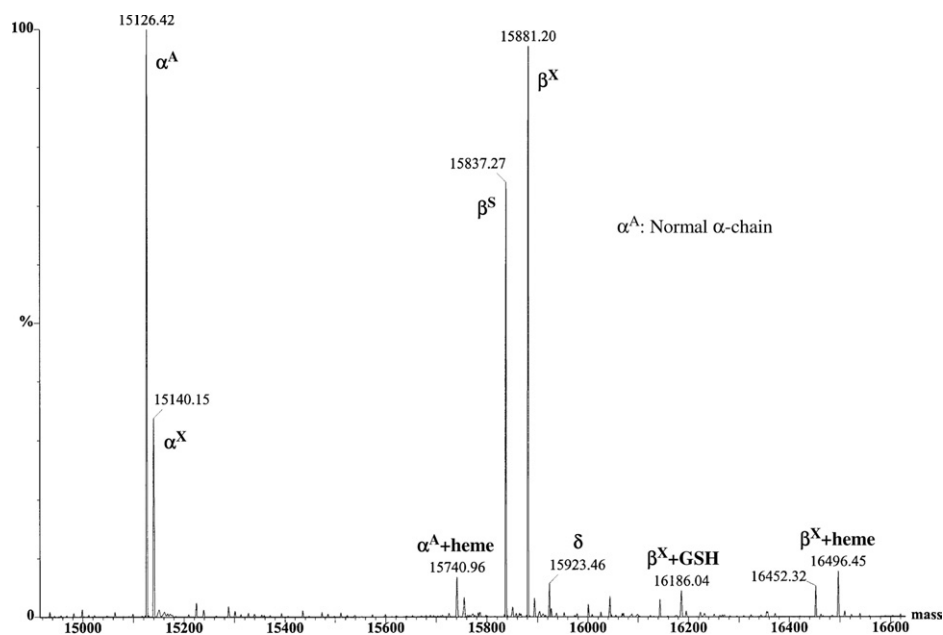


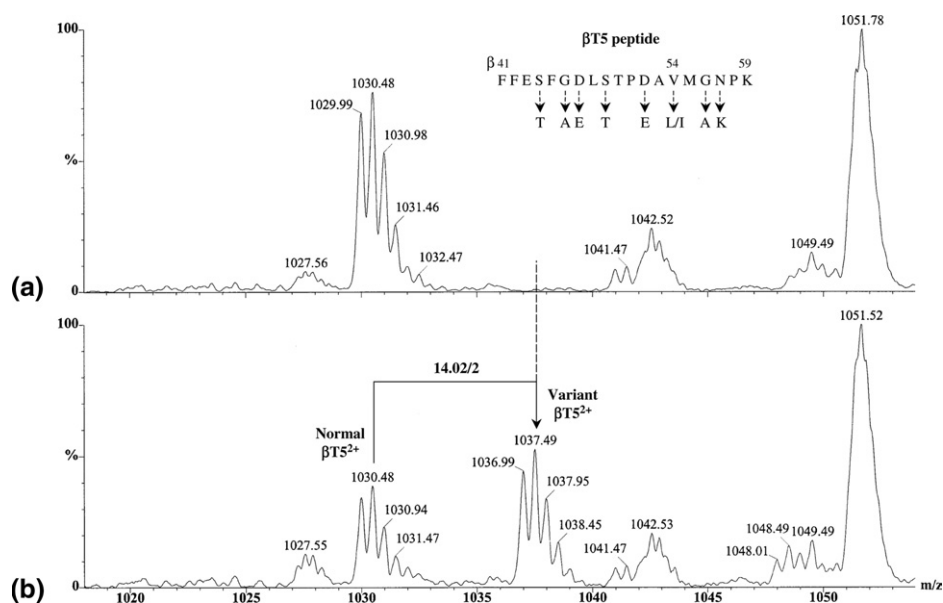
Figure 1. Cation-exchange HPLC trace of the abnormal blood sample. Peak names are those assigned by the ce-HPLC system. A0: normal hemoglobin.



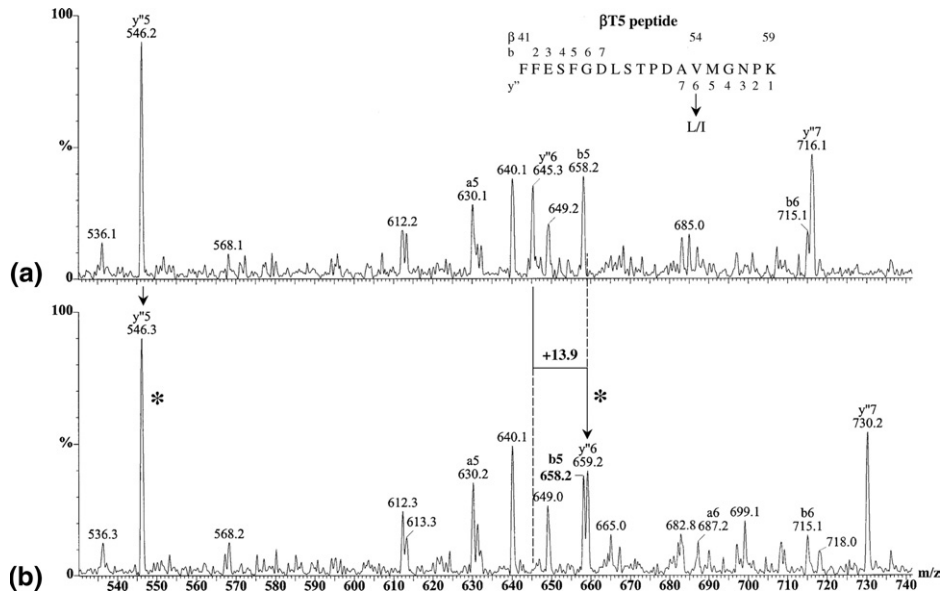
**Figure 2.** Maximum entropy deconvoluted ESI mass spectrum of the 500-fold diluted and denatured abnormal blood sample obtained by the triple quadrupole instrument. It shows the presence of a 14 Da heavier than normal  $\alpha$ -chain variant ( $\alpha^X$ ), and two  $\beta$ -chain variants, one 30 Da lighter ( $\beta^S$ ) and the other 14 Da heavier than normal ( $\beta^X$ ). Masses are experimental. The sequence masses of the normal  $\alpha$ - and  $\beta$ -chains are 15,126.38 and 15,867.24 Da, respectively. GSH: glutathione.

conditions (ECD energy = 5%, corresponding to ECD cathode potential =  $-4.35$  V) (data not shown) did not allow differentiation between the isomeric amino acids. Interestingly,  $z_6$  ions were not observed in that mass spectrum. At higher electron energies (ECD energy = 15%, corresponding to ECD cathode potential =  $-14.35$  V) (Figure 5), additional fragmentation was detected. A peak corresponding to the  $z_6'$  ion ( $m/z_{\text{meas}}$  644.3446,

$m/z_{\text{calc}}$  644.3436) was observed, as was a peak corresponding to ( $z_6 \bullet - 29$ ), (see insets). (Note that  $z'$  ions are the result of hydrogen abstraction by radical  $z \bullet$  ions. In the present case, apparently the  $z_6 \bullet$  ions either fragment producing  $w$  ions, or hydrogen abstraction occurs resulting in  $z_6'$  ions). No peak corresponding to ( $z_6 \bullet - 43$ ) was observed at  $m/z$  600. As described above, the  $z_6 \bullet - 29$  ion constitutes a secondary  $w$  ion originat-



**Figure 3.** Diagnostic region of mass spectra obtained by the triple quadrupole instrument from 30-min tryptic digests of (a) a normal control sample and (b) the abnormal sample showing that the mutation in the heavier than normal  $\beta$ -chain variant occurs in the  $\beta T5$  peptide.



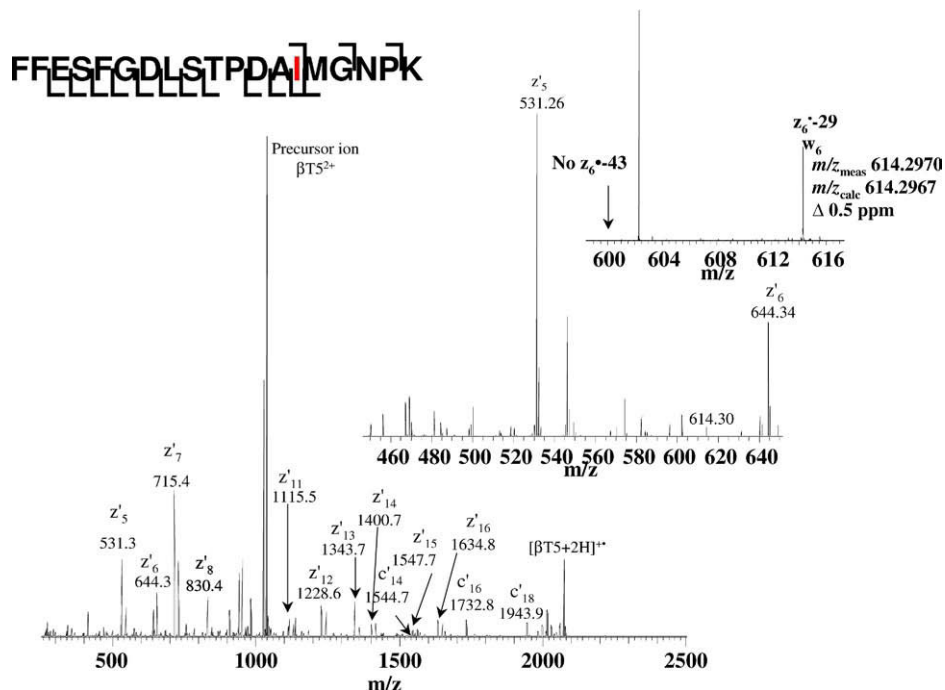
**Figure 4.** Diagnostic region of product ion spectra obtained using the triple quadrupole from (a) the normal  $\beta T5^{2+}$  peptide ion and (b) the variant  $\beta T5^{2+}$  peptide ion. The 14 Da mass-increase between (a) and (b) at  $y^*_6$  identifies the mutation as either  $\beta 54\text{Val}\rightarrow\text{Leu}$  or  $\beta 54\text{Val}\rightarrow\text{Ile}$ .

ing from an isoleucine residue (Scheme 1). To confirm our assignment, we performed HECD on the synthetic peptides FFESFGDLSTPDAIMGNPk and FFESFGDLSTPDALMGNPk (Figure 6). As for the  $\beta T5^{2+}$  ions, HECD of  $[M + 2H]^{2+}$  ions of FFESFGDLSTPDAIMGNPk (Figure 6a) resulted in a peak corresponding to  $(z_6 \bullet - 29)$  ions. No peak corresponding to  $(z_6 \bullet - 43)$  ions was observed. HECD of  $[M + 2H]^{2+}$  ions of FFESFGDLSTPDALMGNPk (Figure 6b) resulted in a peak corresponding to  $(z_6 \bullet - 43)$

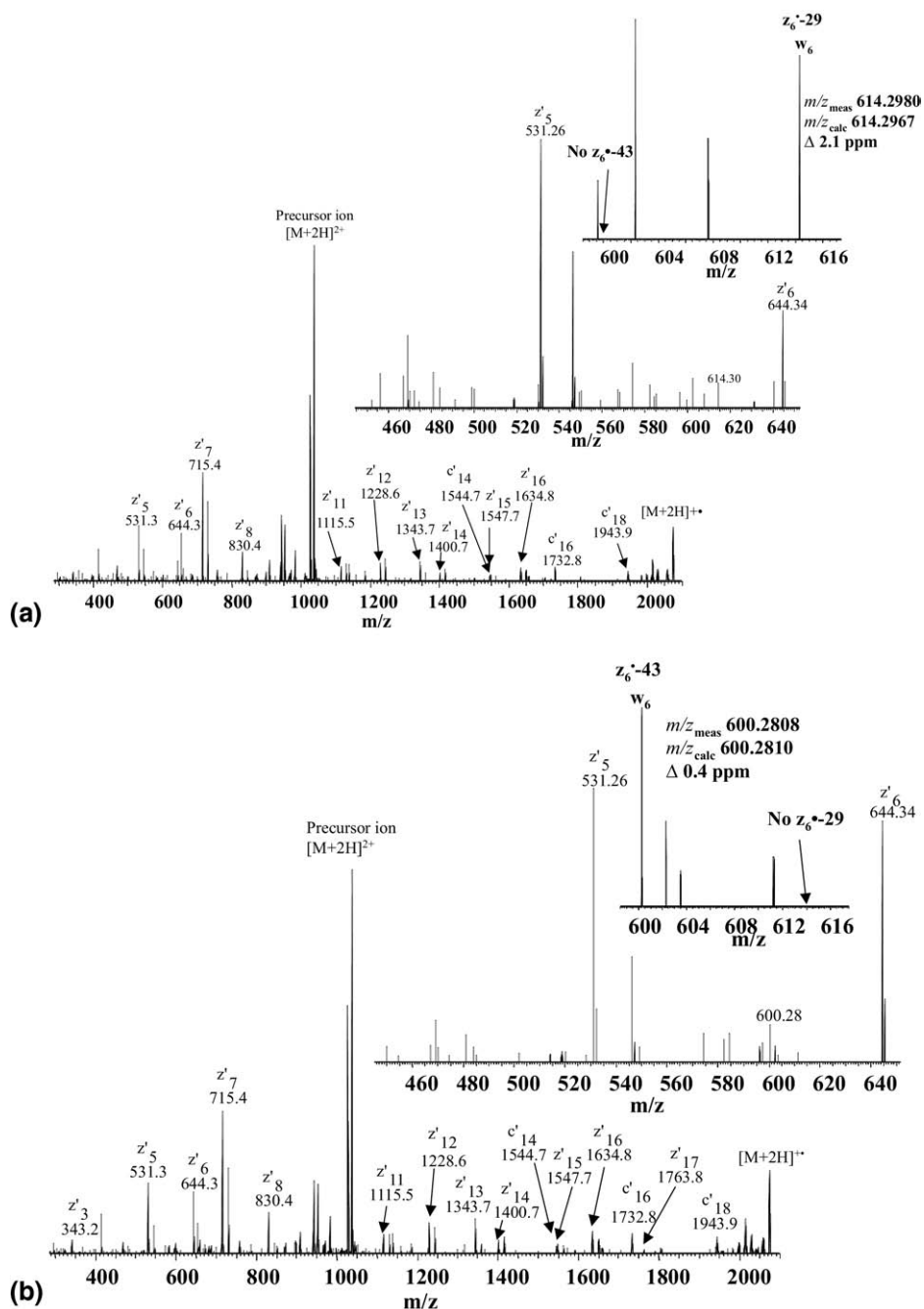
ions. No peak corresponding to  $(z_6 \bullet - 29)$  ions was observed. The mutation is thus identified as  $\beta 54(D5)\text{Val}\rightarrow\text{Ile}$ . This mutation has not been described previously [1], and we have named it Hb Askew.

### Discussion

As a stand alone technique ce-HPLC may presumptively, but never positively, identify any variant. Mass



**Figure 5.** HECD mass spectra of the variant  $\beta T5^{2+}$  peptide ion obtained using the FT-ICR instrument. The insets show the detection of the  $w_6$  ( $z_6 - 29$  Da) ion which identifies the mutation as  $\beta 54(D5)\text{Val}\rightarrow\text{Ile}$ .



**Figure 6.** HECD mass spectra of the  $[M + 2H]^{2+}$  ions of the synthetic peptides (a) FFESFGDLSTP-DAIMGNPK and (b) FFESFGDLSTPDALMGNPK. The insets show the detection of the  $w_6$  ions.

spectrometry has been shown to be a powerful complementary technique, which can detect and identify phenotypically silent mutations as well as those that are easily detected by ce-HPLC [8]. In this study, the mutation  $\beta 54(D5)Val \rightarrow Ile$  is silent by ce-HPLC and was only discovered because it occurred together with other detectable mutations in the same patient, so prompting further investigation. The aim of the UK antenatal screening program is to detect Sickie Hb,  $\beta$ -thalassaemia trait, or one of the clinically significant variants, such as Hb C, D-Punjab, E, O-Arab, and Lepore, which are

known to interact with Sickie to cause sickling. The initial 3 min analysis of the intact Hb chains supported the likely presence of the Sickie variant and ruled out the presence of any of the above known interacting variants. The results also demonstrate the utility of HECD for distinguishing between Leu and Ile residues in human hemoglobinopathies obviating the need for DNA analysis. The variant was initially identified as  $\beta 54Val \rightarrow Leu$  or Ile by triple quadrupole mass spectrometry. Low-energy CID on the triple quadrupole and FT-ICR instruments, and conventional ECD could

not resolve the ambiguity as backbone sequence fragments from the isomers have identical masses. HECD results in secondary fragmentation of the amino acid side-chains allowing Leu and Ile to be distinguished. In the present case, we identified the valine to isoleucine mutation at position 54 in the  $\beta$ -chain following detection of the  $w_6$  ( $z_6 \bullet - 29$ ) ion from the peptide FFESFG-DLSTPDAIMGNPK, and the absence of a  $w_6$  ( $z_6 \bullet - 43$ ) ion that would have implied the tryptic peptide FFESFGDLSTPDALMGNPK. The assignment was confirmed by performing HECD on the synthetic analogues of these peptides. The codon for the  $\beta 54$ Val is GTT. The codons for Ile are ATT, ATC, and ATA, and those for Leu are TTA, TTG, CTT, CTC, CTA, and CTG. Thus we conclude that the Askew variant is the result of a single base change in the nucleotide codon, GTT $\rightarrow$ ATT.

## Acknowledgments

JPW thanks the Warwick University Research Development Fund for a grant and Professor Peter J. Sadler for helpful discussions. HJC gratefully acknowledges the Wellcome Trust (074131) for funding.

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