Prediction of Product Ion Isotope Ratios in the Tandem Electrospray Ionization Mass Spectra from the Second Isotope of Tryptic Peptides: Identification of the Variant β131 Gln→Glu, Hemoglobin Camden

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Many human hemoglobin variants occur in heterozygotes; that is, the variant and normal hemoglobins are present in the same sample. In a procedure for rapidly identifying such variants by mass spectrometry, mutations that increase the mass by 1 Da require a special approach. One of the steps in this procedure involves digesting the denatured hemoglobin with trypsin and analyzing the resulting peptide mixture by mass spectrometry to identify the mutant peptide. Generally the mutant peptide ion can then be selected as the precursor and sequenced by tandem mass spectrometry to identify or confirm the mutation. However, with heterozygotes in which the mass of the variant is 1 Da higher than normal, the first isotope of the mutant peptide occurs at essentially the same mass as the second isotope of the normal peptide, precluding analysis of the mutant peptide on its own. Product ions from the second isotope of a peptide are doublets, 1 Da apart. The way in which the relative abundance of the components in these doublets varies with the elemental composition of the product ions was predicted from the isotopic abundance of the elements and agreed well with experimental data. These results were applied to the identification of a variant that increases the mass by 1 Da in a heterozygote—that is, β131 Gln→Glu, hemoglobin Camden. (J Am Soc Mass Spectrom 2007, 18, 1493–1498) © 2007 American Society for Mass Spectrometry

Hemoglobin (Hb) exists in the blood cells of vertebrates as a noncovalently assembled tetramer of α- and β-chains ($\alpha_2\beta_2$), in which each chain is associated with a heme group. Its primary function is to deliver oxygen to the organs of the body. Abnormalities in the sequence of one of these chains (15–16 kDa) can seriously interfere with the function of the assembled tetramer. In adult human Hb, approximately 1000 α- and β-chain abnormalities (variants) have been described and many more are possible. Most variants arise from a single base change in the nucleotide codon for one of the chains. Some variants are clinically significant, whereas many function normally. Nevertheless, once a variant has been detected, it is prudent to identify it, particularly if it occurs in a potential parent or in a patient with an unexplained blood abnormality.

Most variants are detected in hospital hematology laboratories by analyzing blood using charge-sensitive phenotypic methods, such as cation-exchange high-performance liquid chromatography or isoelectric focusing. Although these methods can presumptively identify a variant, they do not identify novel variants or positively identify any variant. Unambiguous identification requires protein sequencing or DNA analysis. A procedure for routinely identifying Hb variants by electrospray ionization mass spectrometry (ESI-MS) has been described previously [1, 2]. Briefly, there are three steps in this procedure. The first step involves analyzing blood diluted in a denaturing solvent to determine the mass (molecular weight) of the variant chain and assign the variant to the α- or β-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, which is necessary for only roughly 50% of samples, involves sequencing the variant tryptic peptide by tandem mass spectrometry (MS/MS). To minimize sample preparation and analysis times, all the steps in this procedure involve no chromatographic separation of components either before or during analysis. It has been found in practice that the number of interferences between the 30 or so tryptic peptides in the digest mixture is remarkably low, allowing >95% of the variants encountered in practice to be identified in this way.

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1044-0305/07/$32.00
doi:10.1016/j.jasms.2007.05.005

Published online May 17, 2007
Received April 4, 2007
Revised May 10, 2007
Accepted May 11, 2007

Most variants submitted for analysis occur in heterozygotes (i.e., the variant and normal chains occur together in the same sample). In these cases, the abundance of the variant can vary from a few percent to roughly 50% of total Hb. There are many variants with masses that differ from normal by only 1 Da, including several that are clinically significant. The intact normal and variant chains in such variants cannot be resolved from one another by MS in the first step. Nevertheless, they can be detected at this stage provided their abundance is >10% of the normal chain because the mass of the combined normal and variant chains can be determined very accurately (to within ±0.05 Da standard deviation in 15–16 kDa) [2].

Variants with masses that are 1 Da lower than normal are readily detected in the spectrum from a tryptic digest (step 2), where the first isotope of the variant peptide appears as a separate entity that is resolved by the mass spectrometer from the normal peptide. Therefore, when necessary, sequencing by MS/MS can be undertaken on the monoisotopic ion of the variant peptide. The resulting product ion spectrum can then be compared with that from the monoisotopic ion of the normal peptide from a control sample, and thus the mutant amino acid can be positively identified. Bearing in mind that the mass change is only 1 Da, interpretation of the resulting tandem mass spectrum is considerably simplified by selecting the monoisotopic ion as the precursor for MS/MS. In this way, all the product ions are single peaks.

However, the situation is more complicated when the variant has a mass that is 1 Da higher than normal. In these cases, the monoisotopic ion of the variant peptide has essentially the same mass as the second isotopic ion of the normal peptide, and it is not possible to select only the monoisotopic ion of the variant peptide for sequencing by MS/MS. Consequently, the product ions from the second isotope of the normal peptide ion are doublets, 1 Da apart. One component of the doublet is monoisotopic and contains only the first isotope of the elements constituting the product ion. The other component contains one of the second isotopes of the elements in the ion. Therefore, to identify where the mutation occurs in the product ion spectrum from the normal plus variant ions, it is necessary to know the relative abundance of the components in the doublet from the second isotope of the normal peptide. An approximate relation between the relative intensities of the components in such doublets was used to obtain information on the elemental composition of small molecules [3] and also to illustrate the performance of a novel magnetic sector-time-of-flight tandem instrument [4]. Of course, separation of the variant Hb or variant peptide by chromatographic means would allow selection of the variant peptide ion, but would considerably increase analysis time. Here we show how the relative abundance of the doublet ions in the tandem mass spectrum from the second isotope of a peptide can be rigorously deduced from their elemental composition and applied to the identification of the variant Hb Camden β131 Gln→Glu [5].

Experimental

The procedures for identifying variants in blood samples by mass spectrometry have been previously described in detail elsewhere [1, 2]. Briefly, 10 μL of whole blood samples [in ethylene diamine tetraacetic acid (EDTA) anti-coagulant] were diluted 50-fold with 490 μL of water to give a stock solution. Then, 20 μL of the stock solution was diluted a further tenfold with 180 μL of 5:4 acetonitrile:water containing 0.2% formic acid (solution A). After desalting, this solution was introduced at 5 μL/min into the ESI source of a triple quadrupole mass spectrometer (Quattro Ultima, Waters Corporation, Manchester, UK) for measuring the masses of the intact globin chains. Data were acquired for 3 min.

Tryptic digests were prepared as follows [1]. First, 100 μL of the stock solution was denatured by mixing with 20 μL of 50% aqueous acetonitrile containing 0.5% formic acid. Then, 6 μL of 1 M ammonium acetate solution was added, followed by 5 μL of a 5 mg/mL solution of trypsin (Sigma, T1426). The resulting solution was then incubated at 37 °C for 30 min, after which aliquots were diluted tenfold with solution A and introduced into the mass spectrometer at 5 μL/min. Data were acquired for 3 min.

Tandem mass spectra were acquired directly from the tryptic digest solutions as prepared earlier. The resolution of the first quadrupole was set to resolve the isotopic species, such as that shown in Figure 1 for $\beta T13^{2+}$ ions. Product ion resolution was unit mass as shown in Figures 2 and 3. The collision gas was argon at an indicated Pirani gauge pressure of 2.4×10^{-3} mbar in the collision cell and the collision energy was 18 V. Solutions were introduced at 5 μL/min and data were acquired for 15 min.

Prediction of Isotopic Abundances in the Product Ion Spectra from the Second Isotope of Organic Molecules

The second isotope of an organic molecule of elemental composition $C_wH_xN_yO_z$ contains one heavy isotope, either an atom of ^{13}C or ^{2}H or ^{15}N or ^{17}O, the other atoms being ^{12}C, ^{1}H, ^{14}N, and ^{16}O. For example, a molecule that contains one ^{13}C atom has the composition $^{12}C_{w-1}{}^{13}C_1\,{}^{1}H_x\,{}^{14}N_y\,{}^{16}O_z$ and one that contains a ^{2}H atom has the composition $^{12}C_w\,{}^{1}H_{x-1}\,{}^{2}H_1\,{}^{14}N_y\,{}^{16}O_z$, and so forth. The ions from such molecules are not resolved from one another on instruments typically used for this work. Consequently, when they are subjected to MS/MS analysis, the resulting product ions will contain either one atom of a heavy isotope or will consist

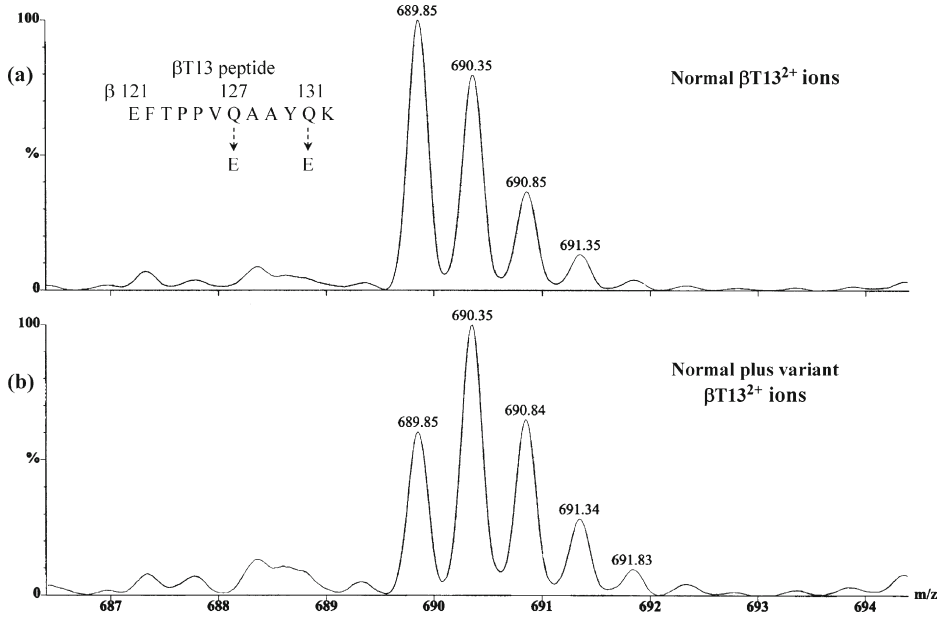

Figure 1. The $\beta T13^{2+}$ ion region of the spectrum from tryptic digests of (**a**) a normal hemoglobin and (**b**) the abnormal heterozygote.

entirely of the light isotopes (i.e., will be monoisotopic). Thus, instead of obtaining a single peak for each product ion, as would be obtained when the first isotopic peak is selected as the precursor, each product ion will occur as a doublet. The probability that the product ions will contain a heavy isotope may be calculated as follows.

The ratio (R) of the second isotope to the first isotope in a molecule of composition $C_wH_xN_yO_z$ is given by [6]

$$R = wc/(1 - c) + xh/(1 - h) + yn/(1 - n) + zo_1/(1 - o_1 - o_2) \quad (1)$$

in which carbon is assumed to consist of ^{13}C and ^{12}C in the ratio c:(1 − c); hydrogen of ^{2}H and ^{1}H in the ratio h:(1 − h); nitrogen of ^{15}N and ^{14}N in the ratio n:(1 − n), and oxygen of ^{18}O, ^{17}O, and ^{16}O in the ratios o_2:o_1:(1 − o_1 − o_2). The isotopic ratios were calculated from the

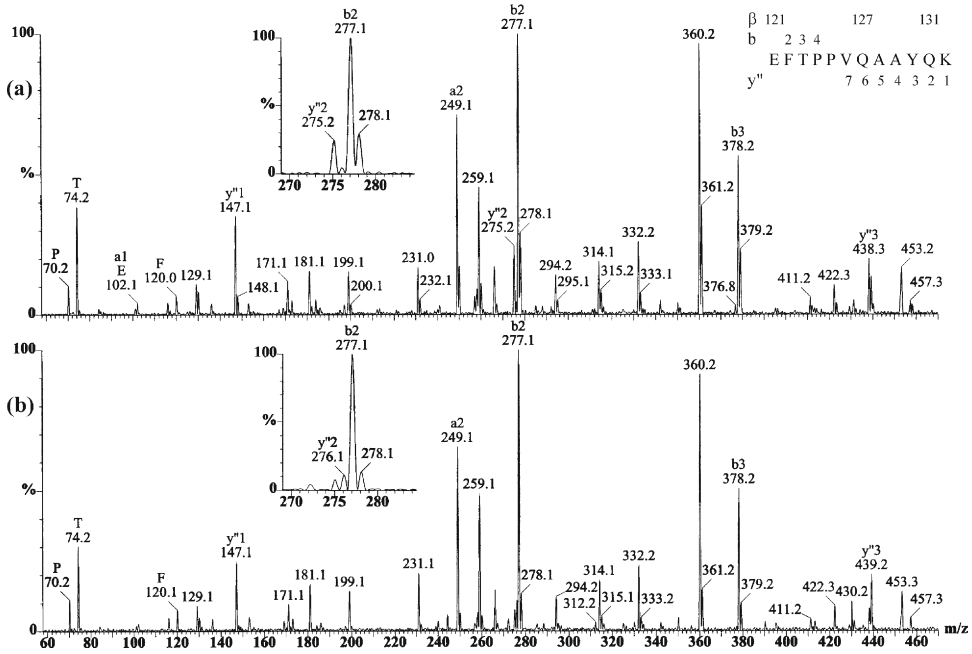

Figure 2. The lower *m/z* region of the product ion spectrum from (**a**) the second isotope of the normal $\beta T13^{2+}$ ion from a control and (**b**) the second isotope of the normal $\beta T13^{2+}$ plus the first isotope of the variant $\beta T13^{2+}$ ions from the abnormal heterozygote.

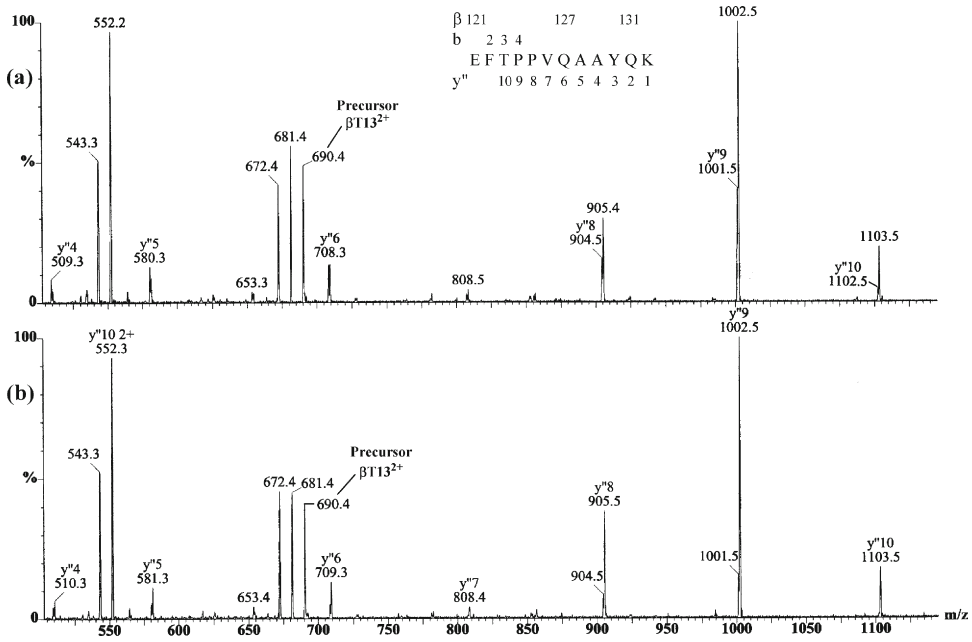

Figure 3. The upper *m/z* region of the spectra shown in Figure 2.

mole fractions of the isotopes of the elements [7] and are as follows: c/(1 − c) = 0.010816; h/(1 − h) = 0.000115; n/(1 − n) = 0.003653; and $o_1/(1 - o_1 - o_2) = 0.000381$. Suppose the second isotopic ion of the molecule $C_wH_xN_yO_z$ is selected as the precursor for MS/MS analysis and produces product ions of composition $C_pH_qN_rO_s$.

From eq 1 above, the proportion of precursor ions containing a ^{13}C atom is wc/(1 − c)R and the probability of a ^{13}C atom occurring in the product ion is p/w. Therefore, the contribution to the product ion by ^{13}C atoms is pc/(1 − c)R. Similarly, the proportion of precursor ions containing a ^2H atom is given by xh/(1 − h)R and the probability of a ^2H atom occurring in the product ion is q/x. Thus the contribution to the product ions by ^2H atoms is qh/(1 − h)R and so forth.

It follows that the proportion (D_H) of heavy isotopic product ions in the doublet is given by

$$D_H = [pc/(1 - c) + qh/(1 - h) + rn/(1 - h) + so_1/(1 - o_1 - o_2)]/R \quad (2)$$

The proportion of the light (monoisotopic) product ion is $D_L = 1 - D_H$.

Substituting the values for c/(1 − c), h/(1 − h), and so forth in eq 2 above gives

$$D_H = [0.010816p + 0.000115q + 0.003653r + 0.000381s]/R \quad (3)$$

in which

$$R = 0.010816w + 0.000115x + 0.003653y + 0.000381z \quad (4)$$

Results and Discussion

Routine analysis of a blood sample by cation-exchange HPLC revealed a variant Hb that eluted before the normal Hb and was present with similar abundance to the normal Hb. This result implies the presence of a heterozygote in which the mutation causes a negative charge change from normal. The denatured blood sample was analyzed by ESI-MS, when the mass of the β-chain was found to be 15,867.78 Da. This mass, 0.54 Da higher than normal, is consistent with the presence of a normal β-chain plus a variant β-chain with a mass that is 1 Da higher than normal [2]. Two globin chains that differ in mass by 1 Da cannot be resolved by MS and their combined mass is the abundance weighted mean of the two chains. Possible mutations that can give 1 Da mass increase by a single base change in the nucleotide codon are Asn→Asp, Gln→Glu, and Lys→Glu, all of which cause a negative charge change in the Hb molecule. However, the mutation Lys→Glu abolishes a trypsin cleavage site and thus is detected, and identified, by the creation of a "new" double peptide. Therefore, the technique described herein is not appropriate in this case.

The spectrum of a 30-min tryptic digest showed that the mutation occurred in the βT13 peptide. Figure 1 shows the $\beta T13^{2+}$ ion region of the tryptic digest from (a) a normal control and (b) the abnormal sample. The peak at *m/z* 689.85 from both samples contains only the monoisotopic ion from the normal peptide and the peak at *m/z* 690.35 from the abnormal sample contains a mixture of the first isotope of the abnormal peptide and the second isotope of the normal peptide. There are two possible mutations in this peptide that can give 1 Da mass increase by a single base change in the nucleotide codon: Hb Complutense, β127 Gln→Glu, and Hb Cam-

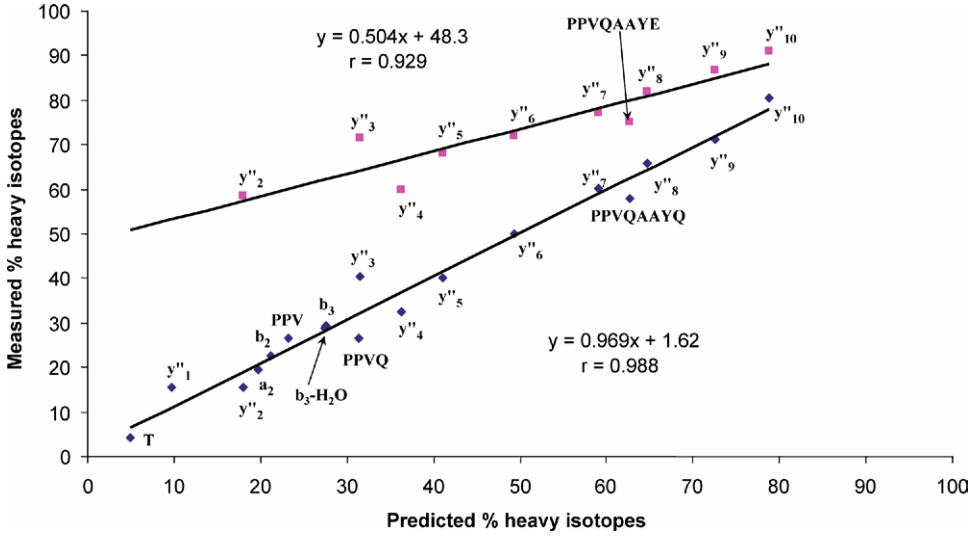

Figure 4. Measured versus predicted proportions of heavy isotope containing ions in product ions observed in the tandem mass spectra from (♦) the second isotope of the normal $\beta T13^{2+}$ ion and (■) the second isotope of the normal $\beta T13^{2+}$ ion plus the first isotope of the variant $\beta T13^{2+}$ ion.

den, β131 Gln→Glu. Therefore, MS/MS analysis was undertaken to distinguish them.

Figures 2 and 3 show respectively the lower and higher *m/z* regions of the tandem mass spectra from (a) the second isotope of the normal $\beta T13^{2+}$ ion from the control sample at *m/z* 690.35 and (b) the first isotope of the variant peptide plus the second isotope of the normal peptide also at *m/z* 690.35. For clarity, the dominant y''^{2+}_9 product ion at *m/z* 501.8 has been omitted. It can be seen in the spectrum from the second isotope of the normal $\beta T13^{2+}$ ion that the product ions are doublets in which the relative intensity of the higher mass component gradually increases as the *m/z* of the product ion increases. The lower line in Figure 4 shows the measured proportion of heavy isotope containing ions in the product ion spectra from the second isotope in the normal $\beta T13^{2+}$ ion plotted against the predicted proportion using eq 3 above. Linear regression analysis (n = 18) gave y = 0.969x + 1.62 with a correlation coefficient (r) of 0.988, and shows good agreement between the measured and predicted proportions of the heavy isotope containing ions. The upper line in Figure 4 shows the proportion of heavy isotope containing ions from the second isotope of the normal $\beta T13^{2+}$ ions plus those ions from the monoisotopic variant $\beta T13^{2+}$ ion plotted against the predicted proportion according to eq 3. Linear regression analysis of these data (n = 10) gave y = 0.504x + 48.3, r = 0.929.

When the spectra from the normal and abnormal samples are compared, it can be seen that there is a marked increase in relative intensity of the higher mass component of the doublet at y''_2 (Figure 2 and insets) and at all subsequent y″ ions. This identifies the mutation as β131 Gln→Glu, Hb Camden, because y''_2 corresponds to β131 in the β-chain. Had the mutation been β127 Gln→Glu, the lower mass component of the doublet would have remained dominant up to and including y''_5, whereas y''_6 to y''_{10} would have appeared similar to the doublets shown in Figure 3b. It can also be seen that the higher mass components of y''_1, b_2 and b_3, actually decrease relative to the low mass component between Figure 2a and b, because these product ions from the mutant peptide do not include the mutation and therefore do not contribute to the higher mass component. This means that there would be a more dramatic increase in the intensity of the heavy isotope containing ion at y''_6 in the case of β127 Gln→Glu than would be inferred from the lower line in Figure 4.

The reason that the measured fractions of y''_3 and y''_4 are respectively significantly higher and lower than predicted is not understood (Figure 4). MS/MS analysis of the monoisotopic $\beta T13^{2+}$ ion from several samples showed the presence of a minor, unidentified, product ion at *m/z* 439.3, one *m/z* unit higher than y''_3. This "background" ion would appear to increase the fraction of the heavy isotope, but attempts to quantitatively allow for it were unsuccessful. A "background" ion at *m/z* 509.3 would account for a lower than predicted fraction of the heavy isotope for y''_4. The deviation of y''_3 was similarly higher than both straight-line fits and y''_4 was similarly lower. Because the deviations from predicted are similar for normal and variant samples, both y''_3 and y''_4 appear to be usable in identifying the variant.

The slope of the line from the Hb Camden heterozygote in Figure 4 is 0.50, which is consistent with the variant being present at 50% abundance. The intercept with the *y*-axis (48.3%) is also consistent with this value. These values are slightly higher than the value determined by HPLC (45%) and slightly lower than the measured fraction of the variant implied from the measured mass of the intact β-chains (54%).

An alternative method of treating variants that increase the mass by 1 Da would be to separate the

variant peptide by chromatographic means. In this way, MS/MS analysis could be performed solely on the variant peptide and there would be no need to compute the product ion isotope ratios from the second isotope of the normal peptide. However, once these ratios have been derived for a particular peptide, they can then be used whenever a mutation occurs in that peptide in the future—that is, the calculations only have to be done once for a given peptide. Furthermore, adding a separation step would increase analysis time and it would still be necessary to sequence the separated variant peptide by MS/MS and compare the results with those from the normal peptide. Overall, the method described earlier for dealing with the few variants that increase the mass by 1 Da does not appear to be significantly more time consuming than separating the variant by chromatographic means.

Conclusions

We have shown that the proportion of the heavy isotope of the major product ions from the second isotope of the normal $\beta T13^{2+}$ ion agrees well with theory. The plots in Figure 4 were used to positively identify the mutation β131 Gln→Glu in a heterozygote and could be used to identify the mutation β127 Gln→Glu. Product ion data similar to those obtained from βT13 were also obtained from the second isotopes of the normal βT3 and βT4 ions and showed good agreement with theory. In principle, this technique could be used to confirm the other mutations that cause a 1 Da mass increase over normal in the β-chain. Fortuitously, there are only seven such mutations that are likely to occur on genetic grounds and, with all of them, the associated tryptic peptide contains only one Gln→Glu or Asn→Asp mutation. Thus, tandem mass spectrometry would not be strictly necessary for identification and would be required for confirmation only if deemed necessary.

The technique could also be applied to similar mutations in the α-chain, but more careful scrutiny of the tryptic peptides would be required to detect the presence of the mutation, because the variant would generally be less abundant (<25%) than that in the case of the β-chain variants. Fortunately only five α-chain variants that produce a 1 Da increase in mass are likely to occur on genetic grounds and three of these occur in small tryptic peptides containing only one likely mutation. However, the other two occur in the large αT9 peptide and the change in the relative intensity of the isotope peaks would be difficult to detect in routine work.

Acknowledgments

J. P. Williams thanks the Wellcome Trust VIP award for research funding.

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