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The Use of On-line Capillary Electrophoresis/ Electrospray Ionization with Detection via an Ion Trap Storage/Reflectron Time-of-flight Mass Spectrometer for Rapid Mutation-site Analysis of Hemoglobin Variants

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Capillary electrophoresis/electrospray ionization using an ion trap storage/reflectron time-of-flight mass spectrometer detector (CE/ESI-IT/reTOF) is used to provide a rapid and sensitive method for analyzing structural variants in the hemoglobin (Hb) β-chain. The Hb α- and β-chains are separated and the β-chain is digested by trypsin. The digest is analyzed by CE/ESI-IT/reTOF where a comparison of the total ion electrophorograms and mass spectra of the mutant and normal hemoglobins (Hbs) can detect the presence of a mutation site. In addition, collision-induced dissociation in the vacuum interface – skimmer region can be used to pinpoint the identity of such a site. The unique capability of the CE/ESI-IT/reTOF system for accurately detecting fast separations with narrow peaks that may be under 1 s full width at half maximum is demonstrated. The speed of this system is essential for resolution of the large number of peaks that are separated in a short time duration using CE separations. © 1997 by John Wiley & Sons, Ltd.

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Since the discovery of its first variant in 1956,[1] hemoglobin (Hb) has been an important subject for biological research and clinical practice. The presence of abnormal hemoglobins can cause severe diseases directly related to the structure of the Hb. Most of the more than 700 structural variants[2] known to date are single amino acid substitutions in either the α- or β-chains, although chain elongation, deletion and other mutations may be found. The conventional detection of hemoglobinopathies relies on electrophoresis[3] and isoelectrofocusing,[4] which are labor intensive and time consuming, often requiring several days to complete.

More recently, reversed-phase high-performance liquid chromatography (RP-HPLC) of either Hb or its tryptic digests has been used to identify Hb variants when the analysis time is typically under 1 h.[5] The analysis of Hb was significantly improved by Shelton *et al.*[6] who used large-pore C_4 columns together with simplified acetonitrile–water–trifluoroacetic acid (TFA) gradients to separate and identify the whole globin chains from normal and variant hemoglobins. A more definitive method used tryptic digestion of Hb with HPLC separation for identification of variant globins based upon a comparison of the tryptic fragments produced.[7,8] In the tryptic fragments of Hb there are 29 digestion peaks for normal adult hemoglobin (HbA), four of which, αT12, αT13, βT10, and βT12 are insoluble and precipitate out during the digestion preparation; two other single amino acid products, αT8 and βT8, are not detected with a UV absorbance detector at 200 nm.[9] Substitution of amino acids between normal Hb and one of its variants results in a change of the position and number of the fragments. The HPLC method provides an important advantage over conventional electrophoresis in terms of the time of analysis. However, HPLC is unable to efficiently resolve all peptides in Hb tryptic digests, and overlapping and unresolved peaks may make identification difficult. Nevertheless, much work has been done developing HPLC methods for the characterization of clinical Hb mutations.[7]

The recent development of capillary electrophoresis (CE)[10,11] has provided a complementary method to HPLC and gel electrophoresis. The high sensitivity and superior resolving power of CE has led to its use in solving important problems in the biological and biomedical fields. Perrett *et al.*[9] studied the tryptic mapping of some common and rare Hb variants using CE separation with UV detection. They readily observed the abnormal peptide fragments which could be identified by comparison between the normal and abnormal tryptic map profiles. Various other researchers, including Casragbola[12] and Ong,[13] have used CE for the separation of globin chains in a rapid identification of variant hemoglobins where separations could be performed within 10 min.

In order to unambiguously prove the presence of a hemoglobin mutation and to identify the variant sites, mass spectrometry has been successfully combined to separation techniques such as HPLC or CE, either off-line or on-line. The use of mass spectrometry in Hb analysis by Matsuo *et al.*[14] incorporated field desorption and fast-atom bombardment mass spectrometry (FAB-

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MS) in the analysis of the tryptic digests of α-, β-, and δ-chains. In subsequent work, Ferranti and co-workers[15] used CE separation of globin chains and tryptic digests with detection by FAB-MS to characterize hemoglobinopathies. In this work, the fractions from CE were collected and sequentially determined off-line by mass spectrometry. The obvious disadvantage of this method is that it is extremely difficult to collect the fast-eluting electrophoretic peaks, and the small sample amount was not sufficient for FAB mass analysis for some fragments. More recently, several groups[16–19] have coupled microbore HPLC on-line with electrospray ionization mass spectrometry (ESI-MS) to identify various Hb mutations. The use of microbore columns improved the analysis time compared with previous HPLC work but is still considerably longer than the time-frame that can be achieved by using a CE separation. In addition, much improved resolution could be achieved using CE as compared to HPLC. Other mass spectrometric methods have also been used for the analysis of Hb variants. Biemann's group[20] used matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) without separation to analyze the proteolytic digests of the normal and cross-linked Hb β-chains. Other groups[21–25] have used a mass spectrometer without separation, directly applying tandem mass spectrometry (MS/MS) and collision-induced dissociation (CID) on the entire Hb protein or separated α- and β-chains, to identify the variation sites in a series of abnormal hemoglobins. The mass analysis provides unambiguous identification for most of the fragments and confirms the variant sites inside the specific chains. However, the mass fragments of the entire multiply-charged Hb molecule result in sequence information for ~29% of the molecule[21] and it can only be used to confirm certain common mutations that lie within the well-known tryptic mapping regions.[17] In addition, the resulting MS/MS or CID spectrum is complicated and it requires extensive calculation and comparison for accurate analysis, especially where mutations may result in a shift of only a couple of mass units.

This work reports on the analysis of globin chain digests in several variant hemoglobins using on-line CE/MS with amine-coated fused-silica capillaries where the CE anodic end was directly applied as the microelectrospray needle. The results demonstrate that using the CE capability for fast separation, combined with mass analysis, a rapid and sensitive method could be developed in the screening and detection of abnormal Hbs. Since the electrophoretic peaks from CE are as narrow as 1 s, a fast mass detector that can acquire the data with sufficient speed is required in order to maintain the quality of the total-ion electrophorogram (TIE) peaks and the high CE resolution. The system utilized in these experiments is an ion trap storage/reflectron time-of-flight (IT/reTOF) mass spectrometer that is capable of acquiring a single mass spectrum at a speed of greater than 10 Hz,[26] thus maintaining the separation efficiency of the CE. This is an important advantage in preserving high resolution separations coupled to mass analysis. In addition, the storage properties of the ion trap and the nonscanning nature of the IT/reTOF system result in a very high duty cycle even at the data acquisition rate required in these experiments. This method provides improved sensitivity in detection over a broad mass range for detection of low intensity signals in these CE separations.

EXPERIMENTAL

Preparation of Hb

The Hb protein was prepared in a manner similar to the method reported by Perrett *et al.*[9] Hemolysates were prepared from blood by washing erythrocytes three times with an equal volume of normal saline buffer. Washed red cell samples (0.5 mL) were centrifuged to remove any additional impurities and the supernatant liquid was aspirated. The cells were lysed by diluting with 2.5 mL distilled water, and centrifuged at 8000 *g* for 15 min to isolate the cell membrane and other solid materials. The supernatant liquid was then added drop-by-drop into a cold acid acetone (conc. 2%) solution to precipitate the globin and to be dehemed; the tube was shaken vigorously during this procedure. After centrifugation at 3000 *g* for 10 min, the supernatant liquid was removed and acetone was added to wash the precipitate. This procedure was repeated twice before the proteins were vacuum dried. The prepared globin was redissolved in deionized water for further use.

Separation

The separation of Hb chains was performed with an HPLC system (Model Rabbit-HP, Rainin Instruments Co. Inc., Emeryville, CA, USA) using a C_{18} 4.6 mm × 30 cm column. The Hb chains were separated using a gradient from 45% to 55% acetonitrile containing 0.1% trifluoroacetic acid (TFA). Detection was performed using a UV absorption detector at 214 nm and the eluent was further identified by mass analysis. The sample was vacuum dried and tryptic digestion was applied to the α- and β-chains separately. Subsequently, the β-chains of both the normal and variant Hbs were digested by TPCK-treated trypsin with a substrate to enzyme ratio (50:1) in NH_4HCO_3 solution at pH 8.4 for 24 hours at 37 °C. The digests were then vacuum dried and reconstituted in the CE buffer.

Capillary electrophoresis was performed on a home-built apparatus using a ±30 kV high voltage power supply (Model CZE 1000R, Spellman High Voltage Electronics Corp., Plainview, NY, USA). In order to minimize the adsorption of peptides to the capillary wall, the inner wall of the capillaries was coated by a method similar to that described by Moseley and co-workers[27] with minor modification. A 60 cm capillary (105 μm o.d., 40 μm i.d.) was rinsed with 1 M NaOH solution for 4 h, followed by flushing with H_2O for 10 min, 5 M HCl solution for 30 min, and H_2O for another 10 min. The capillary was then dried with pure nitrogen gas at 90 °C overnight. Subsequently, it was washed with toluene for 30 min and 2.5% (v/v) 3-aminopropyl-trimethoxy silane (APS) coating solution (toluene) was pumped through the capillary for 12 h. The capillary was conditioned with the CE buffer for 4 h prior to use. The buffer used in all of the experiments was 10 mM ammonium acetate with slightly varying pH, depending on the different samples separated. All of the CE separations were performed in the negative-ion mode with an electrical field strength of ~–300 V/cm. A variable wavelength UV detector (Model SC100, Thermo Separation Products, Fremont, CA, USA) with the wavelength set at 198 nm was

initially used to monitor the CE separation process. The samples were injected using the electrokinetic method which loads approximately 50–100 fmol protein digests for each run. The injection, separation, and data acquisition procedures were remotely controlled by a personal computer (Model P5-66, Gateway 2000 Inc., N. Sioux City, SD, USA), where an ADC-16 analog board was applied to collect the UV signal at a frequency of 7 Hz where custom software controlled all procedures.

On-line sheathless CE/MS

The mass spectrometer used in these experiments was an IT/reTOF system as described in previous work.[28,29] It consists of a reflectron TOF mass analyzer (Model D1450) coupled to a front end quadrupole ion trap storage device (Model C-1251, R. M. Jordan Co., Grass Valley, CA, USA). The pressures inside the ion trap main chamber and the TOF tube are 1×10^{-5} Torr and 2×10^{-7} Torr, respectively. In the IT/reTOF, ions are stored for a specific period of time in the ion trap with a preset RF voltage of 1100 V and a frequency of 1.1 MHz on the ring electrode. A helium buffer gas is added to the trap at a pressure of $\sim 10^{-3}$ Torr in order to enhance trapping of ESI-produced externally-injected ions. Subsequently, a DC pulse voltage of 400 V is applied to the endcap of the trap to eject ions into the reTOF for mass analysis. Ions were detected by a 25 mm triple microchannel plate detector (Model C-2501, R. M. Jordan Co.).

The sheathless microelectrospray interface was constructed by using the capillary end as the electrospray needle as reported previously.[30] Briefly, the capillary tip was etched from a dimension of 105 μm o.d. to a fine diameter with concentrated hydrofluoric acid after 1 cm of polyimide coating had been removed. The outer surface of this tip was silver coated by using the electroless plating process,[31] which includes degreasing in CH_2Cl_2, surface sensitization in a sensitizer solution, and finally insertion into the electro-plating solution of silver nitrate. This silver coating provided electric contact for both electrospray and capillary electrophoresis, by inserting the capillary tip into a 2 cm s.s. tube (125 μm i.d., 250 μm o.d.) connected to the electrospray power supply. One end of the capillary was set at around –13 kV, while the needle was at an electrospray voltage of ~4 kV so that the total CE separation voltage was around –17 kV. The needle formed a stable electrospray plume with a resulting mass signal, provided the outer surface was periodically recoated with silver.

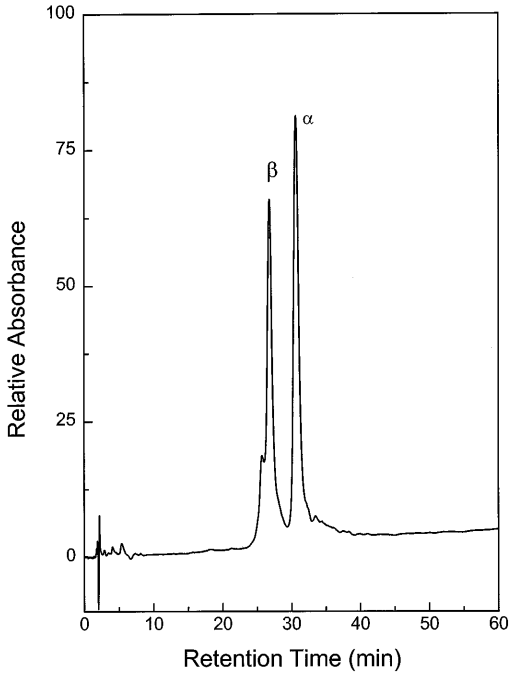

Figure 1. HPLC separation of HbA globin chains. Conditions: C18 column (30 cm, 4.6 mm i.d.), Gradient: 45–55% acetonitrile containing 0.1% TFA in 60 min.

The mass signals were collected using a 250 MHz transient recorder (Model 9846, Precision Instruments Inc., Knoxville, TN, USA) embedded in a P5-66 personal computer, where the data processing was also conducted. This data acquisition system could acquire data at 25 Hz for a CE separation for over half an hour, using software developed in our laboratory.[26] A time-of-flight expansion range of 0 to 150 μs, which corresponds to an *m/z* range of 0–1500, was generally used as the mass acquisition period. Mass calibration was performed using three standard peptides, viz. bradykinin, angiotensin I and Leu-Enk-Arg, according to the following equation, $(m/z)^{1/2} = aT + b$, where T is the flight time.

CID in the vacuum interface – skimmer region

In order to detect the mutation site inside the specific fragment, CID of the CE-separated fragment was performed by raising the focusing lens voltage from 100 V to 150 V–200 V. When the target digest fragment was about to elute from the capillary, the focusing lens voltage was rapidly increased in this way so that dissociation occurred in the region between the focusing lens and the skimmer.

Mass analysis of HPLC-separated globin chains

The confirmation of the mass values for the collected HPLC-separated Hb chains was performed by MALDI-MS using a linear time-of-flight mass spectrometer (R. M. Jordan Co.). The spectra were acquired using 355 nm radiation from a DCR-11 Nd:YAG laser system (Spectraphysics, Mountain View, CA, USA). The matrix used was α-cyano-4-hydroxycinnamic acid, which was prepared in acetonitrile, deionized water and TFA (50:49:1). The mixture of protein and matrix (1:1) was placed on the probe tip and allowed to dry, before being subjected to mass analysis.

Materials and chemicals

Ammonium bicarbonate, TFA, ammonium acetate, acetonitrile, toluene, acetone, sodium hydroxide, hydrochloric acid, silver nitrate, formaldehyde, ammonium hydroxide, 3-aminopropyl-trimethoxy silane, angiotensin I, bradykinin, and Leu-Enk-Arg were purchased from Sigma (St. Louis, MO, USA). TPCK-treated trypsin was purchased from Promega (Madison, WI, USA). These materials were used without further purification. The water was filtered and deionized prior to use by a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). HbA was obtained from Sigma Co., while sickle cell anemia (HbS) and HbE samples were obtained as a gift from the World

Laboratory (Ann Arbor, MI, USA). Fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA).

RESULTS AND DISCUSSIONS

The goal of this work is to pinpoint mutations in variant hemoglobins. In order to simplify this task we have chosen to separate the α- and β-chains of Hb before digestion so that the mutation can be initially identified as being in one or the other of the protein chains. The tryptic digestion of the α- and β-chains of Hb together provides 29 peaks, so that it is preferable to separate the proteins before digestion to simplify the assignment of the mutation. This becomes especially important in many variant Hbs where the mutation may result in a mass difference of only 1 Da or where minor chains, such as γ- and δ-chains, may be present and make the

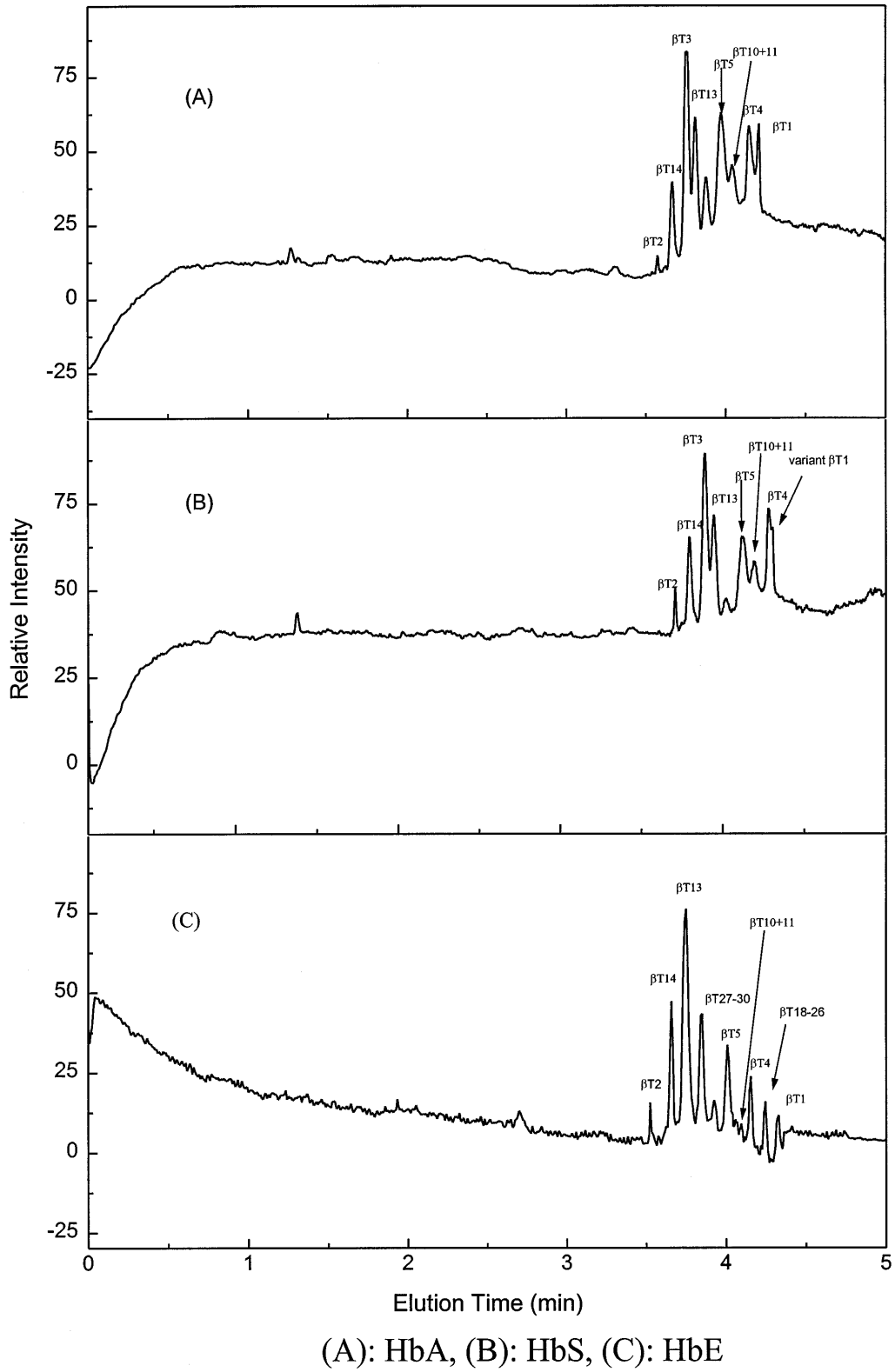

Figure 2. CE separation with UV absorption detection of the Hb digests for (a) HbA, (b) HbS, (c) HbE. Conditions: V_{CE} = –17 000 V, Capillary: APS coated, 60 cm, 40 μm i.d., 105 μm o.d. Buffer: 10 mM ammonium acetate, pH = 4.0.

resulting data particularly difficult to interpret for unknowns. In the case of the total digest separated by CE there may be as many as 10 peaks within a 1 min period and it would be difficult to distinguish abnormal from normal Hb using only total ion-electropherograms in cases where only small variations in structure

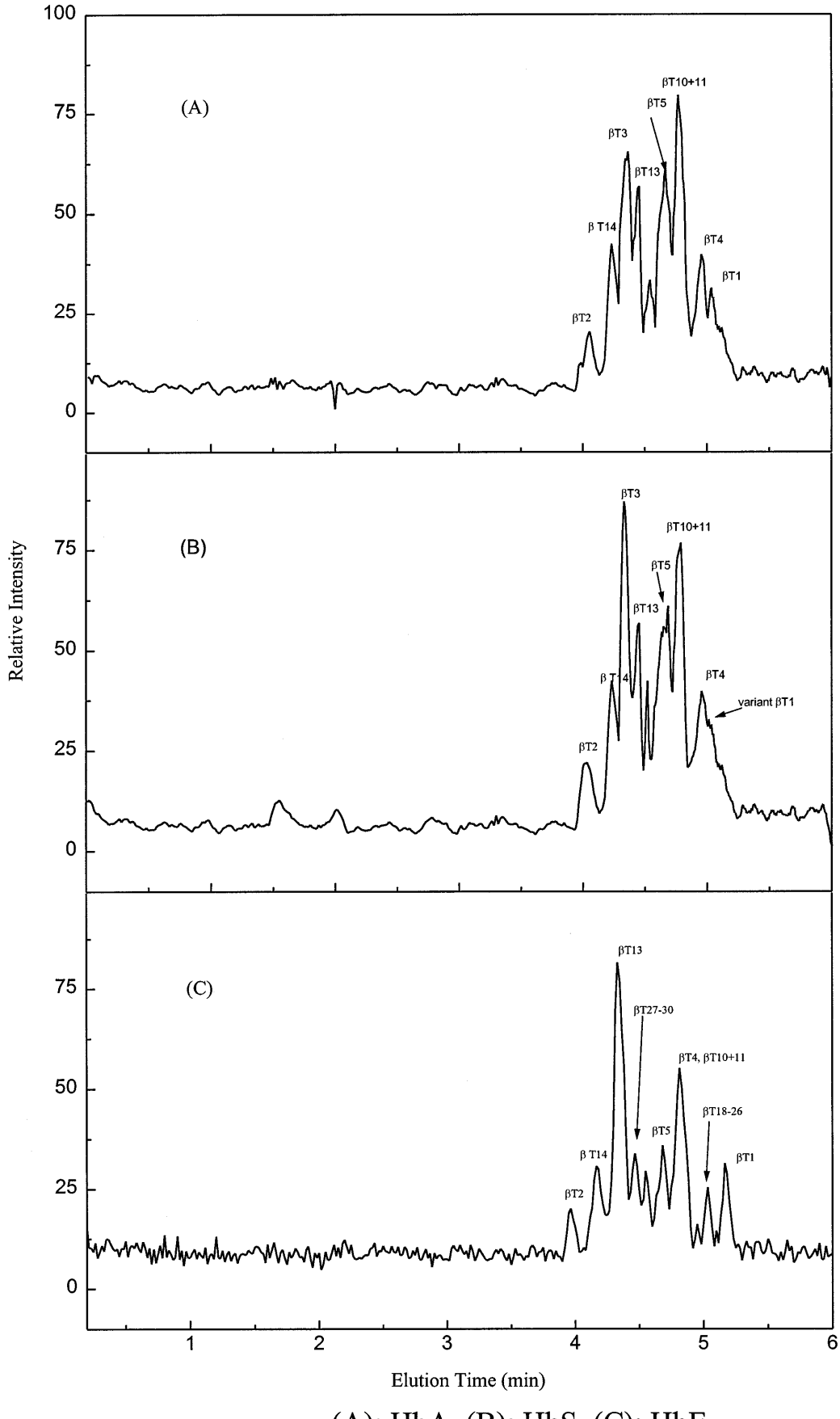

Figure 3. The TIE of the CE/MS of Hb digests for (a) HbA, (b) HbS, (c) HbE. Conditions: V_{CE} = –13 000 V, V_{ESI} = 4 000 V. Other conditions are the same as those in Fig. 2.

Table 1. Comparison of the observed and calculated values of *m/z* fortryptic digests of the Hb β chain in CE/MS

Peptide	Sequence	*m/z* Calculated	*m/z* Observed	Accuracy	Migration time (min)
βT1	Val-His-Leu-Thr-Pro-Glu[a]-Glu-Lys	952.1	952.4	0.03%	4.05
βT2	Ser-Ala-Val-Thr-Ala-Leu-Trp-Gly-Lys	932.1	932.3	0.02%	4.23
βT3	Val-Asn-Val-Asp-Glu-Val-Gly-Gly-Glu[b]-Ala-Leu-Gly-Arg	1314.4	1314.1	–0.02%	4.36
βT4	Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg	1274.5	1274.4	–0.01%	4.46
βT5	Phe-Phe-Glu-Ser-Phe-Gly-Asp-Leu-Ser-Thr-Pro-Asp-Ala-Val-Met-Gly-Asn-Pro-Lys	2059.3	2059.5	0.01%	4.67
βT6	Val-Lys	245.3	not detected		
βT7	Ala-His-Gly-Lys	411.5	not detected		
βT9	Val-Leu-Gly-Ala-Phe-Ser-Asp-Gly-Leu-Ala-His-Leu-Asp-Asn-Leu-Lys	1669.9	not detected		
βT10+11	Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Arg-Leu-His-Val-Asp-Pro-Glu-Asn-Phe-Arg	2547.8	2546.4	–0.05%	4.78
βT13	Glu-Phe-Thr-Pro-Pro-Val-Gln-Ala-Ala-Tyr-Gln-Lys	1378.5	1378.2	–0.02%	4.95
βT14	Val-Val-Ala-Gly-Val-Ala-Asn-Ala-Leu-Ala-His-Lys	1149.4	1149.5	0.01%	5.03
βT15	Tyr-His	318.3	not detected		

Calculated *m/z* values:
[a] HbS: The substitution of glutamate in HbA by valine causes the resulting fragment to be found at *m/z* 922.1, with a migration time at 4.98 min.
[b] HbE: The substitution of glutamate by lysine in this peptide results in an additional cleavage point under tryptic digestion. The resulting two fragments have *m/z* values of 916.0 (βT18–26, eluting at 5.04 min.) and 415.5 (βT27–30, eluting at 4.47 min.), where the total of the two digest products is 1331.5.

were involved. Although a mass spectrometer detector can detect these small differences, interpretation will be complicated by co-eluting peaks and other unseparated background contaminants.

Figure 1 shows the separation of the α- and β-chains of Hb using HPLC with a C_{18} column (30 cm × 4.6 mm) and gradient elution (45–55% acetonitrile in 60 min). The α-chain is observed at 30.63 min. and the β-chain at 26.82 min. A number of minor peaks, which are also observed in the chromatogram, were not identified but could result in background in our experiment if not separated out using HPLC. This separation was performed with ~10 μg of initial sample. A C_{18} column was used in the HPLC separation as opposed to the C_4 column used in earlier work by Shelton.[6] It was found that the use of C_{18} resulted in some sample loss, but that the C_4 column applied in these experiments did not provide a satisfactory separation. The eluent was fraction collected following separation and the mass confirmed by MALDI-MS. The β-chains for various Hbs were determined as β^A (normal Hb), *m/z* 15 867; β^S (sickle-cell anemia), *m/z* 15 837; and β^E, *m/z* 15 866, respectively. In addition, the collected fractions of the small background peaks were also subjected to mass analysis so that their presence in the β-fraction could be monitored.

Following separation of the globin chains of Hb by HPLC, the fraction was dried and subjected to trypsin digestion. The β-chain digests were then separated by CE and initially detected by UV absorption detection. In Fig. 2 is shown the CE electrophorograms of the tryptic digests of the β-chains of three forms of Hb. In Fig. 2(a) is shown the electrophorogram of normal hemoglobin as the standard and in Fig. 2(b) and (c) are shown the electrophorograms of the β-chains of HbS and HbE respectively. Although the electrophorograms are similar, there are clearly differences in the profiles. Peaks that are different from the HbA standard and suspected of containing a mutation site are marked by an arrow. The variation in the Hbs may only be a single amino acid, as in these cases, where the chain variations in HbS and HbE are β6 (Glu→Val) and β26 (Glu→Lys), respectively. Nevertheless, the use of CE can successfully separate and discriminate between the small changes in digest peaks. In these cases such separation may be due to the change in charge distribution, since glutamate is negatively charged while valine is basically neutral and lysine is positively charged at neutral pH. The sharp separations demonstrate that the coating on the capillary inner surface provides a positive layer that repels the peptide successfully under the acidic conditions. The separation generally was on the order of 1.5×10^4 theoretical plates in these experiments. The optimum separation varied depending on the buffer pH and the concentration of the samples and the migration times also varied somewhat according to the conditions. Nevertheless, relatively similar electrophorograms can be observed for the different Hb variants as expected.

In comparison, in Fig. 3 is shown the total-ion electrophorogram of Hb digest obtained using on-line CE/MS. Most of the peaks observed correspond to the CE separation with UV absorption detection of Fig. 2. There are some differences in terms of peak width and migration times. The differences in migration times observed in CE/MS versus UV absorption detection were due to the UV detector window being situated 6 cm before the capillary anode. The slightly broader peak width in the TIE can be explained, according to McLafferty and coworkers,[32] by the fact that when the capillary tip was exposed to atmosphere during electrospray, the solvent was easily evaporated due to the small needle dimension. This evaporative effect at the capillary tip subsequently induces a flow inside the capillary. Furthermore, it was demonstrated by Mann,[33] and later by McLafferty,[32] that the electrostatic drawing of the buffer solution and sample by the electric field between the needle and the transit stainless steel capillary results in a constant flow in the direction of electrospray, even without the addition of the CE voltage. Nevertheless, it is noteworthy that more than 10 peaks in the electrophorogram were separated in 1–2 min and the entire separation was completed in less than 6 min where many of the peaks are as narrow as 1–2 s. The TIEs of Fig. 3 illustrate the capability of the

IT/reTOF to acquire spectra for even the fastest separation. The non-scanning characteristics of the IT/reTOF, allows data acquisition at a rate of 0.04 s/spectrum, limited ultimately by the data system. In these experiments, a 0.25 s data acquisition time was used in order to compromise between preserving the CE resolution and the mass sensitivity. In Table 1 is listed a comparison of the calculated and observed mass values of the tryptic digests of HbA in the CE/MS experiment.

In Fig. 4 are shown the mass spectra corresponding to the T1 digest products of HbA and HbS. As reported in Shackleton's[17] earlier experiments in the LC/MS of hemoglobin digests, the major ESI-produced ions of the Hb protein digests were doubly-charged. Such doubly-charged ions are indeed observed as shown in Fig. 4 and can be confirmed as being doubly-charged using the resolution of the IT/reTOF where the relative separation of the isotopic peaks in the doubly-charged ions is half that of the singly-charged ions, as expected. We examined the mass spectra of the particular TIE peaks that appear to shift between the analysis of HbA and HbS. This mutation site was substantiated by the mass spectra where a mass shift of *m/z* 15 was observed for

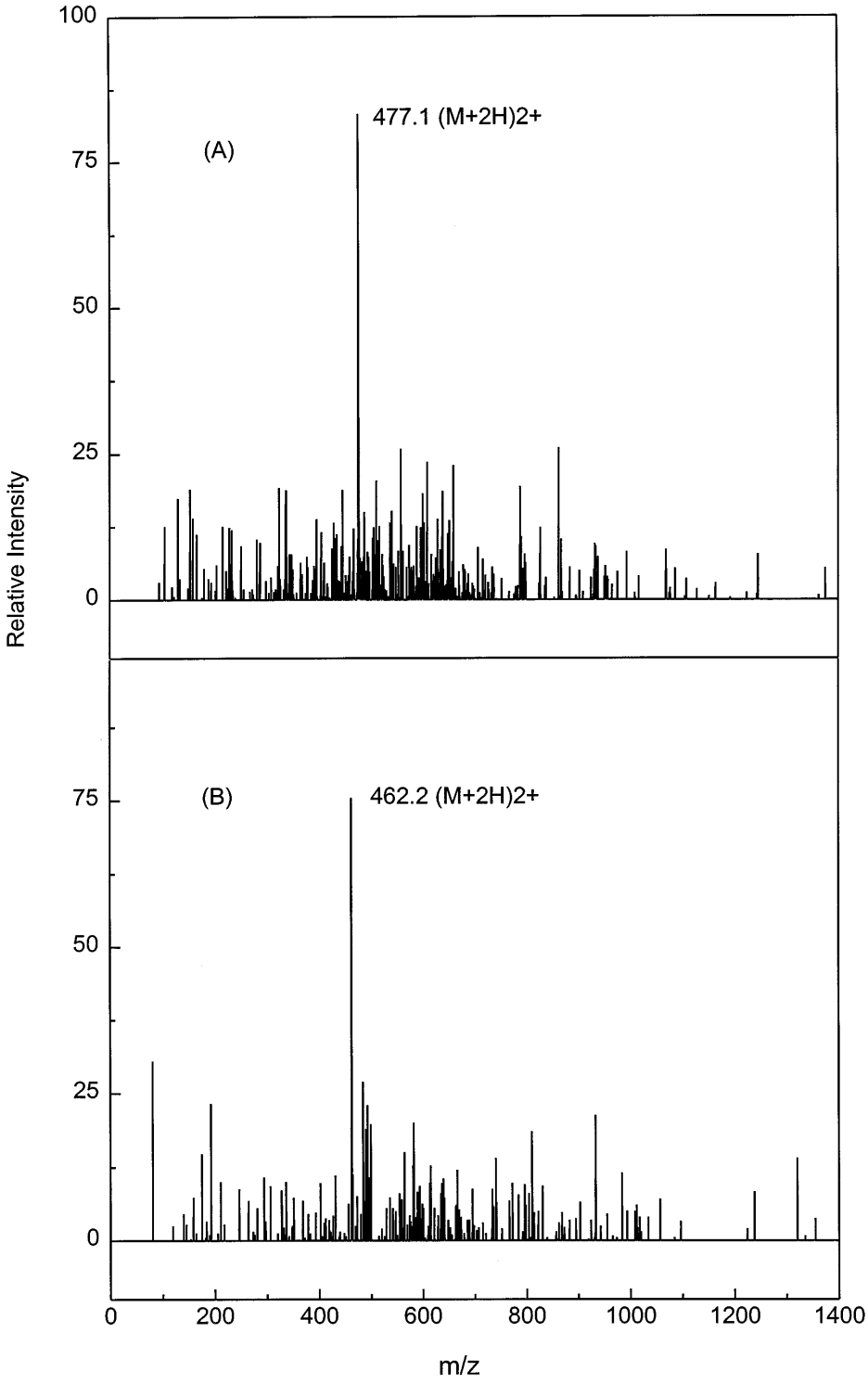

Figure 4. Mass spectra of the βT1 fragment in HbA and HbS obtained in CE/MS experiment (a) HbA βT1 fragment, (b) HbS variant βT1 fragment ion. Single mass spectrum acquisition time: 0.25 s, $V_{\text{Focusing Lens}}$ = 100 V.

doubly-charged ions. This corresponds to a mass shift of 30, which is expected for the substitution of glutamate by a valine. The mass resolution in these experiments is ~2000 and the mass error is less than 0.05% as shown in Table 1. Thus, it appears that this method clearly demonstrates the expected mutation site and identity of this sample as sickle cell anemia.

In the HbE electrophorogram with UV detection and in the TIE of Figs 2 and 3, there were several additional digest peaks observed compared to the case of HbS and HbA. Since the glutamate is substituted by a lysine in the HbE, it would be expected that trypsin would cut the protein in this position resulting in two additional fragments. In a comparison of the mass spectral data of the CE separation of the tryptic digests of HbA and HbE, two extra peaks at *m/z* 916 and *m/z*

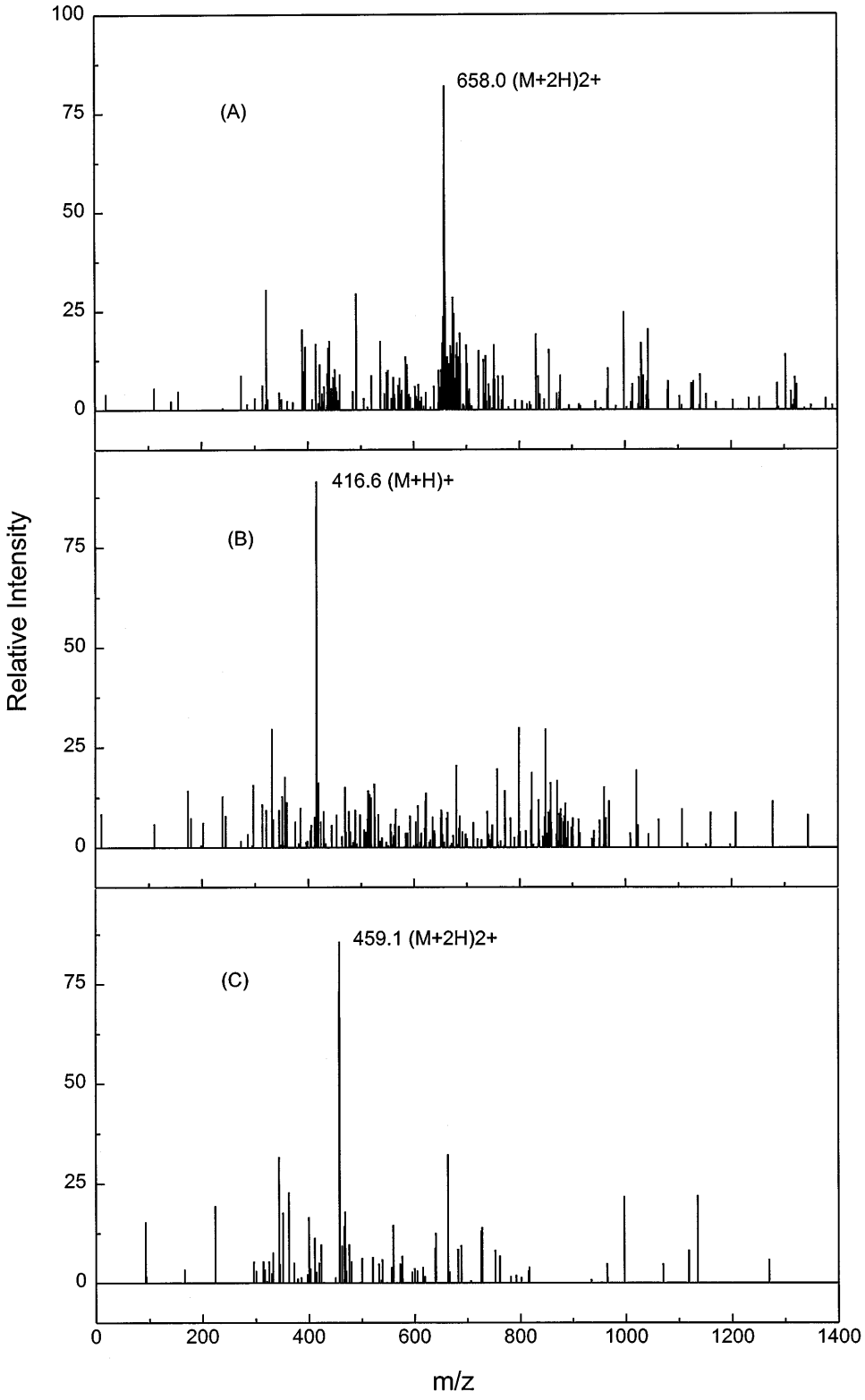

Figure 5. Mass spectra of the βT3 fragment in HbA and corresponding fragments in HbE obtained in CE/MS experiment where (a) corresponds to HbA βT3 fragment ion, (b) to HbE βT27–30 fragment, and (c) to HbE βT18–26 fragment. These two fragment ions result from the tryptic digestion at the lysine residue at β26 position in HbE.

415.5 were observed in HbE β-chain, which correspond to the β18–26 and β27–30 fragments, respectively. The sum of these two fragments is equal to 1331.5, which is the mass value of the βT3 peptide plus a water molecule if this lysine position was not cleaved by trypsin. The mass spectra for these fragments are shown in Fig. 5.

In order to pinpoint the mutation site detected in these specific fragments, collision-induced dissociation was performed for the HbS βT1 fragment in the region between the focusing lens and skimmer. Although this method can not isolate the parent ion for fragmentation, the CE separation provided a preselection for the specific ions. Shackleton *et al.* previously recorded CID data from Hb variants by changing the nozzle-to-skimmer voltage and inducing considerable fragmentation in the parent ion.[18] In the present experiment, when the βT1 was about to elute from the capillary in the second run, immediately following the initial CE/MS experiment, the focusing-lens voltage was raised from 100 V to between 150 V and 200 V. In Fig. 6 (b) is shown the HbS βT1 fragment CID spectrum. This was obtained using a storage time of 0.5 s and signal averaging the spectrum at least eight times. Thus, a total effective time of 4 s was used to examine the CID spectrum. An increase in storage time was needed to enhance the signal-to-noise ratio in these experiments. In the spectrum, the dominating fragment ions are of the y- and b- series. The y_3 and y_6^{2+} ions are 30 mass units less than those in the HbA (see Fig. 6 (a)), as a result of the substitution of the glutamic acid by valine (Val-His-Leu-Thr-Pro-Val-Glu-Lys vs. Val-His-Leu-Thr-Pro-Glu-Glu-Lys) whereas the y_1, y_2, and b_2 ions are the same as those in HbA, as expected. From these data, it is quite clear that the β6 position is where the substitution occurs.

CONCLUSION

CE/ESI using an IT/reTOF mass detector has been used to provide a rapid and sensitive method for analyzing structural variants in hemoglobin. Tryptic digests of variant Hbs have been analyzed by this method in which a comparison of the TIEs and mass spectra of the mutant and normal Hbs can detect the presence of the mutation site. In addition, CID in the vacuum interface – skimmer region has been used to pinpoint the identity of such a site in HbS, following

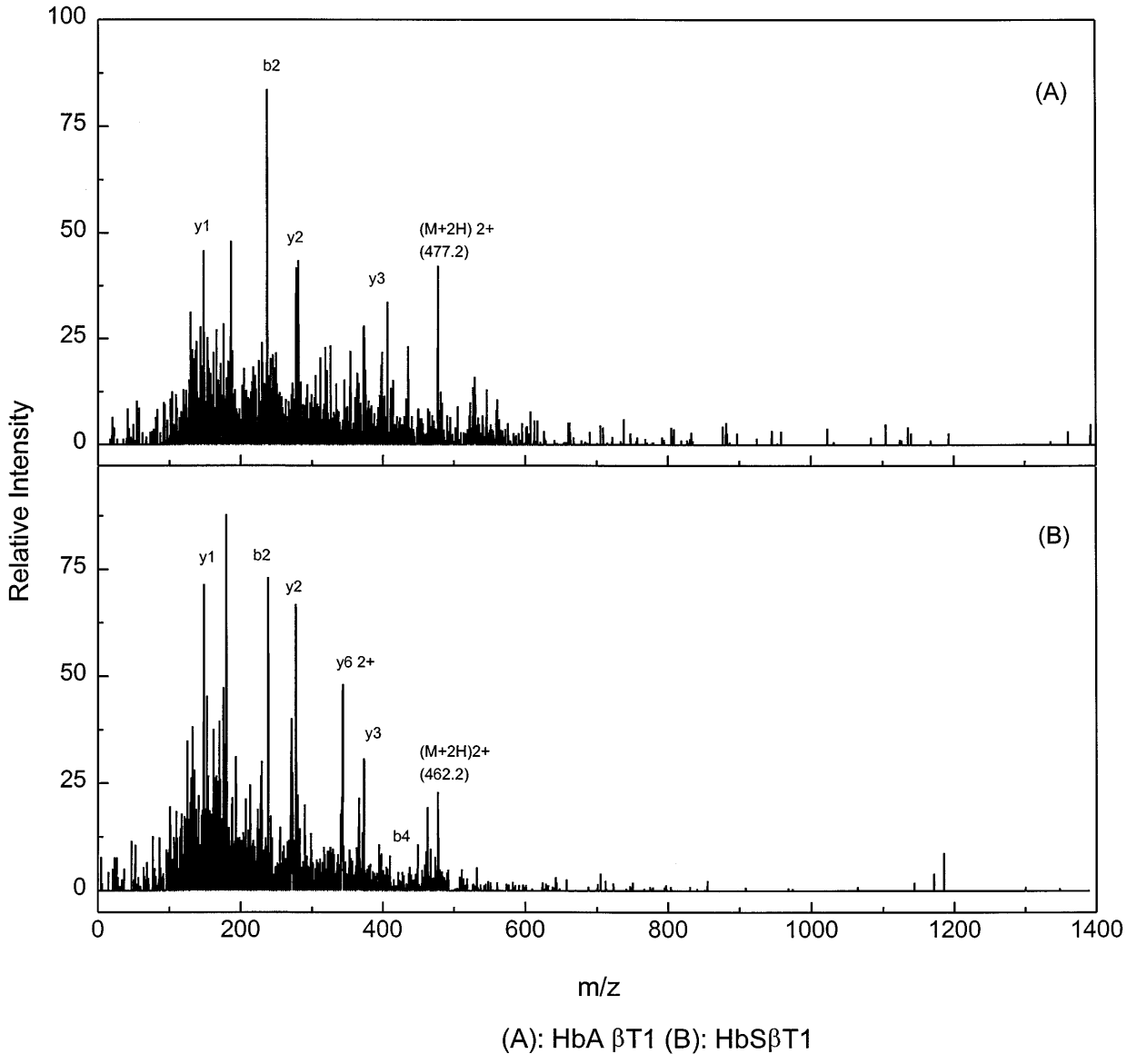

Figure 6. CID spectra of the βT1 fragment in (a) HbA and (b) HbS during the CE/MS experiment.

separation of the peptide digest fraction by CE. The CE/ESI-IT/reTOF/MS method is shown to have unique capability for efficiently and accurately detecting fast separations with narrow peaks that may be under 1 s FWHM. The speed of this system is critical for the isolation of the large number of peaks that must be separated in a short time duration in these CE experiments.

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