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Research Article

Discovery of a biomarker for β-Thalassemia by HPLC-MS and improvement from Proton Transfer Reaction – Parallel Ion Parking

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ABSTRACT

β-thalassemia is a quantitative hemoglobin (Hb) disorder resulting in reduced production of Hb A and increased levels of Hb A_2. Diagnosis of β-thalassemia can be problematic when combined with other structural Hb variants, so that the separation approaches in routine clinical centers are not sufficiently decisive to obtain accurate results. Here, we separate the intact Hb subunits by high-performance liquid chromatography, followed by top-down tandem mass spectrometry of intact subunits to distinguish Hb variants. Proton transfer reaction-parallel ion parking (PTR-PIP), in which a radical anion removes protons from multiply charged precursor ions and produces charge-reduced ions spanning a limited *m/z* range, was used to increase the signal-to-noise ratio of the subunits of interest. We demonstrate that the δ/β ratio can act as a biomarker to identify β-thalassemia in normal electrospray ionization MS1 and PTR-PIP MS1. The application of PTR-PIP significantly increases the sensitivity and specificity of the HPLC-MS method to identify δ/β ratio as a thalassemia biomarker.

Introduction

Humans produce six normal hemoglobin (Hb) polypeptide subunits (ε, ζ, α, β, γ, δ) from embryogenesis to adulthood. Production of α subunits is controlled by two duplicated genes located on chromosome 16, and β-like subunits (β, ε, γ, and δ) are encoded by a gene cluster located on chromosome 11 [1,2]. Normal adult Hb (Hb A) is a tetramer comprised of two α and two β ($\alpha_2\beta_2$) subunits, each bound to a prosthetic heme group that reversibly binds oxygen for transport throughout the body [1–4]. Hb A_2 ($\alpha_2\delta_2$) is also present in low abundance (<3.5 % total Hb) in all adults along with small amounts (<1%) of fetal hemoglobin, Hb F ($\alpha_2\gamma_2$) [2].

Hemoglobinopathies, heritable blood disorders related to abnormal production of Hb, are a significant health concern among 7 % of the worldwide population [5]. They are mainly divided into two categories: (1) structural Hb variants and (2) thalassemia syndromes [1,2]. Most Hb variants occur when a missense mutation causes a single amino acid substitution in a globin protein subunit, resulting in a structurally abnormal Hb tetramer, such as sickling hemoglobin, Hb S (β E6V) [2,6,7]. Thalassemia syndromes are characterized by mutations that cause reduced synthesis of α or β subunits, resulting in under production of Hb [1,2,8]. The severity of symptoms depends on the nature of the mutation(s). In β-thalassemia, mutations that allow production of reduced quantities of β globin are classified as β^+, whereas mutations that completely inactivate a β globin gene, causing no β globin production, are classified as β^0. Thalassemia major, the most severe form of the disorder, occurs when both alleles have thalassemia mutations (β^0/β^0 or β^+/β^0 genotypes). Individuals with thalassemia minor (β^0/β or β^+/β), also called "carriers", have only one damaged gene and are usually clinically asymptomatic. Symptoms of thalassemia intermedia

Abbreviations: Hb, Hemoglobin; PTR-PIP, Proton transfer reaction-parallel ion parking; Hb A, Normal adult Hb; IFCC, International Federation of Clinical Chemistry and Laboratory Medicine; MS, Mass spectrometry; *m/z*, Mass-to-charge ratios; FT-ICR, MS Fourier transform ion cyclotron resonance mass spectrometer; ESI, Electrospray ionization; FA, Formic acid; ACN, Acetonitrile; MCW, Methanol/chloroform/water; AUC, Areas under the curve; XIC, Extracted ion chromatograms; TIC, Total ion chromatogram; ROC, Receiver operating characteristic; S/N, Signal-to-noise ratios; IQR, Interquartile range; Se(c), Sensitivity, the probability of a true positive); Sp(c), Specificity, the probability of a true negative); J, Youden Index; TP, True positive; FN, False-negative; FP, False-positive; TN, True negative; HbA1d, Hb β with glutathione; PTM, Post-translational modification; CID, Collision-induced dissociation; ETD, Electron-transfer dissociation.

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(β^0/β^+ or β^+/β^+) fall between the extremes of the major and minor forms [2].

The presence of high levels of Hb F in the months following birth usually masks symptoms of β-thalassemia for the first six months of life. Once severe anemia develops and associated physical symptoms manifest (typically within the first two years), patients with β-thalassemia major are easily diagnosed. These patients require regular blood transfusions and lifelong, ongoing, medical care to prevent life-threatening complications, such as iron overload [9]. Individuals with β-thalassemia intermedia are often diagnosed later in life and usually have mild to moderate anemia that does not require regular blood transfusions [2,9]. Since β-thalassemia minor carriers are often asymptomatic, many are unaware that they possess an altered gene for the disorder. However, it is important they can be accurately diagnosed to ensure proper treatment for mild anemia, and for family planning purposes, especially among populations with high carrier rates. When both parents are carriers of thalassemia minor, there is a 50 % chance that offspring will also be carriers and a 25 % risk of homozygous thalassemia (major or intermedia) with each pregnancy [9]. Moreover, coinheritance of thalassemia syndromes with other hemoglobinopathies can result in a broad range of phenotypes. Sickle cell trait carriers with β -thalassemia have a clinical course like that of sickle cell disease. Striking ethnic variation in phenotype, ranging from asymptomatic to transfusion-dependence, has also been observed among individuals with identical mutations in the β globin gene promotor region [10].

Results of hematological tests among carriers of β-thalassemia minor, though usually abnormal, are inconsistent and can be difficult to distinguish from iron-deficient anemia without further testing. However, detection of elevated levels (greater than3.5 %) of Hb A_2 is a hallmark of thalassemia minor [2,3,11–13]. Decreased β subunit synthesis relative to that of the δ subunit increases the proportion of Hb A_2, but there are several modulating factors that influence empirically determined Hb A_2 levels [14]. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) has been working to standardize Hb A_2 quantitation since 2004, but difficulties remain [11]. Quantitative Hb profiles are usually generated by cation-exchange high-performance liquid chromatography (HPLC), gel, or capillary electrophoresis. Accurate visual interpretation of the chromatogram is essential, but not always possible because several prevalent Hb variants or their glycated adducts (e.g., Hb E, Hb S1c) are known to coelute with Hb A_2, leading to inaccurate peak area measurements [3,11,15]. Given the low relative abundance of Hb A_2 and the aim of measuring small changes in its abundance, it is important to ensure that the chromatogram is free of interference from Hb variants.

Mass spectrometry (MS), on the other hand, is an attractive alternative that can be employed to identify and quantify proteins or peptides based on their observed mass-to-charge ratios (m/z) and peak magnitudes. For example, relative quantification of Hb subunits was achieved by bottom-up MS of signature peptides in multiple reaction monitoring mode. Measured α/β or δ/β peptide ratios were used as surrogate markers of HbA_2 and distinguished β-thalassemia trait carriers [16,17]. Stable isotope-labeled peptides have also been used as internal standards for more accurate quantitation of Hb subunits [18,19]. However, co-inherited Hb sequence variants can interfere with the accuracy of these methods because they target specific tryptic peptides. Top-down MS, in which the proteins are analyzed intact, has also been applied to determine Hb subunit abundances. Helmich et al. showed that the intact α/β ratio is a poor marker [20], but α/(β + γ) and δ/α ratios have been used to differentiate β-thalassemia patients [21,22].

Recently, He et al. demonstrated that the intact δ/β ratio was significantly higher in β-thalassemia samples measured by top-down MS with a 21 T (T) Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS) [23]. The δ/β ratios considered all detected charge states generated by positive electrospray ionization (ESI) and were calculated from m/z-to-mass deconvolved spectra [24]. However, the low relative abundance of δ subunits and overlap of δ isotopic peak clusters with those generated by adducts of the β subunit often confounded the deconvolution algorithm. Although this issue did not prevent accurate classification of β-thalassemia samples, mean δ/β ratios nearly double and greater specificity was achieved when additional sample cleanup steps were implemented to remove salts. Here, we expand upon this previous work and explore the use of proton transfer reactions (PTR) [25,26] and parallel ion parking (PIP) [27] to improve detection specificity for Hb subunits of low abundance.

Experimental section

Materials

Patient whole blood samples were provided by Associated Regional and University Pathologists (ARUP) Laboratories in compliance with University of Utah (IRB_00102396) and Florida State University Institutional Review Boards. In total, 14 β-thalassemia minor samples and 21 non-β-thalassemia patient samples were analyzed. LC-MS grade formic acid (FA) was obtained from Thermo Scientific Pierce. LC-MS grade water, 2-propanol, and acetonitrile (ACN) were obtained from Honeywell Burdick & Jackson. Amicon® Ultra 0.5 mL 30 k Da molecular weight cutoff centrifugal filters were acquired from MilliporeSigma. The PTR reagent, perfluoromethyldecalin, was obtained from Oakwood Chemical.

Sample preparation

For sample analyses, 100 μL of whole blood was centrifuged at 1000 × *g* for 10 min at room temperature. The plasma was discarded, and an aliquot of 20 μL of the remaining aggregated red blood cells was lysed by dilution with 180 μL of water (volume ratio of 1:9). Cell debris was removed by centrifugation at 13,500 × *g* for 5 min. The cell lysate solution was then precipitated by methanol/chloroform/water (MCW) [28]. The protein pellet was washed twice with 450 μL of methanol, briefly dried under a stream of nitrogen, and reconstituted in 1200 μL of solvent A (95 % water, 4.7 % ACN, 0.3 % FA; v/v). To remove larger proteins that could interfere with chromatography,100 μL of reconstituted protein solution was diluted to 500 μL with solvent A in a 30 k Da Amicon@ filter device and centrifuged at 14,000 × *g* for 15 min. 1 μL of the eluate was injected for each HPLC-MS experiment.

Liquid chromatography

The HPLC system (ACQUITY M−Class; Waters, Milford, MA) was operated at 2.5 μL/min for sample loading/trapping onto a custom-fabricated 360 μm o.d. × 150 μm i.d. fused silica microcapillary trap column packed 2.5 cm with PLRP-S resin (5 μm particles, 1000 Å pore, Agilent Technologies, Palo Alto, CA). Samples were washed with 95 % solvent A for 10 min. Reversed-phase separation was performed with a 360 μm o.d. × 75 μm i.d. fused-silica microcapillary analytical column packed 17 cm with the same PLRP-S resin. Samples were eluted over 40 min at a flow rate of 0.3 μL/min using the following gradient: 5–30 % solvent B (47.5 % ACN, 47.5 % 2-propanol, 4.7 % water, and 0.3 % formic acid; v/v) in 5 min, 30–50 % B in 30 min, 50–75 % B in 5 min. Immediately following separation, samples were directly ionized by ESI with 15 μm fused-silica PicoTip emitter (New Objective, Woburn, MA) packed with 3 mm PLRP-S resin.

Mass spectrometry

Prior to experimentation, external mass calibration was performed with Hb A α subunit (most abundant isotopologues of the 13 + to 19 + charge states) separately for m/z ranges 300 to 2000 and 600 to 2000. Each sample was analyzed in technical triplicate with (PTR-PIP MS1) and without (normal MS1) the use of PTR and PIP for a total of six experiments per sample. Mass spectra were acquired with a custom-built

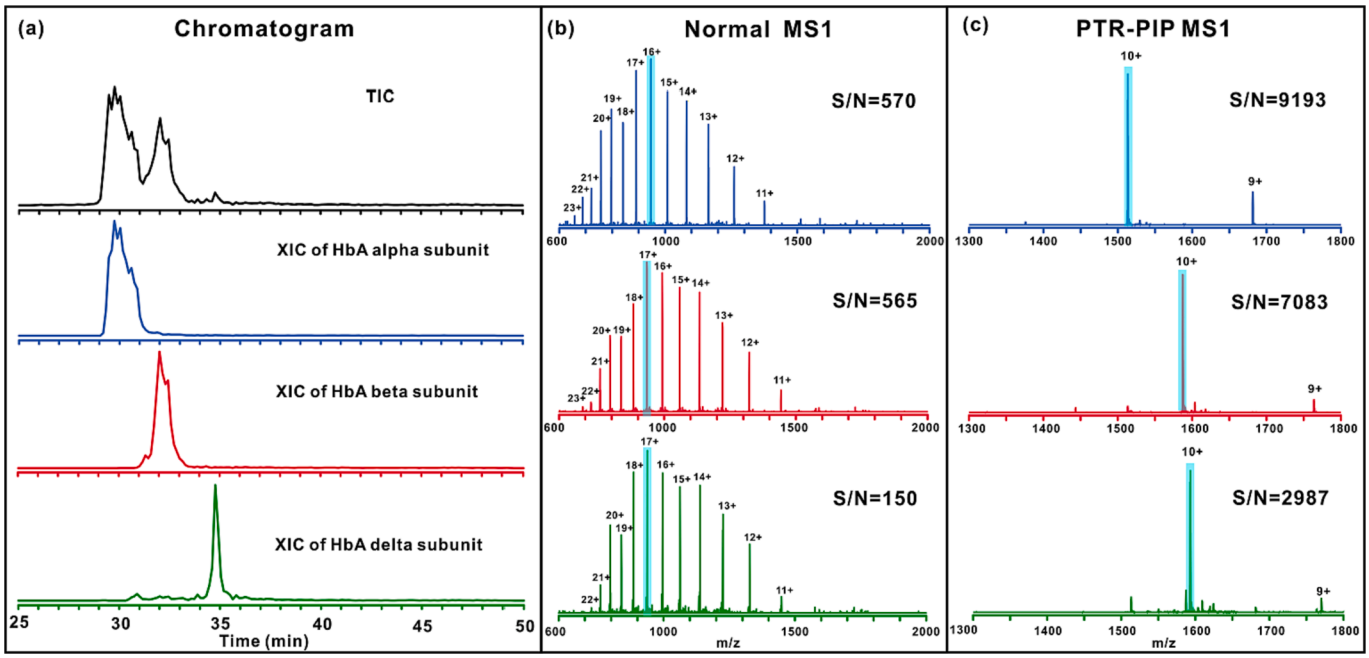

Fig. 1. HPLC-MS analysis of intact Hb α (blue), β (red) and δ (green) subunits purified from a β-thalassemia patient blood sample. (a) HPLC-MS total ion chromatogram (TIC) and extracted ion chromatograms (XIC) for each Hb subunit. (b) MS1 spectra depicting the positive ESI charge state distributions for Hb subunits ("normal MS1)". (c) MS1 spectra taken after precursors are subjected to 100 ms PTR with PIP ("PTR-PIP MS1"). The S/N ratios for the most abundant charge states (highlighted) in each spectrum are indicated.

21 T FT-ICR mass spectrometer operated with Xcalibur software (Thermo Fisher Scientific, Waltham, MA) [29]. The ESI source voltage was biased at 2.8 kV, and the inlet capillary was heated to 325 °C. Broadband mass spectra were acquired from *m/z* 600 to 2000 as the sum of four 0.76 s time-domain acquisitions (corresponding to 300 k resolving power at *m/z* 400) taken in magnitude mode. The precursor automatic gain control target was set at 1 million charges. For PTR-PIP experiments, the automatic gain control target for perfluoromethyldecalin reagent anions was set to 1 million and required a 20 ms injection period before reaction with precursor analyte at a ratio of 1:1. PIP AC voltage waveforms were applied only during PTR scan functions, as previously described [30,31]. The waveform included frequency components corresponding to *m/z* 1500–2500, with a normalized activation amplitude biased at 0.15. The waveform also included a reagent activation window (centered at *m/z* 512, 20 Th window), for which the amplitude was lowered to 0.065. The entire precursor ion population (no isolation) was exposed to the PTR reagent and PIP waveforms for 100–125 ms [31].

Data analysis

All data were processed in.raw file format (Thermo Fisher Scientific) in reduced profile mode (i.e., noise baseline-subtracted). Data were analyzed by use of Xcalibur 3.0 software (Thermo Fisher Scientific). To determine δ/β ratios, areas under the curve (AUC) of extracted ion chromatograms (XIC) for Hb subunits (α, β, δ, etc.) were obtained by use of the embedded peak detection function and manually verified or refined as needed. For normal MS1 experiments, XICs were generated from the most abundant isotopologues of the six most abundant charge states (19 + to 14 +) with a mass tolerance of 0.2 Th. For PTR-PIP MS1 experiments, XICs were similarly generated from the charge-reduced 10 + product ions. The box-and-whisker plots were created in R with the geom_boxplot function of the ggplot2 package [32]. Mann-Whitney U tests were used to determine whether there was a statistically significant difference in the δ/β ratio for the two groups. Receiver operating characteristic (ROC) curves and Youden criteria were used to determine the diagnostic performance and obtain the optimal cutoff points for this methodology.

Results and discussion

Signal-to-noise ratio improvement

Reagents for PTR act as Brønstead bases and the reactions are used to reduce the charge of analytes in the gas-phase by abstracting protons from them inside the mass spectrometer, increasing their *m/z* prior to mass analysis [25,26]. Coupling PTR with PIP provides a means to control PTR kinetics and stop further charge reduction once analytes are charge-reduced to selected *m/z*-ranges, which concentrates the multitude of charge states produced by ESI into just a few charge states [27]. This procedure maximizes signal magnitude and significantly increases effective sensitivity [30,31]. The PTR and PIP capabilities applied here were recently described by Weisbrod et al. and involve mild kinetic activation of the PTR reagent to slow the overall rate of reaction, and exposure of all electrosprayed analytes to the PTR reagent without any prior precursor ion selection/isolation [31].

Exemplar HPLC-MS chromatograms and mass spectra from a β-thalassemia patient blood sample are shown in Fig. 1. The center panels depict the "normal MS1" positive ESI charge state distributions of the α, β, and δ subunits (shown as the post-FT spectral average of nine data acquisitions over the elution period). Their signals are spread among isotopic peak clusters carrying 10 + to 23 + charges, with the signal-to-noise ratios (S/N) of the base peak (highest magnitude) isotopic peak clusters ranging from 570 (α subunit) to 150 (δ subunit). For the spectra on the right, the Hb subunit cations were exposed to perfluoromethyldecalin reagent anions for 100 ms to reduce their charge via proton-transfer. Throughout the 100 ms PTR period, a PIP AC voltage waveform including frequency components corresponding to the product ion parking window (*m/z* 1500–2500) and reagent anions (*m/z* 512) was applied to the x-rods of the quadrupole linear ion trap, where the reactions occur. When the Hb subunit cations undergo PTR, their *m/z* values increase, and their frequencies of oscillation come into

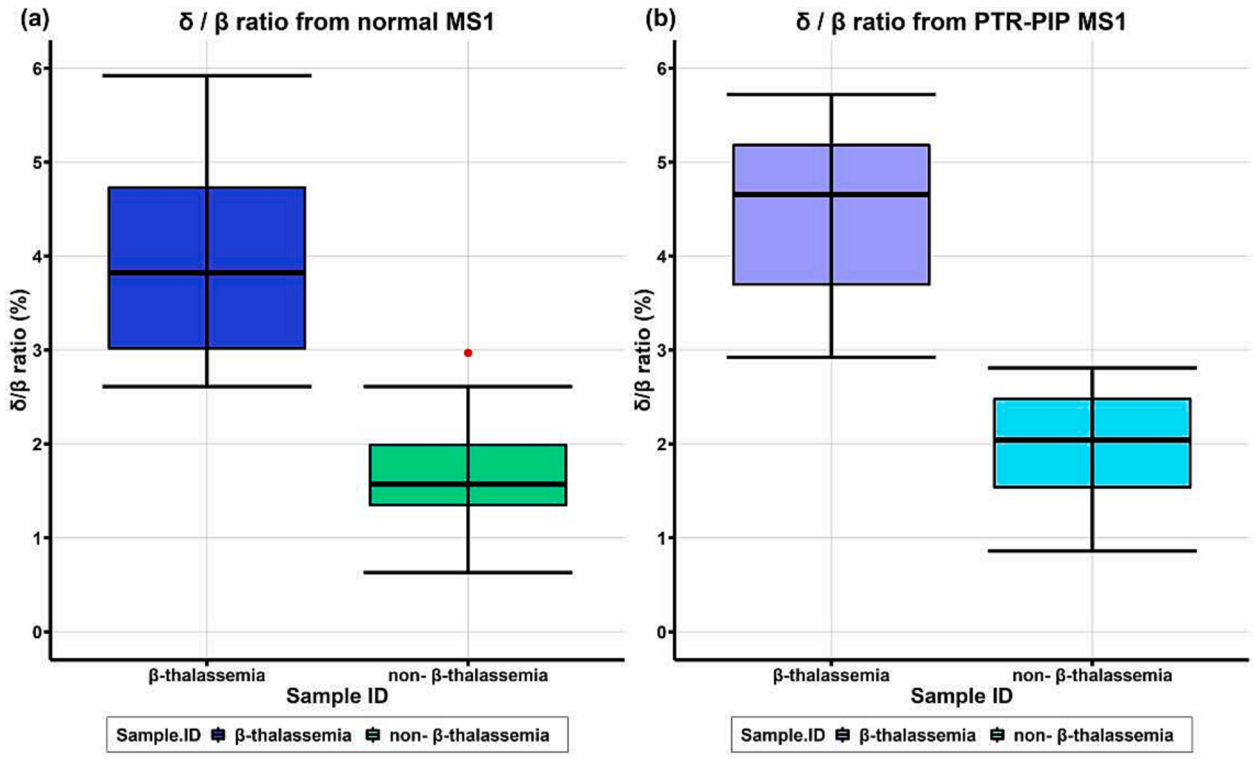

Fig. 2. Box-and-whisker plots for the δ/β ratio from normal MS1 and PTR-PIP MS1 experiments. (a) Normal MS1 with *P* value of 8.36×10^{-8}. (b) PTR-PIP MS1 with *P* value of 3.45×10^{-9}.

resonance with the applied waveform. This process occurs when their *m/z* reaches the 1500–2500 range, at which point the ions are activated, increasing their velocity relative to reagent anions, and substantially slowing the rate of further charge reduction [27]. As a result, the Hb subunit signals were "parked" in just two charge states (10 + and 9 +), simplifying the spectra. Further, there was a concomitant increase in the S/N of the base peak isotopic peak clusters ranging from approximately 12 to 20-fold for the 3 subunits shown. This step provides significant analytical advantages for intact protein analysis by MS, especially for the less abundant proteins in a sample.

Identification of β-thalassemia samples from δ/β ratio

In carriers of β-thalassemia minor, expression of one of the two β globin genes is reduced or eliminated, increasing the proportion of δ subunits relative to β subunits [14]. We previously demonstrated that FT-ICR MS can be used to measure the relative abundance of intact Hb subunits to determine the δ/β ratio and diagnose β-thalassemia minor [23]. However, δ /β ratio determination for non-β-thalassemia samples was difficult in some cases due to the lower relative abundance of the δ subunit. This problem, combined with mass spectral interference from

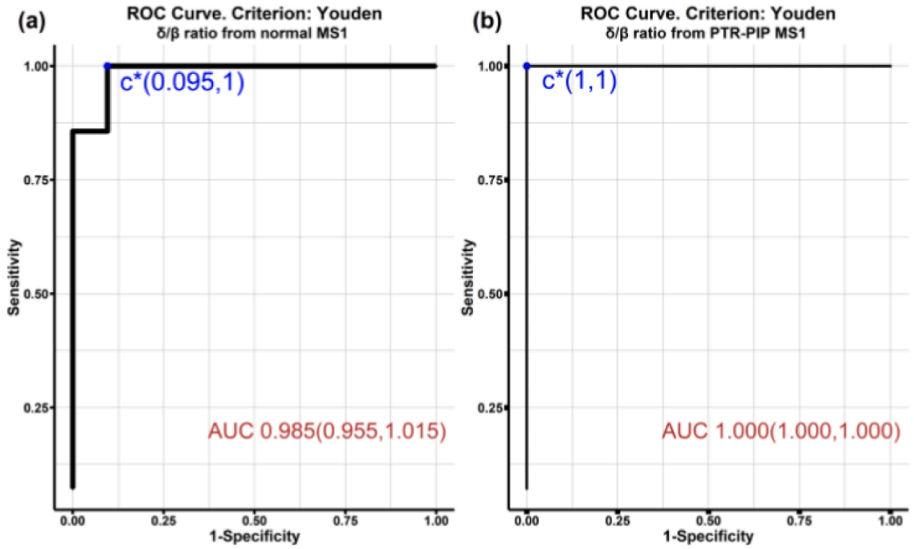

Fig. 3. ROC plots for both normal MS1 and PTR-PIP MS1. (a) ROC plot of normal MS1, the cutoff point (c*) determined by Youden criterion is at 2.61% with a sensitivity of 100% and a specificity of 90.5% (2 FP and 19 TN). (b) ROC plot of PTR-PIP MS1, the cutoff point (c*) determined by Youden criterion is at 2.92% with a sensitivity of 100% and a specificity of 100% (no FN or FP).

the salt adducts of the more abundant Hb subunits, caused inaccurate *m/z*-to-m deconvolution. For example, for the Hb SD sample, there is a δ subunit eluted shown in Fig. S1**(a)** MS1 raw data but no deconvolved masses matched with the δ subunit in Fig. S1**(b),** which shows the deconvolved masses. Here, we used the AUC of each subunit's XIC to calculate the δ/β with increased accuracy by use of the equation:

$$\delta/\beta = AUC(\delta)/AUC(all\beta\ \text{subunits}) \quad (1)$$

With clinical chromatographic assays, the presence of common Hb sequence variants can lead to over- or under-estimation of Hb A_2 [15]. Usually this result is not an issue, because cases of coinherited hemoglobinopathies are rare. However, in parts of the world where hemoglobinopathies are common and considered a major public health concern, the inability to accurately measure Hb A_2 has led to mistaken risk prediction for genetic counselling [33]. Top-down MS can achieve molecular specificity akin to gene sequencing for rapid diagnosis of structural hemoglobin variants [23,34]. Expanding its role in diagnosis of thalassemic disorders provides opportunities to easily identify compound heterozygous cases, and distinguish β-thalassemia from structural variants with thalassemic phenotype, such as Hb E (β E26K) [35]. To that end, our aim was to demonstrate accurate δ/β ratio measurement in the presence of Hb variants.

The patient samples we analyzed included 14 cases of β-thalassemia minor and 21 "non-β-thalassemia" samples. The AUC of XICs for all β subunits including variant subunits were considered in δ/β ratio calculations. Average δ/β ratios determined from normal MS1 and PTR-PIP MS1 experiments, each performed in triplicate, for all samples are listed in **Table S1**. Average δ/β ratio distributions for β-thalassemia and non-β-thalassemia samples measured with and without PTR-PIP are represented as box-and-whisker plots in Fig. 2. The lone outlier (average δ/β ratio > Q3 + 1.5*IQR) was Hb AE, which had an average δ/β ratio of 2.97 % under normal MS1 conditions. This result was expected because the Hb E gene mutation introduces a partially active alternate splicing site resulting in a small proportion of anomalous mRNA, thus, reduced β globin synthesis [35]. On the other hand, with PTR-PIP MS1, there are fewer charge states (most charge-reduced product ions are 10 + and 9 +) leading to more accurate determination of AUC of XICs. In Fig. 3 (b), the maximum δ/β ratio is still related to the Hb AE sample, but is no longer an outlier because only one charge state with significantly increased S/N ratio was used to generate the XIC.

Because there is no evidence that the measured δ/β ratios are normally distributed or have consistent scales, the Mann-Whitney *U* test, which is a nonparametric test for ordinal data, was chosen to determine if there are statistically significantly differences between these two groups [36]. The *P* value is 8.36×10^{-8} for normal MS1 and 3.45×10^{-9} for PTR-PIP MS1. Ergo, the *P* values are<0.05, indicating that the null hypothesis is rejected and δ/β ratios are significantly different between β-thalassemia and non-β-thalassemia samples. The similar δ/β ratio ranges shown in the box-and-whisker plots for these two groups from normal MS1 and PTR-PIP MS1 signify that PTR-PIP MS1 can preserve the quantitation information for intact proteins of interest.

ROC analysis reveals improved sensitivity, specificity and cleaner cutoff points with PTR-PIP

The receiver operating characteristic (ROC) curve is used to determine the diagnostic performance of a diagnosis method [37,38]. It is a plot of sensitivity (Se(c), the probability of a true positive) versus 1-specificity (1-Sp(c), Sp(c) is the probability of a true negative) for the possible cutoff point (c) of the variables [38]. A larger area under the ROC curve (AUC-ROC) indicates a better distinguishing method [39]. The Youden Index (J) is commonly used for determining the optimal cutoff point (c*), for which J is maximized at c* [38,40]. J = 1 indicates the absolute separation of variables measured in two different groups and J = 0 means complete overlap [38]. The indexes are defined by the following equations:

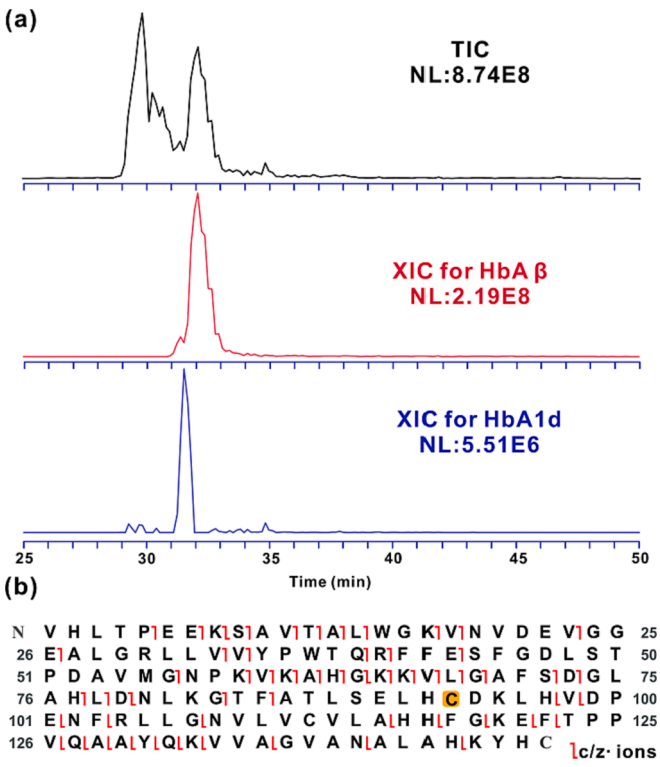

Fig. 4. (a) XIC of Hb A β and HbA1d from one β-thalassemia sample. (b) Sequence coverage for HbA1d, indicating that 93C is the binding site for glutathione. NL = base peak magnitude (normalization level).

$$Se(c) = \frac{n(TP)}{n(TP) + n(FN)}$$

$$Sp(c) = \frac{n(FP)}{n(FP) + n(TN)}$$

$$J = Se(c) + Sp(c) - 1$$

in which n(TP), n(FN),n(FP), and n(TN) stand for the numbers of true positive, false-negative, false-positive, and true negative at each possible cutoff point (c) [37,40,41,42]. The ROC curve and AUC-ROC are generated by Package 'OptimalCutpoints' in R with Youden criterion [43,44]. ROC plots for both normal MS1 and PTR-PIP MS1 are illustrated in Fig. 3. The δ/β ratio cutoff points are 2.61 % with a sensitivity of 100 % and a specificity of 90.5 % (with 2 FP and 19 TN) from normal MS1, and 2.92 % with a sensitivity of 100 % and a specificity of 100 % from PTR-PIP MS1. The AUC-ROC for normal MS1 data is 0.985 which was dramatically different compared to 1.000 from PTR-PIP MS1 data, indicating that the δ/β ratio calculated by PTR-PIP MS1 leads to more accurate identification of β-thalassemia.

Observation of Hb β with glutathione (HbA1d)

S-glutathionylation of Hb β takes place at the 93C position as the β molecule ages, resulting in the addition of 305.0682 Da [2]. Because δ/β ratio is used to identify β-thalassemia and the post-translational modification (PTM) happens at the β subunit, it is important to determine if HbA1d influences the results. Fig. 4**(a)** shows the XIC for Hb A β and HbA1d from one β-thalassemia sample, indicating that they are coeluting during the HPLC separation process. The precursor ion at *m/z* 889.42 (associated with the 305.0682 Da mass addition) was subjected to ETD for a targeted run for Hb ARiyadh sample. The RMS ppm error for the intact HbA1d is 0.71 ppm (based on the seven highest magnitude isotopic peaks for 14 + to 19 + precursor ions). The sequence coverage is

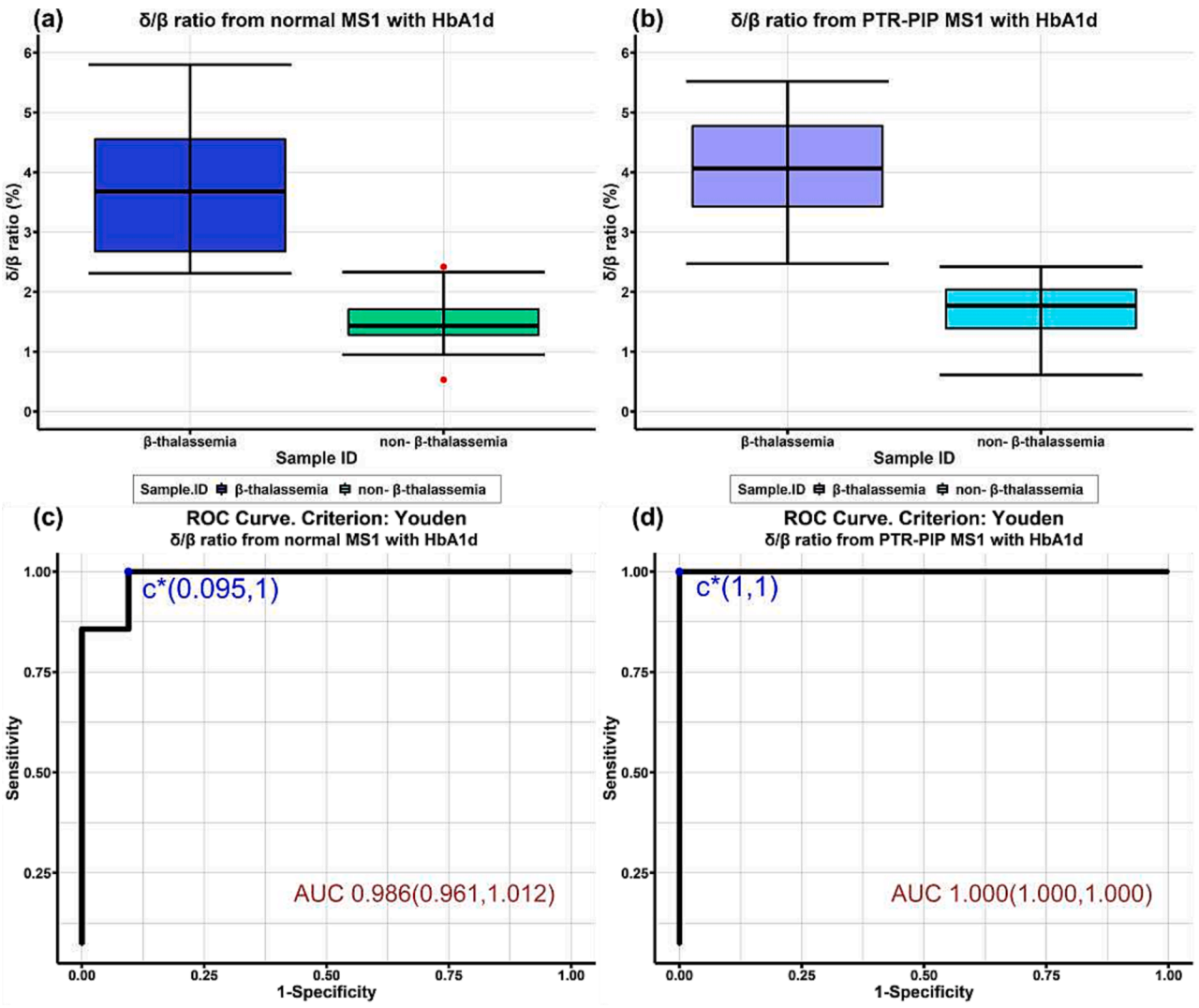

Fig. 5. Box-and-whisker (a), (b) and ROC plots (c), (d) of δ/β ratios with HbA1d included from normal MS1 and PTR-PIP MS1.

shown in Fig. 4**(b)**: the observation of z67 and z49 confirms that the glutathione binds to cysteine at 93rd position of Hb β subunit.

The δ/β ratios with HbA1d counted are listed in **Table S2**. Box-and-whisker and ROC plots of δ/β ratios with HbA1d counted as denominator are listed in Fig. 5. From Fig. 5 (a) and (b), the δ/β ratio ranges for normal MS1 and PTR-PIP MS1 are well separated, with two false-positive results and one upper outlier (both related to Hb AE samples) in normal MS1 (P value is 1.04×10^{-8} from Mann- Whitney U test), and no false-positive results in PTR MS1 with P value of 8.62×10^{-10} from Mann- Whitney U test. The ROC results are shown in (c), (d), the cutoff point for normal MS1 is 2.31 % with a sensitivity of 1, a specificity of 0.90, and AUC-ROC is 0.986. On the other hand, the cutoff point for PTR MS1 is 2.47 % with sensitivity and specificity of 1 and AUC-ROC of 1. The results with HbA1d counted also indicate that δ/β ratio can be used as a biomarker for differentiation of β-thalassemia in normal MS, and PTR-PIP MS1 can increase the sensitivity and specificity comparing with normal MS1.

The Mann-Whitney U test is also used to determine if the HbA1d will influence the δ/β ratio used for diagnosing β-thalassemia. The P values were 0.45 for β-thalassemia samples and 0.27 for non-β-thalassemia samples in normal MS1, and 0.27 for β-thalassemia samples and 0.06 for non-β-thalassemia samples in PTR MS1. The P values are all greater than 0.05, indicating that HbA1d does not affect the calculated δ/β ratios.

Perspectives and conclusions

The number of samples was 14 for β-thalassemia and 21 for non-β-thalassemia. Ergo, the sample size is enough to test the feasibility of our method, but a larger sample size is needed to obtain a clear cutoff point with fewer false-positive results. It is noticeable that the P value of 0.06 for non-β-thalassemia samples in PTR-PIP MS1 with and without HbA1d is close to 0.05; it is not possible to predict if the HbA1d will affect the δ/β ratio when the sample size is large. Therefore, the influence of HbA1d should be taken into consideration when the sample size expands. The box-and-whisker and ROC plots illustrate that the δ/β ratio is a reliable biomarker to distinguish β-thalassemia in normal MS1 with a cutoff point of 2.61 % and in PTR-PIP MS1 with a cutoff point of 2.92 %. The charge states of precursor ions are concentrated into fewer charge states (usually-one or two) in PTR-PIP MS1 experiments, not only improving the S/N ratios for the Hb subunits but also improving quantification information of the subunits with better sensitivity and specificity. With regard to clinical feasibility, a straightforward diagnostic approach is highly desirable. We demonstrated that δ/β ratios calculated from HPLC-MS experiments can serve as a biomarker to identify β-thalassemia, and that the PTR-PIP technique improves the sensitivity and specificity. Moreover, fragmentation methods such as collision-induced dissociation (CID) and electron-transfer dissociation (ETD) can provide MS2 product ion spectra to identify Hb variants. We

recently published a paper featuring the chimeric ion loading technique [45] in which CID and ETD fragments are simultaneously detected to successfully site-localize the mutation sites for Hb variants (even for heterozygotes) with a shorter data acquisition time and improved sequence coverage [34]. In conclusion, with HPLC top-down tandem MS, it is possible to obtain the relative quantification of δ and β subunits to characterize β-thalassemia, and simultaneously identify Hb variants.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Extended tables and figures (PDF). Patient samples list; Scale-expanded MS1 of δ subunit at charge state 18+ and partial deconvoluted results; δ/β ratio from normal MS1 and PTR-PIP MS1; δ/β ratio from normal MS1 and PTR-PIP MS with HbA1d counted. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2023.01.004.

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