provided high sensitivity for PCa detection in this referral group of men.

This work was supported by the Deutsche Forschungsgemeinschaft (GZ Ha3168 1/1). The study design was based on a proposal by Diagnostic Oncology for Roche Diagnostics. Specimen recruitment, data generation, and database management were performed by Diagnostic Oncology under the sponsorship of Roche Diagnostics. This collaboration led to the submission (P990056) and the premarket approval of the Elecsys total PSA Assay to the Food and Drug Administration. Roche Diagnostics provided the database to Dr. Partin and associates for analysis. Roche Diagnostics facilitated the writing of the manuscript by supporting inquiries regarding the database and participating in review of the conclusions of the manuscript.

References

High-Throughput Analysis of Hemoglobin from Neonates Using Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry, Urban A. Kiernan,1,2 Jeff A. Black,3 Peter Williams,2 and Randall W. Nelson1*

(1 Intrinsic Bioprobes, Inc., 625 S. Smith Rd., Suite 22, Tempe, AZ 85281; 2 Department of Chemistry and Biochemistry, Arizona State University, Tempe, AZ 85281; 3 Perkin Elmer Life Sciences-Norton, 3985 Eastern Rd., Norton, OH 44203; * author for correspondence: fax 480-804-0778, e-mail rnelson@intrinsicbio.com)

The risks associated with sickle cell and other untreated hemoglobinopathies have led to mandatory newborn screenings in 41 of the 50 states in the US (1). Preliminary screenings are often performed by electrophoresis at alkaline pH on cellulose acetate, with follow-up analysis of abnormal samples by acid electrophoresis on citrate agar (2). Alternatively, isoelectric focusing (IEF) or HPLC can be used in the screenings (3), but other techniques offer advantages.

In the past decade, new mass spectrometry (MS) approaches, including matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, have been applied to the study of proteins (4). MALDI-TOF MS offers a potent means of analyzing constituent chains of hemoglobin A (HbA) directly from samples as small as a single red blood cell (5). Mass accuracy and resolution are sufficient to determine the presence of point mutations within chains differing in mass by as little as ~20 Da. This ability allows detection of the β6Glu→Val variant (~30 Da lower in molecular mass) during direct analysis of the intact chains. Moreover, the site of mutation can be determined by combining MALDI-TOF MS with specific enzymatic digestion, i.e., “mass mapping”.

Thus, MALDI-TOF MS has the potential to be used in place of conventional approaches (6) for HbAS/HbS screening in the general population. To date, however, the mass spectrometric approaches have not been taken to the high-throughput mode necessary for efficient population screening, nor have they been applied to neonatal blood samples, which contain roughly equal portions of HbA and HbF. We report here progress in the development of a high-throughput mass spectrometric screening approach capable of screening the hemoglobin of neonates at rates of ~100 samples/h. In a blind analysis, 96 neonate blood samples were prepared for both intact protein molecular mass determination and mass mapping using parallel robotics (96-well format) and analyzed by MALDI-TOF MS for point mutations. Results from these analyses were later confirmed by IEF.

We studied 96 neonatal heel-stick/blotter-paper samples isolated in individual wells of a 96-well titer plate. The samples were extracted and processed in parallel by soaking each disk in 200 μL of 1 mL/L trifluoroacetic acid (TFA) for 3 min with gentle mixing in a robotic workstation (Multimek 96; Beckman). The TFA liberated protein from the paper and disrupted the interactions between hemoglobin subunits. The acidified hemoglobin solutions were diluted 500-fold in a mixture of 1 mL/L TFA and 1 mmol/L N-octylglucoside and dispensed onto 96-well-formatted MALDI-TOF MS targets for either intact protein analysis or tryptic mass mapping.

For intact protein analysis, samples were prepared in parallel on the robotic workstation by depositing 2 μL of diluted hemoglobin solution and 2 μL of MALDI matrix solution [saturated sinapinic acid in a 1:2 (by volume) mixture of acetonitrile–2 mL/L aqueous TFA] on a 96-well-format hydrophobic/hydrophilic contrasting MALDI-TOF MS target (7); samples were then allowed to air dry. The MS analysis was performed on a Bruker Biflex III MALDI-TOF mass spectrometer operating in linear delayed-extraction mode with 19.00 kV full accelerating potential. Draw-out pulses of 1.700 kV (300-ns delay) were used for parent chain analysis. Mass spectra were acquired automatically by summing 5–10 laser shot spectra while gauging spectral quality with fuzzy logic routines (8).
For tryptic mass mapping, tryptic digests of the 96 neonatal samples were prepared simultaneously on a 96-well-format trypsin-activated MALDI-TOF MS target. Targets were prepared according to previously described protocols (7, 9, 10). In brief, gold-plated 96-well-format MALDI-TOF MS targets were contrasted (hydrophobic/hydrophilic surfaces) with self-assembled monolayers of dithiobis[succinimidylpropionate] (Pierce Chemical) and octadecyl mercatan (Aldrich).

Trypsin (Sigma) was covalently immobilized on the dithiobis[succinimidylpropionate]-active surface through a 30-min incubation with a trypsin solution (0.01 g/L) in 0.1 mol/L Na2HPO4 buffer. After trypsin linkage, targets were washed in 0.1 mol/L Na2HPO4 buffer followed by rinses with doubly distilled water. After the final rinse, the targets were air dried, and each reaction site was individually coated with 2 μL of 20 mmol/L Tris-HCl and allowed to air dry. The Tris-HCl stabilized the trypsin after immobilization and was a compensating buffer for hemoglobin mapping (producing a final pH of ~8–9).

Mass mapping used 2-μL aliquots of diluted hemoglobin solution deposited in parallel (using the pipetting workstation) onto the trypsin-active sites on the target and digested for 10 min in a humidified environment at 37 °C. The digestions were terminated by the addition of 4 μL of acidified MALDI matrix solution (saturated aqueous solution of α-cyano-4-hydroxycinnamic acid in 330 mL/L acetonitrile–4 mL/L TFA) to each spot (using the robotic workstation) and allowed to air dry. Mass maps (100 laser shots/sample) were acquired in reflectron mode using an instrument setting of full accelerating potential of 19.00 kV, an ion-mirror voltage of 20.00 kV, and a draw-out pulse voltage of 2.65 kV (300-ns delay).

Mass spectrometric results obtained in the blind analysis were confirmed by IEF gel electrophoresis using a previously reported method (11).

A precursory analysis of all 96 hemoglobin samples was performed to determine the molecular mass of the intact chains. This analysis required, on average, <1 min/sample with parallel sample preparation (~5 min for 96 samples) and automated data acquisition (~45 min for 96 samples). The resulting parent-ion mass spectra displayed a mass accuracy within 0.01% and precision (CV) ≤0.03%. Three distinct profiles were observed as displayed in Fig. 1A. Ninety-two neonate samples exhibited “normal” hemoglobin profiles, showing signals attributable to wild-type α, β, and γ-chains of hemoglobin (Fig. 1A, bottom spectrum). Three of the samples yielded profiles that were essentially the same as the normal profiles, with the exception of an additional signal at a mass ~30 Da lower than that of the wild-type β-chain (Fig. 1A, middle spectrum). This peak “splitting” is generally indicative of a sample heterozygous for a point mutation. One sample showed only a signal from a β-chain shifted approximately ~30 Da from the wild type, indicating a patient homozygous for the point mutation (Fig. 1A, top spectrum). Both of these β-chain profiles are consistent with the presence of the βαGlu→Val variant.

Mapping was performed in parallel using trypsin-activated MALDI-TOF MS targets followed by automated data acquisition (the data were evaluated manually because we had no automated analysis software devoted to the task). Mass accuracies of the monoisotopic ion signals of the digest fragments were within 0.002% and maintained a precision (CV) ≤0.005%. Again, three different profiles were observed with regard to the β-chain in digest fragment 1–30. The bottom spectrum in Fig. 1B is representative of maps obtained for 92 of the samples. (A), all traces display signals from α-, β-, and γ-chains; an enlargement of the mass region of interest is also shown. (Bottom), MS trace of a neonatal sample containing the wild-type β-chain. This spectrum is representative of 92 of the samples in this analytical run. (Middle), spectrum representative of three samples containing sickle cell trait (HbAS). The wild-type and mass-shifted (approximately ~30 Da) β-chain signals are indicated. (Top), mass spectrum of a sample from an individual with sickle cell anemia (HbS) showing only a single signal from a mass-shifted (approximately ~30 Da) β-chain. (B), MS trace representing tryptic digest profile of a homozygous wild-type sample with a signal present from the wild-type β-chain1–30 digest fragment. This spectrum is representative of 92 of the 96 samples. (Middle), MS trace representing three of the tryptic maps that indicated the presence of HbAS. The spectrum contained signals from both the wild-type β-chain1–30 fragment and a mass-shifted (approximately ~30 Da) β-chain1–30. (Top), MS trace of the only spectrum that indicated the presence of the mass-shifted (approximately ~30 Da) β-chain1–30 fragment and absence of the wild-type signal, indicating HbS. (Inset), signal monitored at m/z 2228.150.
Monoisotopic signals were observed at \( m/z \) 3126.504 and \( m/z \) 3161.652, which were attributable to \( \alpha \)-chain\(_{4-90} \) and \( \beta \)-chain\(_{1-30} \) digest fragments, respectively. The middle spectrum in Fig. 1B is representative of three of the maps. The additional signal at \( m/z \) 3131.675 in the presence of the signal at \( m/z \) 3161.652 is consistent with the presence of HbAS. A third map was observed for only 1 of the 96 samples and is shown in the top spectrum of Fig. 1B. Only the signal at \( m/z \) 3131.675 was observed, consistent with the presence of HbS. To further localize the site of mutation, a second signal at \( m/z \) 2228.160 was monitored (Fig. 1B, inset). This signal was attributable to the \( \beta \)-chain\(_{9-30} \) fragment resulting from tryptic cleavages at residues 8 (Lys) and 30 (Arg). The absence of an additional signal, either split or shifted by approximately \( -30 \) Da, indicated that the mutation was present in the first eight residues of the \( \beta \)-chain and was most likely attributable to the \( \beta_6 \mathrm{Glu} \rightarrow \mathrm{Val} \) substitution. The mapping results were in agreement with those of the precursory screen: the same samples were found to contain the variant hemoglobins. Additionally, these results were later confirmed by IEF (results not shown).

The \( m/z \) 3161.652 signal can be monitored for other hemoglobin variants. HbC and HbE are hemoglobin variants caused by \( \beta_6 \mathrm{Glu} \rightarrow \mathrm{Lys} \) and \( \beta_2 \mathrm{Glu} \rightarrow \mathrm{Lys} \) mutations, respectively, each producing an approximately \( -1 \) Da shift from the wild-type \( \beta \)-chain\(_{1-30} \) tryptic fragment. [This mass shift is not readily apparent in the precursory screen of the intact hemoglobin chains, which, in passing, is a good reason to map all hemoglobin samples rather than just those suspected (from the precursory screen) to contain mutations]. The mutations will either broaden the \( m/z \) 3161.65 isotope distribution (heterozygous) or cause a wholesale shift of the isotope distribution in the homozygous (normal) samples, each by an approximately \( -1 \) Da shift in mass. Such shifts are easily recognized in reflectron mass spectra when the surrounding signals are used as internal calibrants. Additionally, both mutations would produce a new cleavage site for trypsin (Lys is introduced in the sequence), which after digestion will yield mass maps containing new tryptic fragments. Previous studies on blood samples of compound heterozygotes (individuals containing both HbS and HbC) yielded the discussed mass shifts and cleavage fragments (12). After further investigation, neither HbC nor HbE mutations were observed during the screening of the hemoglobins from the neonatal samples in this study.

In conclusion, MALDI-TOF MS provides a rapid and sensitive means of determining protein molecular masses with high reproducibility and accuracy; it also lends itself to the analysis of protein mixtures and, in particular, the individual subunits of hemoglobin. Given these abilities, precursory MALDI-TOF MS screening of hemoglobin samples from neonates yielded profiles of the intact hemoglobin chains that readily detected the approximately \( -30 \) Da shift in the \( \beta \)-chain molecular mass associated with HbAS and HbS. Mass mapping with trypsin-active MS targets was subsequently used to localize the site of mutation to the \( \beta \)-chain\(_{1-8} \) fragment, consistent with \( \beta_6 \mathrm{Glu} \rightarrow \mathrm{Val} \). Application of both approaches, as previously suggested by Chace (13), yielded a highly comprehensive analysis of the hemoglobins from neonates for HbAS/HbS. Moreover, other hemoglobinopathies, namely HbC and HbE, which differ from the wild-type \( \beta \)-chain by approximately \( -1 \) Da, may be screened via the same mass mapping approach. Using the parallel sample preparation and mapping protocols, we screened samples at rates as high as \( \sim 100 \) samples/h. These high rates of analysis suggest the possible use of this MALDI-TOF MS-based approach for the efficient, large-scale screening of hemoglobins in neonates.

This study was supported in part by Grants 2 R44 GM56603-01, 2 R44 GM56580-01, and R01 GM55872 from the NIH. The contents of this report are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. We would also like to thank Dr. Dobrin Nedelkov for critical reading of the manuscript. Additional data and figures showing results from this study are available at the Clinical Chemistry Online web site (clinchem.org/content/vol48/issue6/).

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