Poster Note 64730

Development of a High-Throughput Hemoglobinopathies Workflow Using High Resolution Accurate Mass Analysis

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ABSTRACT

A high-throughput hemoglobinopathies workflow is presented which is designed to perform research to automated data acquisition and processing for normal human alpha and beta chains, as well as profiling for abnormal hemoglobin chains. The presentation demonstrates a single method to profile samples regardless of complexity and the data acquisition scheme facilitates sequence detection for small mass deviations from normal hemoglobin chains. A large set of samples were used to evaluate the workflow, consisting of normal human hemoglobin (from dilute DBS) as well as aliquots spiked with known amounts of bovine hemoglobin. Spiking ranges were ca. 9-fold for both conditions (human:bovine and bovine:human). Results show excellent reproducibility with %CVs less than 6% across the 25 samples per spiking level and %RDS for measured AUC ratios were less than 10% for the expected SNP ranges and 20% for the extreme spiking ranges.

INTRODUCTION

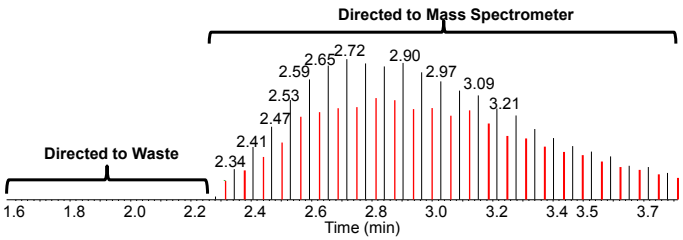
Hemoglobin profiling incorporates detecting, characterizing, and determining relative quantitation of all hemoglobin chains arising from normal protein sequence translation as well as sequence variants consisting of SNPs, truncations, and omissions. In addition, PTMs associated with glycation must be detected and quantified. Currently there are over 1000 reported hemoglobin forms prohibiting effective screening methods incorporating targeted/biased detection methods. Recently, mass spectral detection based on HRAM MS and AIF has emerged as a suitable method to perform hemoglobinopathies due to the advances in resolution and reproducible product ion detection over large dynamic ranges.

MATERIALS AND METHODS

Hemoglobin samples were created directly from a stock of whole blood from a healthy donor. A 10 µL aliquot was diluted 100-fold with 90:10:0.1 solvent of H_2O/MeOH/Acetic Acid and loaded into separate wells of a cation exchange plate. A bovine hemoglobin (purchased from Sigma Aldrich) stock solution and used to spike into selected sample wells at known concentrations to increase the complexity. The sample set was comprised of 20-25 replicates of neat human plasma, 9:1 human/bovine, 7:3, 5:5, 3:7, 1:9, and neat bovine hemoglobin. The plate was washed to remove salts and small molecules, and then desired molecular weight range was displaced and collected using an ammonium formate solution.

All experiments were performed on a Thermo Scientific™ Q Exactive™ Focus mass spectrometer equipped with a Thermo Scientific™ Vanquish ™ UHPLC autosampler and pump, with the peak tubing running through a 6-port divert value. The autosample loaded 20 µL of the extracted hemoglobin samples directly onto an Sephacryl™ S100 SEC column (GE Healthcare Life Sciences) for concentration washing for 1.5 minutes, then the divert valve was switched to the mass spectrometer for data acquisition for 3.5 minutes prior to diverting the LC flow back to waste to clean and condition the column for the next injection. (Figure 1)

Figure 1. Representative data acquisition strategy for the routine hemoglobin profiling experiment showing the chromatographic profile for direct sample loading, washing, and data analysis using alternating HRAM MS (black lines) and AIF (red lines) scan events.



The data acquisition sequence consisted of alternating high resolution/accurate mass (HRAM) MS and all ion fragmentation (AIF) events for the 3.5 minutes of data acquisition. Data acquisition parameters were consistent for both MS and AIF scan events including AGC = 5e6, max ion fill times of 100 msec, 10 microscans, and a resolution setting of 70,000 (FWHM) at *m/z* 200. Figure 2 shows representative spectra from the HRAM MS (2A) and AIF spectra (2B) from the human hemoglobin.
All data processing was performed in the Pinnacle software (Optys Technologies). Automated qualitative and quantitative data analysis utilized a FASTA file of the reported alpha and beta human and bovine hemoglobin sequences as downloaded from the UniProt database. (www.uniprot.org) Search parameters used partial sequence considerations as well as dynamic modifications for oxidation, deoxy, and hexose. A mass tolerance of ± 10 ppm was used for precursor and product ion isotopic determination.

Figure 2. Example data for neat human whole blood analysis following sample preparation and LC analysis where 2A shows full scan HRAM MS data and 2B shows the AIF spectrum. Each spectrum is one scan consisting of 10 µscans. The peaks labeled in blue font are from the alpha chain and red font represents charge states attributed to the beta chain.

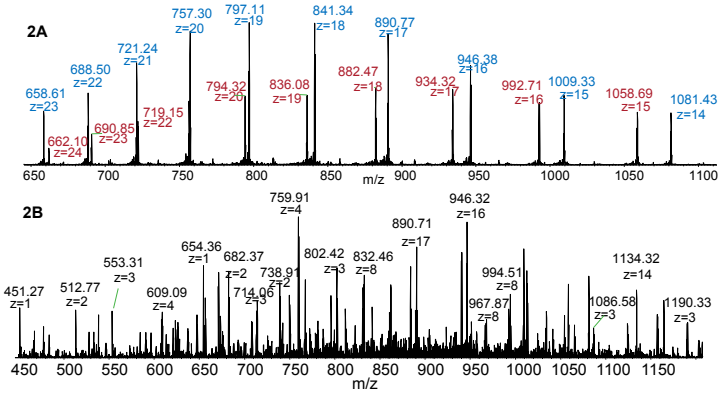


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Figure 3. Automated protein sequence determination in the Pinnacle software based on default Uniprot sequences and partial cleavage assumptions. The insets shows the matched isotopic profiles used for sequence determination of the partial sequence for the +16 charge state for the alpha chain and +17 charge state for the beta chain. Matching algorithms compare empirical isotopic and charge state m/z values with theoretical values for all possible sequences (assuming partial and PTMs) to identify the highest scoring match.

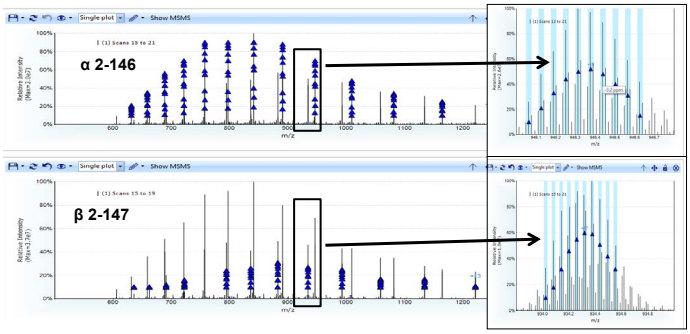
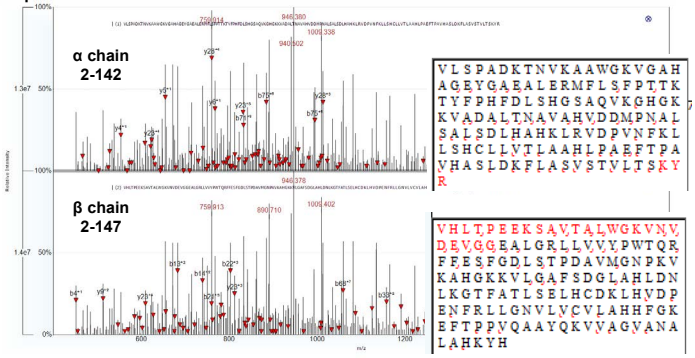


Figure 4. Automated AIF data analysis for the refined alpha and beta chain sequences. The matched product ions are marked in red triangles based on 10 ppm mass tolerance, charge state determination, and isotopic distribution for the proposed sequence (Fig. 3). Peptide diagrams for each chain are displayed in the inset with b- and y-type coverage maps indicated.



RESULTS

Successful hemoglobinopathies routines must confidently determine normal hemoglobin chains or variants. Therefore sample prep and data acquisiton must facilitate integrated and reliable search strategies. The knowledgebase compiled from the analysis of normal plasma (Figs. 2-4) are used as the first data set to screen for presence/absence of variants, and then facilitate characterization.

To simulate the increased complexities of variant hemoglobin samples, normal human plasma was spiked with known amounts of bovine hemoglobin, doubling the number of chains. While the mass shifts between human and bovine hemoglobin chains are not as close as some reported human variants, the increased complexity does stress the independent data acquisition routines for both precursor charge state determination and fragmentation analysis. Thus the determination of sequence specific fragments from each chain become key in characterizing hemoglobin content.

Finally, the routine must be reproducible and robust to handle the sample load facing most institutions. Therefore a larger number of replicates were prepared and analyzed to determine reproducibility. A set of 25 samples per spiking mixture was created in two 96-well plates and processed in a bulk manner. The resulting sample analysis was acquired in an automated manner and evaluation of response factors and carry-over was measured by randomizing the autosampler injection sequence. Of critical importance was the ability to reproducibly detect all hemoglobin chains, maintain mass accuracy for the majority of charge states per hemoglobin chain, determine the chain ratios, and measured the relative abundance of human to bovine ratios.

Figure 5. HRAM MS analysis of a hemoglobin mixture from human and bovine. The spiked bovine levels were ca. 1:1 with estimated human amounts. The charge states are labeled by the color coded key. Due to the narrow mass difference, not all detected charge states are labeled. The inset shows a narrow mass range to demonstrate the precursor distribution identification between the different alpha and beta chains.

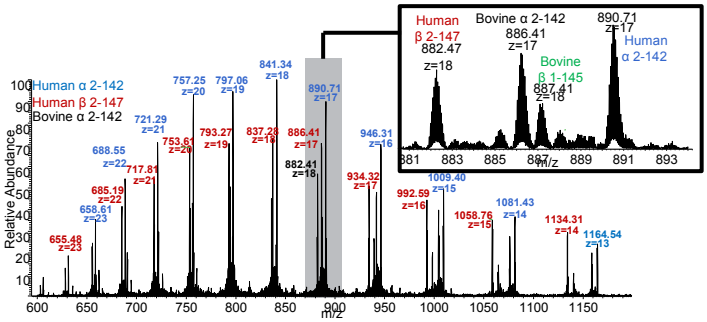


Figure 6. Comparative detection levels for the human and bovine hemoglobins from the different spiking levels used in the study. The displayed mass range covers the +17 and +18 precursor charge states for the alpha and beta chains, respectively. The spiked ratios are labeled for each spectrum (human:bovine).

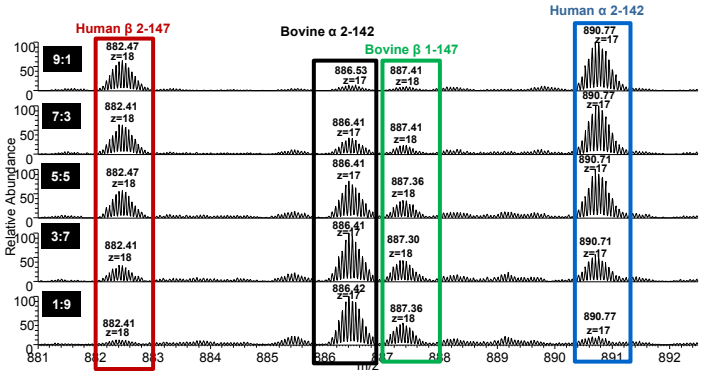


Figure 7. Comparative AIF spectra covering different spiking levels of human:bovine. The labeled fragment ions are matched based on sequences identified in Figure 4 and 5. Nomenclature used to label fragment ions indicate species, chain, fragment type, and position. The predominant fragments from human hemoglobin are labeled in the 9:1 spectrum and the predominant bovine hemoglobin fragments are labeled in the 1:9 spectrum.

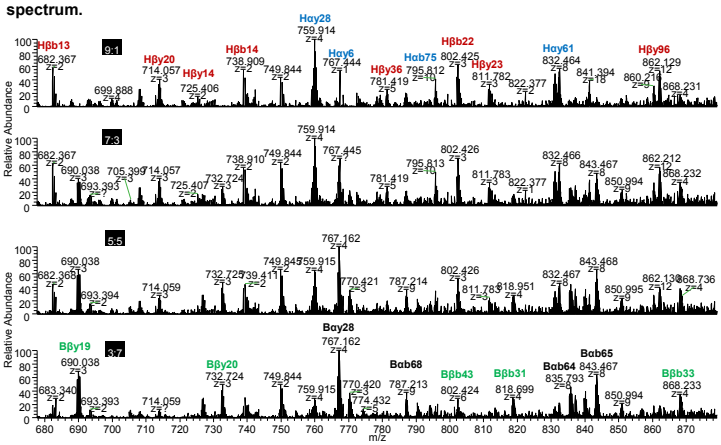


Figure 8. Evaluation of the workflow reproducibility based on the analysis of 25 replicate samples. The coefficient of variance was determined from measured AUC values for each hemoglobin chain per sample. Two representative plots are shown for the hemoglobin mixtures of 7:3 (Fig. 8A) and 3:7 (Fig. 8B) human/bovine. Reported hemoglobin chain AUC values were based on summed charge states and isotopes, as presented in Fig. 3. The %CVs are labeled by each chain. The trend shows excellent agreement for the species response, as the AUC trends track across the 25 samples. In addition, the AUC ratios between chains from the same species are consistent.

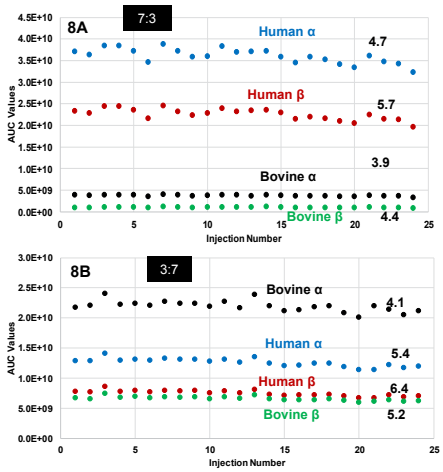


Figure 9. Summary report for quantitative hemoglobin spiking analysis. Histogram shows average AUC values and error bars for each chain per spiking ratio. In addition, the AUC ratios and %RSDs is reported between human and bovine. The ratios were corrected based on the estimated 1:1 ratios.

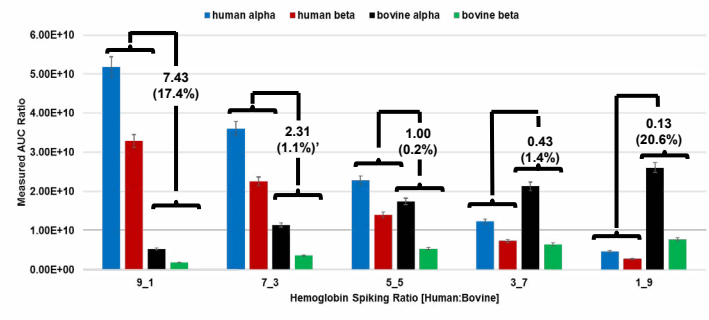
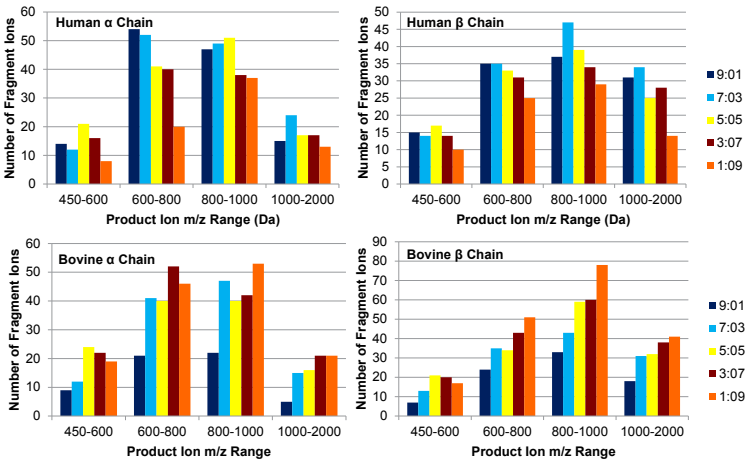


Figure 10. AIF product ion detection per hemoglobin chain across the different spiking ratios. The histograms are broken down into product ion m//z range. The key at the right is conserved for all histograms. Fragment ion confirmation was based on mass accuray, correct charge state and product ion isotopic distribution overlap (determined based on dot product correlation coefficients) for b- and y-type ions. The Pinnacle software also determines uniqueness of fragment ion association across the four hemoglobin chains.



CONCLUSIONS

The presentation shows an integrated workflow for high-throughput hemoglobinopathies research using the Q Exactive Focus mass spectrometer. The results demonstrate an effective method to combine robust sample preparation, delivery into the mass spectrometer, qualitative and quantitative data acquisition, and routine data processing. The workflow enables 384 samples to be analyzed per day with automated data processing on a single autosampler/LC stack. Specific attributes relative to different workflow aspects include:

- Sample preparation
  - Method can support DBS or plates
  - Plate methods require ca. 8 minutes for clean up
- Introduction into the mass spectrometer
  - Utilization of 6-port valve to load on column, and wash salts prior to diverting flow to ion source
  - Coupled with sample preparation steps, increased ruggedness of ion source
- Data acquisition
  - Alternating HRAM MS and AIF enabled many data points to be acquired across the 1.5 minute elution profile
  - AIF increased fragment ion detection from a wide range of precursor charge states
  - Use of 10 microscans significantly increased spectral quality for automated sequencing
- Data processing
  - The Pinnacle software leverages known protein sequences to expedite data characterization and facilitates screening for truncations, PTMs, and sequence variants
  - Precursor and product ion isotopic profiling was used to increase confidence in assigning hemoglobin sequence to each chain

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