Mass Spectrometry of Hemoglobin Variants

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Hemoglobinopathies or genetic variants of hemoglobin caused by a single polynucleotide polymorphism typically result in a single amino acid substitution in the globin chain. One common variant is hemoglobin S responsible for sickle-cell trait, where valine replaces glutamine in position 6 of the beta chain.

Two classic techniques used to identify hemoglobinopathies in the clinical laboratory, cation exchange (CE)-HPLC and capillary zone electrophoresis (CZE), are oftentimes insufficient to identify specific variants. We use a top-down mass spectrometry (MS) approach, along with the CE-HPLC and CZE results, to identify and predict which chain contains which amino acid substitution based on the mass differences at the intact subunit level. We will use a bottom-up MS approach to confirm the amino acid substitution and location at the peptide level.

Top-down MS approach. Washed whole blood samples, including known variants for proof of principle and unidentifiable specimens via CE-HPLC and CZE, were lysed in water (1:100 v/v) and centrifuged to pellet the insoluble material. 5 uL hemolysate was loaded onto a Zorbax 300 SB-C3 column (2.1 x 100 mm, 1.8 micron Agilent) at 70°C with a precolumn 1260 Infinity LC inline filter (2.1 mm Agilent) using a PAL HTS-xt autosampler (Leap Technologies). Hemoglobin subunits were separated by reversed-phase chromatography using a UFLC XR (Shimadzu) during an 11 min gradient of 44.5% - 48% B (A, water with 0.2% TFA; B, ACN:MeOH 9:1 with 0.2% TFA) at a flow rate of 0.2 mL/min. Samples were detected using a TripleTOF 4600 quadrupole time-of-flight mass spectrometer (AB SCIEX) with Analyst TF 1.6 software. Data 550-1400 m/z were accumulated in TOF MS mode at 0.500 ms/cycle, number of time bins 16, 1.055 ms pause between mass ranges, and Q1 transmission at 530.000 Da being 100%. MS settings were CUR 30, GS1 40, GS2 40, ISVF 5500, TEM 550, CE 10, and DP 100. Deconvolution of spectra was conducted in Analyst TF 1.6 or PeakView 2.1 with Bayesian

Protein Reconstruct or Reconstruct Protein, respectively. The settings for the output mass range were Start mass 15000 Da, Stop mass 16500 Da, Step mass 1.00 Da, and Charge agent/Adduct H^+. In PeakView, additional settings were Start m/z 750, Stop m/z 1280, and Input spectrum isotope resolution Resolved (30000).

Out of a total of 66 patient specimens, we present eight cases, including a proof of principle case with hemoglobin Athens, GA. The dilute-and-shoot top-down MS approach indicated a shift in mass of the beta chain of -28.0 Da. Three additional cases that were assigned to be hemoglobin Athens, GA by CE-HPLC, yielded two more confirmations of and one rule-out for Athens, GA. This approach was also used to identify a variant (experimental alpha Δmass = 26.1) that co-eluted with hemoglobin A_0 in HPLC and co-migrated with hemoglobin A in CZE. The hint that a variant existed was a tiny shoulder on the A_2 peak in both HPLC and CZE. Finally, two cases of G-Philadelphia (calculated alpha Δmass = 14.07) and a G-Philadelphia/S heterozygote were also investigated.