Free Light Chain Analysis Via Proteolytic Cleavage and Liquid Chromatography Tandem Mass Spectrometry

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Abstract:
In 1847, Dr. Bence Jones described excess protein in the urine of a patient with cancer. Noting that the protein, later discovered to be immunoglobulin light chains, precipitated when warming urine from 40 to 60^{o}C, he thus developed the first method for detecting a cancer marker in bodily fluids. Today, light chains are characterized or measured by clinical labs using techniques such as gel electrophoresis and immunoassay. Recent literature has questioned the validity of quantitative results measured by immunoassay. Therefore we developed a liquid chromatography tandem mass spectrometry method to measure κ and λ free light chains, which employed proteolysis.

Introduction:

Serum free light chain (sFLC) quantitation is recommended by the International Myeloma Working Group (IMWG) for evaluation and management of multiple myeloma and related plasma cell disorders (1). The above recommendation was spurred by the demonstrated clinical utility of an automated serum free light chain assay that was initially developed in 2001 (Freelite®, The Binding Site, Ltd, Birmingham, UK). This assay utilizes polyclonal antibodies that are specifically designed to bind regions only exposed on free light chains and do not have affinity for immunoglobulin heavy chain-bound light chains (2). In 2011 a second commercially available automated sFLC assay was released, the N-latex FLC assay (Siemens Healthcare Diagnostics, Marburg, Germany), and is currently available to clinical laboratories outside the U.S.

Despite the importance of sFLC measurement, many concerns have arisen regarding the accuracy, precision and reproducibility of these immunoassays. These findings include instrument-to-instrument variability with the Freelite® assay when the same reagents are used, as well as discrepancies when measuring the same sample at different laboratories (3). Studies have also shown discrepant results between these two commercial immunoassays which are thought to

be due to the differing specificities and affinities of the antibodies used (3). It was also observed that both immunoassays have shown evidence of lot-to-lot variability and linearity issues (3). Recently, a mass spectrometry method was developed to characterize monoclonal gammopathies; however this method looked at monoclonal proteins consisting of both heavy and light chains and did not explore quantitation of free light chains alone (4). In this study they first reduced intact monoclonal proteins to separate light chains from their respective immunoglobulin heavy chains, and then demonstrated the presence of distinct peaks with masses consistent with heavy and light chains (4). This study was followed up with a publication describing use of the same method to characterize the polyclonal kappa and lambda light chain repertoire in serum (5). Neither of these approaches aimed to specifically quantitate free light chains, as the method described involved a reduction step that would preclude differentiation between free and bound light chain. Therefore, our objective was to develop a liquid chromatography tandem mass spectrometric method that quantitates κ and λ free light chains. Our method utilizes ultracentrifugation and proteolytic cleavage, followed by analyses of κ and λ signature peptides from the respective constant regions using multiple reaction monitoring (MRM).

Experimental:

Informatics

Protein accession numbers were acquired from the Universal Protein Resource (UniProt) database. Skyline software (MacCoss Lab Seattle WA) was used to aid in peptide selection (5). FASTA formats of the constant region sequences of κ and λ light chains were inputted into Skyline. The κ protein accession used in this effort was P01834. The λ sequences encompassed the 1, 2, 3 and 7 isoforms corresponding to the respective accessions P0CG04, P0CG05, P0CG06 and A0M8Q6. It is noted that many of tryptic peptides for the λ sequences have overlap between the different isoforms. Protein BLAST searches also revealed for the λ sequences that there is tryptic peptide overlap with proteins lambda-like polypeptide 1 (accession P15814) and lambda-like polypeptide 5 (accession B9A064).

Samples

Sample preparation for LC-MS/MS analysis included ultracentrifugation using an Amicon 30 kDa MWCO filter (EMDMillipore Waltham, MA) to separate free light chains (MW approximately 25 kD) from intact immunoglobulins (MW approximately 150,000 kD). Forty

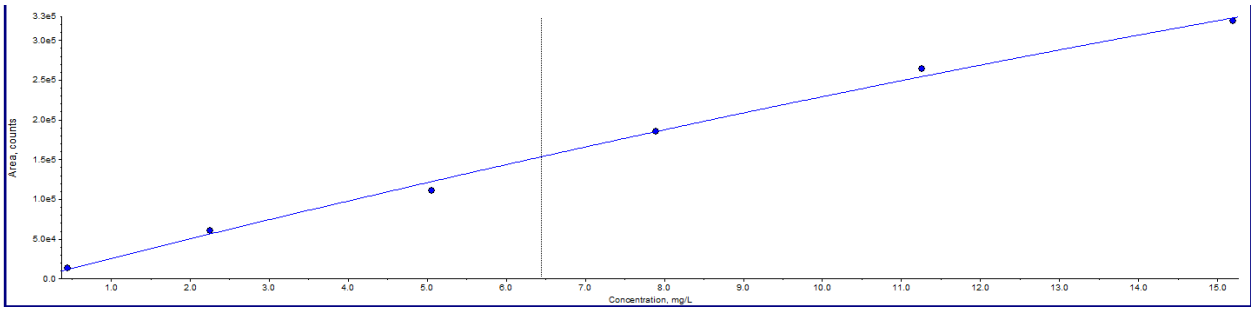
microliters of the ultrafiltrate containing the free light chains was diluted with 100 μL of 0.1M ammonium bicarbonate, reduced using 10 μL of 200 mM dithiothreitol (Sigma Aldrich, St. Louis, MO) at 60^0C and alkylated at 25^oC in the dark using 10 μL of 1M iodoacetamide (Sigma Aldrich). Following reduction and alkylation the samples were digested using 15 μL of a 1 mg/mL trypsin (Sigma Aldrich) and were allowed to digest at 37^oC for 2 hours after which 15 μL of ≥95% formic acid (Sigma Aldrich) was added. Following digestion, samples were added to glass auto-sampler vials with 200 μL glass inserts, capped and inserted into a 4^oC cooled auto-sampler of a XR HPLC system (Shimadzu Scientific, Japan) that was interfaced with a QTRAP 6500 mass spectrometer (AB SCIEX, Framingham, MA). A C18 Kinetex 3x50 mm column (Phenomenex Brea, CA) was used for peptide separation. A 10 minute chromatographic method employed linear-gradient chromatography using water with 0.1% formic acid as mobile phase A and acetonitrile with 0.1% formic acid as mobile phase B. Calibrators used for LC-MS/MS analysis were from the Freelite® kit (The Binding Site, Ltd, Birmingham, UK), where the calibrator concentrations for the κ light chains ranged from 0.57 to 17.1 mg/L and the λ light chain concentrations 0.45 to 15.2 mg/L. Waste human urine samples from the University of Minnesota Medical Center, Fairview, will be selected from patients with known free urinary κ or λ protein as observed on urinary protein gel immunofixation. Samples will be stored at -20^oC until analysis.

Results:

Figure 1 shows an example of a calibration curve observed for the ADGSPVK peptide during λ FLC analysis by the LC-MS/MS method ($r = 0.9969$). It is noted that this preliminary work and results are void of a stable isotope labeled internal standard. However, even in the absence of an internal standard, measurement of the Freelite® κ and λ calibrators demonstrated excellent linearity.

Based on this promising preliminary data, further work will be done to optimize the assay with an isotope-labeled internal standard. Once the assay is optimized, we will compare our LC-MS/MS method to current immunoassay methods used in clinical laboratories by assaying waste patient serum and urine samples with both methodologies.

Figure 1.



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