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 60ºC, 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

Quantitation of serum free light chain (sFLC) proteins are recommended by the International Myeloma Working Group (IMWG) for evaluation and management of multiple myeloma and related plasma cell disorders (1). In 2001 a polyclonal immunoassay specifically designed to bind regions only exposed on free light chains (Freelite®, The Binding Site, Ltd, Birmingham, UK) which does not have affinity for immunoglobulin heavy chain-bound light chains was introduced to clinical laboratories (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.

Many concerns have arisen regarding the accuracy, precision and reproducibility of these immunoassays. In a study of the Freelite® assay, issues cited included instrument-to-instrument variability with the same reagents, same sample discrepant results measured at different laboratories, and linearity issues. This study also found contrasting results between these two commercial immunoassays which are thought to be due to the differing specificities and affinities of the antibodies used (3).

Studies using mass spectrometry methods and relative abundance have been developed to characterize intact monoclonal proteins consisting of heavy and light chains, and polyclonal kappa and lambda light chain repertoire in serum, which included both free and bound light chains (4,5). 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

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 mL of 0.1M ammonium bicarbonate, reduced using 10 mL of 200 mM dithiothreitol (Sigma Aldrich, St. Louis, MO) at 60ºC and alkylated at 25ºC in the dark using 10 mL of 1M iodoacetamide (Sigma Aldrich). Following reduction and alkylation the samples were digested using 15 mL of a 1 mg/mL trypsin (Sigma Aldrich) and were allowed to digest at 37ºC for 2 hours after which 15 mL of ≥95% formic acid (Sigma Aldrich) was added. Following digestion, samples were added to glass auto-sampler vials with 200 mL glass inserts, capped and inserted into a 4ºC 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. Remnant human urine samples from the University of Minnesota Medical Center, Fairview, were selected from patients with known free urinary κ or λ protein as observed on urinary protein gel immunofixation using a Hydrasys System (Sebia® Electrophoresis Norcross, GA). Samples were stored at -20ºC until analysis.

Protein Survey Scans

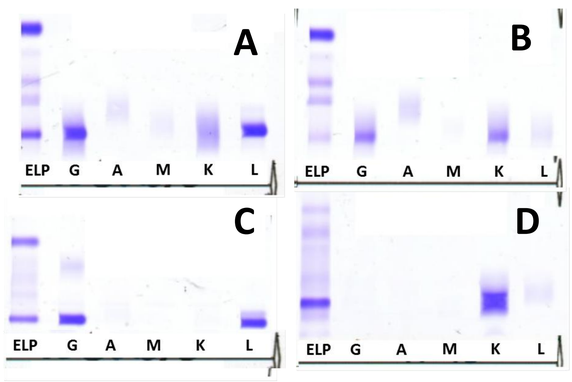
Data were generated using a QTRAP 6500 EMS, ER and EPI method that selected the three most intense ions during a cycle from digests of the highest Freelite® calibrators, which were subjected to the proteolysis as described above. The data was then exported as a Mascot Generic File (MGF) and uploaded into the Mascot (Matrix Science Inc. Boston, MA) database search engine, where it was searched against the Swiss-Protein Database.

Informatics and Transition Generation

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 and lambda-like polypeptide 5 (respective accessions P15814 and B9A064).

Results

Immunofixation Monoclonal Examples



Gel A: Serum IgG Lambda, Gel B: Serum IgG Kappa
Gel C: Urine IgG Lambda, Gel D: Urine Kappa Free Light Chain

Sample Preparation

1. Ultracentrifugation of urine sample with 30,000 Da MWCO filter.
2. 40 µL Sample
3. 100 µL 0.1M ammonium bicarbonate
4. 10 µL 0.2M DTT
   - Incubate at 60ºC for 1 hour
5. 10 µL 1M iodoacetamide
   - Incubate at room for 0.5 hour
6. 15 µL Trypsin
   - Incubate at 37ºC for 2 hours
7. 15 µL Formic Acid

Protein Survey Results

Kappa Calibrator Summary for Protein Survey Scan

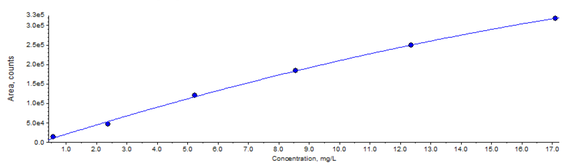
1	ALBU_HUMAN	Serum albumin OS=Homo sapiens
2	TRFE_HUMAN	Serotransferrin OS=Homo sapiens
3	MOT1_HUMAN	Monocarboxylate transporter 1 OS=Homo sapiens
4	PCDA4_HUMAN	Protocadherin alpha-4 OS=Homo sapiens
5	LRIG1_HUMAN	Leucine-rich repeats and immunoglobulin-like domains protein 1 OS=Homo sapiens
6	NCKX1_HUMAN	Sodium/potassium/calcium exchanger 1 OS=Homo sapiens
7	PLCE_HUMAN	1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon OS=Homo sapiens
8	TV23A_HUMAN	Golgi apparatus membrane protein TVP23 homolog A OS=Homo sapiens

Lambda Calibrator Summary for Protein Survey Scan

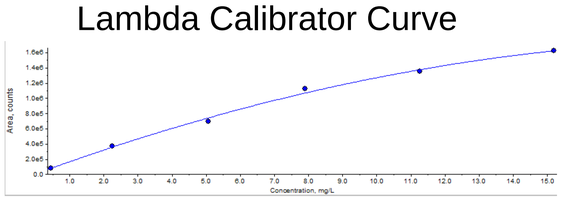
1	A1AT_HUMAN	Alpha-1-antitrypsin OS=Homo sapiens
2	ALBU_HUMAN	Serum albumin OS=Homo sapiens
3	IGHA1_HUMAN	Ig alpha-1 chain C region OS=Homo sapiens
4	TRFE_HUMAN	Serotransferrin OS=Homo sapiens
5	ROBO3_HUMAN	Roundabout homolog 3 OS=Homo sapiens
6	NAPEP_HUMAN	N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D OS=Homo sapiens
7	ZFP30_HUMAN	Zinc finger protein 30 homolog OS=Homo sapiens
8	ZNT8_HUMAN	Zinc transporter 8 OS=Homo sapiens

No light chain protein observed for either digest. Also noted the presence of alpha-1-antitrypsin in the calibrator. Therefore we used Skyline software to generate a transition list.

Kappa Calibrator Curve

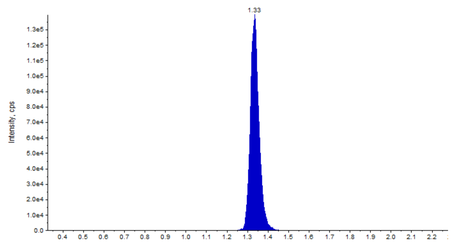


Lambda Calibrator Curve



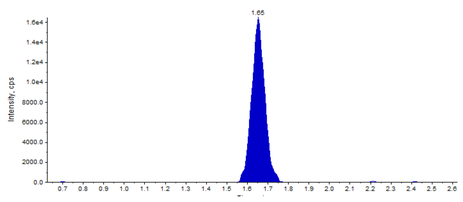
All calibrator curves were generated in the absence of an internal standard.

Urinary Free Light Chain Example Kappa

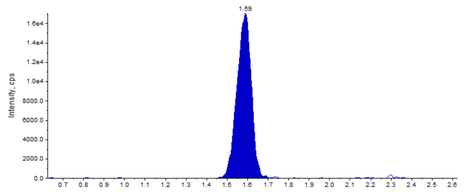


Free Kappa Light Chain value was 26.2 mg/L (SFNR)

Urinary Free Light Chain Example Lambda



Lowest Calibrator Lambda



The highest patient sample intensity was lower than the lowest calibrator. The amount of lambda free light chain was estimated to be 0.400 mg/L where the lowest calibrator is valued at 0.468 mg/L in this instance. (ADSSPVK)

Preliminary Comparison Results

Kappa Light Chain

- The kappa light chain result for the sample above was found to be 26.2 mg/L by LC-MS/MS.
- Freelite® immunoassay yielded a value of 602.5 mg/L.
- In general we observed a similar trend for all other urine samples containing light chains with immunoassay yielding values 10x higher than LC-MS/MS.

Lambda Light Chain

- The lambda light chain result for the sample above was found to be 0.4 mg/L by LC-MS/MS.
- Freelite® immunoassay yielded a concentration of 10.6 mg/L.
- Therefore, we see potentially large discrepancies for both kappa and lambda light chains between the two different methodologies.

Same Freelite® calibrators were used in both systems: Immunoassay and LC-MS/MS.

Conclusions

- Quantitation of kappa and lambda free light chains in urine appears promising.
- Several peptides from both kappa and lambda isoforms have shown reasonable signal and linearity.
- Preliminary results for patient samples using LC-MS/MS show large discrepancies when compared to immunoassay results.
- This difference should not be due to a calibration difference as the same calibrators were used.
- This should not be due to differential detection of bound light chain or light chain multimers, as the ultrafiltrate was analyzed with both the Freelite® and LC-MS/MS methods.

Future Work

Assay refinement will encompass the following aspects:

- Validation of peptides and internal standards
- Assess ultracentrifugation process
  - 30 kDa vs. 50 kDa MWCO filters
  - Needs to be performed as dimers could be present in specimens
- Large study comparison
  - Freelite vs. N-latex vs. LC-MS/MS
- Potentially expand to serum measurements
  - Measure serum values by LC-MS/MS
  - Compare results to Freelite, N-latex

References

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