Development of a Multiplexed Mass Spectrometry-Based Apolipoprotein Panel Assay for Plasma and Serum

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Overview

Purpose: Development of a SRM MS multiplexed assay for an apolipoprotein (Apo) panel.

Methods: Novel software was used to predict target peptides and transitions for assay development.

Results: A small cohort of clinical serum samples from stroke and healthy (control) patients was interrogated with the SRM MS assay. Two apolipoproteins were significantly differentially expressed across collection time points in serum samples from all stroke patients.

Introduction

The relative levels of various apolipoproteins can be important biomarkers for heart disease, stroke, Alzheimers, diabetes and metabolic syndrome. Typically, these proteins are individually measured in blood by immunoassay. Apolipoproteins are clinically more feasible to measure than other markers such as cholesterol since they are not influenced by fasting states. In addition, most patients have multiple risk factors that interact and potentiate disease phenotypes, such that heart disease, cerebrovascular disease, and obesity are risk factors for each other and influence each other greatly. Therefore, a panel of multiple apolipoproteins may help to individualize risk assessment in each patient and help to monitor therapeutic efficacy of medical therapy.

The availability of a multiplexed assay that could simultaneously and quantitatively measure a panel of apolipoproteins would be an extremely useful clinical research tool. Mass spectrometry-based selective reaction monitoring (SRM) is rapidly becoming a preferred technology for the development of quantitative protein or peptide assays for clinical research. SRM assays deliver high sensitivity, selectivity and throughput, and when taken together, these parameters provide a breakthrough quantification methodology. In this report, we describe the application of novel software, Pinpoint, to the development of an SRM-based assay for a comprehensive panel of apolipoproteins. Pinpoint software facilitates iterative development steps to optimize the choices of target peptides and transitions. Once the multiplexed apolipoprotein panel SRM assay was optimized, the method was used to interrogate a small cohort of stroke patients (n=5) and healthy patients (control) (n=5) serum samples collected at various time points.

Methods

Clinical samples

Serum samples from stroke and normal patients were collected with full consent and approval (Fig. 1). Blood samples were collected into tubes which did not contain anti-coagulant. Samples were centrifuged at 1,500 rpm for 15 minutes at 4 °C and then frozen at -70 °C. Samples were transported frozen and were thawed immediately before processing. Samples from stroke and healthy control patients were processed in a random order to prevent systematic errors and variations from experiment to experiment.

Sample preparation

Serum samples were thawed and prepared by diluting serum 1:4 v/v with 8 M GuHCl/150 mM Tris/10 mM DTT pH 8.5. Samples were incubated at 37 °C for 60 minutes and then cooled to room temperature. Alkylation was performed by addition of 500 mM iodoacetic acid/1 M Tris pH 8.5 to each sample to give a final concentration of 40 mM and incubated in the dark at room temperature for 60 minutes. The reaction was quenched with the addition of 2M DTT to a final concentration of 5mM. Post quench, 30 mg samples were diluted to 120 µL with the addition of 50 mM Tris/5 mM $CaCl_2$ pH 8.0 and 0.3mg of sequencing grade enzyme (Promega) was added to each sample and incubated at 37 °C for 24 hours. The digestion reaction was quenched with the addition of TFA to 1% and heavy (R or K) peptide standards were spiked into each sample. Samples were then desalted on-line utilizing the autosampler in which the sample loop had been replaced with a 1mm x 10mm trapping column. Peptides eluted from the trapping column were then introduced directly into the LC set up.

SRM assays

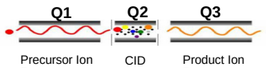
SRM assays were developed on Thermo Scientific TSQ Vantage and TSQ Quantum Ultra triple quadrupole mass spectrometers (Fig. 2), Surveyor MS pump, Micro autosampler and IonMax Source equipped with a low flow metal needle. Samples were analyzed in triplicate. Reversed-phase separations were carried out on a 1 mm X 50 mm Thermo Scientific Hypersil Gold 1.9µm C18-particle column. Solvent A was LC-MS grade water with 0.2% (v/v) formic acid. Solvent B was LC-MS grade 30% (v/v) acetonitrile with 0.2% (v/v) formic acid (Optima grade reagents, Thermo Fisher Scientific). Thermo Scientific Pinpoint software (available upon request) was used for targeted protein quantification. The Pinpoint™ software algorithm was used to predict candidate peptides and selection of fragment ions for SRM assay design, build MS instrument method and peptide quantitation. For the workflow described herein, differentially expressed peptides that were identified in previous LC-MS/MS discovery libraries of archived MS data were imported directly into Pinpoint software. Transitions were chosen based on the predominant fragments observed in the discovery data (at least 4 transitions per peptide). Peptides were identified by co-eluting light and heavy transitions derived from synthetic peptide standards. Quantification was relative to a known amount of heavy peptide.

Results

FIGURE 1. Clinical samples.

Number of patients	Blood Collection times	Sample type
5 (S1-S5)	Upon admission (T1) 1 day after admission (T2) 2 days after admission (T3)	Stroke
5 (N1-N5)	Upon admission (T1) 2 months after admission (T2)	Normal

FIGURE 2. A triple quadrupole mass spectrometer functions as a set of highly SELECTIVE and SENSITIVE mass filters to perform selected reaction monitoring (SRM) targeted assays.



1. A "proteotypic" target peptide is selected by hypothesis driven (chosen from publications or databases) or from discovery data.
2. Q1 is set to transmit only the parent *m/z* of the selected peptide.
3. CID fragmentation is induced in Q2
4. Q3 is set to transmit only a selected fragment ion.
5. SRM analyses for many targeted proteins can be carried out during an LC run.

FIGURE 3. Archived libraries of high-resolution LC-MS/MS data (discovery data) were mined by Pinpoint software to allow efficient selection of target peptides and transitions for SRM assays.

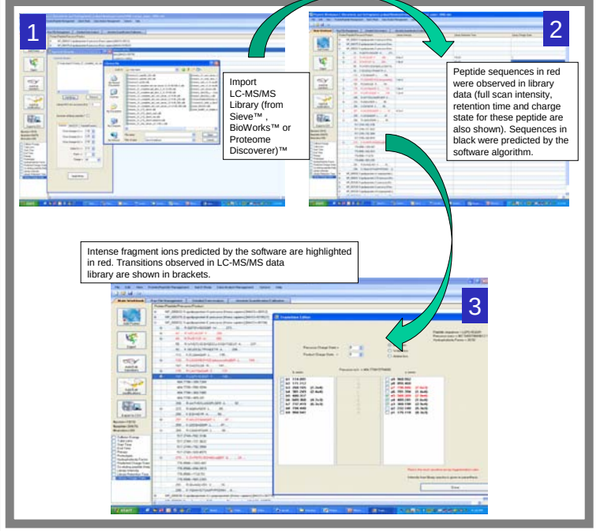
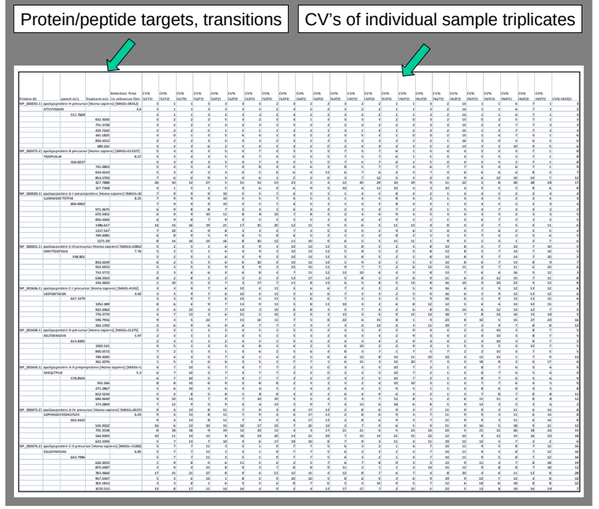


FIGURE 4. A multiplexed SRM assay for 9 apolipoproteins was developed and applied to interrogate serum samples from stroke and normal patients. The CV's for triplicate injections were <15% in most cases.



Conclusions

- A multiplexed assay for a panel of 9 apolipoproteins was developed.
- The assay was used to interrogate a small cohort of stroke and normal healthy (control) clinical samples.
- Sample preparation consisted of digestion of raw serum followed by LC-MS/MS. No prefractionation was required.
- The LOQ of the target peptides neat and in serum matrix was 50 attomoles and 500 attomoles, respectively.
- CV's of triplicate injections were below 10% in most cases.
- Both Apo IV and Apo CII were observed to be significantly differentially expressed. These apolipoproteins have been associated with cerebral infarction (1,2).

References

1. Ogawa H, Nishikawa T, Fukushima S, Sasagawa S.Studies on stroke-prone spontaneously hypertensive rats (SHRSP) fed a high-fat and high-cholesterol diet--changes in serum concentrations and distributions of apolipoproteins A-I, A-IV and E. Nippon Eiseigaku Zasshi. 1989 Jun;44(2):622-30.
2. Konemori G, Kawanishi M, Nakamoto A, Hiraoka M, Kajiyama G. Application of the box plot to evaluate serum lipid levels and apolipoprotein levels in patients with cerebral infarction and diabetes mellitus Nippon Ronen Igakkai Zasshi. 1990 Jan;27(1):22-7

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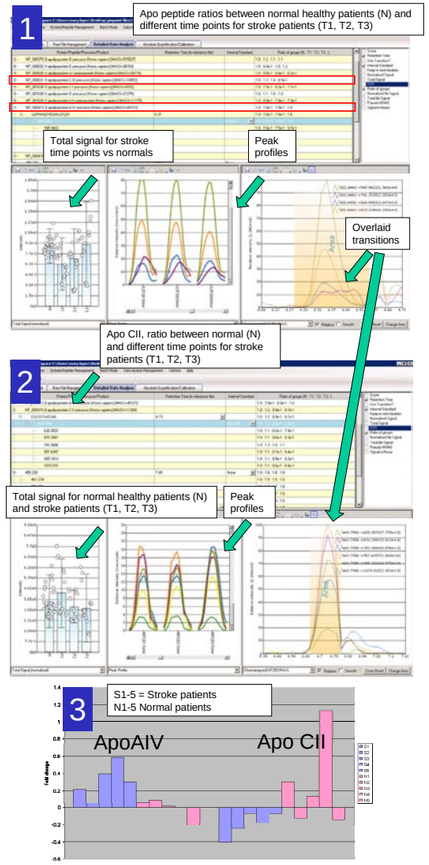
FIGURE 5. Two apolipoproteins from the panel of 9 were differentially expressed in the stroke versus normal samples.



Statistical analysis of the SRM assay data indicated that 2 of the 9 apolipoproteins monitored were differentially expressed over the 3 time points assayed in the stroke patient samples with respect to the normal healthy patient samples (Panels 1,2 on left). Trends between time points were computed for the stroke patient samples using intensity ratios and these were compared with the trends between time points for the normal patients. Student T-test was used to calculate the significance of peptide ratio differences amongst stroke and control patients. Relative Apo CII levels in stroke patients decreased in all 5 patient samples from admission (T1) through day 1 (T2) and day 2 (T3) blood sample collections, see Panels 2 and 3. While, Apo AIV levels increased from the initial blood draw upon admission (T1) and continued to increase in samples collected on day 1 (T2) and day 2 (T3) see Panels 1 and 3.