Serum profiling: The analysis of Therapeutic proteins and diagnostic markers for lysosomal storage disorders by 'label free' LC-MS

Waters

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OVERVIEW

- The feasibility of monitoring therapeutic proteins in serum samples over time is demonstrated
- A Linear response for alpha galactosidase A, between 10 and 500 fmol injected on a 75µM analytical column is presented
- The data from patient samples illustrates the potential for monitoring global protein concentration changes across multiple conditions

INTRODUCTION

Fabry disease is an X-linked recessive glycolipid storage disorder, caused by deficient activity of the lysosomal enzyme alpha-galactosidase A (alpha gal A). In affected patients progressive accumulation of the glycolipid substrate for this enzyme, globotriaosylceramide, occurs within vulnerable cells and tissues. Patients may show ocular deposits, febrile episodes, burning pain in the extremities and skin lesions (angiokeratomas). Administration of recombinant alpha-gal A has been shown to alleviate symptoms of the disease and is used as a long-term enzyme replacement therapy in patients with a confirmed diagnosis of Fabry's disease.
Monitoring circulating levels of alpha gal A can determine the uptake and metabolism of the replacement enzyme and could potentially allow the therapeutic level of the drug to be determined in patient samples, allowing the dose of the drug to be adjusted to maintain safe, cost effective levels of the treatment.
Here we detail a preliminary investigation into the potential of LC-MS for analyzing digested samples of human serum, containing spiked levels of recombinant alpha gal A. We have determined the limit of detection of the enzyme, present in the complex serum background, and have established the linear dynamic range over which measurements can be made. Finally, we have investigated the potential for relative quantification of alpha gal A from patient serum samples.

METHODS

Sample preparation.
The human serum samples and alpha gal A were diluted, and solubilized by incubation at 80°C for 15 mins in 0.1% RapiGest™ SF (Waters, Milford, MA) before reduction with 5 mM dithiothreitol and alkylation with 10 mM iodoacetamide. The proteins were then digested with 1% (w/w) sequence grade trypsin overnight (16 hr). RapiGest SF was cleaved by the addition of HCl, followed by centrifugation, and the supernatant subsequently collected. Samples were diluted with 0.1% formic acid to an appropriate concentration prior to analysis.

Experimental.
Experiments were conducted using a reversed phase gradient (5 to 40% acetonitrile in 120 minutes) on a Waters® CapLC™ System (Waters, Milford, MA) coupled to a Q-Tof™ mass spectrometer (Waters Micromass Manchester UK). Standards and samples were run in duplicate and triplicate, respectively. The Q-Tof mass spectrometer was programmed to acquire data as describes previously (1), where the energy applied to the collision cell is alternated between a low (10 eV) and elevated (23-33 eV) energy. Protein identifications and quantitative information were extracted by the use of specialized algorithms, and searching a Human species-specific database.

RESULTS

Initial feasibility experiments were conducted to determine the limits of detection for alpha gal A in the presence of a complex matrix, i.e. digested human serum.

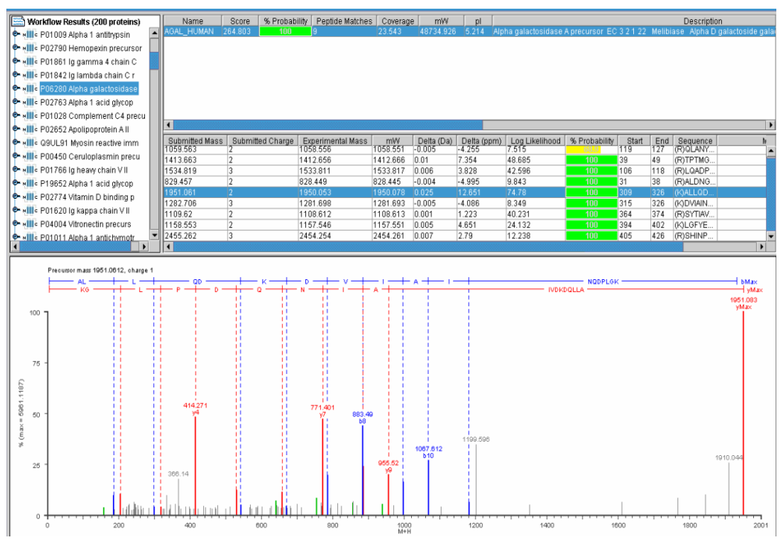

Figure 1. Identification of alpha gal A from a species specific database, spiked into human serum at the 100fmol level The search uses the elevated energy fragment ion data

Relative quantitation at the protein level

A tryptic digest of alpha gal A was spiked at the 10 to 500 fmol level in 0.2 µg/µl of serum. The result of these experiments which identify the proteins based on the high-energy fragment ion data, shown in Figure 1, prior to performing any quantification, is presented in Figure 2.

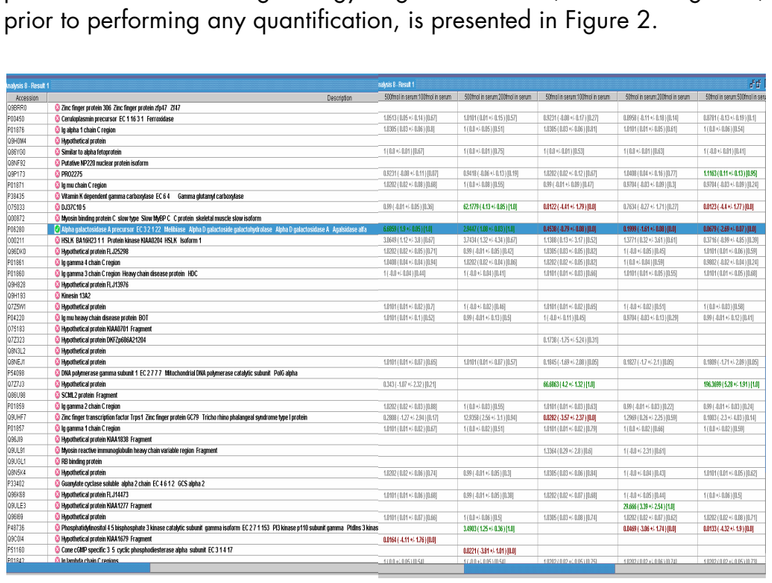

Figure 2. Protein identification based relative quantification of alpha gal A in undepleted human serum. Each sample was analyzed in duplicate. Human serum albumin was specified as the internal standard.

An alternative method of quantification is illustrated in Figure 3, where the sum of the intensities of three peptides from alpha gal A which replicated across all conditions is divided by the sum of the intensities of three peptide of human serum albumin or Ig gamma 1 chain and is displayed as a function of the amount of alpha gal A injected on column.

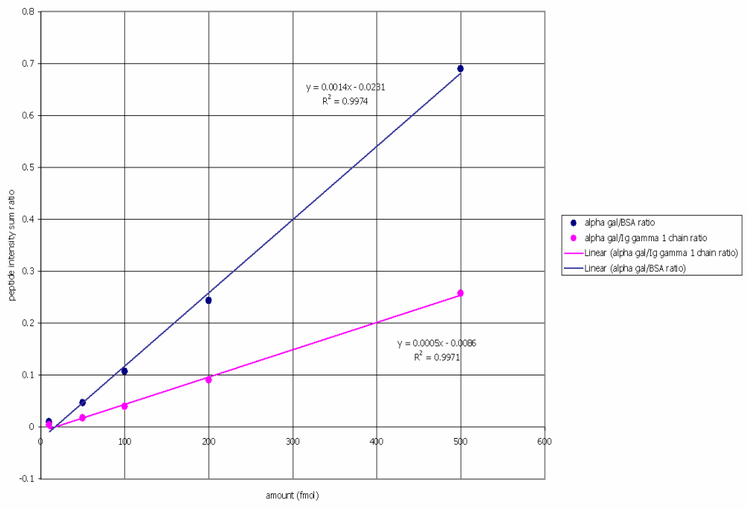

Figure 3. Relative quantification of alpha galactosidase spikes in undepleted digested human serum. Quantification was performed by using summed intensities of identified peptides from the low-energy data set that replicate across all conditions.

Relative quantitation at the peptide level

Identification of proteins can also be conducted from the peptide exact mass only. In this instance, the data sets are clustered based on their exact mass and retention time (EMRT). An example is shown in the upper pane of Figure 4, where the log intensity ratio is depicted as a function of mass. A selector tool is used to select the peptides of interest which can be followed by a peptide mass fingerprint type search using the peptide mass information, illustrated in the bottom pane of Figure 4.

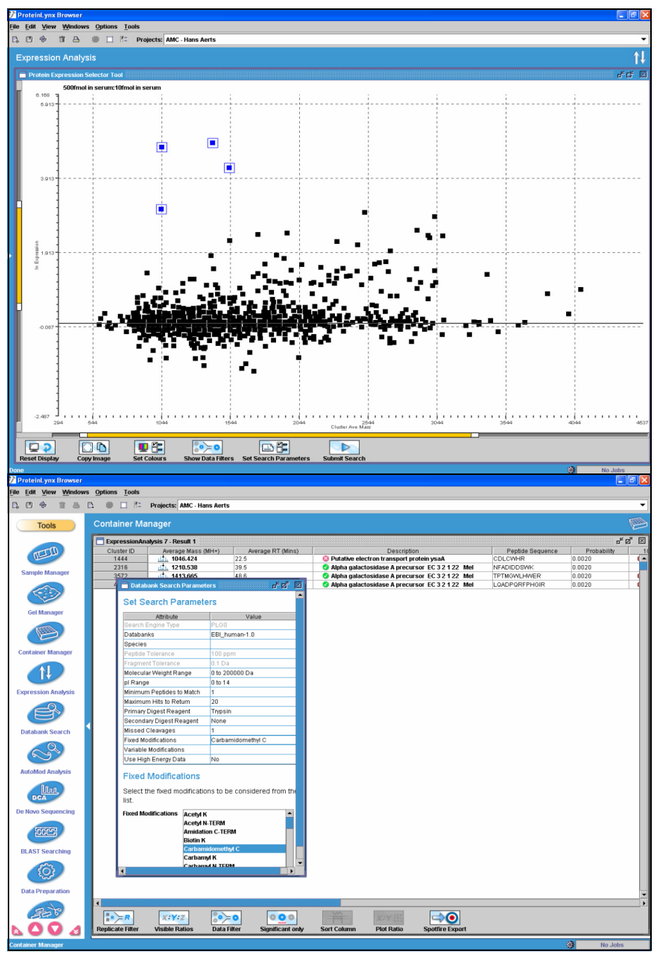

Figure 4. Exact mass retention time browser – upper pane – showing the log intensity ratio of 10 fmol of alpha galactosidase vs. 500 fmol injected on column and the low energy peptide mass finger print search result for 10 fmol of alpha galactosidase– bottom pane.

Alternatively, the log intensities of the matching peptides between two conditions can be plotted as a function of each other. Figure 5, shows a scatter plot of the human serum containing 50 fmol vs. that with 100 fmol of alpha gal A injected on column. Statistical analysis was conducted to identify statistically-significant expression differences.

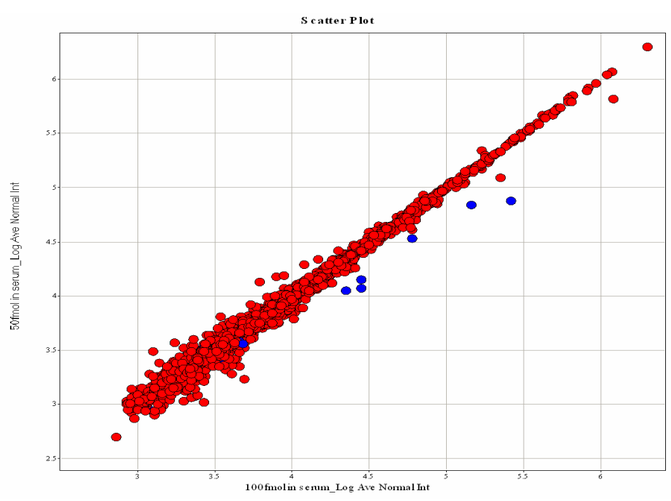

Figure 5. Average peptide intensity ratio for duplicate injections from a 50 fmol sample of alpha galactosidase vs. 100 fmol of alpha galactosidase, both present in a background of human serum. Significant intensity changes determined using a student t-test and the coefficient of variation of the intensity were evaluated and are indicated by the blue circles.

Monitoring Protein Expression changes in Patient Serum

Alpha gal A was administered to a patient over a prolonged period of time and the protein changes on a global level were monitored. Figure 6 shows the probability and expression ratio as a function of protein identity, when comparing condition one (time = 0 hr) vs. condition 6 (time = 12.25 hr). The identified down regulated proteins are colored blue and were selected to construct a fold change profile, which is shown in Figure 7.

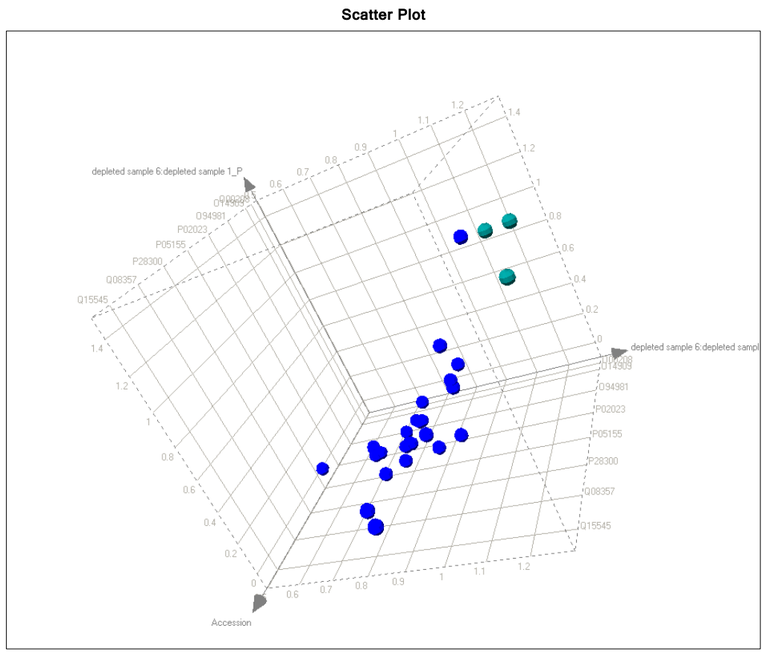

Figure 6. Shows the identified acute phase reactant proteins during alpha galactosidase infusion over time (12 hrs) plotted by expression ratio and probability as a function of accession number for condition one (time = 0 hr) vs. condition six (time = 12.25 hr).

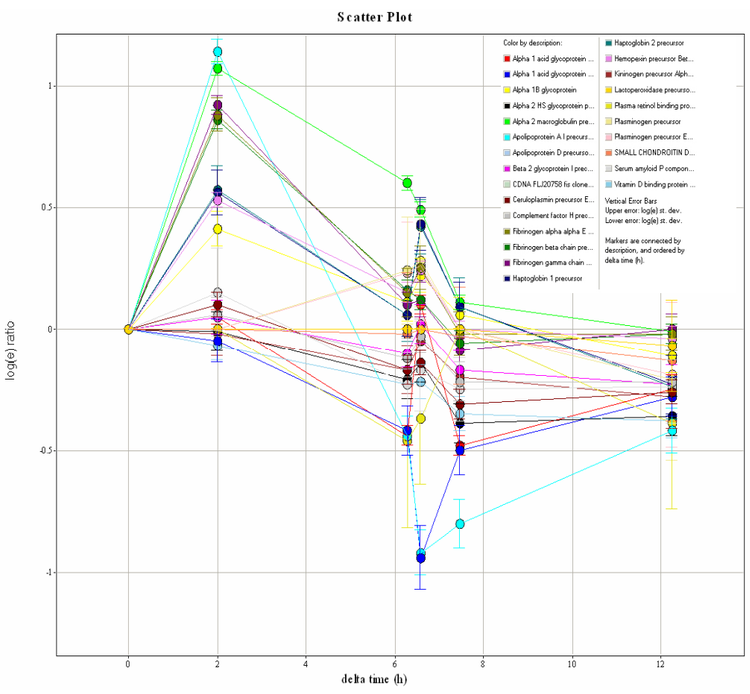

Figure 7. Acute phase plasma protein response as a function of time, illustrating global protein expression monitoring.

Conditions one (time = 0 hr) and two (time = 2 hr), were also analyzed by creating log intensity plots and evaluating the data, based on fold change, including statistical filtering. The colored circles in Figure 8 represent peptides exhibiting a significant change from the control.

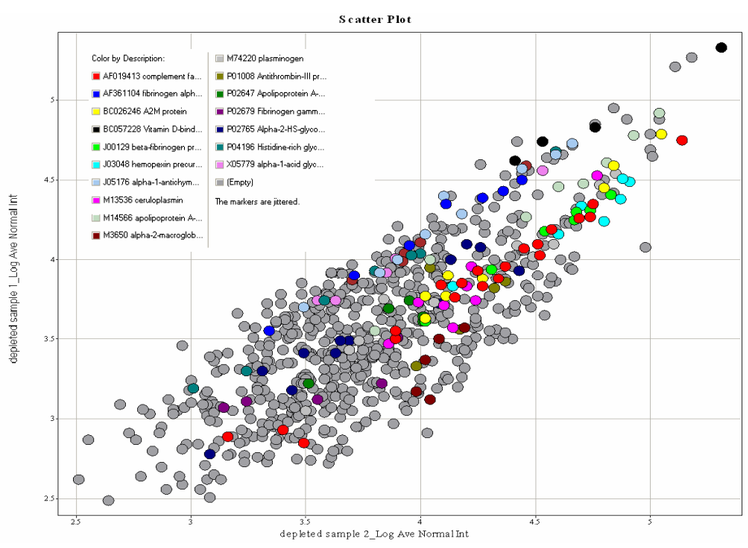

Figure 8. Average peptide intensity ratio for triplicate injections for condition one (time = 0 hr) vs. condition two (time = 2 hr). Significant intensity changes determined by a student t-test and coefficient of variation change in intensity were evaluated and are colored according to the legend above.

CONCLUSIONS

- The data presented shows the feasibility of monitoring the concentration changes of therapeutic proteins in complex serum samples over time
- The results indicate good limits of detection (10fmol on column); whilst limits of quantitation and linearity for alpha gal A have been shown between 10 and 500 fmol injected on column
- The data illustrate the potential of the method for the monitoring of global protein concentration changes across multiple conditions
- Further works is currently ongoing on the identification and quantification of alpha gal A in real patient samples

REFERENCES

1. McKenna *et al.* A Novel Approach to Protein Identification: A Direct Comparison to Traditional Mass Spectrometric Techniques. 52nd meeting of the ASMS. Poster presentation (2004)

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