Monitoring of the Therapeutic Protein, alpha galactosidase A, in Human Serum using Liquid Chromatography Coupled to Electrospray Mass Spectrometry

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Introduction

Fabry disease is an X-linked recessive glycolipid storage disorder that is 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. In male patients this results in characteristic skin lesions (angiokeratomas) over the lower trunk. In addition, patients may show ocular deposits, febrile episodes, and burning pain in the extremities. Death results from renal failure, cardiac or cerebral complications of hypertension or other vascular disease. Administration of recombinant alpha-gal A has been shown to alleviate symptoms of the disease and has been used as a long-term enzyme replacement therapy in patients with a confirmed diagnosis of Fabry's disease.

Investigation and monitoring of circulating levels of alpha gal A can provide information upon the uptake and metabolism of the replacement enzyme and could potentially allow the therapeutic level of the circulating drug to be determined in patient samples. This would allow the dose of administered drug to be adjusted accordingly, to maintain safe, cost effective levels of the treatment.

Here we detail a preliminary investigation into the potential of LC-MS for analyzing tryptically digested samples of human serum, containing spiked levels of recombinant alpha gal A. In this work 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 in human serum from patient samples.

Methods

Sample preparation
The human serum samples and alpha gal A were diluted, and solubilized by incubation at 80ºC 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 2 hr reversed phase gradient (5 to 40% acetonitrile over 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 & Discussion

Initial feasibility experiments were conducted to determine the limits of detection for alpha gal A in the presence of a very complex matrix, i.e. digested human serum. To this extent, 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, prior to performing any quantification is presented in Figure 1.

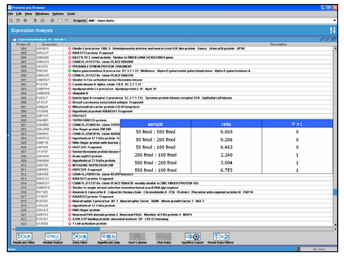


Figure 1. Protein identification based relative quantification of alpha gal A in undepleted human serum. Each sample was analyzed in duplicate. Human serum albumin was specified as the internal standard.

An alternative way of quantification is illustrated in Figure 2, 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

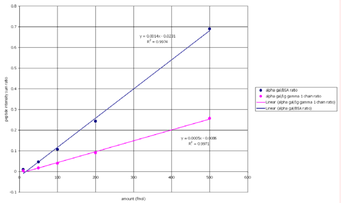


Figure 2. Relative quantification of alpha galactosidase spikes in undepleted digested human serum by using summed intensities of identified peptides from the low-energy data set that replicate across all conditions.

An example of the high-energy fragmentation spectra which led to the identification and the relative quantification of alpha gal A is shown in Figure 3.

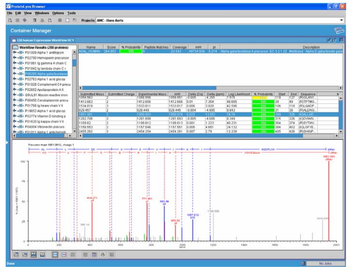


Figure 3. Identification of alpha gal A at the 100fmol level using high-energy fragment ion data

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.

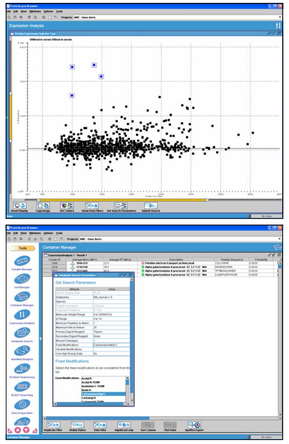


Figure 4. Exact mass retention time browser – upper pane – showing the long intensity ratio of 10 fmol of alpha galactosidase vs. 500 fmol injected on column and the low energy peptide mass finger print search result for 10 fmol of alpha galactosidase– bottom pane.

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 a 50 fmol vs. 100 fmol injection of alpha gal A on column. Statistical analysis was conducted on the data sets to identify expression differences that we deemed significant.

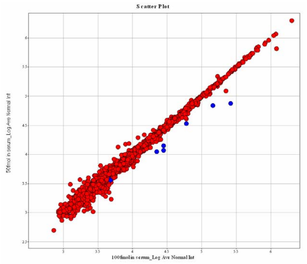


Figure 5. Average peptide intensity ratio for duplicate injections from a 50 fmol sample of alpha galactosidase vs. 100 fmol of alpha galactosidase. Significant intensity changes determined using a student t-test and the coefficient of variation of the intensity were evaluated and are indicated by the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

Monitoring protein expression change 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 turquoise and were selected to construct a fold change profile, which is shown in Figure 7.

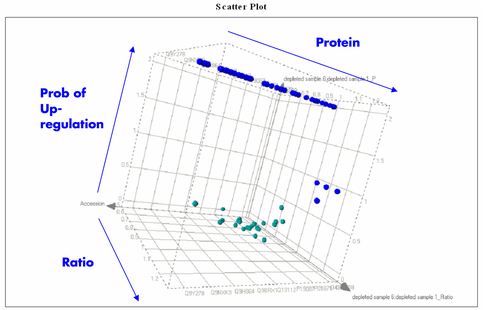


Figure 6. Identification of expressed acute phase reactant proteins generated during alpha galactosidase infusion over a prolonged period of time (12 hrs) plotted by expression ratio and probability as a function of accession number for condition one (time = 0 hr) vs. condition six (time = 12.25 hr).

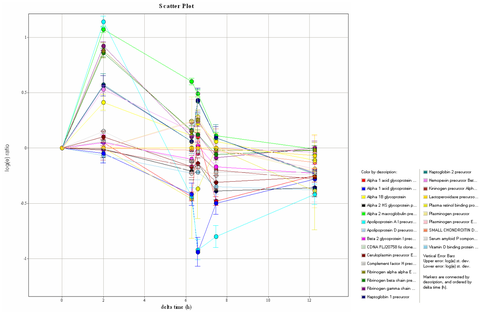


Figure 7. Acute phase plasma protein response as a function of time, illustrating global protein expression monitoring for down regulated identified proteins.

Conditions one (time = 0 hr) and two (time = 2 hr), were also visualized 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.

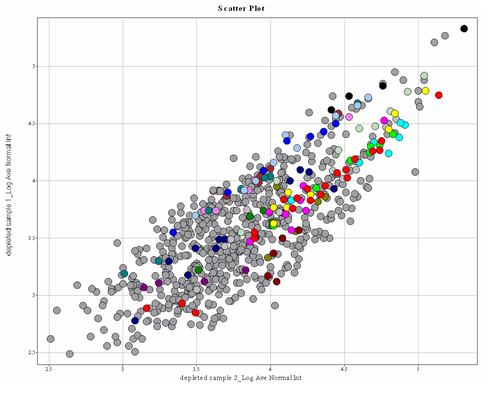
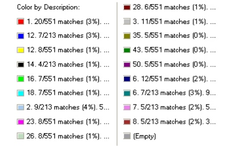


Figure 8. Average peptide intensity ratio for triplicate injections for condition one (time = 0 hr) vs. condition two (time = 2 hr). Significant intensity changes determined by a student t-test and coefficient of variation change in intensity were evaluated and are colored according to the legend opposite.



Conclusions

- The data presented shows the feasibility of monitoring the changes in concentration of therapeutic proteins in complex serum samples over time
- The results indicate good limits of detection and linearity for alpha gal A with 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

Acknowledgement
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Reference
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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