



## EXPERIMENTAL

### Sample preparation

Patient serum samples – control, pre-, and post-treatment – were diluted prior to depletion with an affinity removal system column that removes the 6 or 12 most abundant serum proteins. The depleted samples were denatured with RapiGest™ SF (0.1%), reduced (10-mM DTT), alkylated (10-mM IAA), and enzymatically digested with trypsin, 1:50 (w/w) enzyme/protein ratio. RapiGest SF was removed by the addition of 2  $\mu$ L concentrated HCl, followed by centrifugation, and the supernatant was collected. Samples were diluted with 0.1% formic acid to an appropriate final working concentration prior to analysis.

### Activity measurements

The enzyme activity for chitotriosidase was biochemically determined with 4-methylumbelliferyl  $\beta$ -D-N,N',N''-triacetylchitotriose substrate assay.

### LC/MS<sup>E</sup> conditions

LC/MS<sup>E</sup> quantification experiments were conducted using a 1.5 hr reversed-phase gradient at 300 nL/min (5% to 40% acetonitrile over 90 min) on an Identity<sup>E</sup> High Definition Proteomics™ System, using a nanoACQUITY UPLC® System with a NanoEase™ Atlantis® C<sub>18</sub> 3  $\mu$ m, 75  $\mu$ m x 15 cm nanoscale LC column. Samples were run in triplicate. The Identity<sup>E</sup> System also included a SYNAPT™ MS Mass Spectrometer, which was programmed to step between normal (5 eV) and elevated (15 to 35 eV) collision energies on the gas cell, using a scan time of 1.5 s per function over m/z 50 to 1990.

### LC/MS/MS (MRM) conditions

MRM analysis was performed on a Xevo™ TQ (triple quadrupole) Mass Spectrometer coupled to a nanoACQUITY UPLC System configuration as described above. The gradient consisted in this instance of 1% to 50% acetonitrile in 30 min. The MS1 and MS2 transition windows were 1 Da, the collision energy approximately 15 to 20 eV, and the dwell time was 25 ms.

Two synthetic peptides, containing a <sup>13</sup>C-isotopically labeled arginine and corresponding to T26 (SFTLASSSDTR) and T38 (YPLIQTLR), were obtained from New England Peptide (Gardner, MA) and spiked at a concentration of 5 nM in the affinity depleted plasma samples.

### Identity<sup>E</sup> and Verify<sup>E</sup> quantification informatics

The LC/MS<sup>E</sup> data were initially processed, peak detected, time-aligned, and searched with the use of dedicated algorithms residing with ProteinLynx Global SERVER™ v.2.4, including a protein and peptide ion accounting search algorithm included within the Identity<sup>E</sup> System.

A method to assess the molar and absolute amount of proteins can be employed during the search of the data-independent LC/MS<sup>E</sup> data. It is based on the linear relationship between ESI signal response and the molar concentration of the protein infused. The inclusion of an internal standard of known concentration, therefore, enables a response factor to be calculated, which allows estimation of the concentration of all identified proteins within the sample mixture.

MRM transitions were selected on the basis of the experimentally-derived Identity<sup>E</sup> search results using Verify<sup>E</sup> Software. Only unique, proteotypic MRM transitions were considered. More detail on peptide selection rules is provided in the Results section.

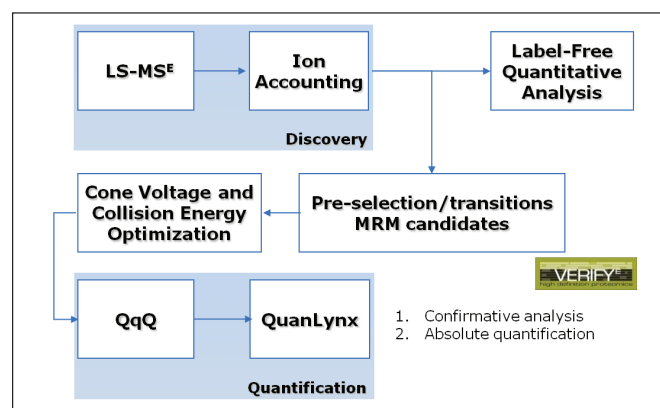


Figure 2. Verify<sup>E</sup> discovery-based workflow.

## RESULTS AND DISCUSSION

### Quantitative LC/MS<sup>E</sup> experiments

The absolute concentration and enzyme activity of chitotriosidase was initially estimated for four patient plasma samples based on the LC/MS<sup>E</sup> experiments and compared with the results of the 4-MU assay, as shown in Figure 3. In two of the patients' samples (C and E), the methods were not in agreement and it could be concluded that the LC/MS-based method underestimates the enzyme concentration. Interestingly, however, these samples have a relatively high suggested activity based on the 4-MU assay substrate experiments.

Further validation of the identification and quantitative results obtained through the LC/MS<sup>E</sup> experiments was achieved through quantitative MRM-based triple quadrupole LC/MS/MS experiments. Based on the qualitative LC/MS<sup>E</sup> results, the best proteotypic peptides of chitotriosidase and associated MRM parameters were determined, as shown in Figure 4. In short, C-, M-, and N-terminal Q-containing peptides were excluded. Furthermore, only multiply-charged precursor ions and singly-charged product ions were considered, as well as peptides that had a certain amino acid sequence length and product ions within a given mass range.

Ultimately, the two most suitable peptides and two transitions per peptide were selected based on precursor and product ion intensity from the remaining MRM transitions.

The two best chitotriosidase MRM candidate peptides were found to be SFTLASSSDTR and YPLIQTLR, as shown in Figure 5. Verify<sup>E</sup> Software was used to automatically generate MRM methods for the Xevo TQ Mass Spectrometer. Moreover, <sup>13</sup>C-isotopically labeled arginine variants of the peptides of interest were synthesized and used as internal standards for absolute quantification.

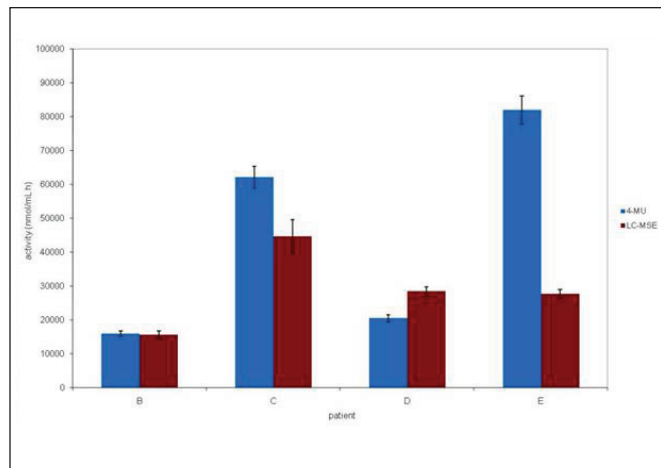


Figure 3. Plasma chitotriosidase activity accessed by means of 4-MU substrate assay (blue) and absolute quantification by means of LC/MS<sup>E</sup> (red).

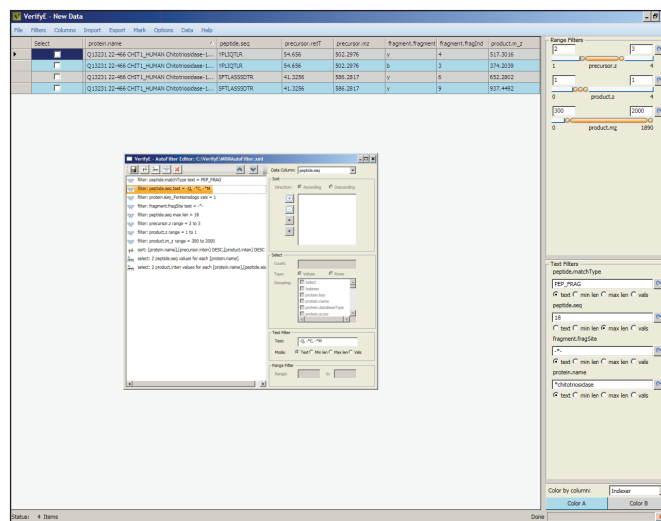


Figure 4. Verify<sup>E</sup> Software browser and proteotypic MRM selection editor/results filter.

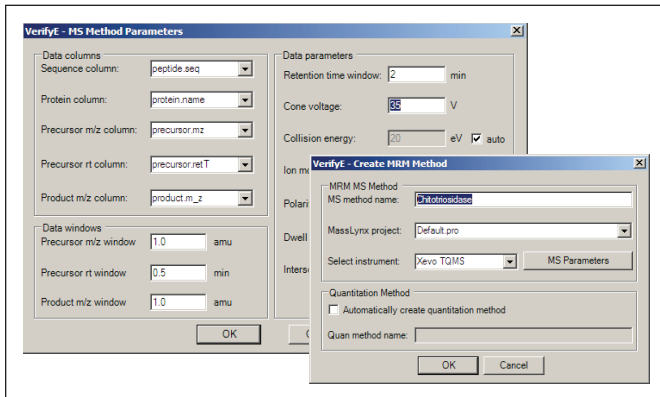


Figure 5. Verify<sup>E</sup> Software MRM method creator for the Xevo TQ Mass Spectrometer.

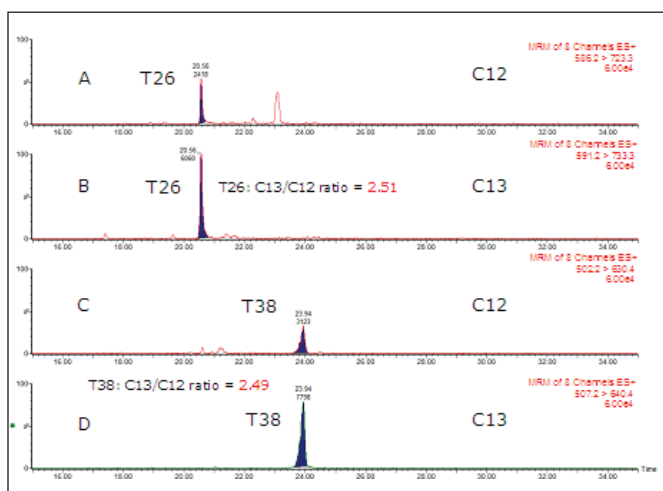


Figure 6. MRM chromatograms for SFTLASSSDTR (T26), YPLIQTLR, (T38), and <sup>13</sup>C-isotopically labeled internal standards.

The Verify<sup>E</sup> Software MS method editor includes all typical MRM parameter settings, such as cone voltage, collision energy, dwell times, and interscan delays, but also includes the required peptide characteristics for successful and robust proteotypic peptide MRM transitions.

The obtained LC/MRM-MS/MS chromatograms of interest are shown in Figure 6. As expected, the <sup>13</sup>C-isotopically labeled internal standards co-elute with the peptides of interest and allow for the absolute concentration calculation based on the <sup>13</sup>C/<sup>12</sup>C ratio.

Next, the calculated peptide concentrations were averaged and converted into a chitotriosidase enzyme activity, as shown in Figure 7.

All applied activity measurement methods were in good agreement for the sera analysis of patients B and D. However, the results seem to imply that the 4-MU assay substrate experiments overestimate the chitotriosidase enzyme activity, rather than the LC/MS<sup>E</sup>-based method underestimating it. It is of significant interest that there are differences in specific activity among individual Gaucher patients, i.e., the hydrolytic activity towards substrate per mole of enzyme is not constant.

This has led to the development of a new biochemical assay. With this new method, the enzyme activity of chitotriosidase is on average approximately 10% to 20% lower; however patient-dependent, providing values are very close to the concentration LC/MS-based methods.

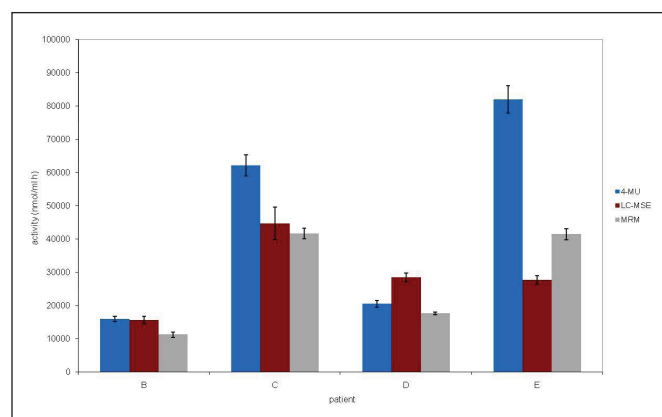


Figure 7. Plasma chitotriosidase activity accessed by means of 4-MU substrate assay (blue), absolute quantification by means of LC/MS<sup>E</sup> (red), and absolute quantification by means of triple quadrupole MRM LC/MS/MS (grey).



## CONCLUSIONS

- LC/MS<sup>E</sup> experiments were used to identify and quantify the known Gaucher disease marker, chitotriosidase, in patient sera.
- LC/MRM-MS/MS experiments were derived from these initial experiments using Verify<sup>E</sup> Software.
- This allowed a robust MRM method to be established that could also be configured to look at other protein analytes in patient sera, providing a cost-effective alternative compared to traditional biochemical assays.
- The activity of chitotriosidase was compared to a fluorescence-based biochemical assay; and for two patients there was good agreement between the MS and biochemical assays.
- Two patients showed distinct differences in the levels of chitotriosidase due to isoform and patient gene variants.
- Genomic sequencing was used and subsequently resulted in development of a new and improved fluorescence assay.

## References:

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2. Hollak CEM, van Weely S, van Oers MHJ, Aerts JMFG, Marked elevation of plasma chitotriosidase activity, a novel hallmark of Gaucher disease, *J. Clin. Invest.* 93 (1994) 1288-1292.
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4. Silva JC, Gorenstein MV, Li GZ, Vissers JPC, Geromanos SJ. Absolute quantification of proteins by LCMS<sup>E</sup>, a virtue of parallel MS acquisition, *Mol. Cell. Proteomics* 5 (2006) 144-156.

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**Waters Corporation**  
34 Maple Street  
Milford, MA 01757 U.S.A.  
T: 1 508 478 2000  
F: 1 508 872 1990  
[www.waters.com](http://www.waters.com)

