Assay for the Simultaneous Measurement of Multiple Amyloid Beta Isoforms

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Introduction

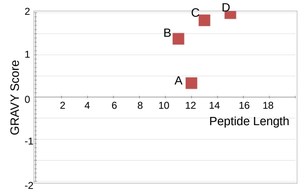
Alzheimer's disease (AD) remains the most common form of dementia and evidence suggests amyloid-β (Abeta) plays a central role in AD pathogenesis. Three major forms of the Abeta peptide are produced, which are Abeta38, Abeta40 and Abeta42. The most abundant form is Abeta40. Though less abundant, Abeta42 is more hydrophobic and prone to form toxic oligomers, and is the species of particular importance in early plaque formation. Thus, the length of the hydrophobic C-terminal seems to be critical for the oligomerization and neurotoxicity of the Abeta peptide. Here we investigated the physiology of Abeta isoforms in humans by simultaneously measuring the isoforms' *in vivo* metabolic rates. Traditionally, Abeta isoforms are purified by immunoprecipitation in a serial fashion for quantitation using LC/MS. Recently, we developed a way to quantitate all isoforms from the same sample, including the ones to which there are no antibodies yet. This is done by analyzing the terminal peptides after protease digestion of the immunoprecipitates.

Method Outline

- The Abeta standards were produced by feeding a cell line that over-produces Abeta using $^{13}C_6$ labeled leucine to obtain media labeled to 0, 1.25, 2.5, 5, 10 and 20%.
- Human volunteers were intravenously given $^{13}C_6$ labeled leucine over nine hours and 6 ml samples of CSF were obtained through a lumbar catheter every hour.
- Immunoprecipitations using a mid-domain antibody HJ5.1 and 1 ml of cell culture media standard and 1 ml human CSF were performed at RT for 2 hours followed by elution in formic acid .
- Speed-vacuum dried Abeta was resuspended and digested with proteases.
- Digests were separated and detected by LC/MS and examined for the incorporation of $^{13}C_6$ labeled leucine.
- The percent of $^{13}C_6$ leucine incorporated into Ab was determined by area ratios from MRM experiments.

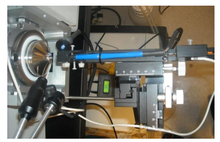
DAEFRHDSGYEVHHQ KLVFFAEDVGSN KGAIIGLMVGGVVIA
KGAIIGLMVGGVV
KGAIIGLMVGG

Peptide	Gravy Score
(A). KLVFFAEDVGSN	0.33
(B). KGAIIGLMVGG	1.38
(C). KGAIIGLMVGGVV	1.82
(D). KGAIIGLMVGGVVIA	1.99

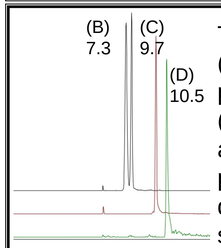


The figure shows the sequence of Abeta and the peptides generated after processing with Lys-N Metalloendopeptidase. Due to the high GRAVY scores of the peptides (extremely high hydrophobicity), the C-terminal fragments generated upon Lys-N cleavage of Abeta, i.e. Ab28-x, were naturally not well suited for analysis by HPLC-MS/MS until now.

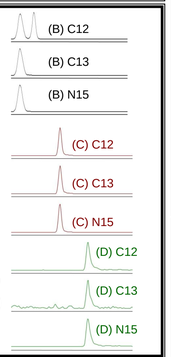
Results

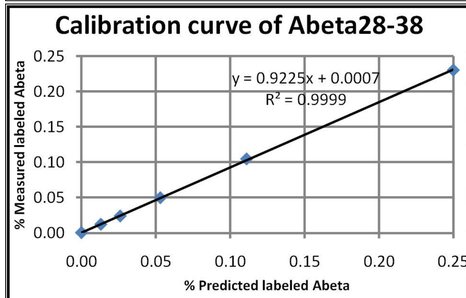


The Abeta peptides elute poorly from columns and a column heater (Blue color), Phoenix S & T, Inc. Chester, PA) was used to heat the column to 80 °C. This helped to improve the peak shapes and reduced excessive back pressure.

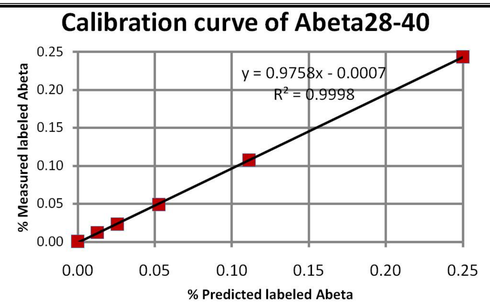


The chromatographic trace (left) of the C-terminal peptides for (B) Ab29-38 (black), (C) Ab29-40 (brown) and (D) Ab29-42 (green). The peptides were separated and detected from the same sample. The figure to the right shows the detection of the unlabeled Abeta (top), newly synthesized C13-labeled Abeta (middle) and N15 synthetic Abeta internal standard.

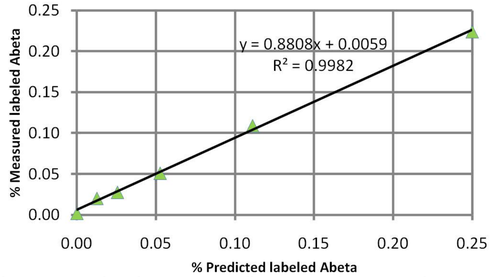




The standard curve shows Abeta38 quantitation. The percentage measured Abeta was calculated from peak area of labeled and unlabeled Abeta28-38 specific chromatographic peaks.

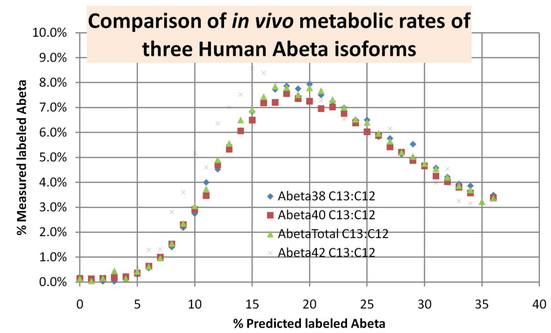


The standard curve figure above shows the quantitation of Abeta40 using the isoform specific peptide from the $^{13}C_6$-labeled Abeta media. The standard curve of the percentage measured vs. predicted ratios were plotted and indicated high linear correlation (R2= 0.99) and reproducibility and demonstrated an LOD <1% $^{13}C_6$ labeling.



The standard curve figure above shows the quantitation of Abeta42 using the isoform specific peptide from the $^{13}C_6$-labeled media.

This quantitation demonstrated an *in vivo* labeling experiment in humans. Participants were admitted to the Washington University GCRC. A $^{13}C_6$-labeled leucine solution was infused through an IV for 9 hours. Six ml of CSF was obtained through the lumbar catheter every hour. All 37 sampled CSF were processed as described with immunoprecipitation of Abeta digested with Metalloendopeptidase (Lys-N), and analyzed with a TSQ Vantage (ThermoFisher, San Jose, CA), coupled to an Ultra NanoLC-2D (Eksigent Technologies, Dublin, CA). The percent labeled Abeta at each hour time point were determined from Abeta isoforms, which were all measured from the same sample.



The figure above demonstrates that the metabolism of the Abeta isoforms could be monitored and their production and clearance rates calculated. It shows the metabolism time profile of Abeta38 (blue), Abeta40 (red) and Abeta42 (purple). It is advantageous to measure all the Abeta isoforms in the same sample rather than using the traditional method of isolating individual isoforms with specific antibodies in a sequential fashion. Measuring the C-terminal peptide unique to all the isoforms from the same sample ensure that non-specific interaction with antibody is eliminated. Using the stable isotope kinetics, we can compare the metabolism of the different isoforms of Abeta and consider them as a diagnostic tool for AD. In this CSF set of samples analyzed above, the synthesis and clearance curves for Abeta38, ABeta40 and Abeta42 were similar. The curves demonstrate a rapid production and clearance volunteer. This allows us to directly test different AD hypotheses, including whether Abeta42 production rate will be increased relative to Abeta40 in familial mutation carriers. It is thought that the Abeta peptide ratio 42/40 correlates with amounts of phospho-Tau in patients with AD. The simultaneous measurement of these isoforms will demonstrate whether the 42/40 ratio and / or the metabolic rates determination are relevant for an early diagnosis of AD.

Conclusions

We can now measure *in vivo* Abeta38, Abeta40 and Abeta42 production and clearance rates simultaneously. The pathophysiology of AD can be directly measured in humans with CSF Aβ metabolism studies. Aβ metabolism results will allow for a clearer picture of the pathophysiology of the brain in AD. This information will likely lead to improved diagnostic testing and more precise pharmacodynamic testing of disease modifying treatments.

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