



Quantitative LC-MS/MS Assay Development for Alzheimer's Disease Serum Protein Biomarkers Using a Library of Proteolytic MS Spectra

Authors

Bob Xiong, Kojo Abdul-Hadi, Lily Li

Introduction

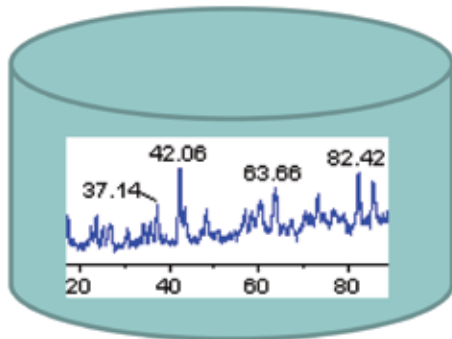
Developing a quantitative LC-MS/MS assay for a serum protein biomarker typically requires a protein standard upfront to identify and prioritize proteolytic peptides, which then serve as proxy analytes for the underlying protein in a quantitative assay. However, protein standards may not be readily available, especially for less well-studied proteins. The lack of protein standards, therefore, presents a challenge for assay development because the selection of peptides becomes difficult without protein standards. As an alternative to peptides established from protein standards, predicted peptides can be explored for assay development. We present here a novel approach to identifying specific peptides for proteins of interest by comparing the predicted chromatographic retention time with the observed retention time in a MS spectral library. Retention time-matched putative peptides are then utilized for quantitative assay development. As a proof of concept, the tryptic peptides IPTTFENGR of Apo-D and SGTDVDAANLR of CASP-3 (Alzheimer's disease biomarkers) were shown as a positive and negative example, respectively.



Material and Method

Human serum was depleted using MARS spin column (Agilent, Hu-6) to remove the six most abundant proteins including albumin, IgG, IgA, transferrin, antitrypsin, and haptoglobin. Depleted serum was digested with trypsin (Roche, sequencing grade enzyme) followed by protein precipitation. Extracted tryptic peptides were loaded onto a LC-MS system (Shimadzu coupled with API 5000 or QSTAR). Q1 MS (API 5000) and TOF-MS (QSTAR) scans were collected under a linear LC gradient condition (0.25% B/min gradient rate, i.e. 5-50% mobile phase B containing 50:50/MeOH:Acetonitrile, 0.1% formic acid, for 180 minutes) on a RP column (Synergi, Polar-RP, 250x4.6mm). Raw data were processed using Trans-Proteomic Pipeline (TPP, available from Seattle Proteome Center) to extract peaks in each scan (2 seconds). Serum-specific peaks were obtained by subtracting peaks in the water-based control digest from those in the serum-based digest using predefined values for retention time tolerance, mass tolerance, and intensity differential. Putative peptide peaks (i.e. peaks of certain mass-to-charge ratio or m/z) were identified by comparing the predicted and observed retention time. Synthetic peptides were used to confirm the identified proteolytic peptides. Stable isotope labeled synthetic peptides with discernable mass shift were used to serve as internal standard and calibration standard for quantitative assay development.

Library construction of MS spectra of human serum tryptic peptides



Human serum MS peak Library

- Tryptic peptides (MARS depleted serum)
- LC-MS full scan of tryptic peptides
- Background-subtracted peak library (six-million peaks)
 - threshold of peak intensity (100,000 cps)
 - intensity fold difference (10-fold stronger than background)
 - mass tolerance (0.7 amu)
 - retention time tolerance (18 sec)
 - peptide containing M, C, or N-terminal Q excluded



Filtered Tryptic Peptides of Apo-D and CASP-3

Apolipoprotein D (Apo-D, P05090)

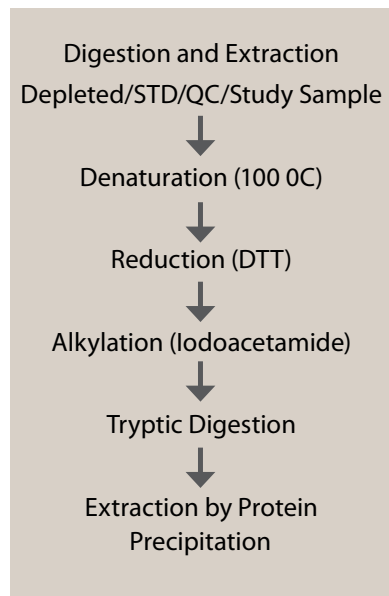
MVMLLLLLSALAGLFGAAEGQAFHLGKCPNPPVQENFDVN
 KYLGRWYEIEKIPPTTFENGRCIQANYSLMENGKIKVLNQE
 LRADGTVNQIEGEATPVNLTEPAKLEVKFSWFMPSPAPYWI
 LATDYENYALVYSCTCIIQLFHVDFAWILARNPNLPPETV
 DSLKNILTSNNIDVKKMTVTDQVNCPKLS

Caspase-3 (CASP-3, P05090)

MENTENSVDSKSIKNLEPKIIHGSESMDSGISLDNSYKMD
 YPEMGLCIIINNKNFHKSTGMTSRSGTDVDAANLRETFRN
 LKYEVRNKNDLTREEIVELMRDVSKEDHSKRSSFVCVLLS
 HGEEGIIFGTNGPVDLKKITNFFRGDRCSRSLTGPKPLFII
 QACRGTELDGCIETDSGVDDDMACHKI PVEADFLYAYSTA
 PGYYSWRNSKDGSWFIQSLCAMLKQYADKLEFMHILTRVN
 RKVATEFESFSFDATFHAQKQIPCIVSMLTKELYFYH

Sample Preparation

- For human serum spectral library construction, 14 μ L of healthy human serum (pooled, BioChemed) was depleted (MARS) to remove the top six most abundant proteins. The depleted sample was concentrated by 5K MWCO filter device.
- For quantitative assay, calibration standard (STD) and QC samples were prepared by adding stable isotope labeled peptide in 25-fold water diluted human serum. Study samples were diluted 25-fold with water.
- All samples were then processed for protein digestion and peptide extraction.





MS Method

LC-TOF MS and LC-MS

Scans of tryptic peptides were achieved using LC-TOF MS on QSTAR® and LC-MS on API 5000™

HPLC Separation

- Column – Applied Biosystems Polar RP (250 x 4.6 mm)
- Mobile Phase A: 95/5, H₂O/ACN (0.1% formic acid)
Mobile Phase B: 50/50 MeOH/ACN (0.1% formic acid)
- Injection Volume: 20 µL
- Gradient

TIME (min)	%B
0.1	5
10.0	5
190.0	50
191.0	90
210.0	90
250.0	5

QSTAR® XL (Applied Biosystems/Sciex)

- TOF Q1 Full Scan: *m/z* 100 – 2500
- Declustering Potential: 60 V

AB Sciex Sciex API 5000™

- Q1 MS Scan: *m/z* 100 – 1250
- Declustering Potential: 75 V

LC-MS/MS

HPLC Separation

- Column – Varian MetaSil C18 (50 x 2.0 mm)
- Column Heater: 40°C
- Mobile Phase A: 95/5, H₂O/ACN (0.1% formic acid)
Mobile Phase B: 50/50 MeOH/ACN (0.1% formic acid)
- Injection Volume: 10 µL
- Gradient

Time (min)	%B	Flow Rate (ml/min)
0.00	0	0.6
2.00	25	0.6
2.75	90	0.6
3.50	90	0.6
3.60	0	0.6
4.50	0	0.6

Mass Spectrometry

- API 5000™ (Applied Biosystems/Sciex)
- MRM: six transitions (see MRM Transitions table)
- Declustering Potential: 75 V
- Collision Energy: analyte dependent (see MRM Transitions table)



MS Method (continued)

MRM Transitions

Peptide ID	Peptide Sequence	Function	MW (Da)	Precursor m/z	Sequence Ion m/z	CE (eV)
P05090_L	IPTTFENGR	Endogenous	1034.2	$M+2H]^{+2}$ 518.1	824.6	30
P05090_H1	IPTTFENGR*	Calibration Standard	1044.2	$M+2H]^{+2}$ 523.1	834.6	30
P05090_H2	IP*TTFENGR*	Internal Standard	1050.4	$M+2H]^{+2}$ 526.1	834.6	30
P42574_L	SGTDVDAANLR	Endogenous	1118.2	$M+2H]^{+2}$ 560.1	758.4	25
P42574_H1	SGTDVDAANLR*	Calibration Standard	1128.0	$M+2H]^{+2}$ 565.0	768.5	25
P42574_H2	SGTDVDAANL*R*	Internal Standard	35.8	$M+2H]^{+2}$ 568.9	775.6	25



Results and Discussion

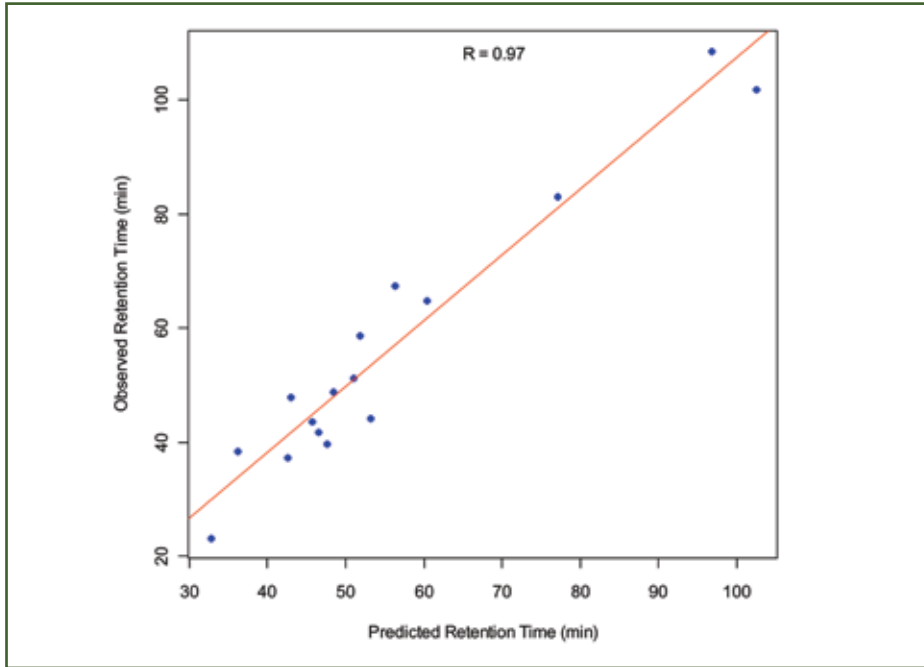
Predicted and Observed Retention Time for 16 Synthetic Peptides

ID	Peptide		Pred. (min)	Obs. (min)	Diff. (min)
	Length (A.A)				
1	7		32.7	23.17	9.53
2	10		42.5	37.34	5.16
3	13		36.2	38.55	-2.35
4	8		47.6	39.84	7.76
5	9		46.4	41.82	4.58
6	9		45.7	43.75	1.95
7	9		53.1	44.15	8.95
8	13		42.9	48.03	-5.13
9	13		48.4	48.81	-0.41
10	11		50.9	51.39	-0.49
11	9		51.7	58.69	-6.99
12	13		60.3	64.92	-4.62
13	7		56.3	67.52	-11.22
14	10		77.1	83.07	-5.97
15	20		102.4	101.99	0.41
16	16		96.7	108.7	-12

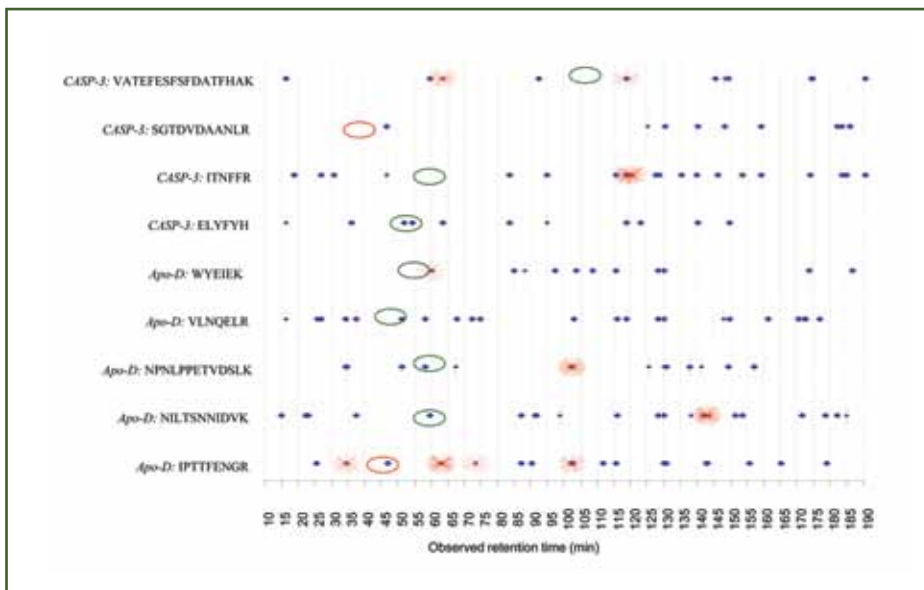


Results and Discussion (continued)

Correlation of Predicted and Observed Retention Time for 16 Synthetic Peptides



Sunflowerplot of Observed Retention Time for Putative Tryptic Peptides of Apo-D and CASP-3



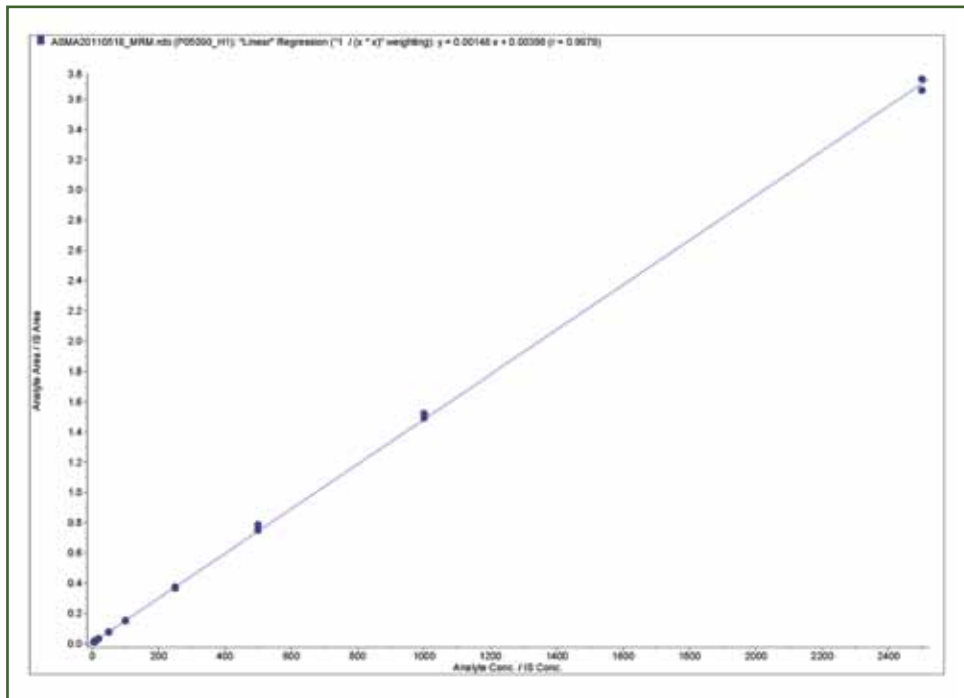


Results and Discussion (continued)

Predicted Retention Time for Tryptic Peptides of Apo-D and CASP-3

Protein	Peptide Sequence	Predicted RT (min)	Observed RT (±6 min)	PTested with Synthetic Peptide
Apo-D	IPTTFENGR	45.7	✓	✓
	NILTSNNIDVK	57.6	✓	
	NPNLPPETVDSLK	57.4	✓	
	VLNQELR	46.8	✓	
	WYEIEK	55	✓	
CASP-3	ELYFYH	55.2	✓	
	ITNFFR	58.9		
	SGTDVDAANLR	37.5		✓
	VATEFESFSDFATFHAK	105.8		

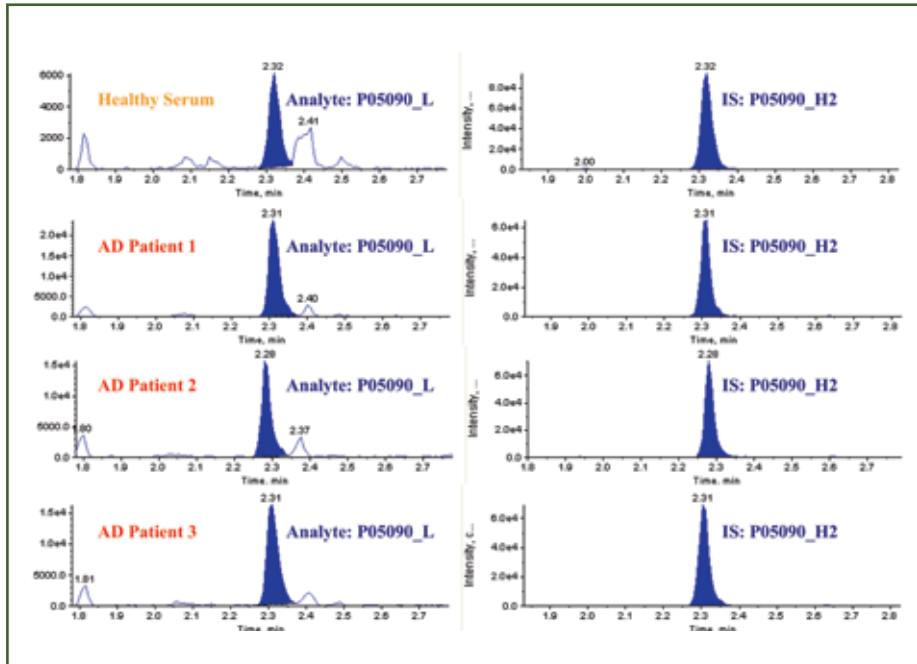
Standard Curve for the Apo-D Peptide IPTTFENGR (P05090_H1)



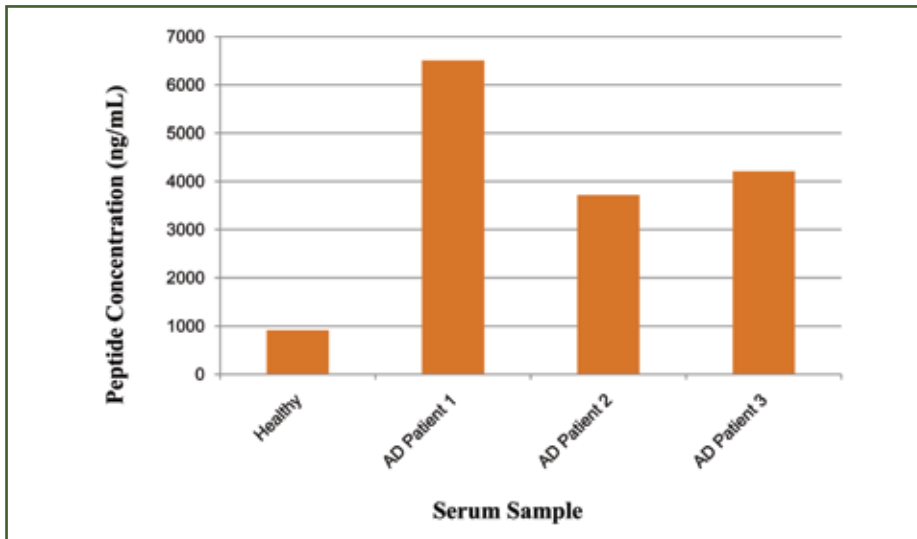


Results and Discussion (continued)

Chromatograms for the Apo-D Peptide IPTTFENGR (P05090_L and P05090_H2) in Test Samples



Apo-D Peptide IPTTFENGR (P05090_L)





Results and Discussion (continued)

Library of MS spectra of human serum tryptic peptides

Over six millions of unique peaks were identified in the tryptic digest of human serum using the criteria described in the current study (i.e. peak intensity counts ≥ 100000 cps, retention time tolerance between background and serum = 18 seconds, peak intensity differential ≥ 10 -fold if retention time difference between background and serum < 18 seconds).

Correlation of predicted and observed retention time

Using a modified version of a published method for predicting the retention time of 16 synthetic peptides, a correlation of 0.97 between the predicted and observed retention time was obtained, which was consistent with the reported value (0.94) based on 108 tryptic peptides (Tripet, B. et. al. 2007).

Tryptic peptide IPTTFENGR of Apo-D was more abundant in AD patients

The predicted retention time for the tryptic peptide IPTTFENGR ($[M+2H]^{+2}$, m/z 518.1 at 45.7 min) of Apo-D matched the retention time of the MS peak of a similar mass-to-charge ratio (m/z 518.0 at 47.1 min). Synthetic peptide confirmed the identity of the tryptic peptide. When monitored in healthy serum (pooled, BioChemed) and three AD patients, the concentration of the peptide was significantly higher in the latter, which was expected of the Apo-D protein in AD patients.

Tryptic peptide SGTDVDAANLR of CASP-3 was not detected as predicted

The predicted retention time for the tryptic peptide SGTDVDAANLR ($[M+2H]^{+2}$, m/z 560.1 at 37.5 min) of CASP-3 did not match the retention time of any MS peaks of similar mass-to-charge ratio in the library. Synthetic peptide failed to confirm the tryptic peptide in serum and the peptide was not detected in either healthy serum or AD patients.



Conclusion

With the construction of a library of MS spectra for tryptic peptides of human serum proteins and protein sequence database, it is feasible to identify and prioritize peptides for quantitative LC-MS/MS assay development. The ability to develop quantitative assays without using protein standards represents an exciting opportunity to explore quantitative LC-MS/MS methods for serum protein biomarkers that are poorly studied due to lack of protein standards.

Reference

Tripet, B., Renuka Jayadev, M., Blow, D., Nguyen, C., Hodges, R. S. and Cios, K. J. (2007) Proteomic data mining using predicted peptide chromatographic retention times. *Int. J. Bioinformatics Research and Applications*, 3(4): 431-45.