

Demonstrating Instability of Peptide Biomarkers in Human Blood Samples Using Time-course Mass Spectrometry

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Introduction

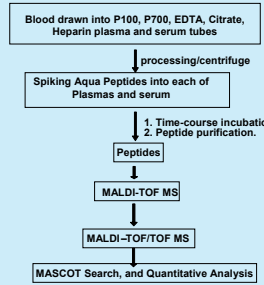
Human serum and plasma, with rich information about disease status, have recently garnered extensive research activities in proteomics-based biomarker discovery (1,2). Protein and peptide biomarkers in blood samples have been discovered in cancers (3-6), and other diseases (7-8).

Yet, intrinsic proteolysis causes general variability and instability of serum and plasma protein samples (9). During blood collection and sample preparation, proteins are digested by proteases, generating *ex vivo* peptides (10). The stability of peptide biomarkers is still questionable. In this study, an AQUA (absolute quantification) peptide, a synthesized peptide incorporating a stable isotopically labeled residue, was used as a biomarker, by spiking a known quantity into a blood sample. Its sequence, therefore, its chemical and biochemical properties, is identical to its native counterpart while its molecular mass can easily be distinguished from its native counterpart due to the isotopic-labeled residue. To monitor the stability of a biomarker in the blood samples, we incubated the spiked samples for a series of time periods up to 72 hours, and subsequently measured the AQUA peptide using MALDI-TOF MS. The results indicated that the spiked AQUA peptides and thus other potential peptide biomarkers, are decreasing over time. The generated smaller peptides increase during early incubation, and decrease later, suggesting a sequential degradation by the intrinsic peptidases in the blood samples. As AQUA peptides were initially intended to be used as an internal control for absolute quantification of proteins or peptides, the instability of AQUA peptides suggests that a careful control and selection of blood samples is required for accurate quantitation.

Experimental Procedures

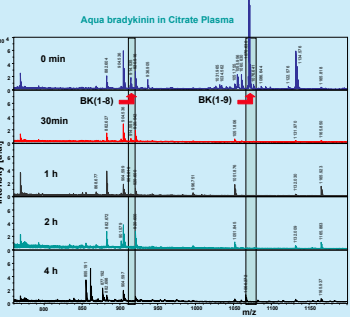


- BD™ P100™ tube contains:
- A broad-spectrum protease inhibitor cocktail
 - K₂EDTA as anti-coagulant.
 - A mechanical separator, which provides a robust barrier between plasma and cells.
- BD™ P700™ tube:
- Contains a proprietary Dipeptidyl Peptidase IV (DPP-IV) protease inhibitor.
 - Provides immediate stabilization of GLP-1.
 - K₂EDTA as anti-coagulant.

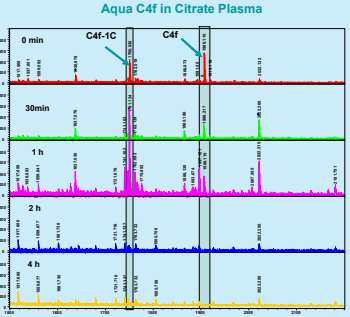


- Spiking an AQUA peptide into serum, EDTA, heparin, citrate, BD™ P100 and P700 samples collected from the same subject.
- Monitoring peptide biomarker by time-course incubation of samples, followed by MALDI-TOF MS (Ultraflex II, Bruker-Datomics) analysis.
- Monitoring the AQUA peptide intensity as an indicator of the stability of the sample proteome.
- Sequencing by TOF/TOF MS and monitor peptide fragments, which provides mechanistic and dynamic information of proteolytic degradation.

Results



- Bradykinin, BK(1-9), is a potential biomarker of cardiovascular disease (9).
- Spiked AQUA BK (1-9) (at 107.64 m/z) is no longer detectable after incubation for 30 min.
- Its fragment through truncation of one C-terminal residue AQUA BK(1-8), can be observed at "Time 0", suggesting extreme instability of the BK.
- Similar degradation of the BK was also observed in serum and Heparin plasma.

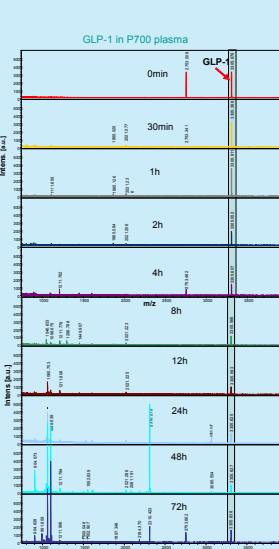


- C4f is the primary peptide generated, both *in vivo* and *ex vivo*, from Complement Component 4.
- The intensity of AQUA C4f (1906.15 m/z) decreases over time, and essentially disappears after 2 hr incubation in citrated plasmas.
- Its fragment, C4f-1C: the peptide truncated by one residue at the C-Terminus can be also observed at "Time 0", increases over time up to 1 hour, and then decreases thereafter.
- Similar degradation of the AQUA C4f was observed in serum, and Heparin and EDTA plasmas.

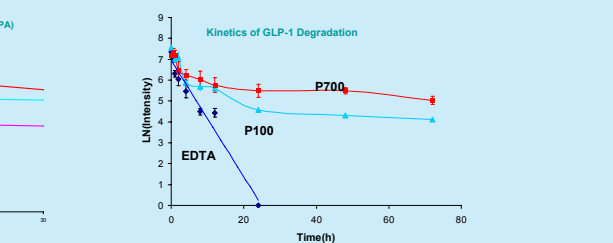
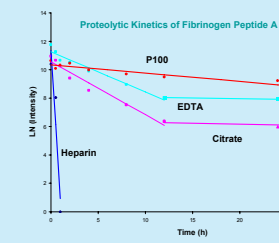
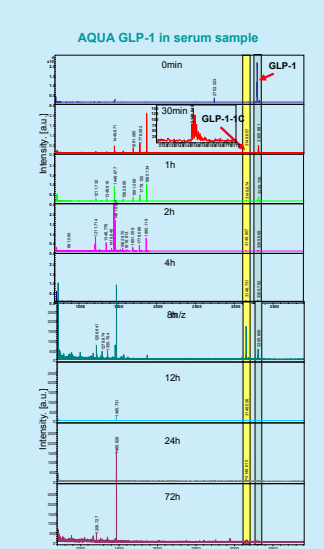
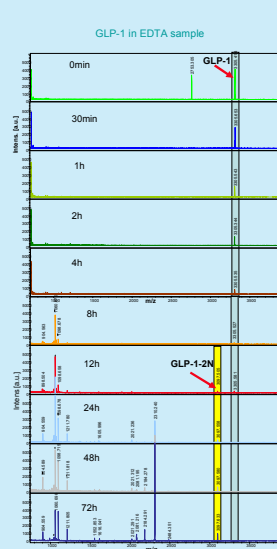
Half-life of AQUA Peptides in blood samples*

Peptide	T _{1/2} (h)	Serum	Heparin	Citrate	EDTA	P100	P700
FPA	T ₁	ND	6.64	1.86	2.46	11.85	ND
	T ₂	ND	ND	58.97	64.18	ND	ND
GLP-1	T ₁	0.35	ND	1.01	9.53	16.35	25.86
	T ₂	ND	ND	ND	15.79	ND	ND
C4f	T ₁	ND	0.31	0.34	1.96	2.157	ND
	T ₂	-0.38	-0.38	-0.38	8.7	7.16	ND
BK (1-9)	T ₁	ND	ND	ND	2.46	ND	ND
	T ₂	ND	ND	ND	1.86	ND	ND

- *Footnote: ND = not observed under the experimental conditions, ND = not determined.
- T₁ represents the half-time of the first, fast degradation, and T₂ represents the half-life of the second, slower degradation.
 - The same peptide displays different stabilities in different samples:
 - Half life varies among the samples.
 - Half life varies along the incubation time, which may be reflected by mechanistic changes.
 - P100 provides benefit for a general stabilization of the peptides tested in this study.
 - P700 provides the best stability for GLP-1.



- GLP-1, at 3305.68 (m/z), is a peptide biomarker of Type II diabetes and other metabolic diseases.
- A decrease in peptide intensity was observed over time in both serum and plasma.
- GLP-1 is no longer detectable after incubation for 12 hours in serum, 24 hours in EDTA.
- The peptide was still detected after incubation for 96 hours in P700 (shown above), and 72 hours in P100 samples (data not shown).
- Stability with record to sample for GLP-1 is in the order: P700 > P100 > EDTA > Heparin - Citrate > Serum.
- Digestion of this peptide was observed by truncation of both the N-terminal and the C-terminal residues.
- GLP-1-1C, shortened by one C-terminal residue at 3148.67 m/z can be seen in serum.
- GLP-1-2N, shortened by two N-terminal residues at 3097.55 m/z, can be seen in EDTA. This peptide is generated likely by DPP-IV activity. This peptide was not detected in P700 sample, suggesting that DPP-IV is effectively inhibited in P700 samples.
- The concentrations of both shortened peptides, when detectable, increased during early incubation, then decrease as a function of time, typical of a processive or sequential enzymatic reactions.



- A linear relationship of Ln (Intensity) versus reaction time is observed:
 - Indicates that the degradation of the peptide follows a first-order like reaction.
 - The slope indicates an apparent rate constant of the degradation.
 - The apparent half-life of the peptide can be determined.
- In the Heparin sample, the AQUA FPA degradation is very fast; it was no longer detectable after 30 min incubation. Degradation of the peptide in serum is even faster than in heparin.
- In EDTA and Citrate plasmas, two essentially linear phases are observed:
 - Indicating a fast degradation mechanism at early time, followed by a slower mechanism.
 - The later slow degradation phase may be caused by feedback inhibition by the proteolytic product, or other unidentified mechanistic changes.
- In P100, AQUA peptides are stabilized by protease inhibitors:
 - The fast phase of degradation was not observed, suggesting an inhibition on the early phase.
 - The stability of peptides is essentially in the order: P100 > EDTA > Citrate > Heparin > Serum.
 - P700 sample provides the best stabilization of GLP-1.

Conclusions

- Intrinsic peptidase activity causes peptide variability and instability by truncation from both ends of a peptide biomarker.
- Protease and peptidase inhibitors appear to improve peptide stability.
- Peptide biomarkers, are in general, in unstable serum and common plasma samples.
- Peptide digestion caused by intrinsic peptidases follows a first-order consecutive reaction:
 - Ln(Con.) versus reaction time is a linear relationship.
 - Intrinsic peptidases shows substrate specificity.
- Overall, P100 displays a general stabilization of peptide biomarkers:
 - In general, peptide stability is in the order: P100 > EDTA > Citrate and Heparin > serum.
 - P100 may provides a more robust sample for peptide biomarker discovery, validation, and potential future clinical applications.
 - P700 provides specific inhibition of DPP IV and best stabilization of GLP-1.

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