

Metabolic Peptide Biomarkers: From Sample to Spectrum

Jizu Yi, Zhaoxia Liu, David Craft

BD Diagnostics, 1 Becton Drive, Franklin Lakes, NJ 07417, USA

Introduction

Incretins, including Glucagon-Like Peptide-1 (GLP-1) and Glucose-Dependent Insulinotropic Peptide (GIP), stimulate insulin release in a glucose-dependent manner in healthy individuals, with potential therapeutic value in treatment of type II diabetes (1). However, both GLP-1 and GIP are subject to digestion by Dipeptidyl Peptidase IV (DPP IV), which cuts and removes the N-terminal two residues of incretin peptides. Also other yet-defined peptidases can degrade the peptides nonspecifically. Other metabolic peptidases, such as Glucagon and Ghrelin, are also subject to instability in blood samples (2). As biomarkers, the stability of these metabolic peptides under ex vivo conditions is critical for their clinical applications (3-5).

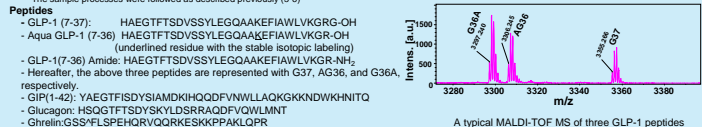
In this study the stability of GLP-1, GIP, Glucagon and Ghrelin was investigated by spiking these peptides into blood samples to assess the effects of anticoagulants and protease inhibitors. For GLP-1 stability, stable isotopically labeled peptide was used as an internal control in MALDI-TOF MS analysis. The loss of full-length GLP-1 peptides (7-36A, and 7-37) was found to be caused by not only DPP IV activity but also a yet-identified carboxypeptidase activity. Further digestion of the GLP-1 fragments was due to the intrinsic peptidase activities. Time-course kinetics analysis indicates that the substrate specificity of the intrinsic peptidase activity is related to sample types, and the serum intrinsic peptidase activity is broader than that in plasma samples. To stabilize these metabolic peptides, a cocktail of protease inhibitors was included into the blood collection tubes. Our results demonstrate that the utility of both DPP IV and peptidase inhibitors significantly increases the half-life of GLP-1, GIP, Glucagon and Ghrelin peptides.

Methods and Procedure

Blood Collection and Plasma/Serum Preparation

Human blood from non-healthy individuals was directly drawn into a variety of plasma and serum tubes, including EDTA, P700™, and P800™ tube (BD, NJ) ("For Research Use Only").

The sample processes were followed as described previously (5-6)



the residue is modified by a chemical modification with a *n*-octanoyl group

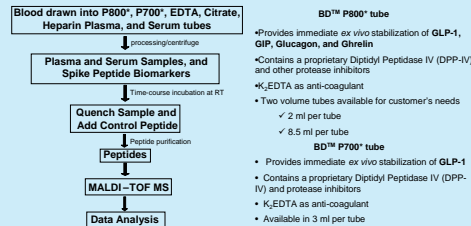
Sample Preparation for Time-Course MALDI-TOF Mass Spectrometric Analysis

- Specified peptides were spiked into thawed serum or plasma samples to a final ~40 fmol/μL
- Samples were incubated at RT in a time-dependent manner
- At specified time period (0 - 96 hours), the samples were quenched with addition of 40 μL of ACN with 0.2% TFA
- At specified time period (0-72 hours), the samples were quenched with a final concentration of 10% ACN and 0.2% TFA
- The quenched samples were extracted using Zip-Tip C18 (Millipore), followed by MALDI-TOF MS analysis

Time-Course experiments

Specified peptides were spiked into a thawed serum or plasma samples to a final concentration of ~40 fmol/μL

- Samples were incubated at RT in a time-dependent manner
- At specified time period (0 - 96 hours), the sample was quenched with addition of 40 μL of ACN with 0.2% TFA
- The quenched samples were supplied for extraction using Zip-Tip C₁₈ (Millipore), followed by MALDI-TOF MS analysis



MALDI-TOF MS

Performed on an Ultraflex II MALDI-TOF MS (Bruker-Daltonics, Germany) as described previously (7)

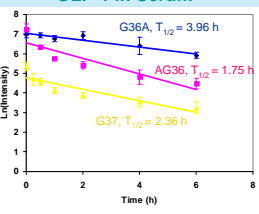
- Peptide samples were mixed in 1:1 ratio with 5 mg/mL of CHCA solution
- The final spectrum was obtained from accumulation of 30 qualified spectra; each of these was from 100 laser shots under fixed laser power
- The sample site targeted by the laser was moved automatically after each of 100 shots to prevent the over-burn of samples
- The final spectrum was calibrated externally

Stability and Kinetic Analysis

A roughly linear relationship is observed with: $\ln(R) = -k_{obs}t + C$, where R = the ratio of peptide intensity to its control; or $\ln(I/I_0) = -k_{obs}t + C$ when a control peptide is not necessary. The linear relationship suggests that degradation occurs according to a first-order reaction in degradation (2)

the half-life of a peptide is determined by $t_{1/2} = \ln(2)/k_{obs}$

GLP-1 in Serum



The half-life of three GLP-1 peptides (G37, G36A, AG36) are 2.36, 3.95, and 1.75 hours, respectively.

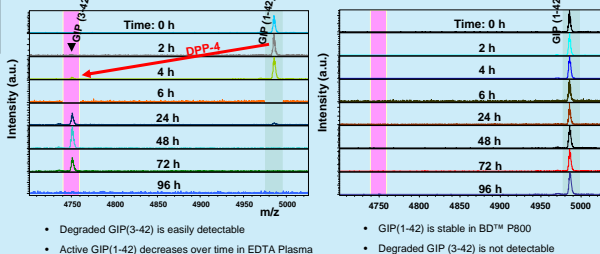
G36A has the best stability among the three peptides, suggesting that the amidation on its C-terminal residue stabilizes the peptide

G37 with C-terminal R residue is more stable than AG36 with C-terminal A residue, verifying our previously reported results that the positively charged residue can provide more stability of the GLP-1 peptide

Above observations indicate that the C-terminal end contributes to the stability of GLP-1

The short half lives of three GLP peptides suggest that extreme ex vivo (and in vivo) instability of GLP-1 in ex vivo serum sample.

GIP: Its Instability and Stabilization in Plasma



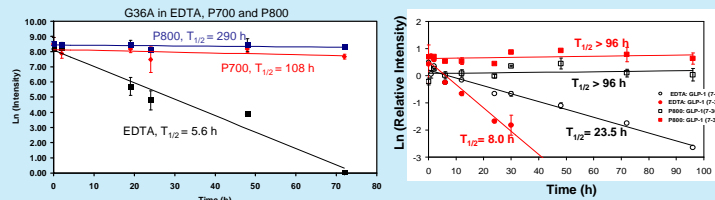
- Degraded GIP(3-42) is easily detectable
- Active GIP(1-42) decreases over time in EDTA Plasma
- GIP(1-42) is stable in BD™ P800
- Degraded GIP(3-42) is not detectable

Conclusions

- Intrinsic peptidase activities cause the instability of metabolic peptides:**
 - DPP IV digests both active GIP and GLP-1 peptides (GLP-1 (7-37) and GLP-1 (7-36A)) by remove the N-terminal two residues
 - Carboxypeptidase activity also removes the first C-terminal residue of GLP-1 peptides
 - The generated fragments are subject to further digestions by intrinsic peptidases
- Peptidase activities show substrate specificity**
 - Serum and plasma behave differently in terms of substrate specificity
 - Plasma samples may contain a different set of peptidases from those in serum
- Peptide digestion caused by intrinsic peptidases follows a first-order reaction**
 - $\ln(I/I_0)$ or $\ln(Rel In)$ versus reaction time is a linear relationship
 - The kinetic analysis provides a quantitative measurement of peptide stability ($T_{1/2}$)
- Both BD P700 and P800 provide stabilization of GLP-1**
- P800 stabilizes also additional three metabolic peptides: GIP, Glucagon and Ghrelin**
- Time-course MALDI-TOF MS provides a feasible method for investigation of peptide biomarker stability under preanalytical processes**

Results

Incretin Peptides Are Stabilized in the Inhibited Plasma Samples



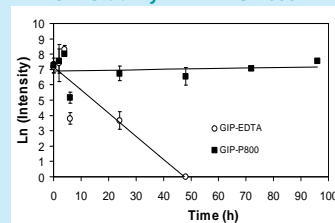
The relative intensity of GLP-1(7-37) and GLP-1(7-36A) to AG36 was compared using time-course MALDI-TOF MS

- Both G36A and G37 are stabilized in P800 plasma for > 4 days
- The half-life of G37 and G36A in EDTA plasma is 8 and 23.5 h
- Digestion of GLP-1 peptides in EDTA is subject dependent
- The half-lives of GLP-1 peptide in serum and plasma samples are summarized as Table 1.

The stability of G36A in EDTA, P700, and P800 samples was compared

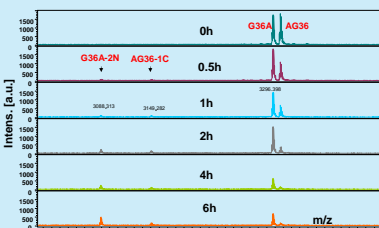
- The peptide is not stable in EDTA sample, However, it is stabilized in both P700, and P800 samples
- G37 and AG36 are also stabilized in both P700 and P800
- G37 is more stable than AG36 in the same sample, providing another evidence that positive end residue provides a better stability

GIP Stability: EDTA VS P800



- $T_{1/2}$ of GIP is greater than 4 days in P800
- $T_{1/2} < 5$ h in EDTA samples

Mechanistic Analysis of GLP-1 Digestions G36A and AG36 in Serum



- The time-course experiment indicates spiked peptides decrease in a time-dependent manner
- DPP IV activity was observed
 - Evidenced by the observation G36A-2N, representing the peptide with truncated two N-terminal residues from G36A
- Carboxypeptidase activity was also observed
 - Evidenced by the observation of a fragment peptide, G36-1C, representing the peptide with truncated one C-terminal residue from AG36

References

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