Ex vivo Stabilization of GLP-1 and GIP in Human Plasma

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Summary

Incretin peptides, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), possess multiple physiological roles that make them both important peptide biomarkers for metabolic disorder research. Both of these peptides are subject to intrinsic proteolytic degradation and metabolism in human plasma, e.g. by Dipeptidyl Peptidase-IV (DPP-IV). Ex vivo stabilization of full length GLP-1 and GIP is critical for their utility in diagnostics and/or drug development. We directly investigated the stability of these peptides and their metabolites in conventional serum, EDTA plasma, or EDTA plasma with protease inhibitors as stabilizers. Using spiked blood samples, peptides were monitored by time-course MALDI-TOF MS. Mass spectrometry allows detection of the intact peptides for kinetic analysis as well as identification of the cleaved peptides. The results indicate that degradation of GLP-1 and GIP fit an observed sequential first-order reaction with the greatest stability (longest half-life) in the inhibited plasma sample. Identification of the cleaved peptides revealed that N-terminal two amino acid residues and C-terminal residues were truncated, indicating that both intrinsic DPP-IV and exo-carboxypeptidase contribute to the digestion of active GLP-1 ex vivo. Further, our study demonstrates that stabilization of both GLP-1 and GIP was accomplished by including a cocktail of enzyme inhibitors in a blood-collection tube, allowing accurate measurement for biomarker development. We also conducted an evaluation of two commercially available ELISA kits for GLP-1 and GIP demonstrating some kits are sensitive to peptidase degradation while others are not.

Key words: plasma peptide, stabilization, time-course MS, ELISA, GLP-1, GIP, incretin.

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Introduction

Glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are two major human incretin hormones that stimulate insulin release in a glucose-dependent manner, called “incretin effect” (1-3). GLP-1 and GIP contribute to approximately 60%-70% of the total postprandial insulin response in healthy individuals, and potentially have a therapeutic value in treatment of type II diabetes (4, 5). However, the incretin effect is significantly decreased in patients with type 2 diabetes, delaying and reducing insulin release after oral glucose administration. It is well documented that GLP-1 and GIP are rapidly digested by the enzyme dipeptidyl peptidase-IV (DPP-IV) after their secretion (4, 6). The half-life of these two peptides in vivo is very short: approximately 2 minutes for intact GLP-1 and 5 minutes for intact GIP (3).

Active GLP-1 has two forms differing in their C-terminal ends: a 30–amino-acid-residue peptide with the C-terminal residue aminated (GLP-1 (7-36A)) and a 31–amino-acid-residue peptide with the C-terminal end extended with a glycine residue (GLP-1(7-37)) (Fig. 1). Both forms are derived from a larger protein called proglucagon, and secreted by L cells located predominantly in the distal gastrointestinal (GI) tract.

GIP, a 42–amino-acid-residue peptide (Fig. 1), is proteolytically derived from the ProGIP protein (3) and is secreted by endocrine K cells mainly present in the proximal gastrointestinal (GI) tract (duodenum and proximal jejunum), and circulated in blood veins.

A peptide therapy is largely dependent on accurate ex vivo measurement of the peptide. Current methods for peptide quantification mainly include antibody-based immunoassay (e.g. ELISA) and mass spectrometry (MS)-based analysis. The immunoassay is dependent on the specificity and affinity of the detecting and capture antibodies binding to the targeted peptide.

While an immunoassay provides high sensitivity that is largely needed in clinical applications, detection monoclonal antibodies typically recognize a specific 6-8 amino acid sequence and may or may not be sensitive to degradation. On the other hand, MS-based analysis with its high resolution specifically detects the intact peptides. Although MS-based assays are less sensitive, the detection of the targeted peptide as well as its daughter fragments generated from the proteolytic digestion allows the mechanism of degradation to be studied.

As a living tissue, blood contains an extensive array of enzymes (e.g. proteases) that retain their activity ex vivo. This ex vivo proteolytic activity may cause protein instability resulting in assay variability (7). We have investigated the stability of spiked incretin peptides in blood to assess the effect of different anticoagulants as well as protease inhibitors. Purified peptides including: GLP-1(7-37), GLP-1(7-36A), stable isotopically labeled GLP-1(7-36), or active GIP(1-42) were spiked into plasma or serum (8). In this paper we identify loss of full-length GLP-1 and GIP peptides signal due to digestion by DPP-IV. Further GLP-1 was also cleaved by an un-identified exo-carboxypeptidase, which removed the amino acid residue on its C-terminus in serum. Time-course kinetics allowed us to measure the stability (half-life) of each peptide. We found that the stability of each peptide is related to the amino acid sequence, as well as the C-terminal aminidation. Also, peptide stability is largely dependent on the sample tubes into which blood is drawn, and addition of DPP-IV and other enzyme inhibitors (BD™ P800*) increases the stability of both GLP-1 and GIP.

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Methods and Procedures

Blood Collection and Plasma/Serum Preparation

Human whole blood from healthy individuals was directly drawn into P800*, K₂EDTA or serum BD Vacutainer® tubes (BD, Franklin Lakes, NJ) by venipuncture. Plasma samples were centrifuged or processed immediately. For serum, the tube was allowed to clot at room temperature for 60 min and centrifuged, as described in our previous publication (7).

GLP-1 and GIP Peptides

Peptides used in this study include three GLP-1 isoforms [GLP-1 (7-36) amide (G36A), Aqua GLP-1 (7-36) (AG36), and GLP-1 (7-37) (G37)] and one full-length GIP (1-42) (GIP) were all purchased from Sigma (sigma-aldrich.com). The sequence of each peptide can be found in Fig. 1. AG36, an isotopically labeled peptide, was synthesized by Sigma-Genosys (The Woodland, Texas) with lysine (K²⁰) labeled with stable isotopic ¹³C₆ and ¹⁵N₄, resulting in a 10 Da higher shift than its natural counterpart (Fig. 1). The three peptides were easily resolved in MALDI-TOF MS with monoisotopic signals at 3296.7, 3305.4, and 3355.3 m/z for G36A, AG36, and G37, respectively.

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

Into 10 µL of thawed P800, EDTA plasma, or serum, each of which was pooled from the same three healthy individuals, 1 µL of GLP-1 or GIP solution was spiked to a final concentration of ~ 40 fmol/µL. The spiked samples were incubated in a temperature-controlled chamber at 25 ± 1°C in a time-dependent manner. At specific time intervals between 0 and 72 hours, the samples were quenched with the addition of ACN and TFA, to a final concentration of 10% ACN and 0.2% TFA, as well as AG36 (20-40 fmol/mL) for MS-based GLP-1 analysis; or 50% ACN, 30% acetone and 0.2% TFA for MS-based GIP assays. GLP-1 peptides were extracted from the quenched sample using a Zip-Tip C18 (Millipore). The GIP samples were centrifuged under 125,000 x g, speed vacuum dried and re-suspended in 10% ACN, 0.1% TFA solution, followed by Zip-Tip purification. A 1.0 µL aliquot of the eluted peptide solution was mixed with 1.0 µL CHCA matrix and spotted onto a MALDI target, air-dried, and analyzed by MALDI-TOF MS (Ultraflex II, Bruker-Daltonics) (8).

Sample Preparation for Time-Course Enzyme-linked Immunosorbent Assay (ELISA)

Blood from consenting subjects (n = 4) was collected into multiple P800 and K₂EDTA BD Vacutainer® tubes by venipuncture. The tubes were immediately centrifuged at 1300 RCF for 20 minutes and the resulting plasma from each tube type was pooled into secondary tubes. The pooled plasma was subsequently spiked with either GLP-1 or GIP to a final concentration of ~ 800 pmol/mL. Peptide stability at 25 ± 2°C was assessed on plasma aliquots collected at 7 time intervals (0, 2, 6, 12, 24, 48, and 72 hours) post spiking. These aliquots were frozen and stored at -80°C until analysis.

ELISA Analysis

ELISA kits specific for measuring total GLP-1, active GLP-1, and total GIP peptides were purchased from LINCO Research (St. Charles, MO). The detection active GLP-1 antibody recognizes specifically both active G36A and G37. The GIP assay is for total GIP measurement, however we also tested if it can distinguish between the active GIP(1-42) and inactive GIP(3-42).

Incretin Peptide Sequences

GLP-1 (7-37): HAEGFTSDVSSYLEGQAAKEFIAWLVKGR-OH
GLP-1(7-36A): HAEGFTSDVSSYLEGQAAKEFIAWLVKGR-NH₂
AQUA GLP-1: HAEGFTSDVSSYLEGQAAKEFIAWLVKGR-OH
GIP: YAEGTFISDYSIAMDKIHQQDFVNWLLAQKGKKNDWKHNITQ

Figure 1. Primary Sequences of GLP-1 and GIP. The underline indicates the residue with the stable isotopic label.
Results and Discussion

Intrinsic plasma proteolysis has been well characterized in our previous reports (7, 9). Plasma endoproteases digest proteins at specific residues (e.g. Arg or Lys by thrombin) releasing parent peptides, which can further be digested by exopeptidases, truncating either or both C- and N-terminal residues to form daughter peptides (7). These proteolysis-caused modifications of a peptide can easily be observed using MALDI-TOF MS due to its high resolution and high mass accuracy. Both the parent and its derived daughter peptides are detectable in a single spectrum (9). Herein, we developed a method using MALDI-TOF MS, to measure incretin peptide stability under ex vivo sampling conditions (8).

Digestion of GLP-1 in serum and plasma samples

We first investigated ex vivo stability of GLP-1 with our time-course MALDI-TOF MS method to monitor peptide changes over time (8, 9). Two peptides of active GLP-1; G36A and AG36 (Fig. 1), were simultaneously spiked into either serum or plasma. The spiked peptides and their fragments were identified by high resolution MALDI-TOF MS. During time-course incubation of GLP-1-spiked serum, we observed both decreasing intensity of the two active GLP-1 parent peptides and increasing intensity of GLP-1 fragments, specifically G36A-2N and G36-1C (Fig. 2). The former peptide (G36A-2N) is formed through the truncation of the N-terminal two residues by DPP-IV; the later peptide (G36-1C) is formed through the truncation of one C-terminal residue, likely by a carboxypeptidase. Along with the increase of these fragments, which were detected in as early as 30 minutes of incubation, the spiked parent peptides’ signal intensity diminished rapidly until it was undetectable after four hours (Fig. 2).

As EDTA plasma has been widely used for active and total GLP-1 measurements, we have also evaluated GLP-1 stability in EDTA plasma using our time-course MS approach. As shown in Fig. 3A, both active forms of GLP-1 (7-36A) and GLP-1 (7-37), relative to the control peptide (AG36), decreased during time-course incubation within EDTA plasma. Meanwhile, fragments GLP-1 (9-37) (G37-2N) and GLP-1 (9-36A) (G36A-2N) were observed increasing in intensity over time during the incubation. These -2N fragments clearly indicated DPP-IV activity in EDTA plasma contributing to the instability of GLP-1. Similar results were observed when two active GLP-1 peptides were spiked into citrate- or heparin-plasma (data not shown). All of these results clearly indicate that both DPP-IV and exocarboxypeptidase activities contribute to ex vivo instability of GLP-1.

Figure 2. Digestion of GLP-1 by DPP-IV and carboxypeptidase activities. G36A and AG36 were spiked into and mixed with serum sample in 1:10 ratio (v/v). The sample was incubated at r.t. and aliquots were withdrawn for peptide extraction and MALD-TOF MS analysis at indicated time points. Both daughter peptides G36A-2N and G36-1C were detected after incubating for 30 min.
Ex vivo stabilization of GLP-1 with DPP-IV and peptidase inhibitors

To stabilize GLP-1 peptide, we screened, selected, and optimized a cocktail of inhibitors using the internal method previously described (also for GLP, Glucagon, and Ghrelin). The cocktail was included into an evacuated blood collection device BD™ P800™ to prevent the degradation at the point of phlebotomy (7). Using the inhibited P800 plasma, we demonstrate that both GLP-1 active peptides (G36A and G37) were stabilized without significant changes in their intensities relative to the Aqua control over a four-day incubation at room temperature (Fig. 3B). Supporting the observed stabilization of active GLP-1 peptides in P800, neither the -2N or -1C fragments were detected in P800 plasma samples (see Figs. 2, 3A, 3B). Although these studies were performed with spiked GLP-1, these results suggest that ex vivo stabilization of GLP-1 can be achieved using P800 for up to 4 days (Fig. 3B).

For a more quantitative comparison of the GLP-1 stability in P800 and EDTA samples, the ratios of GLP-1 intensity to control (AG36) intensity were plotted versus incubation time (Fig. 4). The results indicate that logmatic intensity (natural log) versus the incubation time is linear, suggesting that degradation of GLP-1 by DPP-IV is fitted to a first-order reaction. Therefore, the half-life of GLP-1 can be determined according to the first-order kinetics model (9). The results show that the half-life of either active GLP-1 forms in P800 plasma is more than three days while the same peptide has a half-life shorter than 24 hours in EDTA plasma (Fig. 4). Also, note that in this EDTA vs. P800 comparison, samples were pooled from the same three subjects.

Figure 3A. Instability of GLP-1 in EDTA plasma. Both G36A and G37 were spiked into EDTA plasma and incubated at r.t. At indicated time points, aliquots were withdrawn and AG36 was added as control for MS analysis. While G36A and G37 decreased over incubation time, G36A-2N and G37-2N increased.

Figure 3B. Stabilization of GLP-1 in P800 by enzymatic inhibitors. The experiments were carried out as described in Fig. 3A. Both G36A and G37 were stable over incubation time, and their fragments, G36A-2N and G37-2N, were not detectable for up to four and two days, respectively.
For a complementary comparison, the stability of active GLP-1 was further evaluated with an ELISA assay in which the detecting monoclonal antibody (mAb) recognizes the two N-terminal residues of active GLP-1 peptides. Using the inhibited P800 plasma, we demonstrate that the concentration of GLP-1 active peptide (G36A) was consistent within 48-hours at room temperature, compared to the fast drop in concentration of the peptide in an EDTA control within the first couple of hours (Fig. 5). Even at time “O” a significantly lower GLP-1 signal was observed in EDTA, reflecting the degradation of active GLP-1 during sampling and/or incubation during the ELISA assay (Fig. 5). These ELISA-based results complement the MS work and further demonstrate that ex vivo stabilization of GLP-1 was accomplished using P800 for blood collection.

Stability of GLP-1 in whole blood samples

Under typical clinic settings, blood tubes drawn with sample specimens may not be processed immediately, and in some cases the whole blood tubes may be transported to a centralized lab for processing and analysis. This time variability in un-inhibited blood could cause major variations in analytic results. To test if GLP-1 is stable during this dwell time between blood collection and centrifugation, we spiked the GLP-1 peptides into whole blood EDTA and whole blood P800 samples. After time-dependent incubations at room temperature the blood specimens were centrifuged, and the spiked peptides from the separated plasma samples were extracted and analyzed as previously described. The results indicated that G36A and G37 have 9.9, and 12.5 hours of half-life in P800 whole blood while in EDTA whole blood the two peptides display much shorter half-life of 1.3 and 1.9 hours, respectively (Fig. 6). The results further suggest that P800 provides greater stability than EDTA in whole blood, but centrifugation as early as possible after blood collection is recommended as GLP-1 is more stable in plasma than in whole blood (Figs 4 and 6).

**Active GLP-1 measured by MS**

![Active GLP-1 measured by MS](image)

**Figure 4. GLP-1 stability comparison in EDTA and P800 plasma by MS.** G36A and G37 were spiked into EDTA and P800 plasma samples withdrawn from same three healthy individuals. The peak heights of GLP-1 relative to control were fitted to the first-order degradation and half-life of the GLP-1 was then determined.
Figure 5. GLP-1 stability comparison in EDTA and inhibited plasma by ELISA: G36A was spiked into EDTA and P800 plasma samples obtained from same four healthy subjects, and incubated at room temperature. At indicated periods of time, aliquots were withdrawn, frozen and stored below -70°C prior to testing. The samples were later thawed and active GLP-1 concentrations determined using ELISA.

Figure 6. GLP-1 stability in whole blood. Both G37 and G36A were spiked into EDTA and P800 whole blood. After incubation at r.t. for indicated time periods, the blood specimens were centrifuged, and plasma was taken for MS-based peptide analysis.
**Digestion and Stabilization of GIP**

We have carried out similar time-course MS and ELISA experiments to evaluate stability of GIP (1-42) in EDTA and our P800 plasma sample from the same healthy individual. In the time-course MALDI-TOF MS experiments, the spiked GIP (1-42) in EDTA plasma (Fig. 7A) decreases over time until an undetectable after 24 hours. Meanwhile, its fragment GIP (3-42) generated by the removal of two N-terminal residues (-2N) of GIP (1-42) was detected after only two hours of incubation, and its signal intensity increased over time until GIP (1-42) was no longer detectable (Fig. 7A). Similar to GLP-1 -2N degradation, GIP (1-42) was also degraded by DPP-IV in EDTA plasma (2, 3, 6, 10-13). The intensity of GIP (1-42) in P800 plasma, did not decrease (Fig. 7B); nor were its fragment GIP (3-42) detectable in the first three days of incubation (only a very small peak after four days) (Fig. 7B). In another experiment in which EDTA and P800 samples were pooled separately from three healthy individuals and control peptide was added after quenching for analysis, similar observations indicated the stabilization of active GIP peptide in P800 sample versus the instability of GIP (1-42) in EDTA and serum samples (Fig. 7C). In both cases, the half-life of GIP was increased at least four-fold in P800 compared to serum or EDTA (Fig. 7C). While we observed sample-to-sample variation (14) of GIP in serum and EDTA, we also observed constantly stable GIP in individual P800 samples. All of these results indicated that the DPP-IV activity was inhibited in P800 plasma samples, and full-length GIP was stabilized for more than four days.

Again for comparison, we used ELISA for GIP measurement after time-course incubation. By comparing the results between P800 vs EDTA plasma, we observed that the concentration of GIP in both samples stayed steady without significant changes over 72 hours of incubation at room temperature (Fig. 8). These ELISA results were consistent with those observed using MS analysis of P800 sample, but were inconsistent with MS-based results using EDTA samples. The disparity between MS and ELISA methods for the EDTA samples are due to the lack of specificity of the detection antibodies in the ELISA assay to the active GIP (1-42) N-terminus. The ELISA kit performed as advertised since the manufacturer makes no claims of its specificity to distinguish between GIP (1-42) and GIP (3-42). That being said, a better appreciation for the biological significance between active and total analytes such as GIP and distinguishing between the two (e.g., by MS-based method) will play an important role in pharmacokinetic and pharmacodynamic studies in the near future.

![Figure 7A](image_url)

**Figure 7A**

**Figure 7. Stability analysis of GIP in serum and plasma samples by MS:** in EDTA plasma (A), in P800 plasma (B). The first-order kinetics analysis of GLP-1 in pooled P800, EDTA and serum samples (C), where the control peptides were added after time-course incubation and relative signals of GIP were determined for half-life ($T_{1/2}$) calculation.
GIP in P800

Figure 7B

GIP time course

Figure 7C

T₁/₂ > 96 h

T₁/₂ = 22.4 h

T₁/₂ = 20.6 h

Intens. [a.u.]
Conclusions

Both GLP-1 and GIP are two important metabolic peptides that are potentially used for biomarker development. While both peptides are not stable in traditional serum or plasma samples, likely due to proteolytic digestion by DPP-IV and other peptidases, both peptides are stabilized in P800 plasma sample with half-lives of greater than four days. The stabilization provides a foundation for accurate measurement of these metabolic peptides in real clinical settings.

Figure 8. Stability comparison in EDTA and P800 plasma samples by ELISA: GIP was spiked into EDTA and P800 plasma obtained from four healthy subjects. The plasma was incubated at room temperature. At indicated time points, aliquots were withdrawn, frozen and stored below -70°C prior to testing. The samples were later thawed and active GLP-1 concentrations determined using ELISA.
References


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