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Immunoprecipitation and liquid chromatographic–mass spectrometric determination of the peptide glucose-dependent insulinotropic polypeptides GIP_{1-42} and GIP_{3-42} from human plasma samples
New sensitive method to analyze physiological concentrations of peptide hormones

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Abstract

The gastrointestinal peptide glucose-dependent insulinotropic polypeptide (GIP_{1-42}) is one of the incretin hormones regulating glucose-induced insulin secretion from the endocrine pancreas. GIP_{1-42} is a substrate of the circulating enzyme dipeptidyl peptidase IV, which removes the N-terminal peptide Tyr–Ala resulting in the inactive polypeptide GIP_{3-42}. Hither to existing immunoassays do not enable a separate quantification of active and inactive forms, respectively. Therefore, we developed a highly specific and sensitive LC–MS assay for the identification and quantification of GIP_{1-42} and GIP_{3-42}. Total GIP was immunoprecipitated from crude plasma samples using a C-terminally directed antibody. Thus, peptides were purified and concentrated prior to LC–MS analysis. The present immunoprecipitation–LC–MS assay enables the quantification of active and inactive GIP over a concentration range from 5 to 350 pmol/l in human plasma samples. Since this range covers the basal and postprandial levels of GIP, the method is applicable to the determination of concentration changes and changes in the ratio of active and inactive forms of GIP in human plasma.

Keywords: Immunoprecipitation; Peptides; Polypeptides; Hormones; Enzymes; Dipeptidyl peptidases

1. Introduction

Glucose-dependent insulinotropic peptide (GIP) is a gastrointestinal peptide hormone which is involved in the regulation of gastric acid secretion, blood glucose levels and body fat [1,2]. The 42 amino acid peptide is secreted postprandially by gut cells and acts costimulatory to glucose on insulin release by pancreatic beta cells [3]. In 1993 Mentlein et al. [4] demonstrated that GIP and glucagon-like peptide 1 (GLP-1), a related gastrointestinal peptide hormone, are rapidly hydrolyzed by the exopeptidase dipeptidyl peptidase IV (DP IV). The release of the N-terminal dipeptide of GIP and other peptide hormones by this enzyme leads to an inactivation of GIP that can be blocked by inhibitors of DP IV [5,6].

A further enlightenment of GIP metabolism and DP IV inhibition in particular makes a sensitive and

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highly specific assay mandatory, capable of discriminating between the active and inactive form of GIP. Although a number of GIP radioimmunoassays have been described so far, none of them can distinguish between the active and inactive form of GIP [7–11]. On the other hand, a more specific LC assay presented by Mentlein et al. [4] lacks sensitivity, as major drawback, making UV detection inadequate for GIP plasma level quantitation. Basal plasma level of total GIP have been found to be 10 to 50 pmol/l. Plasma concentrations may increase to levels between 200 and 400 pmol/l after stimulation.

This work presents an assay using mass spectrometry for discrimination between active and inactive GIP. In order to achieve the required sensitivity in the low picomolar range, a sample preparation step is necessary to preconcentrate and prepurify the crude plasma sample. As shown earlier [12–14], immunoprecipitation meets these requirements and has been implemented successfully in combination with mass spectrometric methods for qualitative determination of beta-amyloid peptides, transthyretin variants and neurotensin. This report describes, for the first time, the use of immunoprecipitation as a sample preparation method for simultaneous LC–MS quantification of different forms of peptide hormones in human plasma samples.

2. Experimental

2.1. Chemicals and solvents

Ultrapure analytical water was purified in a Purelap Plus System (USF). Methanol (HPLC grade) and formic acid (90%) were obtained from Baker (Griesheim, Germany). All standard peptides: GIP_{1-42} (peptide content: 72.24%, purity: 98%), GIP_{3-42} (peptide content: 74.9%, purity: 98%) and [Arg^{18}, Glu^{21}]GIP_{1-42} and valine-pyrrolidide were synthesized in our laboratory. The peptide content of the synthetic human GIP_{1-42} and GIP_{3-42} was determined by quantitative amino acid analysis. All other chemicals used were commercially available and of analytical grade.

The polyclonal rabbit-anti-C-GIP antibody was produced by Biogenes (Berlin, Germany). The peptide used for immunization contained the C-terminal part of GIP (15 amino acids) and was synthesized in our laboratory using standard methods of peptide chemistry.

2.2. Standards and plasma inhibitor cocktail

Standard plasma samples were prepared by spiking 1.9 ml plasma (EDTA or citrate plasma) with appropriate amounts of the analytes and the internal standard in phosphate-buffered saline (PBS)/Tween buffer solutions.

To stabilize the analytes in plasma samples we used 100 μl of an inhibitor cocktail per 1 ml plasma. The inhibitor solution described by Pietsch et al. [15] has been modified by addition of our specific DP IV inhibitor valine-pyrrolidide to protect the active peptides to DP IV-catalized N-terminal degradation. The composition is a mixture of 0.4 mg aprotinine (Sigma, Deisenhofen, Germany), 500 mg glutathione (Fluka, Deisenhofen, Germany), 2 mg sodium azide (Merck, Darmstadt, Germany), 2 mg streptomycine sulfate (Sigma), 250 mg disodium EDTA (Sigma) and 10^{-3} mol/l valine-pyrrolidide in 9 ml water and of 2 mg dithiothreitol (DTT) (Fluka), 5 mg phenylmethanesulfonic acid fluoride (PMSF) (Sigma) and 20 mg chloramphenicol (Sigma) in 1 ml ethanol. Finally 600 mg sucrose (Sigma) were added.

2.3. Immunoprecipitation

Magnetic beads (20 μl suspension containing approximately $6.7 \cdot 10^8$ beads/ml) with covalently bound secondary anti-IgG-antibodies (Dynabeads M-280; sheep anti-rabbit IgG, Dynal, Hamburg, Germany) were washed three times with 1 ml buffer (PBS/Tween, pH 7.8, Sigma). For separating the beads from solution a magnetic separation stand (MagneSphere, Promega, Mannheim, Germany) was employed. Thereafter, the beads were then incubated with the primary anti-C-GIP antibodies (20 μl, 0.26 mg/ml) in PBS/Tween buffer overnight at 7°C for binding. After washing, 1.9 ml plasma sample (EDTA or citrate plasma) stabilized by the conserva-

tion solution and containing an appropriate amount (400 pmol/l) of internal standard was added to the magnetic beads and incubated for 4 h by the magnet. The supernatant was removed carefully and the precipitated peptides were eluted with 50 μl of 80% methanol containing 0.5% formic acid (15 min). The supernatant containing the peptides was transferred to an autosampler vial for LC–MS analysis (see Fig. 1).

2.4. HPLC–MS equipment and conditions

The HPLC system was a series 200 microgradient pump (Perkin-Elmer, Überlingen, Germany) equipped with a series 200 autosampler (Perkin-Elmer) with a 100 μl sample loop and 50 μl syringe. Separation was performed using an UltraSep ESD Prot column (Sepserv, Berlin, Germany/100 mm×1 mm I.D., particle size 5 μm) protected by a guard column UltraSep ESD Prot. Methanol–water (90:10, v/v) containing 0.1% formic acid (A) and methanol–water (10:90, v/v) with 0.1% formic acid (B) were used as eluents. The gradient elution was performed as follows:

- 0–10 min 100% B with 40 μl/min
- 10–15 min 100% B to 70% A and 30% B with 40 μl/min
- 15–30 min 100% A with 20 μl/min
- 30–35 min 100% B with 40 μl/min.

A total of 40 μl samples were injected. Autosampler and column were kept at room temperature.

The HPLC system was coupled to a quadrupole mass spectrometer PE Sciex API 150 EX equipped with an Ionspray (atmospheric pressure ionization) source (both from Applied Biosystems, Weiterstadt, Germany). All samples were analyzed in the positive ion mode. The spray voltage was set at 5600 V. The optimization of the mass spectrometric settings was carried out by continuos flow injection (20 μl/min) of a standard solution of GIP_{1-42} with the single ion monitoring m/z 997.7 using a microliter syringe pump (Harvard Apparatus, Holliston, MA, USA).

Fig. 2 shows the electrospray ionization (ESI) mass spectra of GIP_{1-42}, GIP_{3-42} and the internal standard. We used a modified GIP_{1-42} with two amino acid replacements in the sequence (His to Arg in position 18 and Asp to Glu in position 21) as an internal standard. For LC–MS quantification in the selected ion monitoring mode the mass traces of the $[M+5H]^{5+}$ ions of the three analyzed peptides were used.

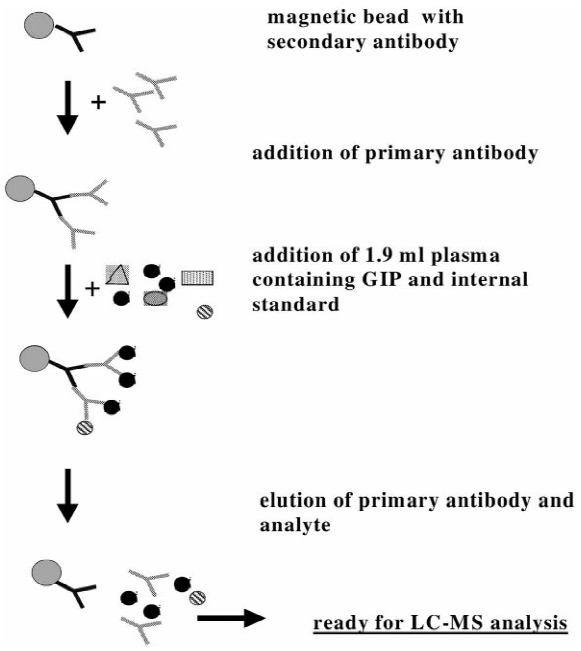

Fig. 1. Principle of the used immunoprecipitation method.

3. Results and discussion

Performing the LC separation under the conditions described $GIP_{1-42,}$ GIP_{3-42} and the internal standard coelute after approximately 21 min and the respective $[M+5H]^{5+}$ peaks could be detected simultaneously. Both, GIP_{1-42} and GIP_{3-42} were quantified based on peak areas relative to the internal standard. The chromatograms from the LC–MS measurements of standard samples of GIP_{1-42} and GIP_{3-42} in 80% methanol with 0.5% formic acid without sample preparation and preconcentration shows linearity in a concentration range from 100 pmol/l to 5 nmol/l with a variability less than 20%. Quantification was

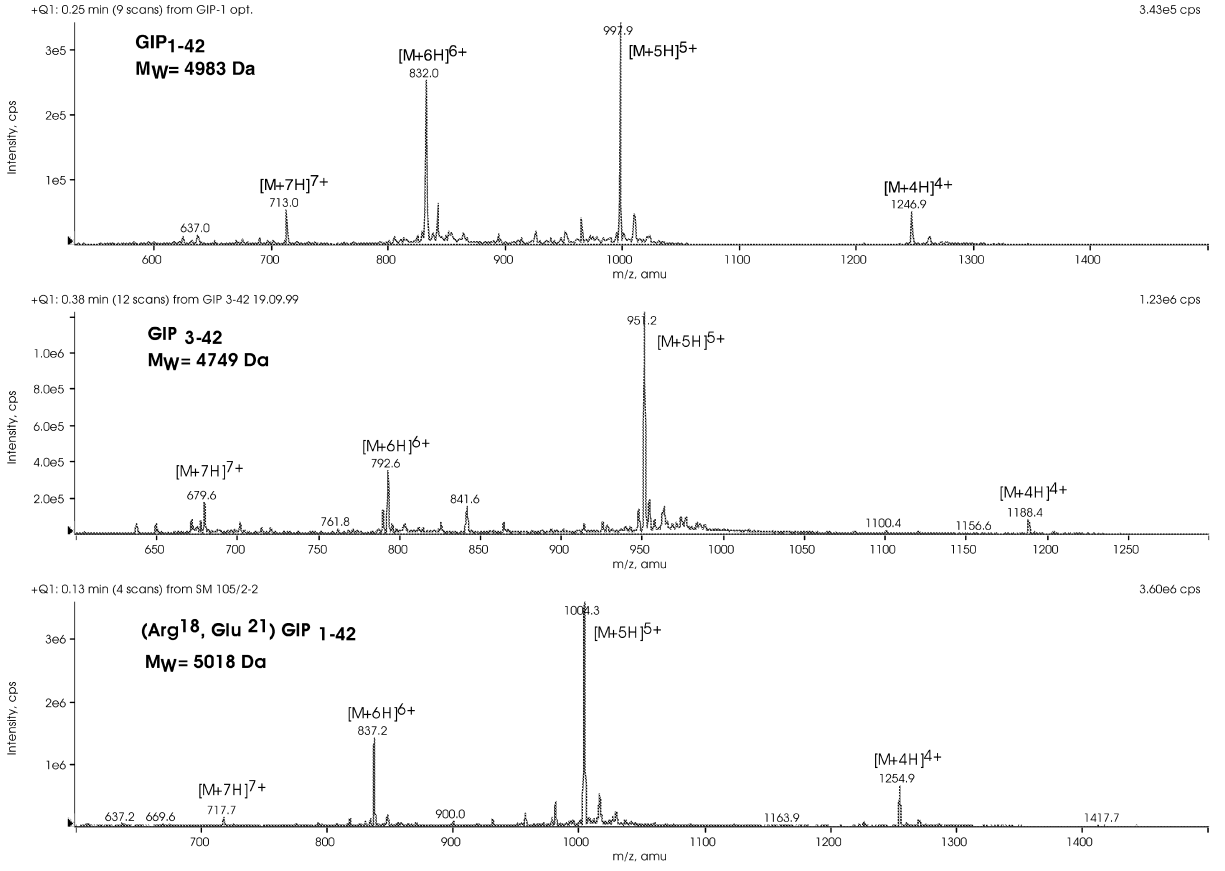

Fig. 2. ESI mass spectra of GIP_{1-42}, GIP_{3-42} and the internal standard. The concentration of the substances was approximately 10 μg/ml in methanol–water (80:20, v/v) with 0.1% formic acid. The infusion rate was 20 μl/min. M_w is molecular mass.

possible by detecting the $[M+5H]^{5+}$ ions (data not shown).

For the determination of the analytes in plasma, we spiked sets of plasma calibration and quality control samples with GIP_{1-42} or GIP_{3-42} ranging from 5 to 350 pmol/l. The internal standard was added to achieve a final concentration of 400 pmol/l. The samples were prepared according the immunoprecipitation method described above. The lower limit of quantification was found to be 5 pmol/l, whereas the limit of detection (S/N 3:1) is 3 pmol/l. The higher limit of quantification is 350 pmol/l. The recovery rate after immunoprecipitation is approximately 75%.

Fig. 3 shows calibration curves of GIP_{1-42} and GIP_{3-42}. Plasma samples spiked over a range from 5 to 250 pmol/l were prepared and measured in duplicate runs of each concentration. Tables 1 and 2 show the results of quality control samples. The inaccuracy was better than ±15%. The precision (expressed as relative standard deviation (RSD)) was less than 11% of both quality control concentrations.

Fig. 4 represents an example for selected ion monitoring (SIM) LC–MS chromatograms of the three peptides coeluting at 21.5 min in a spiked plasma sample.

Thus, the method is highly selective and can clearly discriminate between the active (1-42) and

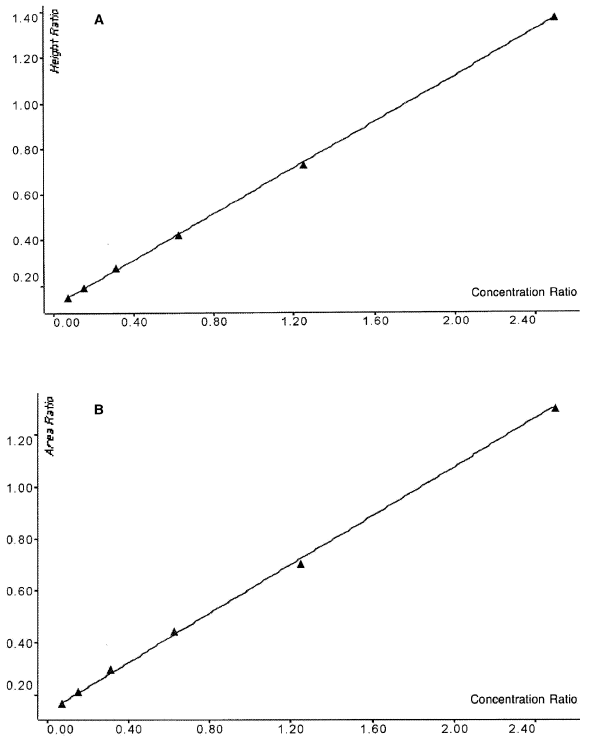

Fig. 3. Calibration curves of GIP_{1-42} (A) and GIP_{3-42} (B) in a range of 5 to 250 pmol/l in human plasma after immunoprecipitation. The correlation coefficient for curve A was 0.999 and for curve B 0.998.

inactive (3-42) forms of GIP. Negative control samples (plasma blank) do not indicate any overlapping interference.

4. Conclusion

An assay for simultaneous quantification of the peptide hormone GIP_{1-42} and its truncated form GIP_{3-42} was developed. With the combination of sample purification and preconcentration by means of immunoprecipitation and LC–MS analysis it is possible to determine these peptides at physiological plasma concentrations. In contrast to currently available GIP assays, the active form of GIP, i.e., GIP_{1-42} can be discriminated selectively from inactive peptide GIP_{3-42} truncated by DP IV. The assay was successfully applied during a clinical study with a selective inhibitor of DP IV to characterize the effect of an orally administered DP IV inhibitor on the metabolism of GIP after meal stimulation. Fig. 5 gives an example of the determination of GIP_{1-42} and GIP_{3-42} in vivo, showing the increase of GIP after meal stimulation. The inactive GIP_{3-42} is blocked by the application of DP IV inhibitor [16]. Furthermore, this assay can be modified in order to quantify several gastrointestinal peptide hormones simultaneously.

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Table 1
Results of the GIP_{1-42} determination from quality control samples in different sample batches

Nominal concentration (pmol/l)	Calculated concentration (pmol/l)	Accuracy (%)	Mean (pmol/l)	SD (pmol/l)	RSD (%)
166	147	88.5			
	185.3	111.6	172.9	18.4	10.62
	186.6	112.4			
41.6	38.8	94.7			
	42.6	103.9	42.6	3.1	7.38
	46.5	113.5			

Table 2
Results of the GIP_{3-42} determination from quality control samples in different sample batches

Nominal concentration (pmol/l)	Calculated concentration (pmol/l)	Accuracy (%)	Mean (pmol/l)	SD (pmol/l)	RSD (%)
166	150.3	90.5			
	178.4	107.5	171	14.8	8.66
	184.2	110.9			
41.6	39.4	96.2			
	40.4	98.6	41.9	2.9	6.85
	45.9	112.1			

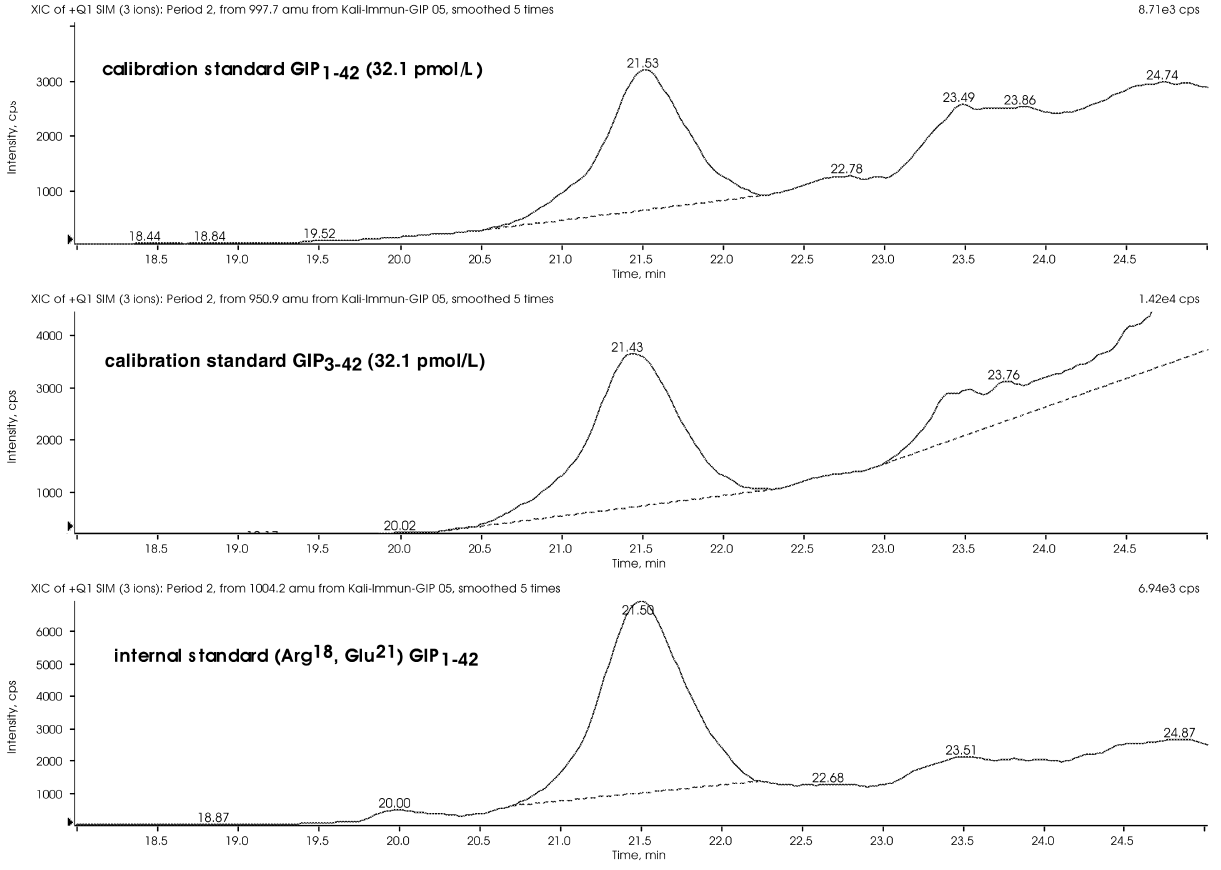

Fig. 4. Representative selected ion monitoring LC–MS chromatograms of a plasma calibration sample after immunoprecipitation under the described conditions.

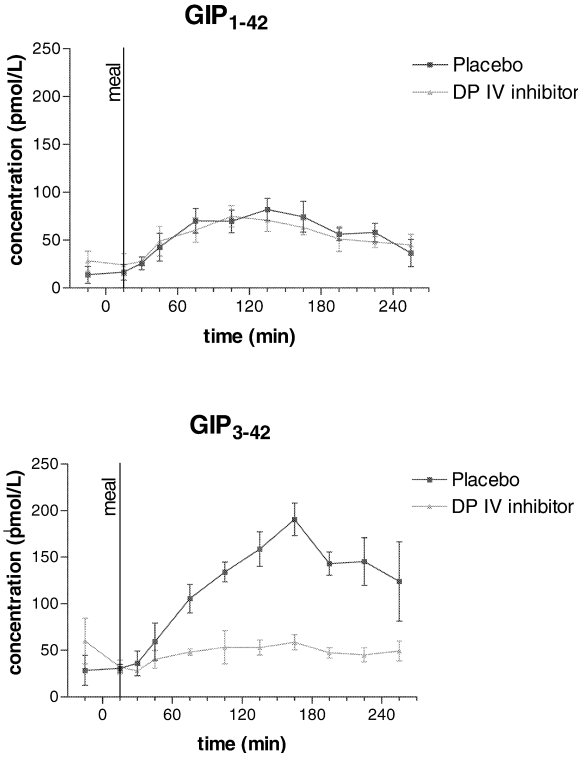

Fig. 5. Example for the determination of GIP_{1-42} and GIP_{3-42} in the course of a clinical study. Graphs: mean±SEM ($n=4$).

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