The isolation and sequencing of human gastric inhibitory peptide (GIP)

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Human GIP 1–42 and fragments of human GIP corresponding to GIP 10–42, GIP 11–42, and GIP 17–42 were isolated from acid–ethanol extracts of human small intestines with the aid of an anti-GIP serum specific for the extreme C-terminal portion of the GIP molecule. The full sequence of human GIP has been established by Edman degradation of these peptides and fragments thereof by automatic gas-phase sequencing. Human GIP differs from porcine GIP at residues 18 and 34. The sequence of human GIP is thus: Tyr-Ala-Glu-Gly-Thr-Phe-Ile-Ser-Asp-Tyr-Ser-Ile-Ala-Met-Asp-Lys-Ile-His-Gln-Gln-Asp-Phe-
5 10 15 20
Val-Asn-Trp-Leu-Leu-Ala-Glu-Lys-Gly-Lys-Lys-Asn-Asp-Trp,Lys-His-Asn-Ile-Thr-Gln. Amino acid
25 30 35 40
residues 18 and 34 are Arg and Ser, respectively, in porcine GIP.

Human *Gastric inhibitory peptide* *Intestine* *Isolation* *Sequence*

1. INTRODUCTION

The intestinal peptide gastric inhibitory peptide (GIP) was first isolated from porcine upper small intestine on the basis of its ability to inhibit histamine-stimulated gastric acid secretion in the dog Heidenhan pouch [1]. Porcine GIP contains 42 amino acid residues [2,3]. The peptide is a potent releaser of insulin in experimental animals [4] and in man [5,6] provided that the blood glucose is above basal levels. Plasma levels of immunoreactive GIP (IRGIP) are elevated after an oral glucose load or a meal in normal man. This increase after a meal is below normal in newly diagnosed insulin-dependent diabetics [7]. GIP, therefore, could be of use as a glucose-dependent insulin-releasing peptide in at least some diabetics. The isolation and sequencing of human GIP were undertaken to ensure that the optimal peptide could be available in the event of GIP being of diagnostic or therapeutic use in man. We report here the isolation and sequencing of human GIP from post-mortem human small intestines.

2. MATERIALS AND METHODS

2.1. *Isolation of GIP*

Human small intestines were obtained post mortem, rinsed and rapidly frozen in contact with solid CO_2. The intestines were kept frozen at $-20°C$ until extracted. Two intestines (A,B) were extracted separately. The intestines were collected 12 and 6 h post mortem, respectively.

The deep-frozen intestines were broken into small pieces with a hammer while at $-20°C$ and homogenized while frozen in 4 vols (w/v) of 0.3% phoshoric acid in 80% (v/v) ethanol. After homogenization the extracts were stirred for 30 min at 4°C. The tissue was collected by centrifugation and reextracted in 4 vols (w/v) of 0.3% phosphoric acid in 65% (v/v) ethanol. The supernatants from the two extractions were pooled.

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Peptides were defatted and precipitated by adding 4 vols (v/v) acetone to the ethanolic extracts and by centrifuging after 30 min stirring at room temperature. The crude peptide precipitates were dissolved in 10% acetic acid and clarified by centrifugation at 10000 × *g* for 30 min followed by filtration through a 0.45-μm Millipore filter. The peptides in the dissolved acetone precipitates were separated by reverse-phase and ion-exchange HPLC on a Spectra Physics SP 8700 solvent delivery system fitted with a Knauer Spectralphotometer 8700 and a Spectra Physics SP 4100 computing integrator.

The distribution of IRGIP during purification was followed by a radioimmunoassay using the anti-GIP rabbit serum R65, standards of highly purified porcine GIP [3] and ^{125}I-labelled porcine GIP. Standards (0–727 pM) or unknowns were incubated with ^{125}I-labelled GIP plus R65 (final dilution 1:8250) for 24 h and the free ^{125}I-labelled GIP separated from antibody-bound ^{125}I-labelled GIP with ethanol [8]. The supernatants containing the free ^{125}I-labelled GIP were counted. The sensitivity of the assay was 3 pM standard (0.3 fmol/tube). The anti-GIP serum R65 reacts with the extreme C-terminus of porcine GIP [3], and does not react with gut peptides other than GIP [8].

2.2. *Sequence analyses*

Edman degradation of peptides was performed with an Applied Biosystems model 470A gas-phase sequencer as in [9,10] with several modifications. A fourth solvent (S1 = n-heptane) was included for washing the filter for 30 s after coupling with PITC. To obtain the methylated derivatives of Asp and Glu, the conversion of amino acid anilinothiazolinones to phenylthiohydantoins (PTH-a.a.) was carried out with 1 N methanolic HCl (R4) instead of 25% trifluoroacetic acid. The sequencer program was adjusted to this reagent as shorter drying times were necessary. The PTH-a.a. were dissolved in approx. 0.25 ml methanol and dried in a Savant vacuum centrifuge for 10 min at 45°C. Dried PTH-a.a. were redissolved in 25 μl methanol containing MTH-tyrosine as internal standard. The PTH-a.a. were identified and quantified by reverse-phase HPLC on an IBM cyano column [11] in a Hewlett Packard liquid chromatograph model 1084B equipped with a variable UV-detector model 79875. Details of the procedure are described in the legend to fig.1. The PTH-a.a. were measured at 263 nm whereas the dehydro derivatives of serine and threonine were detected at 314 nm.

2.3. *Preparation of GIP fragments*

To obtain sufficient peptides for sequencing the C-terminal part of GIP the following GIP fragments isolated from intestine B and identified by partial sequencing were mixed: GIP 17–42 (650 pmol), GIP 10–42 (200 pmol) and GIP 11–42 (150 pmol). The peptides were dried and redissolved in 0.1 ml of 50 mM *N*-ethylmorpholine buffer (adjusted to pH 8.0 with acetic acid) containing 65 pmol *Armillaria mellea* protease, and incubated for 16 h at 37°C. *A. mellea* protease, which specifically cleaves at the N-terminal side of lysine residues, was purified from *A. mellea* [13]. The *A. mellea* protease digest was fractionated by HPLC (fig.2).

One nmol GIP was cleaved at the tryptophan residues by oxidation of tryptophan to oxindolylalanine and methionine to methionine sulfox-

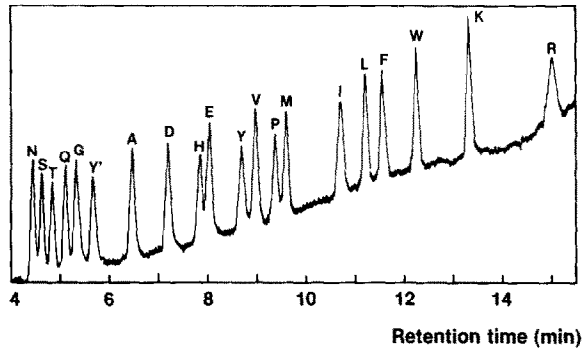

Fig.1. HPLC separation of PTH amino acids on the IBM cyano analytical column (25 × 0.46 cm) equipped with a Permaphase ETH guard column (5 × 0.46 cm, Dupont). Solvent A was 16 mM sodium acetate (pH 5.6) and solvent B acetonitrile/methanol (9:1, v/v). The column was equilibrated with 15% B and eluted with a linear gradient of 15–51.4% B from 0 to 15.30 min. The sample consisted of 5 μl methanol containing 12.5 pmol of each of the PTH amino acids. The flow rate was 1.5 ml/min and the oven temperature 45°C. The PTH amino acids were detected at 263 nm at 1.5 mAUFS (corresponding to attn.-2^1 at the Hewlett Packard 79850B LC terminal). Peak Y' corresponds to MTH-tyrosine used as internal standard. The detection limit was 1.7 pmol, defined as 2-times the noise level.

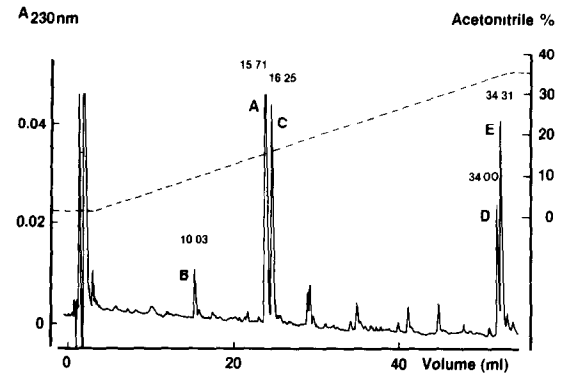

Fig.2. HPLC separation of GIP fragments obtained by cleavage of a mixture of GIP 17–42 (650 pmol), GIP 10–42 (200 pmol), and GIP 11–42 (150 pmol) with *A. mellea* protease. The digest was fractionated on a 5 μm Waters, Nova Pak C-18 column (15 × 0.39 cm) with a linear gradient as indicated. Solvent A, 0.1% trifluoroacetic acid in H_2O; solvent B, 0.07% trifluoroacetic acid in 50% (v/v) acetonitrile. Flow rate 1.5 ml/min. Peptides corresponding to major peaks (A–E) were sequenced.

ide by treatment with dimethylsulfoxide in hydrochloric acid followed by cyanogen bromide cleavage in formic acid [12]. The digest was fractionated by HPLC.

3. RESULTS

3.1. *Isolation of GIP*

The dissolved clarified acetone precipitates were fractionated separately on a 22.4 × 250 mm column of LiChrosorb 10RP 18. The part of the effluent containing IRGIP was concentrated in vacuo and the peptides purified on a 4.6 × 250 mm column of Nucleosil 5 μC-18.

The elution patterns of extracts A and B from the two reverse-phase columns were similar (fig.3,4A,B). The peptide with IRGIP from extract A was isolated by freeze-drying from the effluent of the second C-18 column (fig.3B) and partially sequenced. The IRGIP peptide was found to be intact GIP contaminated with another peptide. The corresponding fraction from extract B (pool 2, fig.4A) was therefore purified on a 7.5 × 75 mm column of Waters SP-5PW. The fraction which eluted at 39.46 min (fig.4C) contained IRGIP and was freeze-dried to remove acetonitrile and ammonium formate.

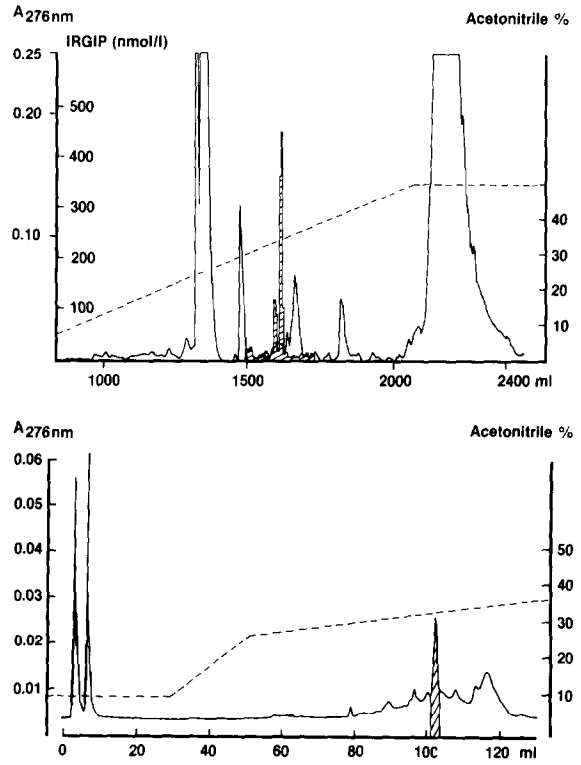

Fig.3. Isolation of partially purified GIP from human small intestine A. Panel A, 230 ml dissolved acetone precipitate were applied at 10 ml/min to a 22.4 × 250 mm column of LiChrosorb 10 RP18 equilibrated with 5% (v/v) acetonitrile in 100 mM ammonium formate (pH 3.3). After application of the sample (absorbance trace not shown) the column was eluted with a gradient of 10–50% acetonitrile in ammonium formate for 200 min. Fractions were collected every 30 s. Panel B, the concentrated IRGIP-containing fractions from the previous column were applied in 1.4 ml to a 4.6 × 250 mm column of Nucleosil 5 μ C-18 equilibrated at 2 ml/min with 10% (v/v) acetonitrile in 100 mM ammonium formate (pH 3.3). The column was eluted with a gradient of acetonitrile in ammonium formate as indicated. Fractions corresponding to the absorbance peaks were collected.

Extract B contained a considerable amount of IRGIP which eluted before the main peak of GIP 1–42 on the first reverse-phase HPLC separation, i.e., in pool 1 (fig.4A). This material was fractionated on a column of Waters SP 5PW, as described for the isolation of GIP 1–42 (fig.4). Two broad zones of IRGIP were recovered, which eluted later than GIP 1–42. The fractions contain-

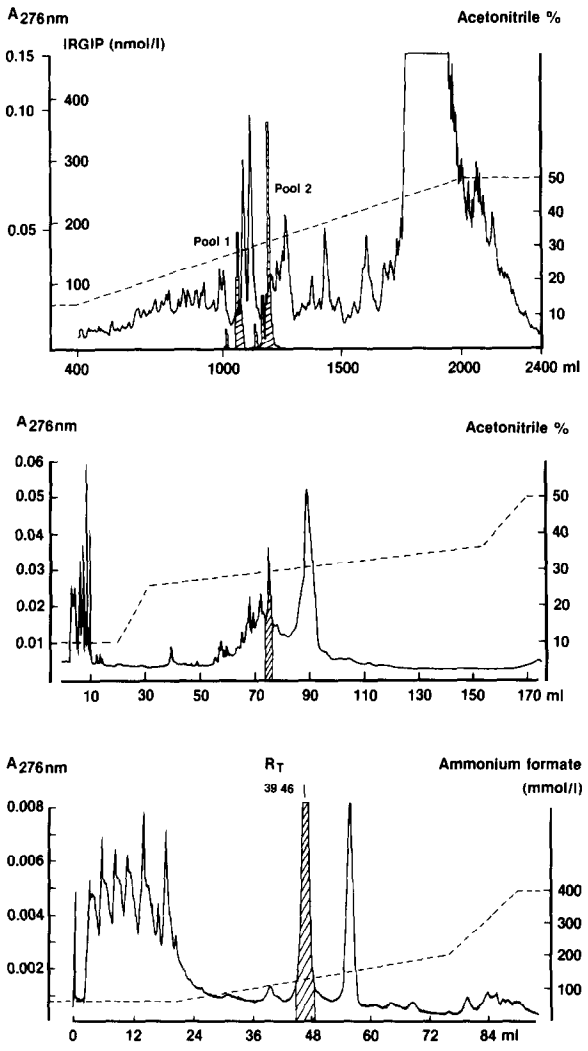

Fig.4. The isolation of GIP and GIP fragments from human small intestine B. Panel A, 400 ml dissolved acetone precipitate were applied at 10 ml/min to a 22.4 × 250 mm column of LiChrosorb 10 RP18 equilibrated with 5% (v/v) acetonitrile in 100 mM ammonium formate (pH 3.3, absorbance trace not shown). Other details as in fig.3A. Panel B, the concentrated IRGIP-containing fractions from the previous column were applied in 3 aliquots of 1.3 ml to a 4.6 mm × 250 column of Nucleosil 5 μ C-18 equilibrated at 2.0 ml/min in 10% (v/v) acetonitrile in 100 mM ammonium formate (pH 3.3). The column was eluted with a gradient of acetonitrile in ammonium formate as indicated. Fractions corresponding to the absorbance peaks were collected. Panel C, the concentrated IRGIP-containing fractions from the previous column (shaded area, panel B) were applied in 6 aliquots of 1.6 ml to a 7.5 × 75 mm column of Waters SP-5PW equilibrated with 50 mM ammonium formate (pH 5.0) in 20% (v/v) acetonitrile at 1.2 ml/min. The column was eluted with a gradient of ammonium formate (pH 5.0) in 20% (v/v) acetonitrile as indicated. Fractions corresponding to the absorbance peaks were collected.

ing these zones were separately purified on a Waters 5 μNova Pak C-18 column (not shown). The material containing IRGIP was pooled and freeze-dried to give a total of 1.0 nmol mixed GIP fragments. The total yield of IRGIP from the two extractions was approx. 4 nmol out of which about 1 nmol were GIP fragments (table 1).

3.2. *Sequence analyses*

By sequencing the intact GIP from extract A the main part of the structure, 31 out of 42 residues, was elucidated (fig.5, table 2). During the sequence analysis of intact GIP a contaminating peptide with an N-terminal sequence of Met-Gln-Ile-Phe-Val- was identified.

By use of Dayhoff's Protein Segment Dictionary [14] the contaminating peptide was identified as human ubiquitin. This peptide was present in 15–20% of the amount of GIP. Since the sequence of human ubiquitin is known [15] this contamination did not cause any serious trouble in the elucidation of the GIP sequence; in fact, the sequence of human ubiquitin 1–26 was confirmed. However, the presence of ubiquitin in the GIP preparation was the main reason that the GIP sequence could not be read beyond residue 31.

The strategy for the sequencing of the C-terminal part was based on the following observations: (i) the human GIP sequence 1–31 showed a strong homology with porcine GIP which meant that a tryptophan residue (with high molar absorption at 230 nm) could be expected in the C-terminal part; (ii) model experiments with porcine GIP digested with the N-terminal lysine-specific *A. mellea* protease showed that porcine GIP 37–42 could be detected with antibody R65; (iii) the model experiments with porcine GIP showed that the digestion with *A. mellea* protease could be carried out specifically and in high yields, and that peptide retention times in the HPLC system used

Table 1

Recovery of IRGIP from extracts of human small intestine

(I) Extract A (841 g small intestine)

Stage	Volume (ml)	GIP (nmol/l)	Total GIP (nmol)	Yield (%)
Acid-ethanol extract	2700	6.4	17.3	100
Dissolved acetone precipitate	230	78.6	18.1	105
Large-scale HPLC, pool 1	12	566	6.8	39
Small-scale HPLC, fraction R_t 50.39 min	3	0.9	2.7	16

(II) Extract B (610 g small intestine)

Stage	Volume (ml)	IRGIP (nmol/l)	Total IRGIP (nmol)	Recovery (%)
Extract	3600	6.3	21.9	100
Dissolved acetone precipitate	400	29	11.6	53
Large-scale HPLC				
Pool 1 (GIP fragments)	35	94	3.3	
Pool 2 (intact GIP)	25	176	4.4	35
Small-scale HPLC (pool 2)				
Fraction R_t 36.10	3	750	2.2	10
SP HPLC				
Fraction R_t 39.46	3	333	1.0	4.5

(fig.2) could be predicted rather accurately from the peptide amino acid composition as in [16].

The *A. mellea* protease digest of the mixture of human GIP 17–42, GIP 10–42, and GIP 11–42 from extract B (pool 1, fig.4B) was fractionated by HPLC (fig.2), and peptides isolated from pools A–E were sequenced. Fragments A and B represented the GIP sequences 33–36 and 37–42,

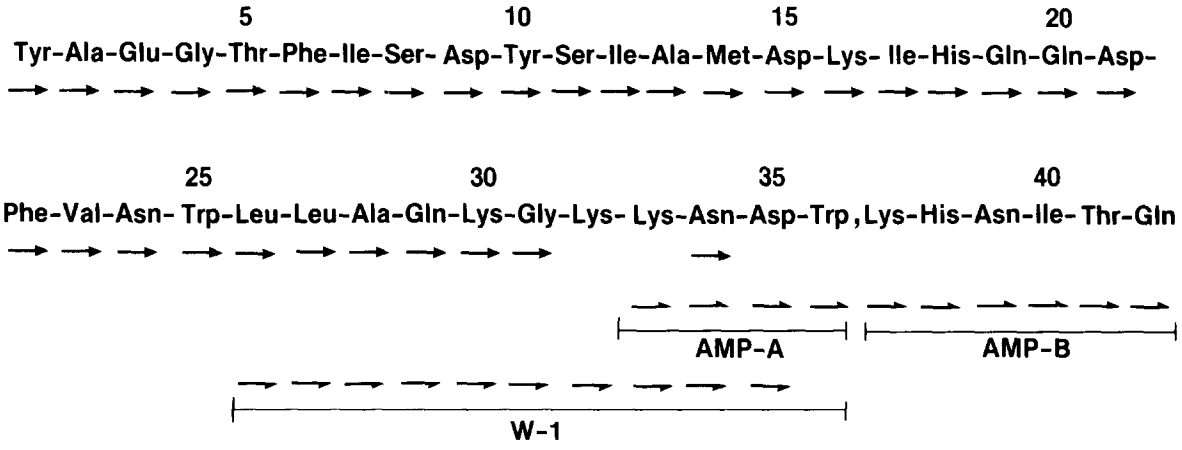

Fig.5. Amino acid sequence of human GIP. The sequence was deduced from the Edman degradation of intact GIP (⟶) (table 2), *A. mellea* protease-fragment A (AMP-A) and B (AMP-B) (⟶), and fragment W-1 (⟶) (table 3).

Table 2

Sequence analysis of intact human GIP

Cyclus no.	Residue no.	PTH-a.a.	Yield (pmol)
1	1	Tyr	1768
2	2	Ala	1362
3	3	Glu	981
4	4	Gly	934
5	5	Thr	491
6	6	Phe	1059
7	7	Ile	772
8	8	Ser	142
9	9	Asp	385
10	10	Tyr	625
11	11	Ser	93
12	12	Ile	318
13	13	Ala	517
14	14	Met	439
15	15	Asp	227
16	16	Lys	294
17	17	Ile	226
18	18	His	57
19	19	Gln	145
20	20	Gln	116
21	21	Asp	137
22	22	Phe	171
23	23	Val	172
24	24	Asn	144
25	25	Trp	70
26	26	Leu	189
27	27	Leu	260
28	28	Ala	138
29	29	Gln	63
30	30	Lys	95
31	31	Gly	64
32	32	–	–
33	33	–	–
34	34	–	–
35	35	Asp	77

Amount applied, 2000 pmol; initial yield, 88.4%; average repetitive yield, 89.2%

respectively (table 3). By peptide sequencing with the gas-phase sequencer the 'carryover' from step N-1 is usually 5–8% (unpublished). However, the carryover observed by sequencing fragment A was approx. 25%, and a minor part of the peptide material isolated from pool A (fig.2) could have been the peptide of Lys-Lys-Asn-Asp-Trp (GIP 32–36) resulting from incomplete cleavage between Lys 32 and Lys 33. Fragments D and E represented the GIP sequences 16–29 and 17–29, respectively (not shown). Fragment C contained the sequence of Lys-Asn-Asp-X. This sequence probably represents a peptide with a derived form of tryptophan in position X.

To confirm the structure around residue 32, intact GIP from extract B was cleaved at the tryptophan residues, and the digest was fractionated by HPLC as described in the legend to fig.2. A peptide fragment (W-1) eluting with a retention time of 19.76 min was isolated and sequenced. Although the yield from the tryptophan cleavage was only 80 pmol (about 8%), 10 out of 11 residues could be identified, thus confirming the sequence from position 26 to 35 (table 3).

Based on the above sequence analyses, the primary structure of human GIP could be deduced (fig.5). Due to the small amount of human GIP available, the peptide bond between Trp 36 and Lys 37 has not been confirmed by peptide sequencing, and no total amino acid analysis has been carried out to support the sequence. However, considering the fragmentation procedures used and the strong homology of human GIP to porcine GIP (40 out of 42 residues are identical) the presence of 'extra' amino acid residues between position 36 and 37 seems rather unlikely although not theoretically impossible.

4. DISCUSSION

In view of the difficulty in obtaining fresh human intestine, our work was carried out with post-mortem tissue. The nature of the starting material was probably responsible for the small amount of IRGIP extracted from the tissue and for the heterogeneity of this IRGIP. This heterogeneity of the IRGIP led to the isolation and sequencing of several 'natural' GIP fragments. Despite the small amounts of IRGIP present in the starting material (approx. 20 nmol/intestine) and its heterogeneity, the full sequence of human GIP has been deduced.

Table 3
Sequence analysis of GIP fragments

Fragment	Cyclus no.											Amount applied (pmol)	Initial yield (%)
	1	2	3	4	5	6	7	8	9	10	11		
A. mellea protease fragment A	Lys-	Asn-	Asp-	Trp								800	36
Yield (pmol)	285	168	124	28									
A. mellea protease fragment B	Lys-	His-	Asn-	Ile-	Thr-	Gln						80	59
Yield (pmol)	47	26	67	59	15	5							
W-1	Leu-	Leu-	Ala-	Gln-	Lys-	Gly-	Lys-	Lys-	Asn-	Asp-		80	45
Yield (pmol)	36	33	26	13	6	18	4	5	12	3			

The full sequence of human GIP differs from that of porcine GIP in that Arg 18 is replaced by His 18 and Ser 34 by an Asn 34. Both of these changes might represent a single base change at the DNA level. The effect of these changes on the biological activity of human GIP compared to porcine GIP cannot be predicted. It can, however, be concluded that these changes can account for the different reactivity of human GIP in plasma with different anti-porcine GIP sera [17] and the slight differences observed between porcine and human IRGIP on reverse-phase HPLC [18].

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