Enzyme Immunoassay of Gastrin in Human Plasma

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A sensitive and specific double-antibody enzyme immunoassay (EIA) for a gastrin-like immunoreactive substance (G-IS) in human plasma was developed. For competitive reactions, the gastrin antibody was incubated with gastrin standard (or sample) and β-D-galactosidase labeled synthetic C-terminal gastrin I fragment (residue 2—17). Free and antibody-bound enzyme hapten were separated using an anti-rabbit IgG coated immunoplate. Activity of the enzyme on the plate was fluorometrically determined. The present immunoassay allows detection of 1 to 20 fmol/ml (2.1 to 42 pg/ml) of gastrin. The levels of G-IS determined in human plasma were 7.8±1.6 pg/ml before lunch and 26.4±8.4 pg/ml after lunch.

Keywords gastrin enzyme immunoassay; β-D-galactosidase-gastrin I (2—17); fluorogenic substrate; sensitive; specific; second antibody coated immunoplate

Gastrin was first detected by Edkins[1] in 1905 in extracts from pyloric antral mucosa as an acid secretion stimulating compound. Gregory and Tracy[2] purified and sequenced from pig antral mucosa two heptadecapeptide gastrins (gastrin I and II) which differed only in the presence of sulfate on the tyrosine of gastrin II. In the 1970s, several molecular forms of tissue gastrin such as big big gastrin, big gastrin, little gastrin and mini gastrin were characterized.[3,4]

In 1967, McGuigan[5] reported radioimmunoassay (RIA) of gastrin using antibodies to the C-terminal tetrapeptide of gastrin and ^{3}H-acetyl tetrapeptide of gastrin. Later, RIAs of gastrin were developed by several groups[6,7] using gastrin-^{125}I. However, in terms of safety, sensitivity and ease of handling, RIA methods are still less than satisfactory. In 1984, Geiger *et al.*[8] reported an enzyme immunoassay (EIA) using human gastrin–peroxidase conjugate and gastrin antibody coated immunoplate. In 1987, Moroder *et al.*[9] reported an EIA for gastrin using gastrin I (2—17)–peroxidase conjugate. In the present study, we wish to report a highly sensitive and specific EIA for gastrin using β-D-galactosidase labeled gastrin I (2—17) as a marker antigen, a second antibody-coated immunoplate as a B/F separator and 4-methylumbelliferyl β-D-galactopyranoside (MUG) as a fluorogenic substrate.

Materials and Methods

Materials Synthetic human gastrin I (G17), big gastrin (G34), tetragastrin (G4), cholecystokinin (CCK-33) and somatostatin were purchased from Peptide Institute Inc. (Osaka, Japan). Gastrin I (2—17) [G17 (2—17)] was purchased from Cambridge Research Biochemicals (Cambridge, England). Human gastrin II was purchased from UCB Bioproducts SA (Belgium). Other synthetic peptides [secretin, gastrin releasing peptide (GRP), vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI)] were supplied from Prof. H. Yajima (Kyoto University, Kyoto, Japan).

β-D-Galactosidase (β-gal) (from *Escherichia coli*) was purchased from Boehringer Mannheim Corp. (Mannheim, Germany). Bovine serum albumin (BSA), polyoxyethylene sorbitan monolaurate (Tween 20), *N*-(ε-maleimidocaproyloxy)succinimide (EMC-succinimide) and MUG were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Goat anti-rabbit IgG (Cappel, 0612-0081) was purchased from Cappel Laboratories (Malvern, PA, U.S.A.).

Antiserum to gastrin (i600/001) was purchased from UCB Bioproducts SA (Belgium) and was reconstituted to 80 ml with an assay buffer (0.05 M phosphate buffer, pH 7.0, containing 0.5% BSA, 1 mM $MgCl_2$ and 250 kIU/ml aprotinin). All other chemicals were of analytical reagent grades.

Preparation of Plasma Extracts Human plasma samples were obtained from 5 healthy volunteers. Blood (10 ml) was collected in a chilled tube containing 0.5 ml aprotinin–EDTA mixture (5000 kIU aprotinin and 12 mg EDTA). After centrifugation (3000 rpm, 4°C, 20 min), plasma was stored at −40 °C until use. Human plasma (0.5 ml) samples were diluted 5-fold with 4% acetic acid (AcOH), pH 4.0, and loaded on reversed-phase C_{18} cartridges (Sep-Pak C_{18}, Millipore Corp, Milford, MA, U.S.A.). After washing with 4% AcOH (10 ml), the gastrin-like immunoreactive substances (G-ISs) were eluted with 70% acetonitrile (MeCN) in 0.5% AcOH, pH 4.0 (2 ml). Eluates were concentrated by spin-vacuum evaporation, lyophilized, reconstituted to 100 μl with the assay buffer and subjected to EIA. Recovery of this extraction procedure of G17 was 83±6%.

Preparation of Enzyme-Labeled Antigen, β-Gal-gastrin I (2—17) Gastrin I (2—17) [G17 (2—17)] was conjugated with β-gal by EMC–

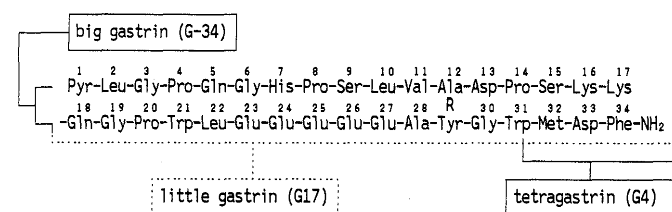

Fig. 1. Structure of Gastrin

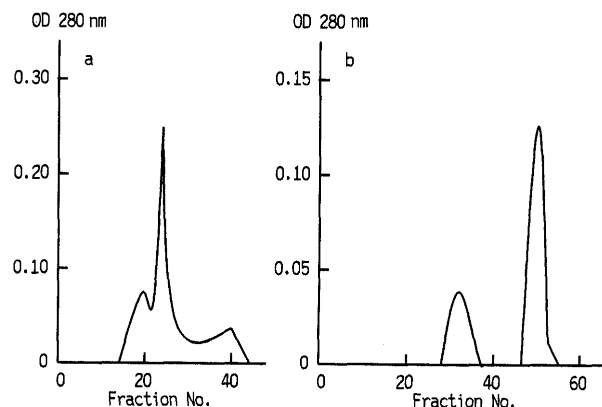

Fig. 2. Purification of β-D-Galactosidase Labeled G17 (2—17)

a: Gel-filtration of EMC-G17 (2—17) on Sephadex G-15. Column, 1.0×64 cm; fraction, 1.8 ml; elution, 0.05 M phosphate buffer pH 7.0. b: Gel-filtration of β-D-galactosidase-G17 (2—17) on Sephacryl S-300. Column, 1.5×57 cm; fraction, 1.8 ml; elution, 0.05 M phosphate buffer pH 7.0 containing 1 mM $MgCl_2$.

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succinimide according to the method of Kitagawa *et al.*[10] G17 (2—17) (0.4 mg) was dissolved in 0.05 M phosphate buffer, pH 7.0 (0.5 ml), and an aliquot of tetrahydrofuran (50 μl) containing EMC–succinimide (0.2 mg) was added. The mixture was stirred at 20 °C for 60 min, then applied to a Sephadex G-15 column (1.0 × 64 cm) pre-equilibrated with 0.05 M phosphate buffer, pH 7.0. The column was eluted with the same buffer. Individual fractions (1.8 ml each) that showed absorbance at 280 nm were collected. The pooled G17 (2—17)–EMC fractions (No. 23—25 in Fig. 2a) were combined with β-gal (3.2 mg) by stirring at 20 °C for 60 min. The β-gal conjugate was applied to a Sephacryl S-300 column (1.5 × 57 cm) pre-equilibrated with 0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$ and was eluted with the same buffer. Individual fractions (1.8 ml each) that showed absorbance at 280 nm were collected. The pooled β-gal-G17 (2—17) fractions (No. 28—37 in Fig. 2b) were stored at 4 °C after addition of 0.2% BSA and 0.1% NaN_3.

Assay Procedure for Gastrin For assay, the above-mentioned assay buffer was used. Second antibody-coated immunoplates were prepared as previously reported[11] using Microwell Maxisorp F8 plates (Nunc, Roskilde, Denmark) and anti-rabbit IgG. A test tube containing 100 μl of gastrin–antiserum, i600/001 (final dilution 1/128000) and each sample (or standard, 100 μl) was incubated at 4 °C for 24 h and then the diluted enzyme-labeled antigen (50 μl) was added. The test tube was further incubated at 4 °C for 24 h. The antibody–antigen solution (100 μl) from each test tube was added to the second-antibody-coated immunoplate. The plate was incubated at 25 °C for 4 h, then washed 4 times with a washing buffer (0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20) and 0.1 mM MUG (200 μl) in a substrate buffer (0.05 M phosphate buffer, pH 7.0, containing 1 mM $MgCl_2$) was added to each well. The plate was again incubated at 37 °C for 3 h. The resulting fluorescence intensity (λ ex 360 nm, λ em 450 nm) of each well was measured with a MTP-100F microplate reader (Corona Electric, Ibaraki, Japan).

HPLC of Plasma Extracts HPLC was performed using a reversed phase C_{18} column (Cosmosil 5C18AR, Nakalai Tesque, Kyoto, Japan). The HPLC consisted of a model 610 dual pump system (Millipore Corp, Milford, MA, U.S.A.). The plasma samples (2 ml) purified by the Sep-Pak C_{18} cartridge mentioned above were reconstituted to 200 μl with 0.1% trifluoroacetic acid (TFA) and applied to the column. G-ISs were eluted with a linear gradient of MeCN (from 15% to 50% in 35 min) in 0.1% TFA. The flow rate was 1 ml/min and the fraction size was 1 ml. The fractions were concentrated by spin-vacuum evaporation, lyophilized, reconstituted to 100 μl with the assay buffer and submitted to EIA.

Results

Standard Curve The typical calibration curve for this EIA of gastrin is shown in Fig. 3. A linear displacement of enzyme-labeled G17 (2—17) by synthetic G17 was obtained, when plotted as a semi-logarithmic function from 0.3 fmol/ml to 200 fmol/ml of G17. The minimum amount of gastrin detectable by the present EIA system was 1 fmol/ml (0.04 fmol/well).

Specificity of the Antiserum, i600/001 Immunospecificity of the antiserum (i600/001) was examined by EIA using β-gal-G17 (2—17). The displacement curves of various gastrin related peptides are shown in Fig. 3. G34 and G17 (2—17) exhibited 100% cross-reactivity as compared with synthetic G17. Gastrin II exhibited approximately 5% cross-reactivity as compared with synthetic G17, and the displacement curve was parallel to that of G17. CCK-33 also inhibited the binding of β-gal-G17 (2—17) with the gastrin–antibody, but reduced cross-reactivity (less than 0.1%), while carboxy-terminal tetrapeptide (G4) did not inhibit the binding of β-gal-G17(2—17) with the gastrin–antibody. Secretin, VIP, PHI, GRP and somatostatin hardly inhibited the binding of β-gal-G17 (2—17) with the gastrin–antibody. Thus, it was shown that the gastrin–antiserum, i600/001, could recognize the central region of G17, and could distinguish gastrin from other gastrointestinal peptides.

Measurement of Gastrin-Immunoreactive Substance (G-IS) in Plasma The proposed gastrin EIA was applied to

Table I. Levels of Gastrin-IS in Human Plasma

Subject[a]	Age	Gastrin-IS (pg/ml)	
		Before meal	After meal
1	23	9.6	35.0
2	34	8.5	30.0
3	23	8.3	14.0
4	26	5.5	22.0
5	34	7.1	31.0
Mean ± S.D.		7.8 ± 1.6	26.4 ± 8.4

Gastrin IS: gastrin-like immunoreactive substance. *a*) All were males.

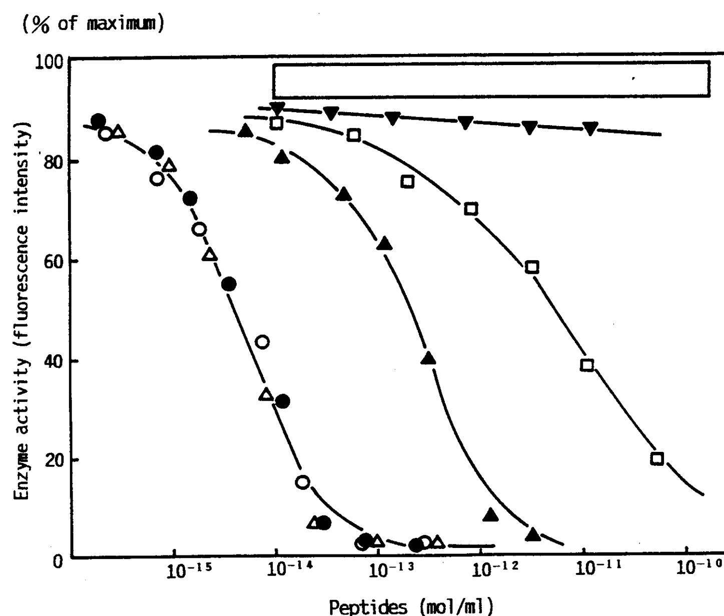

Fig. 3. Inhibition Curves of Various Peptides in EIA by Competition between β-D-Galactosidase Labeled G17 (2—17) and Various Gastrointestinal Peptides toward Antiserum i600/001

●, G17; ▲, gastrin II; △, G34; ○, G17 (2—17); ▼, G4; □, CCK-33; ▭, GRP, VIP, somatostatin, PHI, secretin.

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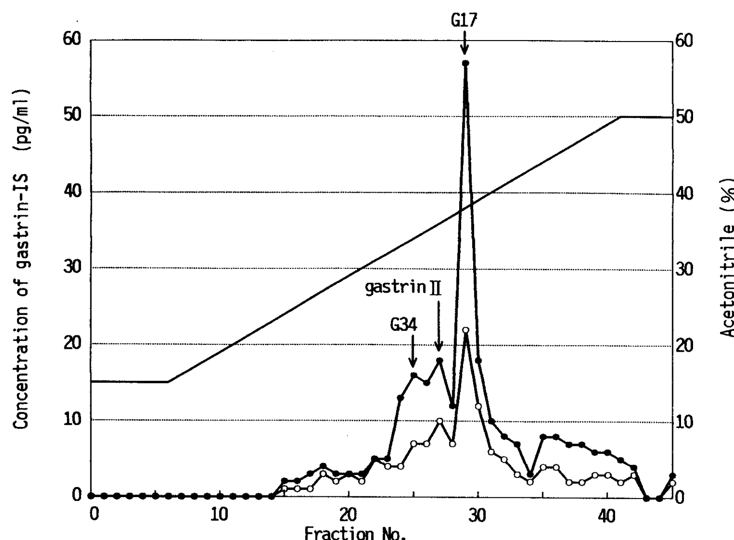

Fig. 4. Elution Profiles of Plasma Extracts by HPLC on a C_{18} Column

G-ISs extracted from plasma by Sep-Pak C_{18} were dissolved in 0.1% TFA and injected onto HPLC. The MeCN gradient is indicated by the straight line. Fractions were lyophilized and their content of G-IS was measured. Synthetic G17, G34 and gastrin II were run in separate chromatographies under the same conditions and their volumes are indicated by arrows. Column, Cosmosil 5C18AR (4.6 × 150 mm), flow rate, 1 ml/min; fraction; 1 ml. ○, before meal; ●, after meal.

the determination of gastrin in human plasma from 5 male volunteers. Concentrations of G-IS in human plasma before and after lunch were 7.8±1.6 pg/ml and 26.4±8.4 pg/ml, respectively (Table I).

HPLC of Plasma Extracts Human plasma extracts were subjected to reversed phase HPLC in order to study the molecular variants of G-IS present in human plasma. Human plasma extracts by a Sep-Pak C_{18} cartridge were applied to reversed phase HPLC on a C_{18} column, and the elution profiles revealed the presence of one main immunoreactive peak that was eluted at the position corresponding to that of standard G17 with two minor peaks eluted earlier at positions corresponding to those of standard G34 and gastrin II.

The elution profiles of plasma extracts before and after lunch were essentially the same (Fig. 4).

Discussions

Using β-gal-labeled G17 (2—17) as a marker antigen, an anti-rabbit IgG-coated immunoplate as a B/F separator and MUG as a fluorogenic enzyme substrate, we developed a highly sensitive and specific EIA for the quantification of gastrin. Since 1967, RIA methods developed for gastrin have been widely used, however these methods have several disadvantages with the use of radioisotopes. Several investigators reported an EIA for gastrin which would retain the advantages of the RIA system and minimize these disadvantages. In 1984, Geiger *et al.*[8] reported an EIA for gastrin using peroxidase–human gastrin conjugate as a marker antigen and gastrin–antibody coated immunoplate, with a detection limit of 0.1 ng/well of gastrin. In 1987, Moroder *et al.*[9] reported an EIA for gastrin essentially the same as Geiger's with a gastrin detection limit of 10^{-9} M. The EIA reported here, using β-gal-labeled antigen, a second antibody-coated immunoplate as a B/F separator and MUG as a fluorogenic substrate, is highly sensitive (1 fmol/ml (0.04 fmol/well)) and specific for quantification of gastrin and a sharp standard inhibition curve was obtained.

We applied the present assay system to the determination gastrin in human plasma. Using the extraction procedure with a Sep-Pak C_{18} cartridge from human plasma (0.5 ml) combined with this EIA, a gastrin level was determined. The levels of gastrin in human plasma from 5 healthy volunteers were 7.8±1.6 pg/ml before lunch and 26.4±8.4 pg/ml after lunch, which were slightly lower than those recently reported by Rakic and Milicevic.[12]

In this study, we identified the presence of immunoreactive gastrin in human plasma extract. The major peak of immunoreactive gastrin was eluted at the position identical to G17 with two minor peaks at positions identical to synthetic G34 and gastrin II. Thus, it was proven that the G-IS in human plasma is identical to authentic G17.

This simple and sensitive EIA will be useful for the measurement of human gastrin in biological fluids and tissues.

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