Enzyme Immunoassay of Motilin in Human Plasma

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A sensitive and specific double-antibody enzyme-linked immunosassay (EIA) for detecting a motilin-like immunoreactive substance (M-IS) in human plasma has been developed. In competitive reactions, the motilin antibody was incubated with a plasma sample (or motilin standard) and β-δ-galactosidase-linked synthetic motilin. Free and antibody-bound enzymes were separated using an anti-rabbit IgG-coated immunoplate. Enzyme activity on the plate was determined by fluorophotometric analysis. This immunassay allows the detection of 20 to 200 fmol/ml (54 to 540 pg/ml) of motilin. The mean level of M-IS detected in human plasma was 53.9 ± 25.6 pg/ml.

Keywords: motilin enzyme-linked immunosassay; motilin-β-δ-galactosidase; fluorophotometric detection; secondary-antibody-coated immunoplate.

Motilin, a powerful inducer of motor activity in the fundus and antral pouch of the stomach, was first isolated from the small intestinal mucosa of pigs in 1971 by Brown.1 Its amino acid sequence was determined three years later.2 Motilin has no effect on gut motility during digestion, but induces the interdigestive myoelectric complex during interdigestive periods.3,4 In 1987, the cDNA encoding human motilin was determined by Seino et al.5

Radioimmunoassays (RIAs) for detecting motilin have been developed by several groups6–8 using 125I-motilin. However, safety concerns and difficult processing render RIA methods unsatisfactory. In this report, we describe a sensitive and specific enzyme-linked immunosassay (EIA) for detecting motilin, using motilin linked β-δ-galactosidase (motilin-β-gal) as a marker antigen, a secondary-antibody-coated immunoplate and 4-methylumbelliferyl β-D-galactopyranoside (MUG) as a fluorogenic substrate.

MATERIALS AND METHODS

Materials: Synthetic porcine motilin which has a sequence identical to human motilin, motilin fragment peptides9 and other synthetic peptides [secretin, gastrin releasing peptide (GRP), vasoactive intestinal peptide (VIP) and peptide histidine isoleucine (PHI)] were supplied by Prof. H. Yajima (Kyoto University, Kyoto, Japan). Synthetic gastrin I, cholecystokinin (CCK-33) and somatostatin were purchased from Peptide Inst. Inc., (Osaka, Japan).

β-δ-Galactosidase (β-gal from Escherichia coli) was purchased from Boehringer Mannheim Corp. (Mannheim, Germany). Polyoxethylene sorbitan monolaurate (Tween 20), N-(ε-maleimidocaproyloxy)succinimide (EMC-succinimide), 4-methylumbelliferyl β-D-galactopyranoside (MUG) and bovine serum albumin (BSA) 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.). An antiserum to motilin (602) was purchased from UCB Bioproducts SA (Allieu, Belgium) and was reconstituted to a final volume of 100 ml with an assay buffer (0.05 M phosphate buffer, pH 7.0, containing 0.5% BSA and 250 KIU/ml aprotinin). All other chemicals were of analytic reagent grades.

Preparation of Plasma Extracts: Human plasma samples were obtained from healthy volunteers at 40 min intervals after lunch. Blood was collected in a chilled tube containing 500 KIU/ml aprotinin and 1.2 mg/ml ethylenediaminetetraacetic acid (EDTA). After centrifugation (1670 g, 4°C, 20 min), plasma was stored at −40°C until assayed. Human plasma samples (0.5 ml) were diluted five-fold with 4% acetic acid (AcOH), pH 4.0, and loaded on a reversed-phase C18 cartridges (Sep-Pak C18, Millipore Corp., Milford, MA, U.S.A.). After washing with 4% AcOH (10 ml), M-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 an assay buffer, and subjected to EIA. When 20 pg/ml synthetic motilin was added to hormone-free plasma prepared by the method of Tai and Chey,10 the recovery of motilin was 96 ± 14%.

Preparation of Enzyme-Linked Antigen, Motilin-β-gal: Human motilin was conjugated with β-gal by EMC-succinimide according to the method of Kitagawa.11 Motilin (0.4 mg) in 0.05 M phosphate buffer, pH 7.0 (0.5 ml), was mixed with EMC-succinimide (0.2 mg) in tetrahydrofuran (50 µl) at 20°C for 60 min. The EMC-motilin thus obtained was purified via separation through a Sephadex G-15 column (1.0 × 64 cm) using 0.05 M phosphate buffer, pH 7.0, to elute the column. The purified EMC-motilin fractions were combined with β-gal (3.0 mg) by mixing them at 20°C for 60 min. The β-gal conjugate was then applied to a Sephacryl S-300 column (1.5 × 57 cm) and eluted with 0.05 M phosphate buffer, pH 7.0, containing 1 mM MgCl2. The fractions containing peak β-gal activity were collected and stored at 4°C after the addition of 0.2% BSA and 0.1% NaN3.

Assay Procedure for Motilin: The assay buffer mentioned above was used for a plasma sample assay. Secondary-antibody-coated immunoplates were prepared as previously reported using MicroWell MaxiSorB 8
plates (Nunc, Roskilde, Denmark) and anti-rabbit IgG.
A test tube containing 100 μl of motilin-antiserum, i602
(final dilution: 1/120000) and 100 μl of sample (or stan-
dard) were mixed and incubated at 4 °C for 24 h. Twenty-
fold diluted enzyme-linked antigen (50 μl) was then added,
and the test tube was incubated at 4 °C for an additional
24 h. One hundred microliters of the antigen–antibody
solution for each sample was added to the secondary-
antibody-coated immunoplate. The plate was incubated
at 25 °C for 4 h, washed 4 times with a buffer (0.01 M
phosphate buffer, pH 7.2, containing 0.15 M NaCl and
0.05% Tween 20), and then 200 μl of 0.1 mM MUG in
substrate buffer (0.05 M phosphate buffer, pH 7.0, contain-
ing 1 mM MgCl2) was added to each well. The plate was
again incubated at 37 °C for 3 h. The fluorescence intensity
(λex 360 nm, λem 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 C18 column (Cosmosil 5C18,
Nacalai 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 a
Sep-Pak C18 cartridge described above were reconstituted
to 200 μl with 0.1% trifluoroacetic acid (TFA) and passed
through the column. M-ISs were eluted with a linear gradient of MeCN (from 5% to 50% over 45 min) in 0.1%
TFA. The flow rate was 1 ml/min and the fraction size
was 1 ml. Eluted fractions were then concentrated by
spin-vacuum evaporation, lyophilized, and reconstituted
to 100 μl with an assay buffer prior to undergoing EIA.

RESULTS

Standard Curve A typical calibration curve for the
motilin EIA is shown (Fig. 2). When plotted as a semi-
logarithmic function, a linear displacement of enzyme-
linked motilin by synthetic motilin was noted, between
20 and 200 fmol/ml. The minimum amount of motilin
detectable by this EIA system was 20 fmol/ml (0.8 fmol/
well), approximately the same sensitivity as obtained
using the RIA methods.6–8)

Specificity of the Antiserum, i602 Immunospecificity
of the antiserum (i602) was examined by EIA using
motilin-β-gal. The displacement curves of various motilin-
related peptides are shown (Fig. 2). The amino-terminal
motilin fragment (1–16) exhibited 100% cross-reactivity
to synthetic motilin, but the carboxy-terminal fragment
(6–22) exhibited reduced cross-reactivity (less than
0.1%). Secretin, VIP, PHI, GRP, gastrin, CCK and
somatostatin minimally inhibited the binding of motilin-
β-gal with the motilin-antibody. Thus, the motilin-
antiserum, i602, recognizes the amino-terminal region
of motilin, and can distinguish motilin from other gastroin-
testinal peptides.

M-IS Levels in Plasma The proposed motilin EIA was
used to determine the level of M-IS in human plasma from
three male volunteers. The mean concentration of M-IS
in human plasma, measured 40 to 240 min after lunch
was 53.9 ± 25.6 pg/ml (Table I).

HPLC of Plasma Extracts Human plasma extracts
were subjected to reversed-phase HPLC to assess the
presence of M-IS molecular variants in human plasma.
Extracts from human plasma (1 ml) were passed through
a reversed-phase HPLC using a C18 column. The elution
profiles revealed the presence of one main immunoreac-
tive peak, eluted at a position corresponding to that of
standard motilin, and several minor peaks (Fig. 3).

Table I. Motilin-IS Levels in Human Plasma

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age</th>
<th>Motilin-IS (pg/ml)</th>
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<tbody>
<tr>
<td>1</td>
<td>35</td>
<td>45.5 ± 19.7 (n=6, range 25 to 82)</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>36.0 ± 19.2 (n=6, range 15 to 70)</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>79.3 ± 22.9 (n=6, range 54 to 120)</td>
</tr>
<tr>
<td>Mean ± S.D.</td>
<td></td>
<td>53.9 ± 25.6 (n=18)</td>
</tr>
</tbody>
</table>

Motilin-IS: motilin-like immunoreactive substance. a) All were males.

Fig. 2. Competitive Inhibition of Motilin and Various Peptides in the
EIA

Fig. 3. HPLC Elution Profiles of Plasma Extracts

M-ISs extracted from plasma by a Sep-Pak C18 cartridge were dissolved in 0.1% TFA and passed through an HPLC column. The MeCN gradient is indicated by the straight line. Fractions were lyophilized and the M-IS content of each fraction was measured. Synthetic motilin and motilin fragment peptides were run in separate chromatograms under the same conditions, and the fractions containing these compounds are indicated by the arrows. Column: Cosmosil 5C18AR (4.6 x 150 mm), flow rate: 1 ml/min, fraction volume: 1 ml.
DISCUSSION

Using β-gal-linked motilin as a marker antigen, anti-rabbit IgG-coated immunoplates and MUG as a fluorogenic enzyme substrate, we developed a highly sensitive and specific EIA for quantitating motilin. Since 1975, RIA methods developed for motilin have been used widely, however, these methods have several disadvantages due to the use of radioisotopes. The EIA detailed in this report retains the advantages of the RIA system while minimizing the disadvantages. This EIA is highly sensitive (20 fmol/ml (= 0.8 fmol/well)) and specific for motilin, and the sharp standard inhibition curve obtained was linear between 20 and 200 fmol/ml.

We applied the EIA to determine the concentration of M-IS in human plasma. The M-IS levels in human plasma from 3 normal subjects determined after lunch had an extensive range (53.9 ± 25.6 pg/ml) and were slightly lower than those reported by Preston et al.13) using RIA methods.

In this study, we identified immunoreactive motilin in human plasma extract using an EIA. The major peak of M-IS in human plasma was eluted at an identical position to that of synthetic motilin, with several minor peaks (Fig. 3). Thus, this simple and sensitive EIA is likely to be useful for measuring M-IS in biologic fluids and tissues.

REFERENCES