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Ultrasensitive detection of pepsinogen I and pepsinogen II by a time-resolved fluoroimmunoassay and its preliminary clinical applications

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Abstract

A fast and highly sensitive assay for pepsinogen I (PG I) and pepsinogen II (PG II) by using time-resolved fluoroimmunoassay (TRFIA) detection technique has been developed for the determination of serum PG I and PG II against gastrointestinal diseases. On the noncompetitive assay, one monoclonal antibody (McAb) coated on wells was directed against a specific antigenic site on the PG I or PG II. The McAb, called as labelling McAb, was prepared with the europium-chelate of *N*-(*p*-isothiocyanatobenzyl)-diethylenetriamine-*N*,*N*,*N*,*N*-tetraacetic acid and directed against a different antigenic site on the PG I or PG II molecule. After bound/free separation by washing, the fluorescence counts of bound Eu^{3+}–McAb were measured. The levels of PG in sera from patients or healthy volunteers were determined by PG I and PG II TRFIA using the autoDELFIA$_{1235}$ system. The measurement ranges of PG I-TRFIA were 3.5–328.0 $\mu g\,L^{-1}$ and those of PG II-TRFIA were 2.0–55.0 $\mu g\,L^{-1}$. The within-run and between-run CVs of the PG I-TRFIA were 1.9% and 4.7%, respectively, and those of PG II-TRFIA were 2.1% and 3.8%, respectively. The recovery rates of PG I-TRFIA and PG II-TRFIA were 102.7% and 104.6%, respectively. The detection limitations of PG I and PG II were 0.05 $\mu g\,L^{-1}$ and 0.02 $\mu g\,L^{-1}$, respectively. The dilution experiments showed the percentage of expected value of PG I-TRFIA was 93.2–102.3% and of PG II-TRFIA was 97.3–110.6%. The cross-reacting rate between PG I and PG II was negligible. The linear correlation of radioimmunoassay (RIA) and TRFIA measurements resulted in a correlation coefficient as 0.926 of PG I and as 0.959 of PG II. The europium-labelling McAbs were stable for at least one year at −20 °C, and the results of the TRFIA with same reagents were reproducible over one year as well. The means of 1600 healthy volunteers were $162.4 \pm 52.1\ \mu g\,L^{-1}$ for serum PG I, $11.7 \pm 6.8\ \mu g\,L^{-1}$ for serum PG II, and 13.8 ± 7.4 for the PG I/PG II ratio. The normal ranges of Serum PG I levels for healthy volunteers were 58.2–266.6 $\mu g\,L^{-1}$, and those of serum PG II levels were less than 25.3 $\mu g\,L^{-1}$. The availability of a highly sensitive, reliable, and convenient PG-TRFIA method for quantifying PG will allow investigations into the possible diagnostic value of this analysis in various clinical conditions, including gastric carcinoma, duodenal ulcer, gastric ulcer and gastritis. The sensitivity and reproducibility of the assay were satisfactory for clinical applications.

Keywords: Time-resolved fluoroimmunoassay (TRFIA); Pepsinogen I; Pepsinogen II; Monoclonal antibody; Analysis

1. Introduction

In recent years, with the application of immunoassay in clinical medicine, *Helicobacter pylori* infection, duodenal ulcer, gastritis and gastric cancer are often diagnosed with non-endoscopic methods, such as pepsinogen (PG) [1–3]. PG includes pepsinogen I (PG I) and pepsinogen II (PG II), which differ by their structural and immunological characteristics. PG I and PG II are synthesized in the gastric mucosa and secreted into the gastric lumen, where they are converted into the active enzyme under acidic conditions. But some of PG also enters the blood circulation [4].

Given the potential role of PG as a prognostic marker in gastrointestinal disease, it is essential to have a reliable and highly sensitive quantitative method for its measurement in sera from patients [5]. Both the radioimmunoassay (RIA) using rabbit antibodies and the enzyme-linked immunosorbent assay

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(ELISA) using monoclonal antibodies (McAbs) were used to measure pepsinogens. The radio-labelling pepsinogens used in RIA appeared to be relatively unstable from weeks to months and the ELISA was not sensitive enough [6–9]. In this report, we used the method of time-resolved fluoroimmunoassay technique (TRFIA) to measure PG I and PG II, which is more sensitive than any reported RIA or ELISA [10,11]. The TRFIA is based on the use of lanthanide (such as europium) chelate labeling with unique fluorescence properties. The fluorescence lifetime of the specific signal is several orders of magnitude longer than the non-specific background. This enables the label to be measured at a time when the background has already decayed. The special fluorescence properties of lanthanides are long in decay time, large in stokes' shift, sharp in emission peak, and high in fluorescence intensity. These properties contribute a lot to the increase of the signal-to-noise ratio. The labeled compounds of TRFIA have a high specific activity and good stability with a minimal influence on biological activity. Its general robustness and automation friendliness have also led to its widespread use in clinical screening and diagnostics. PG I and PG II are regarded as a marker in the early diagnosis of gastric carcinoma and in the mass screening in high risk population [12,13]. This report describes the development of a sensitive and specific TRFIA for PG I and PG II using McAbs and Eu chelates labeling, and the distribution of serum PG I and PG II levels in healthy volunteers, peptic ulcer, and gastric cancer patients were also reported. This assay has the advantages as high sensitivity, convenient manipulation, wide detection ranges, high stability, and non-radioactive labeling, so that PG I and PG II-TRFIA could be used as an important non-invasive tool for elucidating the possible diagnostic role of PG in sera in vitro.

2. Experimental

2.1. Chemicals and instrumentation

Diethylenetriaminepentaacetate (DTPA), Bovine serum albumin (BSA), Tris, and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). PD-10 column and Sepharose CL-6B column were from the Pharmacia Co. (England). The 96-well polystyrene microtitre plates were obtained from Nunc International (Denmark). Europium-labeling kit (1244-302), including N^1-(*p*-isothiocyanatobenzyl)-diethylenetriamine-N^1,N^2,N^3,N^4-tetraacetic acid (DTTA), was purchased from Perkin-Elmer (USA). β-Naphthoyltrifluoroacetone (β-NTA) was synthesized in our laboratory. Monoclonal antibodies to human PG I and PG II and PG I and PG II radioimmunassay (RIA) kits were obtained from Chinese Institute of Cancer (Beijin, China). Q2 anion exchange chromatography, DEAE-52 chromatography, and gel filtration HPLC were purchased from Bio-Rad Co. (USA). Pure water was produced by Barnstead Equipment. Other reagents used were of analytical reagent grade.

A model DU-650 spectrometer from Beckman (German), at 280 nm, was used for detection of proteins during collection of the antibodies in the purification process. AutoDELFIA$_{1235}$, from Perkin-Elmer, was used to measure Eu^{3+} fluorescence in microtiter wells.

2.2. Blood samples

Healthy volunteers: Serum samples were obtained from 1600 subjects free from upper abdominal complaints and without evidences of gastroduodenal disorder, liver diseases and renal diseases after health examination.

Patients: the blood samples from patients were collected from endoscope examinations and histologic examinations.

2.3. Purification of PG and calibrators

The surgically resected stomach tissues were free from the invaded part. The PG I and PG II were purified by using DEAE-52 chromatography, gel filtration HPLC, and Q2 anion exchange chromatography, as described previously [8]. PG I calibrators of 0, 5, 10, 50, 100, and 300 μg L^{-1} were prepared by diluting highly purified PG I in assay buffer (0.05 mol L^{-1} Tris–HCl, pH 7.8, 0.9% NaCl, 0.2% of purified BSA, 0.01% Tween-20, 20 μmol L^{-1} DTPA, and 0.05% sodium azide). PG II calibrators were 0, 5, 10, 20, 30, and 50 μg L^{-1} in the same buffer.

2.4. Preparation of solid antibody

The polystyrene microtiter plates were coated with the captured monoclonal antibodies diluted in coating solution (50 mmol L^{-1} Na_2CO_3–$NaHCO_3$, pH 9.6) by incubating overnight at 4 °C, and blocked with the blocking buffer for 2 h. After the blocking solution were removed, the wells were dried in a high vacuum, and the plates were sealed and stored at −20 °C, as described previously [14].

2.5. Preparation of enhancement solution

The luminescent enhancement system was a solution mainly containing 2-naphthoyltrifluoroacetone (β-NTA). In 1 L of enhancement solution, it contains 15 μmol β-NTA, 50 μmol tri-*n*-octylphosphine oxide, and 1 mL Triton X-100, pH 3.2 [15].

2.6. Labelling McAbs with Eu^{3+}

The buffer for McAbs was exchanged to the labeling buffer (pH 8.5, 50 mmol L^{-1} Na_2CO_3–$NaHCO_3$ 0.155 mol L^{-1} NaCl) by PD-10 column. Europium-chelate of DTTA (0.2 mg into 100 μL H_2O) was added to the 1.0 mg of McAbs in 500 μL labelling buffer (pH 8.5), then mixed gently and incubated overnight at room temperature for 18 h. Separation of the labelled antibody from unreacted chelate and aggregated McAb was performed by gel filtration using a Sepharose CL-6B (1× 40 cm) column with an elution buffer of 50 mmol L^{-1} Tris–HCl (pH 7.8) containing 0.9% NaCl and 0.05% sodium azide. The fractions were collected by 1.0 mL per fraction. The concentration of Eu^{3+} was determined from an aliquot, which was diluted with enhancement solution (1:1000). The fluorescence was measured in the microtitration wells (200 μL $well^{-1}$). The signal was compared to the signal of stock standards diluted 1:100 in enhancement solution. The fractions from the first peak with the highest Eu counts were pooled and characterized. Labelled

McAbs were preserved by rapid freezing and drying in a high vacuum after dilution with elution buffer containing 0.2% BSA as a stabilizer. The labelled McAbs were stored at −20 °C.

2.7. PG I and PG II assay protocol

The procedures for TRFIA of serum PG I and PG II were same, which was performed by using two-step non-competitive "sandwich-type" technique. In brief, 25 μL of Calibrators (samples) and 200 μL of the assay buffer were pipetted into coated microtiter wells. The plates were incubated at 25 °C with shaking for 1 h, washed twice (washing solution containing 0.05 mol L^{-1} Tris–HCl, pH 7.8, 0.02% Tween-20, and 0.05% sodium azide), then 200 μL of 50-fold diluted Eu^{3+}–McAb solution in assay buffer was added. The plates were incubated again at 25 °C with shaking for 1 h. After washed 6 times, 200 μL of enhancement solution were dispensed into each well. The plates were shaken for 5 min before fluorescence readings. All the procedures were auto-controlled by autoDELFIA$_{1235}$, with the software designed in our lab. The calibration curve and calculation of the concentrations in the unknown samples were performed automatically by using Multicalc software program, where a spline algorithm on logarithmically transformed data was employed.

2.8. RIA of serum PG I and PG II

Fifty Sera samples were assayed by RIA kits according to the kit instructions and the results were compared to those of measurement by the TRFIA.

3. Results and discussion

3.1. Labelling yield

The anti-PG I IgG and anti-PG II IgG were Eu-labeled as described above and the average labelling yields were 8.6 Eu^{3+}/McAb and 12.3 Eu^{3+}/McAb, respectively, giving high sensitivity with low background (<1000 cps).

3.2. Calibration curve, detection limition and precision

The calibration graphs of PG I and PG II were found to be linear over the concentration. The line equation for the calibration curve of PG I was $y = 1190.4x + 1092.6$ and that of PG II was $y = 5594.6x - 4839.4$ [y—response counts (cps); x—concentration (μg L^{-1})]. With 25 μL of serum samples, the measurement ranges of PG I-TRFIA were 3.5–328.0 μg L^{-1} with ED25, ED50, and ED80 being 11.34 ± 0.2 μg L^{-1}, 38.73 ± 0.8 μg L^{-1}, and 132.3 ± 2.9 μg L^{-1}, respectively. The measurement ranges of PG II-TRFIA were 2.0–55.0 μg L^{-1} with ED25, ED50, and ED80 being 7.84 ± 0.2 μg L^{-1},16.2 ± 0.7 μg L^{-1}, and 34.6 ± 1.3 μg L^{-1}, respectively. The limit of detection, defined by the concentration of PG I and PG II corresponding to the fluorescence of the zero calibrators plus two S.D., were 0.05 μg L^{-1} and 0.02 μg L^{-1}, respectively. Assessed at various PG I concentrations from 5 to 300 μg L^{-1}. The intra-assay and inter-assay coefficients

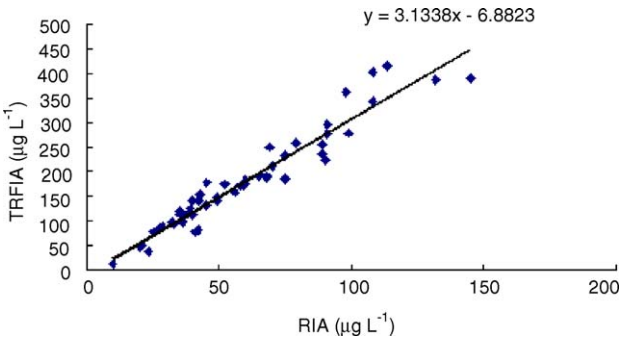

Fig. 1. The linear correlation of PG I TRFIA and RIA.

of variation (CVs) of PG I-TRFIA were 1.9% and 4.7%, respectively; and those of PG II-TRFIA were 2.1% and 3.8%, respectively, assessed at various PG II concentrations from 5 to 50 μg L^{-1}. The cross-reactivity was checked between PG I and PG II and no cross-reactivity was found. Samples with relatively high PG I (PG II) concentrations were analyzed at various dilutions and calculated the percentage of expected value. The diluting buffer was the same as the calibrator buffer. The percentage of expected value of PG I-TRFIA was 93.2–102.3% for all dilutions, and that of PG II-TRFIA was 97.3–110.6%. Recovery was checked by supplementing purified PG I at 80 and 20 μg L^{-1}, respectively, in serum, and PG II at 30 and 10 μg L^{-1}, respectively. By analysis, the average recovery rates of PG I-TRFIA and PG II-TRFIA were 102.7% and 104.6%, respectively. No high-dose hook effect of PG I-TRFIA was observed at 18,000 μg L^{-1}, and at 5000 μg L^{-1} for PG II-TRFIA.

3.3. Analysis of samples

The serum PG I and PG II concentrations were assayed in 50 samples with the RIA kits, which were plotted against TRFIA determinations in Figs. 1 and 2. The linear correlation of both measurements resulted in a correlation coefficient 0.926 of PG I ($y = 3.1338x - 6.8823$, $n = 50$), and 0.959 of PG II ($y = 0.9686x + 0.0332$, $n = 50$).

The means of 1600 healthy volunteers were 162.4 ± 52.1 μg L^{-1} for serum PG I, 11.7 ± 6.8 μg L^{-1} for serum PG II, and 13.8 ± 7.4 for the PG I/PG II ratio. The normal ranges of serum PG I concentrations of healthy volunteers, calculated as means ±2S.D., were 58.2–266.6 μg L^{-1}. The low range was

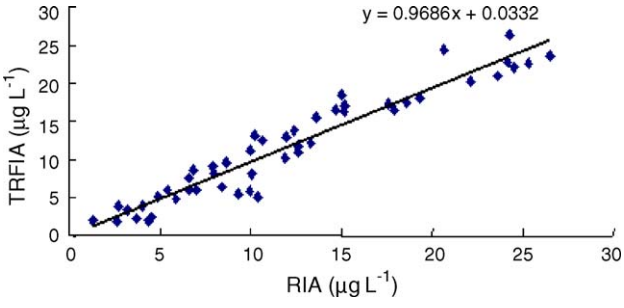

Fig. 2. The liner correlation of PG II TRFIA and RIA.

Table 1
Serum PG concentrations and the ratio of PG I/PG II in healthy volunteers, gastric ulcer and duodenal ulcer patients, gastric cancer patients

Diagnosis	No.	PG I means ($\mu g\,L^{-1}$)	PG II means ($\mu g\,L^{-1}$)	PG I/PG II Ratio means
Healthy volunteers	1600	162.4	11.7	13.8
Gastric ulcer	23	226.0	21.5	10.5
Duodenal ulcer	48	272.5	23.7	11.4
Gastric cancer	30	131.2	24.6	5.3

the same as reported in some references [16–18], and the normal range of serum PG II concentrations were less than 25.3 $\mu g\,L^{-1}$. The cut-off points were 6.0 for the PG I/PG II ratio. There were strong positive correlations between serum PG I and PG II levels in volunteers. The serum levels of both PG I and PG II were significantly higher in the gastric ulcer and duodenal ulcer than in any other groups. The gastric cancer group had a lower concentration of serum PG I and a lower ratio of PG I/PG II in comparison with the healthy volunteers group (see Table 1).

Gastroscopy and examination of biopsy are normally required for diagnosis of gastric diseases, such as gastritis, peptic ulcer, gastric cancer. This is both costly and inconvenient for the patients, and especially not suitable for mass screening. There is a need for a simple method to reduce the endoscopy workload. Many research efforts have been focused on PG [1,16–19]. PG is the inactive precursor of pepsin. This molecule is synthesized primarily in the chief and mucus cells of the gastric glands and is secreted into the gastric lumen where it is converted to the active form under acidic conditions [4]. The secretory ability of gastric mucosa differs due to the change of the chief cells when atrophic gastritis, peptic ulcer and gastric cancer occur. Since the serum levels of PG I and PG II reflect primarily the number of chief cells and accessory cells in the gastric fundus, it is believed that the determination of the concentrations of PG I and PG II in serum, as well as the ratio of PG I/PG II, may be very useful in the evaluation of the degree of atrophic gastritis and the diagnosis of peptic ulcer. The serum PG I levels in patients with gastric cancer, atrophic gastritis might be lower, while it might be higher in patients with peptic ulcer. The atrophic gastritis and *Helicobacter pylori* infection are considered as risk factors for the development of malignancy; therefore, the determination of PG in serum may also be useful as a screening tool in populations at high risk from stomach cancer. Recent research found that PG II was also associated with breast cancer [20,21]. So the availability of a highly sensitive, reliable, and convenient method for quantifying PG could be a useful tool in those various clinical applications.

To our best knowledge, this is the first time that PG-TRFIA, as a highly sensitive and specific immunofluorometric method with McAbs, is established by using the dissociation-enhancement immunofluorometric principle. In the assays, the fluorescence of the labelled McAbs after binding reaction is enhanced by the addition of enhancement solution. The low pH of enhancement solution efficiently dissociates the europium from the labelled compound. The free Eu^{3+} rapidly forms a new, highly fluorescent chelate inside a protective miscelle with components of the enhancement solution. The fluorescence of the lanthanide chelate is amplified 1–10 million times by the enhancement technique. The technique has been developed to increase the sensitivity of the analysis. The time-resolved principle is applied in fluoroimmunoassays to eliminate background interferences. These technologies created the high-sensitivity PG-TRFIA. The stability of the chelate allows long-term storage of labelled McAbs. The combination of the solid-phase chemistry and the new nonisotopic labeling has particularly facilitated the current automation level in the field of immunoassays.

4. Conclusion

Our experimental results indicate that the newly developed PG-TRFIA is more sensitive than RIA or ELISA [8,9], and the assay also provides wider dynamic working ranges and better reproducibility. The results of the TRFIA with same reagents were reproducible for one year. Two-step incubation in our assay can reduce the interference of assay system. An excellent correlation was found between the PG-TRFIA and PG-RIA. In addition, the 2-h automatic protocol makes the assay be simple to operate. The random handling errors can also be reduced significantly. Only as small as a 25 μL of serum sample is enough for simultaneous measurement, which is very useful for mass screening since several determinations are usually performed on each sample. The concept is extremely simple to be applied from the user's point of view because the $AutoDELFIA_{1235}$ time-resolved fluorometer enables fast and simple assays.

From the Results, the sera with either low or high PG I levels in patients have their values. We have finished almost 2000 case studies and whenever we found that the subject has a plasma PG I, PG II, and PG I/PG II ratio with values beyond the normal ranges, we advise the subject for endoscope examination. We observed a correlation between the increase of PG I or PG II serum level and gastric ulcer or duodenal ulcer, as well as a correlation between the decrease of serum PG I level or PG I/PG II ratio and severe atrophic gastritis or gastric cancer. Through the analysis of data, we observed that, in many dyspeptic patients, including early gastric cancer patients, the serum PG level could be either higher or lower than the normal ranges. As a result, we believe that PG should be regarded as one of the indicators of gastric mucosa function. There are many factors that affect the level of serum PG and the specificity of PG is poor. Also the detection of PG in sera may provide an early warning of developing gastric cancer to patients, but it will be inappropriate to use it as an indicator of gastric cancer when the values of the serum PG I level or PG I/PG II ratio are lower than the normal ranges. The best utilization of this technology is mass screening in high-risk population, find potential patients with gastrointestinal diseases, and suggest them for further endoscope examinations or biopsy diagnosis. It will be beneficial not only for the diagnosis of atrophic gastritis or gastric cancer, but also for the early discovery of gastric ulcer, duodenal ulcer or gastritis as well. We will perform further research on this issue.

In summary, we presented a quantitative and fast immunological assay for measuring PG I and PG II levels in serum. An

intrinsically fluorescent stable Eu chelate with high activity is used as the labeling. The assay is suitable for potential automation and is simpler than RIA. Therefore, it has a great value for immunoassay development of PG. We believe that the availability of this highly sensitive method will facilitate the further studying of PG.

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