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BRIEF ARTICLE

Simultaneous detection of different serum pepsinogens and its primary application

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Abstract

AIM: To develop the simple, rapid and sensitive dual-label time-resolved fluoroimmunoassay for pepsinogens in human serum.

METHODS: Based on two-site sandwich protocol, monoclonal antibodies (McAbs) against pepsinogen Ⅰ (PG Ⅰ) and PG Ⅱ were co-coated in 96 microtitration wells, and tracer McAbs against PG Ⅰ and PG Ⅱ were labeled with europium (Eu) and samarium (Sm) chelate, respectively. Diluted serum samples of Eu^{3+}- and Sm^{3+}-McAbs were added into microtitration wells simultaneously. After washing, fluorescence of bound Sm^{3+} and Eu^{3+} tracers was detected.

RESULTS: The detection limit was 0.2 μg/L for PG Ⅰ and 0.05 μg/L for PG Ⅱ. The assay range was 5.0-320.0 μg/L for PG Ⅰ and 1.0-55.0 μg/L for PG Ⅱ. The average recovery rate was 102.7% for PG Ⅰ and 98.8% for PG Ⅱ. Sera from healthy controls and patients with gastric disease were analyzed. The PG detected by dual-label assay was in good agreement with that detected by single-label assay or by enzyme-linked immunosorbent assay.

CONCLUSION: Dual-label assay can provide high-throughput serological screening for gastric diseases.

Key words: Serum pepsinogen; Simultaneous detection; Time-resolved fluoroimmunoassay

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INTRODUCTION

There is considerable interest in developing methods to simultaneously quantify two or more analyses of one sample in a single assay[1,2], which is advantageous to the confidence level of results, especially in cases when the ratio of compounds gives important information[3,4]. Dual-label has potential applications in various fields such as microbiology, molecular biology, drug analysis, and clinical research. Fluorescence immunoassay, like other immunoassays involving non-isotopic labeling, has been well accepted as a stable, inexpensive, rapid, and sensitive

method. However, conventional fluorescent labeling has a limited success in assay of multiple analytes because of its high background, short decay time and broad spectrum, which make it difficult to be distinguished between its emission bands[5,6]. Up to now, fluorescent lanthanide is a favorable choice owing to its narrow emission peak at different wavelengths. Its lifetime ranges 50-1000 μs (over four decades longer than the average background duration) depending on the temperature and the solvent presented[7]. These features can be utilized for optimization of the measurement conditions to get the maximal sensitivity and to minimize the signal spillover. The europium ion (Eu^{3+}), is the lanthanide mainly used in time-resolved fluoroimmunoassay (TRFIA)[8]. Eu^{3+} and terbium ion (Tb^{3+}) form the most efficient fluorescent chelates, but Tb^{3+} requires fluorinated aliphatic β-diketone for simultaneous detection[9], rather than β-naphthoyltrifluoroacetone (β-NTA) used in the enhancement solution optimized for Eu^{3+} detection. β-NTA is also applicable to samarium ion (Sm^{3+}) excitation, which has thus been suggested that Sm^{3+} can be used as a counterpart to Eu^{3+} with the same enhancement formulation (enhancement solution or DELFIA inducer) in a dual-label system[10].

Human pepsinogens originating from gastric mucosa can be classified into two immunochemically distinct groups: pepsinogen I (PG I) and PG Ⅱ[11], which are mostly secreted into the gastric lumen and nearly 1% of them are leaked into the blood circulation. Serum PG levels reflect the morphological and functional status of gastric mucosa. Human pepsinogens have a diagnostic value for various gastroduodenal disorders, especially for peptic ulcer and atrophic gastritis, which have been widely discussed[12,13]. The PG I /PG Ⅱ ratio can provide even better information on the extent of chronic gastritis than gastric intubation[14].

Since PG I and PG Ⅱ serve as useful predictors in early diagnosis of gastric cancer and in mass screening of populations at a high risk of gastric cancer[15,16], a reliable and sensitive method is needed to detect PG I and PG Ⅱ in human sera. In our previous study[17], a fast and highly sensitive TRFIA was developed to measure serum PG I and PG Ⅱ. The present study was to evaluate the dual-label TRFIA for simultaneous detection of PG I and PG Ⅱ in human serum.

MATERIALS AND METHODS

Chemicals and instruments

Diethylenetriaminepentaacetate (DTPA), bovine serum albumin (BSA), Tris and Triton X-100 were purchased from Sigma (St. Louis, MO, USA). PD-10 column and sepharoseCL-6B column were from the Pharmacia Company (Chalfont St Giles, UK). Q2 anion exchange chromatography, DEAE-52 chromatography, and gel filtration HPLC were purchased from Bio-Rad Company (Hercules, USA). Pure water was produced by Barnstead Equipment (Dubuque, Iowa, USA). Ninety six -well polystyrene microtitre plates were obtained from Nunc International (Roskilde, Denmark). Eu-labeling reagent 1244-302 and Sm-labeling reagent 1244-303, both including N'-[*p*-isothiocyanatobenzyl]-diethylenetriamine-N^1, N^2, N^3, N^4-tetraacetic acid, were purchased from Perkin-Elmer (Waltham, Massachusetts, USA). β-NTA was synthesized in our laboratory. Two counterparts of monoclonal antibodies (McAbs) to human PG I and PG Ⅱ respectively for capture and detection were obtained from Chinese Institute of Cancer with a purity of over 95% (Beijing, China). Enzyme-linked immunosorbent assay (ELISA) kits for detection of PG I and PG Ⅱ were from Biohit Plc(Helsinki, Finland). AutoDELFIA1235, from Perkin-Elmer (Waltham, Massachusetts, USA), was used to measure Eu^{3+} and Sm^{3+} fluorescence in microtiter wells. All other reagents used were of analytical grade.

Reagent solutions

Labeling buffer contained 50 mmol/L Na_2CO_3-$NaHCO_3$ (pH 8.5), and 0.155 mol/L NaCl. Elution buffer contained 50 mmol/L Tris-HC1 (pH 7.8), 0.9% NaC1, and 0.05% NaN_3. Assay buffer contained 50 mmol/L Tris-HCl (pH 7.8), 0.9% NaC1, 0.2% BSA, 0.05% NaN_3, 20 μmol/L DTPA, and 0.1% Tween-20. Washing solution was a Tris-HCl buffered saline solution (pH 7.8) containing 0.9% NaCl, 0.2% Tween-20, and 0.05% NaN_3. Enhancement solution was a 0.1 mol/L acetate-phthalate buffer (pH 3.2) containing 0.1% triton X-100, 15 μmol /L βNTA, and 50 μmol/L tri-*n*-octylphosphine oxide.

Serum samples

Serum samples, collected from healthy volunteers who had no upper abdominal complaints and evidence of gastroduodenal disorder and liver diseases, were stored at -20℃. Blood samples were collected from patients at endoscopic and histological examinations. This study was conducted with the approval of the Ethics Committee of Jiangyuan Hospital Affiliated to Jiangsu Institute of Nuclear Medicine.

Immobilization of McAbs

Five micrograms of the capture McAbs to PG I and PG Ⅱ in 200 μL of 50 mmol of Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) was co-immobilized in each well and incubated overnight at room temperature. After washing, 200 μL of 1 g/L BSA in 50 mmol of Na_2CO_3-$NaHCO_3$ buffer (pH 9.6) was added to block the coated surface for 2 h. After the blocking solution was removed, the plates were dried in a high vacuum, and then stored at -20℃ in a sealed plastic bag with desiccant.

Labeling of McAbs with Eu^{3+}- and Sm^{3+}-chelates

McAbs to human PG I (PG I McAbs) and PG Ⅱ (PG Ⅱ McAbs) were labeled with Sm^{3+}- and Eu^{3+}-chelates, respectively. The buffer for McAbs was replaced with the labeling buffer. Five hundred micrograms of PG I McAbs was gently mixed in 200 μL of labeling buffer with 250 μg of Sm^{3+}-chelates in 100 μL of the same buffer. After an 18-h incubation with continuous gentle shaking at room

temperature, free Sm^{3+}-chelates and aggregated McAbs were separated from Sm^{3+}-McAbs conjugates using a 1 cm × 40 cm column packed with sepharose CL-6B (lower 20 cm), eluted with a descending elution buffer, and collected with 1.0 mL per fraction. The concentration of Sm^{3+}-conjugates in collected fraction was measured with fluorescence, and diluted with an enhancement solution (1:1000). The fluorescence in microtitration wells (200 μL per well) was detected by comparing the signal of samples to that of stock standards diluted at 1:100 in an enhancement solution. The fractions from the first peak with the highest Sm^{3+} count were pooled and characterized. PG Ⅱ McAbs were labeled with Eu. The labeled McAbs were rapidly lyophilized under high vacuum after dilution with an elution buffer containing 0.2% BSA as a stabilizer, and stored at -20℃. No loss of immunoreactivity was observed during a 6-mo storage period.

Purification of PG and calibrators

Surgically resected stomach tissues were free from the invaded part. PG Ⅰ and PG Ⅱ were purified by DEAE-52 chromatography, gel filtration HPLC, and Q2 anion exchange chromatography, as previously described[18]. The purity of PG Ⅰ was over 98% and that of PG Ⅱ was over 95.0%. Calibrators were prepared by diluting them with the assay buffer containing 0, 5, 10, 50, 100, 300 μg/L of highly purified PG Ⅰ and 0, 5, 10, 20, 30, 50 μg/L of highly purified PG Ⅱ, respectively.

Assay procedure

Dual-label TRFIA was performed to detect PG Ⅰ and PG Ⅱ simultaneously in serum with a one-step "sandwich-type" protocol. In brief, 25 μL of calibrators (samples) and 200 μL of 50-fold diluted Eu^{3+} and Sm^{3+} tracer McAbs solution in assay buffer were pipetted into the coated microtitration wells. The plates were incubated with continuous shaking for 2 h at 25℃. After washed 6 times, 200 μL of enhancement solution was added into each well. The plates were shaken for 5 min before fluorescence reading. All procedures were automatically performed by $_{auto}$DELFIA$_{1235}$ with the software designed in our laboratory. Calibration curve was plotted and concentrations in unknown samples were measured using Multicalc software program, where a spline algorithm on logarithmically transformed data was employed. ELISA was performed with a kit following its instructions.

Statistical analysis

Data about PG Ⅰ or PG Ⅱ were expressed as mean ± SD. The limit of detection was defined by the concentration of PG Ⅰ or PG Ⅱ corresponding to the fluorescence of the zero calibrators plus two SD. The average intra- or inter-assay coefficient of variation (CV) was calculated for the precision of the assay. The recovery rate was evaluated by comparing the measured and theoretical values. Regression analysis was used to display the linearity and correlations. Differences in patients with gastric disease and healthy controls were analyzed using paired *t*-test. $P < 0.05$ was considered statistically significant. Analysis of data was performed using SPSS 13.0 (Chicago, IL, USA).

Table 1 Precision of dual-label assay for PG Ⅰ and PG Ⅱ in serum of controls[1]

	PG Ⅰ (μg/L)		PG Ⅱ (μg/L)	
	mean ± SD	CV (%)	mean ± SD	CV (%)
Serum pool 1				
Within-run (n = 10)	43.2 ± 1.36	3.2	5.23 ± 4.62	4.8
Between-run (n = 6)	42.8 ± 2.28	5.1	5.65 ± 6.45	6.7
Serum pool 2				
Within-run (n = 10)	105.0 ± 1.88	2.3	11.7 ± 3.66	3.9
Between-run (n = 6)	103.8 ± 2.73	3.6	10.5 ± 4.55	5.1
Serum pool 3				
Within-run (n = 10)	198.2 ± 5.31	6.3	22.0 ± 3.88	4.6
Between-run (n = 6)	186.7 ± 4.35	5.4	21.2 ± 5.71	8.3

[1]Served as controls. PG: Pepsinogen; CV: Coefficient of variation.

RESULTS

Kinetics, detection limits and precision

The calibrators covered a range of 10-300 μg/L of PG Ⅰ and 2-50 μg/L of PG Ⅱ. The serum samples from healthy controls and patients with chronic atrophic gastritis and peptic ulcer were incubated for different periods of time (60, 90, 120, 150 min) at different temperatures (25℃, 37℃). Both calibrators and serum samples reached a plateau value around 120 min at 25℃ and around 60 min at 37℃, respectively. In this study, the incubation time was 120 min and the temperature was 25℃ for the assay on $_{auto}$DELFIA$_{1235}$.

The calibration curves of PG Ⅰ and PG Ⅱ were linear over the concentration. The equation was y = 46.4x + 383.2 for the calibration curve of PG Ⅰ and y = 2198.2x + 5189.1 for the calibration curve of PG Ⅱ, where y indicates the response counts (cps), x indicates the concentration (μg/L). With 25 μL of serum samples, the measurement range of PG Ⅰ, ED_{20}, ED_{50}, and ED_{80} was 5.0-320.0, 19.87 ± 4.3, 64.32 ± 6.2, and 176.0 ± 12.9 μg/L, respectively, and the measurement range of PG Ⅱ, ED_{20}, ED_{50}, and ED_{80} was 1.0-55.0, 3.546 ± 2.2, 9.746 ± 4.7, and 23.79 ± 6.3 μg/L, respectively.

The limit of detection was 0.2 μg/L for PG Ⅰ and 0.05 μg/L for PG Ⅱ. The average intra-assay CV of the calibrators was 4.6% for PG Ⅰ and 5.3% for PG Ⅱ. The intra- and inter-assay CV of serum-based controls was summarized in Table 1. The results showed that the assay had a good precision not only for the calibrators but also for the clinical samples.

The cross-reactivity between anti-PG Ⅰ antibody to PG Ⅱ and anti-PG Ⅱ antibody to PG Ⅰ was detected. No interference between them was found. The result showed that the specificity of the assay was good.

Analysis of samples

The correlations between dual-label and single-label assay are shown in Figure 1. The correlation ratio between

Table 2 Serum PG level and PG Ⅰ/PG Ⅱ ratio in healthy controls and patients with duodenal ulcer, gastric ulcer, atrophic gastritis, superficial gastritis and gastric cancer (mean ± SD)

Diagnosis	*n*	Age (yr)	PG Ⅰ (μg/L)	PG Ⅱ (μg/L)	PG Ⅰ/PG Ⅱ ratio
Healthy controls	500	41.5 ± 18.8	150.3 ± 45.1	10.4 ± 8.4	14.5 ± 4.3
Patients with duodenal ulcer	112	43 ± 14.2	252.9 ± 84.5[d]	18.4 ± 16.0[d]	13.7 ± 8.0
Patients with gastric ulcer	44	37 ± 11.2	221.2 ± 91.7[d]	15.6 ± 12.4[b]	14.2 ± 7.1
Patients with atrophic gastritis	21	48 ± 9.7	89.5 ± 51.2[d]	12.9 ± 9.39	6.9 ± 6.2[b]
Patients with superficial gastritis	76	35 ± 7.4	175.3 ± 45.8[a]	12.7 ± 10.1	13.8 ± 5.3
Patients with gastric cancer	126	55 ± 12.2	157.1 ± 81.9	15.6 ± 14.4[b]	13.4 ± 7.8

[a]$P < 0.05$, [b]$P < 0.01$, [d]$P < 0.001$ *vs* healthy controls.

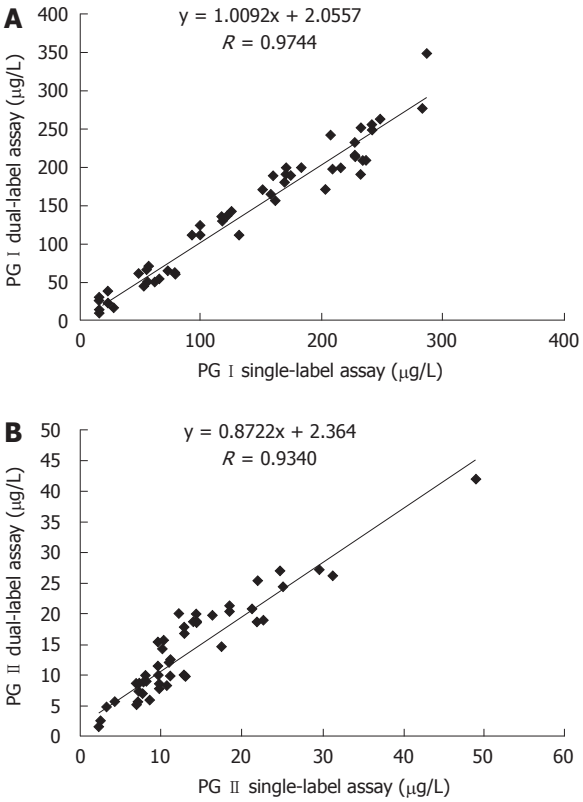

Figure 1 Correlation between single-label and dual-label assay of pepsinogen (PG) Ⅰ (A) and PG Ⅱ (B) in human sera (*n* = 50).

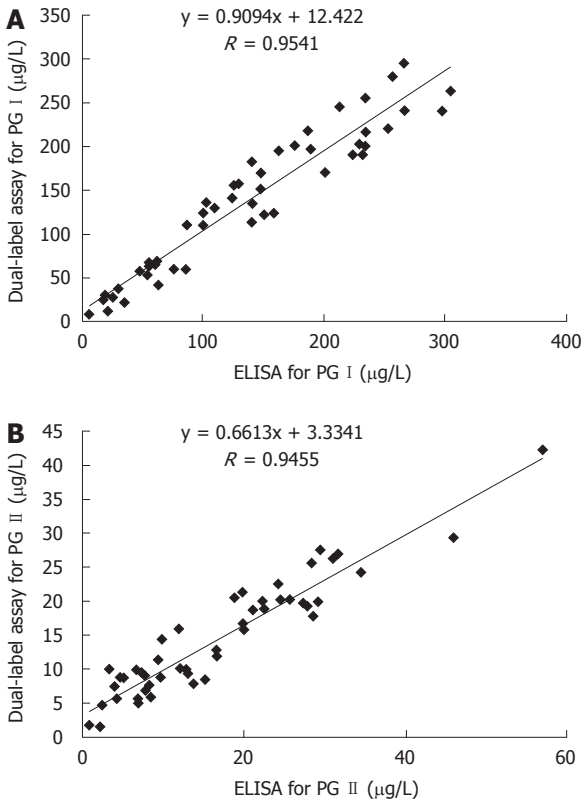

Figure 2 Correlation between enzyme-linked immunosorbent assay (ELISA) and dual-label assay of PG Ⅰ (A) and PG Ⅱ (B) in human sera (*n* = 50).

single-label and dual-label assay for PG Ⅰ and PG Ⅱ was 0.9744 and 0.9340, respectively. The correlations between dual-label assay and ELISA for PG Ⅰ and PG Ⅱ are shown in Figure 2. The correlation ratio between ELISA and dual-label for PG Ⅰ and PG Ⅱ was 0.9541 and 0.94550, respectively. The results indicate that the dual-label assay is in agreement with ELISA or with single-label assay.

The serum samples were analyzed by dual-label assay (Table 2). The normal range of serum PG Ⅰ in healthy controls was 60.1-240.3 μg/L. The range of serum PG Ⅱ was lower than 27.2 μg/L. The cut-off point was 5.9 for the PG Ⅰ/PG Ⅱ ratio.

The serum PG level was lower or higher than its normal range in dyspeptic patients including those with gastric cancer. The distributions of PG Ⅰ and PG Ⅱ value and PG Ⅰ/PG Ⅱ ratio in patients with gastric cancer and in those with duodenal ulcer are shown in Table 3. The difference in PG Ⅰ value was relatively small. The serum PG Ⅰ level was remarkably higher in peptic ulcer patients, especially in those with active duodenal ulcer than in healthy controls. The increased PG Ⅰ level would be a high risk factor for duodenal ulcer, and a remarkably low serum PG Ⅰ level could exclude the diagnosis of peptic ulcer[19].

DISCUSSION

Eu^{3+} chelate is the most commonly used label in time-resolved fluorometry-based analysis because of its higher fluorescence yield and lower background than other lanthanide complexes. Tb^{3+} chelate usually has a longer decay time and a higher fluorescence yield than Sm^{3+} chelate, and their fluorescence is less sensitive to aqueous quench-

Table 3 PG Ⅰ and PG Ⅱ values and PG Ⅰ/PG Ⅱ ratio in patients with gastric carcinoma and duodenal ulcer *n* (%)

	n	PG Ⅰ (μg/L)			PG Ⅱ (μg/L)		PG Ⅰ/PG Ⅱ ratio	
		< 60	60-240	> 240	≤ 27	> 27	≤ 6	> 6
Patients with gastric cancer	126	17 (13.5)	88 (69.8)	21 (16.7)	109 (86.5)	17 (13.5)	25 (19.8)	101 (80.2)
Patients with duodenal ulcer	112	0	69 (61.6)	43 (38.4)	100 (89.2)	12 (10.8)	3 (2.7)	109 (97.3)

ing. However, the relatively shorter emission wavelength of Tb^{3+} chelate (545 nm) makes it more prone to interference (e.g. phosphorescence) derived from plastic or glass materials. Additionally, it is required to use an aliphatic β-diketone to enhance the fluorescence of Tb^{3+} in immunoassay for DELFIA-type of multiple analytes[9]. Considering these factors, we selected Eu^{3+} and Sm^{3+} as labels in the present study.

As the Sm photoluminescence yield is lower than that of Eu, Sm^{3+} is usually used as a tracer in assays not requiring a great sensitivity. The detection limit for PG Ⅰ in dual-label assay is 0.2 μg/L, whereas that of single-label assay is 0.05 μg/L[17]. The sensitivity and precision for PG Ⅰ can be improved significantly by increasing Sm^{3+} label yield. The labeling reaction between PG Ⅰ McAbs and Sm labeling reagent can be prolonged with a suitable excess of the Sm labeling reagents, which may help to get a higher Sm^{3+} label yield. Despite this, the detection sensitivity for PG Ⅰ with a limit of 0.2 μg/L is more than adequate for measuring the PG Ⅰ concentration in clinical samples.

Direct passive absorption of two or more binders is still the routine method for multi-analyte immunoassay (MAIA)[20]. When only the sandwich-type configuration is employed in MAIA, it is necessary to prepare an activated surface binder (e.g. antibody) in order to get a high sensitivity. In this study, the anti-PG Ⅰ and anti-PG Ⅱ antibodies were co-coated simultaneously in a strip well, which was found to be beneficial and economical for a suitable fluorescence by adjusting the concentration of coated antibodies. The total concentration of the co-coated antibodies which can achieve favorable results was no more than 5 μg/mL (1000 ng per well) in this study.

Samples with a relatively high PG Ⅰ or PG Ⅱ were analyzed at various dilutions. The diluting buffer was identical to the calibrator buffer. The percentage of expected value was 96.3%-101.7% for PG Ⅰ and 98.1%-109.6% for PG Ⅱ. No hook effect of dual-label assay was found at a relatively high PG Ⅰ or PG Ⅱ concentration. Recovery was identified by supplementing PG Ⅰ calibrators at 20 μg/L and 100 μg/L, and PG Ⅱ calibrators at 5 μg/L and 50 μg/L. The average recovery rates for PG Ⅰ and PG Ⅱ were 102.7% and 98.8%, respectively, showing that the analytical accuracy is satisfactory for clinical use.

Different analytes (i.e. t-PSA/f-PSA, AFP/CEA) can be detected at present by dual-label TRFIA[3,21] and the corresponding instruments are commercially available. In this study, the human PG detected by dual-label TRFIA was similar to that detected by ELISA and single-label TRFIA. The sensitivity, measurement range and stability of dual-label TRFIA were substantially better than those of ELISA. Unlike radioimmunoassay or ELISA[22,23], dual-label TRFIA can measure the concentration of PG Ⅰ and PG Ⅱ, as well as the ratio of PG Ⅰ/Ⅱ, thus reducing the random handling errors and increasing the clinical confidence level of PG Ⅰ/PG Ⅱ ratio. Direct labeling of immunoreagents with lanthanide chelates and lack of overlapping between Eu^{3+} and Sm^{3+} chelates allow a rapid assay. In addition, 25 μL of samples is enough for the simultaneously detection of PG Ⅰ and PG Ⅱ.

In summary, dual-label TRFIA can serve as a high-throughput tool for the detection of serum PG and has good prospects of clinical application.

COMMENTS

Background

Non-invasive serum pepsinogen (PG) test provides much information on intestinal metaplasia, atrophic gastritis, as well as *Helicobacter pylori* infection and peptide ulcer, which has a significant clinical value for the mass screening of patients at a high risk of gastric cancer. A reliable and effective detection method covering a wide concentration range with good sensitivity for PG is required. Furthermore, simultaneous determination of PG Ⅰ and PG Ⅱ can improve the confidence level of the PG Ⅰ/PG Ⅱ ratio.

Research frontiers

In this study, the authors found that multi-analyte immunoassay could increase the throughput and reduce the overall cost per test. A simple, rapid and sensitive dual-label time-resolved fluoroimmunoassay (TRFIA) for pepsinogens in human serum was developed.

Innovations and breakthroughs

TRFIA is a sensitive technique used in analysis of trace substances. Compared with traditional methods, such as radioimmunoassay or enzyme-linked immunosorbent assay (ELISA), dual-label assay can reduce random handling errors and increase the confidence level of PG Ⅰ and PG Ⅱ (especially for the PG Ⅰ/PG Ⅱ ratio). Only 25 μL of serum samples is enough for each test, which is useful for mass screening.

Applications

The PG detected by dual-label TRFIA was in good agreement with that detected by single-label assay or by ELISA. The analytical accuracy, precision and stability are satisfactory for its use in clinical practice. Dual-label TRFIA may serve as a high-throughput tool for the detection of serum PG and has good prospects of clinical application.

Peer review

The analysis of pepsinogens in serum/plasma of patients is a well established method to identify subjects at a higher risk of developing gastric cancer. The described method for analyzing Pep-Ⅰ and Pep-Ⅱ simultaneously seems to have similar parameters in relation to sensitivity and specificity as EIA or ELISA. The manuscript is well written.

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