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A magnetic nanoparticle-labeled immunoassay with europium and samarium for simultaneous quantification of serum pepsinogen I and II

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ABSTRACT

Objective: To develop a novel immunoassay for the simultaneous determination of serum pepsinogen I (PG I) and pepsinogen II (PG II) by combining established methods of time-resolved fluoroimmunoassay (TRFIA) and magnetic nanoparticles separation.

Materials and methods: This new immunoassay method was characterised by immobilising primary antibodies on the surface of magnetic particles and labelled with stable fluorescent chelates of europium and samarium.

Results: Using magnetic nanoparticles, the TRFIA immunoassay exhibited broad dynamic assay ranges for PG I with detection limit of 0.33 ng/mL, while for PG II with detection limit of 0.38 ng/mL. Cross-reactivity between PGs I and II were <15. The intra- and inter-assay coefficient variations of the method were <3%, and the recoveries were in the range of 97–103% for serum samples. Bland–Altman analysis of 124 serum samples showed good consistency with a commercial TRFIA kit. For PG I, the mean (95% confidence interval) difference was 0.97 (–14.3–12.3) ng/mL, whilst for PG II the difference was 0.6 (–4.4–3.2) ng/mL.

Conclusions: Our data suggest that the method is feasible and could be developed into a platform for the routine clinical determination of PG I and PG II levels in human serum.

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KEYWORDS
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Introduction

Pepsinogen (PG), the protease precursor, is secreted by the gastric mucosa of the stomach specifically. According to biochemical properties and immunogenicity, pepsinogen can be divided into two subgroups, pepsinogen I (PG I) and pepsinogen II (PG II) [1,2]. PG I is mainly secreted by fundic gland cells and mucus cells, whereas PGII is secreted by cells in the pyloric and Brunner's glands [3]. Normally, about 1% of PG enters into the blood circulation through the blood capillary of the gastric mucosa, and the content of serum PG is very stable; when pathological changes in gastric mucosa happen or pepsinogen secreting cells are involved, the content of serum PG will be changed [4]. The serum content is closely related with gastrointestinal diseases, like gastric ulcer, duodenal ulcer and atrophic gastritis. The non-invasive markers PGI and PGII and their ratio have high clinical value on estimating the state and function of gastric mucosa [5,6], and has been used as early screen for gastric cancer [7]. More than 30 million data analyses showed that the sensitivity of PG was 77%, specificity of 73%. Compared to other markers, PG has a potential value for screening and early diagnosis of high-risk groups of gastric cancer.

Currently, pepsinogen can be determined by the latex-enhanced nephelometry, chemiluminescence, enzyme immunoassay and time-resolved fluorescence immunoassay (TRFIA). Although some methods can be automated, they need large instruments and space, and the costs are also more expensive. TRFIA using lanthanide complexes chelates as the labels was used as an ideal immunoassay method when it was first reported [8]. The labels used in this immunoassay are rare earth metals such as europium (Eu^{3+}), samarium (Sm^{3+}) and terbium chelates, which can generate strong fluorescence with long decay times, large Stokes shifts and sharp emission profiles [9,10]. As the chelates of these lanthanides have fluorescence peaks at different wavelengths and are clearly distinguishable from one another, the combined use of several chelates enables double- or even multiple-label immunoassays to simultaneously quantitate several indicators contained in a single sample. Due to its unique advantages, TRFIA has been viewed as a highly sensitive method and employed in numerous applications in the biomedical sciences [11–14].

Magnetic nanoparticles have been successfully applied in many areas of research, such as cell separation [15,16], bimolecular detection [17,18], DNA extraction [19,20] and various immunoassay methodologies [21,22]. One of the most important advantages of magnetic beads is that they conveniently separate the target

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molecules coated on the beads from unbound analytes in the mixture. Furthermore, the concentration of analytes could be captured quickly within a short time by an external magnetic field. Therefore, magnetic nanoparticles with bioactive molecules such as antibodies are very useful tools for immunoassays; however, the applications of magnetic nanoparticles in TRFIA have not been widely investigated.

We herein attempted to develop a novel immunoassay for the simultaneous measurement of serums PG I and PG II, which is characterised by the use of magnetic particles as a solid-phase and the use of cations of europium and samarium (Eu^{3+} and Sm^{3+}). We tested the method on routine clinical samples from 124 patients.

Materials and methods

Reagents: Bovine serum albumin (BSA), diethylenetriaminepentaacetic acid (DTPA), 4-morpholineethanesulfonicacid (MES), N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), proclin-300 and Tween-20 were purchased from Sigma (St. Louis, MO, U.S.A.). Magnetic particles were obtained from JSR Life Sciences (Tokyo, Japan). Monoclonal anti-PG I antibodies and anti-PG II antibodies were obtained from Medix Biochemica (Turku, Finland). Eu^{3+}- and Sm^{3+}-labelled kits were obtained from Perkin-Elmer Wallac (Turku, Finland). PD-10 and Sepharose CL-6B was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, U.S.A.). All other chemicals used were of analytical reagent grade and ultra-pure water obtained using a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) was used throughout the experiments.

Serum samples and comparison method: Serum samples were kindly provided by Jiangsu Jiangyuan Hospital (Jiangsu Province, China) with PG I and PG II values measured by PG I/ PG II quantitative determination Kit (TRFIA) (Wuxi Jiangyuan Industrial Technology and Trade Corporation, China). Of 124 clinic patients, 51 were female and 73 were male; the age ranged from 19 to 74. Precise clinical data were anonymised, but 20 had been diagnosed with a duodenal ulcer. Fifty-one of 124 patients were positive for PG I and 56 patients were positive for PG II measured by TRFIA Kit. All the patients were diagnosed on the basis of characteristic clinical features and confirmed by laboratory tests. These serum samples were stored at –20 °C.

Coating conjugate preparation: The magnetic particles (10 µL, 1 mg/mL) were firstly activated using the mixture solution of EDC (300 µL, 50 mg/mL) and NHS (300 µL, 50 mg/mL). After incubation for 30 min at room temperature, the mixture solution was washed three times with phosphate buffer solution with tween-20 (PBST). Under slight stirring, 50 µg of anti-PG I antibody was added and incubated overnight at 4 °C. Then, the magnetic particles were separated by an external magnetic field and washed three times with PBST to remove the unbounded Abs by magnetic separation process. The non-specific sites on magnetic particles were blocked by using 2% BSA solution for 30 min at room temperature. The obtained magnetic particles–antibody conjugates were stored at 4 °C for further use. The anti-PG II antibody was conjugated to magnetic particles using a similar method.

Antibody labelling with Eu or Sm chelates was carried out according to the manufacturer's instructions. Briefly, the PD-10 column was used to exchange buffers for Mc Abs of PG I or PG II to pH 8.5 (50 mmol/L Na_2CO_3–$NaHCO_3$ 0.155 mol/L NaCl). About 2 mg/mL Sm-chelate of N^1-(p-isothiocyanatobenzyl)-diethylenetriamine-N^1, N^2, N^3, N^4-tetraacetic acid were added to the 2 mg/mL PG I Mc Abs labelling buffer (pH 8.5) 2 mg/mL Eu-chelate of N^1-(p-isothiocyanatobenzyl)-diethylenetriamine-N^1, N^2, N^3, N^4-tetraacetic acid were added to the 2 mg/mL PG II Mc Abs labelling buffer (pH 8.5), and then incubated overnight at 4 °C. The labelled antibodies were separated from the free chelate and aggregated Mc Abs through gel filtration on a column of Sepharose CL-6B (1 cm × 40 cm) with an elution buffer of 50 mmol/L Tris-HCl pH 7.8 containing 0.9% NaCl, and 0.05% sodium azide as a preservative. The concentration of Eu^{3+} or Sm^{3+} in the fractions was obtained by measuring their fluorescence after appropriate dilution with the enhancement solution. The collected protein factions were preserved by rapid freezing and drying in a high vacuum after dilution with an elution buffer containing 0.2% BSA as a stabiliser. The labelled Mc Abs were stored at –20 °C.

Preparation of PG I, PG II Standards: The concentrations of PG I in the six mixed standards were prepared by diluting highly purified PG I antigen (1 mg/mL) in standard buffer (50 mmol/L pH7.8 Tris-HCl buffer containing 2 g/L BSA and 1 g/L NaN_3) both as 0, 10, 50, 100, 200 and 300 ng/mL. The concentrations of PG II in the six mixed standards were prepared by diluting highly purified PG II antigen (1 mg/mL) in standard buffer both as 0, 5, 10, 15, 25 and 40 ng/mL.

The proposed immunoassay for the simultaneous determination of PG I and f PG II was performed based on a sandwich-type immunoassay format by combining a TRFIA assay and immunomagnetic separation. Initially, 5 µL of magnetic nanoparticles coated with anti-PG I antibody, 5 µL of magnetic nanoparticles coated with anti-PG II antibody, 75 µL of Sm^{3+}-labelled anti-PG I antibody and 75 µL of Eu^{3+}-labelled anti-PG II antibody were added sequentially into the analytes. After 15-min incubation with continuous gentle stirring at room temperature (25 °C), the plate was positioned on the magnet for 5 s and the supernatant was discarded. After removing the free substances and rinsing with washing buffer six times, 200 µL of enhancement solution was added and then the immunocomplexes were resuspended in enhancement solution and the mixtures were incubated for 5 min at room temperature with stirring. Finally, the fluorescence

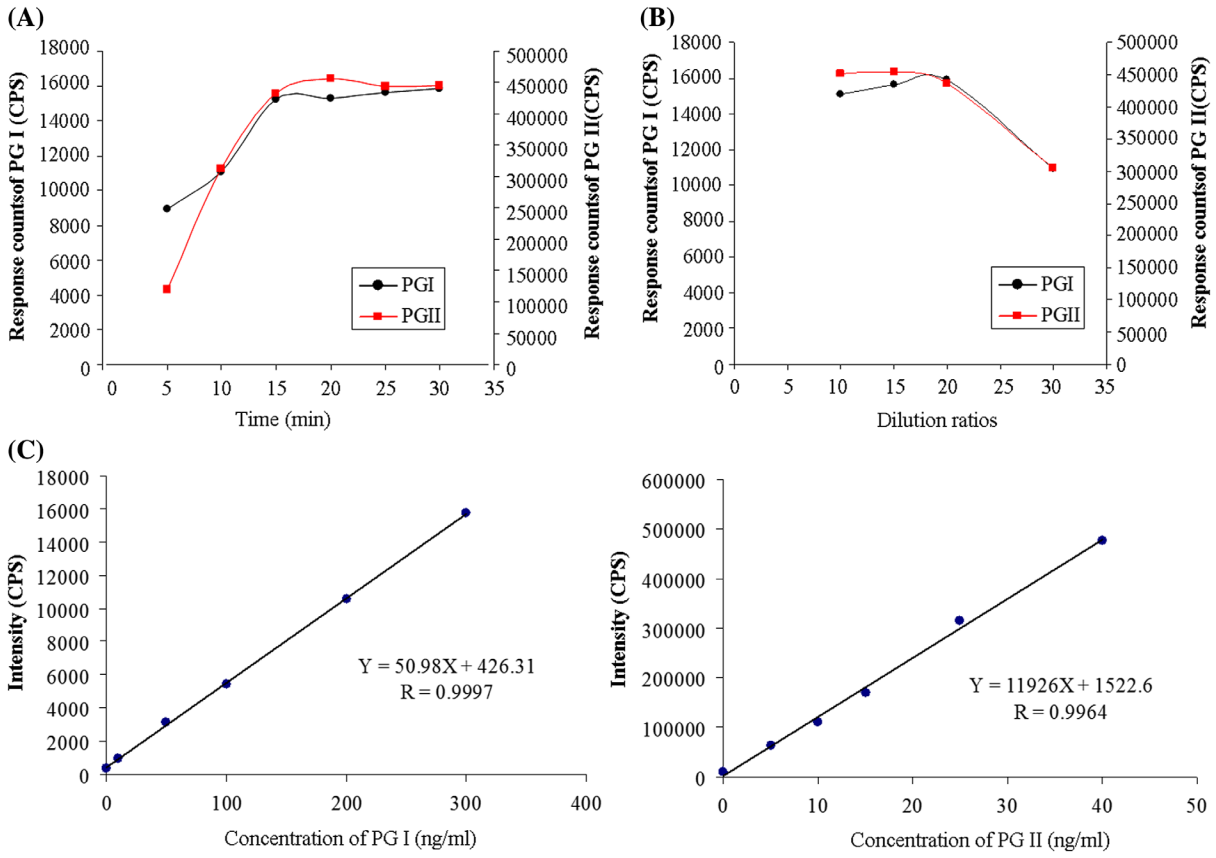

Figure 1. (A) Influence of incubation time on fluorescence intensity. The curves correspond to a series of incubation times (from 5 to 35 min), 50 µL of PG I/PG II standards (300 ng/mL and 40 ng/mL), 10 µL of magnetic particles (1 mg/ mL), 75 µL of Eu^{3+} (or Sm^{3+})-labelled antibody (dilution ratio of 1: 20). (B) Influence of the dilution ratio of Eu^{3+} (or Sm^{3+})-labelled antibody on fluorescence intensity. The curves correspond to a series of dilution ratios of Eu^{3+} (or Sm^{3+})-labelled antibody (from 1: 10 to 1: 30), 50 µL of PG I/PG II standards (300 ng/mL and 40 ng/mL), 10 µL of magnetic particles (1 mg/mL), incubation for 15 min at room temperature. (C) Calibration curves for the magnetic nanoparticle-based TRFIA for the simultaneous measurement of PG I and PG II standards.

signal was measured using the AutoDEFIA1235 Multilabel Counter. The fluorescence of Sm^{3+} was measured at an excitation wavelength of 340 nm and an emission wavelength of 642 nm. The fluorescence of Eu^{3+} was measured at an excitation wavelength of 340 nm and an emission wavelength of 615 nm.

Statistical analysis: Data are presented as mean and standard deviations (SD). Standard curves were obtained by plotting the fluorescence intensity (Y) against the sample concentration (X) and fitting a logistic equation using Origin7.5 SR1 (Microcal, U.S.A.). Pearson's linear regression was used to display the linearity and correlations. Serum samples measured using the proposed method and a commercial TRFIA kit were compared by Bland and Altman analysis [23]. $p < 0.05$ was considered statistically significant. All analyses were performed on SPSS 17.0.

Results

The analytical performance was notably influenced by several reaction parameters, including reaction temperature, incubation time, concentration of magnetic particles and the dilution ratios of Eu^{3+}- or Sm^{3+}-labelled antibody. As it is known that temperature has a great effect on the immunoreaction between antigens and antibodies, a contrast test was carried out at room temperature (25 °C) and 37 °C, which are temperatures frequently used for incubation. Standards at five different concentrations were tested with the same reaction conditions at different temperatures. The results showed that the reaction time was significantly reduced but the slope of the standard assay curve showed no significant difference between data at 25 and 37 °C (data not presented). Therefore, to simplify operations and instruments, a temperature of 25 °C was considered as the optimal reaction temperature for practical applications.

The effect of different incubation times (5, 10, 15, 20, 25 and 30 min) were compared by measuring PG I (300 ng/mL) and PG II (40 ng/mL) standards. As results shown in Figure 1(A), long incubation times indeed enhanced the sensitivity of the assay; however, when the incubation time exceeded 15 min, the fluorescence intensities of the PG I and PG II standard points reached a dynamic equilibrium. Thus, 15 min was selected as the incubation time, which was shorter than that for conventional TRFIA (which usually needs incubation of ≥1 h) [24,25]. Incubation time reduction was probably attributable to the application of magnetic particles, which distributed evenly in the sample to allow antigen molecules to be

more accessible, the reaction to be faster and dynamic equilibrium to be reached more quickly.

The sensitivity of an immunoassay is generally related to the dilution ratios of its antibodies. Therefore, the effects of various dilution ratios (1:10, 1:15, 1:20 and 1:30) of Eu^{3+}- or Sm^{3+}-labelled antibody were determined. The fluorescence intensity increased with decreasing dilution ratio of Eu^{3+}-labelled anti-PG II antibody/ Sm^{3+}-labelled anti-PG I antibody (Figure 1(B)). However, the fluorescence intensity tended to reach a maximum at the dilution ratio of 1:20. Hence, Eu^{3+}-labelled anti-PG II antibody/Sm^{3+}-labelled anti-PG I antibody of 1:20 were selected as the optimum because satisfactory signal intensity was achieved.

Analytical sensitivity and linear range: Under optimised experimental condition, a series of PG I/PG II standards with different concentrations were measured simultaneously and the calibration curves for PG I/PG II are illustrated in Figure 1(C). Both the calibration curves of PG I and PG II were linear over the concentration with a correlation coefficient of 0.99 for both. The linear regression equation of PG I was adjusted to Y = 50.98X + 426.31, with a lower detection limit of 0.33 ng/mL (defined as the concentration corresponding to blank fluorescence intensity plus two standard deviations, n = 20). The linear regression equation of PG II was adjusted to Y = 11926X + 1523, with a lower detection limit of 0.38 ng/mL. No high-dose hook effect was observed within these linear ranges. These results suggested that the sensitivity reached permitted determination of very low levels of PG I or PG II.

The specificity of the assay for PG I/PG II was evaluated by measuring the cross-reactivity with each other. Results showed that there was no cross-reactivity with each other (less than 1%). The reproducibility of the immunoassay method was estimated by intra-assay and inter-assay coefficients of variation (CV). Results are shown in Table 1. The inter-assay variations were 0.9–2.8% for PG I and 1.1–2.6% for PG II, while the intra-assay variations were 1.0–2.2% for PG I and 0.8–1.3% for PG II, indicating an acceptable reproducibility. The mean recoveries obtained by the same method via spiking three different concentration levels of PG I and PG II standards into maternal serum controls. The recoveries of PG I and PG II varied from 99.2 to 100.2% and 97.1 to 101.3%, respectively (Table 2). The data suggested that the recovery of the proposed immunoassay was satisfactory.

Clinical application of the established assays: The reliability of this immunoassay system was investigated by analysing 124 clinical serum samples. Results were compared with those obtained from a commercially available TRFIA kit by Bland and Altman analysis (Figure 2). The mean difference was –0.97 ng/mL (95% confidence interval [CI] –14.27–12.33) and with a standard deviation of 6.8 ng/mL for PG I. The mean difference was –0.6 ng/mL (95% CI –4.4 to 3.2) with a standard deviation of 1.94 ng/mL for PG II. Only 7.3% (9/124) samples of PG I and 4.8% (6/124) samples of PG II were outside the limits of agreement of one standard deviation from the mean, indicating good consistence between our proposed method and commercial TRFIA kit. The PG II results clearly showed two non-overlapping populations with better agreement in those with lower levels.

Discussion

Although time-resolved fluoroimmunoassay (TRFIA) is a promising immunoassay method, some limitations in the conventional TRFIA still remain. For example, specific antigen or antibody reagents and interactions may not absorb fully (and so be immobilised) on the plastic surface of 96-well microplates and so could be washed out. However, use of magnetic nanoparticles beads can minimise the loss of specific antigens or antibodies in the plate-washing process. Nanoparticles can provide a comparably large surface area available for reaction within a small sample volume due to their high surface

Table 1. Inter-assay and Intra-assay reproducibility.

	Sample	Theoretical value (ng/mL)	Observed value[a] (ng/mL)	CV (%) n = 10
Inter-assay	PG I	60	61.17 ± 1.51	2.8
		120	121 ± 1.63	1.3
		185	184.7 ± 1.63	0.9
	PG II	6	6.06 ± 0.07	1.1
		11	10.84 ± 0.21	1.9
		17	16.77 ± 0.44	2.6
Intra-assay	PG I	60	61.39 ± 1.34	2.2
		120	121.2 ± 1.31	1.1
		185	184.6 ± 1.83	1.0
	PG II	6	6.14 ± 0.05	0.8
		11	11.13 ± 0.14	1.3
		17	17.12 ± 0.14	0.8

[a]Mean value ± standard deviation.

Table 2. Recoveries determined via spiking three different concentration levels of PG I and PG II standards into maternal serum controls.

Sample	Serum concentration (ng/mL)	Spiked standard (ng/mL)	Observed value[a] (ng/mL)	Theoretical value (ng/mL)	Recovery (%) n = 10
PG I	152.6	10	161.98 ± 2.18	162.6	99.6
	152.6	100	253.16 ± 1.26	252.6	100.2
	152.6	300	448.75 ± 3.57	452.6	99.2
PG II	12.3	5	17.52 ± 1.23	17.3	101.3
	12.3	15	27.94 ± 2.21	27.3	102.4
	12.3	40	50.77 ± 2.36	52.3	97.1

[a]Mean value ± standard deviation.

to volume ratio. This enables more antibodies to be coupled to the surface, leading to a reduction in the consumption of reagents and enabling the immobilisation of more antibodies.

Combining the advantages of magnetic particles with double-label TRFIA, we established a magnetic particle-based TRFIA method. The proposed immunoassay method was characterised by immobilising primary antibodies on the surface of magnetic particles and labelled with stable fluorescent chelates of Eu^{3+} and Sm^{3+}, a process that brings several advantages. The application of magnetic particles as both the immobilisation matrix and a separation tool effectively improves sensitivity and significantly reduces the analysis time via a homogenous format. Additionally, this method shows broad dynamic assay ranges and less consumption of reagents compared with the conventional method. Our serum results reflect those of a published reference range measured by a latex immunoassay [26] of approximately 26–153 ng/ml for PG I and <30 for PG II. The anonymised nature of our serum samples from a clinical setting indicates our assay can determine levels of the PGs markedly higher than these reference levels. Others have used a chemiluminescence and other assays to report similar levels [1,27].

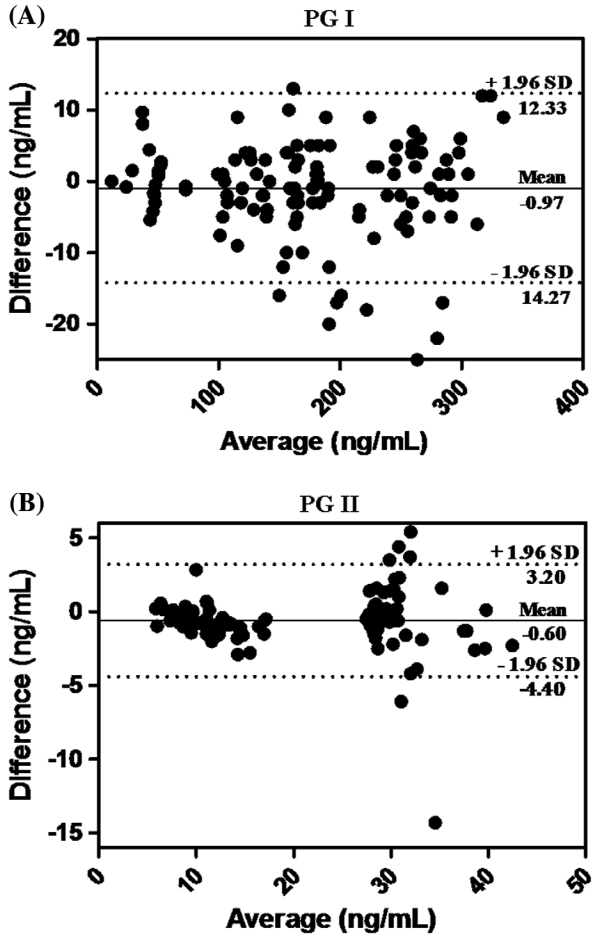

Figure 2. Bland and Altman plot for PG I (A) and PG II (B) in 124 serum samples detected by the proposed method and commercial TRFIA kits.

As the magnetic nanoparticle-based dual-label TRFIA has been developed as a highly sensitive method, it has been employed in numerous applications for simultaneous determination of multiple analytes. In this study, our novel immunoassay method was applicable to the simultaneous determination of PG I and PG II. As results show, it was confirmed that the cost of reagents is lower and the assay procedure is simpler than the conventional method. Moreover, it shows good properties for simultaneous detection of PG I and PG II with acceptable reproducibility, specificity and recovery. Furthermore, the proposed method established here, when applied to the determination of PG I and PG II levels in serum samples, showed good comparison with a commercial TRFIA kit, suggesting it could be developed into commercially available kits for the clinical application.

Here, we described a developed TRFIA method for the simultaneous determination of PG I and PG II by immobilising primary antibodies on the surface of magnetic nanoparticle beads. High sensitivity, satisfactory specificity, broad dynamic assay ranges, short analytical time and cost-effectiveness of such novel immunoassay are exhibited according to the results described above, indicating its potential application value. Based on this investigation, it is worthwhile to perform further studies on the application of this simultaneous assay system and develop other biomarkers using the same platform in clinical laboratories. This work represents an advance in biomedical science because high sensitivity, satisfactory specificity, broad dynamic assay ranges, short analytical time and cost-effectiveness of such novel immunoassay are exhibited.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Summary table

What is known about this subject

- The serum content of PG I and PG II is closely related with gastrointestinal diseases
- TRFIA is an established immunoassay method
- Magnetic nanoparticles have been successfully applied in many areas of research

What this paper adds

- We combined TRFIA method and magnetic nanoparticle separation into a new method with good performance criteria
- Levels of PG I and PG II in serum can be simultaneously determined at levels above the reference range

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