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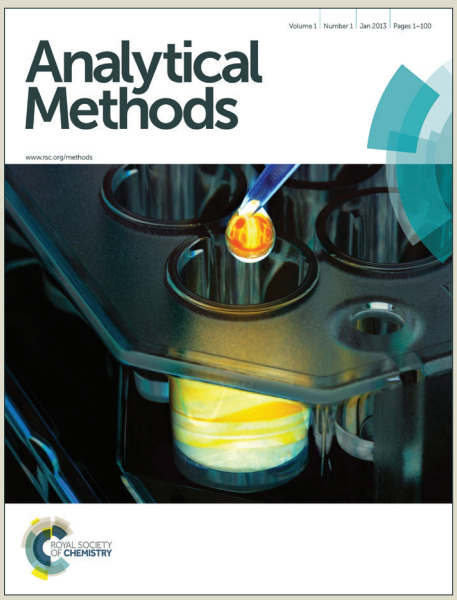

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Fluorescent Immunochromatographic Assay for Rapid and Sensitive Detection of Human Prealbumin in Serum

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Abstract

Protein-energy malnutrition is a significant problem among hospitalized patients. Prealbumin, a plasma protein, is commonly used in clinical practice to assess nutritional status. Prealbumin is also a powerful predictor of mortality risk in dialysis patients. Variation in prealbumin concentration provides valuable information regarding malnutrition and diagnostic applications. Fluorescent microspheres, which combine with anti-human prealbumin monoclonal antibodies, were introduced into an immunochromatographic assay for quantitative detection of human prealbumin in serum. A sandwich immunoassay was developed and the fluorescence intensity of the test line in the test strip was proportional to the prealbumin content in the specimens. The fluorescence intensities of the test and control lines were recorded using a commercial fluorescent strip reader. Results showed that the limit of detection of prealbumin reached 1.0 ng/mL within 20 min with a good linear range of 8.0 ng/mL to 110.0 ng/mL. Serum specimens can be diluted 5000 times to avoid matrix interference. The average intra- and inter-assay recoveries ranged from 95.7% to 102.8% and 95.3% to 105.6% respectively, with corresponding variation coefficients of 3.3% to 4.3% and 4.1% to 9.9%. The test strip showed no cross reaction with hemoglobin and albumin. A significantly good agreement was observed between the test strip and immunoturbidimetric assay The developed novel assay in this study is a sensitive, specific, reproducible, time-saving, inexpensive, and quantitative method for detection of human prealbumin in serum.

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Keywords: fluorescent microspheres, immunochromatographic test strip, quantitative detection, prealbumin.

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1. Introduction

Protein-energy malnutrition (PEM) is a common problem among hospitalized patients, the incidence of malnutrition in hospitals can exceed 50%.[1,2] Malnutrition has been associated with increased risks of in-hospital morbidity and mortality, prolonged hospitalization, and increased expenditure and use of health care resources.[3,4] Moreover, patient nutritional status plays a vital role in the recovery from illness.[5,6] Thus, a sensitive and reliable marker is needed to indicate the nutritional status of each patient.

Among biochemical markers, prealbumin (PA), a negative acute phase reactant in plasma proteins, is commonly used in clinical practice to assess nutritional status. PA is also a powerful predictor of mortality risk in dialysis patients.[7,8] PA concentration in serum is sensitive to the early phases of decreased nutrition because of its short half-life (1.9 days).[9] PA has been reported to significantly decrease when a person suffers from malnutrition, acute hepatitis, cirrhosis or severe hepatitis. PA concentration >170 mg/L has been suggested to indicate low or no risk for malnutrition; PA concentration = 110 mg/L to 170 mg/L is considered moderate risk, which requires less intensive nutritional therapy requirement; and PA concentration < 110 mg/L is considered high risk, requiring major nutritional therapy.[10,11] Once the malnutrition has been cured, the PA concentration would rapidly return to normal. PA detection has been clinically useful, particularly in initial screening and monitoring of nutritional recovery.[12,13]

The reported methods for detection of human PA are mainly include electrophoresis,[14]

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radial immunoassay,[15,16] immunoturbidimetric assay,[17,18] latex-enhanced immunoassay,[19] enzyme-linked immunosorbent assay (ELISA),[20] immunoresoance scattering spectral assay,[21] chemiluminescence immunoassay,[22] and high-performance liquid chromatography (HPLC).[23] The sensitivities of electrophoresis, radial immunoassay, immunoturbidimetric assay, and latex-enhanced immunoassay are low, whereas those of ELISA, immunoresoance scattering spectral assay, chemiluminescence immunoassay, and HPLC are high. However, the highly sensitive techniques require long detection time. Therefore, the development of a highly sensitive, time-saving, inexpensive, and convenient method for assaying PA is significant for clinical detection.

In the present study, fluorescent microspheres (FMs) were introduced as signal reporters into an immunochromatographic assay to quantitatively detect human serum PA. FMs lateral flow immunoassay (FMs-LFIA) is a screening method used for onsite testing because of several advantages, such as high detection sensitivity, wide linear range, small specimen amount, short detection time, and high stability.[24-26] The sensitivity and reliability of the FMs-LFIA were verified by comparing this method with the immunoturbidimetric assay. To our knowledge, our study is the first to report a novel and sensitive method based on FMs to detect human serum PA.

2. Materials and Methods

2.1 Reagents and materials

PA human antigen was provided by GenWay Biotech, Inc. (San Diego, USA). Mouse

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anti-human PA monoclonal antibody (anti-PA mAb) was obtained from Shanghai MabStar, Inc. (Shanghai, China). Rabbit anti-human PA polyclonal antibody (anti-PA pAb) and real human serum specimens were provided by Beijing Zhongsheng Jinyu Diagnosis Technology Co., Ltd. (Beijing, China). The limit of detection of the ELISA using these mAb and pAb to analyze the human prealbumin was 0.1 ng/mL. Donkey anti-mouse antibody was provided by Wuxi Zodolabs Biological Technology Co., Ltd. (Wuxi, China). Artificial serum was obtained from Huzhou InnoReagents Co.,Ltd. (Zhejiang, China). FMs (diameter = 175 nm, excitation wavelength = 470 nm, emission wavelength = 525 nm, COOH = 443 μeq/g) were purchased from Merck (Darmstadt, Germany). The 135 nitrocellulose (NC) membrane (The pore size and flow rate of the NC membrane is 8 μm and 120-150s/4cm, respectively.), absorbent pad, sample pad and conjugate pad were purchased from Millipore (Bendford, MA, USA). Hemoglobin and albumin from human serum, 2-(N-morpholino) ethanesulfonic acid (MES), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA). All solvents and other chemicals were of analytical reagent grade.

2.2 Apparatus

The F-380 fluorescence spectrophotometer was supplied by Tianjin Gangdong Sci.&Tech Development Co., Ltd. (Tianjin, China). A fluorescence strip reader was purchased from Shanghai Huguo Science Instrument Co., Ltd. (Shanghai, China). The Multiskan spectrum microplate reader was purchased from Thermo Fisher Scientific Inc.

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(Massachusetts, USA). BioDot XYZ platform combined with a motion controller, BioJet Quanti3000k dispenser and AirJet Quanti3000k dispenser for solution dispensing were supplied by BioDot (Irvine, CA, USA). Vacuum drying oven was purchased from Shanghai Fuma Test Equipment Co., Ltd. (Shanghai, China). An automatic guillotine cutter was purchased from Hangzhou Fenghang Technology Co., Ltd. (Hangzhou, China).

2.3 Preparation and characterization of FMs-mAb

FMs-mAb were prepared using previously described methods with slight modifications.[27,28] About 0.15 mg of FMs and 15 μL of 1.0 mg/mL freshly prepared aqueous solution of EDC (dissolved in MES buffer) aqueous solution were slowly added to 3.0 mL of 0.05 mol/L MES buffer (pH 6.0). After 1 min sonication, 15 μL of 0.5 mg/mL anti-PA mAb was added and gently stirred at room temperature for 2 h. The mixture was blocked with 300 μL of 10% BSA (w/v) for 30 min. The unreacted anti-PA mAb and FMs-mAb were separated by centrifugation at 9000 rpm for 10 min. Afterward, the supernatant was discarded, The precipitate was resuspended in a 300 μL solution containing 0.02 mol/L Na_2HPO_4 at pH 5.5, 5% sucrose (w/v), 3% trehalose (w/v), 0.1% NaN_3, 1% BSA (w/v), and 1% Tween-20. Resuspended conjugates were stored at 4 °C in the dark for further use. FMs and FMs-mAb characterizations were analyzed using the F-380 fluorescence spectrophotometer and Multiskan spectrum microplate reader.

2.4 Preparation of FMs-LFIA test strip

The test strip consisted of four parts, namely, sample pad, conjugate pad, NC membrane,

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and absorbent pad. The conjugate pad was pretreated with 0.02 mol/L phosphate buffer (pH 5.5), which was composed of 0.5% (w/v) polyvinylpyrrolidone, 3% sucrose, 1% BSA (w/v), and 0.5% Tween-20, The pad was subsequently dried at 30 °C for 12 h. Anti-PA pAb (0.5 mg/mL) and donkey anti-mouse antibody (0.5 mg/mL) were immobilized on NC membrane as the test and control lines, respectively. The treated NC membrane was dried at 30 °C for 2 h. Finally, the sample pad, conjugate pad, NC membrane, and absorbent pad were assembled as the FMs-LFIA test strip (Fig. 1).

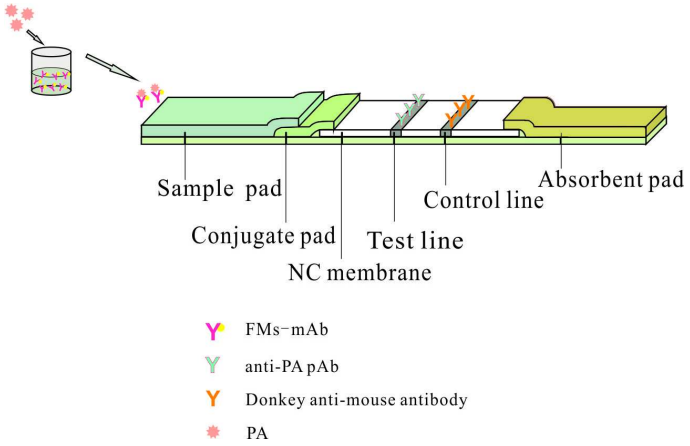

Fig. 1 Schematic of the sandwich FMs-LFIA test strip.

2.5 Immunoassay procedure

A sandwich immunoassay for human PA was performed on the FMs-LFIA test strip. Up to 2 μL of FMs-mAb and 100 μL of specimens were pipetted in ELISA wells and incubated for 3 min to allow formation of FMs-mAb-PA complexes. Afterward, 100 μL of the complexes were added to the sample well of the test strip. The complexes migrated across the NC membrane, these complexes were then captured by anti-PA pAbs that were immobilized on the test line. Unbound FMs-mAb, which were captured by donkey anti-mouse antibody,

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were immobilized on the control line. The fluorescence intensity (FI) of the test line (FI_T) was proportional to the PA content in the specimens.

The FI_T and FI_C (fluorescence intensity of the control line) values, as well as the FI_T/FI_C ratio were recorded using a fluorescence strip reader. All experiments were performed in triplicate.

2.6 Optimization of parameters

To determine the most appropriate pH value, 0.05 mol/L MES buffer was adjusted to different pH values (5.0, 6.0, 7.0, and 8.0) with 0.1 mol/L of HCl or 0.1 mol/L NaOH. The ideal amount of antibodies conjugated on FMs was optimized by adding different volumes (9, 15, 24, and 30 μL) of 0.5 mg/mL anti-PA mAb. The optimal detection time was determined on the basis of the kinetic curves by plotting the FI_T/FI_C ratio against time. The FI_T/FI_C ratio was recorded every 2 min after the specimens were added into the sample well.

2.7 Quantitative standard curve of the FMs-LFIA test strip

PA was diluted by adding artificial serum to prepare a serial standard dilution at different concentrations: 0.0, 1.0, 6.0, 10.0, 20.0, 40.0, 60.0, 80.0, 100.0, and 110.0 ng/mL. The strip FI_T/FI_C ratio was measured with a fluorescence strip reader after adding the specimen for 20 min. The quantitative standard curve was constructed by plotting the FI_T/FI_C ratio against the PA concentrations.

2.8 Assay validation

Method specificity was determined by adding hemoglobin and albumin at 40 μg/mL and

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10 μg/mL concentrations to the artificial serum, respectively. The accuracy and precision of the FMs-LFIA test strip were verified by analyzing the recovery and coefficient of variation (CV) of the intra- and inter-assay. Three spiked concentrations of PA samples at 16, 30, and 50 ng/mL were analyzed for recovery studies. All experiments were performed in triplicate.

2.9 Comparative evaluation with immunoturbidimetric assay

To evaluate the reliability and sensitivity of the FMs-LFIA test strip, eight human serum pools were analyzed both with the test strips in our laboratory and the immunoturbidimetric assay at the Second Affiliated Hospital to Nanchang University. In brief, 10 μL of blank sample or PA samples and 225 μL of PBS buffer were pipetted into the cuvettes. After a 5 min incubation at 37 ℃, 75 μL of goat anti-PA antibody was added and then incubation at 37 ℃ for another 5 min. The absorbance of the blank sample (A1) and PA samples (A2) were measured at 340 nm. A standard curve was constructed by plotting the ΔA (ΔA=A1－A2) against concentrations of PA. Concentrations of human serum specimens were calculated from the standard curve.

3. Results and Discussion

3.1 Characterization of FMs and FMs-mAb

FMs-mAb were obtained by coupling the anti-PA mAb amino group with the carboxyl group on the FMs surface. The F-380 fluorescence spectrophotometer was used to characterize the fluorescence properties of free FMs and FMs-mAb. The maximum emission peaks of FMs-mAb and free FMs were in the similar position. However, the FMs-mAb

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intensity was approximately 0.25 times lower than that of the corresponding free FMs (Fig. 2A), because part of the fluorescence signal was shielded by the antibody on the FMs surface. The Multiskan spectrum microplate reader was used to detect the optical signal and confirm whether the anti-PA mAb were conjugated with FMs. Figure 2B shows the ultraviolet visible spectra of the FMs-mAb, free FMs, and anti-PA mAb. The results confirmed that the anti-PA mAb were successfully coupled on the FMs surface, because the FMs-mAb exhibited anti-PA mAb characteristic peaks at 280 nm position.

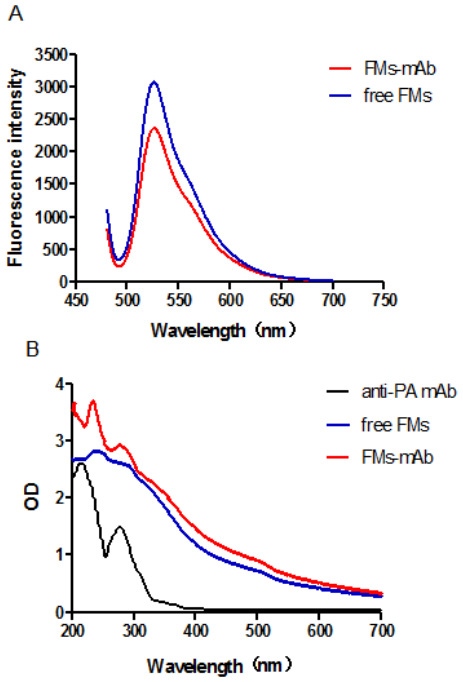

Fig. 2 (A) Fluorescent intensities of free FMs and FMs-mAb. (B) Ultraviolet visible spectra of anti-PA mAb, free FMs, and FMs-mAb.

3.2 Optimization of FMs-LFIA strip parameters

The optimum pH and amount of anti-PA mAbs were determined by comparing the FI_T/FI_C ratios at different pH values (5.0, 6.0, 7.0, and 8.0) with different volumes (9, 15, 24,

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and 30 μL) of anti-PA mAbs, respectively (Fig. 3A and 3B). The results showed that the highest FI_T/FI_C ratio was detected at pH 6.0 with 15 μL of anti-PA mAb. The optimal detection time was obtained by plotting the FI_T/FI_C ratio against time. Figure 3C shows that the FI_T/FI_C ratio remained constant after the 20 min reaction. Thus, the optimal detection time was 20 min.

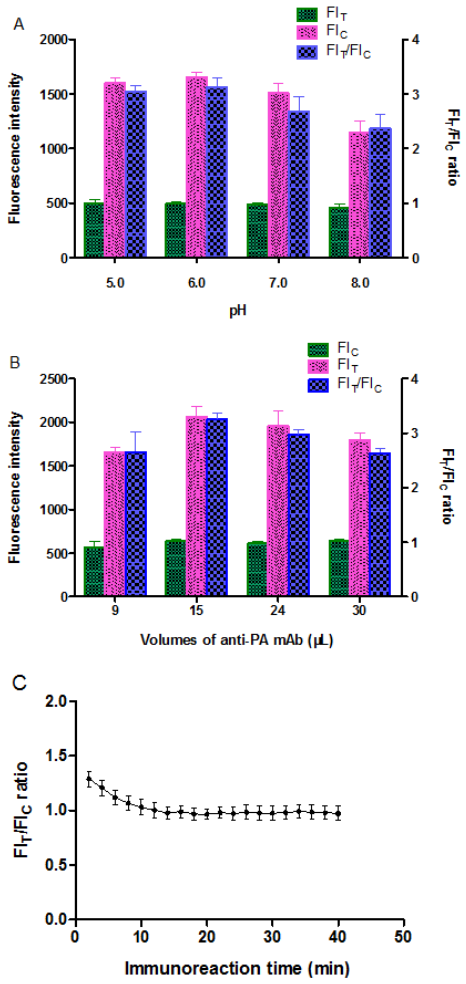

Fig. 3 Effects of pH value and amount of anti-PA mAb on FI_T/FI_C. (A) in different pH values (5.0, 6.0, 7.0, and 8.0). (B) in different volumes (9, 15, 24, and 30 μL) of anti-PA mAb at 0.5 mg/mL concentration. (C) Immunoreaction dynamics of FI_T/FI_C ratio at different detection times. Error bars are based on triplicate measurements.

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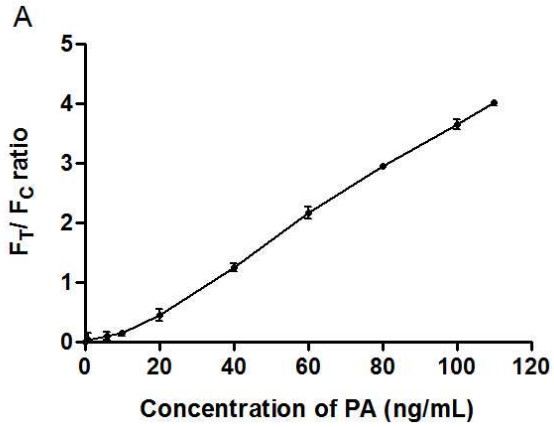
3.3 Linear range and sensitivity of the test strip

The quantitative standard curve of the FMs-LFIA test strip was constructed by plotting the FI_T/FI_C ratios against various PA concentrations under optimal experimental conditions. As shown in Figure 4, the standard curve exhibited a good linear range from 8.0 ng/mL to 110.0 ng/mL, with a correlation coefficient ($R^2 = 0.99$). The mean and standard deviation (SD) of the blank sample were both zero. Therefore, the limit of detection (LOD) of the FMs-LFIA based on mean plus threefold SD of the blank sample cannot be calculated. The FMs-LFIA assay sensitivity was 1.0 ng/mL (Table 1).

Notably, the serum specimens in this study can dilute up to 5000 times. The high serum dilution ratio did not only prevent matrix interference in the serum effectively but also reduced the required amount of human serum.



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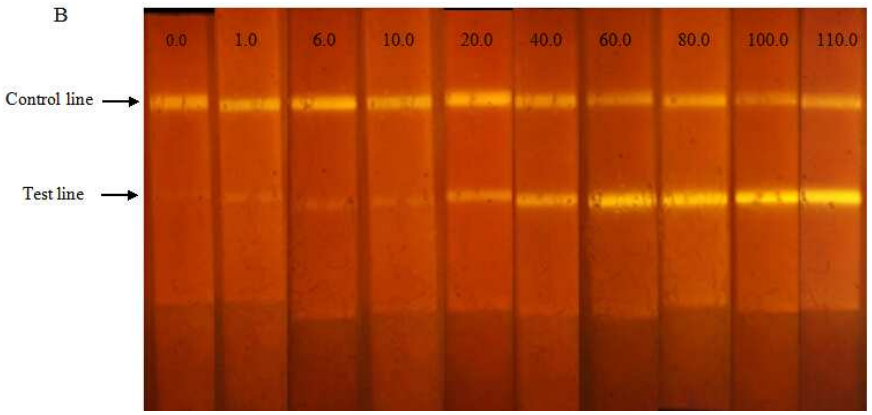

Fig. 4 Standard curves for PA quantitative analysis with a series of concentrations at 0.0, 1.0, 6.0, 10.0, 20.0, 40.0, 60.0, 80.0, 100.0, and 110.0 ng/mL. (A) The quantitative standard curve was constructed by plotting the FI_T/FI_C ratio against the PA concentrations. Error bars are based on triplicate measurements. (B) Fluorescent pictures of test and control lines at different PA concentrations.

Table 1 FI_T/FI_C Signal intensity.

Concentration (ng/mL)	FI_T/FI_C Signal intensity		
	Mean	SD	CV (%)
0.6	No	No	No
0.8	No	No	No
1.0	0.04	0.004	10.0
2.0	0.08	0.007	8.75
6.0	0.1	0.06	6.0
10.0	0.15	0.007	4.67
20.0	0.45	0.05	11.1

3.4 Specificity and accuracy of the test strip

Normal levels of hemoglobin and albumin in human serum ranged from 110 to 200 g/L and 30 to 50 g/L, respectively. After 5000 times dilution, the hemoglobin and albumin levels

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were 22 to 40 μg/mL and 6 to 10 μg/mL, respectively. Thus, the 40 μg/mL of hemoglobin and 10 μg/mL of albumin were selected for the specific assay. These two kinds of protein showed no cross reaction with the test strip (Fig. 5).

The intra- and inter-assay results of the recovery studies are listed in Table 2. The average recoveries for intra- and inter-assay ranged from 95.7% to 102.8% and 95.3% to 105.6%, respectively, with corresponding CVs of 3.3% to 4.3% and 4.1% to 9.9%.

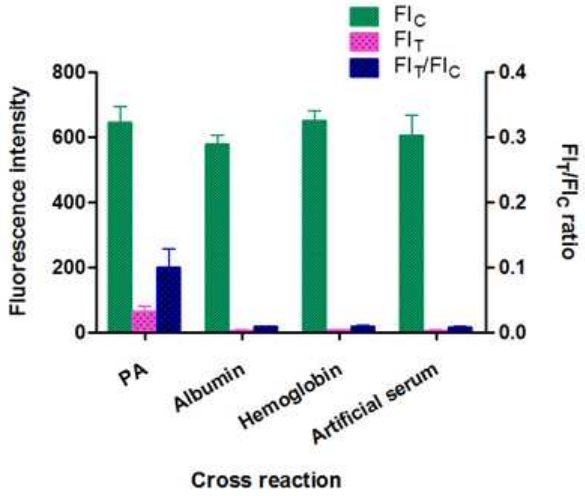

Fig. 5 Cross reaction with hemoglobin and albumin at 40 μg/mL and 10 μg/mL concentrations, respectively. PA at 1.0 ng/mL concentration was used as positive control, whereas artificial serum was used as negative sample.

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Table 2 Three spiked concentrations (16, 30, and 50 ng/mL) of PA were analyzed for intra- and inter-assay recovery studies.

Sample (ng/mL)	Intra-assay				Inter-assay [b]			
	Mean [a] (ng/mL)	Recovery (%)	SD	CV (%)	Mean (ng/mL)	Recovery (%)	SD	CV (%)
16	16.5	102.8	0.7	4.2	16.9	105.6	0.86	5.1
30	28.7	95.7	0.94	3.3	28.6	95.3	1.2	4.1
50	97.8	97.8	2.1	4.3	49.2	98.4	4.9	9.9

[a] Mean values of triplicate measurements.

[b] Inter-assay was completed for three days in a row, three times per day, with triplicate measurements at each concentration.

3.5 Comparison study of the test strip with immunoturbidimetric assay

To validate the FM-LFIA strip, eight human serum pools were analyzed with the test strip and immunoturbidimetric assay, a conventional PA detection method in the hospital. As shown in Figure 6, the correlation coefficient (R^2 = 0.94) is in a very good agreement between the two methods.

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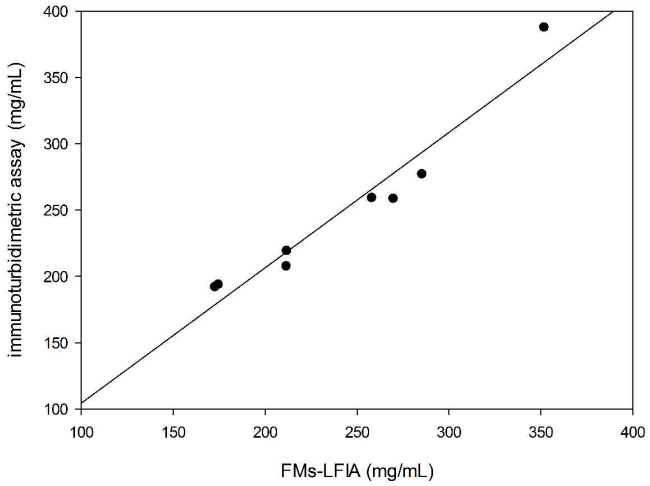

Fig. 6 Method comparison between the FMs-LFIA test strip (X-axis) and immunoturbidimetric assay (Y-axis) for PA detection in human serum specimens.

4. Conclusions

A sensitive, specific, time-saving, inexpensive, and quantitative immunochromatographic test strip for human PA screening in serum was successfully developed. This method effectively prevented the matrix interference in serum specimens because of the high dilution ratio. Under optimal conditions, the LOD reached 1.0 ng/mL within 20 min, with a good linear range from 8.0 ng/mL to 110.0 ng/mL. Intra- and inter-assay CVs were < 10%, which is acceptable for immunoassays. Furthermore, this novel method is more suitable for point-of-care human PA diagnostics than immunoturbidimetric assay.

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