J Fluoresc (2015) 25:885–890
DOI 10.1007/s10895-015-1568-3

ORIGINAL PAPER

Development of Fluorescence-Linked Immunosorbent Assay for Paeoniflorin

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Received: 7 January 2015 / Accepted: 10 April 2015 / Published online: 21 April 2015

Abstract In this study, we developed a fluorescent immunoassay approach to detect paeoniflorin (PF) using a fluorescently labelled monoclonal antibody. The PF-specific antibody was purified by the caprylic acid-ammonium sulfate method and protein G Sepharose 4 Fast Flow column and then labelled with fluorescein isothiocyanate (FITC). The FITC-labelled monoclonal antibody was highly specific for PF, with less than 0.076 % cross-reactivity to seven structurally related compounds. The FITC-labelled monoclonal antibody was then used to develop an indirect competitive enzyme-linked immunosorbent assay (icELISA) and indirect competitive fluorescence-linked immunosorbent assay (icFLISA), respectively. FLISA is simple, rapid and sensitive, with a 500-fold lower limit of detection (LOD) compared with conventional ELISA. Finally, using a variety of standards, FLISA was validated. We observed a strong correlation between the results determined by either FLISA or conventional HPLC for the quantification of PF levels (R^2=0.9927). Collectively, this study shows that the icFLISA method can be successfully applied for the detection and quantification of PF in medicines and biological samples.

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Keywords Paeoniflorin (PF) · Fluorescence-Linked Immunosorbent Assay (FLISA) · Enzyme-Linked Immunosorbent Assay (ELISA) · Determination · Fluorescently labelled monoclonal antibody

Introduction

Paeoniflorin (PF), a monoterpene glycoside isolated from the Chinese herb Radix Paeoniae alba, has a broad spectrum of anti-inflammatory, antirheumatic, immunomodulatory [1], analgesic [2] and neuroprotective pharmacological properties [3]. For quality control to analyze the index component of peony, there are currently a number of methods used for the determination of paeoniflorin in medicines and biological samples, including high-performance liquid chromatography (HPLC) [4], ultra-performance liquid chromatography-electrospray tandem mass spectrometry (UPLC-ESI-MS/MS) [5], and HPLC/MS/MS [6, 7], which display high sensitivity, reliability and validity for use in studies.

In recent years, enzyme-linked immunosorbent assays (ELISAs) using monoclonal antibodies (MAbs) have been utilized as a valuable method for the qualitative or quantitative detection of related compounds in natural products for a variety of purposes. This method of detection has multiple benefits, including minimal pre-treatment of samples prior to the assay, as well as being inexpensive, fast, highly specific, and sensitive assays [8, 9].

In our ongoing study of the production of MAbs against naturally occurring bioactive compounds, we have produced MAbs for the detection of baicalin [10], geniposide [11], puerarin [12]. Using these MAbs, we have also developed indirect competitive enzyme-linked immunosorbent assays (icELISA) for the determination of these compounds in medicines and biological samples.

In the conventional ELISA, the following steps that required almost 4 h are necessary: (i) coating antigen; (ii) blocking step to reduce non-specific adsorption; (iii) primary antibody reaction; (iv) secondary antibody reaction; (v) enzyme-substrate reaction. However, in order to deal with a large number of serial plant and biological samples, a simple, rapid, and sensitive immunoassay is required. Fluorescently labeled antibodies have been widely used and easily generated through the chemical conjugation of organic fluorophores to validated antibodies. In the fluorescence-linked immunosorbent assay (FLISA) method, time- and cost-consuming secondary antibody reaction and following enzyme-substrate reaction can be avoided, making it possible to complete the assay within 3 h [13]. The fluorescence intensity sent by fluorescein labelled antibody is detected in FLISA, while in ELISA the absorbance would be tested, which relied on the enzyme-substrate reaction with cascade amplification effect. A FLISA method is more sensitive [14] and faster than the conventional method of ELISA and requires minimal manipulation [15].

In the present study, we have utilized a specific monoclonal antibody against PF to develop a FLISA method for the measurement of PF. For use in our FLISA system, we have assembled both the capture antibody and the fluorescently conjugated detection antibody. This system enables the high throughput measurement of PF in medicines and biological samples, with an enhanced detection range of PF.

Materials and Reagents

Paeoniflorin (PF) and other compounds were purchased from Dingguo Changsheng Biotechnology Co. Ltd. (Beijing, China). Various Chinese medicinal granules were purchased from Huarun Sanjiu Pharmaceutical Co., Ltd (Guangdong, China). All other chemicals and reagents were of analytical grade and were purchased from Sinopharm Chemical Reagents Beijing Co., Ltd. (Beijing, China).

Instrument

A CLARIOstar fluorescence microvolume assay technology instrument was purchased from BMG Labtech (Germany), and an ELx 800 microplate reader was obtained from Biotek (USA).

Experimental Methods

Purification of anti-PF MAb

In our previous study, we obtained the monoclonal antibody against PF from a hybridoma created through the fusion of splenocytes immunized with PF-bovine serum albumin and conjugated with the HAT-sensitive mouse myeloma cell line SP2/0. For this study, monoclonal antibodies against PF were purified by the caprylic acid-ammonium sulfate method [16] and protein G Sepharose 4 Fast Flow column (GE healthcare Bio-Sciences AB, Uppsala, Sweden) [12], and the antibody content was quantified using the Bradford Protein Assay Kit (Biotopped, Beijing, China).

Antibodies Labelled with Fluorescence

The monoclonal antibody was labelled with fluorescein isothiocyanate (FITC), as described in previous reports with some modifications [17]. After dilution with 0.025 M carbonate buffer saline solution (pH=9.0, consisting of 0.5 M Na_2CO_3 and 0.5 M $NaHCO_3$) to 1 mg/mL, the purified monoclonal antibody in a dialysis bag (molecular weight cut-off:12, 000–14,000, Beijing Blodee Blotechnology Co., Ltd) was dialyzed in 0.1 mg/mL FITC (molecular weight: 389.4) solution with a 10-fold volume of antibody solution with stirring for 12 h at 4 °C. The antibody was then dialyzed in 0.01 M phosphate buffer saline (PBS) for 12 h at 4 °C. The PBS was changed every 2 hours in order to remove the free fluorescein.

Analytical Procedure

Indirect ELISA and icELISA

We examined the reactivity of the FITC labelled antibody to PF- bovine serum albumin (PF-BSA) conjugates using indirect ELISA. PF-BSA conjugate (1 $\mu g \cdot mL^{-1}$, 100 μL/well) was adsorbed in the wells of a 96-well immunoplate for 1 h, which was then treated with 200 μl of blocking buffer (PBS containing 1 % gelatin) for 1 h to reduce non-specific adsorption. The plate was washed three times with washing buffer (PBS containing 0.05 % Tween 20; PBST), and FITC labelled antibody (100 μL/well) was added to each well and incubated for 1 h. The plate was then washed three times with PBST and incubated with 100 μl of a 10,000-fold diluted peroxidase-labeled goat anti-mouse IgG solution for 0.5 h, followed by an additional 3 washes with PBST. Then 100 μl of the substrate solution (0.1 mM citrate buffer (pH=4.0) containing 0.75 % (v/v) H_2O_2 and 2 $mg \cdot mL^{-1}$ 3,3′,5,5′-tetramethylbenzidine (TMB)) was added to each well, and the plate was incubated for 15 min. The reaction was then terminated by adding 50 μl of 2 M sulfuric acid to each well. All reactions were carried out at 37 °C. The absorbance was determined at 450 nm using a Biotek ELx 800 microplate reader.

In order to analyze the inhibitory activity of FITC labelled antibody against PF, we utilized icELISA. The same protocol as for the indirect ELISA was used until the blocking step.

The plate was washed three times with PBST, and 50 μL of various concentrations of PF in PBS were incubated with 50 μL of FITC-labelled MAb solution for 1 h. The subsequent process was the same as that described for indirect ELISA.

To evaluate the specificity of the FITC-labelled antibody, the cross-reactivities (CRs) of the purified FITC-labelled antibody against various compounds were calculated using the formula developed by Weiler and Zenk [18]

Fluorescence-Linked Immunosorbent Assay (FLISA)

For the indirect FLISA, black microtiter plates (FluoroNunc, MaxiSorp, Roskilde, Denmark) were coated and treated as described for the indirect ELISA protocol. Subsequently, various concentrations of FITC-labelled antibody (100 μL/well) were incubated for 1 h at 37 °C. After washing the plate three times with PBST, the fluorescence was measured at an excitation wavelength of 494 nm and an emission wavelength of 518 nm using a fluorescence microplate reader (CLARIOstar, BMG Labtech, Germany).

For indirect competitive FLISA (icFLISA), we utilized the same procedure as for the indirect FLISA until the blocking step. After washing the plate three times with PBST, various concentrations of PF (50 μL) in PBS were incubated with 50 μL of purified FITC-labelled MAb solution for 1 h at 37 °C to observe the competition between PF and PF-BSA. After washing the plate three times with PBST, the remaining fluorescence was measured with a fluorescence microplate reader.

Assay Variation

Intra- and inter-assay variations were tested by evaluating relative standard deviations (RSD, %) of different prescriptions. All measured values were indicated for three replicate wells of each sample on three separate plates.

Correlations Between icFLISA and HPLC for the Determination of PF

Sample Preparation

Based on the document described in *Treatise on Febrile Diseases* of the Han dynasty, the five traditional Chinese medicine prescriptions were produced using Chinese medicinal granules and diluted with distilled water, followed by filtration through a 0.45 μm polyethersulfone (PES) membrane prior to quantification. PF was dried to constant weight and accurately weighed before being dissolved in distilled water, and then further diluted in PBS prior to filtration through a 0.45 μm microporous membrane. The concentrations of PF in the five traditional Chinese medicine prescriptions were compared by HPLC and icFLISA methods in parallel.

Chromatographic Conditions

An Agilent series 1260 HPLC instrument (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode-array detector, an autosampler and a column compartment was used for analyses. The sample was separated on a Zorbax SB-C18 column (5 μm, ϕ 4.6×150 mm, Agilent), with the mobile phase consisting of CH_3CN (A) and water containing 0.1 % acetic acid (B). A gradient program was used as follows: 10 % A in the first 5 min, linearly increased to 25 % A at 15 min, then linearly decreased to 10 % A at 16 min using a mobile phase flow rate of 1.0 mL/min. The chromatogram was recorded at 230 nm and the spectral data for all peaks were obtained in the range of 190–400 nm. The column temperature was kept constant at 30 °C.

Data Analysis

The experimental results are expressed as the mean±SEM. For analysis of the results, all the data obtained from the microplate reader were handled by the Origin Pro 9.0.

Results and Discussion

Indirect ELISA and icELISA

The reactivity of the FITC conjugated PF MAb compared with the PF-BSA conjugate was analyzed by indirect ELIS A. The concentration of each fluorescently labelled MAb positively correlated with the absorbance value as shown in Fig. 1, which suggests that the labelled antibody could detect the PF-BSA conjugate and could be used to detect PF in the ELISA method.

Subsequently, we investigated a competitive binding assay in which the anti-PF fluorescently labelled MAb bound either free PF or a PF-BSA conjugate adsorbed onto a polystyrene microtiter plate with ELISA. Under these conditions, the linear range of the assay extended from 2 to 320 ng•mL^{-1}, as indicated in Fig. 2. The sensitivity of the antibody was satisfactory, with an IC_{50} value of 39.2 ng•mL^{-1} in buffer.

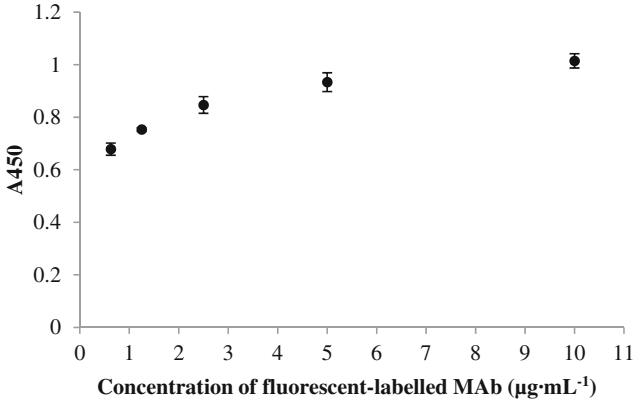

Fig. 1 Reactivity of fluorescent-labelled MAb to PF–BSA conjugates in indirect ELISA, various concentration of fluorescent-labelled MAb were added to each well of a 96-well plate coated with PF–BSA conjugates

Cross-Reactivity (CR) Tests for the Assessment of Specificity

To determine the specificity of the anti-PF FITC-conjugated MAb, CR tests were carried out using the formula established by Weiler and Zenk [18]. As shown in Table 1, the CRs of the fluorescently labelled MAb against albiflorin, a isomer of PF was extremely low (0.076 %). Moreover, we detected no CR with other compounds (<0.01 %). Therefore, the FITC-conjugated MAb for PF has a high specificity for the detection of PF, and it can be used to assay the concentration of PF in various samples with little influence from the other compounds in the medicines or prescriptions.

Indirect FLISA and icFLISA

We applied this fluorescently-labelled MAb in the rapid and simple fluorescence immunoassay. In this FLISA, the time-and cost-consuming secondary antibody reaction and the subsequent enzyme-substrate reaction were avoided because the FITC-labelled anti-PF MAb enabled direct detection using a fluorescence microplate reader.

Table 1 Cross reactivities (%) of anti-PF MAb against various compounds using icFLISA

Compound	Cross reactivities (%)
paeoniflorin	100
albiflorin	0.076
ginsenoside Re	<0.01
gentiopicrin	<0.01
geniposide	<0.01
baicalin	<0.01
catalpol	<0.01
artemisinin	<0.01

The indirect FLISA was carried out using a 96-well black microtiter plate. As shown in Fig. 3, the concentration of the fluorescently labelled MAb was positively correlated with the fluorescent intensity value in a linear manner.

The competitive inhibitory activities of the FITC-labelled MAb against PF were analyzed in an icFLISA as shown in Fig. 4. In this icFLISA, the linear range of PF concentrations ranged from 0.01 ng•mL^{-1} to 100 ng•mL^{-1}. As expected, the limit of detection (LOD; 0.004 ng•mL^{-1}) for PF determination in the FLISA was found to be 500-fold lower than that in conventional ELISA (2.26 ng·mL^{-1}). We hypothesize that this improvement in LOD was derived from the highly sensitive fluorescence of FITC detected by the fluorescence microplate reader compared with that of the enzymatic chromophore. These results suggest that in addition to a simple and rapid immunoassay, a sensitive immunoassay (Table 2) could be developed using fluorescently labelled MAbs instead of unlabelled monoclonal antibodies.

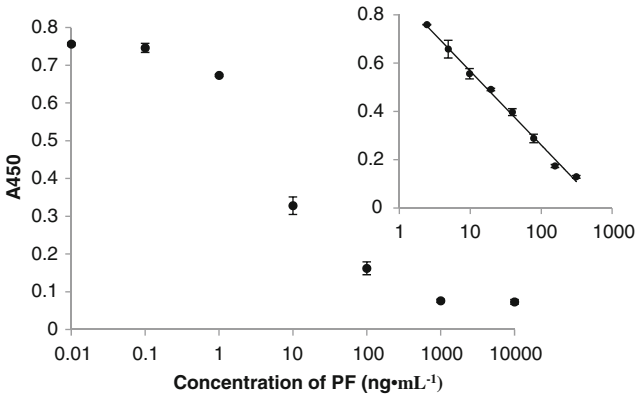

Fig. 2 Standard curve of inhibition by PF using the MAb in ELISA. Various concentrations of PF were incubated with anti-PF MAb in a 96-well plate precoated with PF-BSA (1 μg•mL^{-1}). Error bars: ±1 std dev; the data represent the mean of five replicates. The linear regression equation was y=-0.133ln(x)+0.872 with R^2=0.9952

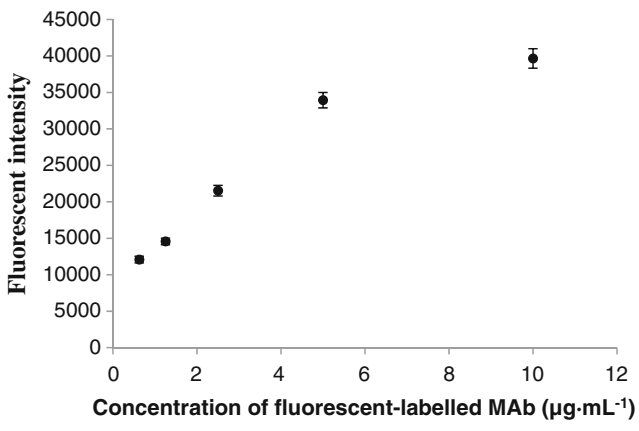

Fig. 3 Reactivity of fluorescent-labelled MAb to PF–BSA conjugates in indirect FLISA, various concentration of fluorescent-labelled MAb were added to each well of a 96-well black microtiter plate coated with PF–BSA conjugates

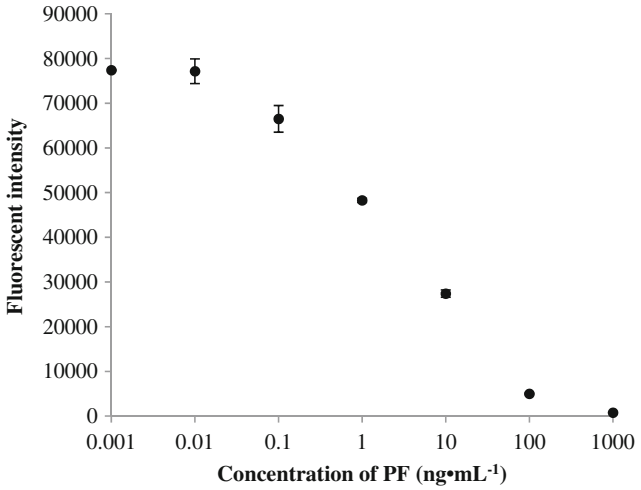

Fig. 4 Standard curve of inhibition by PF using the MAb in FLISA. Various concentrations of PF were incubated with fluorescent-labelled MAb in a 96-well FLISA plate precoated with PF-BSA (1 μg•mL^{-1}). *Error bars*: ±1 std dev; the data represent the mean of five replicates

Precision and Accuracy of FLISA

The accuracy and variation of the assay were evaluated using the relative standard deviations (RSDs) of intra- (well to well) and inter- (plate to plate) assay data from the FLISA using the FITC-labelled PF MAb. A standard curve for the FLISA was established with dilutions of PF in PBS, which allowed for the calculation of RSDs in three replicate wells (intra-assay) and across multiple plates (inter-assay). Table 3 shows that the intra-assay RSDs were less than 4.91 % and the inter-assay RSDs were lower than 6.67 %, which suggests that this assay is very accurate and stable.

Correlations Between icFLISA and HPLC for the Determination of PF

The concentrations of PF in the five traditional Chinese medicine prescriptions were measured using icFLISA and compared to results we acquired by testing with HPLC, allowing us to verify the reliability of our method. The correlation was then calculated from a plot developed by regression analysis based on the values obtained from FLISA and HPLC, as indicated in Fig. 5. The concentrations of PF in the five traditional Chinese medicine prescriptions as measured by our FLISA method were highly consistent with those obtained by HPLC from the same samples (R^2=0.9927).

HPLC method was developed for determination PF in the medicines and biological samples with the characteristics of simultaneous detection a variety of ingredients in samples. The FLISA method is more sensitive, faster for analyzing multiple samples simultaneously with simple sample preparation. Therefore, this FLISA method has a specific advantage in detection the trace amounts of PF in the biological samples.

From our results, it is apparent that this FLISA method can be applied to quantify PF in medicines. This technique will be very useful and convenient for product quality control and quantification in the future.

Table 2 The difference between FLISA and ELISA

	FLISA (ng•mL^{-1})	ELISA (ng•mL^{-1})
Liner range	0.01–100	2.0–320.0
LOD	4*10^{-3}	2.26
IC50	2.20	39.20

Table 3 Intra- and inter-day PF precision in PBS

Samples	The mean concentration of PF (mg•mL^{-1})	RSD (%)	
		Intra-assay	Inter-assay
A	4.41	1.55	6.67
B	5.26	2.25	5.73
C	6.29	4.91	5.6
D	5.62	2.55	5.21
E	3.37	3.13	4.78

A-E represented five chinese medicine priscriptions respectively. A:Shaoyao Gancao Decoction, B:Shaoyao Gancao Fuzi Decoction, C: Si Ni Powder, D: Huang Qin Decoction, E: Guizhi Fuzi Decoction

Conclusions

The results of the present study demonstrate that our newly-developed FLISA assay based on a fluorescently labelled MAb against PF provides a simpler, more efficient and more

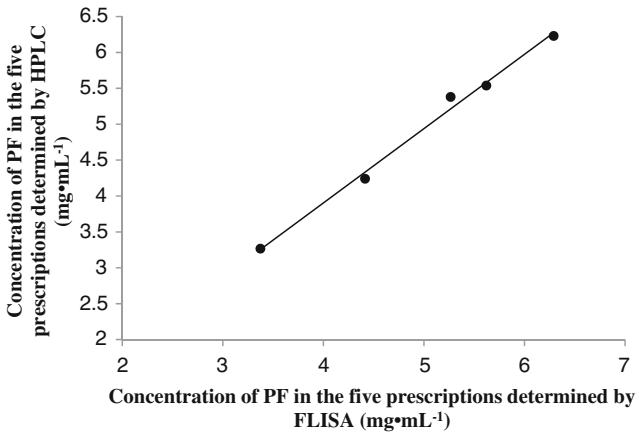

Fig. 5 Correlation between the concentration of PF determined by FLISA and HPLC. The linear regression equation was y=1.0348x - 0.234 with R^2=0.9927

sensitive approach to quantify PF content than conventional ELISA. In the future, FLISA can be also used to assay the concentrations of PF in biological samples, as well as to investigate the pharmacokinetics of PF in different tissues to explore the targets of PF in vivo.

Acknowledgments This research was supported by the National Natural Science Foundation of China (81473338, 81373542), the National Key Basic Research Development Program (973 program) (2011CB505101) and the Classical Prescription Basic Research Team of Beijing University of Chinese Medicine. This manuscript has been thoroughly edited by a native English speaker from an editing company (Boston Professional Group Editing).

The authors declare no conflicts of interest.

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