ORIGINAL ARTICLE

Development of a Fluorescence-Linked Immunosorbent Assay for Baicalin

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Abstract Previously, we developed an indirect competitive enzyme-linked immunosorbent assay (icELISA) for baicalin (BAL) and used this assay to investigate the pharmacokinetics of BAL in mice. In this study, an anti-BAL monoclonal antibody (MAb) was purified by the caprylic acid method and then labelled with fluorescein isothiocyanate (FITC). Subsequently, an indirect competitive fluorescence-linked immunosorbent assay (icFLISA) was developed to detect baicalin (BAL) using FITC-labelled anti-BAL MAbs. Characterization of the assay demonstrated an effective BAL measurement range of 6.4 ng/mL to 500 μg/mL (R^2=0.997). The relative standard deviations (RSDs) for both intra-assay and inter-assay repeatability and precision were below 10 %. This icFLISA for BAL is simple, rapid and sensitive, with a 390-fold larger linear range and a 2-fold lower limit of detection (LOD) compared with the previously developed icELISA. We observed a strong correlation between the results determined by the icFLISA and icELISA methods. Overall, this study provides a useful method for detecting BAL in medicines, enabling in vivo visualization research.

Keywords Baicalin · Fluorescence-linked immunosorbent assay · Enzyme-linked immunosorbent assay · Traditional Chinese medicine · Fluorescently labelled monoclonal antibody

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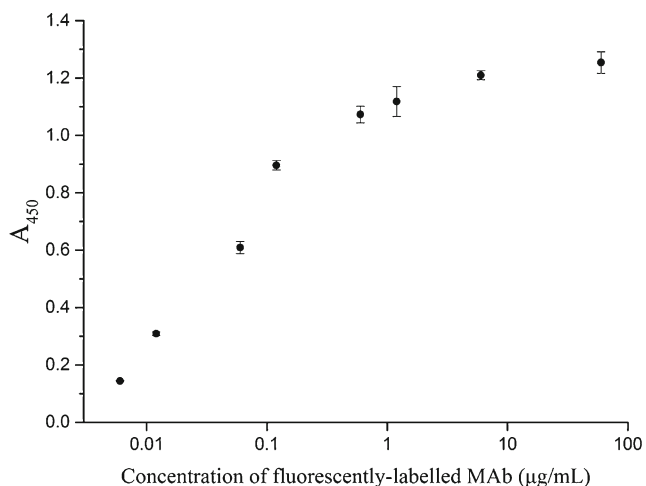
Introduction

Baicalin (BAL), a bioactive flavonoid isolated from the root of Scutellaria baicalensis, is known to have multiple biological functions, including antioxidant, anti-tumour, anti-ischemic, and anti-inflammatory properties [1, 2]. In our previous study, we developed an enzyme-linked immunosorbent assay (ELISA) for BAL and investigated the pharmacokinetics of BAL in mice [3, 4]. These studies provided a good approach for the analysis of small amounts (1–5 μL) of blood samples obtained from small experimental animals.

One drawback of conventional ELISA is that the five indispensable steps, including fixation of the coated antigen, blocking, incubation with the capture antibody and detection antibodies, and substrate reaction, require almost 4.5 h. In contrast, a fluorescence-linked immunosorbent assay (FLISA) takes only 3 h to complete because the time-consuming enzyme-substrate reaction necessary for ELISA can be avoided. ELISAs often expose the samples to environmental temperatures. As a result, the edge effect, which is the difference in temperature between the outer and inner wells, is one of the factors resulting in inter-experimental error. In FLISA, samples are exposed to environmental temperatures for shorter periods of time, minimizing the error compared with ELISA [5]. FLISAs have been developed and
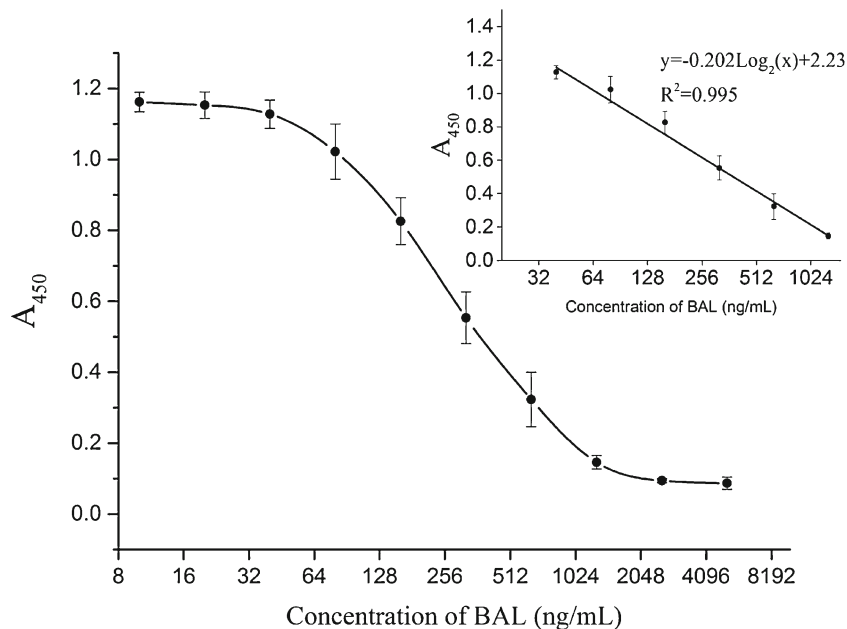
Fig. 1 Reactivity of fluorescently labelled MAbs to BAL–BSA conjugates in iELISA. Various concentrations of fluorescently labelled MAb were added to each well of a 96-well plate coated with BAL–BSA conjugates

applied to analyse the constituents of herbs and foods [6–8].

Recently, we developed an indirect competitive FLISA (icFLISA) for paeoniflorin and succeeded in detecting its content in traditional Chinese herbal medicines [6]. This work demonstrated that this method is simple, rapid and sensitive, with a 500-fold lower limit of detection (LOD) compared with conventional ELISA. These results indicated that the developed icFLISA method was better able to detect the trace amounts of PF in the samples. Therefore, an icFLISA holds promise as a method for the determination of BAL. This study uses a fluorescently labelled anti-BAL monoclonal antibody (MAb) to develop an icFLISA method for the measurement of BAL.

Experimental

Materials and Reagents

Baicalin was purchased from Shanghai Standard Biotech Co., Ltd. (Shanghai, 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 CLARIO star fluorescence microvolume assay technology instrument (Filters: 240–750 nm or 240–900 nm for FI, FP, TRF; 240–750 nm for LUM; Spectrometer: 220–1000 nm for ABS) was purchased from BMG Labtech (Germany), and an ELx 800 microplate reader (Wavelength range: 400 to 750 nm) was obtained from Biotek (USA). A CE7200 UV-Visible Spectrophotometer (Double Beam Wavelength Scans, 190–900 nm) was purchased from CECIL Instruments (England).

Sample Preparation

Radix Scutellariae extract was prepared by aqueous extraction followed by alcohol precipitation. Dachaihu decoction and xiaochaihu decoction were produced using Chinese medicinal granules diluted with methanol. These decoctions were then diluted with deionized water prior to detection by icELISA and icFLISA.

Fig. 2 Standard curve of BAL inhibition of the MAb ELISA signal. Various concentrations of BAL were incubated with anti-BAL MAb in a 96-well plate precoated with BAL-BSA; the data represent the mean of five replicates

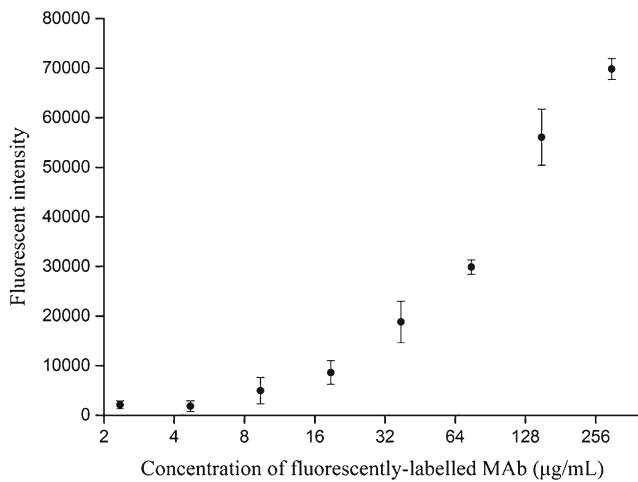$y = -0.202 \log_2(x) + 2.23$
$R^2 = 0.995$

Fig. 3 Reactivity of the fluorescently labelled MAb to BAL–BSA conjugates in iFLISA. Various concentrations of fluorescently labelled MAbs were added to each well of a 96-well black microtiter plate coated with BAL–BSA conjugates

Purification of the Anti-BAL MAb

In our previous study, we obtained the anti-BAL MAb from a hybridoma created through the fusion of splenocytes immunized with BAL-ovalbumin and conjugated with the HAT-sensitive mouse myeloma cell line SP2/0. The conventional caprylic acid purification method enables large scale purification, requiring short processing time and minimal buffer preparation [9]. Therefore, anti-BAL MAbs were purified by the caprylic acid method.

Fluorescent Antibody Labelling

Fluorescent labelling of antibodies has conventionally been accomplished through the chemical conjugation of organic

fluorophores. The MAb was labelled with fluorescein isothiocyanate (FITC, Amresco chemical company, conjugation grade) as described in previous reports, with some modifications [10]. First, 4 mg of anti-BAL MAb powder was measured and diluted to 10 mg/mL with a 0.025 M carbonate buffer saline solution (pH=9.0, consisting of 0.5 M Na_2CO_3 and 0.5 M $NaHCO_3$). A 60 μL volume of FITC solution (1 mg/mL in DMSO) was added to the MAb solution, mixed thoroughly and incubated in the dark at room temperature for 6 h. To remove the unbound FITC, the mixture was passed through a Sephadex G-50 column equilibrated with 0.005 M phosphate buffer (PB). Then, the effluent was detected by a spectrophotometer to calculate the concentration of the FITC-labelled anti-BAL MAbs and the F/P molar ratio, defined as the ratio of moles of FITC to moles of protein in the conjugate. To determine this ratio, it is necessary to determine the absorbance of the conjugate sample at 280 and 495 nm.

iELISA and icELISA

The icELISA protocol used to measure the BAL concentrations was previously established by our group [3]. We examined the reactivity of the FITC labelled anti-BAL MAbs to BAL-bovine serum albumin (BAL-BSA) conjugates using an indirect ELISA (iELISA). The BAL-BSA conjugate (1 μg/mL, 100 μL/well) was adsorbed in the wells of a 96-well immunoplate, 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 wash buffer (PBS containing 0.05 % Tween 20; PBST), and FITC-labelled anti-BAL MAbs (100 μL/well) were 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-labelled goat anti-mouse IgG

Fig. 4 Standard curve of BAL inhibition of the MAb FLISA signal. Various concentrations of BAL were incubated with the anti-BAL MAb in a 96-well plate precoated with BAL-BSA; the data represent the mean of five replicates
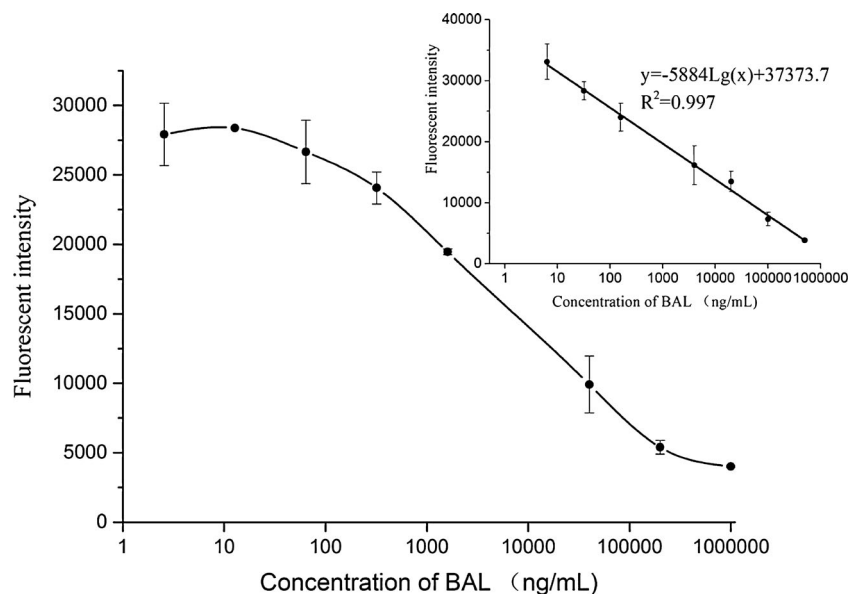
Table 1 The characteristics of the FLISA and ELISA methods

	FLISA (ng/mL)	ELISA (ng/mL)
Liner range	6.4–500000	16–1280
IC50	1687.5	153.8
LOD	6.1	15.0

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/mL 3, 3',5,5'-tetramethyl benzidine (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 sulphuric 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.

The inhibitory activity of the FITC-labelled antibody against BAL was analysed by icELISA. The same protocol was used as for the iELISA until the blocking step. The plate was washed three times with PBST, and 50 μL of various concentrations of BAL in PBS were incubated with 50 μL of anti-BAL MAb solution for 1 h. The subsequent process was the same as that described for the iELISA.

iFLISA and icFLISA

For the indirect FLISA (iFLISA), black microtiter plates (FluoroNunc, MaxiSorp, Roskilde, Denmark) were coated and treated as described for the iELISA protocol. Subsequently, various concentrations of FITC-labelled anti-BAL MAbs (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 485 nm and an emission wavelength of 520 nm using a fluorescence microplate reader (CLARIOstar, BMG Labtech, Germany).

The indirect competitive FLISA (icFLISA) used the same procedure as the iFLISA until the blocking step. After washing the plate three times with PBST, various concentrations of BAL (50 μL) in PBS were incubated with 50 μL of purified FITC-labelled anti-BAL MAb solution for 1 h at 37 °C to observe the competition between BAL and BAL-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 the relative standard deviations (RSD, %) of BAL samples at different concentrations. All of the reported values were determined from three replicate wells on three separate plates.

Correlations Between icFLISA and icELISA for the Determination of BAL

Three different BAL concentrations were determined by the icFLISA and icELISA methods in parallel.

Determination of BAL in Traditional Chinese Medicines

The concentrations of BAL in the Radix Scutellariae extract and two prescriptions containing Radix Scutellariae without pretreatment were measured by icFLISA.

Data Analysis

The experimental results are expressed as the mean ± SEM. All of the data obtained from the microplate reader were analysed using Origin Pro 9.0.

Results and Discussion

Fluorescent Antibody Labelling

The optimum F/P ratio is between 0.3 and 1.0 according to the Sigma product information. Lower ratios will yield low signals, while higher ratios will result in high background. However, F/P ratios outside the optimal range have been successfully used to develop FLISAs [11]. To obtain the F/P ratio and antibody concentration, we placed the conjugate sample in a quartz cuvette and read the absorbance of the conjugate sample at 280 and 495 nm using a spectrophotometer.

Table 2 Intra- and inter-assay precision of the icFLISA for BAL

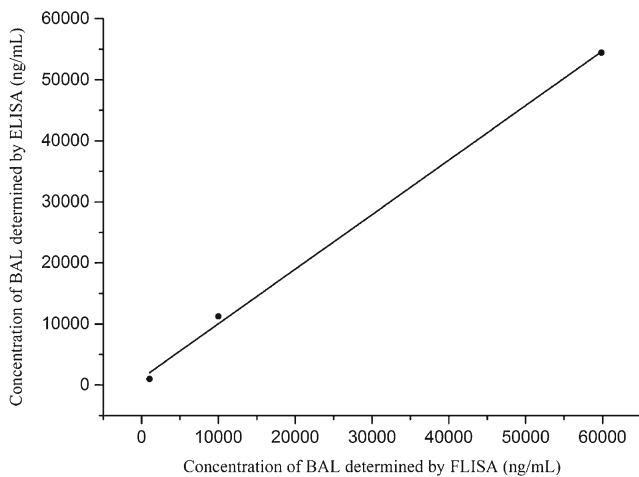| BAL (ng/mL)	Intra-assay		Inter-assay	
	Concentration (ng/mL)	RSD (%)	Concentration (ng/mL)	RSD (%)
1000	1145.66±14.98	1.31	1038.67±80.75	7.78
10000	9809.62±101.38	1.03	10002.39±921.47	9.21
40000	34733.88±1103.27	3.18	33625.81±1620.53	4.82
200000	203017.53±5903.10	2.91	191377.93±20589.96	10.76

Fig. 5 Correlation between the BAL concentrations determined by FLISA and ELISA. The linear regression equation was $y=0.894x+1091.2$, with $R^2=0.9969$

The concentration of the FITC-labelled anti-BAL MAbs and the F/P ratio were calculated as 0.19 mg/mL and 2.57, respectively. Although the F/P ratio is outside the optimal range of 0.3 to 1.0, the subsequent results showed that these FITC-labelled anti-BAL MAbs were successfully implemented in the icFLISA.

IELISA and icELISA

The reactivity of the FITC-labelled anti-BAL MAbs compared with the BAL-BSA conjugate was analysed by iELISA. The concentration of the FITC-labelled anti-BAL MAb was positively correlated with the absorbance value, as shown in Fig. 1, suggesting that the labelled antibody can detect the BAL-BSA conjugate and can be used to detect BAL in the FLISA method.

Subsequently, the BAL icELISA procedure was established. Figure 2 demonstrates the competitive inhibition between anti-BAL MAbs and BAL-BSA resulting from increasing BAL concentrations. This provides a calibration curve for the BAL concentration in the icELISA. Under these conditions, a linear relationship was observed between optical density and concentration in the range of 16 ng/mL to 1.28 μg/mL. The sensitivity of the antibody was satisfactory, with an IC_{50} value of 153.8 ng/mL in buffer.

IFLISA and icFLISA

The iFLISA was carried out using a 96-well black microtiter plate. As shown in Fig. 3, the concentration of the fluorescently labelled MAb exhibited a positive linear correlation with the fluorescent intensity.

The competitive inhibitory activities of the FITC-labelled anti-BAL MAb were analysed in an icFLISA, as shown in Fig. 4. In this icFLISA, the linear range of BAL concentrations ranged from 6.4 ng/mL to 500 μg/mL. As expected, the linear range of the icFLISA (6.4–500000 ng/mL) was 390-fold greater than that of the ELISA (16–1280 ng/mL). The limit of detection (LOD) for BAL determination in the icFLISA (6.1 ng/mL) was found to be 2-fold lower than that in conventional icELISA (15 ng/mL) (Table 1). Our results suggest that using fluorescently labelled MAbs instead of unlabelled MAbs could not only improve the simplicity and speed of the immunoassay but also enhance its sensitivity.

Precision and Accuracy of the icFLISA

The precision 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 icFLISA using the FITC-labelled anti-BAL MAbs. A standard curve for the icFLISA was established with dilutions of BAL in PBS, enabling the calculation of RSDs in sextuplicate wells (intra-assay) and across multiple plates (inter-assay). Table 2 shows that the intra-assay RSDs were less than 3.18 % and that the inter-assay RSDs were lower than 10.76 %, suggesting that the precision of this assay is acceptable and stable.

Correlations Between icFLISA and icELISA for BAL Determination

In our previous study, we developed an icELISA method for BAL determination and used that method to investigate the pharmacokinetics of BAL in mice [3, 4, 12]. A linear correlation was obtained for the BAL concentrations in the range from 34.69 to 2220.00 ng/mL. The regression equation was $y=1.5557-0.4028\,Lg\,(C)$, with a correlation coefficient of 0.9936. These analytical results of the icELISA method were

Table 3 Total BAL concentrations in various traditional Chinese medicines determined by icFLISA

Sample	Composition of Kampo medicine	Concentration of BAL (μg/mL)
Radix Scutellariae extract	Radix Scutellariae	9.961±0.773
Dachaihu decoction	Radix Bupleuri, Radix Scutellariae, Rhizoma Zingiberis Recens, Rhizoma Pinelliae, Fructus Ziziphi Jujubae, Paeonia Lactiflora, Fructus Aurantii Immaturus, Rheum Rhabarbarum	317.225±11.571
Xiaochaihu decoction	Radix Bupleuri, Radix Scutellariae, Rhizoma Zingiberis Recens, Radix Ginseng, Rhizoma Pinelliae, Radix Glycyrrhizae Praeparata, Fructus Ziziphi Jujubae	302.294±18.840

consistent with those of an HPLC method used to determine BAL pharmacokinetics in studies of traditional Chinese medicine (Gegen Qinlian Decoction) in mice.

Therefore, we used the conventional icELISA assay as a comparison for the icFLISA method. Three BAL samples with different concentrations were measured using icFLISA and compared to the icELISA results, allowing us to verify the reliability of the new method.

The correlation was then calculated from a plot developed by regression analysis based on the values obtained from FLISA and ELISA, as indicated in Fig. 5. The concentrations of BAL as measured by our icFLISA method were highly consistent with those obtained by icELISA from the same samples (R^2=0.9969).

Determination of BAL in Traditional Chinese Medicines

We applied the newly developed method to determine the concentrations of BAL in Radix Scutellariae extract and in two prescriptions containing Radix Scutellariae (Table 3). These results demonstrate that this icFLISA method can be applied to quantify BAL content in medicines. This convenient technique will be highly useful for product quality control and quantification in the future.

Conclusions

In this study, FITC-labelled BAL-MAbs were used to develop an icFLISA to detect the BAL content in traditional Chinese medicine. Moreover, the linear range for BAL determination in FLISA (6.4–500000 ng/mL) was 390-fold larger than that observed in conventional ELISA (16–1280 ng/mL). In the future, this FLISA method can also be used to assay the concentrations of BAL in biological samples, as well as to investigate the pharmacokinetics of BAL in different tissues to explore the targets of BAL in vivo.

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