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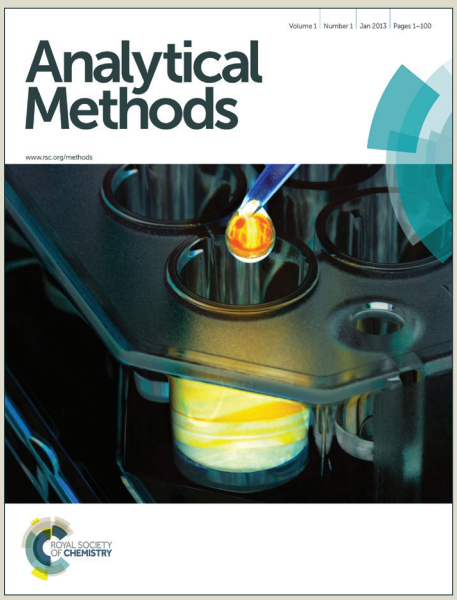

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Novel artificial antigen synthesis for antibody production and development of an indirect competitive ELISA of cyanocobalamin

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Abstract:

In the current work, cyanocobalamin was coupled to Bovine Serum Albumin (BSA) and ovalbumin (OVA) by CDI method to produce artificial antigens. Then a monoclonal antibody (MAB) against cyanocobalamin was acquired. In comparison with other reported antibodies, the MAB showed good specificity toward cyanocobalamin. Cross reactivity was found to be less than 0.01% with other compounds of vitamin B, except for three analogs of cobalamins which indicated from 0.27% to 2.31%. An indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) for the detection of cyanocobalamin was developed based on the MAB. Under the optimum conditions, the limit of detection was 0.2 ng mL^{-1} with a linear working range of 2–100 ng mL^{-1} (R^2=0.993). The spiked samples were detected with recovery ranging from 86.02–110.54%, and the coefficients of variation were from 2.62% to 13.7%. Six commercially available vitamin supplements were tested by ELISA method, after purification with immunoaffinity column, the samples were validated by HPLC with UV detection at 361 nm. The results obtained also showed good correlation between ELISA and HPLC (R^2=0.998). Thus, the method proposed had been proved to be a trustworthy tool to quantify cyanocobalamin in vB_{12} tablets supplements.

Keywords: cyanocobalamin, ELISA, immunoaffinity column, HPLC

1.Introduction

The B_{12} vitamins, or cobalamins, are some organic complex containing central cobalt ion in a corrin ring.[1] Cobalamins consist of four major forms which were cyanocobalamin, adenosylcobalamin, hydroxocobalamin and methylcobalamin respectively (Fig. 1).[2, 3] As an essential nutrient for human health, B_{12} vitamins play an important role in the synthesis of DNA and development of the myelin sheath, especially in blood delivery system and nervous system, deficiency of B_{12} vitamins may lead to pernicious anemia or irreversible neurological damage.[4, 5] However, excessive intake of B_{12} vitamins may cause allergic reaction or anaphylactic shock.[6] Besides, they are such vitamins that have great influences on human health at the nanogram level, and a moderate consumption of B_{12} vitamins is crucial in acceptable daily intake.[7-9]

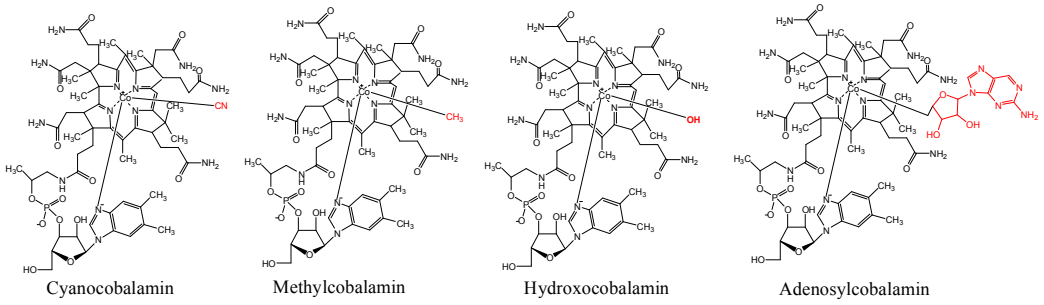

Fig. 1 The structures of cyanocobalamin, methylcobalamin, hydroxocobalamin and adenosylcobalamin respectively

Mostly, B_{12} vitamins are found in animal foods including meat, milk and eggs products but practically free from plant foods. In other words, B_{12} vitamins deficiency more inclined to vegetarians or some older peaple who was unable to absorb naturally occurring B_{12} vitamins. In order to avoid suffering B_{12} vitamins deficiency, the B_{12} vitamin supplements or fortified foods were suggested to be taken to meet their requirments. One B_{12} vitamin that was widely added to foods or vitamin tablets is cyanocobalamin, which with a salient characteristic of higher stability than other cobalamins, so cyanocobalamin is well-known as vB_{12}.[10] Actually, owing to the high-price and easy-decomposition of vB_{12} during processing and storage, only a low level of vB_{12} was exist in the products, and vB_{12} deficiency was unavoidable even they had taken them.[11] Thus, a rapid and accurate method for the determination of vB_{12} in varies vitamin supplements should be set up imminently.

Although many traditional analytical methods had been employed in detecting vB_{12}, such as high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS),[12-15] biosensor method,[16] spectrophotometry or fluorescence assay,[17, 18] they could hardly avoid complex samples pretreatment procedure and matrix effects, coupling with the expensive instruments make them difficult to popularize. Microbiological assay (MBA) was used as a dominant detection method by many countries which showed highly sensitive,[19-21] however, time-consuming, demanding specialized technical input and low precision limited the development of its use. Furthermore, the enzyme linked immunosorbent assay (ELISA) is a rapid method with the characteristics of accuracy, specificity, efficiency and almost free of matrix effects in miscellaneous samples.[9, 22] In this study, the MAB specifically against vB_{12} was obtained. After a full optimization procedure, the ic-ELISA for detecting vB_{12} was established and sample

detection results were confirmed by IAC-HPLC method. The results showed good correlation between ELISA and IAC-HPLC method, it also revealed that the ic-ELISA method is a promising tool for vB_{12} detection.

2. Experimental

2.1 Materials and reagents

Cyanocobalamin (vB_{12}), Vitamin B_1, B_2, B_5, B_6, B_8, Bovine serum albumin (BSA), N, N'-carbonyldiimidazole (CDI), Ovalbumin (OVA), 3,3-,5,5-tetramethylbenzidine (TMB) and goat anti-mouse IgG-HRP, Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIC) were all purchased from Sigma Chemical Co,. Ltd (USA). Methylcobalamin, Hydroxocobalamin and Adenosylcobalamin were obtained from Wuhan Dahua Pharmaceutical Co,. Ltd (China). Dimethyl sulfoxide (DMSO), Ethanol (CH_3CH_2OH) and Trifluoroacetic acid (CF_3COOH) were obtained from Damao Chemical Reagent Co., Ltd. (Tianjin, China). Acetonitrile (CH_3CN) for HPLC use was obtained from Tedia company (USA). All other chemicals involved were of analytical reagent grade. immunoaffinity column was made in our lab Microwell plates and Millipore Millex-GP (22 μm) were purchased from COSTAR Co,. Ltd (USA) and Merck Millipore Ltd (Germany). Vitamin B_{12} samples were purchased from a large supermarket.

2.2 Instruments

Microplate Reader (MKS) used for ELISA was obtained from Thermo Co,. Ltd (ShangHai), UV-VIS spectrophotometer was model 2600 and obtained from Shanghai Analytical Instrument, Agilent HPLC 1100 accompanied with Agilent ZORBAX Eclipse Plus C18 (4.6*150 mm, 3.5 μm particle size) column used for samples analysis was purchased from Agilent Co,. Ltd (USA). High-speed centrifuge used was an Eppendorf 5804 R instrument (Germany).

2.3 Buffers and solutions

Phosphate-buffered saline (PBS pH 7.4) was prepared by dissolving 2.9 g of Na_2HPO_4, 8.0 g of NaCl, 0.2 g of KCl and 0.2 g of KH_2PO_4 in 1000 mL of Distilled water, PBS with 0.05%

Tween-20 (PBST). Block solution was prepared by dissolving 5 g of skimmed milk in 100 mL distilled water.

All experiments were performed in compliance with administration of affairs concerning experimental animals approved by the State Council on October 31, 1988 in China and laboratory animal ethics review committee of Jiangxi province.

2.4 Preparation of the complete antigen

VB_{12} was conjugated to BSA and OVA by CDI (N, N'-carbonyldiimidazole) method. CDI was used to activate the hydroxyl of vB_{12} and then conjugated to a carrier protein (Fig. 2). Briefly, 2.3 mg of vB_{12} and 3.6 mg of CDI were dissolved in 250 μL anhydrous DMSO in a 5 mL vial and carried out the activation reaction at 30□ for 3h under continuous stirring in the dark. The obtained solution was added slowly to a BSA solution (6 mg BSA dissolved in 1.3 mL PBS, pH 7.4), then stirred for 18h at 30□in the dark and the obtained solution (vB_{12}-BSA) was dialyzed against distilled water at 4□for 3 days (2 changes per day). Similarly, vB_{12}-OVA was treated as described above. For identifying the complete antigens, both of the conjugates were scanned respectively by a UV spectrophotometer, finally the conjugates were freeze-dried and stored at -20□ until further use.

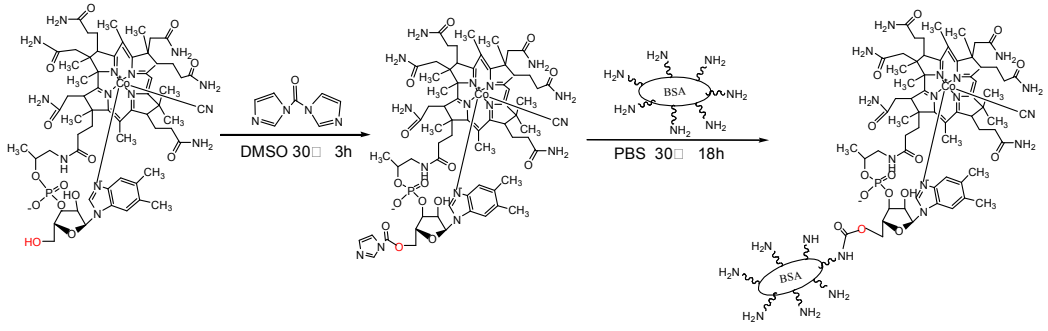

Fig. 2 Synthesis of vB_{12} artificial antigen

2.5 Production of monoclonal antibody against vB_{12}

The immunogens (vB_{12}-BSA) synthetized by CDI method were used to produce MAB. For the primary immunization, vB_{12}-BSA was emulsified with isopyknic Freund's complete adjuvant,

then six Balb/c female mice (8–10 weeks old) were immunized with BSA-vB_{12} (60 μg protein per mice) subcutaneously at multiple sites (10–15 sites). Three weeks later, the immunization was boosted with vB_{12}-BSA emulsified with Freund's incomplete adjuvant, another booster immunization were performed at a 3 week interval. The titer of antiserum was determined by ic-ELISA, in which the vB_{12}-OVA was used as coating antigen. After four boosters, one of the mice exhibited the highest titer, then it was immunized with 30 μg of BSA-vB_{12} through the tail veins as a final dose for fusion experiment. 3 days later the mouse was sacrificed for spleens and the splenocytes was fused with the SP2/0 myeloma cells by hybridoma technique. Finally the hybridomas were selected in a HAT medium and cultured in 96-well plates, culture supernatants from each well were detected by ic-ELISA with the aim of screening the positive hybridomas which could produce antibodies specific to vB_{12}.

2.6 Titration of MAB

The checkerboard procedure was performed in order to determine the optimal dilution of coating antigen and antibody by ic-ELISA. In short, microplates were coated with 110 μL of vB_{12}-BSA at various concentrations (0.5, 1, 2, 4 μg mL^{-1}) in PBS at 37□ for 2 h in the dark, after 3 times' washing with PBST solution, 320 μL per well of 5% skimmed milk were added as the blocking solution for 2 h incubation at 37□, MAB from hybridoma cell culture supernatant was diluted in a series of standards (1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400) with PBS and added to the corresponding plates for 40 min at 37□, after washing 3 times with PBST solution, 100 μL of IgG-HRP was diluted at 1:2500 and added to each well for 40 min, 100 μL of TMB solution was added to the plates and incubated for 8 min, then stop the reaction by using 2 mol L^{-1} H_2SO_4 (50 μL per well) and recorded the absorbance at 450 nm.

2.7 ELISA Procedures

Ic-ELISA procedure was carried out as mentioned below. The plates were coated with 110 μL of vB_{12}-BSA in PBS at 37□ for 2 h in the dark, after 3 times' washing with PBST solution, 320 μL of 5% skimmed milk were added to per well for 2 hours' incubation and then washed with PBS-T solution, 50 μL of the optimized concentration of MAB and 50 μL of a serial concentrations of

vB_{12} standard solutions or samples were added to each well for 40 min at 37□. The plates were washed with PBST solution, incubated for 40 min with 100 μL of IgG-HRP , washed and dried. TMB was added for 8 min (100 mL per well) and stopped the reaction by 50 μL of 2 mol L^{-1} H_2SO_4, and finally measured at 450 nm.

2.8 Standard Addition

2.8.1 Samples Preparation

The vitamin supplement samples for spike experiment were purchased in supermarket and proved to be free of vB_{12}. 10 tablets were exactly weighed and ground by mortar to a uniform powder, then the average weight of one tablet was weighted and transforred into a brown flask. 50 mL distilled water was added and spiked at three different concentrations (1, 2 and 4 μg $slice^{-1}$) with vB_{12}, and the content was sonicated at 42□in the dark. After 30 min' incubation, it was transferred to a volumetric flask and adjust to the 100 mL volume by distilled water (the concentrations of vB_{12} were 10, 20 and 40 ng mL^{-1}). The solution was mixed well and centrifuged at 10000 rpm for 15 min, and then the supernatant was transferred into 50 mL tube in the dark.

2.8.2 For ELISA analysis and HPLC analysis

The samples solution obtained above were detected directly by ic-ELISA. However, for HPLC, the solution should be processed to avoid suffering from matrix effects. For this reason, the immunoaffinity column for vB_{12} was applied to purify and concentrate the sample extracts. Briefly, 1 mL of obtained samples solution with different concentrations were dissolved in 20 mL distilled water for clean-up by immunoaffinity column (let the solution drain by gravity at room temprature). After 2 times' washing with 10 mL distilled water, 3 mL methanol was used to elute antigen (vB_{12}) into a 10 mL glass tube, and evaporate eluate completely by water-bath at 65□. 1 mL ultra pure water was added to the each tube, shaken and filtered through a 0.45 μm filter, then 100 μL solution was injected into chromatography with the operation conditions as below (Table 1).[23, 24]

Table 1 Chromatographic conditions for gradient elution of vB_{12}. Mobile phase A consisted of 0.025% (CF_3COOH) in doubly distilled water, mobile phase B was acetonitrile absolutely, flow rate was 1 mL min^{-1} with running time of 30 min at 25℃.

Time/min	Mobile phase A (%)	Mobile phase B (%)
0–3.5	100	0
3.5–11	75	25
11–19	65	35
19–20	90	10
20–30	100	0

3 Result and Discussion

3.1 Characterization of vB_{12} conjugates

The UV spectrum were recorded from 200 nm to 400 nm , from the scanning diagram, the characterization absorption peaks of vB_{12} and BSA were exhibited at 361 nm and 278 nm respectively. However, the absorption peaks of vB_{12} conjugates not only contained the characteristic absorption peaks at 361 nm (vB_{12}), but also had the absorption peeks at 278 nm (BSA), which suggested the vB_{12}-BSA conjugates were successfully synthesized (Fig. 3). Similarly, the vB_{12}-OVA conjugates were also proved to be synthesized successfully.

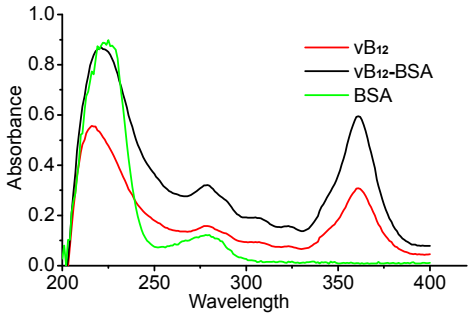

Fig. 3 UV absorption spectra of vB_{12}, BSA, and vB_{12}-BSA

3.2 Monoclonal antibody titer

According to expectation, fusion experiment was performed and the specific MAB was obtained successfully, the subclass of MAB was κ IgG2a and the titer of antibody was determined by checkerboard method (Fig 4). The diagram indicated that 1 μg mL^{-1} of the antigen and the antibody titer of 1:3200 was the best concentrations in ELISA respectively.

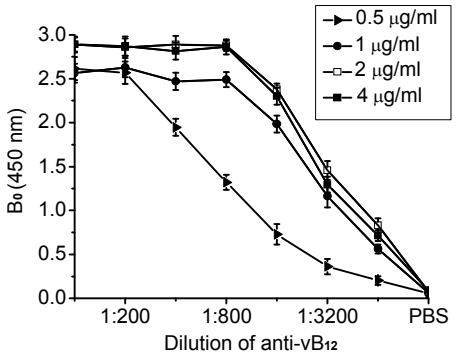

Fig. 4 Checkerboard curve of antigen-antibody of vB_{12}

3.3 Optimization of ELISA conditions

As vB_{12} was easily dissolved in water or ethanol and decomposed in strong acid or alkali, several influence factors such as pH, alcohol and ionic strength to ELISA were examined, each condition were performed 4 times and then the half inhibition concentration (IC_{50}) of different conditions were obtained. Besides, B_0/IC_{50} usually as an important factor to judge the performance of ELISA, higher ratio means higher sensitivity.[25, 26] As shown in Fig 5, when the pH was 6.5, B_0/IC_{50} value was the highest (0.097), and the IC_{50} reduced to the minimum (10.51 ng mL^{-1}). The existence of ethanol could inhibit the interaction between antibody and antigen (Fig. 6). The IC_{50} increased gradually when the concentration of ethanal increased from 0 to 30%, and the higher concentration of ethanol, the higher value of IC_{50}. For ionic strength (Fig. 7), the highest B_0/IC_{50} value (0.090) was obtained when the salt concentration was around 100 mmol L^{-1}, the IC_{50} was 13.3 ng mL^{-1}, however, water solution (pH 6.5) seemed to be the most sensitive. So water solution at pH value of 6.5 was chosen as the optimal conditions. Under the optimum conditions the dose-response curve was obtained (Fig. 8), the limit of detection (IC_{10}) was 0.2 ng mL^{-1}, and the linear range was from 2 ng mL^{-1} to 100 ng mL^{-1}.

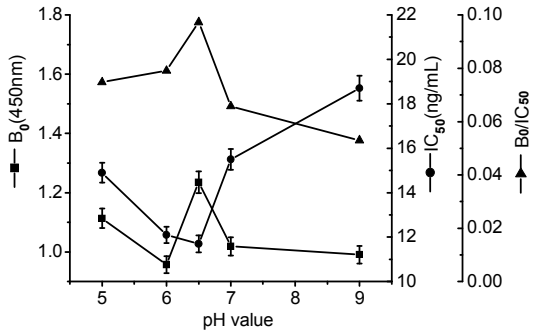

Fig. 5 Effects of pH on ELISA

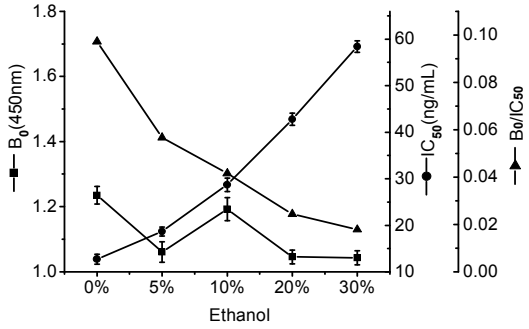

Fig. 6 Effects of ethanol concentration on ELISA

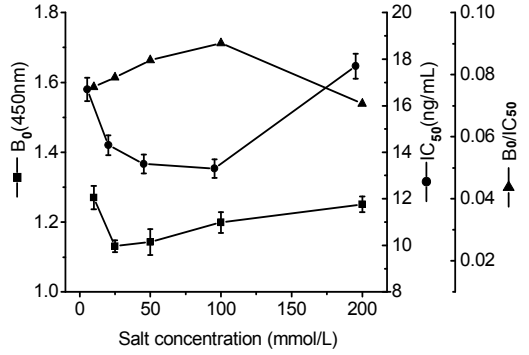

Fig. 7 Effects of ion concentration on ELISA

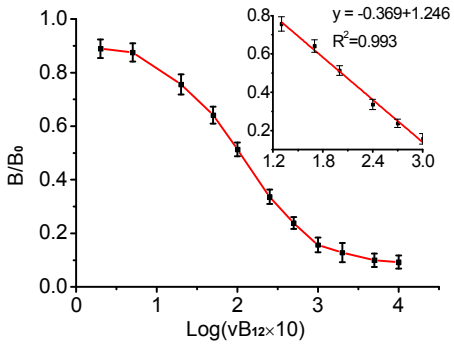

Figure. 8 The competitive inhibition curve and calibration curves in the linear range (inset) for vB_{12}

3.4 Cross-reactivity and sensitivity

As described above, vB_{12} (cyanocobalamin) is more similar to its related compounds in structure (Fig 1), which could lead to high cross reactivity. Thus, with the aim of testing the specificity of MAB, the cross reactivity of vB_{12} was measured by ic-ELISA. As the data shown in Table 2, the cross reactions of the other three compounds were all lower than 3%, hydroxocobalamin revealed little high cross-reactivety of only 2.31%, methylcobalamin, adenosylcobalamin showed the cross-reactivety of 0.53% and 0.27% respectively. No cross reactivity was detected with other related vitamin B compounds, which showed the MAB was highly specific against cyanocobalamin.

Table 2 Cross-reactivity of various of vitamin B compounds (n=4)

Forms of vitamin B	*50%B/B$_0$ (ng ml^{-1})*	*Cross reactivity (%)*
Cyanocobalamin	10.51	100
Methylcobalamin	1975.63	0.53
Hydroxocobalamin	446.60	2.31
Adenosylcobalamin	3948.51	0.27
Vitamin B_1	NR	<0.01
Vitamin B_2	NR	<0.01
Vitamin B_5	NR	<0.01
Vitamin B_6	NR	<0.01
Vitamin B_8	NR	<0.01

NR: no response

3.5 Preparation of immunoaffinity column

The vB_{12} monoclonal antibodies obtained were coupled with CNBr-activated sepharose 4 fast flow, then the immunosorbent was treated with ethanolamine buffer (0.1 mol L^{-1}, pH 8.0) at room temperature. After 3 hours' incubation, the gel was washed by PBS (0.01 mol L^{-1}, pH 7.4) to remove the unreacted antibody. The gel was washed with Tris-HCl buffer (0.1 mol L^{-1}, pH 8.0) and sodium acetate buffer (0.1 mol L^{-1}, pH 3.6) for three times and stored in PBS containing 0.02% (v/w) sodium azide at 4℃overnight. Finnaly the gel was washed by 20% ethanol and filled in 1 mL injector for further use.

3.6 ELISA Analysis and HPLC Confirmation

ELISA was usually used as a tool of qualitative and half-quantitative determination, so each of the establishment of ELISA method should be confirmed by some more accurate instrument methods.[27] In this work, HPLC method was implemented and vB_{12} standard addition recoveries experiments were performed to compare with the ELISA method (table 3). As the data shown, good recoveries were obtained from each method. The ELISA method showed recovery rates of 86.02% to 110.54% and CVs of 2.62–13.7%. Meanwhile, the average recoveries of HPLC method ranged from 78.42% to 102.94%, and it showed more credible and accurate than ELISA's with a coefficient of 0.19% to 2.94%. Besides, some results acquired by ELISA method were little higher than the HPLC method's, and this distinction may cause by nonspecific adsorption of reagents used during the ic-ELISA procedure. The data also indicated that the ELISA method for the detection of vB_{12} was almost free of the matrix effects, and it was more efficient in screening and detecting vB_{12} samples, which the HPLC method can't attain.

Table 3 Recoveries of vB_{12} in Blank Vitamin Samples by ELISA and HPLC. Intra-assay CV was determined by 6 repealicates on one day, and inter-assay CV was determined by 4 repealicates, once a day.

vB_{12}	*Fortified (ng ml^{-1})*	ELISA				HPLC	
		Intra-assay		Inter-assay			
		Recovery (%)	*CV (%)*	*Recovery (%)*	*CV (%)*	*Recovery (%)*	*CV (%)*
Sample 1	10	92.89	10.50	93.53	7.41	102.94	2.94
	20	110.54	7.36	108.2	8.29	93.65	2.18
	40	98.41	2.62	93.65	4.80	88.87	2.47
Sample 2	10	86.02	4.01	90.38	9.37	78.43	2.64
	20	88.32	6.27	93.40	11.06	95.23	2.67
	40	97.90	3.81	94.02	6.36	87.96	0.19
Sample 3	10	92.53	13.7	89.49	10.28	91.47	0.70
	20	102	3.12	104.4	5.61	92.23	2.85
	40	100.4	13.4	96.05	6.52	92.73	2.60

3.7 Unknown Samples Detection

Six vB_{12} supplements (solid samples) were tested by ELISA and validated by HPLC for further evaluation (Table 4). The consistency was obtained between the two methods (R^2=0.998, Fig. 9), which indicated the ELISA method offer an accurate approach for detecting vB_{12} in real samples. Besides, the results also showed that some of determined values are exactly lower than their labeled values such as sample 5, which was proved to be no content. Nevertheless, most of the commercially available vB_{12} supplements were qualified.

Table 4 unknown samples detection by ELISA and HPLC (n=6).

vB_{12} supplements	*Labeled (μg slice^{-1})*	ELISA		HPLC	
		Tested (μg slice^{-1})	*CV (%)*	*Tested (μg slice^{-1})*	*CV (%)*
Sample 1	1.87	2.72±0.023	7.66	2.22±0.02	1.04
Sample 2	2.51	2.99±0.73	8.15	2.96±0.04	0.12
Sample 3	1	1.09±0.11	9.78	0.98±0.01	0.67
Sample 4	3	3.56±0.09	2.53	2.88±0.01	0.40
Sample 5	1.3	0	0	0	0
Sample 6	25	20.76±2.16	10.41	22.2±0.25	1.14

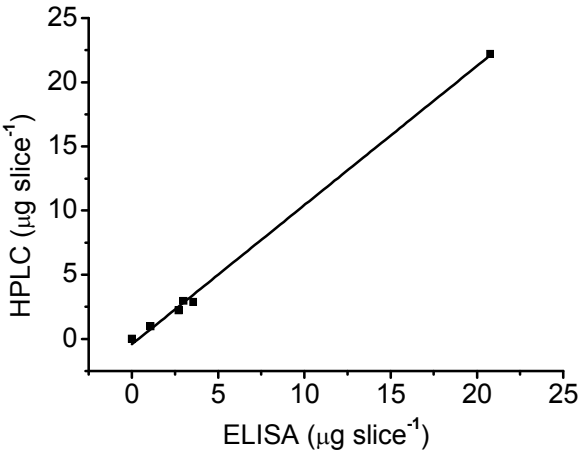

Fig. 9 Correlation between the ic-ELISA and HPLC methods for authentic samples.The regression equation was $y = 1.084x - 0.417$, $R^2=0.998$

Conclusion

VB_{12} is a small molecular that can't initiate an immune response itself, so it needs to be coupled with a big carrier such as protein to become a complete antigen.[28] In previous study of vB_{12} conjugates, acid hydrolysis procedure was performed to product functional group (carboxyl groups) and then reacted with protein by EDAC method. However, the procedure of acid hydrolysis might destroy the structure of vB_{12} if reaction condition was not well controlled,[29] and finally might lead to the low specificity and affinity of acquired antibody. For this reason, the synthesis of vB_{12} conjugates in this paper choose the hydroxyl as functional group to coupled with BSA, which could maintain the integrity of vB_{12} and product the antibody with high specificity. For L.Sagaya's study the antibody against vB_{12} was with the limit of detection (LOD) of 10 ng mL^{-1}, and the cross-reactivity of adenosylcobalamin, hydroxocobalamin and methylcobalamin were 32.8%, 14.6% and 27.6%. Whereas the MAB achieved in this approach have higher sensitivity and specificity than L.Sagaya reported before.[9] The average recoveries of vB_{12} from vitamin solid samples were from 86.02% to 110.54%, which were satisfactory when compared with HPLC. Thus, as a promising method, it might provide fast and reliable results for the determination of vB_{12}.

Acknowledgements

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