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ORIGINAL ARTICLE

A Rapid Quantitative Chemiluminescence Immunoassay for Vitamin B12 in Human Serum

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SUMMARY

Background: **Vitamin B12 is very important for the human body. Early diagnosis of vitamin B12 deficiency is essential because it is associated with many problems such as fatigue, lethargy, depression, poor memory, breathlessness, headaches, pale skin, mania, psychosis, etc. Most of the vitamin B12 molecules in serum bind to specific proteins as a complex; so, although researchers have developed some sensitive methods to quantify vitamin B12, few can be used for human serum detection of vitamin B12 because of disturbed releasing process. In this study, we are aiming to develop a rapid and accurate chemiluminescence immunoassay (CLIA) for human serum testing.**
Methods: **In this study, we studied and optimized labelling of biotin molecules to vitamin B12 binding protein which had been extracted from hog gastric mucus, labelling of vitamin B12 molecules to horse radish peroxidase (HRP), releasing of vitamin B12 from its bound state in serum, concentrations of reagents, and incubation times.**
Results: **The developed method shows good performance and thermo-stability. The limit of detection (LOD) is 41 pg/mL, the recovery rate is 90.7% - 107.4%. The intra-assay CV is 4.4% - 8.0% and the inter-assay CV is 4.5% - 12.1%. Cross reactivity (CR) values are 49.4% for hydroxocobalamin and lower than 0.1% for vitamin B1, B2, B3, B9, and C. The method comparison results show that the correlation coefficient (R^2) is 0.91, with a slight negative bias of -4.1% [95% limits of agreement (± 1.96 SD); -39.6% to 31.6%].**
Conclusions: **The developed method shows high sensitivity and specificity and good correlation using a Beckman Coulter instrument while the whole test needs only 40 minutes. It can fully satisfy the very busy clinical labs.**
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KEY WORDS

vitamin B12 binding protein purification, vitamin B12 releasing, vitamin B12 quantification, CLIA

INTRODUCTION

Four vitamers of vitamin B12 are all red-colored crystals and water-soluble which are involved in the metabolism of all cells in the human body. It is essential for the transformation of methyltetrahydrofolate to tetrahydrofolate for DNA synthesis, fatty acid, amino acid metabolism and so on [1-3]. Its status is commonly estimated using the abundance of the vitamin in serum, typically with approximately 200 pg/mL set as the threshold for diagnosing deficiency [4]. At levels lower than normal, a range of symptoms such as fatigue, lethargy, depression, poor memory, breathlessness, headaches, and pale skin, among others, may be experienced [5,6]. Vitamin B12 deficiency can also cause symptoms of mania and psychosis [7,8]. Since a deficiency of this vitamin may lead to so many problems as described above, early diagnosis is necessary.

In recent years, in spite of some sensitive methods for vitamin B12 such as HPLC, which are quite expensive and time consuming, and radioimmunoassay, where both assays are labor intensive and harmful to the environment and to the human health [9-11], few convenient and rapid methods have been developed for human serum detection. The CLIA method is widely used nowadays because of its simple, cheap, and sensitive attributes with high-throughput [12]. In this study, a rapid competitive immunoassay was established for the quantification of serum vitamin B12.

MATERIALS AND METHODS

Reagents and materials

Vitamin B12 is obtained from TCI. Streptavidin coated magnetic beads were prepared by the method described in the previous paper from our lab. PMPI (p-maleimidophenyl isocyanate) was purchased from Sigma. EZ-Link™ Sulfo-NHS-LC-Biotinylation Kit and HABA solution are purchased from Thermo Fisher. EAH-Sepharose 4B was purchased from GE Healthcare. Streptavidin was purchased from NeuroPeptide in China. HRP was obtained from BBI solutions. Auto magnetic beads chemiluminescent analyzer was purchased from Zecheng Biotechnology in China.

Preparation of VB12-HRP conjugate

We dissolved different amounts of vitamin B12 and a certain amount of PMPI in DMSO, and let it react at room temperature overnight. We dissolved HRP in 0.05 M phosphate buffer, pH8.5. We added the reacted solution into the prepared HRP solution. The mixed solution was incubated for 3 hours at room temperature and then removed the excess PMPI and impurities with a desalination column.

Preparation of biotinylated VB12 binding protein conjugates

Isolation of VB12 binding protein

VB12 binding protein was extracted from hog gastric mucus. The covalent attachment of vitamin B12 to Sepharose 4B was performed as described in a previously paper [13]. The hog gastric mucus was added to 0.1 M Tris-acetate, pH 9.2, and stirred for 30 minutes, then centrifuged at 20,000 g for 30 minutes. The supernatant was filtered and centrifuged again and then immediately the supernatant was subjected to affinity chromatography prepared above. The fraction eluted by 0.1 M potassium phosphate, pH 7.5, 7.5 M guanidine-HCl was then collected [14]. The collected fraction containing binding protein was dialyzed against 0.1 M phosphate buffer, 0.5 M NaCl, pH 7.0 before the next conjugation step.

Conjugate preparation

We dissolved a certain amount of EZ-Link Sulfo-NHS-LC-Biotin in 0.1 M phosphate buffer, pH 7.0 and added it into the dialyzed solution prepared above. We incubated the mixed solution for 2 hours at room temperature and then removed the excess biotin and impurities using a desalination column.

Development and optimization of the CLIA method

For the vitamin B12 competitive immunoassay, upon mixing the biotinylated binding protein with a serum sample containing vitamin B12, the molecules react. After a short incubation, the enzyme conjugate was added. The addition of the enzyme conjugate, results in a competition reaction between the VB12-HRP and the vitamin B12 in the sample because of a limited number of binding sites on vitamin B12 binding protein. After adding in the magnetic beads, the biotinylated binding proteins were immobilized on the magnetic beads' surface. A 4-parameter curve can be generated by utilizing five different known concentration standards, and the concentration of an unknown sample can be ascertained. In this study, some vital factors such as vitamin B12 binding protein concentration, VB12-HRP type and concentration, and incubation time were studied and optimized. For vitamin B12, particularly, the release process was also investigated due to its binding state to the binding proteins in human serum.

Method performance

Limit of detection

Twenty duplicate standard 1 (without vitamin B12) were detected. The concentration calculated by the MEAN-3SD signal value through the standard curve is the LOD.

Precision and recovery

Three samples with low, medium, and high concentrations of vitamin B12 were tested twenty times in one

experiment and once a day on twenty consecutive days to calculate the intra-assay and inter-assay CV. Recovery rate was calculated by adding a certain amount of vitamin B12 to two serum samples with different vitamin B12 concentrations.

Cross reactivity
By adding various concentrations of interfering substances into the serum matrix to evaluate the specificity of the vitamin B12 binding protein, the CR was defined at the point where the reduction in signal corresponded to 50% of the signal achieved in the absence of vitamin B12. The CR values were calculated as follows [15]: CR(%) = (IC_{50} of VB12/IC_{50} of competitor) x 100%

Accelerated stability
The whole kit included SA-magnetic beads, biotinylated binding protein conjugates, VB12-HRP, vitamin B12 standards. All other reagents were incubated at 37°C for 7 days, and the stability was evaluated by comparing the signals on different days.

Methodology comparison
The established method was compared to the vitamin B12 kit purchased from Beckman Coulter. One hundred ninety serum specimens were collected with values that ranged from 86 pg/mL to 1,482 pg/mL.

RESULTS

Characterization of VB12-HRP Conjugates
We coated the intrinsic factor obtained from BBI solutions onto the surface of the microwells. Then, 50 µL of different VB12-HRP ratio conjugates (0.1 µg/mL) were added into the intrinsic factor coated wells separately and no vitamin B12 added to be used as control. It was incubated for 30 minutes and the microwells were washed 5 times to remove the unbound VB12-HRP. The signal values were compared after 100 µL signal reagents were added. The conjugates were prepared successfully, and the results are shown in Figure 1.

Characterization of biotinylated vitamin B12 binding protein conjugates
We coated a series of concentrations of the purified vitamin B12 binding protein onto the surface of the microwells. Then, 50 µL of VB12-HRP conjugates (VB12/HRP mole ratio is 4/1, 0.05 µg/mL) were added into the wells, incubated for 30 minutes and then washed the microwells 5 times to remove the unbound VB12-HRP. The signal values are proportional to the concentration of vitamin B12 binding protein in the coating buffer. The signal values achieve 564,932 when coating with 0.5 µg/mL vitamin B12 binding protein while 2,542 when coating with the negative buffer. The results indicate that the prepared biotinylated conjugates exhibit good biological activity. To estimate the biotinylation efficiency, the biotinylated conjugates are added to a mixture of HABA and avidin solution, and the calculated biotin/protein mole ratio is approximately 4.1.

Development of the CLIA method
Method procedure
We used 30 µL sample, 10 µL stabilizer reagent which contains Tris (2-carboxyethyl) phosphine (TCEP) to stabilize the denatured protein, and 20 µL alkaline solution containing minute amount of potassium cyanide into the tube. We incubated it for 10 minutes to release all the vitamin B12 molecules to free state and convert some vitamer forms of vitamin B12 to cyanocobalamin to ensure equivalent binding to vitamin B12 binding protein, and then neutralized the solution with an acid solution. We added 50 µL biotinylated conjugates into the tube and incubated for 10 minutes, then added 50 µL VB12-HRP conjugates and incubated for 5 minutes. We added 30 µL SA-magnetic beads and incubated for another 5 minutes. The magnetic beads were washed three times, and the instrument read the signals after adding 200 µL signal reagent. We calculated the concentrations of the unknown samples by using the standard curve. The entire procedure was performed at 37°C.

Optimization of biotinylated and VB12-HRP conjugate concentrations
Due to different competing abilities of various ratios of VB12-HRP conjugates, the conjugates with high ratios decreased the signals more. In this study, conjugates labeled by 4:1 of vitamin B12 excess were selected for the further studies because the conjugates showed the biggest value of signal at 0 pg/mL divided by that signal at 1,500 pg/mL and relatively high signal values. The signals are shown in Figure 2. A series of concentrations of biotinylated and VB12-HRP conjugates were investigated. The value of signal at 0 pg/mL divided by that signal at 1,500 pg/mL is acceptable and the signals of standard 1 achieve higher than 400,000 when biotinylated conjugate concentration is 40 ng/mL and VB12-HRP concentration is 800 ng/mL. The results are shown in Table 1.

Optimization of incubation time
For fulfilling the accuracy and rapid requests of clinical testing, the biotinylated and VB12-HRP conjugate incubation times were each studied. The procedure was performed as above except for the incubation time. According to the results and the clinical request of saving time, we chose a 10 minute incubation of biotinylated conjugates and 5 minute incubation of VB12-HRP as the best condition for exhibiting high signals with a relatively low standard deviation. The results are shown in Figure 3 and Table 2.

Vitamin B12 releasing procedure optimization
Commonly, most vitamin B12 molecules in serum bind to proteins forming a bound state. Before detecting the vitamin B12 concentration, a releasing procedure

Table 1. Optimization of biotinylated and VB12-HRP conjugate concentrations.

Biotinylated binding protein (ng/mL)	20			40			80		
VB12-HRP (ng/mL)	200	400	800	200	400	800	200	400	800
Standard 1 (0 pg/mL)	88,532	184,376	252,745	147,453	248,740	449,724	268,475	419,673	529,385
Standard 2 (200 pg/mL)	29,038	95,487	140,584	73,119	159.532	292,861	228,404	383,655	489,048
Standard 3 (400 pg/mL)	11,683	35,856	49,475	34,113	74,975	207,048	164,367	225,827	386,942
Standard 4 (800 pg/mL)	5,593	7,384	12,458	17,063	39,285	96,936	108,503	159,347	228,409
Standard 5 (1,500 pg/mL)	3,364	4,643	6,258	8,708	14,934	15,157	29,275	39,673	48,695

Table 2. Accuracy of the optimized incubation time (n = 10).

Samples	Mean	SD	CV
407 pg/mL	401	22	5.43%
842 pg/mL	872	39	4.45%

Table 3. Optimization of potassium cyanide concentration.

Samples	Beckman Coulter (pg/mL)	Concentration of potassium cyanide			
		0%	0.0025%	0.005%	0.010%
1	488	461	442	480	494
2	459	337	495	467	457
3	494	424	519	518	464
4	482	447	456	483	493
5	493	332	451	461	485
6	464	349	387	439	472
7	462	456	466	467	477
8	481	392	381	422	473
9	477	494	418	510	521
10	468	378	443	471	486
Average	477	407	446	472	482
SD	12.9	57	43	29	18
CV	2.7%	14.1%	9.7%	6.1%	3.8%

should be performed to release all the bound state molecules to a free state. Moreover, there are vitamers of vitamin B12 in serum which exhibit different binding ability and may cause the inaccuracy of the test; therefore, the conversion of hydroxocobalamin to cyanocobalamin by adding a minute amount of potassium cyanide to the releasing buffer is necessary. According to the results, 1.2 M sodium hydroxide solution incubated with samples for 10 minutes at 37°C seems to release vitamin B12 completely with acceptable CV values.

Table 4. Recovery.

Sample	VB12 concentration (pg/mL)			
	Added concentration	Tested	Expected	Recovery
1		276		
	200	503	476	105.7%
	400	726	676	107.4%
2		632		
	200	864	832	103.8%
	400	936	1032	90.7%

Table 5. Intra- and inter-assay precision.

Sample	Intra-assay CV (n = 20)			Inter-assay CV (n = 20)		
	Mean (pg/mL)	SD	CV	Mean (pg/mL)	SD	CV
1	208	16.7	8.0%	213	25.8	12.1%
2	416	21.5	5.2%	408	28.2	6.9%
3	831	36.4	4.4%	838	38.1	4.5%

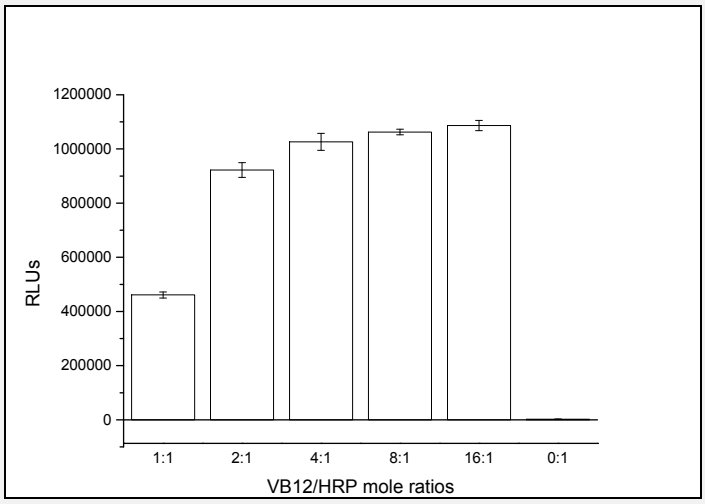

Figure 1. VB12-HRP conjugates confirmation (n = 3).

The results can be seen in Figure 4. The concentration of potassium cyanide was investigated by testing 10 serum samples with almost the same vitamin B12 concentration detected by a Beckman Coulter instrument. The results show that at least 0.005% potassium cyanide is necessary to reduce the deviation. Therefore, 0.005% potassium cyanide in 1.2 M sodium hydroxide releasing solution is chosen because higher concentrations of potassium cyanide are harmful to the human body and the environment.

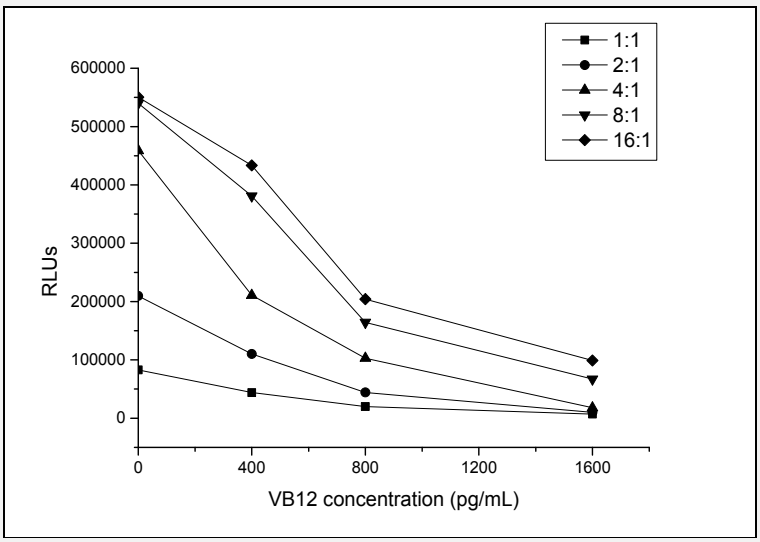

Figure 2. Signals of standards using different VB12/HRP ratio conjugates.

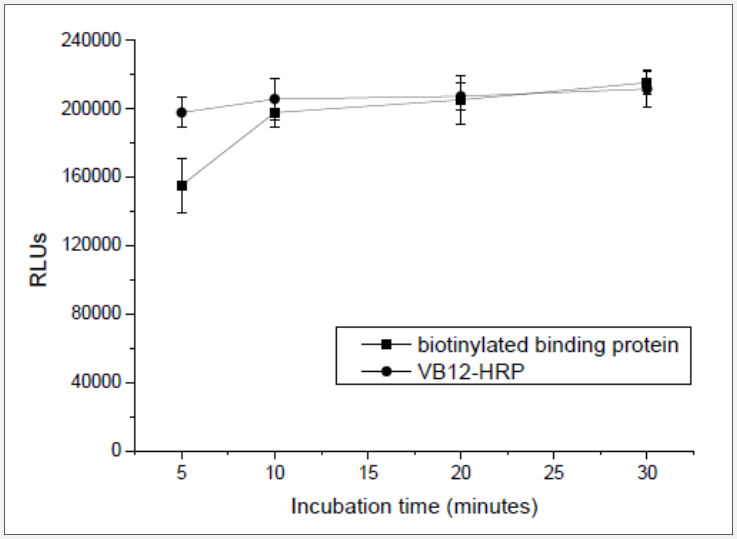

Figure 3. Incubation time optimization (n = 3).

Method performance

The signal values and concentration of vitamin B12 standards are fitted by a 4-parameter logistic equation, the equation is Y = 377,837.5 + (-466,354.8)/(1 + EXP (-(-6.3061 + 1.0233 * Ln(X))) with a regression correlation coefficient of 0.999. The LOD of the method is 41 pg/mL. The recovery rate is 90.7% - 107.4%, and the results are shown in Table 4. The intra-assay CV of the method is 4.4% - 8.0% and the inter-assay CV is 4.5% - 12.1%. The results are shown in Table 5. The CR values

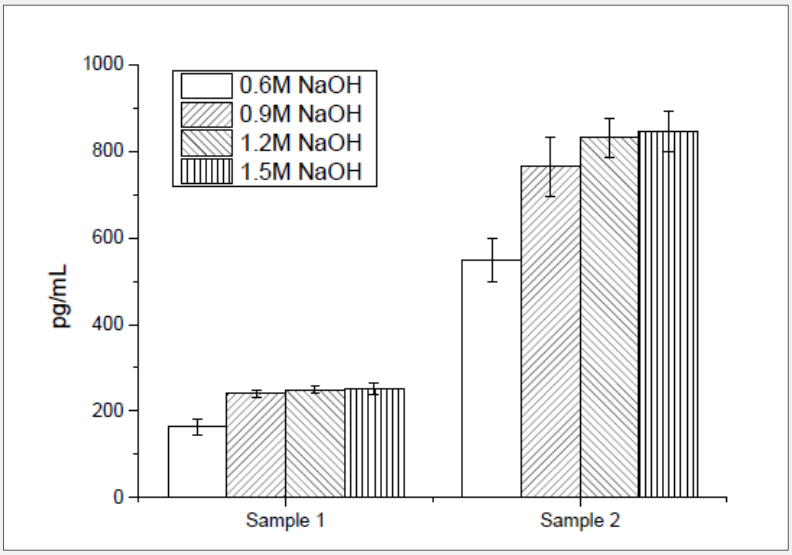

Figure 4. Optimization of NaOH concentration (n = 3).

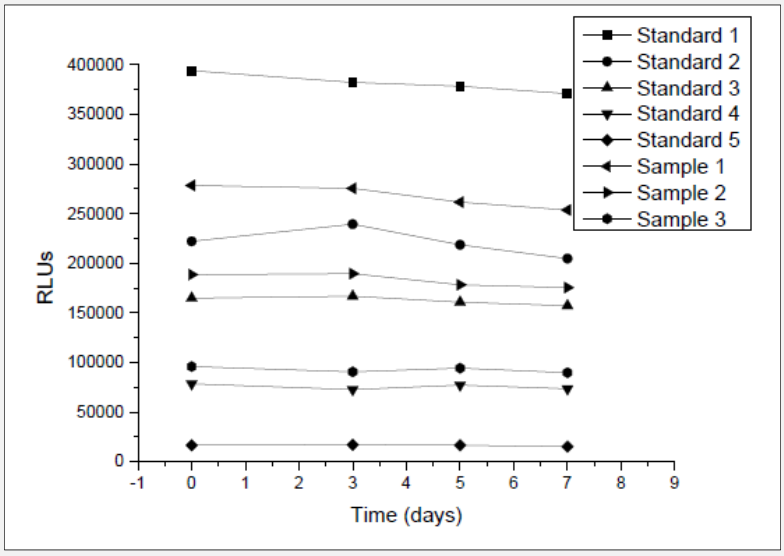

Figure 5. Accelerated studies of the reagents.

are 49.4% for hydroxocobalamin, while lower than 0.1% for other vitamins such as Vitamin B1, B2, B3, B9, and C. The accelerated stability study shows all the components have good thermo-stability. The signals of the standards and samples incubated at 37°C change less than 10% compared to the controls placed at 2 - 8°C. The results are shown in Figure 5.

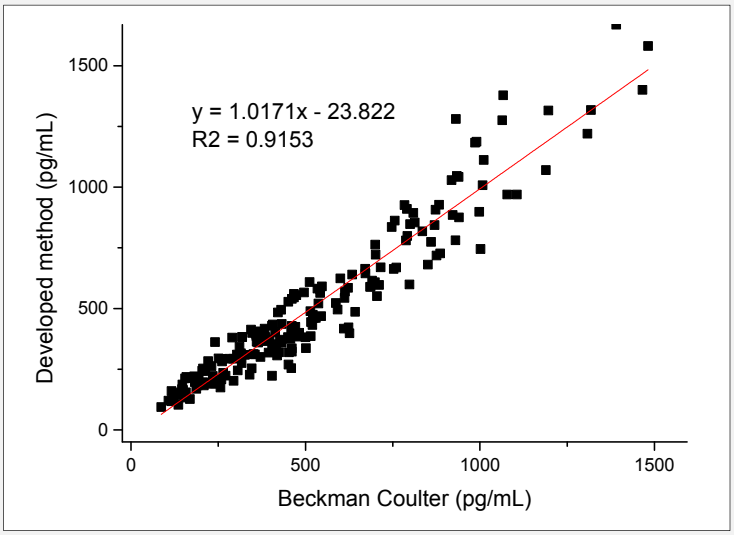

Figure 6. Comparison of the developed method with Beckman Coulter.

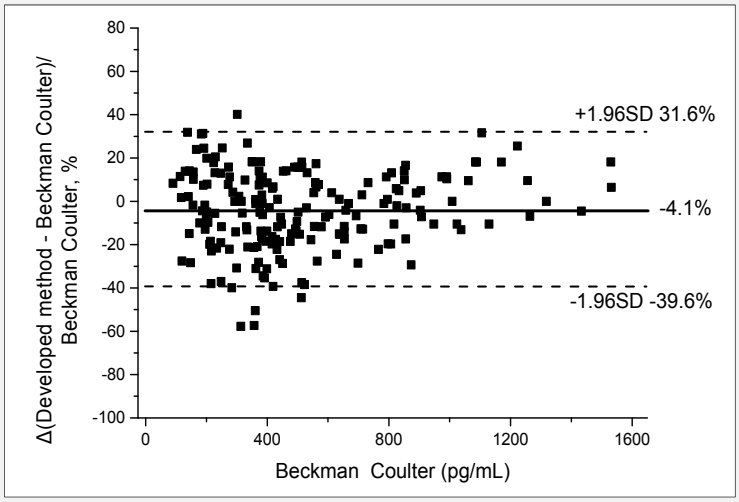

Figure 7. Bland-Altman bias plots for comparison between the developed method and Beckman Coulter. The solid line represents the relative mean difference between paired measurements; dashed lines indicate upper and lower limits of agreement.

Methods comparison

After evaluating the developed method, 190 human serum samples were tested to perform the methods comparison. As shown in Figure 6 and 7, the least square regression equation and the correlation coefficient of the results tested by the developed method and Beckman Coulter were computed, and a slight negative bias of -4.1% [95% limits of agreement (± 1.96 SD); -39.6 to 31.6] show excellent method agreement.

DISCUSSION

Vitamin B12 deficiency is more widespread in the population than has been assumed. Since a deficiency of vitamin B12 may lead to many problems in the human body, early diagnosis is essential [16]. To assess the vitamin B12 status, the serum vitamin B12 test is a widely used method. In recently years, many researchers revealed that methylmalonic acid, homocysteine, and holotranscobalamin (HoloTC) which is the metabolically active fraction of B12, are more sensitive and representative markers of impaired vitamin B12 status. However, some studies suggested that methylmalonic acid levels should be interpreted with caution in some particular populations, such as elderly populations, impaired renal function populations and patients with short bowel syndrome who are at-risk for Small Bowel Bacterial Overgrowth [17, 18]. Meanwhile, a review paper found that the HoloTC immunoassay cannot be used to measure vitamin B12 status any more reliably than total vitamin B12, or to predict the onset of a metabolic deficiency mainly because of the sensitivity of HoloTC and the inconsistent cutoff values [19].

Currently, some sensitive and accurate methods for vitamin B12 quantification have been developed, but most of them are used for testing in pharmaceutical preparations, juices and fortified foods. Few can be used to detect human serum with disturbed vitamin B12 releasing process. This research developed a rapid CLIA for the quantification of human serum vitamin B12. The extraction and purification of vitamin B12 binding protein, biotinylated, and VB12-HRP conjugate preparations, the releasing and converting of vitamin B12, and the incubation times were investigated and optimized. The materials such as VB12-HRP conjugates should be paid more attention as different mole ratio conjugates show different competing abilities. The concentration of NaOH is not as high as it should be while releasing, as signals decrease significantly due to the extreme high concentration of salts. It should be assured that the pH is higher than 7.0 after neutralization, because low pH microenvironment may convert cyanocobalamin to hydroxocobalamin. Researchers also should realize the different vitamers of vitamin B12 in human serum, trying to convert the vitamers to the same structure or to the structure with the same binding abilities is better before the testing. The components of the kit show good performance and thermo-stability, and the limit of detection even slightly lower than that of Beckman Coulter. Method comparison also shows that the developed method has a good correlation with Beckman Coulter. However, the developed method also has some disadvantages. The measuring range is 41 - 1,500 pg/mL, samples lower or higher should be reported as < 50 pg/mL or > 1,500 pg/mL. The test can only be considered as an auxiliary diagnosis, the clinicians should combine it with the overall clinical profile when interpreting the results.

CONCLUSION

The developed method shows high sensitivity and specificity and good correlation with Beckman Coulter by optimizing the conjugate preparations, releasing procedure, reagent concentrations, and incubation times. Moreover, this method can give accurate results in less than 40 minutes, which satisfies the requirements of the very busy clinical labs.

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Declaration of Interest:
No potential conflicts of interest were disclosed.

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