Establishment of a Simple and Convenient Method for Folic Acid Enzyme Chemiluminescence Immunoassay

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Abstract. The enzyme chemiluminescence new immunoassay for folic acid (FA) was established by competition model. Add FA samples to a microtiter plate precoated with the goat anti-mouse IgG firstly, then add enzyme abled FA and FA monoclonal antibody (McAb). The values of CLIA were measured to reflect the quantity of FA. The limit of detection(LOD) of assay is 0.37ng/ mL. The assay shows good correlation during 1～30 ng/mL with correlation coefficient 0.9976. The intra- and inter-assay coefficients of variation are 4.8 %～7.3 % and 6.1 %～12.2 %, respectively. The recovery of folic acid in serum is 90.4 %～113.2 %. Compared with determine value clinically in chemiluminescence immunoassay kit from Roche company, the correlative equation is y = 0.9689x + 0.0228, and correlation coefficient is 0.9780. Various components and kit overall show good stabilities. This method is simple and convenient, and has low LOD value. The method has overcome the shortcomings of the present references. It is easy to apply and has broad clinical application prospect. It lays an experimental foundation for the preparation of Mc Ab against folic acid and the development of domestic kit.

1. Introduction

Folic Acid (FA) and vitamin B12 are used to synthesize the necessary DNA. FA deficiency can lead to giant young red blood cell anemia and white blood cells reduce disease [1, 2]. Now FA deficiency has internationally recognized been the disease risk factors newborn neural tube defects (NTD) [3]. FA excess dose of synthetic folic acid can enter the bloodstream and may produce some toxic effects [4, 5]. The level of FA in the body can also be used as an indicator for the diagnosis and treatment of cancer, malignant blood disease, chronic liver disease and cognitive disorder [6, 7]. Therefore, the determination of FA in human serum is very importance to prevent, diagnose and treat diseases.

Current radiological immunoassay (RIA) method and microbiological method are commonly used to detect folate content in serum. However, RIA is harmful to the environment and people's health. Microbiological method has some limitations, because that the length of the experiment period and the difference between the batch. In recent years, the method of chemiluminescence immunoassay (CLIA)

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has gradually as a clinical method of choice for quantitative detection of folic acid [8], because of its stability, non-toxic, measuring the advantages of rapid, high sensitivity, wide linear range. However, the present market of FA CLIA kits are both used imported kit or imported antibody, which is expensive and restricts the wide use of the clinic. Therefore, FA antibodies localization will greatly reduce the cost of CLIA, effectively promote CLIA in the clinical use of FA, promote FA clinical detection.

This article uses the competition method [9] with coated goat anti mouse IgG to establish FA CLIA method [10]. The innovation point of the established method is that: (1) coated goat anti mouse IgG source is stability, excessive coated is good controllability;(2) the reaction after adding the sample and the enzyme labeled FA will not occur until the antibody is added.(3) the FA in the sample is liquid phase as the same phase of the FA in the enzyme labeled FA, that is the equal competition; (4) competition component of enzyme labeled FA is easy to control by confecting dilution level, to achieve strictly limited; (5) The detection limit of established methods in this paper and the correlation with imported reagent are superior to the existing literature [11];(6) the equipment is not high quality, and can use to ordinary domestic CLIA devices. Innovation based on the above points, this method can satisfy the demand for the clinical detection of FA, establish the foundation for the further development of the preparation of FA McAb and f domestic FA kit. The method has good clinical application prospect, and has a high reference value for readers in the development of domestic FA immunoassay kit.

2. Result and Discussion

2.1. Methodology Establishment

2.1.1. Selection of antibody concentration used for coating, with 0.02 mol/L pH7.4 phosphate buffer (PBS) diluted goat anti mouse IgG to 2.5, 5, 10, 20, 30μg/mL and coated plate, tested by three iodine armour glands original glycine (T3) CLIA kit of Beijing North Institute of Biological Technology(BNIBT). As shown in Fig. 1, the CLIA value of each point was not increased, when the concentration of the coated antibody was 10 g/mL, so the concentration of the coated goat anti mouse IgG was 10μg/mL.

2.1.2. Selection of folate monoclonal concentration, The FA antibody was diluted to 0.125, 0.25, 0.5, 1, 2 μg/mL by phosphate buffer (P BS) (0.02 mol/L pH7.4) with 20% calf serum. As shown in Table 1, when the concentration of FA McAb was 2μg/mL, the concentration of McAb was too high, the difference between low concentration standard points was not obvious. When the concentration of McAb was 0.25μg/mL, the inhibition of the curve is better and linear is better. Taking into account the combination rate, linear and cost factors, the appropriate concentration of FA McAb was 0.25 μg/mL.

2.1.3. Selection of enzyme labeling antigen and dilution, With PBS (0.02mol/L pH7.4) of 20% calf serum to dilute the three enzyme labeled FA antigens (HRP-FA-OVA, HRP-FA-KLH, HRP-FA-BSA) to 1:1000, 1:2000 and 1:4000 respectively, and the standard curve was produced. Results are shown in Table 2, HRP-FA-OVA in different dilution levels, each point rate and the blood value is relatively stable, that can reduce error and long-term transport storage to the analysis result, and linear is better, so the selected enzyme labeled antigen is HRP-FA-OVA and the working concentration is 1:2000.

2.1.4. Selection of reaction time, As the analysis procedure 4.6, reaction time was 0.5, 1, 1.5, 2, 3, 4 h in 37 ℃ respectively, observe the reaction kinetics curves. The experimental results were shown in Fig. 2. With the increase of reaction time, the binding rate of each point no longer increases significantly. When the reaction time is 1h, the binding rate of each point is basically balanced. When the reaction time is 0.5 h, the reaction time is too short, and the error has a great influence on the result. In order to meet the need of clinical rapid detection, 1h is chosen as the reaction incubation time.

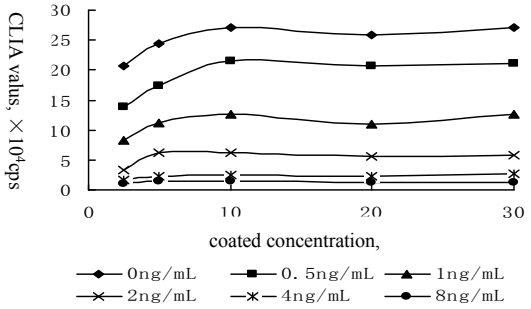


Fig. 1 Selection of coated antibody concentration

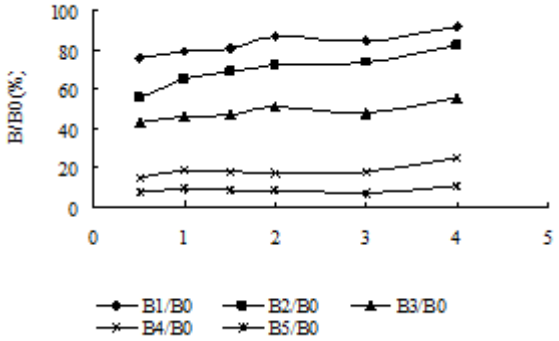


Fig. 2 Selection of reaction time

2.1.5. Selection of sample volume, Sample volume were selected 20μL, 35μL and 50μL with 50μL enzyme labeled antigen and 50μL McAb to react CLIA, investigate the influence of sample volume to detection limit and the standard curve. The results are shown in Table 3, with the increase of the sample volume, the detection limit is reduced, when the sample volume is 50, and the test limit of the curve is better, and the linear correlation is better. Due to the small sampling error of 50μL, the sample volume was selected as 50μL standard+50μL enzyme labeled antigen +50μL Mc Ab.

Table 1. Selecting concentration of FA Mc Ab

C_{McA}[μg/mL]	0.125	0.25	0.5	1	2
B_1/B_0(%)	80.0	85.5	85.3	85.8	85.0
B_2/B_0(%)	65.0	73.4	76.5	77.5	90.0
B_3/B_0(%)	45.0	49.4	59.2	64.1	63.0
B_4/B_0(%)	25.4	28.0	32.8	34.0	36.4
B_5/B_0(%)	12.1	14.3	16.2	18.9	22.6
r value	0.9972	0.9985	0.9968	0.9951	0.9581

Table 2. Selection and dilution level of labeled FA

Dilution level of enzyme labeled FA	HRP-FA-OVA			HRP-FA-KLH			HRP-FA-BSA		
	D1[a]	D2[b]	D3[c]	D1[a]	D2[b]	D3[c]	D1[a]	D2[b]	D3[c]
S_0(×10^5cps)	8.3	5.1	2.9	2.5	2.0	1.0	6.9	7.0	6.5
B_1/B_0(%)	72.6	67.9	62.9	81.3	51.5	53.1	86.5	80.1	70.3
B_2/B_0(%)	61.0	55.6	50.7	63.9	42.0	39.2	75.0	71.4	52.2
B_3/B_0(%)	38.1	32.4	30.2	41.6	22.2	24.9	60.0	44.7	28.4
B_4/B_0(%)	17.9	14.3	12.3	17.5	9.9	8.9	27.5	16.4	5.8
B_5/B_0(%)	9.4	7.8	7.8	9.9	4.4	4.1	10.7	4.3	2.5
r value	0.999	0.999	0.998	0.999	0.995	0.995	0.992	0.988	0.996
Sample 1 [ng/mL]	2.67	3.21	2.82	8.32	5.89	3.80.	7.14	4.19	2.09
Sample 2 [ng/mL]	12.18	12.30	13.14	30.81	21.69	16.13	20.49	9.68	6.70

[a]1: 1000; [b]1: 2000; [c]1: 4000

2.2. Methodology identification

2.2.1. Standard curve, Log-Logit drawing method is used to draw the standard curve and fitting linear regression equation. The standard curve equation is y=-3.21x+ 1.45, and the absolute value of correlation coefficient is 0.9976, as shown in Fig. 3.

Table 3. Effect of sample volume

Smaple volume[μL]	sample enzyme labeled antigen +McAb		
	20+50+50	35+50+50	50+50+50
Limits detection[ng/mL]	0.50	0.42	0.35
r	0.9942	0.9934	0.9983

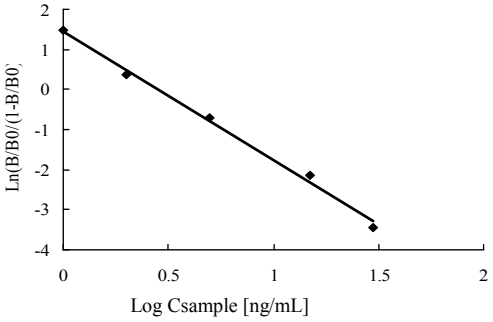


Fig. 3 Standard curve for FA assay

2.2.2. Minimum detection limit, At the same time, CLIA values of 20 "zero" standard are detected, the $\overline{X}$ -2SD value is calculated, and the corresponding concentration is 0.37 ng/mL, which is the minimum detection limit for this method.

2.2.3. Precision, three serum samples of different FA concentrations were tested separately in some holes of one experiment and repeated in different experiments. The experimental results are shown in

Table 4. The inter coefficient of variation (inter-CV) was 4.8% ~ 7.3% and the intra-assay coefficient of variation (CV) was 6.1% ~ 12.2% of this method.

2.2.4. Accuracy, the known concentration standard was added to three serum samples of different FA concentrations, and the recovery rate was measured and calculated. The results were shown in Table 5. The recovery rate of this method was 90.4% ~ 113.2%.

2.3. Methodology comparison.
This analysis method was applied to 52 cases of serum samples and Roche electrochemical immunoassay method compared the test results, the result is shown in Fig. 4, the related equation is y = 0.9689x + 0.0228, correlation coefficient r = 0.9780.

Table 4. Inter - and intra - assay CVs

sample	inter CVs, n=10			intra CVs, n=3		
	$\bar{X}$ /ng· mL^{-1}	SD	CV%	$\bar{X}$ /ng· mL^{-1}	SD	CV%
1	1.51	0.11	7.3	1.47	0.18	12.2
2	3.28	0.23	7	3.02	0.32	10.6
3	11.32	0.54	4.8	10.33	0.63	6.1

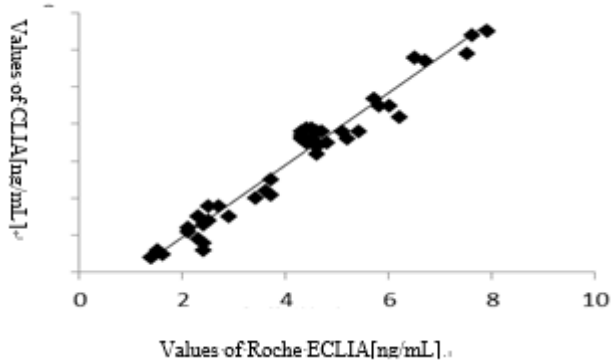


Fig. 4 Comparison between this method and Roche ECLIA

2.4. Stability experiment of kit.
The whole kit (coated plate, enzyme labeled antigen, standard, FA Mc Ab) was at 37 ℃ with the immune analysis to investigate its stability. The results as shown in Table 6, the whole kit at 37 ℃ for 7 days stability is good.

Table 5. Analytical recovery of FA

sample	C_{FA} [ng/mL]			Recovery [%]
	Added concentration	tested value	expected value	
1		1.34		
	2.00	3.78	3.34	113.2
	5.00	6.14	6.34	96.8
	15.00	17.74	16.34	108.6
2		4.23		
	2.00	6.76	6.23	108.5
	5.00	8.34	9.23	90.4
	15.00	18.61	19.23	96.8
3		9.68		
	2.00	10.91	11.68	93.4
	5.00	15.00	14.68	102.2
	15.00	27.15	24.68	110.0

Table 6. Stabilities of FA assay

	0 day	3 days	5 days	7 days
S_0(cps)	550576	610832	500821	511452
B_1/B_0(%)	75.1	73.0	72.5	71.6
B_2/B_0(%)	55.1	63.0	55.2	56.2
B_3/B_0(%)	36.7	36.2	36.3	36.7
B_4/B_0(%)	11.2	11.1	12.5	15.0
B_5/B_0(%)	5.6	5.6	5.3	6.9

3. Conclusion

Due to individual differences and geographical, age and gender, the laboratories should establish the normal range of FA in their respective regions according to the normal groups in their respective regions.

A new method of FA CLIA was established by competition method. Methodology, identification satisfies the basic demands of immunoassay, kit has good overall stability. Clinical serum samples of the test results show that the method is a good correlation with the commercial import FA kit. This method has advantages of simple operation, pollution-free to environment and rapid measurement. It established experimental basis of FA McAb preparation and the development of domestic kits. The kit developed on the basis of this method will fill the blank of the domestic market and has a broad clinical application prospect of market.

4. Experimental part

4.1. Preparation of enzyme labeled FA antigen.

The 1mL of the 10mg/mL BSA, OVA and KLH were respectively reacted with 1mL FA (5mg/mL) and 5mg EDC• HCl to prepare three FA antigens. The enzyme labeled FA antigen was prepared by modified NaIO4-ethylene glycol [10, 12]. According to enzyme labeled antigen: enzyme = 1:1 (mass ratio), dialyze completely with the 0.02 mol/L pH7.4 phosphate buffer (PB) at 4 ℃ . After the dialysis, add same volume glycerin and store in - 20 ℃.

4.2. Preparation of solid phase coated plate.
Diluted goat anti mouse IgG with 0.02mol/L pH7.4 PB to a certain concentration, add to plates (150μL /hole), store at 4 ℃ for the night. The next day, pour the bag to be liquid, pat dry. Add 200μLconfining liquid to each hole and incubate 2 h at 37 ℃. Drain the confining liquid and let it dry.

4.3. Preparation of standard products.
PBS with 20% calf serum was used as a zero-standard solution, by which diluted FA to 0, 1, 2, 5, 15, 30ng/mL concentrations series. Package of 1 ml/bottle, storage avoid light at - 20 ℃.

4.4. Preparation of FA McAb.
FA McAb was prepared by PBS (0.02 mol/L pH7.4) of 20% calf serum. Package with 5 ml/bottle and store at - 20 ℃.

4.5. Preparation of FA labeled antigen.
The self-made three enzyme marker antigens were diluted by PBS (0.02 mol/L pH7.4) of 20% calf serum to the appropriate dilution. 5 ml/bottle packaging and store at - 20 ℃.

4.6. FA enzyme CLIA procedure.
(1) Add 50μL FA or tested samples to goat anti-mouse coated IgG plates each hole; (2) Add 50μL enzyme labeled antigen and 50μL FA McAb, gently shake and incubate 1 h at 37 ℃; (3) Discard the reaction fluid and wash the plate 5 times with detergent liquid, and pat dry; (4) Add 50μL luminescent liquid A and 50μL luminescent liquid B to each hole, and the CLIA value was detected after 10 min

(5) Draw standard curve with the Log - Logit graphic method standard concentration as the abscissa, CLIA value as the ordinate, and fitting of linear regression equation, according CLIA values of samples to calculate the concentrations sample of FA.

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