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Direct microtitre plate enzyme immunoassay of folic acid without heat denaturation of serum

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Abstract

A new and simple method for enzyme immunoassay of folic acid (FA) has been developed, which does not require extraction or heat denaturation of serum. FA-free serum for standards was prepared by a new immunosorbent technique as conventional methods were unsuccessful. The detection limit of the assay is 0.05 ng/ml. Intra- and interassay variabilities ranged between 5–13.3%. Analytical recoveries obtained after spiking with different amounts of FA ranged between 93–110%. We eliminated the interference of endogenous folate binding protein – a major problem in direct FA assay by incubating serum samples (or standards) with FA-HRP conjugate in antibody coated plates at 50° C. Comparison of our data with results obtained by microbiological assay and also by heating samples in alkaline buffer showed good correlation.

Keywords: Folic acid; Direct enzyme immunoassay; Microbiological assay; Folic acid-free serum

1. Introduction

Folic acid (pteroylglutamic acid, PGA, FA) is one of the essential vitamins, the deficiency of which causes megaloblastic anemia. Folic acid deficiency has also been implicated in cervical dysplasia, megaloblastic changes in cervical epithelium, cancer, chronic heart disease and neural tube defects (Butterworth, 1993). In vivo, FA is enzymatically reduced to dihydrofolic acid (DHF) and then to tetrahydrofolic acid (THF), which are then formylated and methylated. 5-methyltetrahydrofolate (5-methyl-THF) is the crucial factor involved in pyrimidine biosynthesis (Krumdieck et al., 1992). Although FA is not the active metabolite, generally in cases of folic acid deficiency it is given in the form of capsules or tablets because, it is the most stable folate analogue (Gregory et al., 1990). It has been shown that, in methotrexate treated psoriatic patients the plasma FA and DHF levels, before and after administration of the drug are significantly different (Handel, 1981). This indicates that plasma FA level is a good clinical marker in therapies using inhibitor of dihydrofolate reductase. The bioavailability of folate depends not only of the intake (dietary or

supplemented) but also on the absorption status and the activity of dihydrofolate reductase (Baugh et al., 1971). The study of clinical significance of different folate metabolites in serum or tissue has long been hampered by the absence of suitable techniques for measuring the individual FA derivatives. Although the serum concentration of FA is low, the availability of suitable method for estimation of FA along with an assay of total folate would not only be useful for clinical diagnosis of folate deficiency but will also be helpful in studying absorption, metabolism and excretion of dietary and supplemented FA in normal and pathological states.

The most commonly used methods for estimation of FA are the microbiological assays. They require the use of both *Lactobacillus casei* which measure total folate, including 5-methyl-THF and FA, and *Streptococcus faecalis* which measures non-methylated folate(s) such as FA, DHF and THF. The procedures are time-consuming, tedious and this has precluded their widespread use. HPLC methods for FA analysis (Chapman et al., 1978; Kohashi and Inouc, 1986) are also complicated rendering them unsuitable for routine use. Folate binding proteins (FBP) have also been widely used for the estimation of total folate and a variety of methods based on FBP has been reported in the literature. These include enzyme-linked ligand sorbent assay (Hansen and Halm, 1988), radioassay (Waxman et al., 1970; Rothenberg et al., 1972; Dunn and Foster, 1973), chemiluminescence (Campbell et al., 1989) and CEDIA (Engel and Khanna, 1992; Van der Weides et al., 1992; Dworschack, 1993). A number of FBP based assay for total folate are now commercially available.

In contrast, specific determination of PGA (FA) in serum has long been hampered by the absence of suitable techniques. Reported radioimmunoassay procedures were cumbersome and lacked sensitivity (Da Costa and Rothenberg, 1971; Handel, 1981). The non-availability of high titre and specific antibody against FA was the major hurdle in developing a suitable immunoassay for FA. In the previous communication (Das Sarma et al., 1995), we have demonstrated the efficiency of ε-aminocaproic acid modified BSA as a carrier protein for raising highly specific antibody against FA. However, development of a reliable and sensitive immunoassay for serum FA requires solution of several other analytical problems. (1) In serum and tissue FA is present in several metabolic forms – differing in the functional groups, oxidation state and the number of conjugated glutamic acid residues present – among which FA is only a minor constituent. (2) The presence of endogenous FBP and (3) the difficulty in the preparation of FA-free serum for standards.

Here, we describe a simple and sensitive ELISA method for the determination of FA in serum. FA-free serum for standards was prepared by a new immunosorbent technique as conventional methods were unsuccessful. We eliminated the interference of endogenous FBP – a major problem in direct FA/folate assay (Rothenberg et al., 1977; Campbell et al., 1989), by performing the immunological reaction at 50° C.

2. Materials and methods

2.1. Preparation of immunosorbent column

Sepharose 4B (10 ml) was activated by cyanogen bromide method according to published procedure (Turkova, 1978). The activated gel was reacted with 0.5 ml (8 mg protein) of purified anti-FA antibody in 0.1 M sodium bicarbonate for 16 h at 4° C and then filtered. Estimation of the protein concentration in the supernatant by the method of Lowry et al. (1951) showed that 0.75 mg of antibody was coupled per ml of gel. Residual active groups were blocked by treating the gel with 0.2 M glycine solution for 4 h at 4° C. The coupled gel was thoroughly washed, alternately with 0.1 M $NaHCO_3$, pH 8.3 and 0.1 M acetate buffer, pH 4.0 (both containing 0.5 M sodium chloride), and preserved in phosphate buffer saline (PBS) in presence 0.01% thimerosal at 4° C. The immunosorbent column (20 × 35 mm bed volume) prepared with this gel was equilibrated with PBS before use.

2.2. Folic acid-free serum

Normal human serum (40 ml), decomplemented at 56° C for 30 min, was passed through the immunoaffinity column maintained at 50° C. The eluted serum was used for preparation of seven FA standards (0.1–10 ng/ml).

For regeneration, the column was washed with PBS, treated with 1 ml of potassium thiocyanate (4 M), and washed exhaustively with PBS. The column could be used for treatment of about 200 ml of serum without significant loss of efficiency.

2.3. Assay procedure

The anti-FA antibody, enzyme conjugate, buffers, coated plates and other reagents used were same as described in the preceding paper (Das Sarma et al., 1995).

Direct ELISA of folic acid

To each well of coated plate, 25 μl of standard or sample was transferred in duplicate and 125 μl of FA-HRP solution in incubation buffer was added. The plate was covered, mixed by swirling and incubated for 2 h at 50° C. The wells were then washed with washing buffer, and 150 μl of substrate solution containing TMB as chromogen was added. After incubation in the dark at room temperature for 20 min, the reaction was stopped by adding 100 μl of 4 N H_2SO_4 to each well in the same sequence as the chromogen solution was added. The contents of the wells were mixed by swirling and absorbance measured at 450 nm with a microplate reader (Anthos Model 2001). The amount of FA in unknown serum samples was estimated from the calibration curve.

ELISA of folic acid in heat denatured serum

To ascertain whether FBP interferes in our direct ELISA procedure, serum samples were also assayed in parallel after heat denaturation (Dunn and Foster, 1973). For this purpose, the serum sample was diluted three fold with lysine buffer (pH 10.6) and heated in a boiling water bath at 100° C for 15 min. After cooling and centrifugation, 50 μl of sample was transferred into each well of an antibody (1/6000 dilution) coated microtitre plate in duplicate. Then 100 μl of enzyme conjugate (1/6000) in incubation buffer was added and incubated for 2 h at 37° C. The rest of the procedure was same as described for the direct method.

2.4. Microbiological assay

Serum samples were diluted five-fold with working buffer (0.05 M sodium phosphate, pH 6.1, containing 0.05% ascorbic acid) and vortexed. After adding two glass balls (2.5 mm diameter) to each tube, the samples were autoclaved at 121° C for 10 min, thoroughly mixed in a vortex mixture and centrifuged at 10,000 rpm for 15 min. FA was assayed in the clear supernatant with *S. faecalis* in a total volume of 10 ml, by the method of Cooperman (1971).

3. Results

The dilution of the coating antibody and enzyme conjugate used in the assay were optimized by preliminary checkerboard titration. Thereafter, the effect of other variables on the assay performance was systematically studied.

3.1. Effect of temperature

The effect of incubation temperature on the assay response was investigated by assaying three control sera (Lyphocheck, Bio-Rad) as calibrators. The target values of these sera containing low, medium and high levels of FA were assigned by microbiological method. Dose-response curves were obtained for the control sera at three different temperatures (37, 50 and 60° C) of immunological reaction. As shown in Fig. 1, the dose-response curves and the FA values obtained at different temperatures of incubation are significantly different (Table 1). The values obtained at 37° C for all the three control sera were very low compared to those obtained at 50° C and 60° C. The values obtained at 50° C for all control sera correlated well with the target values. Consequently, we routinely used 50° C as temperature of incubation.

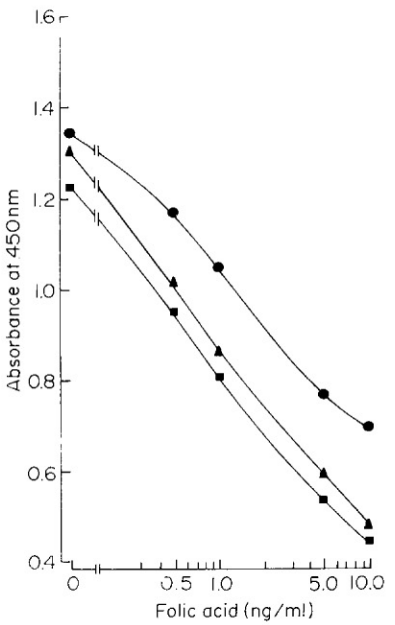

Fig. 1. Effect of temperature on the dose-response curve using 25 μl of serum standard and fixed dilution of coating antibody and enzyme conjugate (1/8000). Temperature of incubation were 37° C (●); 50° C (▲) and 60° C (■). Each value represents the mean of two assays in duplicate.

We have also investigated the effect of temperature on the activity of the enzyme tracer both in solution or bound to solid phase. For this purpose the enzyme conjugate bound to antibody coated solid phase was incubated at 37° C or 50° C for 2 h in incubation buffer. Similarly the enzyme conjugate solution was incubated for 2 h at 37° C or 50° C. There was no significant difference in the activity of the bound enzyme incubated at 50° C and 37° C. However, the activity of the enzyme incubated at 50° C in solution was about 10% lower than in 37° C. Thus, the effect of incubation at 50° C on the activity of the label is negligible.

3.2. Effect of incubation time

We have investigated the effect of incubation time (1–3 h) on the dose-response curve with the three control sera as calibrators. Dose-response curves obtained after 2 and 3 h of incubation was almost superimposable for all the three control sera. However, incubation for only 1 h resulted in much lower FA values in all the three control sera, presumably, due to incomplete release of FA from FBP.

3.3. Folic acid-free serum

We compared the residual FA in a sample of human serum (containing FA 1.2 ng/ml and protein 6.5 g/dl) treated with (a) charcoal (10 g/dl, 18 h at 4° C), (b) AG1 × 8 ion exchange resin (treated twice with 5 g/dl, 8 h at RT) and (c) Sepharose-4B immunosorbent (see materials and methods section). The FA values obtained by microbiological assay were 0.48, 0.42 and 0 ng/ml respectively. The results show that human serum passed through immunosorbent column was completely free of endogenous FA. Charcoal or resin treatment could remove only 60–70% of endogenous FA. Measurement of protein concentration by Lowry's method showed no significant loss of protein in the immunosorbent treated serum. Protein content in charcoal, ion-exchange resin and immunosorbent treated serum was 4.8, 5.2 and 6.3 g/dl respectively. We, therefore, routinely used serum passed through the immunosorbent as diluent for FA standards.

Table 1
Effect of temperature on serum folic acid quantification

Temperature of incubation	Folic acid estimated in control serum [a] (ng/ml)		
	I	II	III
37° C	3.0	0.7	0.15
50° C	6.0	2.0	0.60
60° C	5.6	1.9	0.5

[a] Target values obtained by microbiological assay for control serum I, II and III were 6.5, 2.0 and 0.65 ng/ml respectively.

3.4. Effect of serum concentration

We studied the interference of serum constituents in the present assay by using three serum samples containing low, medium and high concentration of FA. We serially diluted the serum samples with incubation buffer and assayed for their FA content as described in the materials and methods section. The FA levels determined showed a linear relationship with sample dilution, suggesting the absence of any interfering compound in the tested serum samples. The amount of FA estimated was independent of the volume of serum present in the assay mixture in the range examined (6.25–50 μl).

3.5. Standard curve

Fig. 2 depicts an optimised standard curve covering the range 0.1–10 ng/ml. The detection limit determined at 95% of the zero dose signal (B_0) was 0.05 ng/ml (1.25 pg/well). The 50% intercept corresponded to 1 ng/ml (25 pg/well) of FA.

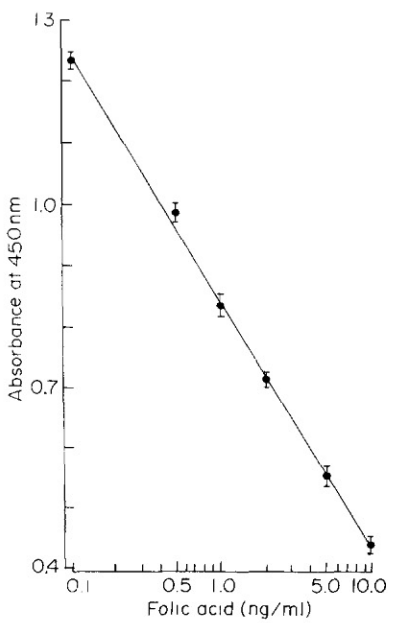

Fig. 2. Standard curve for folic acid estimation in serum. Standards used 25 μl; dilution of coating antibody and enzyme conjugate were 1/8000. Plate was incubated at 50° C for 2 h. Each point represents the mean and standard deviation of four measurements in duplicate.

Table 2
Specificity of assay

Metabolite	Cross-reactivity (%)
Folic acid	100
5-CH_3-THF	< 0.001
5-CHO-THF	< 0.001
DHF	90.0
THF	80.0
Pterine	0.01
Methotrexate	< 0.001

3.6. Specificity of assay

The specificity of the present ELISA was determined by estimating the cross-reaction of several structural analogues of FA. Solutions of these analogues were prepared in FA-free serum. Cross-reactivity was calculated according to the procedure of Abraham (1969). As Table 2 indicates, the cross-reactivity of most FA analogues tested was negligible except for DHF and THF, which had cross-reactions of 90 and 80% respectively. Since DHF and THF was known to be easily oxidised to FA (Handel, 1981), it was of interest to know whether the high cross-reactivity of the two metabolites are due to their oxidation under the conditions of the assay. Since the ultraviolet spectrum of FA has a distinct peak of 349 nm, which is absent in the spectra of DHF and THF, their conversion was conveniently monitored spectrophotometrically. For this purpose serum was spiked with DHF or THF (10 μg/ml), diluted 6-fold with incubation buffer and incubated for 3 h at 50° C. It was observed that more than 98% of DHF and THF was converted to PGA after 2 h (60% after 30 min).

3.7. Precision

Intra- and interassay variabilities were carefully assessed by testing three serum samples covering low, medium and high concentration of FA (Table 3).

Table 3
Precision of the assay

	Folic acid concentration (ng/ml)		
	Low	Medium	High
Intra-assay			
Mean	0.415	2.03	5.62
SD	0.034	0.176	0.323
n	16	16	16
CV%	8.2	8.4	5.7
Interassay			
Mean	0.414	2.2	5.86
SD	0.055	0.27	0.296
n	6	6	6
CV%	13.3	12.3	5.0

Three serum samples were assayed in replicates by direct ELISA. For the interassay variation each sample was measured four times in duplicates on 6 different days.

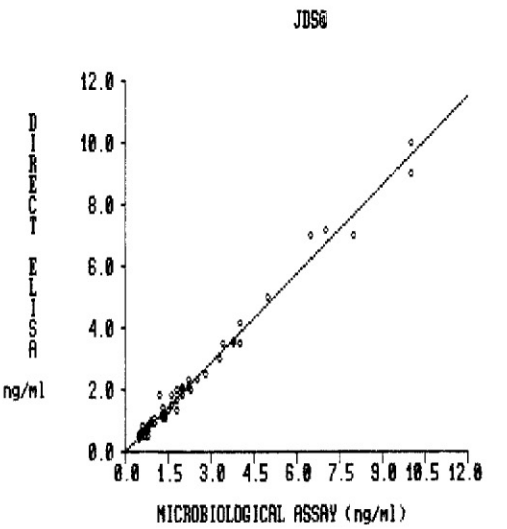

Fig. 3. Correlation between serum folic acid values of 65 samples estimated by microbiological assay using Streptococcus faecalis as test organism and by direct ELISA. The solid line represents the best fit obtained by regression analysis.

3.8. Accuracy

To investigate the analytical recovery in the method, two serum samples containing different levels of endogenous FA were assayed by spiking with different amounts of FA. The analytical recoveries ranged from 93.3 to 110% (Table 4).

In a dilution recovery test, a serum sample with FA content of 2.5 ng/ml was serially diluted with FA-free serum and the measured values for 2-, 4-, 8- and 16-fold diluted samples were 1.3, 0.70, 0.40 and 0.18 ng/ml showing excellent linearity with dilution.

Table 4
Spike recovery tests

Folic acid ng/ml				% Recovery
Endogenous	Added	Calculated	Found	
0.5	0.25	0.75	0.725	96.0
	0.5	1.0	1.0	100.0
	1.0	1.5	1.5	100.0
2.0	0.25	2.25	2.20	93.3
	0.5	2.50	2.5	100.0
	1.0	3.0	3.3	110.0

Two serum samples were spiked with known amounts of folic acid and then assayed by direct ELISA procedure. The values are the mean of duplicate measurements.

3.9. Validation of the method

FA concentrations in 85 serum samples were measured both by microbiological assay and the present direct ELISA (Fig. 3). Since the microbiological assay was less sensitive than the ELISA, serum samples containing FA concentration less than 0.5 ng/ml was not considered. The equation of correlation obtained with 65 serum samples was:

$$\text{folic acid}_{\text{direct assay}} = 0.026\text{ng/ml} + (0.96 \times \text{folic acid}_{\text{microbial assay}});$$

$$r = 0.99$$

The FA values of 35 serum samples obtained with and without prior heat denaturation of the samples, agreed well, the equation of correlation being

$$\text{folic acid}_{\text{direct assay}} = 0.023\text{ng/ml} + (0.99 \times \text{folic acid}_{\text{heat denatured}});$$

$$r = 0.99$$

3.10. Normal values

The concentration of FA in 52 serum samples from normal subjects under random diet was found to be in the 0.5–1.7 ng/ml with a mean ($\pm$SD) of 0.87 ± 0.4 ng/ml. The values are in accord with the range reported in the literature (Grossowicz et al., 1962; Da Costa and Rothenberg, 1971).

4. Discussion

A critical step in the development of direct ELISA for serum FA requires preparation of FA-free serum for serum based standards. Removal of endogenous FA from serum is difficult because, FA is the most stable among the folate compounds and in serum most of the FA is bound to FBP with high affinity. It has also been reported that serum FA cannot be extracted by charcoal treatment (Rothenberg et al., 1977). Our attempts to prepare FA-free serum by treatment with charcoal or ion-exchange resin were unsuccessful because they could remove only 60–70% of FA. The new procedure of passing normal serum at 50° C through an immunosorbent column containing covalently immobilized anti-FA antibody provided an excellent method for preparation of FA-free serum. Our data from the microbiological assay with *S. faecalis* as test organism indicates that the procedure is highly effective in removing endogenous FA from serum. The immunosorbent column can be reused after washing with potassium thiocyanate. We think that the technique may have wide application in the field of immunoassays.

The present direct method for ELISA of serum FA with highly specific anti-FA antibody and FA-HRP as a tracer provided a sensitive and specific technique for determining FA in serum. The remarkable specificity of the antibody eliminates the need for extraction or sample purification step. The detection limit of the assay (0.05 ng/ml) is much lower than that of a published RIA for serum FA (0.1 ng/ml) (Da Costa and Rothenberg, 1971; Handel, 1981) and the accuracy and precision of the assay compares favourably with published methods. The FA levels assayed after serial dilution of serum samples in buffer varied linearly with dilution suggesting the absence of any serum interference. As most of the FA in serum is bound to FBP, it is necessary to release the FA before assay. This was done previously by heating serum samples at 100° C for 15 min in lysine buffer, pH 10.6 (Dunn and Foster, 1973). A comparison of the data for heated and non-heated sample demonstrate that interference due to FBP are fairly eliminated at 50° C incubation performed in our procedure and prior heating of sample at 100° C is not necessary. The specificity studies of the assay demonstrates high cross-reactivity of DHF and THF. This may, be due to oxidation of DHF and THF to FA. Thus the present ELISA of FA estimates total FA, DHF and THF in serum. Similar results has also been observed in the RIA of serum FA (Handel, 1981). The serum FA levels obtained by ELISA with those obtained by microbiological assay using *S. faecalis* as test organism show good correlation between the two methods. The levels of FA concentration obtained in normal human serum is consistent with those reported by various investigators by RIA and microbiological assay (Grossowicz et al., 1962; Da Costa and Rothenberg, 1971).

At present microbiological assay (Cooperman, 1971) using *S. faecalis* (ATCC 8043) is the only available method for estimation of non-methylated folate(S) as microbiological assay using *L. casei* (ATCC 7469) and binding assays using FBP measures the total folate. However, there are several limitations of the *S. faecalis* assay. The present ELISA method for estimation of FA in combination with an assay of total folate would not only be a valuable tool for clinical diagnosis of folate deficiency but also in the study of absorption, metabolism and excretion of dietary and supplemented FA in normal and pathological conditions.

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