RESEARCH PAPER

Received 12 June 1995
Indian J.Pharm. Sci., 1996, 58(1) pp 32-36

Estimation of Folic acid in Multivitamin Formulations by Enzyme Immunoassay

J.DAS SHARMA[1]; C. DUTTAGUPTA[1]; E. ALI[2] AND T.K. DHAR[2]*
[1]Indian Statistical Institute, 203 B.T. Road, Calcutta - 700 035.
[2]Indian Institute of Chemical Biology, 4 Raja S C Mullick Road, Jadavpur, Calcutta - 700 032.

A sensitive ELISA method for estimation of folic acid in multivitamin formulations has been developed. The assay relies of simple sample preparation step followed by dilution of extracted sample. The detection limit of the assay is 0.1 pg/well. Intra- and interassay variation ranged between 2.7 to 5.1%. Analytical recoveries obtained after spiking with different amount of folic acid ranged between 94 to 104%. The present assay procedure is accurate, selective and useful for direct measurement of folic acid in multivitamin formulation.

FOLIC Acid (pteroylglutamic acid, FA) is one of the essential vitamins, the deficiency of which causes megaloblastic anemia. Mammalian cell cannot synthesize folic acid and normal requirement is met from dietary sources. In cases of folic acid deficiency, exogenous supply of this vitamin in the form of vitamin formulations are requires (1,2).

Quality control of multivitamin formulations often require estimation of folic acid. A variety of methods for determining folic acid in multivitamin formulations are available. These include microbiological method using **Streptococcus faecalis** as test organism (3), Colorimetric (4), HPLC (5,6), homogeneous enzyme linked competitive binding assay (7) and voltametry (8,9). These techniques have varying degrees of disadvantages rendering them unsuitable for routine analysis.

Enzyme immunoassay has emerged as an attractive choice in clinical chemistry laboratory because of its ability to selectively quantitate trace levels of important physiological substances (drugs, hormones, vitamins, etc.) without prior sample extraction or purification. Development of sensitive ELISA method require high titre antibodies. Previously, we reported the simple method of raising highly specific antibody against folic acid by using ε -aminocaproic acid modified BSA as a carrier protein and a direct ELISA method for estimation of serum folic acid (10,11). Herein, we describe the development of a sensitive ELISA method for estimation of folic acid in multivitamin formulations by using this antibody.

EXPERIMENTAL

Apparatus

All photometric measurements were made with Hitachi UV-VIS spectrophotometer and Anthos Model 2001 microplate reader.

Materials

Flat bottomed polystyrene microtitre plates (Maxisorp) and 8 channel microplate washer were from Nunc, Denmark. Bovine serum albumin (cat No.2153), horseradish peroxidase (type VI), polyoxyethylene sorbitan monolaurate (Tween 20), dicyclohexylcarbodiimide (DCC), 1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide hydrochloride (EDC). ε-aminocaproic acid, folic acid (pteroylgluta-

* for correspondence

mic acid, FA), N-hydroxysuccinimide (NHS), 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma, St. Louis, USA. All other chemicals and buffer salts were of analytical grade. Multivitamin tablets or capsules of different manufacturers were purchased from local medical stores.

Lyophilized cultures of **Streptococcus faecalis** (ATCC 8043) were obtained from the American Type Culture Collection, Rockville, MD 20852. All culture media were obtained from HI-Media Laboratories (Bombay, India).

Buffers

Coating buffer was sodium potassium phosphate (0.05 M, pH 7.6). Washing buffer was coating buffer containing Tween 20 (0.5 m/l). Post coating buffer was sodium phosphate (0.05 M, pH 7.6) containing BSA (5 g/l) and thimerosal (0.1 g/l). Incubation buffer was sodium phosphate (0.5 M, pH 7.5) containing per liter, 0.15 M of NaCl, 1 g of BSA and 0.1 of thimerosal. Enzyme assay buffer was sodium acetate/citric acid (0.1 M, pH 3.95).

Chromogen

TMB was dissolved in DMSO to give a final concentration of 0.042 M and 8 ml of this solution was added to 1 L of enzyme assay buffer. Just before use, 1.5 ml of aqueous hydrogen peroxide (30 g/l) was added.

Preparation of folic acid - modified BSA conjugate

Bovine serum albumin was modified with ε-aminocaproic acid in presence of EDC as described previously (10). Briefly, to a stirred solution of BSA (500 g) in 10 ml of deionized water, ε-aminocaproic acid (1g) and EDC (300 mg) was added. The reaction was carried out at room temperature for 4 h. The reaction mixture was purified by extensive dialysis against phosphate buffer followed by chromatography over a column of Sephadex G-50. Amino group analysis by TNBS method (12) showed no change in the number of amino groups in modified BSA compared to native BSA.

FA was covalently coupled to modified BSA by the activated ester method (13) using 25 mg of FA, 10 mg of NHS, 23 mg of DCC and 100 mg of modified BSA. The conjugate was purified by dialysis and chromatography over Sephadex G-50. The number of moles of FA bound per mole of modified BSA was approximately 20 as estimated by TNBS method (12).

Immunization procedure

New Zealand white rabbits were immunized with FA-modified BSA as immunogen. Rabbits were given s.c. and i.m. injections of approximately 1 mg of the FA-protein conjugate in 1 ml saline, emulsified with an equal volume of Freund's adjuvant (complete). Booster injections with the same amount of antigen in Freund's incomplete adjuvant were administered after 2 and 4 weeks and thereafter at monthly intervals. After checking the antibody titer by Ouchterlony immunodiffusion in gel, blood was collected by cardiac puncture after 3 months. The antisera was purified by precipitation with ammonium sulphate (50% saturation) followed by dialysis against phosphate buffer saline (0.01 M, pH 7.5). They were passed through a BSA Sepharose immunosorbent column to remove anti-BSA antibody. The purified globulin fractions was stored at -20°C (Ig^G concentration 16 g/l).

Preparation of FA - peroxidase conjugate

FA was covalently linked to horseradish peroxidase by the activated ester method (13). The product was purified through extensive dialysis followed by chromatography over Sephadex G-50. The working dilution consisted of a 50-fold dilution of the conjugate (final concentration of 0.02 g/l) in incubation buffer.

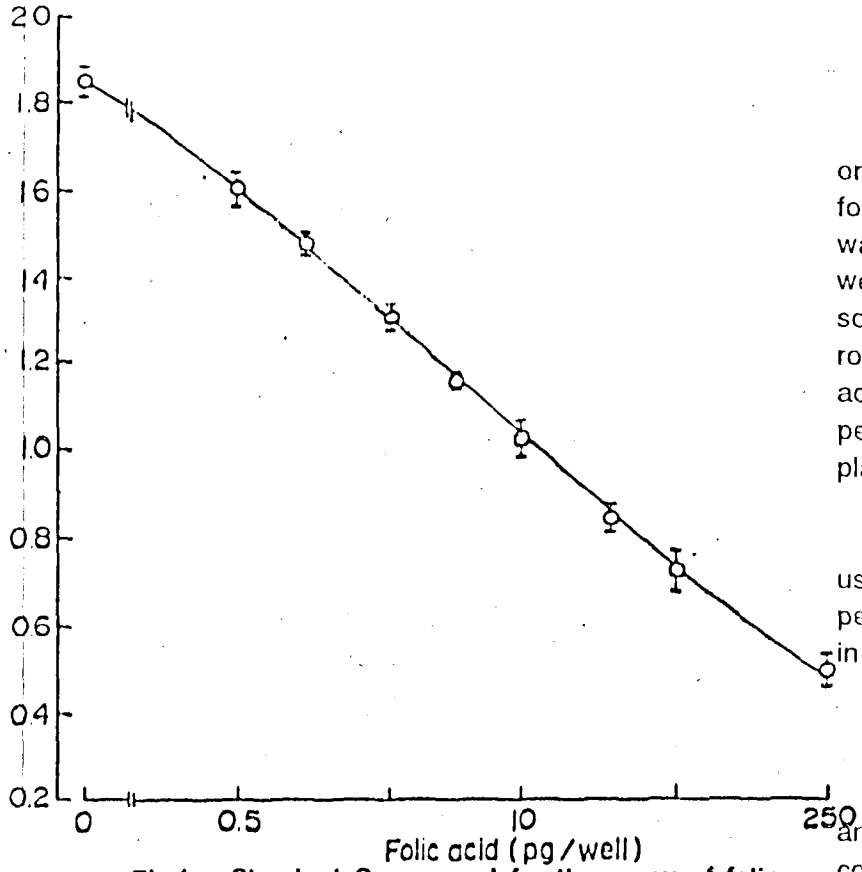

Fig.1.: Standard Curve used for the assay of folic acid. Antibody and Enzyme conjugate dilution used were 1:8000. Each point represents the mean and standard deviations for measurement in duplicate.

Preparation of multivitamin samples

Tablets and capsules of different multivitamin formulation were weighed, powdered and were shaken vigorously with 50-ml of K_2HPO_4 (0.01 M, pH 8.0) for 15 min. The mixture was centrifuged at 8720 g for 5 min and the clear supernatant was decanted. Different working dilutions of the sample were prepared with incubation buffer and used directly for ELISA of FA.

Assay procedure

Enzyme immunoassay - Anti-FA-antibody was treated with glycine- HCl buffer (0.05M pH 2.3) for 15 seconds and immediately diluted at 1:8000 fold with coating buffer. Each well of micrititre plate was coated with 200 µl of diluted antibody and incubated at 4°C overnight. The wells then washed and vacant sites were blocked with post-coating buffer (200 µl) for 3h at 37°C.

To each well of a coated plate, 50 µl of sample or standard and 100 µl of enzyme conjugate (1:8000 fold diluted) was added in duplicate and the plate was incubated for 2 h at room temperature. The wells were washed and 150 µl of TMB substrate solution was added and incubated in the dark at room temperature. After 30 min, the enzymatic reaction was stopped by adding 100 µl of 4N H_2SO_4 per well and the A_{450} was measured in a microtitre plate reader.

Microbiological assay - Microbiological assay using **Streptococcus faeclais** as test organism was performed according to the standard procedure (4) in a total volume of 10 ml.

RESULTS

The assay condition used for immobilization of anti-FA antibody, dilution of antibody and enzyme conjugate to obtain optimal coloration, and the time and temperature of incubation were systematically studied by checker board titration. Both antibody and enzyme dilution at 1:8000 gave steep curve and was used in all subsequent assays. **Fig.1** depicts the standard curve of folic acid in the range of 0.5 to 250 pg/well. The lower limit of sensitivity (distinguishable from blank by twice the SD) was 0.1 pg/well.

Since folic acid content of vitamin formulations were 1-5 mg/tablet or capsule, the sample extracts were diluted 60,000/- fold for assay. At this dilution the FA content of sample could be estimated using the steep linear part of the standard curve. The assayed concentration of folic acid ranged between approximately 8.5 to 85 pg/well.

FA content of five commerical multivitamin formulations (tablets or capsules) were estimated by the present immunoassay procedure. The calculated values were in good agreement with the manufacturer's claim and were also consistent with those determined by microbiological assay (Table -1). This also showed that the present method can

Table 1: Analysis of multivitamin formulations

Name of the vitamin[a]	Claimed	FA/Tablet or Capsule(mg) Found[b] ELISA	Microbiological assay
Becadexamin (Glaxo)[c]	1.0	1.10 ± 0.02	0.94 ± 0.02
Becosule (Pfizer)[d]	1.0	0.98 ± 0.03	1.20 ± 0.04
Anemidox (Merck)[e]	1.5	1.55 ± 0.04	1.40 ± 0.06
Autrin (Lederle)[f]	1.5	1.46 ± 0.06	1.42 ± 0.05
Folvite (Lederle)[g]	5.0	5.20 ± 0.04	4.92 ± 0.07

a -Name of the manufacturer in parenthesis.

b -Mean of 4 determination ± standard deviation.

c -Contains vitamin B_1 5 mg; B_2 5 mg; B_6 2 mg; B_{12} 5 mg; C 75 mg; folic acid 1 mg; nicotinamide 45 mg; pantothenate 5 mg.

d -Contains vitamin A 5000 1U; D_3 400 1U; E 15 mg; B_1 10 mg; B_2 10 mg; B_6 3 mg; B_{12} 5 mg; C 150 mg; folic acid 1 mg; nicotinamide 50 mg; pantothenate 12.5 mg.

e -Contains vitamin B_{12} 15 mg; folic acid 1.5 mg; vitamin D_3 400 1U.

f -Composition same as Anemidox but without vitamin D_3.

g -Contains only folic acid 5 mg.

Table 3: Spike recovery

Name of Vitamin	Folic acid (mg) /tablet/ Capsule Found before spiking[a]	Added	Found after spiking[a]	%Recovery
Anemidox	0.98±0.04	0.2	1.12±0.04	94.91
		0.5	1.50±0.03	101.35
Becosule	1.44±0.03	0.2	1.70±0.05	103.65
		0.5	2.02±0.03	104.00
Folvite	4.98±0.05	0.2	5.20±0.04	100.00
		0.5	5.38±0.05	98.17

a -Mean of four determinations ± SD.

Table 2: Intra and interassay variation in multivitamin samples

	Folic acid (mg)/tablet or capsule[a] Low	Medium	High
Intra-assay			
Mean	0.39	1.47	4.72
SD	0.02	0.04	0.16
n	12	12	12
CV%	5.1	2.7	3.38
Inter-assay			
Mean	0.44	1.47	4.86
SD	0.015	0.04	0.24
n	7	8	8
CV%	3.4	2.7	4.9

a -Fecontin-F, Becosule and Folvite were extracted and further diluted 60,000/- fold with incubation buffer for assay. Fecontin-F (Mundipharma Ltd) contains besides 0.5 mg. folic acid, 100 mg of ferrous glycine sulphate. Composition of Becosule and Folvite is given in Table 1.

be used for selective assay of folic acid in presence of wide range of other vitamins.

Intra and inter assay variation in three multivitamin samples containing low, medium and high concentration of FA are shown in **Table 2**. The variation in every instance was <10 %.

To investigate the analytical recovery of the method, three multivitamin samples containing different concentration of folic acid (1, 1.5 and 5 mg) were analysed after spiking with 0.2 and 0.5 mg of folic acid. The analytical recoveries ranged from 94 to 104% (**Table 3**).

In a dilution recovery test, the extract from one formulation was diluted 2 to 64-fold serially with incubation buffer and assayed by ELISA. The comparison of observed and expected values are shown in **Table 4**.

Table 4: Dilution recovery

Dilution factor	Expected mg/tablet	Observed mg/tablet[a]	Recovery %
—	5	4.980	96.60
1/2	2.5	2.480	99.20
1/4	1.25	1.260	100.80
1/8	0.625	0.628	100.48
1/16	0.312	0.300	96.15
1/32	0.156	0.155	99.35
1/64	0.078	0.080	102.56

a -Recorded values depicts the mean of two determinations in duplicate.

DISCUSSION

Quality control of multivitamin formulation often requires estimation of FA. However, the presence of other vitamins and/or ingredients in large excess interferes with many of the assays. HPLC methods have been traditionally used in analytical chemistry and the method has also found application in the estimation of FA in multivitamin formulation. However, due to interference by other vitamins or compounds present in the preparation, preliminary sample clean-up is generally required. For this reason tedious microbiological method using **S. faecalis** as test organism is still widely used for such estimation. The present ELISA method developed is much simpler and eliminates the tedious and time consuming handling inherent of the existing known methods. The assay relies on simple sample preparation step and can directly quantitate folic acid from the sample. The precision, selectivity, accuracy and ultimate analytical utility of the method have been demonstrated by determining FA content of various commercial multivitamin formulation in the presence of a wide range of other vitamins or ingredients. Apart from general advantages of the microtitre plate enzyme immunoassay, the method has several analytical characteristics rendering it suitable for routine applications. We believe that the present assay method may find wide application for quality control of folic acid content in pharmaceutical and food products.

ACKNOWLEDGEMENTS

The author thanks the Director and the Dean of studies, Indian Statistical Institute for a Research Fellowship to JDS and to the Director, Indian Institute of Chemical Biology for laboratory facilities to JDS for carrying out this work. We also thank Dr. S.N. Banerjee for many valuable suggestions and co-operation extended during this work.

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