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Research Article

Development of Simple Method for the Determination of 5-Methyl Tetrahydrofolate in Dried Blood Spot by High-Performance Liquid Chromatography

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ABSTRACT

Folate (Reduced) or folic acid (oxidized), is a water-soluble vitamin necessary for thymidylate synthase, methionine synthase, serine hydroxymethyltransferase, and folate deficiency associated with megaloblastic anemia, neural tube defect, and coronary heart disease. The available folate data is minimal due to a dearth of suitable, accurate, field-friendly blood collection and estimation methods. Conventional assays like microbiological and competitive protein-binding radioassay methods are used to estimate the total folate in serum and whole blood. In erythrocytes, folate exists mainly as 5-methyltetrahydrofolate polyglutamate and indicates the long-term storage of folate status. Therefore, the study aimed to develop a simple method for estimating 5-methyl tetrahydrofolate (5-MTHF) by high-performance liquid chromatography (HPLC) in dried blood spots (DBS) as an indicator of folate status. The present study results indicate standard 5-methyltetrahydrofolate with different concentrations with linear regression was showed R^2-0.98, and the minimum detection limit of a standard was 20 pg. The inter-and intra-assay variations were found to be less than 10%. A good correlation (R^2-0.964) between the DBS and blood and the recovery of added standard in the DBS sample was more than 80%. A minimum of three DBS punches equivalent to 20µL of blood was required for the analysis, and the competitive protein-binding radioassay showed slightly high folate compared to the HPLC method. The stability of 5-MTHF in DBS was also tested periodically. DBS technique and HPLC method are simple, sensitive, not expansive, and safe compared to the microbiological and competitive binding assay for assessing sub-clinical folate status in a field survey on a large population.

INTRODUCTION

Folate is an active water-soluble vitamin-B_9 one-carbon donor essential for the growth, reproduction, and maintenance of normal body functions. It involved in various biological reactions like thymidylate synthesis, methionine synthesis, carbohydrate metabolism, amino acid metabolism, nucleotide metabolism, serine glycine inter-conversion, a neurotransmitter (serotonin, norepinephrine, and dopamine) synthesis, and folate deficiency is mainly associated with megaloblastic anemia, neural tube defects[1,2] and coronary heart disease.[3] The conversion of 5-methyl tetrahydrofolate from folates in the liver and circulated to the blood and all childbearing age women required to consume 400 µg of folic acid daily to meet recommended dietary allowance (RDA).[4] The chemical form of folic acid is an oxidized state that is not showing cofactor activity due to not found in a significant amount in nature further, the folic acid must be converted into the reduced form of tetrahydrofolate in the presence of dihydrofolate reductase that shows cofactor activity. The unmetabolized folic acid is the beneficial factor that is associated with B_{12} deficiency.[5] The supplementation of natural folate is required to meet RDA through the folate-rich diet. Fortification technology is another method to supplement folic acid, where the chemical form of folic acid is fortified with the staple food that is highly bioavailable

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to reduce the risk of folic acid deficiency and to overcome folate deficiency. It is advisable to take an additional 400 to 800µg/day folic acid supplements or fortified foods to control neural tube defects before pregnancy to achieve baseline folate status in young women. Serum or plasma folate, erythrocyte folate, and homocysteine are used as a biomarker of folate status to reflect the folate intake. Serum folate shows a recent intake of folate, and erythrocyte folate shows a long-term intake of folate. Red blood cell (RBC) folate estimation is technically demanding than the serum due to long-term folate intake, and the deoxyhemoglobin folate maintains a stable state.[6] The natural folate in erythrocytes comprises mainly 5-methyltetrahydrofolate polyglutamate and deconjugation by endogenous plasma γ-glutamyl hydrolase (conjugase), assayable form 5-methyltetrahydrofolate monoglutamate. A high sensitive method is required to estimate the concentration of total folate in the serum and whole blood. Many methods have been developed for the estimation of different folate metabolites in blood. The existing conventional methods like microbiological and competitive protein-binding radioassay are sensitive but require a venous blood sample. Folic acid status data is minimal due to a dearth of field-friendly methods of blood collection and estimation.[7] The DBS has been developed as a simple and effective sampling method for the neonatal metabolic profile.[8] The DBS technique views folate measurement in low-cost transportation, extraction, and blood storage as a natural state.[9] The dried blood spot is a unique method to measure many analytes in biological samples. Therefore, the present study aimed to develop the HPLC method for estimating 5-methyl tetrahydrofolate (5-MTHF) in DBS and establish as an indicator of folate status.[10]

Materials and Methods

Special Whatman filter paper, finger prick lancets, sealable zip-lock cover, silica gel, black paper, potassium-EDTA vacutainers, calibrated paper punch, Waters µ bond pack C_{18}, 3.9x300 mm, syringe filter 0.22 µm and Mili-Q water. Standard 5-Methyltetrahydrofolate, Triton X-100 was obtained from Sigma, and all the chemicals used were of analytical grade unless otherwise specified.

Study Design

Random blood samples have been collected from the subjects with informed written consent through prior permission from the Government General Hospital, Tumakuru, Karnataka, India. The University Ethical Committee approved the research study.

Supplementation

For the pilot study of folic acid supplementation, eight women were included in the study, and this may be sufficient as it is paired sampling. Dexorange capsules contain 500 µg of folic acid, 20.61 mg of zinc sulfate monohydrate, 160 mg of ferric ammonium citrate, and 7.5 µg of vitamin B_{12} were administrated at a single point of time immediately after the collection of a basal blood sample under fasting stage, and again blood samples were collected after one hour.

Inclusion Criteria

Women between the age of 20–40 years were included in the study, healthy women with no clinical signs of deficiency were included.

Exclusion Criteria

Those who were on medications and micronutrient supplementation, those who have attained early menopause were excluded.

Data Collection

The data regarding general information, socio-economic status, and health status were collected by using a questionnaire.

Preparation DBS

Dried Blood Spot (DBS) is a technique where the blood is spotted on a special filter paper. Fresh venous blood was collected in potassium-EDTA tubes, gently mixed. Samples were divided into two portions, one portion without a standard for the folate measurement and the other with the standard for knowing recovery. Then the samples were immediately spotted onto a special Whatman filter paper (9.5 X 5.5 cm), and 12.5 mm diameter circles were printed on cards and the samples' details. All DBS cards were placed horizontally on a rack and air-dried at room temperature (RT) for 2 hours and cards covered with black paper with silica gel placed in sealable zipper bags and stored at -20°C until further analysis.[11-15]

Extraction of 5-Methyl tetrahydrofolate

According to the method of Pfeiffer C M and Gregory III JF (1996)[16] briefly, three numbers of DBS spots were taken from each of the DBS cards by using a standard paper punch. Three DBS spots or 20 µL of blood were placed in a round-bottomed glass tube and extracted with 20 volume of 1% ascorbic acid contains Triton X-100 was added, vortex, and extracted for 15 minutes. The nitrogen gas was passed to maintain an inert atmosphere and incubated for 3 hours at 37°C. Proteins were precipitated with 60% perchloric acid then the sample is frozen and thawed. After centrifugation, the clear supernatant was obtained, filtered through 0.22 µm, and injected onto a C_{18} column attached to the HPLC system.

Measurement of 5-methyltetrahydrofolate by HPLC Method

The HPLC system: Agilent 1100 series, Column: Waters mbond pack C_{18}, 3.9 X 300 mm with guard column, Detector: Fluorescence Ex: 295 nm Em: 365 nm, Mobile Phase: 33 mM phosphate buffer containing 8% acetonitrile pH 2.3, filter through 0.22 µm membrane and degassed, flow rate: 1 mL/min. The 5-methyltetrahydrofolate was calculated as an area under the curve.

Statistical Analysis

The results were subjected to statistical analysis. Origin 8.1 version software and SPSS version 21 software were used for the graphs and statistical analysis.

Results

The fifty blood samples were collected for screening folate status and for folate supplementation, study samples were collected from the subjects with informed written consent from the Government General Hospital, Tumakuru, Karnataka, India. The subjects' basic information was obtained through the questionnaire to elicit the socio-economic and general health status. The age of the subjects varied from 22–40 years, about 62.5% of the subjects were below the age of 30 years and 37.5% of them were above the age of 30 years and 50% were married. The majority of the subjects have belonged to the upper-middle-income group. All the subjects were healthy and not having any known specific diseased conditions. None of the subjects took any medications and micronutrient supplements, but only one subject used to take supplements two weeks before the study. The menopausal stage was not reached by any of the subjects. The subjects have not shown any clinical signs of nutritional deficiency (Fig. 1).

Preparation of Dried Blood Spot

Dried blood spot (DBS) was prepared where venous blood or fingerprick blood was spotted on a special Whatman filter paper (9.5 X 5.5cm), and 12.5 mm diameter circles were printed on cards with sample details. All DBS cards were air-dried at room temperature (RT) for 2 hours and DBS covered with black paper with silica gel in sealable zipper bags and stored at -20^{o} C until analysis (Fig.2).

5-methyltetrahydrofolate (5-MTHF) Extraction

To extract 5-methyltetrahydrofolate, 20 µL blood, 3 dried blood spots were taken in the 20 volume of 1% ascorbic acid-containing Triton-X-100. Incubation at 37^{o}C for 3 hours this approach is allowed to endogenous serum gamma-glutamyl hydrolase activity suggesting that the conversion of 5-methyl tetrahydrofolate polyglutamate to monoglutamate. The samples were precipitated with 60% perchloric acid followed by freeze and thaw, and the supernatant was collected after the centrifugation, and the sample was filtered through a 0.22 µm syringe filter and injected onto HPLC (Fig. 2).

Measurement of 5-methyl Tetrahydrofolate

The 5-methyl tetrahydrofolate was separated with stationary phase C_{18} (3.9 X 300 mm) column and mobile phase phosphate buffer (33 mM) containing 8% acetonitrile (pH 2.3), the detector was used fluorescence Ex: 295 nm Em: 365nm, and flow rate at 1mL/min. The calculation of 5-methyltetrahydrofolate monoglutamate concentration was considered an area under the curve. The results of HPLC chromatograms showing the elution of standard 5-methyltetrahydrofolate followed by the sample and spiking (sample processed with the addition of standard) and showed standard, sample, and spiking are eluting the same retention time at 6.2 min (Fig. 3) and linearity of the standard 5-methyl tetrahydrofolate with different concentrations was R^2-0.98 (Fig. 4). The

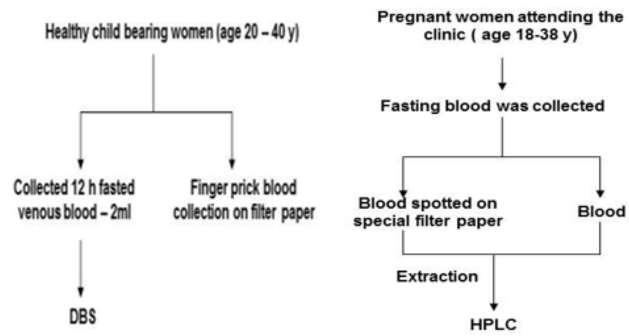

Fig. 1: Flow chart of blood collection

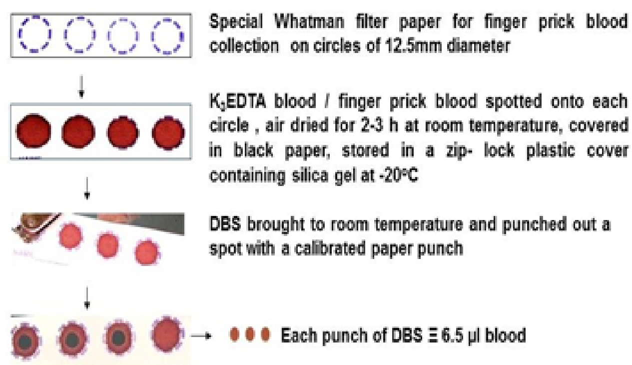

Fig. 2: Preparation of Dried Blood Spot (DBS)

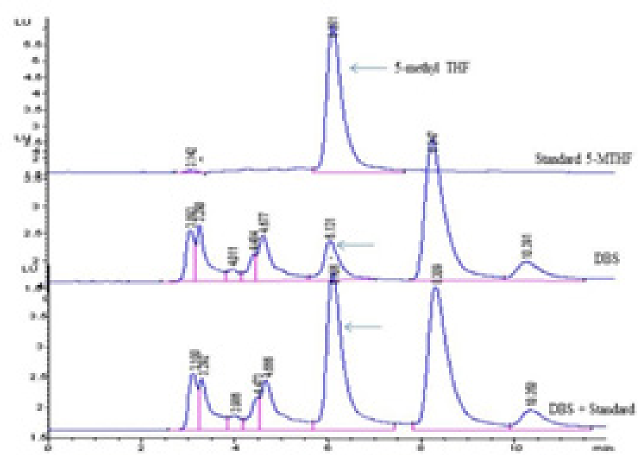

Fig. 3: HPLC chromatograms of standard 5-MTHF, DBS and spiked sample peaks

measurement of 5-methyl tetrahydrofolate in one, two DBS spots were tried and but not showing good signals finally three DBS spots were showed good signals equivalent to 20 μL of blood. The inter and intra assay variations were found to be less than 10%. The addition of standard and extracted in the same method and the results showed the confirmation of 5-methyltetrahydrofolate peak, and results of the recovery of added 5-MTHF in the DBS samples were showed more than 80%. The results showed the extraction of 5-methyl tetrahydrofolate levels increase might be due to the influence of incubation at 37°C for 3 hours. In this method limit of detection (LoD) level of standard 5-Methyl tetrahydrofolate was showed 20 pg. The concentration of folate in both whole blood and dried blood spot in fifty subjects was analyzed by the HPLC method and results showed that regression correlation (R^2-0.964) for fifty samples and the *p*-value was 0.284 indicates there was no significance between whole blood and dried blood spots (Fig. 5).

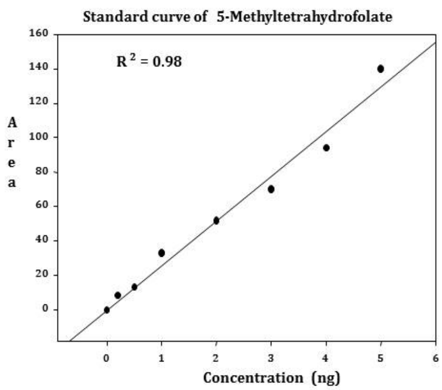

Fig.4: Standard graph of 5-Methyltetrahydrofolate

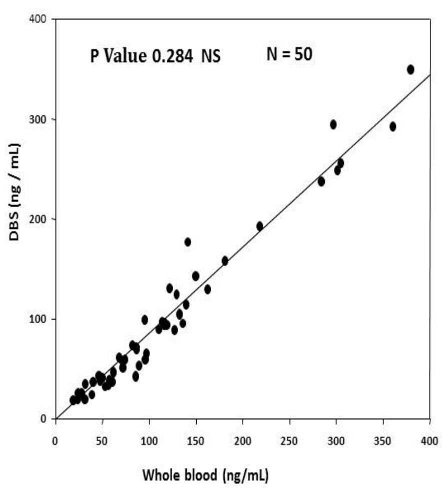

Fig.5: Correlation between 5-MTHF in Blood and DBS samples (*p*-value 0.284, N-50)

Table 1: Comparison of folate in DBS and Finger prick blood by HPLC method

Parameter	*DBS*	*Finger Prick Spot*
5-MTHF (ng/mL)	101.4 ± 44.8	95.6± 27.9

Mean ± SD, N-5, NS p > 0.05

Table 2: Comparison of folate by HPLC method and Competitive protein-binding radioassay in DBS

Parameter	*HPLC*	*Competitive protein-binding radio assay*
5-MTHF (ng/mL)	92.6 ± 30.0	145.3± 59.8

Mean ± SD, N-7 significantly difference by paired t-test p < 0.05

5-methyltetrahydrofolate in DBS vs Finger Prick Blood Spot

In this study, DBS were prepared by the addition of venous blood and fingerprick blood to the special filter paper and determined 5-methyl tetrahydrofolate in DBS and Finger prick spot by HPLC method, and results showed that there was no statistically significant (*p*>0.05) between DBS and finger prick spot sample (Table 1).

5-methyltetrahydrofolate in DBS by HPLC vs. Competitive Protein-binding Radioassay

Protein binding assay and microbiological assay have been used to estimate the folate content in the serum and blood samples. We have estimated the folate in dried blood spots by HPLC and competitive protein-binding radioassay, and results showed statistically significant (*p* <0.05) between HPLC and protein binding assay method. Furthermore, the results showed in DBS low levels of 5-MTHF in the HPLC method, whereas protein binding assay showed high folate levels due to the binding of all forms of folate (Table 2 and Fig. 6).

Folic Acid Supplementation Study

The supplementation of Dexorange capsules contains 500 μg of folic acid was administrated at a single point of time immediately after the collection of a basal blood sample under the fasting condition, and again blood samples were collected after 1-hour. Further, the extraction

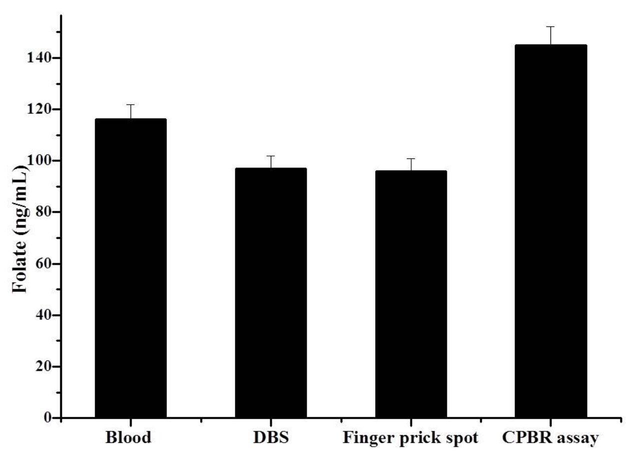

Fig.6: Comparison of an average of 5-Methyl tetrahydrofolate in blood, dried blood spot (DBS), finger prick spot by HPLC method, and competitive protein-binding radioassay (CPBR) in DBS

Table3a: Baseline and final blood folate levels after supplementation.

Name of the sample	*Initial (nM/L)*	*Final (nM/L)*
F1	527	535
F2	222	268
F3	194	370
F4	333	564
F5	120	352
F6	157	167
F7	444	453
F8	472	453
Mean ±SD	309 ±157	395±134

Paired samples t-test p > 0.05

Table3b: Folate levels increased in the folate deficient subjects

Name of the sample	*Initial (nM/L)*	*Final (nM/L)*
F2	222	268
F3	194	370
F4	333	564
F5	120	352
F6	157	167
Mean ±SD	205 ±81	344±147

Paired samples t-test: p < 0.05

of 5-MTHF in Blood and DBS samples was analyzed by the HPLC method. The blood folic acid levels were subjected to statistical analysis and calculated by using paired student test. It was found that the baseline levels ranged from 120 to 527. As the normal folic acid level is 400 nmol/L, five out of eight subjects were deficient. The mean increase in folic acid of 87 nmol/L due to the supplementation was not statistically significant (Paired samples t-test $p>0.05$). Therefore, the null hypothesis, which states that there is no significant difference between the baseline and after supplementation of blood folic acid levels, has been accepted. The absorption rate was not significant after the one-hour administration of folic acid supplementation (Table 3a). However, the mean increase in folic acid of 139 nmol/L due to supplementation was statistically significant (Paired samples t-test, $p < 0.05$) in deficient subjects (Table 3b). Therefore, the rate of absorption was significant after one hour of administration of folic acid supplementation.

Stability of 5-methyltetrahydrofolate

The stability of DBS folate was conducted for one week was measured, and results showed that 25% stability. The folate extraction from DBS with 1% ascorbic acid Triton X-100 0.2 M sodium chloride showed effective folate extraction, and the addition rat plasma source of conjugase showed high folate content compared to endogenous plasma.

Discussion

Folate is a reduced form of the water-soluble vitamin, one-carbon donor, and acts as coenzyme in nucleic acid, protein, and carbohydrate metabolism. The folic acid (oxidized) reduces to tetrahydrofolate in the presence of dihydrofolic acid reductase. Folate cannot synthesize in the body must be obtained from the diet or supplements. The deficiency of folic acid leads to neural tube defects, cardiovascular disease, and megaloblastic anemia.[17,18] The target groups like pregnant women[19] and children are affected due to a lack of external dietary intake through natural folate-rich foods and naturally, folate consists of 5-methyl tetrahydrofolate polyglutamates.[20] Serum and plasm folate is the short-term folate status, and erythrocyte folate indicates the long-term folate status. Assessment of folate was done by different methods such as HPLC,[21] Gas chromatography, microbiological, and protein binding assay. Many methods are available to determine the 5-methyltetrahydrofolate and searching for a simple and accurate method to assess folate status. Folic acid was determined as oxidized form in the tablets, but in the biological samples, folate present as pterylpolyglutamate. In erythrocytes, long-term storage is 5-methyl tetrahydrofolate polyglutamate converted to 5-methyltetrahydrofolate mono glutamate in the presence of conjugase enzyme in the plasma.[22] This study aimed to assess 5-methyltetrahydrofolate status and response after folic acid tablets were administered to women subjects and estimated by the HPLC[23] method. Therefore, this main study focused on determining the 5-methyl tetrahydrofolate in the whole blood, dried blood spot (DBS), fingerprick spots, and comparison between samples and methods. The preliminary data was obtained from the subjects in a questionnaire, subjects age ranging from 22–40 years, married, and upper-middle-income group. All the subjects were healthy, but only one subject used to take supplements two weeks before the study (Fig. 1). In this method, limit of detection[24] level of standard 5-methyltetrahydrofolate was 20pg. Incubation of blood or DBS with 1% ascorbic acid TritonX-100 at 37^{o}C for 3 hours and showed a three fold increase in the 5-MTHF levels suggesting conversion of polyglutamate to monoglutamates of 5-MTHF by the endogenous conjugase (Fig. 2). The HPLC chromatograms showed the elution of standard 5-methyltetrahydrofolate followed by the sample and eluted the same retention time at 6.2 minutes (Fig. 3). The standard paper punch used was calibrated and showed each DBS spot contains 6.5 µL of blood. The results of standard 5-methyl tetrahydrofolate with different concentrations and linearity of the standard showed R^2-0.98. There was a good correlation (R^2-0.964) between the DBS and blood 5-MTHF levels (Fig. 4). The recovery of added 5-MTHF in the DBS samples was more than 80%. The inter and intra assay variations were found to be less than 10%. The folic acid supplementation results showed that five subjects were deficient in folic acid, folate response after administration was significant and overall deference is not significant this may be due to adequate folate in the normal subjects. Any of the subjects did not reach the menopausal stage. The subjects have not shown any clinical signs of nutritional deficiency. The blood folic acid levels were obtained from the samples, baseline, and after supplementation (Table 3a). It was found that the baseline levels ranged from 120 to 527 nmol/L. As the normal folic acid level is 400 nmol/L, five out of eight subjects were deficient. The mean increase in folic acid of 87 nmol/L due to supplementation was not statistically significant (Paired samples t-test $p > 0.05$) Therefore, the null hypothesis states that there is no significant difference between the baseline and after supplementation of blood folic acid levels have been accepted. The rate of absorption was not significant after the one-hour administration of folic acid supplementation). The mean increase in folic acid of 139 nmol/L due to supplementation was statistically significant (Paired samples t-test $p < 0.05$) in deficient subjects (Table 3b). Therefore, the rate of absorption was significant after one hour of administration of folic acid supplementation. 5-methyl tetrahydrofolate in DBS and Finger prick spot by HPLC method and results showed that there was no statistically significant ($p>0.05$) between DBS and finger prick spot sample (Table 1). Results showed statistically significant ($p < 0.05$) between HPLC and protein binding assay method. The results showed in DBS, low levels of 5-MTHF in the HPLC method, whereas protein binding assay showed high folate levels that may be due to the binding of all forms of folate (Table 2). Comparison graphical representation of an average of 5-Methyl tetrahydrofolate in Blood, DBS, Finger prick spot by HPLC method, and competitive protein-binding radioassay (CPBR) in DBS (Fig. 6).

The research reports showed the importance of DBS, suitable methods, folic acid supplementation. Villalpando S *et al*. (2003)[25] indicated the DBS technique is a novel and convenient method in field studies to screen folate content to study under epidemiological conditions. Obeid R *et al*. (2018)[26] revealed RBC folate saturation of 400 µg/day folic acid is not sufficient in the women population. Krishnaswamy K and Nair KM (2001)[27] review showed recommended dietary allowances of folates in Indians men (182 µg/day), women (154.9 µg/day) and pregnant women (145.7 µg/day) and lactating women (165.5 µg/day). The recommended folate intake for prevention of homocysteinemia and neural tube defects 350 µg/day for adults to prevent homocysteinemia and 400 µg/day for pregnancy to prevent neural tube defects. Hyland K and Surtees R. (1991)[28] results showed folates in cerebrospinal fluid by using two methods like microbiological assay (*L.casei*) estimated as total folate and HPLC estimated as a 5-methyltetrahydrofolate and a statistical report showed that the regression was 0.876 and $p < 0.001$ indicates that statistically, significance with two methods means two methods values are different. Gregory III J F *et al*. (1984)[29] studied the detection of folates by using HPLC (Fluorescence) and microbiological assay in food and biological materials. The stability of 5-methyl tetrahydrofolate was observed during the heating compared to tetrahydrofolate, and it revealed the hydrolysis of 5-methyl tetrahydrofolate polyglutamates by the action of conjugase. Fresh rat liver showed 5.51 nmol/g by HPLC with fluorescence detector and showed the sensitivity limit range was picomole. Kalmbach R *et al*. (2011)[30] studied the estimation of 5-MTHF by HPLC affinity column with electrochemical detection of the total folates level, and the results showed that the correlation between HPLC and *L.casei* was 0.979. King M J *et al*. (2012)[31] indicated 1.1 µmol/L RBC folate analyzed by liquid chromatography–mass spectrometry (LC–MS). O'Broin S D *et al*. (1997)[32] presented red cell folate and hemoglobin folate regression was 0.99 and results showed red cell folate males (608.6 nmol/L) and females (620.2 nmol/L). The study showed microbiological assay of the venous control sample folate, and finger stick sample folate regression was 0.92. Giulidori P *et al*. (1981)[33] estimated the 5-methyl tetrahydrofolate in plasma by using a Spectrofluorometric detector and showed recovery of 5-MTHF was 93%. Results exhibited that there was no degradation during boiling with ascorbate. The report showed that the detection limit of folates was 25-50 ng. Leeming RJ *et al*. (1990)[34] measurement of 5-MTHF in serum and RBC by microbiological (*L. casei*) was slow, and radioisotope dilution assay required expensive isotope counting equipment. The report mentioned that the HPLC method was precise and comparable with the microbiological and radioisotope dilution assay. Truswell A S & Kounnavong S (1997)[35] showed serum folates were measured after supplementation of folic acid 100, 500, 1000, and 1500µg/day for 3 weeks and showed 80, 93, 98, and 96% pair of comparison respectively. The study showed an increment value of plasma and red cell folate after folic acid supplementation. Fazili Z *et al*. (2005)[36] experimentally showed deconjugation of pterylpolygluatamate to monogluatamate influenced by the pH, temperature, and time and folates were determined by the LC/MS/MS. Wilson SD and Horne DW (1984)[37] showed the distribution of rat liver folates one of them was 5-methyl tetrahydrofolate 2.35 µg/g, and 37.3% was eluted from the column. According to the covered above reports discussed briefly, after supplementation of folic acid 100 µg to1500 µg/day for 3 weeks serum folates were measured, and 400µg/day folic acid is not sufficient to saturate RBC folate in the women population. The folate was measured by microbiological assay (*L.casei*), HPLC (Fluorescence), microbiological assay, LC-MS/MS' radioisotope dilution assay. There was no degradation during boiling with ascorbate and deconjugation of pterylpolygluatamate to monogluatamate influenced by the pH, temperature, and time. DBS is a novel and convenient method in field studies. From the present study results obtained, it was found that five subjects were deficient in folic acid. In those subjects, the plasma response after administration of folic acid was significant. However, the overall difference in whole blood folic acid not significant. This could be due to adequate folate status in the normal subjects. The stability of DBS folate was conducted for one week was measured and results showed that 25% stability. The folate extraction from DBS with 1% ascorbic acid Triton X-100 0.2 M sodium chloride showed high folate and the addition of conjugase source with rat plasma showed high folate levels compared to endogenous plasma. The levels of 5-MTHF estimated by HPLC in DBS were lower than the folic acid levels by competitive protein-binding radioassay due to the binding of all forms of folate. The study results showed that DBS and HPLC method is a simple, cost-effective, accurate method to assess the folate status in the target population.

Conclusions

5-Methyltetrahydrofolate levels suggesting conversion of polyglutamate to monoglutamates by the endogenous conjugase and showed a 3 fold increase in the incubation of blood at 37°C for 3 hours. There was a good correlation (R^2-0.964) between the DBS and blood. The minimum detection limit of the standard was 20pg. The recovery of added standards in the DBS samples was more than 80%. The inter and intra assay variations were found to be less than 10%. The levels of 5-MTHF estimated by HPLC in DBS were lower than the folic acid levels by radiometric assay. This method is not expansive compared to microbiological and competitive protein-binding radioassay. The DBS technique is a simple, sensitive method for assessing sub-clinical folate deficiency on a large scale in a field survey.

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