OPTIMISATION OF AN ANALYTICAL METHOD FOR THE ANALYSIS OF FOLIC ACID DERIVATIVES IN BIOLOGICAL MATERIALS

BY

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AUTHOR'S DECLARATION

The experimental work presented in this thesis represents the original work by the author, and has not been submitted in any form to any other University. Where the used of other author's work was made, it was duly acknowledge in the text.

The research described in this study was carried out under the supervision of Prof. N. Gqaleni in June 2003 to December 2004.

PD Khanyi (Miss).

I dedicate this work to my sister, Nomvula for her support and words of encouragement.

Above all to the Almighty GOD through HIM things were possible.

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ABBREVIATION

MBA	microbiological assay MBA
BIA	Biomolecular interaction analysis
SPR	surface plasmon resonance
5-MTHFA	5-methlytetrahydrofolic acid
THF	tetrahydofolic acid
5-CH_3-THF	5-methyl-tetrahydrofolate
5-CHO-THF	5-formyl-tetrahydrofolate
DHF	Dihydrofolate
10-HCO-THF	10-formyl-tetrahydrofolate
5, 10-CH2-THF	5, 10-methylene-tetrahydrofolate
ACN	acetonitrile
PAG	pteroylglutamates
FA	Folic acid
HOAc	acetic acid
MeOH	methanol
CO_2	carbon dioxide
H_3PO_4	orthophosphoric acid
HPLC	High Performance Liquid Chromatography
ml	milliliter
mm	millimeter
nm	nanometer
RDA	Recommended Daily Allowance
SAX	strong anion exchange

UV	ultraviolet
w/v	weight per volume
mM	millimolar
µl	microlitre
µg	microgram
C18	Octadecyl
mg	milligram
min	minute
nm	wavelength
^{0}C	degrees Celcius
g/mol	grams per mol
g	grams
v/v	volume per volume
M	Molarity
LC-MS	liquid chromatography-mass spectrmetry
GC-MS	gas chromatography-mass spectrometry

ABSTRACT

Folic acid is a water-soluble, B-complex vitamin influencing a number of biological processes in humans and particularly important in the prevention of neural tube defects (associated with spinal bifida) in unborn children. Reliable analytical methods are therefore needed for quantisation of the amount of total folic acid (FA) in biological materials of quality assurance and regulatory purposes. What is particularly needed are rapid and reliable methods for ensuring that the correct amount of FA is consumed and the degradation rates of these compound is kept at minimum during the extraction process.

Analytical methods for determination of folic acid in biological materials have been around for decades and the most common procedures include microbiological assay; biomolecular interaction analysis (BIA); immunoassay; conventional chromatographic procedure such as thin-layer column chromatography (TLC) and high performance liquid chromatography (HPLC). These procedures were replaced by HPLC, which is more rapid and in many instances yields a better resolution. Current HPLC methods uses C-18 column and reverse phase conditions in combination with ion-pair or ion suppression techniques; fluorescence or electrochemical detector, unfortunately, excitation and emission of folic acid is found not sufficiently to allow physiological levels of the form of the vitamin to be detected. In addition, ion-pair reagent nullifies the mobile phase and interferes with the absorption/ fluoresce spectrum resulting in poor separation. Therefore this study was carried out to address and improve the problems that are in the existing HPLC methods. Currently scarce information is available on the determination of folic acid in biological materials by HPLC with UV detection.

Serum samples were spiked with folic acid standard to check the efficiency of the method. Other wavelengths from 200 nm to 300 nm were attempted for detection of folic acid, in

which the wavelength 250 nm was found to have better absorbance compared to other wavelengths. Folic acid was detected at 250 nm wavelength under isocratic elution using a mobile phase consisting of citrate phosphate buffer: acetic acid: methanol. Folic acid in maize meal was detected at 290 nm using mobile phase containing potassium phosphate containing ascorbic acid/sodium ascorbate mixture and 2-mercaptoethanol under gradient elution.

The mobile phase used for gradient and isocratic elution was suitable for separation of folic acid from other compounds with flow rate of 3 ml/min modified to 1ml/mim to avoid overloading of the column under isocratic elution. For good separation of folic acid under gradient elution the flow rate was set at 0.8ml/min with pH of mobile phase modified from pH 2.2 to pH 2.5. The recovery of folic acid added to human serum was 91% -100% and recovery of folic acid added in unfermented maize meal and fermented maize meal ranged from 55% - 73%. Folic acid level from unfermented maize meal and fermented maize meal ranged between 1.29 - 1.3 μg/g and 1 - 2.1 μg/g respectively.

In conclusion the optimised method in this study gives better analytical results when compared with earlier HPLC method in terms of efficiency, reproducibility and sensitivity for folic acid in human serum and maize meal. However, there is a need to minimise the loss of folic acid during the sample treatment. The outcome of this work indicated that more work has to be done to improve extraction procedure for specific foods with minimum time preparation to sample analysis.

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CHAPTER 1

INTRODUCTION

1.1 FOLIC ACID IN BIOLOGICAL MATERIALS

Vitamins are small organic molecules in the diet that cannot be synthesised by humans or are synthesised at a rate less than that necessary for good health (Julian *et al.*, 1999). They contribute to good health by regulating metabolism and assisting the biochemical processes that release energy from digested food.

Folic acid (FA) is a water-soluble, B-complex vitamin found primarily in organ meats, whole grains and green leafy vegetables (Lane *et al.*, 1995). Folic acid derivatives exist in tissue as at least eight different coenzyme forms which serve as donors and acceptors of 1-carbon fragments in the number of critical enzymatic reactions including the synthesis of purines, pyrimidines, serine, the methyl group of S-adenosyl-methionine and the catabolism of purines and histidines (Brody *et al.*, 1984; Lane, 1995).

Folic acid deficiency leads to impaired cell division and megaloblastic anaemia (Nelson and Davey, 1991; Butterworth, 1993). Folic acid deficiency has also been implicated in the development of cancer. The mechanisms by which folate might protect against cancer are not clear but may relate to its role in DNA methylation and synthesis. It has been hypothesized that folate may modulate cancer risk (Glynn and Albanes, 1994; Mason and Levesque, 1996; Kim, 1999; Duthie ,1999) notably the risk of cervical and colorectal cancer but less well for breast cancer and a rapidly growing number of other cancer sites such as the lung, pancreas, stomach, oesophagus, leukaemia, skin and endometrium (van Dam *et al.*, 2000; Stolzenberg-Solomon *et al.*, 2001; Zhang *et al.*, 1997).

In vivo, FA is enzymatically reduced to dihydrofolic acid (DHF) and then to tetrahydrofolic acid (THF), which is 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 FA deficiency, it is given in the form of capsules or tablets (Gregory *et al.*, 1990a). Clinically, FA has assumed great significance following reports that daily supplementation reduced the risk of neural tube defects (Thornalley, 1993; McLellan, 1992).

In attempting to determine the kinds and amount of folate in biological materials, investigators have encountered a number of obstacles including: the low concentration of FA in most biological materials; the difficulty of separating the numerous derivatives, and their extreme susceptibility to destruction or change in form by heat, light, oxygen, pH, and endogenous conjugase (Baugh *et al.*, 1971a).

Folates are sensitive to heat, strong acid, oxidation and light. They are therefore difficult to extract from food without some oxidation or deconjugation taking place. Because of the complexity of the problem, there are very few high performance liquid chromatography (HPLC) methods available that can be applied generally to the analysis of food. Agreement between HPLC and microbiological methods is mostly poor; one reason being the variable reproducibility of microorganisms to the different forms of folate present in samples (Gregory *et al.*, 1982; Reingold and Picciano, 1982).

AIMS OF THE PROJECT

a) To optimise an HPLC method for analysis of folic acid and its derivatives in biological materials.

b) To apply the optimised HPLC method in the detection of folates in biological materials; unfermented and fermented maize meal and human serum.

c) To compare the HPLC results with those of standard immunoassays (in particular, the chemiluminescence) methods used for analysis of FA in serum in haematology laboratories.

CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

2.1 STRUCTURE OF FOLIC ACID

The B-complex vitamins are essential for human nutrition and, because of their water solubility, excesses of these vitamins are excreted in urine and therefore they rarely accumulate in toxic concentrations (Mildred and Rodriquez, 1978). Folates are a group of vitamins derived from the parent compound, folic acid (pteroylglutamic acid, PGA) Figure 2.1.

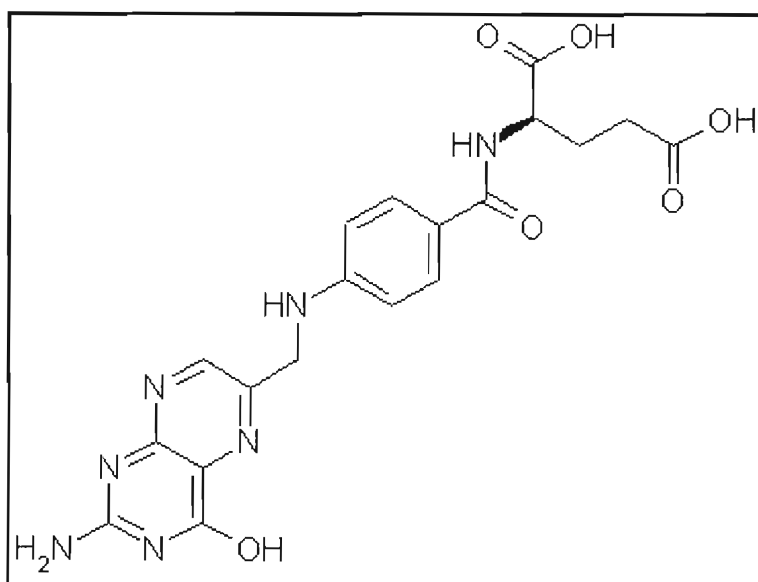

Figure 2.1: chemical structure of folic acid
www.chm.bris.ac.uk/webprojects2002/schnepp/**folic**.html accessed in September 20, 2005

The compounds differ with regard to the state of oxidation of the pterine ring, the number of glutamate residues conjugated to the *para*-amino-benzoic acid moiety and the type of substitution at the 5 and/or 10-carbon position (Blakely, 1969).

2.1.1 NOMENCLATURE

The principal derivatives (Figure 2.2) of folic acid (FA), the oxidized monoglutamate and various names and abbreviation that have been used to identify them are summarised in Table 2.1.

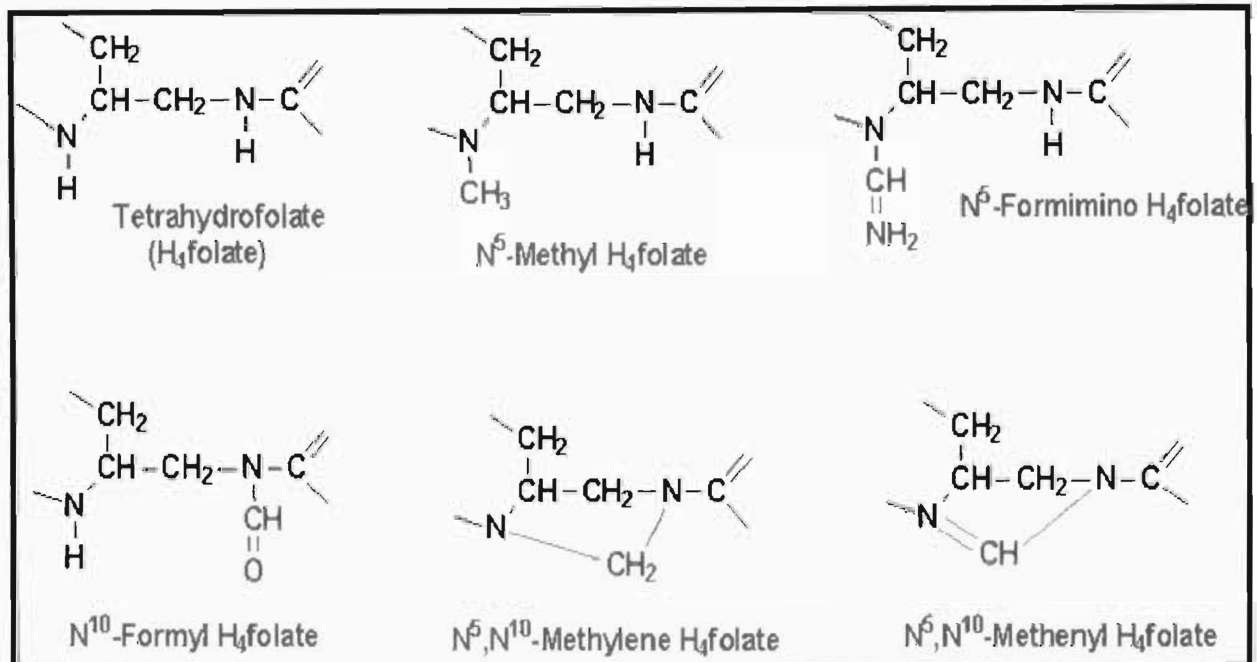

Figure 2.2: Chemical structure of folic acid and its derivatives (www.atozofhealth.com/.../ folate-derivatives.gif; accessed in May 25, 2005).

The nucleus may exist in any one of the three different oxidation/Reduction state- folic acid, the oxidized form; 5,6-dihydrofolic acid (H_2-folic acid) or 5,6,7,8-tetrahydrofolic acid (H_4-folic acid) and the reduced metabolically active parent compound (Figure 2.3).

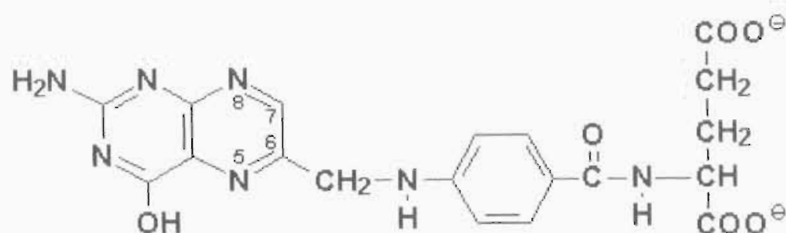

Figure 2.3: Chemical structure of folic acid showing different positions, 7 and 8 carry hydrogens in dihydrofolate (DHF), positions 5-8 carry hydrogens in tetrahydrofolate (THF), (Hawkes *et al.*, 1989)

These compounds usually have any one of the six different 1- carbon (C_1) substitutes in the N^5 and/or N^{10} position (5-methly, 5-formyl, 10-formyl, 5, 10-methylene, 5, 10-methyl, and 5-formimino) of H_4-folic acid. In the naturally occurring folacin, the glutamyl residue of H_4-folic acid is commonly linked in a γ-peptide linkage to a polyglutamyl side chain consisting of one to six γ-glutamyl residues (Baugh and Krumdieck, 1971b). This γ-peptide linkage is readily split by the enzyme,γ-glutamyl carboxypeptidase (commonly referred to as "conjugate") (Bird *et al.*, 1945) which is ubiquitous in both plant and animal tissues.

Table 2.1: Folate nomenclature (Mildred, 1978)

Compound	Abbreviation
Oxidised folate:	
Folic acid	FA
Folic acid glutamates	PGA
Pteroyldiglutamic acid	$PteGlu_2$
Pteroyltriglutamic acid	$PteGlu_3$
Pteroylheptaglutamic acid	$PteGlu_7$

10-formylfolic acid	10-CHO-Folic acid
10-Formy; pteroyltriglutamic acid	10-Cho-PteGlu$_3$

Partially reduced folate:

Dihydrofolic acid	H_2-folic acid
10-formyldihydrofolic acid	10-CHO-H_2-folic acid

Reduced folate:

Tetrahydrofolic acid	H_4-folic acid
5-formyltetrahydrofolic acid	5-CHO-H_4-folic acid
10-formyltetrahydrofolic acid	10-CHO-H_4-folic acid
5, 10-methylenetetrahydrofolic acid	5,10-CH_2-H_4-folic acid
5-methyltetrahydrofolic acid	5-CH_3-H_4-folic acid
5, 10- methyltetrahydrofolic acid	5,10-CH=H_4-folic acid
5-formimidoyltetrahydrofolic acid	5-HCNH-H_4-folic acid

2.2. FUNCTION OF FOLIC ACID

It has been found that the folates influence a number of biological processes in humans and are particularly important in the prevention of neural tube defects (associated with spinal bifida) in unborn children. This has led to the fortification of some foodstuffs with folic acid (Williams, 1994). Only the reduced folates are found naturally in plants and animals. In humans, the predominant form of folate found in blood is 5-methlytetrahydrofolic acid (5-MeTHF) whereas in the porcine species, it has been reported that tetrahydrofolic acid (H_4 Folate) is the major circulating form. Several different forms have been found in foodstuffs,

the most common being 5-MeTHF, 5 and/or 10-formyltertrahydrofolic acid (folinic (CHOTHF) and H_4 folate.

The principal function of folates is to act as coenzymes in the transfer of single carbon atoms in reactions essential to the metabolism of several amino acids (including glycine and methionine) and to nucleic acid synthesis (purine and pyrimidine synthesis) (Stefania *et al.*, 1999). Tetrahydrofolic acid (THF) is essential for the synthesis of 5,10-methylene-tetrahydrofolate, which is a cofactor for thymidylate synthase the enzyme which converts deoxyuridylate into thymidylate in the process of DNA synthesis. Lack of folate therefore decreases the supply of THF, thus limiting the production of DNA. The signs of folate deficiency are therefore seen in rapidly dividing cells, particularly in the bone marrow and folate deficiency typically presents as megaloblastic anaemia (Stefania *et al.*, 1999).

2.2.1 Active form of folic acid

Derivatives in the diet are cleaved by specific intestinal enzymes to monoglutamyl folate for absorption. Most of this is reduced to tetrahydrofolate in the intestinal cell (Figure 2.3) by the enzyme, folate reductase, which uses NADH as a donor of reducing equivalences. Tetrahydrofolate polyglutamate is probably the functional coenzyme in tissue (Figure 2.4).

2.2.2 Folic acid deficiency

Lack of folic acid is globally the most common vitamin deficiency. In underdeveloped countries or in low economic classes deficiency may be caused by infection, haemorrhage, pregnancy or certain drugs. In short, a deficiency may occur if the diet is poor or if an individual is unable to absorb FA. Since blood cell turn over is rapid, folate deficiency is usually seen as megaloblastic anaemia, a condition in which haemoglobin levels are low and

the bone marrow shows abnormally high number of large, abnormal, immature erythrocytes (Rex *et al.*, 1996).

Choi and Mason (2000) have reported that collectively, the evidence from epidemiologic, animal and human studies strongly suggests that folate status modulates the risk of developing cancers in selected tissues, the most notable of which is the colorectum. Folate depletion appears to enhance carcinogenesis whereas folate supplementation above what is presently considered to be the basal requirement appears to convey a protective effect.

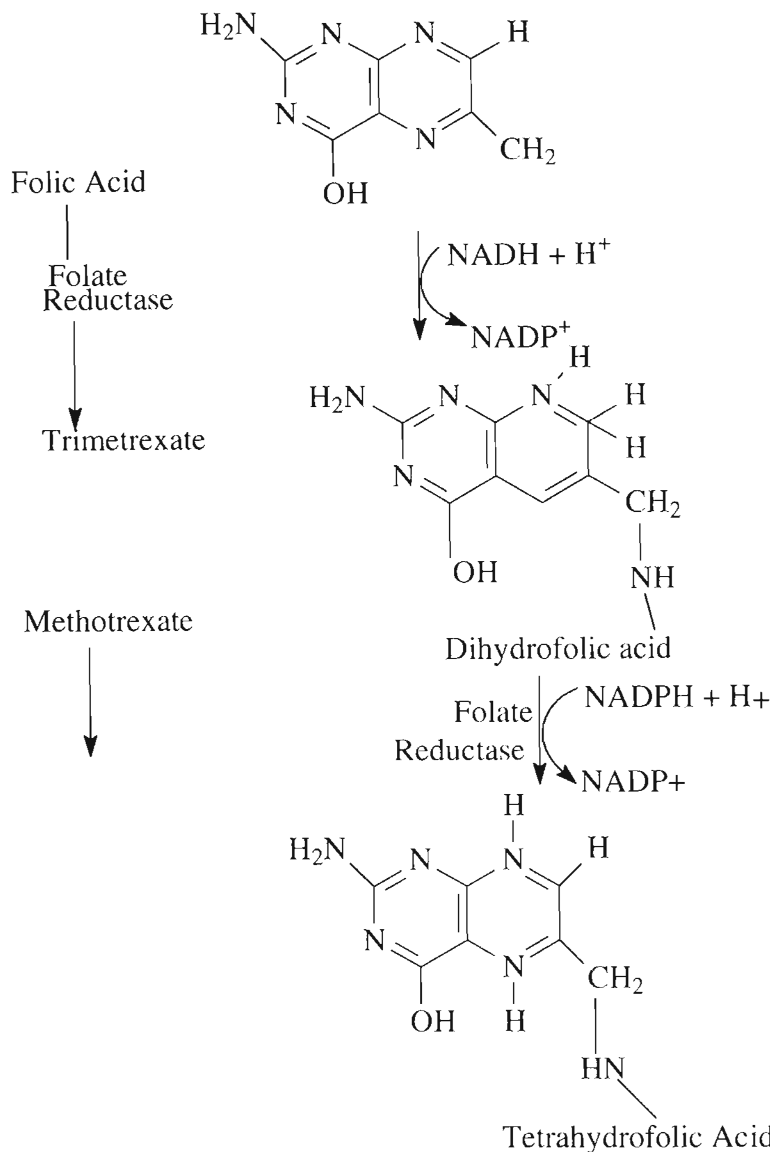

Figure 2.4: Reaction catalysed by dihydrofolate reductase (Rex, *et al.*, 1996).

Cancer involves cells that continue to divide indefinitely. Since cell division requires a net synthesis of nucleic acids, there has been considerable effort to find compounds that will selectively inhibit the formation of nucleic acid and check the uncontrolled growth of cancer. The least successful attempts involve the inhibition of the general types of reaction used in the formation of the purines and pyrimidine rings. For example, nitrogen is transferred twice from glutamine in the formation of the purine ring. Concentrations of the glutamine analogue that are effective in suppressing cancer growth are dangerously toxic, and these compounds are not useful drugs. Another general class of reaction used for nucleotide synthesis is the transfer

of 1-carbon units from tetrahydrofolate derivatives. Such transfer occurs twice during the formation of purine rings and once in the formation of deoxythymidine monophosphate (dTMP). The formation of dTMP is a special case in that it involves a simultaneous reduction of tetrahydrofolate to dihydrofolate. There is a possibility that a compound inhibiting the reduction of dihydrofolate back to tetrahydrofolate would be selectively toxic to dividing cells, since these are the only cells forming deoxynucleotides. Two such compounds are commercially available, *aminopterin* and *methotrexate* which are folate analogues (Figure 2.5).

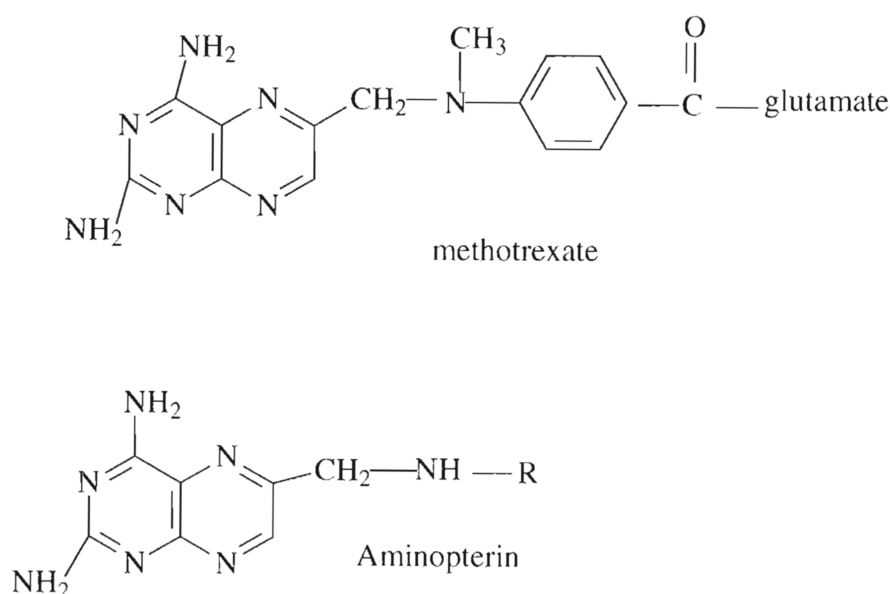

Figure 2.5 Chemical structures of aminopterin and methotrexate (McGilvery, 1970).

The introduction of universal fortification of basic food stuffs poses a number of questions of efficacy and toxicity (Ohmori *et al.*, 1987b) and points to the importance of a reliable assay for serum FA under different treatment regimes. Using a differential microbiological assay method, Matsura *et al.*, (1985) detected folic acid (FA) in sera of subjects given FA in a

number of foodstuffs as well as in aqueous solution. Differential microbiology assays, however, lack sensitivity and are cumbersome to standardize and perform.

2.3 SOURCES

Folate is present in almost all foods. Certain foodstuffs particularly rich in folates include yeast, liver, nuts, green vegetables and chocolate (Herbert 1963; Santini *et al*, 1964). Most food folate exists as reduced, methylated or formylated polyglutamates (Butterworth *et al.*, 1963; Scott and Weir, 1976). Marked losses of folate activity occur during boiling of food, especially in large volumes of water (Herbert, 1963, Hurdle *et al*, 1968). In addition, during preparation of food, some folate derivatives formed are metabolically inactive (Scott and Weir, 1976). Estimates of the total folate in the diet of Western countries are of the order of 200-230 μg per day (Hoppner *et al.*, 1977). The folate content of meat is low. Pork and lamb contain only a few micrograms of folate and most raw beef cuts contain < 10 μg folate/ 100 g. Most fish also contain <15 μg folate/100 g, but extensive variation exist even in recently reported values (e.g., from 3.4 to 26 μg/100 g for salmon). The folate content of chicken meat is in the same range as those of fish (Livsmedelstabeller, 1988; Holland *et al.*, 1991; Scherz and Senser, 1994). The amount of folate in pasteurised milk is about 5 μg/100 g largely composed of 5-methyltetrahydrofolate (Holt *et al.*, 1988; Wigertz and Jagerstad, 1995). Folate is well concentrated in egg yolk, but egg white is a poor folate source (Livsmedelstabeller, 1988; Sherwood *et al.*, 1993). These low levels of folate in food of animal origin are important as far as folate intake from diet is concerned because large amounts of food of animal origin, particularly liquid dairy products, are consumed. The distribution of folates in these foods is not well known, and up-to-date food composition data based on modern analytical techniques are needed to estimate the daily folate intake from diet. Also, the influence of folate distribution and stability or bioavailability is still unclear (Liisa *et al.* 1997).

2.4 FOLIC ACID REQUIREMENTS

Folic acid is required for cell metabolism as well as for the synthesis of purines and pyrimidines, which are components of DNA and RNA. Therefore, when the total number of cells in the body or the rate of cell synthesis increases FA requirement also increase. In the last trimester of pregnancy and in neonates, the number of new cells being synthesized is very large.

Total body stores of FA in normal humans are of the order of 6-10 mg, with the liver being the main storage site. Normal hepatic folate concentrations are greater than 5 µg/g (Chanarin *et al.*, 1966). Normal catabolism and excretion of folate compound is poorly understood, but substantial amount of folates and their breakdown products may be lost in the urine (Johns *et al.*, 1961; Retief and Huskisson, 1969). Folate excreted into bile in high concentrations is reabsorbed by the gut (Pratt and Cooper, 1971). Adult requirement are approximately 50-100 µg daily (Herbert, 1962a). When a physician volunteer, presumably with normal stores, was placed on a virtually folate-free diet, megaloblatsic anaemia developed after 19 weeks (Herbert, 1962b). In two alcoholics, who presumably had marginal folate stores, megaloblastic haematopoiesis occurred after 5-10 weeks on a similar diet (Eichner *et al.*, 1971). Thus, in contrast to vitamin B_{12}, folate stores can be rapidly exhausted within a few months. This accounts for the greater role of decrease dietary intake in the aetiology of folate deficiency, as well as the tendency for folate depletion to develop in states of increase demand, such as pregnancy and haemolytic anaemia.

2.4.1 Folic acid derivatives in food

Due in part to the rapid changes in the C_1 moiety, the state of oxidation and the number of glutamyl residues in the peptide side chain during food preparation and analysis, there are few data on the folacin derivatives in different foods. In addition, as much as 50-95% of the

activity may be destroyed by cooking and/or processing (Hurdle *et al.*, 1968; Deller *et al.*, 1965). The folacin from both plant and animal sources contain formyl and methyl derivatives. Perry (1971) has reported that in a cooked meal, 60% of the folacin derivatives were methyl and 33% were formyl derivatives. Cossins *et al.*, (1972) detected a similar pattern in whole leaf extract of pea seedlings. Those in cabbage (Chan *et al.*, 1973), egg (Butterfield and Callowway, 1972), liver (Noronha and Silverman, 1962; Shin *et al.*, 1972), milk (Dong and Oace, 1975) and orange juice (Dong and Oace, 1973) are predominantly methyl derivatives. Most of the folacin in yeast (Schertel *et al.*, 1965) and soy-beans (Shin *et al.*, 1975) are formyl derivatives. In the early 1960s, Butterworth *et al.*, (1963) and Santini *et al.*,(1964) found three folacin derivatives in food when separated chromatographically: 10-CHO-folic acid (55%), 5-CHO-H_4-folic acid (34%) and folic acid (11%) could be recognised.

2.4.2 Absorption

Folate is absorbed rapidly, primarily from the proximal small intestine (Booth, 1961; Hepner *et al.*, 1968) by a mechanism resembling that of iron and vitamin B_{12} through an active, energy-dependent process for small quantities but by diffusion in unphysiological amounts. Polyglutamates from foods are broken down and reduced to dihydro- and tetrahydrofolates (THF), the latter being the active or co-enzyme form, present in serum, liver and other tissue as 5N-methly THF. Ascorbic acid helps to maintain FA co-enzymes in a reduced or active form.

Folate-binding proteins are present in foods, such as milk (Ghitis, 1967), but virtually nothing is known about the release of folate from food in the gastrointestinal lumen. Most food folates are polyglutamates, which must be hydrolysed, mainly to the monoglutamate form before transported across the intestinal cell (Butterworth *et al.*, 1969). The enzyme which hydrolyses folate polyglutamates ("conjugase") is presented in human jejunum in two isoezyme forms,

one in lysosomes and the other in brush border membranes (Hoffbrand and Peters, 1969). *In vivo* hydrolysis probably occurs at all cell surfaces (Halsted *et al.*, 1975; Rosenberg, 1976). Whether folates are actively or passively transported through the intestine is the subject of much conflicting experimental data (Rosenberg, 1976), but the occurrence of a congenital disorder in which malabsorption is limited only to folate compounds (Lanzkowsky, 1970) suggests that there is a specialised transport mechanism.

The bioavailability of folate in various foods has not been widely studied. Contrary to previous speculation, much of the polyglutamate folate in foodstuffs is available for absorption (Rosenberg, 1976). The folates in bananas, lima beans and yeast were found to better absorbed than those in lettuce, cabbage, wheat germ and orange juice (Tamura and Stokstad, 1973; Tamura *et al.,* 1976) Glucose enhances the intestinal uptake of monoglutamate folate (Gerson *et al.*, 1971).

2.5 ANALYSIS AND DETECTION OF FOLATES

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. Since serum concentrations of FA are low, the availability of a suitable method for estimation of FA, along with an assay of total folate would provide useful information about absorption, metabolism and excretion of dietary and supplemented FA in normal and pathological states. The total folate content of foods is usually determined by a microbiological assay using *Lactobacillus* casei (Finglas *et al.*, 1993). If the quantification of specific chemical forms of folate is of interest, chromatographic method such as ion-exchange or reverse-phase HPLC can ensure the separation and determination of various folates (Gregory, 1989). Regardless of the analytical procedure, the accuracy of the analysis is highly dependent on the merits of the preparation. A key preparative phase is the extraction (Gregory

et al., 1990). Also important is the enzymatic deconjugation of the polyglutamyl folates, which must be optimised for each type of sample (Engelhardt and Gregory, 1990). Because of the low endogenous folate content of many foods and their complex compositions, food extracts must often be concentrated and purified if chromatographic methods are to be employed for the quantification of folates.

2.5.1 Food extraction

The most common method of extraction involves heating a buffered sample homogenate to thermally denature folate-binding proteins and enzymes that may catalyse folate degradation or interconversion (Gregory, 1989). Gregory *et al.* (1990) showed the need for second extraction in the analysis of many foods and the superiority of the Wilson and Horne (1984) extraction buffer (mixed Hepes/Ches buffer, 101 mM ascorbate, 200 mM 2-mercaptoethanol, pH7.85) compared with two buffers of lower pH that contained only ascorbate. Several researchers have reported that treatment of high-protein products with protease and treatment of food item high in starch and glycogen with α-amylase, in addition to the folate conjugase (pteroylpolyglutamate hydrolase) treatment, provided significantly increase measurable total folate concentration (Yamada, 1979; Cerna and Kas, 1983; deSouza and Eitenmiller, 1990). Martin *et al.*, (1990) reported that a triezyme treatment was necessary to determine folate accurately in certain foods. The method included the digestion of food extract with α-amylase and a non-specific protease in sequence with the folate conjugase treatment. All these studies, however, employed a microbiological assay for total folate quantification, and they did not distinguish among the different folate derivatives.

Folic acid in fortified products can be extracted with various buffers in the presence of antioxidants such as ascorbate (Hoppner and Lampi, 1982; Schiefer *et al.*, 1984) or

mercaptoethanol (Clifford and Clifford, 1977). In order to simplify the chromatography, polyglutamyl folates are deconjugated with purified pteroyl-poly γ-glutamyl hydrolase (conjugate). Chicken pancrease conjugates have been reported to yield a diglutamyl folate product after incubation for 10 min (Leichter *et al.*, 1977). Reingold and Piccian, 1982), however, obtained unconjugated FA by incubation for 24 hours with a similar product while Gregory *et al.*, (1984) preferred the use of hog kidney conjugate and a 30 minute incubation. Extracts may be further purified by ion-exchange chromatography (Hopper and Lampi, 1982; Schiefer *et al.*, 1984).

Hoppner and Lampi (1982) used the protease papain to extract FA from food matrices. They found recoveries of FA in milk-based and soy-based infant formula to be 96 and 98%, respectively, Bart and Franklin (1992), however, reported modification and expansion upon the sample preparation procedure from that of Hoppner and Lampi (1982) to obtain quantitative extraction of FA in infant formula and liquid medical products. While Hoppner and Lampi (1982) used a solid-phase extraction column to remove potential interferents from sample preparation before injection into the liquid chromatography system, Bart and Franklin (1992) modified and expanded the procedure by altering sample preparation and replacing the solid phase extraction step with an automated procedure using column switching. They, however, encountered a problem in that the extraction procedure showed a large number of UV absorbing compounds that formed during enzymatic hydrolysis. The papianed extraction procedure of Hoppner and Lampi (1982) was further modified by the addition of a bacterial protease, use of 295 nm as the detection wavelength, the bacterial protease was added to remove interfering peaks in the chromatograms of whey containing samples. Although the procedure was successful in most instances, the chromatogram of some samples displayed interfering peaks even after the addition of the second protease. Consequently, the detection

wavelength was changed to 345 nm, a wavelength at which interfering peaks were not observed. However, the bacterial protease was not removed from the sample preparation because the recovery of FA from some soy-based samples was improved when this protease was used in conjunction with papain.

2.5.2 Methods of analysis of folic acid in biological materials

The most common procedure for the determination of folates in foods is the microbiological assay using *Lacobacillus casei*. Although this assay is highly sensitive, it is time–consuming, labour-intensive, and not consistently reproducible (Hawkes and Villota, 1989). In addition, the growth response of *L. casei* has been reported to vary between folate forms (Ruddick *et al.*, 1978; Hawkers and Villota, 1989; Gregory, 1989) and exhibit inhibition or stimulation because of inherent compounds in foods (Gregory, 1989). These limitations make the microbiological assay an undesirable routine method for folate analysis in foods.

Newman and Tsai (1986) introduced a microbiological assay of folates in 96-well microtiter plates. This microbiological assay for folate in foods measures the growth response of *Lactobacillus rhamnosus* (ATCC 7469) inoculum in sample extracts utilising a 96 well microplate technique. The results were read with an automatic plate reader. This modification decreased reagent costs and shortened the time spent pipetting and manually reading results for samples spectrophotometrically. Their procedure, however, required extensive manipulation of the inoculum and protracted (36-38 h) incubation times, drawbacks that make this procedure less than ideal for routine assays. Bacterial growth could also be compared by measuring the turbidity of samples after a suitable incubation period. An important improvement in folate analysis is the use of glycerol-cryoprotected cultures because they can be grown in large quantities and frozen for later use and require less time and labour to

maintain than serial cultures (Wilson and Horne, 1982). Despite advances in the use of cryoprotected cultures, the assay is not very convenient because the organism remains difficult to grow. Furthermore, cryoprotected cultures lose viability in 2– 3 months, making it difficult to maintain cultures that will yield adequate inoculum levels.

Biomolecular interaction analysis (BIA), a biospecific technique, has primarily been applied to characterise macromolecular interaction (Jonsson *et al.*, 1991; Malmqvist, 1993). The protocol is based on the exchangeable immunosensor chip immobiliser with analyte via a corboxymethyldextran surface. The detection based on surface plasmon resonance (SPR) originating from a refractive index change cause by ligand binding. A microfluidics system facilitates continuous automated analysis, with sensor chip regeneration interpolate between individual sample injections. The principle advantages of this instrumental technique compared with other biospecific techniques include real time measurements, freedom from enzyme or radioisotope labelling requirements and enhanced precision. The technique has been formatted as an inhibition immunoassay for the analysis of biotin and folates in fortified infant formulars (Rose *et al.*, 1997; Bostrom *et al.*, 2000;). In view of its potential for rountine quality control and nutritional labelling applications, BIA technique was evaluated with reference to standard MBA methods. It is worth noting that the MBA method is recognised to be laborious, lengthy, and of relatively poor precision.

Biosensor-based determination of folic acid has also been developed: an immunoassay performed in an optical biosensor system which utilises the phenomenon of surface plasmon resonance (SPR) as the detection principle (Jonsson *et al.*, 1991). Several factors contribute to making the method rapid and easy to use, for example, the sensitive SPR detection, the specific interaction between analyte and antibody and low non-specific binding.

Measurements may be performed in coloured, turbid or opaque solutions and there is no need of any colour reagents or extended incubation times. The biosensor is a continuous flow system with an automated sample handling. A run is completed within 12 h including sample preparation and measurement of calibrants and 40 samples. The biosensor system has been reported to show good performance in various application areas for quantitative as well as qualitative determination in food, e.g antibiotics (Mellgren *et al.*, 1996; Crooks *et al.*, 1998; Mellgren and Sternesjo, 1998,), mycotoxin (van de Gaar *et al.*, 1997), pesticide (Wagner *et al.*, 1995) and food-borne pathogens (Haines and Patel, 1995) but this biosensor based method was still on the validation stage for the quantification of folic acid in fortified food, in terms of accuracy and precision.

In the case of blood tissue, traditionally, serum and erythrocyte folate are analysed by the microbiological assay (Chanarin, 1979). Because of the original technical difficulty of such assays, laboratories adopted/ utilised radiometric competitive binding assay (radioassays) as they became available. It was, however, subsequently found that these newer assays were fraught with problems, particularly with respect to erythrocyte folate estimates (McGown *et al.*, 1978). These difficulties are said to have been corrected in the version of the radioassay, and it was believed that both assays are measure similar folates. Radioassay procedures for vitamin B12 and FA were replaced again by microbiological assay (MBA), an assay that has been used for many years to estimate the concentration of folic acid and its derivatives (Scott *et al.*, 1974; Grosswicz, *et al.*, 1981), MBA is time consuming and subjected to interference (Gunter *et al.*, 1996). Although the radioassay has been used (Dunn and Foster, 1973; Waxman and Scheiber, 1973), their applicability for measuring folate in tissue has been questioned. Shane *et al.* (1980) concluded that radioassays were not suitable for determination of the mixture of folate derivatives normally encountered in biological extracts, and they

suggested that the microbiological assay was preferable as considered up to today. Using a differential microbiological assay method, Colman *et al.* (1975) detected FA in sera of subjects given folic acid in a number of foodstuffs as well as in aqueous solution. The differential microbiological assay, however, showed lacked of sensitivity as well as being cumbersome to standardized and perform.

Most laboratories "estimate' the relative folate content of a tissue or blood sample using a radioisotope dilution competitive binding assay (Gilors and Dunbar, 1987; Lindenbaum and Allen, 1995). Methods that employ the principle of competitive binding for assay of folates using limiting amounts of folate-binding protein are prone to inaccuracy because folate exists in tissue as many as eight different coenzyme forms with different polyglutamate chain length resulting in differences in affinities for folate-binding protein (Gilors and Dunbar, 1987). In addition, radioisotope dilution assays, while sensitive, did not discriminate between unmetabolised FA and other circulating folates in serum. Radiometric procedure are reported to have been used mainly to determine folates in blood, although they are not suitable for the analysis of complex materials because of the varying affinity of different forms of folate towards the binding protein (Waxman *et al.*, 1972; Gregory *et al.*, 1982).

O'Sullivan *et al.* (1992) reported that although radioimmunoassay methods have been developed, they have not been widely used due to difficulties in preparing appropriate antibodies. 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. Das Sarmata *et al.*, (1995) demonstrated the efficiency of e-aminocaproic acid modified Bovine Serum Albumin (BSA) as a carrier protein for raising highly specific antibody against

FA. Development of a reliable and sensitive immunoassay for serum FA, however, requires solution of several other analytical problems. An alternative to the microbiological assays is liquid chromatography (LC).

This technique has been used by numerous researches to separate folates in standards, blood, tissue, and foods. Most sample preparations, however, fail to extract quantitative (95%) levels of FA from foods. The most common extraction involved heating the mixture of sample and buffered antioxidant solution. This procedure is often used to extract folate from milk and infant formula (Selhub, 1989; Day and Gregory, 1981; Gregory *et al.*, 1984; Holt *et al.*, 1988; Engelhardlt, 1988). Engelhardt (1988) reported that less than 80% of added FA is recovered from food when a single extraction step is used.

Separation and quantification of FA and other folates derivatives in food have been reported by Hawkes and Villota (1989). Published methods also include those of Muller (1993) Vahteristo *et al.* (1996) and Pfeiffer *et al.* (1997) which appear to work well for quantification of low levels of native and added folates in foods. These procedure, however, use lengthy multi-enzyme extraction to release bound native folate and convert polyglutamates to monoglutamate forms, extensive sample clean-up and concentration using ion-exchange or affinity column and gradient elution chromatography profile.

Wilson and Horne (1984) reported that central to the understanding of cellular folate metabolism in mammalian tissue is a reliable method for quantitating the various folate derivatives. They reported that such a method would allow investigators to quantitatively examine the effects of various metabolic states (e.g. folate or vitamin B_{12} deficiency) or chemicals (e.g. ethanol, anticonvulsants and chemotherapeutic drugs) on tissue folate

metabolism. The first studies on the growth tissues depended on the differential growth requirements of *Lactobacillus casei, Streptococcus faecalis* and *Pediococcus ceevisiae* (Bird *et al.,* 1969). Subsequently, these procedures were improved by prior separation of extracts by column chromatography (Davidson *et al.*, 1975; Brody *et al.*, 1976; Ratanasthien, *et al.*, 1974) or parallel identification by thin-layer chromatography (Rao and Noronha, 1978). Such procedures are reported to suffer from poor reproducibility and resolution and are time consuming. They were replaced by high performance liquid chromatography (HPLC) which obviates many of these problems and, in most cases, yields greatly improved resolution (Gregory *et al.*, 1984; Duch *et al.*, 1983; Day and Gregory., 1981).

Conventional chromatographic procedures such as thin-layer (Copenhaver and O'Brien, 1969; Scott, 1980) and column chromatography (Buehling *et al.*, 1974; Kas and Cerna, 1976) need a lot of time and are often less reproducible and reliable. These procedures were also replaced by the HPLC which is more rapid and in many instances yields a better resolution. Since 1976, numerous HPLC methods have been proposed (Reed and Archer, 1976; Allen and Newman, 1980; Gregory 111 *et al.*, 1984; Gounelle *et al.*, 1989), but all the systems lack some properties necessary for their application to biological materials. Either they do not allow the separation of all important forms of folates (Chapman *et al.*, 1978; Branfman and McComish, 1978; McMartin *et al.*, 1981; Reingold and Picciano, 1982) or they are not sensitive and specific enough for direct determination of folacins (Reed and Archer, 1976; Allen and Newman, 1980). For this reason, they often needed prechromatographic steps to clean and concentrate the sample (Duch *et al.*, 1983; Gregory 111 *et al.*, 1984; Gounelle *et al.*, 1989) or require microbiological quantification of the collected fractions (McMartin *et al.*, 1981; Wison and Horne, 1984; Kashani and Cooper, 1985;).

High performance liquid chromatography (HPLC) methods that have been developed for analysing folate (Day and Gregory, 1981) generally use C-18 column and reverse phase conditions in combination with ion-pair or ion suppression techniques (Meyer, 1994). The addition of an ion-pair reagent complicates the mobile phase and interferes with the absorption/fluoresce spectrum of the eluting analyte, thus making positive identification difficult. In the past, ion-suppression conditions for folate molecules (pH2.2) gradually degraded the silica of the column, which changed their performance characteristic and nullified the use of automatic peak picking routine on computer-controlled HPLC systems. Birmingham and Greene (1983) reported the use of an HPLC assay employing electrochemical detection suitable for the routine analysis of folate. The assay designed was free from interference from FA, dihydrofolic acid, 5-methyltetrahydrofolic, and methotrexate. This assay has been applied to the quantification of folinic acid in human serum. The disadvantage was that only folinic acid was reported to be successfully detected and in many instances sample preparation required sample derivatization which can lead to degradation of the analyte.

Liisa *et al*, (1997) reported that in foods the distribution of folates is not well known and up-to date food composition data based on modern analytical techniques are needed to estimate the daily folate intake from the diet. The influence of folate distribution of folate stability or bioavailability is still unclear due to the number of methods that have been attempted in the analysis of different forms FA both in foods and human fluid.The attempt by many investigators to separate many folate forms simultaneous in biological samples using HPLC still seems in place up to this day. For example, the reported use of C-18 column and reverse phase conditions in combination with ion-pair or ion suppression techniques by most researchers which said some the procedure employed gradually degraded the silica of the column, which changed their performance characteristic and nullified the use of automatic

peak picking routine on computer-controlled HPLC systems. Such a problem indicates the need to further optimise the method.

It is clear that, despite the effort devoted to developing methods with better performance, there is still a problem. Many methods are time–consuming and the precision achieved may be strongly dependent on the operation of a skilled person. The development and validation of rapid procedures such as HPLC were reported to be in the developmental stages in 1993 (Finglas *et al.*, 1993). Despite progress in the development of HPLC methods the official methods that are currently used for compliance purposes are still microbiological assays. Therefore, there is still a need to optimise a method that is rapid, sensitive and specific, especially since maize meal food is being fortified with folic acid. Maize meal is the most stable food item for most population. There is really a need for a method that will minimise time from sample preparation to HPLC analysis and lessen the amount of FA loss during preparation.

CHAPTER 3

OPTIMISATION OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR FOLIC ACID ANALYSIS

3.1 INTRODUCTION

The goal of high performance liquid chromatography is to separate components of interest within the reasonable period of time into separate bands of peaks as they migrate through the column (Wilson and Walker, 1994). This is achieved by using high pressure to drive the analyte in solution through a packed chromatographic column, causing separation. The analyte under test can then, be identified and quantified using appropriate methods. The major components of the HPLC system include: the mobile phase reservoir, the pump, sample injector, separating column, detector and recorder (Figure 3.1).

3.1.1 Resolution

The identification and quantification of an analyte depends mainly on the performance of the column, the type of the mobile phase used and physio-chemical nature of the analyte. The success of HPLC is also measured by its ability to separate completely (resolve) one analyte from a mixture of similar compounds (Wilson and Walker, 1994). Peak resolution (Rs) is related to the properties of the peaks such that : $Rs = 2\ (t_{RB} - t_{RA}) / W_A + W_B$, where t_{RA} and t_{RB} are the retention times of compound A and B respectively, and W_A and W_B are the base peaks for A and B, respectively.

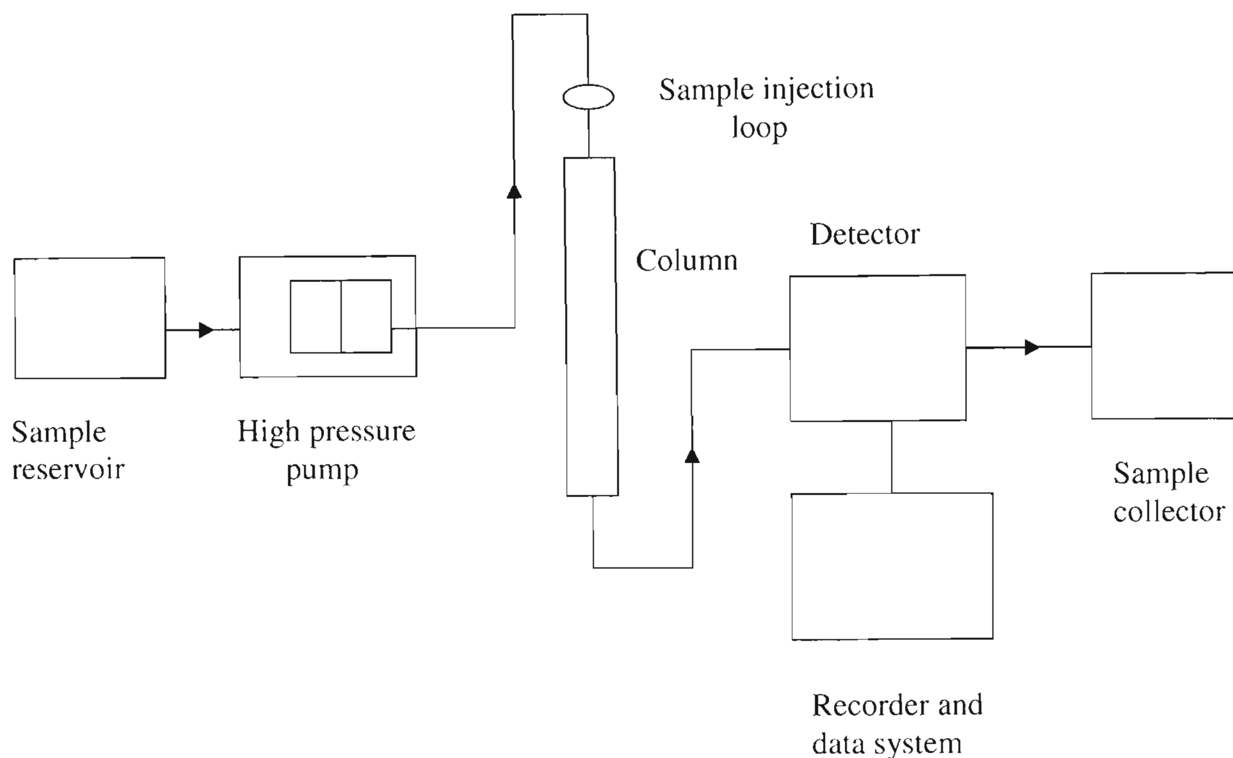

Figure 3.1: The diagram of an HPLC system (Wilson and Walker, 1994)

The Rs value of 1.0 corresponding to 98%, is usually adequate for quantitative analysis (Wilson and Walker, 1994). A chromatography column is considered to consist of a number of adjacent zones in each of which there is sufficient space for the solute to achieve complete equilibration between the mobile phase and stationary phase. Each zone is called a theoretical plate and its length in the column is called the plate height (H), which has dimensions of length. The more efficient the column, the greater the number of theoretical plates that are involved. The number of theoretical plates (N) involved in the elution of a particular analyte is given by: $N = 16\,(t_R / W)^2$

The plate number can be increased by increasing the column length (L) to a limit since length increases with peak broadening. The plate height (height equivalent to a theoretical plate, HETP) is a parameter which is useful for comparative purposes such as operating the column under different conditions. It is expressed as:

$$HETP = L/N = H$$

The maximum number of peaks that can be separated by a specific chromatographic system is called peak capacity (n) and is expressed as:

$$N = 1 + \sqrt{N}/16 \, (\ln V\omega/V\alpha)$$

$V\omega$ and $V\alpha$, are the first and the last peaks, respectively. Peak capacity can be increased by performing a gradient elution. The other major functions necessary for good resolution in a chromatographic system include: selectivity, the ability of the system to discriminate between structurally related compounds, and efficiency, a measure of the diffusion effects that occur in the column to cause peak broadening and overlap. Basically a separation process is considered to be more efficient if the peaks are well separated both at the apices and at the base. In quantitative terms, separation is expressed by two parameters, i.e. 1. Relative retention, t_R (or the separation factor, α) and 2. Resolution, Rs. Relative retention is indicative of the degree of separation at the apices ($\alpha = t_{R1}/t_{R2}$).

Resolution is defined as the difference in retention times of the two peaks divided by the average peak width at the base, expressed in time units:

$$Rs = t_{R2} - t_{R1}/0.2 \, (W_{b1} + W_{b2})$$

Where the subscripts refer to two adjacent peaks, 1 and 2. Since normally, W_{b1} is approximately equal to W_{b2} .

$$Rs = t_{R2} - t_{R1}/ W_b$$

Resolution therefore offers a more accurate description of the quality of separation. The chromatographic separation can be optimised if the relationships between the various interdependent factors such as resolution, capacity factor, relative retention and plate number are understood. The number of theoretical plates (N) involved in the elution of a particular analyte is give by:

$N = 16 (t_R/W)^2$. The number of plates can be increased by increasing the column length (L) to a limit since length increases with peak broadening (Scott, 1977).

3.1.2 Liquid chromatography separation modes

The principle of adsorption chromatography is known from classical column and thin-layer chromatography. A relatively polar material with a high specific surface area is used as the stationary phase, silica being the most popular, but alumina and magnesium oxide are often used. The mobile phase is relatively non-polar (heptane to tetrahydrofuran). The different degree to which various types of molecules in the mixture are adsorbed on the stationary phase provide the separation effect. A non-polar solvent such as hexane elutes more slowly than a medium-polar solvent such as ether. Meyer's rule of thumb is that polar compounds are eluted later than non-polar compounds (Meyer, 1994). Polar means water-soluble and hydrophilic; non-polar is fat-soluble and lipophilic.

3.1.2.1 Ion-exchange chromatography

The stationary phase contains ionic groups (e.g. NR_3^+ or SO_3^-) which interact with the ionic groups of the sample molecules. The method is suitable for separting, e.g amino acids, ionic metabolic products and organic ions (Meyer, 1994).

3.1.2.2 Ion-pair chromatography

Ion-pair chromatography may also be used for the separation of ionic compounds and overcomes certain problems inherent in the ion-exchange method. Ionic sample molecules are 'masked' by a suitable counter ion. The main advantage are, firstly the widely available reversed- phased system can be used, so no ion exchange is needed, and, secondly, acids, bases and neutral products can be analysed simultaneously.

3.1.2.3 Ion chromatography

Ion chromatography was developed as a means of separating ions of strong acids and bases (e.g. Cl^-, NO_3^-, Na^+, K^+). It is a special case of ion-exchange chromatography but the equipment used is different (Meyer, 1994).

3.1.2.4 Normal phase

Adsorption chromatography is usually achieved with non-derivatised silica packing materials. Retention of the analytes is primarily caused by the interaction of polar groups with the silanol on the silica surface. Adsorption chromatography is used for analytes with medium polarity that are soluble in organic solvents. It is not suitable for polar analytes, because these analytes will interact with the silica surface, resulting in poorly shaped peaks or even no elution at all. Variations in separation with silica gels from different manufacturers are caused by differences in shape and pore size, particle size, surface area or types of silanol groups (**http://www.varianinc.com/cgi-bin/nav?products/consum/lccolumns/normal&cid, accessed in May 23, 2005).**

3.1.2. 5 Reverse-phase chromatography

In reverse-phase systems, water cannot wet the non-polar (hydrophobic = water-repellent) alkyl groups and does not interact with them. Hence, it is the weakest mobile phase and gives the slowest sample elution rate. The greater the amount of water in the eluent, the longer the retention time. Sample compounds are better retained by the reverse-phase surface, the less water-soluble (i.e. the more non-polar) they are. The retention decreases in the following order: aliphatics > induced dipole e.g (CCl_4) > permanent dipole e.g. ($CHCl_3$) > weak Lewis bases (ethers, aldehydes, ketones) > strong Lewis bases (amines) > weak Lewis acids (Alcohols, phenols) > strong Lewis acids (carboxylic acids). Also, the retention time increases as the number of carbon atoms increase (Parris, 1984).

Temperature and the nature of the mobile phase are considered the most important during optimisation. When changing temperature, more frequently all molecules will show a similar decrease in retention time or elution volume with increasing temperature, making it less attractive to use temperature programming to improve a separation. Controlling temperature is important in improving the reproducibility of the separation.

In liquid chromatography, the retention characteristics of sample components within a given column are extremely dependent on the chemical composition nature of the mobile phase. A small change in the composition of the liquid mobile phase can cause a drastic change in the sample retention. This represents the most powerful parameter available to the liquid chromatographer who wishes to develop and optimise the separation of chemical mixtures. Important factors to be considered when deciding to change the mobile phase are polarity, pH and/or ionic strength. This is the principle used most in gradient elution (Parris, 1994).

3.1.3 Principle of detection

3.1.3.1 Fluorescence detection

According to Scott (1977), fluorescence is a specific type of luminescence in which molecules are excited by electromagnetic radiation to produce luminescence (termed photoluminescence). If the release of electromagnetic energy is immediate or stops on the removal of the exciting radiation, the substance is said to be fluorescent. Fluorescence, however, has been shown to be extremely effective and detectors based on fluorescence measurement have provided the highest sensitivities available. If electrons are raised, due to absorption of energy, to an upper excited singlet states then such transitions are responsible for the characteristic visible or ultraviolet (UV) absorption spectra observed for such compounds. If the excess energy is not dissipated rapidly by collision with other molecules or by other means, the electron will return to the ground state with the emission of energy in the form of electromagnetic radiation. This effect is called fluorescence. As some energy is always lost before emission occurs, the emitted fluorescent energy is always of longer wavelength than the absorbed or exciting radiation (Scott, 1977).

3.1.3.2. Ultraviolet (UV) detection

Ultraviolet (UV) absorption detectors respond only to those substances that absorb UV light. A great many compounds fall into this category, including all substances having one or more double bonds (π electrons) and substances having unshared (non-bonded) electrons, e.g. all olefins, all aromatic compounds and containing $> C = O$, $C = S$, $-N = O$, $-N = N$ (Scott, 1977). Derivatives of folate differ in the substitution at N^5 and N^{10} position, the state of oxidation and the number of glutamic acid residues (Stokstad and Koch, 1967). Folic acid and folinic acid are both aromatic compounds. When the bond is N-X, X = H that is folic acid, when X= CHO that is a 10-formylfolate or 5-formylfolate (folinic acid).

The aim of the study is to optimise a method for folic acid and folinic acid analyses in human serum using either fluorescence or UV detection on reversed phase HPLC. Reserved phase HPLC will be the method choice because folate compounds are completely soluble in buffer solution. The bioavailability of a FA nutrient cannot be assessed without accurate analytical data. Better analytical methods are therefore needed for the measurement of total folate in maize meal, which include naturally occurring folates and added folic acid. In addition, rapid, sensitive and less costly method is needed for FA assessment which in turn can lead in improvements of food composition data, and can subsequently be implemented for routine analyses.

Over the last few years, a number of HPLC procedures for the separation and quantification of folic acid have been reported usually based on anion exchange (Reed and Archer, 1976; Chapman *et al.*, 1978), ion-pair reverse phase HPLC (Reingold and Picciano, 1982; Kashani and Cooper, 1985) or conventional reserved phase HPLC (Gounelle *et al.*, 1989; Day and Gregory, 1981). None of these methods allows the separation of all FA. Many previously described chromatography systems could not even separate some important folates, e.g 5-CH_3-THF and 5-CHO-THF (Stout et al.,1976 ; Clifford and Clifford, 1977), Pteroylglutamic acid (PteGlu) and dihydrofolic acid (DHF) or 5-CH_3-THF and PteGlu (McMartin *et al.*,1981; Briggs *et al.*,1982). The main aim of this study is to develop a method that will be specific for FA and its derivatives detection since folic acid is considered the most important form of folate and is used mostly in fortification of foods and also as supplements.

3.2 METHODS AND MATERIALS

3.2.1 Reagents

The following materials and reagents were used in this study: folic acid (pteroylglutamic acid) and folinic acid (calcium salt) were both obtained from Sigma (St Louis, USA),

ascorbic acid, sodium ascorbate, dipotassium hydrogen phosphate (K_2HPO_4), phosphoric acid , 2-mercaptoethanol, methanol and acetonitrile were obtained from Merck (Darmstadt, Germany). All solvents were of HPLC-grade, unless otherwise stated. Chemically pure water was further filtered through a 0.45 μm filter and used to prepare buffer solution, solution was filtered again. Solvents (methanol, acetonitrile) and buffer solution were also degassed by sonication before being pumped into the HPLC system.

3.2.2 Preparation of samples and standards

3.2.2.1 Precaution

In order to avoid cross-contamination, all glassware was thoroughly cleaned. Pipettes were allowed to stand in a detergent for 45 minutes or more, washed with tap water, rinsed thoroughly with distilled water and finally dried with acetone. Flasks, beakers and measuring cylinders were soaked in 20% chromic-sulphuric acid fluid, washed well with tap water and finally rinsed thoroughly with distilled water. Glassware could also be kept under ultraviolet light, for photolytic degradation of FA since folic acid tends to stick to the walls of glassware thus resulting in false reading values of FA concentration (Stokstad *et al.*, 1947). Folic acid and folinic acid compounds were always handled in dim light conditions as these compounds are light degradable.

3.2.2.2 Folic acid and folinic acid standard

The method adapted from Masahiro and Kazuo (1986) was used for preparation of folic acid and folinic acid standard , in which folic acid (0.01g), folinic acid (0.01 g) were dissolved in a 100 ml of 0.2 M 2-mercaptoethanol containing 0.1% sodium ascorbate to protect it from oxidative decomposition. They were frozen at – 20 ^{0}C and protected from light until use. The

method adapted from Liisa *et al.* (1996) was also used as a second option for preparation of the standard solution. Folic acid (0.01g) and folinic acid (0.01g) each were separately dissolved in 100 ml of 0.1 M phosphate buffer (pH 7.0 adjusted using phosphoric acid) containing 0.1% (v/v) 2-mercaptoethanol for determination of concentration because these compounds are very unstable without added (2-mercaptoethanol) antioxidant (Blakley, 1969). The rapid degradation would cause shifts in absorbance maxima and give false low results for the purity determination. Standard solutions were then flushed with nitrogen and stored in a freezer (-70 ^{0}C) until required. Internal standard of salicylic acid compound was dissolved in the similar composition of 100 ml buffer solution as FA and folinic acid, according to the method adapted from Liisa *et al.* (1996).

3.2.2.3 Extraction for spiked serum samples with folic cid and folinic acid standards

The extraction method used was adapted from Patrick *et al.* (1996). Human serum sample from one individual was divided into 8 x 1.5 ml Eppendorf, 1 ml of a serum sample from each Eppendorf was spiked with 10 μg/ml of FA standard, other four set of serum sample 1 ml of serum was spiked with 10 μg/ml folinic acid and last set of serum was spiked with salicylic acid (10 μg/ml). All samples were deproteinised by addition of 100 μl (60% v/v) of perchloric acid. The samples were then centrifuged and supernatant was removed to a second clean microfuge tube; 6 M of potassium hydroxide was added to the samples to bring the pH to neutral. The samples were rapidly frozen (-60 ^{0}C) until required.

3.2.2.4 High performance liquid chromatography analysis

Detection of folic acid and folinic acid was carried out using HPLC (Waters Liquid Chromatograph) equipped with a 600E System controller and 991 photodiode array detector with 991 software. Injections were performed using Gilson 401C dilutor and 231 XL

sampling injector equipped with a Rheodyne 7010 injector having a 20 µl sample loop. The HPLC separation column used was a Luna 5µ C18 (2) reverse phase analytical column (250 x 4.60 mm) from (Separation, South Africa).

3.2.2.5 Chromatographic condition for folic acid and folinic acid determination

The method used was adapted from Patrick *et al.* (1996). In this method isocratic elution was used to separate the component FA and folinic acid. The mobile phase comprised the buffer and methanol in different ratios. The buffer solution comprised of citrate phosphate buffer (0.1 M, pH 4.0) adjusted using phosphoric acid: 1% acetic acid: methanol (43:42:15 v/v/v). The flow rate of 3ml/min was modified to 1ml/min because flow rate of 3ml/min was not suitable for the column. The detection was set at 250 nm and 280 nm. Folic acid and folinic acid standard solutions were filtered through Whatman nylon filters (0.45µm) before being injected into the column. In order to identify the ratio that will give the best resolution the mobile phase was changed to different ratios of 50:50; 60:40; 70:30; 80:20; 90:10 buffer: methanol (v/v). The above chromatographic conditions were also used with fluorescence detection in which the wavelength was set at 360 nm and 460 nm for excitation and emission, respectively.

3.2.2.6 Gradient elution

For this part of the study, the mobile phase used was that described in section 3.2.2.5. Standards were prepared according to a method adapted from Liisa *et al.* (1996) in which folic acid (0.01 g) and folinic acid (0.01g) were separately dissolved in 100ml of phosphate buffer (0.1 M; pH 7.0 adjusted using phosphoric acid) containing 0.1% (v/v) 2-mercaptoethanol. The details of the gradient elution are indicated in Table 3.1. Three were

programmes were used. Programme 1: Mobile phase was initial allowed to run at a ratio of 70:30 (v/v) for 8 minutes and was changed for another 8 minutes to a ratio of 75:25 (v/v) buffer: methanol. The mobile phase ratio of 75:25 (v/v) was kept constant for another 10 minutes. Programme 2 : Mobile phase ratio of 75:25 (v/v) buffer: methanol was initial run for 15 minutes and kept constant at 17 minutes. In programme 3; Mobile phase ratio of 75:25 (v/v) was initial run for 5minutes and kept constant for 7minutes. Other ratios of 50:50; 60:40; 80:20; 90:10 were attempted in the same pattern as indicated in Table 3.1

Table 3.1 gradient elution programme

Programme 1

Initial	Flow	% Buffer	% Methanol	Curve
8 min	1ml/min	70	30	
8 min		75	25	6(Linear gradient)
10 min		75	25	11 (Hold)
Programme 2				
15 min	1ml/min	75	25	6(Linear gradient)
17 min		75	25	11 (Hold)
Programme3				
5 min	1ml/min	75	25	6(Linear gradient)
7 min		75	25	11(Hold)

3.2.2.7 Analysis for the spiked serum sample with folic acid and folinic acid.

Spiked serum samples were analysed under chromatographic conditions described in 3.2.2.5. The mobile phase ratio of 70:30 (v/v) was used. Folic acid and folinic acid standards were always run prior to samples for easy identification of an analyte peak and correct retention. A folic acid percentage recovery on the spiked serum under the ratio of 70:30 (v/v) was calculated using equation:

$$\mathbf{Y = 25.025\,x + 0.7}$$

The equations for folic acid, folinic acid and salicylic acid were obtained by plotting the peak areas versus the concentration of working standards. Concentrations of working standard 1 μg/ml, 2 μg/ml, 5 μg/ml, 10 μg/ml, were used. Y denotes the peak area and X represent the concentration of folic acid (unknown) on the equation. Salicylic acid (Internal standard, 10 μg/ml) was also analysed. The use of an internal standard was to assess the efficiency of the extraction method and separation efficiency. Concentration of salicylic acid in serum was calculated using equation in appendix 5. Y in the equation represents peak area; X represents the concentration of unknown in a serum (i.e. salicylic acid).

3.3 RESULTS AND DISCUSSION

Average recovery of folic acid on the spiked serum sample under the ratio of 70:30 (v/v) mobile phase was 91% - 100% (Table 3.2), whereas, average recovery of salicylic acid (internal standard, 10 μg/ml) on spiked serum was 65 ± 3% as shown in Figure 3.2.

Table 3.2: Standard recovery of folic acid concentration on spiked serum sample at 250nm wavelength, flow rate of 1ml/min and mobile phase ratio of 70:30 (v/v)

Samples	Amount Recovered (μg/ml)	% Recovery
Extraction 1	13.96 ± 0.05	113
Extraction 2	12.32 ± 0.1	94
Extraction 3	13.4 ± 0.08	106
Extraction 4	12.12 ± 1.1	91

Single extraction was done per sample in triplicate. The results confirmed the efficiency of the extraction method and separation method. The chromatogram of serum spiked with folinic acid is shown in Figure 3.2. The chromatogram shows unresolved peaks which led to the difficulties in folinic acid identification.

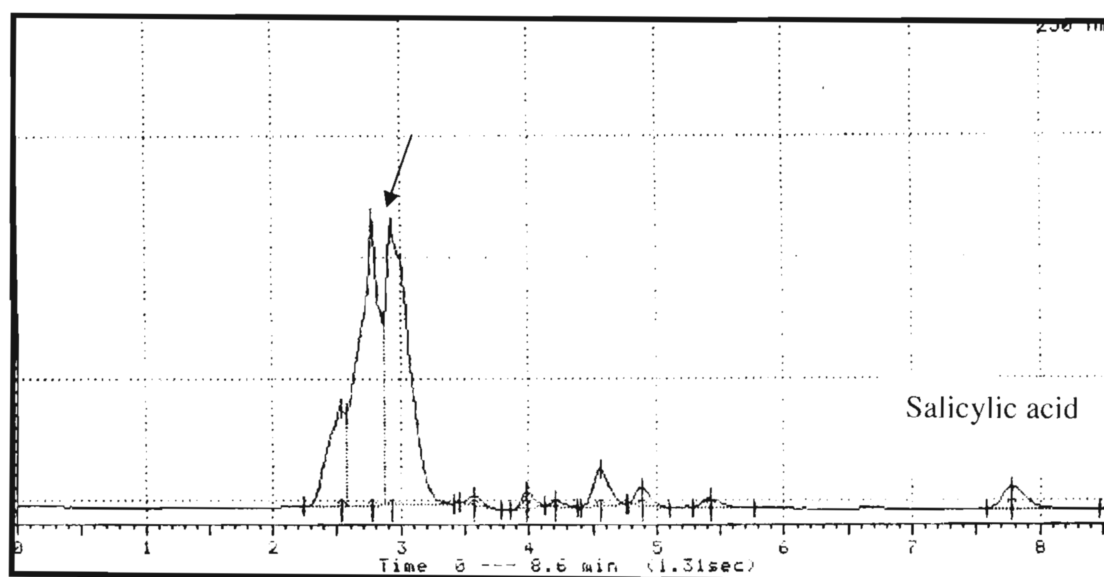

Figure 3.2: HPLC/UV chromatogram for spiked serum with salicylic acid and folinic acid at ratio of 70:30 (v/v) buffer: methanol, flow rate of 1ml/min and wavelength of 250nm. salicylic acid eluted at 7.78min, the arrow on the chromatogram indicates the unresolved peak of folinic acid in serum sample.

Folinic acid standard was mixed with internal standard to check the presence of impurities that could cause interferences which could result in poor separation of folinic acid in serum (Figure 3.3).

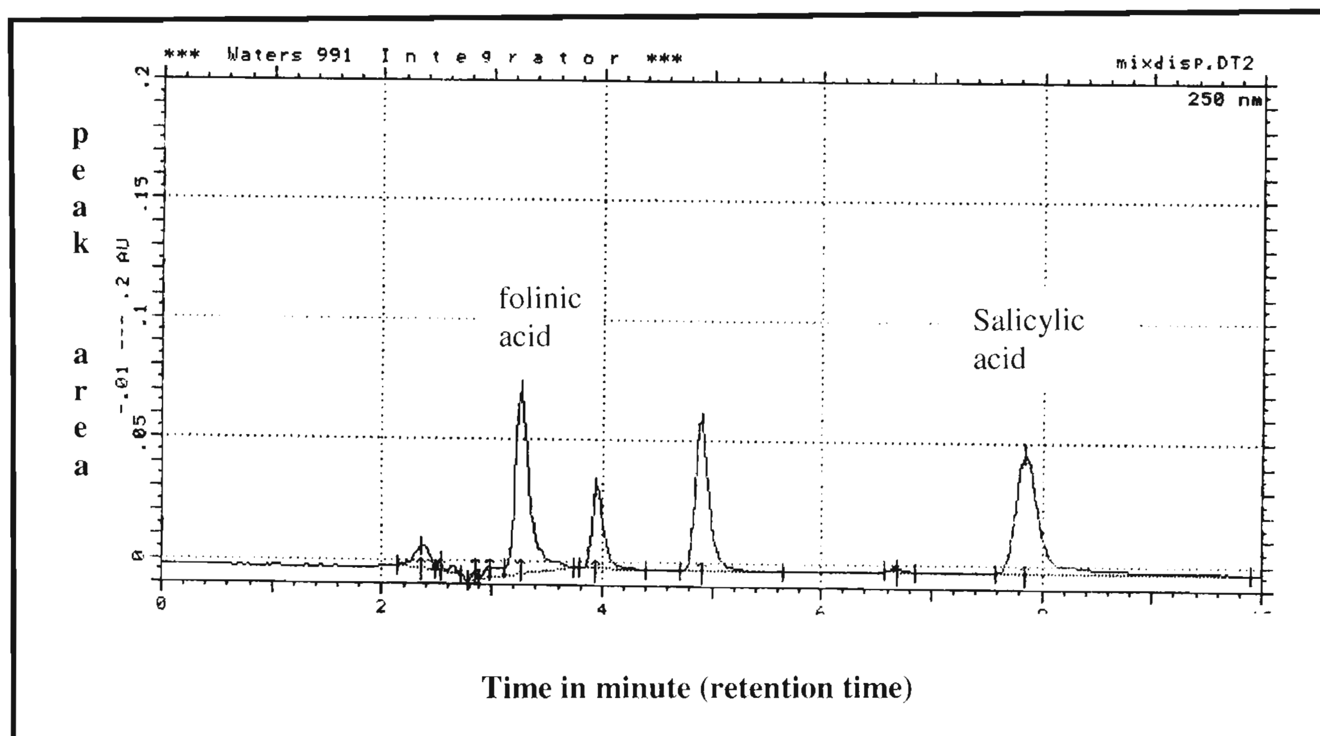

Figure 3.3: HPLC/UV chromatogram shows the separation efficiency between the mixture of folinic acid standard and salicylic acid standard at ratio of 70:30 (v/v) mobile phase, flow rate of 1ml/min and wavelength of 250nm. The first peak at 3.20 minute represents folinic acid and shows no traces of any impurities the last peak at 7.8 represent salicylic acid.

Figure 3.3 confirmed the separation efficiency using the HPLC/UV under the mobile phase that comprised of citrate phosphate buffer (0.1 M, pH 4.0 adjusted using phosphoric acid): 1% acetic acid methanol (43:42:15 v/v) at wavelength of 250 nm at flow rate of 1 ml/min and mobile phase ratio of 70:30. Salicylic acid eluted at 7.8 minutes and folinic acid eluted at 3.20 minutes, whereas the reducing agents peaks eluted at 4 and 4.88 minutes. Salicylic acid peak in the serum eluted at the same time as the standard salicylic acid peak (7.77 min) as expected. Folinic acid in serum sample was expected to elute at 3.20 minute as in Figure 3.3. It seems that analysis of folinic acid in serum would present a problem as in Figure 3.2, although the extraction method and separation proved to be efficiency. The identification of

folic acid and folinic acid was possible with the isocratic elution programme using a ratio of 70:30 (v/v) mobile phase. This ratio (70:30 v/v) gave good separated peak compared to the ratio of 50:50; 60:40; 80:20; 90:10 at maximum absorption of 250 nm wavelength (Figure 3.4).

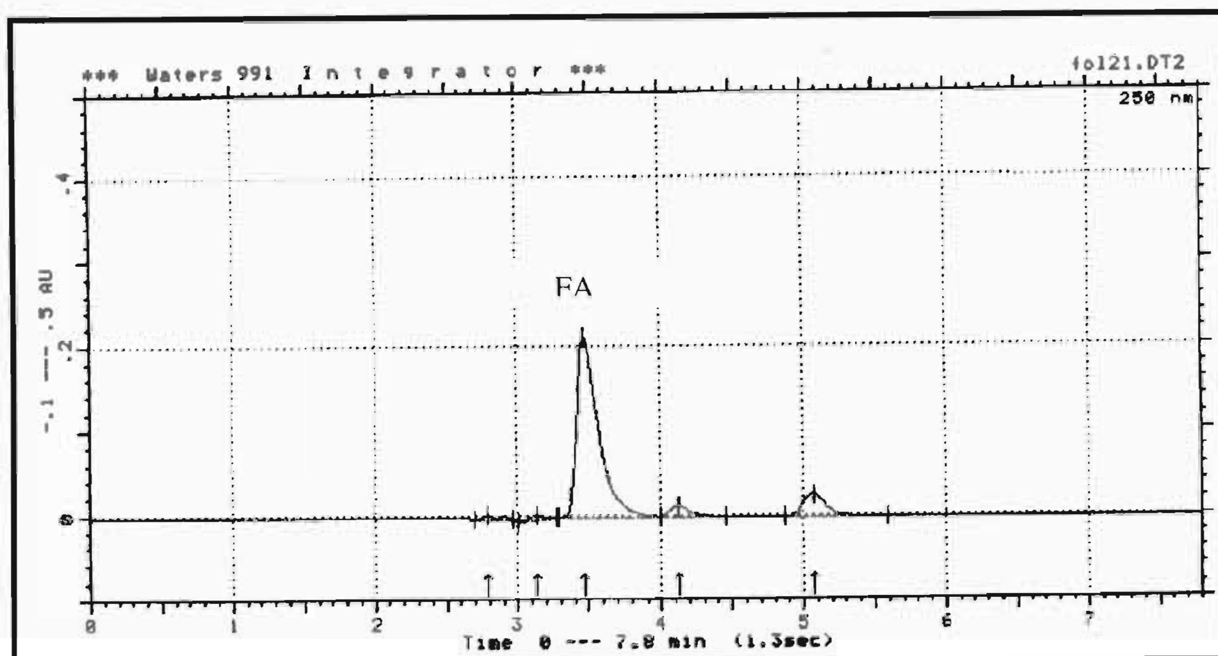

Figure 3.4: good separation of peaks for standard folic acid analysed using HPLC/UV under the ratio of 70:30 (v/v) mobile phase (citrate phosphate: methanol), wavelength of 250nm and flow rate of 1ml/min, the second and third peaks are solvent peaks. The first peak is for folic acid (FA) eluted at 3.48 minutes.

The mobile phase ratio 50:50; 60:40; 80:20; 90:10 were used, they all gave too many noise peaks with difficulties in peak identification of folic acid and folinic acid using HPLC coupled with UV detector at the wavelength of 250 nm. It was noted that the wavelength of 250 nm at the mobile phase ratio of 70:30 (v/v) gave high absorbance peaks over 280nm wavelength using 70:30 v/v. The use of 280 nm in other ratios 50:50; 60:40; 80:20; 90:10 also gave many noise peaks, wavelength of 280nm was therefore not considered. Mobile phase ratio 80:20 (v/v) gave two good separation peaks, it was therefore not considered as a good

ratio because of peak interference. The first peak interfered with the second peak and resulted in two peaks instead of three peaks (Figure 3.5).

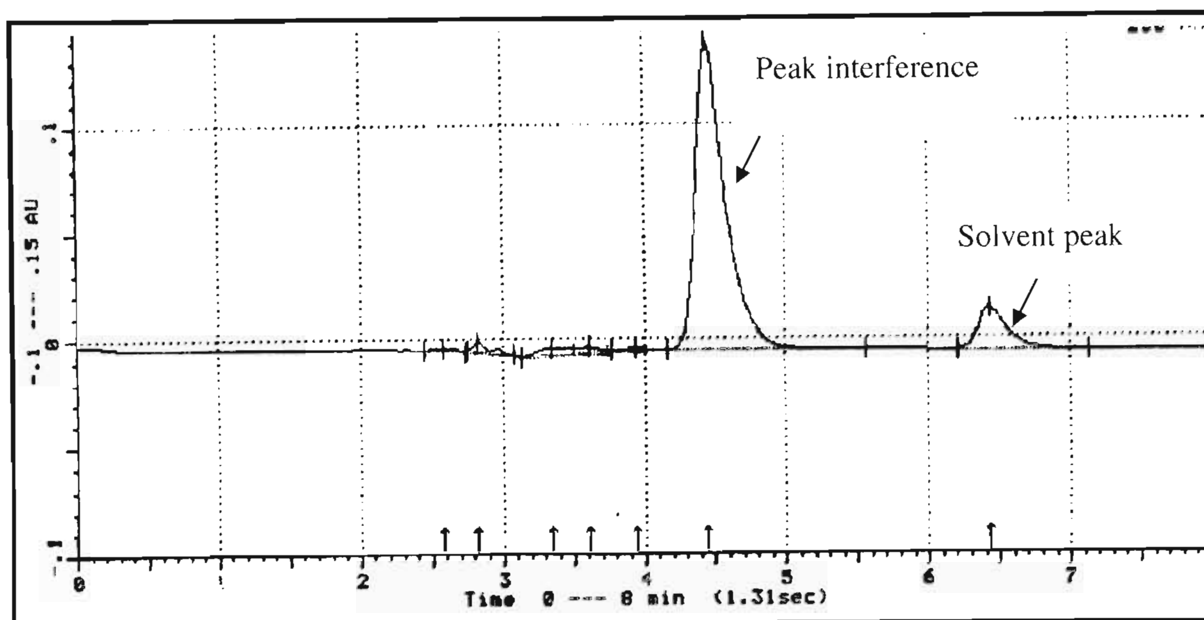

Figure 3.5: Is the typical HPLC chromatogram for folic acid standard at the ratio of 80: 20 (v/v) mobile phase, at a λ of 250nm, flow rate of 1ml/min, chromatography shows significant peak - interference of folic acid peak with first peak of solvent peaks under ratio of 80: 20 (v/v).

Folinic acid standard run under the same condition as folic acid at ratio of 70:30 v/v mobile phase also gave three good separated peaks with the retention time of the first peak at 3.17 min, second peak at 4.12 minute and third peak at 5.02 minute. The different in the elution time of folic acid peak at 3.40 minutes and that of folinic acid at 3.17 minutes at maximum absorption of 250 nm wavelength was noted. The peak for the compound of interest was confirmed by running the blank prior to pure solution of standard folic and folinic acid. The blank run did not show identifiable amount of any folic acid or folinic acid traces. Only two peaks were observed from blank (solvent) chromatogram. The concentration of 100 μg/ml and 10 μg/ml standard was also pumped into the HPLC column to compare the absorbance strength between the highest and lower concentration (10 μg/ml). Comparison of the peak area for 10 μg/ml (0.004459 AU/min) was ten times less than 100 μg/ml (0.044389 AU/min) peak area as expected.

Calibration curves (Appendix 3 and 4) of folic acid and folinic acid showed a linear response (R^2 = 0.99) of peak area versus concentration of folic acid and folinic acid under the ratio of 70:30 mobile phase at wavelength of 250 nm, flow rate of 1ml/min. The detection limit was 1μg/ml for both compounds. Although it was easy to identify folic acid and folinic acid when run separately under the ratio of 70:30, but simultaneously identification of these compound resulted in two unseparated peaks (Figure 3.6).

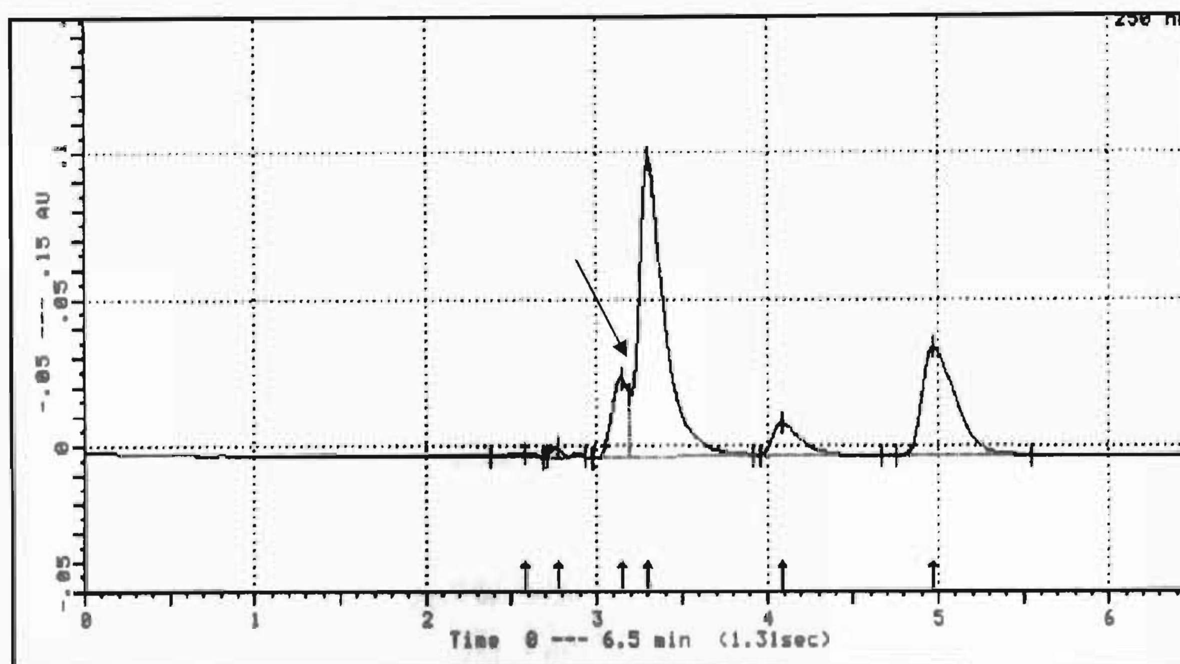

Figure 3.6: Is a HPLC chromatogram for the mixture of standard folic acid and folinic acid separated using the mobile phase ratio of 70:30 at λ= 250nm wavelength, flow rate of 1ml/min,under chromatographic condition described in 3.2.2.5. The unseparated standard of folic acid and folinic acid on the chromatogram are indicated by an arrow, the other two peaks are solvent peaks.

The use of gradient elution under mobile phase ratio 50:50; 60:40; 70:30; 80:20; 90:10 was not efficient and the use of HPLC/FL at a wavelength of 360 nm and 460 nm for excitation and emission was not suitable for separating the mixture of folic acid and folinic acid. The chromatogram mixture of folic acid and folinic acid analysed on HPLC/FL showed a peak

which is similar to the standard folic acid chromatogram in which two peaks were observed at 3.43 minutes and 4.43 minutes. The folinic acid chromatogram showed several peaks with folinic acid peak at 4.68 minutes (Figure 3.7). The problem persisted even after the glassware was soaked and thoroughly washed. Therefore the use of gradient elution in HPLC couple with fluorescence detector was not considered in this study.

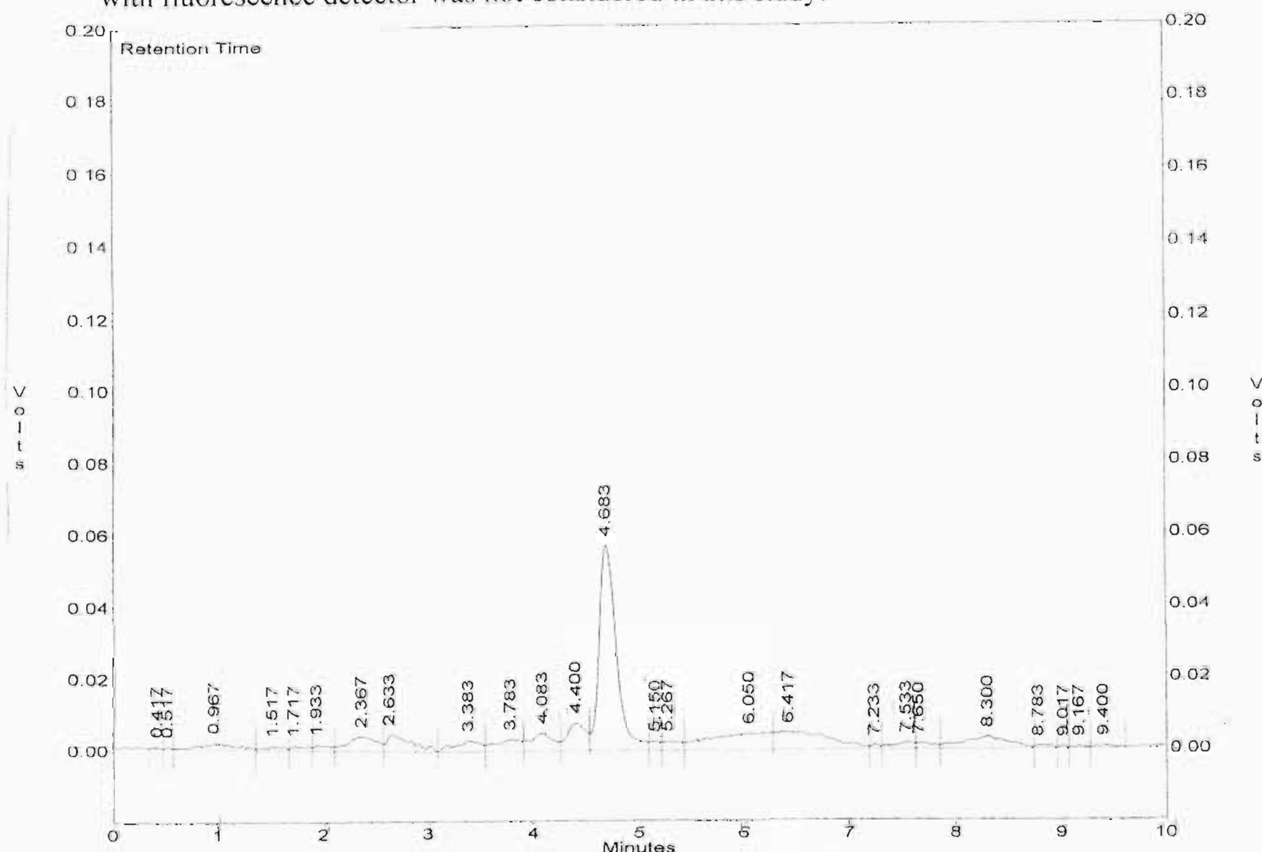

Figure 3.7: Is a HPLC chromatogram for folinic acid standard analysed on the HPLC coupled with fluorescence detector at 360nm for excitation and 460nm for emission wavelength, Folinic acid eluted at 4.68min at a flow rate of 1ml/min.

Although the use of 250 nm wavelength at ratio of 70:30 (v/v) mobile phase in this study was efficiency, there is a need to focus on folinic acid in serum until satisfactory result are achieved because folinic acid is as important as folic acid vitamin. It is also one of the forms of folate that is given as supplements in case of folate deficiency. Stefana *et al.* (1999) reported the detection limit of 0.2 ng per injection for folic acid and 0.8 ng per injection for folinic acid using HPLC/UV at wavelength of 290 nm but did not detect folic acid and folinic

acid using HPLC/FL at 290 nm wavelength. Other folate forms (H_2folate, 5-CH_3-H_4folate, 5-HCO-H_4folate, and 10-HCO-PGA) were detected on both HPLC/UV (290 nm) and HPLC/FL (290 nm and 356 nm) wavelength. Our main aim was to develop a method that will enable identification of both compounds in a sample to minimise cost and time.

Folic acid (FA) constitutes only 1% of dietary folacin (Perry, 1971). It has been the most widely used derivative in absorption studies because of its clinical importance and the availability of the tritium labelled compound. Folic acid has also been used as reference standard in most studies, but there is still little information on the absorption and utilisation of dietary folic acid. This is due to problems for developing a satisfactory method of quantisation of the amount of dietary folic acid absorbed. Extraction method use in this study was efficient as the percentage recovery of folic acid was 91% - 100% although uncertainty still remains with folinic acid, but the suitability of the method can always be evaluated by a series set of data on a similar case of study.

The used of ultraviolet (UV) detector at wavelength of 254 nm, 284 nm, 285 nm, and 280 nm has been reported in which detection limits differed for different wavelength in ng per injection. Hahn *et al*. (1991) reported that the used of fluorimetric detector at 365/450 nm; 295/356; 295/356 gave the lowest detection limits compared to UV detector. During the optimisation process in this study the used of fluorescence detector for the analyses of folinic and folic acid compound gave unsatisfactory results as compared UV detector. Fluorescence detector has a disadvantage that sometimes sample need derivatisation which could lead to some errors as folates compound are sensitive to heat and exposure to light, although the lowest detection limit can be reached. Therefore the study of folic acid is interesting but also complicated because analysis of folic acid has been reported by different investigators using

different numbers of wavelength. Standard preparation of folic acid and folinic acid according to method adapted from Masahiro and Kazuo, (1986) was not efficient under HPLC/UV.

3.4 CONCLUSION

Identification of folic acid and folinic acid is possible with isocratic elution at 250 nm wavelength at a flow rate of 1 ml/min. The mobile phase composition of Buffer: Methanol resulted in the early elution (between 3 to 3.5 minute) of the compounds folic acid and folinic acid which saves analysis time. Although analysis of folinic acid on spiked serum sample presented a problem because of unseparated peaks observed and the impossibility to simultaneous separates folic acid and folinic acid. Such a problem indicates that, these is a continues study where more effort is needed to consider factors such as wavelength, detection limit and improvement on the extraction method that would allow not only easy identification of folic acid but also of folinic acid. The results obtained on optimisation can lead to the useful information about folic acid analysis using HPLC and it utilisation of folic acid in our body.

CHAPTER 4

DETERMINATION AND QUANTIFICATION OF FOLIC ACID IN BLOOD SERUM USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV DETECTION AND IMMUNOASSAY WITH CHEMILUMINESCE

4.1 INTRODUCTION

Folates exist in tissues in at least eight different coenzyme forms (Brody *et al.*, 1984). Normally, folate in our diet is in the form of folic acid, found primary in organ meat, whole grain and green leafy vegetables. The various naturally occurring forms of folic acid (pterolymonoglutamic acid, PGA) function as essential coenzymes in the synthesis of protein and nucleic acid (Baugh *et al.*, 1971). Daily requirements depend on metabolic and cell turnover rate (Herbert and Colman 1988). The estimated safe and adequate daily dietary intakes for adult men is 200 mg and for women 180 mg (NRC, 1989). Folic acid deficiencies lead to impaired cell division and megaloblastic anaemia (Nelson and Davey, 1991).

Humans are unable to synthesise folic acid, but posseses 15 enzymes that carry out transformation of preformed folic acid. Folic acid is reduced in two steps by dihydrofolate reductase to the coenzyme THF, which is subsequently involved in the cyclic metabolism of C-1 fragments (Olson and Doisy, 1981). In serum and tissue, folic acid is present in several metabolic forms which differ in the functional groups, oxidation state and the number of conjugated glutamic acid residues present among which folic acid is only a minor constituent (Butterworth, 1993). The derivatives differ in the substitution at N5 and N10 position, the state of oxidation and the number of glutamic acid residues (Stokstad and Koch, 1967). For analysis of folate some laboratories are currently using chemiluminescence: Access Immunoassay System. This method offers great performance, reliability and efficiency. The principle of chemiluminescence method occurs when a chemical reaction causes a susceptible

molecule to enter an unstable excited state and then return to a stable form with the emission of light. Although fairly wide range of analytical procedures has been developed for the determination of folates in food and biological sample (Santhosh-Kumar *et al.*, 1995; Stokes and Webb, 1999; Dueker *et al.*, 2000), including highly specific HPLC separation techniques coupled with UV and/or electrochemical detection (Seyoum and Selhub, 1993; Bagley and Selhub, 2000), the microbiologic assay using *Lactobacillus casie* is still considered by some investigators to be golden standard method for folate measurement. It has been used routinely to determine the total folate concentration in different matrices (Wright and Phillips, 1985; Gunter *et al.*,1996; Pfeiffer *et al.*, 2001).

Microbiological assay results, however, vary depending on the organism used the presence of antifolate drugs and/or antibiotics in the specimen (Tamura, 1990). Other typical methods for measuring serum folate in clinical laboratories are variations of immunoassays or competitive protein binding assays. Although sensitive these methods lack specificity to distinguish between different forms of folate and they have poor reproducibility (Gunter *et al.*, 1996; Pfeiffer *et al.* 2001). The use of HPLC with either fluorescence or electrochemical detector has been reported (Gregory *et al.*, 1984). Unfortunately, folic acid does not fluorescence sufficiently to allow physiological levels of the vitamin form to be detected. Currently, scarce information is available on the determination of serum folic acid using HPLC coupled with UV. The study therefore aimed to specifically detect and quantify folic acid and its derivatives in human serum and compared the method with a standard immunoassay method. Serum samples of cancer patients and those with non-cancer diseases were used to test the efficiency of the method that was optimized in chapter 3.

4.2 METHOD AND MATERIALS

4.2.1 Sample Collection

The serum samples were previously collected from cancer patients and non-cancer who were hospitalized in King Edward Hospital. Consent and ethical approval for folate analysis was obtained (Ref: H 140/2000). Blood from control (individuals with no known illness) participants was drawn by qualified medical personnel. All patient and individuals with no known illness were above 18 years. Patients were not matched for race, age because the study was not investigating the role of folic acid in cancer, rather to develop a method that will detect folic acid in biological samples.

4.2.2 Reagents and equipment

All solvent were of HPLC-grade, unless otherwise stated. Deionised water was filtering purified through a 0.45 µm filter and used for buffer solution, after which the solution was filtered again. Folates Compounds: Folic acid (pteroylglutamic acid), folinic acid (calcium salt), ascorbic acid, sodium ascorbate, dipotassium hydrogen phosphate (K_2HPO_4) were all purchased from Sigma (St Louis, USA). Phosphoric acid and 2-mercaptoethanol, perchloric acid, methanol, acetonitrile were all obtained Merck (Darmstadt, Germany). Solvent (methanol, acetonitrile) and buffer solution were degassed by sonication before being pumped into the HPLC. Quantisation of folic acid and folinic acid was carried out by HPLC (Waters Liquid Chromatograph) equipped with a 600E System controller and 991 photodiode Array Detector combine with 991 software. Injections were performed using Gilson 401C dilutor and 231 XL sampling injector equipped with a Rheodyne 7010 injector with a 20 µl sample loop. The HPLC column used was a Luna 5 µm C18 (2) reverse phase analytical column (250 x 4.60mm) from Phenomenex (Separation, South Africa).

4.2.3 Sample preparation (adapted from Patrick *et al.*, 1996)

Stored serum samples from -70 °C degrees freezer was thawed to room temperature then 1ml aliquots of that sera were transferred into 5ml microfuge tubes. The samples were deproteinised by addition of 100 µl (60% v/v) perchloric acid. The samples were first mixed using a vortex then centrifuged. Supernatant aliquots were transferred to a second clean 5 ml microfuge tube. To neutralize serum sample 100 µl (6 M) potassium hydroxide was added to bring the pH to neutral. The sera samples were then rapidly frozen and stored at -70 degrees until required.

4.2.4 High performance liquid chromatography

Quantisation of folic acid in the sample was carried out using HPLC. The adapted isocratic HPLC method with mobile phase comprised of citrate phosphate buffer (0.1M, pH4.0 adjusted using phosphoric acid): 1% acetic acid: methanol (43: 42:15 v/v/v) was used. The ratio of 43:42:15 v/v/v was mixed in one reservoir. The wavelength of detection (UV) was 250nm; the flow rate adapted from Patrick, (1996) was modified from 3ml/min to 1ml/mim to avoid overloading of the column. For easy identification of folic acid and folinic acid standard peaks, solutions of these compounds were always ran before any serum sample was analysed. All buffer solutions were filtered through Whatman nylon filters (0.45 µm) before being injected into the column. The concentration of folic acid in serum was determined using equation in appendix 3 as indicated earlier in chapter 3.

4.2.5. Analysis of FA using chemiluminescence method

Stored frozen serum samples were used after being thawed in which 200 µl of the serum samples was transferred into the vials for analysis. The instrument had sample pickup of 50 µl (dead volume). The control (liquid assayed immunoassay control) of known value was run as

a normal specimen prior the samples in which it point was plotted on the Levy Jennings graph. This is the direct testing from the tube in which the sample reacts with reactive ingredients which are found in the commercially prepared reagents, the following Figure 4.1 explain step by step what happens after the sample is being injected to the instrument.

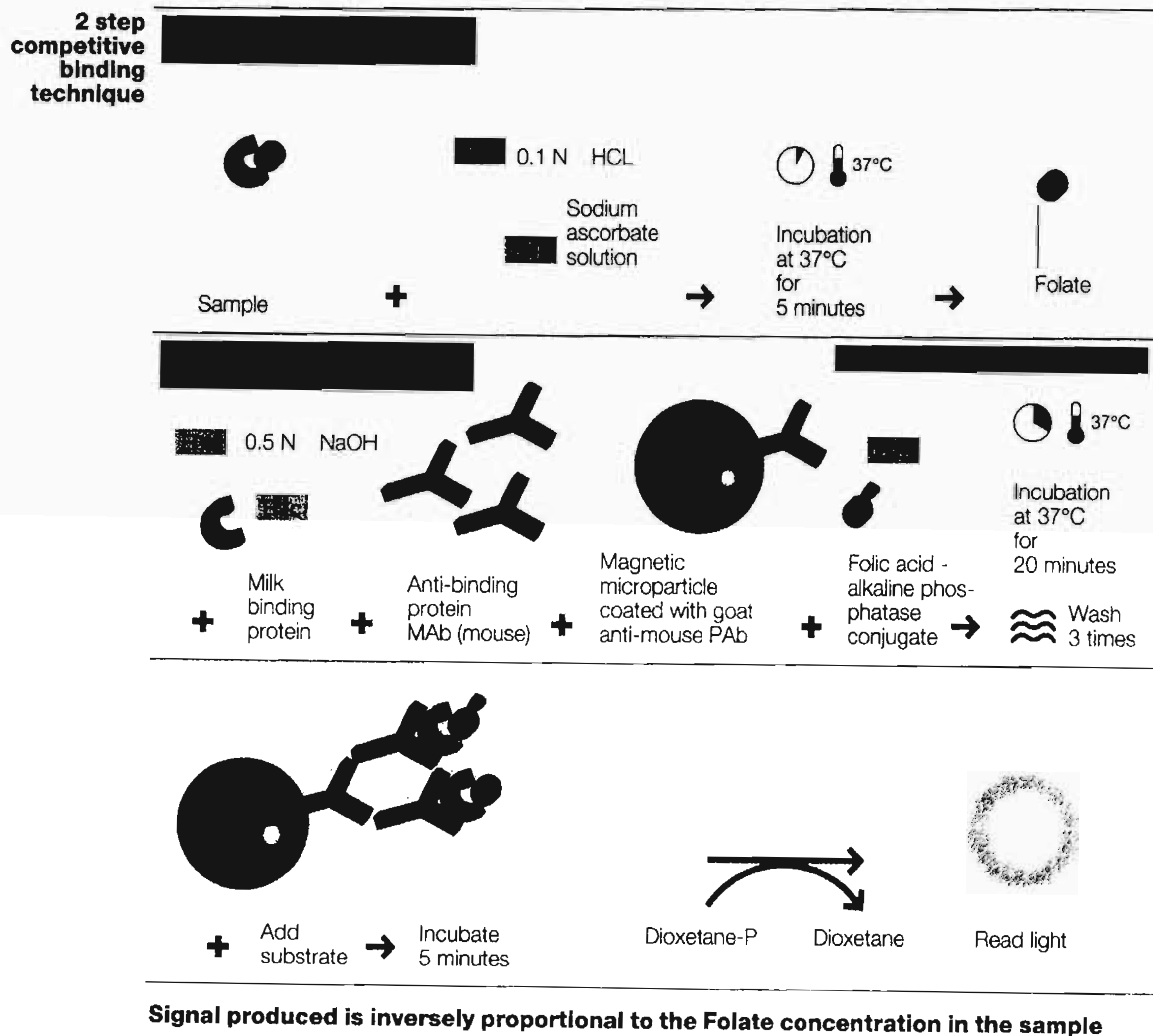

Figure 4.1: Shows analysis of folate in serum using chemiluminescence method (SDP, 1997. Access Folate . Product Catalogue of Sanofi Diagnostics Pasteur).

Automated values are computed by the machine depending on the light emission from each specimen. Access immunoassay system (AIS) has detection limit of 0.5 ng/ml, each sample is analysed for 35 minute.

R1a – Paramagnetic particles coated with goat anti-mouse + folic acid-alkaline phosphatase conjugate.

R1b- Sodium ascorbate solution

R1c- Milk binding protein and monoclonal anti-bindinng protein antibody

R1d -HCl solution

R1e- NaOH solution

4.3 RESULTS

Percentage recovery of folic acid in spiked serum sample was found to be 91% - 100% as reported in chapter 3. Folinic acid was not detected in serum samples. Results in table 4.1 shows that only folic acid was detected in blood serum. The level of folic acid in human serum was between 1 to ± 4 µg/ml. Determination of folic acid in serum was satisfactory at this stage using HPLC couple with UV detection.

Table 4.1 (table continued to page 54) Concentration of folic acid (µg/ml) in blood serum detected using HPLC/UV and Immunoassay

Samples	Amount of folic acid (µg/ml) (HPLC)	Amount of folic acid (ng/ml) (Immunoassay)
FA1	ND	7.2 ± 0.03
FA2	1 ± 0.14	5.6 ± 0.08
FA3	1.09 ± 0.04	6.6 ± 0.03
FA4	1.01	8.4 ± 0.1
FA5	ND	13 ± 0.1
FA6	ND	19 ± 0.2
FA7	1.35 ± 0.02	6.8 ± 0.02

FA8	2.11 ± 0.02	> 20 ng/ml
FA9	1 ± 0.007	9.2 ± 0.03
FA10	3.85 ± 0.05	3.9 ± 0.07
FA11	1.17 ± 0.02	8.05 ± 0.2
FA12	1.17 ± 0.02	6.4 ± 0.02
FA794	ND	2
FA785	2.1 ± 0.03	0.4 ± 0.18
FA767	2.73	0.67
FA106	ND	ND
FA868	3.53 ± 0.08	3.7 ± 0.34
FA762	1.61 ± 0.02	2.35 ± 0.04
FA761	2 ± 0.01	1.74 ± 0.01
FA778	ND	ND
FA786	3.25	1.76 ± 0.12
FA1068	ND	4.1 ± 0.12
FA758	ND	ND
FA813	1.8	2 ± 0.1
FA842	1	5.9 ± 0.53
FA 777	2.63 ± 0.02	1.2 ± 0.21
FA865	3.73 ± 0.08	2.4 ± 0.07
FA772	1 ± 0.06	7.9 ± 0.04
FA1072	2.81 ± 0.12	3.8 ± 0.3
FA1071	1.29	3.6 ± 0.3
FA784	1.649	0.8 ± 0.14

FA798	1 ± 0.006	6.7 ± 0.01
FA1067	2.73	3.9 ± 0.2
FA859	1.6	3 ± 0.08
FA780	1.1 ± 0.02	3.9 ± 1.6
FA858	1 ± 0.02	2.6 ± 0.2
FA869	ND	ND
FA765	2.9 ± 0.075	3.4 ± 0.04

ND = Not detected (below the HPLC detection limit of 1μg/ml), FA – folic acid, 1 to 1072 – is the sample number

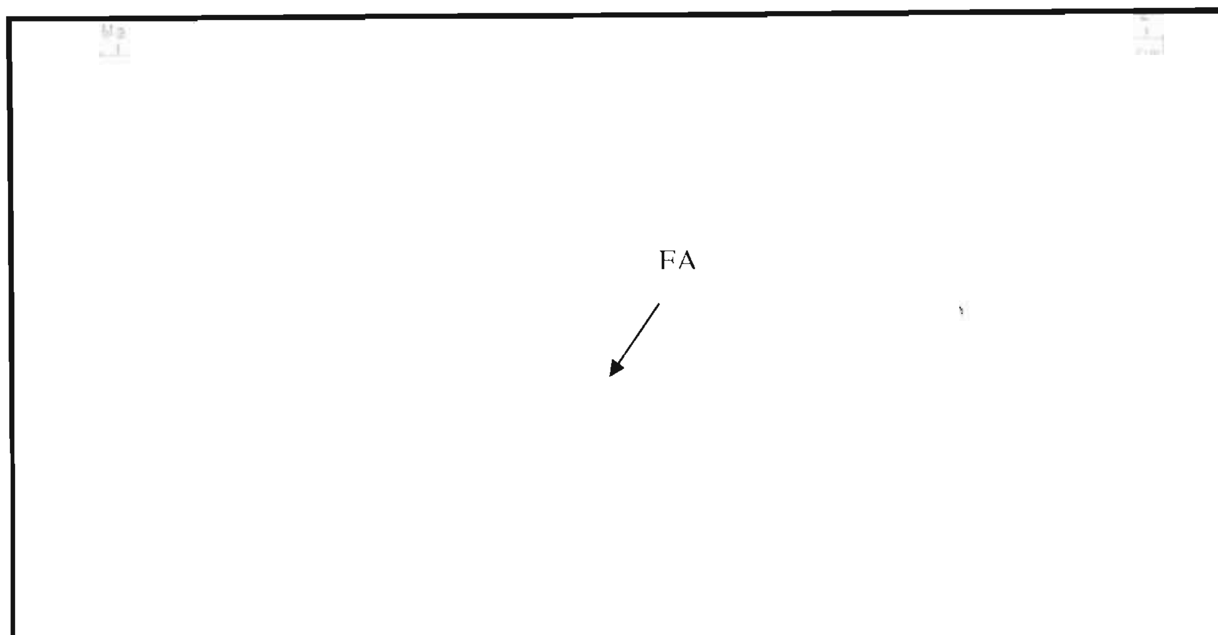

Figure 4.2a: Shows an HPLC chromatogram for folic acid (FA) peak in a serum at 3.47 min under 250nm wavelength, flow rate of 1ml/min and mobile phase ratio 70:30 (citrate phosphate: methanol). No standard matched was analysed for the unspecified peaks in the figure.

Folic acid identification in a serum was based on the retention time and was compared with the standard folic acid peak in (Figure 4.2b). In Figure 4.2a folic acid peak is well separated from other peak components of sample, making it easy for identification.

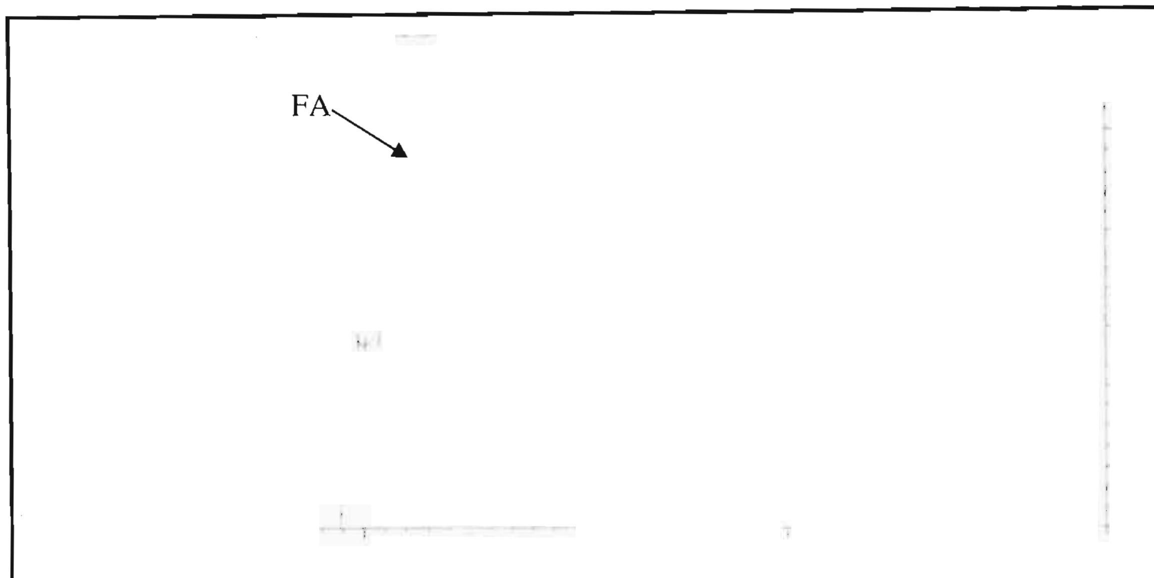

Figure 4.2b: Shows HPLC chromatogram for pure standard of folic acid (FA, 10µg/ml) at 3.46 min , under the wavelength 250nm, flow rate 1ml/min. the other two peaks are solvent peaks, folic acid peak show no traces of impurities or interferences.

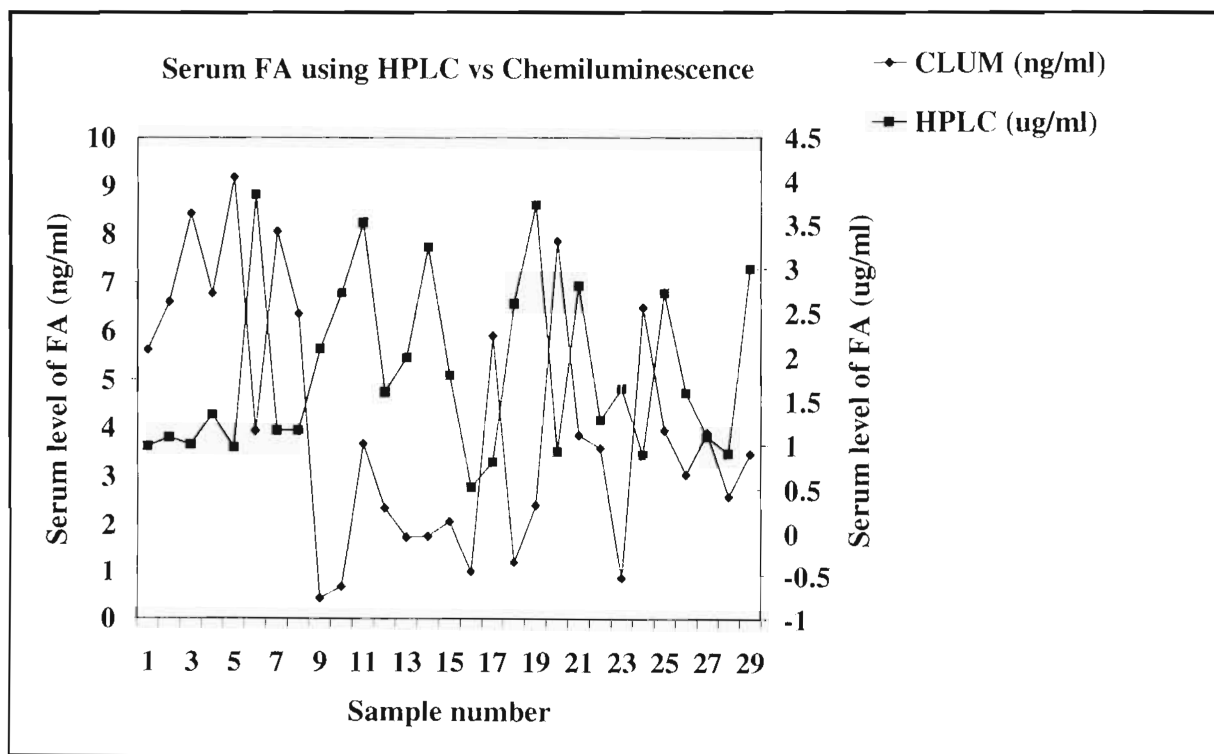

Figure 4.3: Shows the level of serum folic acid analysed using HPLC versus chemiluminescence, HPLC result (in µg/ml) are plotted in the secondary axes while chemiluminescence (in ng/ml) are plotted in the primary axes. A negative correlation coefficient of (-0.455) indicates that as one variable increases, the other decreases, and vice-versa.

The difference in sensitivity between HPLC and Immunoassay: chemiluminescence in the detection of folic acid is depicted in Figure 4.3. A correlation coefficient of minus number obtained in this study indicated an inverse relationship of analysis of serum folic acid between the HPLC method and chemiluminescence assay method resulting in poor agreement.

4.4 DISCUSSION

The HPLC method used in this study is effective for determination of folic acid in human serum, although folinic acid was not detected in all sera. It might be possible that folinic acid in serum is below the detection limit or was not released during the deproteinisation stage of serum sample. Serum folic acid level obtained using HPLC was within 1 to ± 4 μg/ml. The estimate safe and adequate daily dietary intake for adult men is 200 mg and for women 180 mg (NRC, 1989). Certainly the research supports the idea that folic acid supplementation of 200 mg-1000 mg per day is safe for virtually all persons and that this level of supplementation can prevent or correct clinical and subclinical evidence of folate deficiency in the vast majority of persons.

The Recommended Dietary Allowance (RDA) for folate for adults is 400 μg per folic acid equivalents increase to 800 μg for allowance for pregnancy and 500 μg for lactaction (Food and Nutrition Board, 1980). The RDA of 400 μg was based on the assumption that 100-200 μg folic acid equivalents was required to meet the minimum requirement and that the bioavailability of dietary folate was only 25-50%. Folic acid level obtained from serum sample folic acid using chemiluminescence was within ±1 to ± 9 ng/ml. Serum folate level in humans are normally within the range of 5 - 16 ng/ml (11 - 36 nmol/l folic acid activity (PGA equivalents) (Herbert 1999). Lawrence *et al.*, (1999) reported that median serum folate values

in clinical specimens in the US increased from 12.6 to 18.7 ng/ml during the period 1994 - 1998, presumed to be a consequence of the introduction of mandatory cereal fortification (140 μg folic acid/100 g grain) during 1996 - 1998. The difference in results obtained with each method may be attributed to the differences in principle analysis of each method. Such differences could be accepted because folic acid analysed by HPLC is the parent compound for all forms of folate. Folate,is a collective term use for other folate forms which are structurally related and which have similar biological activity to folic acid, such as Tetrahydrofolate; 5–MethylH_4folate, N^5- FormiminoH_4folate, $N1^0$-formylH_4folate, N^5,N^{10}-methylene H_4folate and N^5,N^{10}-methnylH_4folate. The results obtained using immunoassay: chemiluminescence method are always reported as serum folate, it is clear that the results by the chemiluminescence method shows that only a certain form of folate was detected. The method lacks the ability to specify between which form was detected and has attributed to poor agreement between the HPLC and Chemiluminence results.

Christine *et al.* (2004), reported that although it is likely that some or all the formyl-folate in serum is in form of 10-formyltetrahydrofolic acid, folate slowly converts to 5FoTHF at room temperature and is stable only at neutral and alkaline pH. In acidic conditions it concorts to 5, 10 methyltetrahydrofolic acid with complete conversion below pH 2.4, because of that interconversion they did not measured any 10-formyltetrahydrofolic acid using LC/MS method. Nelson *et al.*, (2004), reported the measurement of 5-methyltetrahydrofolic acid human serum. Different researcher's measures the different form of folic acid in human serum using different techniques. The method used in this study is efficient. Modification can be on the sample extraction to be able to detect both folic acid and folinic acid. Stokes (1999) reported folate is extremely prone to oxidation and therefore requires the present of antioxidant such as ascorbic acid and/or 2-mercaptoethanol. Such antioxidant also helps to

recovery folic acid from the frozen serum sample. Contradictary, Stokes (1999) also reported that the use of such compounds can cause interference at the detection stage of the analysis. In this study none of the mentioned antioxidants was used, deproiteinisation of serum sample with perchloric acid did not interfere with folic acid determination.

Folinic acid is present in the serum. Determination of folinic acid in human serum using high-performance liquid chromatography with amperometric detection was reported by Birmingham and Greene (1983), in which folinic acid concentration of 1.28 μg/ml was found after intravenous administration of 10 mg drug, serum sample was collected 5 min after injection of 10 mg iv of folinic acid to the 80 kg human volunteer. Folinic acid concentration in human serum was reported in μg/ml, this unit are comparable with the results obtained in this study particularly with use of HPLC. In academic research laboratory, various HPLC methods for measurement of folate in biological matrices has been developed (Pfeiffer and Gregory, 1996; Bagley and Selhub, 2000). Gas chromatography-mass spectrometry method used to analyse whole-blood showed greater selectivity than previous chromatography methods. Gas chromatography-mass spectrometry, however, was reported to require complex sample preparation including chemical derivatisation which resulted in new sources of experimental error and increases the probability of folate degradation (Cheruppolil, *et al*., 1997; Lin, *et al*., 2002). The study of folic acid and it derivativative is still the question of the day for most researchers, but our aim to optimise the method of analyses was a success with the early elution time of the compound of the interest at less than 4 minutes. Minor modification, however, can be on sample extraction to minimise unwanted interference which was observed in Chapter 3 on spiked serum sample with folinic acid.

4.5 CONCLUSION

Detection of folic acid in sera sample by high performance liquid chromatography (HPLC) with UV detector is possible. The HPLC/UV method used in this study has proven to be efficient for both separation and identification of folic acid in serum. The only disadvantage particular with UV detection is lack of sensitivity to a very low level of folic acid in serum and such problem can be corrected by using an alternative detector. At this stage it is satisfactory to say HPLC/UV method presented in this study can be considered for routine analysis of serum folic acid.

CHAPTER 5

ANALYSIS OF FOLIC ACID IN MAIZE MEAL SAMPLES USING GRADIENT ELUTION HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.1 INTRODUCTION

Compounds that have folate activity are essential nutrients for human beings. Folate is a generic term for compounds naturally occurring in food that have vitamin activity similar to pteroylmonoglutamic acid (folic acid), a synthetic product. The term folic acid and folate are often used interchangeably, but folic acid is approximately twice as bioavailable as the folate naturally occurring in food (Sauberlich *et al.*, 1987). During the past decade or so, folate activity also has been associated with the attenuation of biomarkers for cardiovascular disease and other human health maladies. Because of perceived health benefits, breakfast food cereal and other cereal based products in the United State has been voluntarily fortified with FA and other vitamins for several years. Now, based on recent scientific findings, the fortification of specified cereal products with FA has been made a requirement in the Unite States regulation at the federal level (Food and Drug Administration, 1996). In line with this, South Africa's Department of Health launched South Africa's National Food, Fortification Programme in April 2003. The food fortification regulations came into effect in October 2003, when consumers could expect to find fortified bread and maize on the shelves. Food fortification was an initiative to reduce micronutrient deficiencies in South Africa.

It has been estimated that between 80 and 90% of breakfast cereals consumed are fortified with folic acid. Most folic acid fortified products contain between 125 and 200 μg/100g. Some brands are fortified at a substantially higher level (333 μg/100 g). Fortification of bread is less widespread and it is mainly the soft grain varieties that are fortified to a level of approximately 120 μg/100 g (Department of Health 2000). The Committee on the Medical

Aspects of Food and Nutrition Policy (COMA, 2000) reviewed the role of folic acid in the prevention of disease (Department of Health, 2000). They suggested a number of options including universal fortification of flour with folic acid at the following levels 140 µg/100 g of flour, 200 µg/100 g, 240 µg/100 g, 280 µg/100 g or 420 µg/100g as a means of reducing the number of neural tube defects (NTD) affected pregnancies. Maize meal being the stable food item for most populations, is also currently fortified with folic acid (SA Department of Health Strategic Plan, 2000). Pteroylglutamic acid (PGA, folic acid) is used in fortification due to its stability (Ranhotra and Keagy, 1995). Although folic acid is used in fortification, natural folates may also be present in the product to be fortified (Rader *et al.*, 1998). Synthesis of B-complex vitamins, partial digestion of starch and proteins and release of enzyme are brought about during fermentation (Reedy *et al.*, 1980). Increase of vitamin B_{12} and folic acid and the synthesis also of a few antibiotics have been noted, particularly in fungal fermentation (Van Veen and Steinkraus, 1970).

The method of choice for determination of folate activity in foods has been microbiological, based on the growth of various microorganisms (Rader *et al.*, 1998). Ndaw *et al.*, (2001) also reported that most widely used analytical procedure for the determination of the total folate content of foods is still the microbiological assay using *Lactobacillus casei* as test organism, where a growth response of the organism to the mixture of folates present is measured turbidimetrically (Keagy *et al.*,1985; Finglas *et al.*, 1993). Even with the development of semi-automated procedures including the microtitration format (Newman and Tsai, 1986; Horne and Patterson, 1988). The microbiological approach, however, is both time–consuming and demanding in execution. In addition, to the response of the organism to the different folates forms it is not always identical. There are existing HPLC methods that have been use for analysis of folates in foods and drinks such as juice, meat, fish, lettuce (Erik *et al.*, 2001), but nothing has been reported on the analysis of folic acid in maize meal. Maize meal has

been for many years analysed using microbiological assays. Reliable analytical methods are, therefore needed for quantisation of the amount of total folic acid (FA) in maize meal. For quality assurance and regulatory purposes, what is especially needed are rapid and reliable methods for ensuring that the correct amount of FA is consumed and the degradation rates of these compound is kept at minimum during extraction process. For products fortified with folic acid, compliance can be demonstrated by using analytical methods that are either specific for folic acid or those that can detect a broader range of folates. The simultaneous presence of several folate forms at low concentration level also puts great demands on the method of analysis.

The aim of the study is to optimise an HPLC method for detecting and quantifying folic acid and folinic acid in maize meal for routine analysis of this particular source of food.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and reagents

All chemicals used in this study were of Analar grade supplied by BHD chemicals (Poole, England) unless otherwise specified. Folic acid compound (pteroylglutamic acid), folinic acid compound, (HG) hogs kidney conjugase (carboxylpeptidase), phosphoric acid were purchased from Sigma (St Louis, USA), Five different brands of maize meal were purchased from the local super market (South Africa), Strata solid phase extraction purchased from Merck (Darmstadt, Germany).

5.2.2 Extraction and cleanup

5.2.2.1 Sample preparation (method adapted from Liisa *et al.*, 1996)

Samples (1.5 g) from five different brands of maize meal (purchased in a local Supermarket) were weighed into 5 x 50 ml Beckman tubes (sample were 1 x 4 per brand), homogenized in 6-8 ml of extraction buffer (75mM K_2HPO_4 containing 52mM ascorbic acid/ sodium ascorbate mixture and 0.1% (v/v) 2-mercaptoethanol adjusted to pH 6.0 using phosphoric acid). The mixture was flushed with nitrogen for 5 minutes. The tubes were capped and homogenised, heated in boiling water for 10 minutes then cooled rapidly in ice water, centrifuged at 11000g for 30 minutes at $4^{O}C$. The extract was transferred into small vials adjusted to pH 4.9 with acetic acid followed by addition of 0.8ml HK conjugase for deconjugation of folic acid. These extracts was then kept in a water bath at $37^{O}C$ for 2 hours, then into boiling water bath for 5 minutes to inactivate the enzymes and subsequent cooling in ice-water. Extracts were further purified in a SAX cartridge. A modified method of Gounelle *et al.*, (1989) from Liisa *et al.*, 1996 was used for purification of sample, in which 10ml of 0.01M phosphate buffer containing 0.1% 2-mercaptoethanol (pH 7.0, conditioning buffer) was applied to the column before passing the sample through. 15μl of 2-

mercaptoethanol was added to the sample before it was applied on a SAX column. Sample was passed through slowly and the column was washed again with (2 X 1.5 ml) conditioning buffer. Finally folic acid was eluted with 2.5ml of 0.1M sodium acetate containing 10 % (w/v) sodium chloride and 1% (w/v) ascorbic acid and was then kept frozen until the HPLC analysis. Fermented maize meal was prepared from each brand of maize meal by boiling 400ml water in boiling container followed by addition of mash maize meal (40g of maize meal dissolved in 100ml of water) into boiled water. The suspension was cooked for 10 minutes, allowed to cool at room temperature and then transferred to a fermented vessel followed by addition of sugar (3g). Fermentation took place in about three to four days at room temperature. 5 ml to 6 ml of fermented maize meal was transferred into 50 ml Beckman tubes, and was extracted as described above.

5.2.2.2 Spiked sample

One brand of maize meal was chosen (Inyala maize meal, from Premier Foods, Sandton, SA) in which (1.6g x 3) of maize meal was put in three Beckman tube, spiked with (6.25μg/ml) of folic acid to assess the suitability of the extraction method which in turn increases the reliability of peak identification. Samples were spiked before the extraction and clean-up procedure. Recoveries of folic acid on spiked samples was calculated as follows: (Concentration of folic acid measured in spiked sample – concentration of folic acid measured in unspiked sample) / (concentration of folic acid added in spike sample) X 100 = % recovery.

5.2.3 HPLC analysis

Method adapted from Liisa *et al.* (1996) was used with modification. Gradient elution programme with mobile phase of acetonitrile and 30mM potassium phosphate buffer (pH

2.2) which was modified to (pH 2.5) was used for separation of folic acid in maize meal samples. The run time was 15 minutes and the time between injections was 10 minutes. The gradient was started at 10% acetonitrile with a lag of 4 minutes, after which the acetonitrile proportion was raised to 24% within 8 minutes and again back to 10% after 3 minutes, the flow rate was set at 0.8 mL/min. The detection limits in this chromatographic system was 1μg/ml for both folic acid and folinic acid compound.

5.3 RESULTS

5.3.1 Percentage recovery of folic acid (6.25µg/ml) from spike sample (1.5g)

The percentage recovery of folic acid concentration on spiked maize meal was found to be 73% ± 0.09, whereas percentage recovery of folic acid on spiked fermented maize meal sample was 55% ± 0.05. The concentration of folic acid in the sample was calculated using the equation in appendix 1. Efficiency of the method was observed when the blank run showed no traces of FA, FN and interfering peaks. The instrument or technique response was stable (no drift observed), no sample loss was suspected or noted during the experiments. Standard folic acid also showed no traces of impurities Figure 5.1

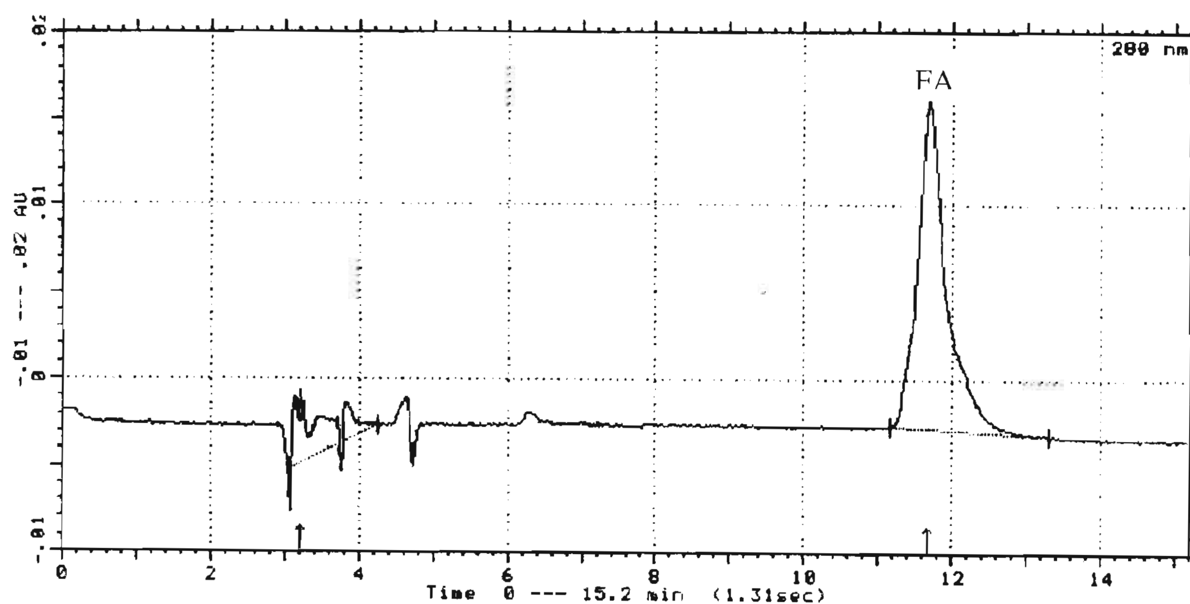

Figure 5.1: an HPLC chromatogram of FA standard (10µg/ml) at 290nm wavelength, mobile phase (ACN: 30mM K_2HPO_4), flow rate of 0.8ml/min, gradient elution has allowed good identification of folic acid peak without any traces of impurities and interfering peaks at 11.65min using pH 2.5.

When FA standard (100 µg/ml) was run against standard concentration of 10 µg/ml (FA), FA concentration 10µg/ml was ten times less than the concentration of 100 µg/ml. The use of the internal standard was therefore omitted in this chapter. Table 5.1: Show the results of folic

acid concentration obtained after maize meal samples were extracted, cleaned up and finally analysed by HPLC/UV. Ace maize meal contained 1.47 ± 0.005 of folic acid , while impala maize meal contained 1.8 ± 0.15 of folic acid, Induna and inyala contained 1.3 ± 0.003 and 1.29 ± 0.43 respectively. Folic acid was not detected in super maize meal sample

Table 5.1: Concentration of folic acid in maize meal (µg /g) analysed using high performance liquid chromatography

Brands of maize meal	Folic acid (µg/g), unfermented maize meal	Folic acid (µg/g), fermented maize meal
Ace	1.5 ± 0.005	2.1 ± 0.334
Impala	1.8 ± 0.15	1
Induna	1.3 ± 0.43	1.1
Inyala	1.29 ± 0.003	2.1 ± 0.024
Super maize-meal	ND	ND

ND- not detected (below the detection limit of 1 µg/ml)

Elution of folic acid is shown in (Figure 5.2). The elution time of folic acid peak from sample in Figure 5.2 was compared to standard FA peak (Figure 5.2a) in which samples showed a small peak of folic acid with the similar retention time to pure standard folic acid. Increased in folic acid concentration was observed in fermented maize meal prepared from inyala and ace maize meal and decrease in fermented maize meal prepared from impala and induna maize meal whereas nothing was detected in super-maize meal brand. Folinic acid was not detected in the entire brands of fermented maize meal (Table 5.1).

The lowest concentration of standard folic acid shown in Figure 5.2a was chosen to avoid false results from the sample that could have occurred if highest standard concentration (10μg/ml) was injected.

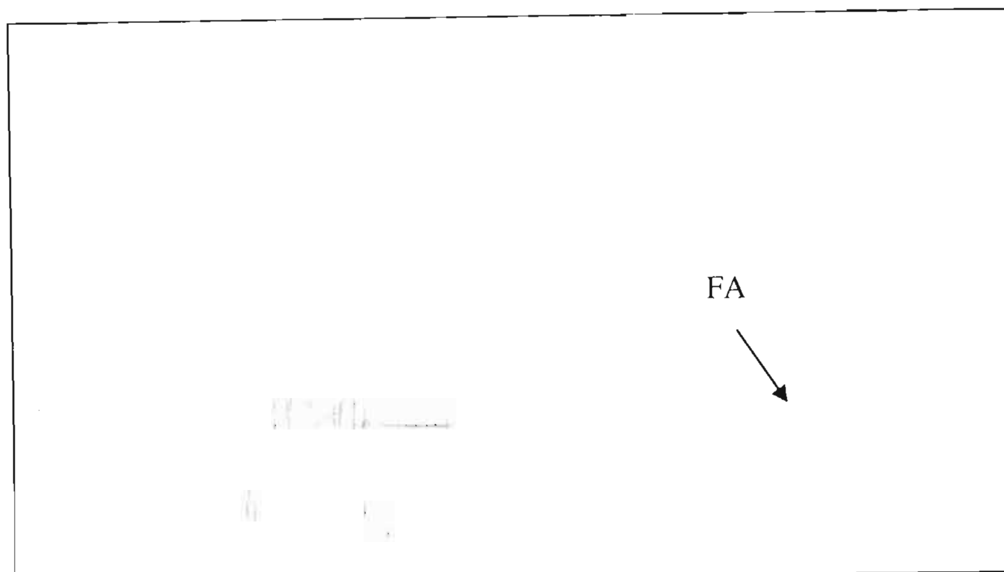

Figure 5.2: Folic acid peak from the sample maize meal is indicated by an arrow, folic acid eluted at 10.75min at wavelength of 290nm, flow rate of 0.8ml/min using HPLC gradient elution, Sample showed small peak of folic acid due to the low level of folic acid in maize meal.

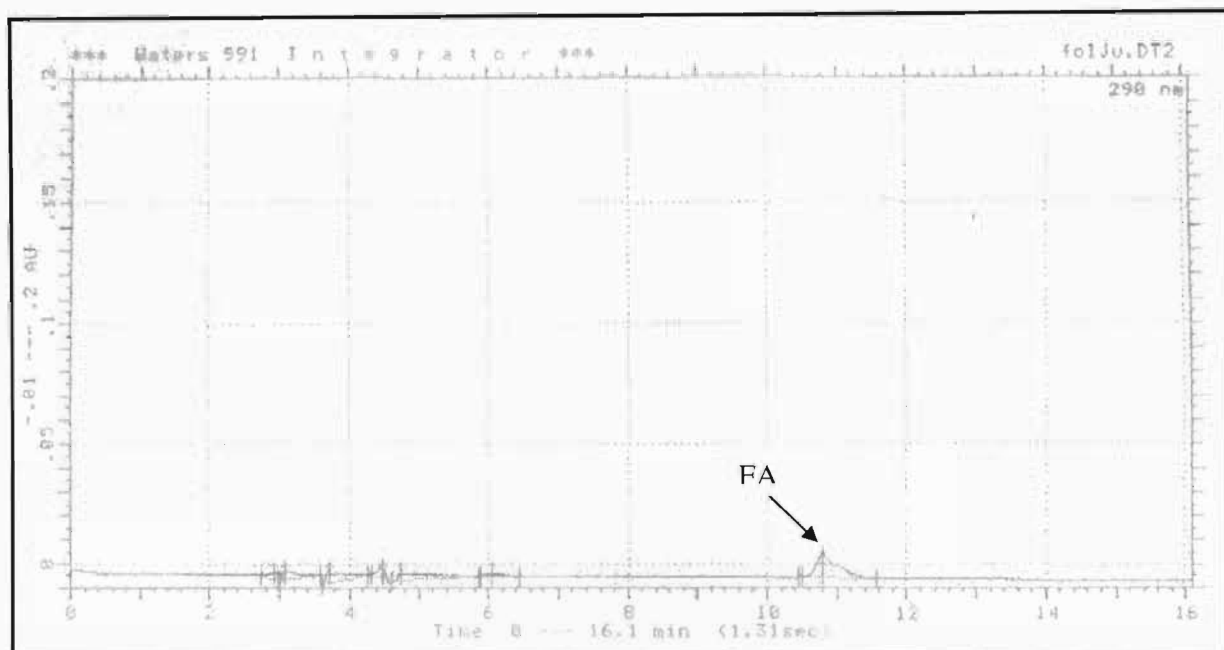

Figure 5.2a: Reverse-phase chromatogram of standard folic acid (2μg/ml). UV detection was at 290nm, mobile phase (ACN: 30mM K_2HPO_4), flow rate of 0.8ml/min, gradient elution has allowed good identification of folic acid peak without any traces of impurities and interfering peaks at 10.80 minutes.

5.4 DISCUSSION

Adjusting the pH2.2 from the method by Liisa *et al.* (1996) to pH 2.5 resulted in early elution of folic acid, this was advantageous in our attempt to minimised analysis time and that has allowed more samples analyses in one day. The percentage recovery of folic acid concentration from spiked maize meal was 73%, the assumption was that the 27% of folic acid that was not recovered was lost because of other components present in maize meal and due to sample processing. Percentage recovery of folic acid concentration from spiked fermented maize meal was 55%. During this study the use of chicken pancrease conjugase was omitted from the method adapted from Liisa *et al.* (1996) because it was not commercially available and the omission did not have any effect on the results, because the percentage recovery of folic acid was above 50% for both maize meal and fermented maize meal. Such percentage is at least acceptable . Jesse *et al.*, (1984) reported recovery of added folates for cow's milk to be 71.9 ± 7.3 for H_4folate; 96.6 ± 4.6 for 5-CH3-H_4folate and did not determine 5HCO-H_4folate and folate. In cabbage the recovery of added folates was 79 ± 0.0 for H_4folate, 107.2 ± 8.1 for 5-CH3-H_4folate, 73.0± 9 for 5HCO-H_4folate and 98.4 ± 1.0 for folate using HPLC fluoremetric detection.

Krumdieck *et al.*, 1992 reported recovery of approximately 100% for various folates, using their differential cleavage method. Their recovery values were determined by adding much higher level of folate compounds such that precision would be improved and relative losses due to entrapment in insoluble sample residues would be minimized. However they did not give a report on their detection limits. While Christine (2004) reported that the recovery of five different folate added to white bread, rice and spaghetti were between 85 and 107% using the trienzymes extraction and affinity chromatographic purification. Although such results indicate efficiency of they method, the disadvantage is that the procedure is long and

tedious and such methods were therefore not adapted to this study. It is clarify the percentage recovery for different food materials and manipulation. It is reported that the degree of loss could be influenced by environmental factors, including pH, oxygen content, metal ion concentration, antioxidant levels, duration and product: water ratio (Gregory, 1989; Hawkes and Villota, 1989). However, precautions were taken during this study but such factors seem to remain a challenge for further research to be able detect more folic acid derivatives.

According to Ball (1998), literature on the extent and mechanisms of folate loss during processing is limited, as 50 to 95% of folate activity may be destroyed by cooking and/or processing. In addition, loss is attributed in part to the rapid changes in the C_1 moiety, the state of oxidation and the number of glutamyl residues in the peptide side chain during food preparation and analysis. There are few data on the folic acid derivatives in different foods (; Deller *et al.*, 1965; Hurdle *et al.*, 1968). Further complications arise from the instability of several folates during sample preparation and the complexity of the sample matrices to be analysed.

Problems associated with food folate analysis have been reviewed previously (Hawkes and Villota, 1989; Martin, 1995). Folacin from both plant and animal sources contain formyl and methyl derivatives, these could suggest the necessity to derivatise some form of folate which can lead to more forms of folate detection without changing the chromatographic condition. Perry (1971) has reported that in cooked meal 60% of the folacin derivatives were methyl and 33% were formyl derivatives. Cossins *et al.*, (1972) detected a similar pattern in whole leaf extract of pea seedling. Those in liver (Noronha and Silverman, 1962 and Shin *et al.*, (1972), orange juice (Dong and Oace, 1973), milk (Dong and Oace, 1975), egg (Butterfield and Callowway, 1972), and cabbage (Chan *et al.*, 1973) are predominantly methyl derivatives. But most of the folacin in yeast (Schertel *et al.*, 1965) and soy-beans (Shin *et al.*, 1975) are formyl derivatives.

In the early 1960s Butterworth *et al* (1963) and Santini *et al.*, (1964) found three folacin derivatives in food when they separated them chromatographically: 10-CHO-folic acid (55%), 5-CHO-H_4-folic acid (34%) and folic acid (11%). Cereal grain products are reported to provide up to 70% of the daily energy intake. Because cereal products enjoy such wide acceptability as daily staple foods, they have been used successfully as base carriers for fortification (Austin, 1978). In South Africa, the milling industry has voluntarily enriched maize meal with riboflavin and niacin. However, the monitoring of this practice is inadequate (Aggert *et al.*, 1989) and its impact is largely unknown (Nutrition committee to the Minister of health), an Integrated Nutrition Strategy for South Africa, Pretoria, Department of Health, 1994). Despite the effort devoted to develop folate methods with better performance, there are still problems to be solved. (Finglas, van de Berg and de Froidmont-Gortz, 1996; Seale and Finglas, 1995) reported that unwanted interference from the sample matrix suggest necessity use of extensive sample extraction and clean-up.

It is known that fermentation is subjected to the action of micro organism and/or enzyme to give biochemical and significant modification of the quality of food referred to as fermented food. The expectation was an increase in folic acid concentration in all fermented maize meal. The challenge again could be to study in details the behaviour of some components in maize meal during fermentation which might have great influence on the stability of folic acid and folinic acid, because food fermentations continues to be primarily important in developing countries where the lack of resources limits the use of techniques such as vitamin enrichment of foods. (Chaven and Kadam 1989) even reported that technology of producing many fermented foods from cereals remains a household art and prospects for applying advanced technologies to fermented foods.

In this study an increase in folic acid level in all fermented maize meal brands was expected because increase of vitamin B_{12}, folic acid and the synthesis also of a few antibiotics, are reported to have been noted in fermented food (Van Veen and Steinkraus, 1970). Gregory *et al.*, (1990) reported incomplete extraction of folic acid from some food, which they attributed to interaction of folate with insoluble residues or retention by physical entrapment. The purpose of this study was 2-fold: first, to detect and analyze folic acid and folinic acid content in maize meal as it is the stable food item for the majority of South African population, and second, to optimise HPLC method for analysis of folic acid in maize meal. At this stage only the determination of folic acid was possible using method described here, the use of HPLC method for analysis of maize meal is satisfactory and can always be improved to excellence.

5.5. CONCLUSION

Although it possible to identify folic acid in maize meal and fermented maize meal using HPLC/UV, there is still a need to minimise the loss of folic acid and folinic acid during the sample preparation. Peak identification was based on the retention time.

Our objective to detect and identify folic acid peak on maize meal was achieved. The results from this study can be useful to improve some areas which can lead to more data reported which in turn the consistence can be measured, especially for the determination of folinic acid before HPLC can be routinely used for analysis of other folates forms in maize meal. The outcome of this work also indicates that more work is still needed to determine the best extraction procedure to use with specific foods.

GENERAL DISCUSSION AND CONCLUSION

Nutrition is the keystone for prevention of numerous diseases. The view expressed in the editorial by Kretchmer, (1994) that the public should be thoroughly informed of the role of nutrition in disease prevention and health maintenance, whether through the media, various avenues of medical services, or other means is excellent. Thus, we have a real responsibility as Researchers to ensure nutrition in food we eat is kept at it maximum in whatever forms the food is consumed (e.g. fermented maize meal) and also that the presence of nutrition (vitamin folic acid) in our body is sufficient. That can only be ensured by quantifying and being able to detect the specific nutrition (in our study vitamin folic acid) using reliable methods. The chromatographic procedure described in this study can be helpful in obtaining some information concerning the folic acid and folinic acid stability in maize meal as well as in human serum, can also lead to the good evaluation of analysis method. In turn results could be use for improvement and promoting the consumption of good quality nutritional food by the community.

- However, during this study it was possible to achieve a chromatographic specific to identification of folic acid and determination of the folic acid content both in maize meal and human serum.
- During optimization it was noted that retention of the compounds studied was very sensitive to even small changes in pressure reading which is why the standard solution was run every day prior to sample analysis which assisted in peak identification.
- It was well established that reported folic acid values obtained in human serum using HPLC in this study still has poor agreement with biological assay in our case immunoassay: chemiluminescence. However, the disadvantage of chemiluminescence method is that it is mostly use in hospital laboratories for the determination of folate in

human serum and cannot be use for food analysis, whereas HPLC/ UV described here was used for both food and human fluid material. The HPLC method described here may also serve as the basic for a collaborative study, which, upon it success may provide an official method applicable to the analysis of folic acid in both maize meal and human serum products analysis.

In general analysis of folic acid and its derivatives in food is still tedious especially sample treatment, maybe due to the lack of validated methods to characterize and quantify more than one forms of folic acid simultaneously. The assessment of folic acid and its derivatives losses during sample preparation is still incomplete. This should be the central aspect for further investigation.

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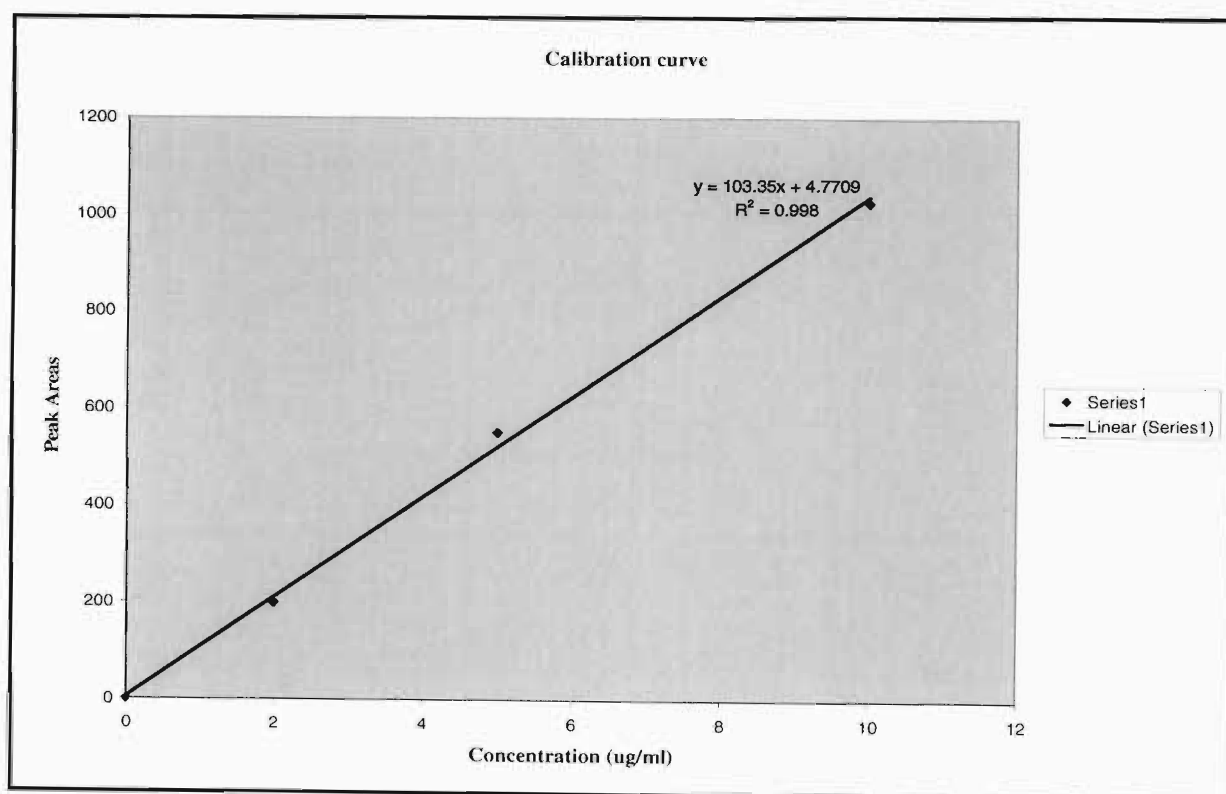
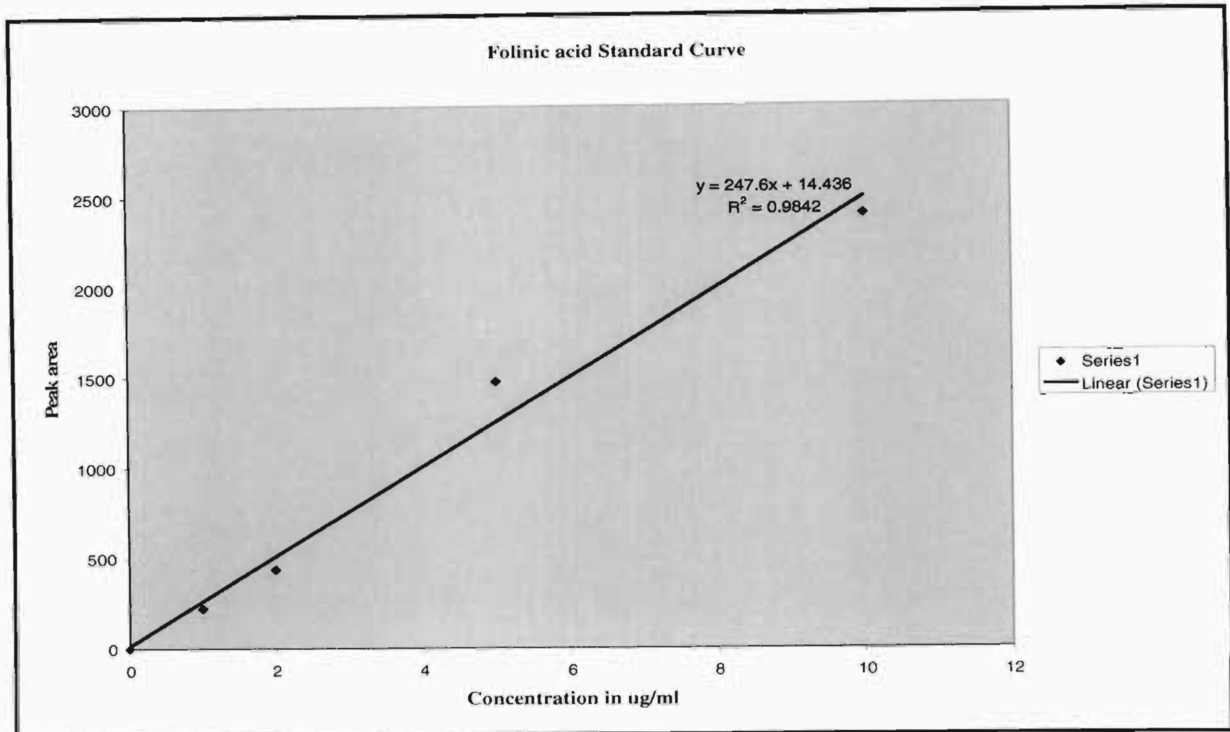
APPENDIX 1



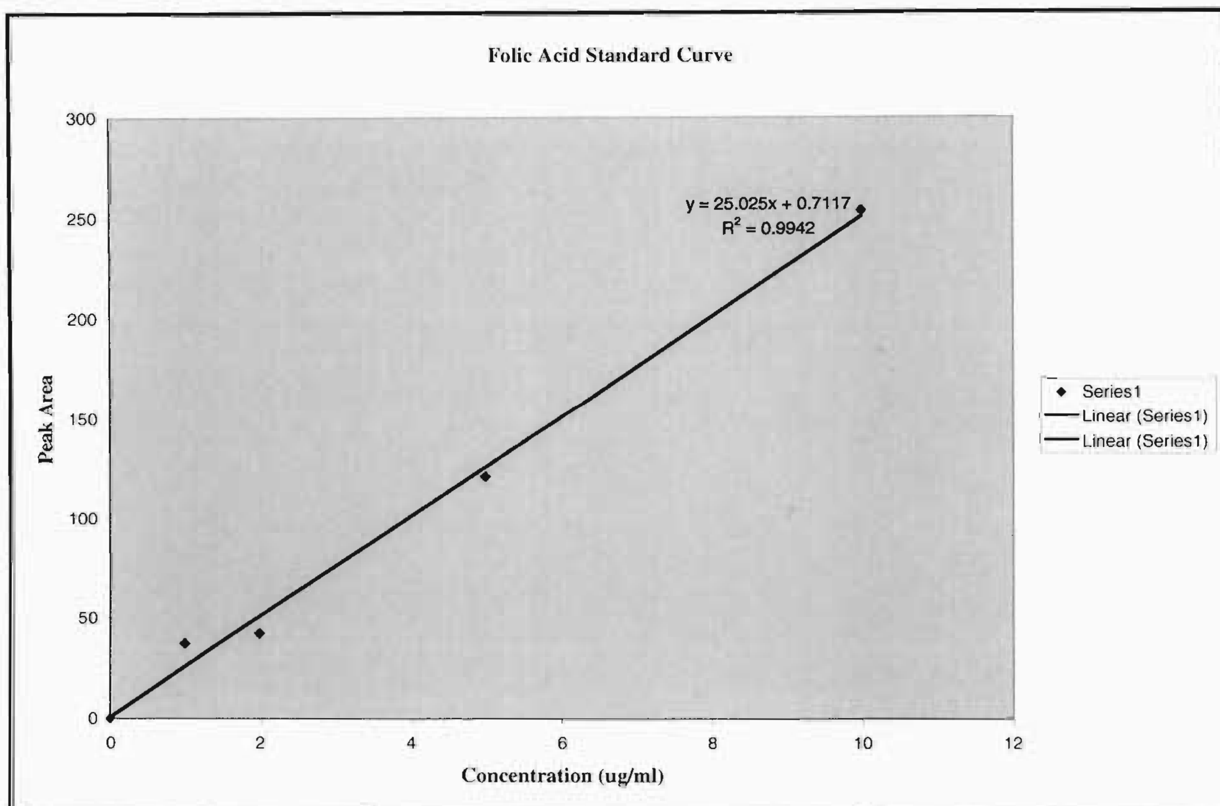
Standard curve for determining folic acid concentration in maize meal using HPLC

APPENDIX 2



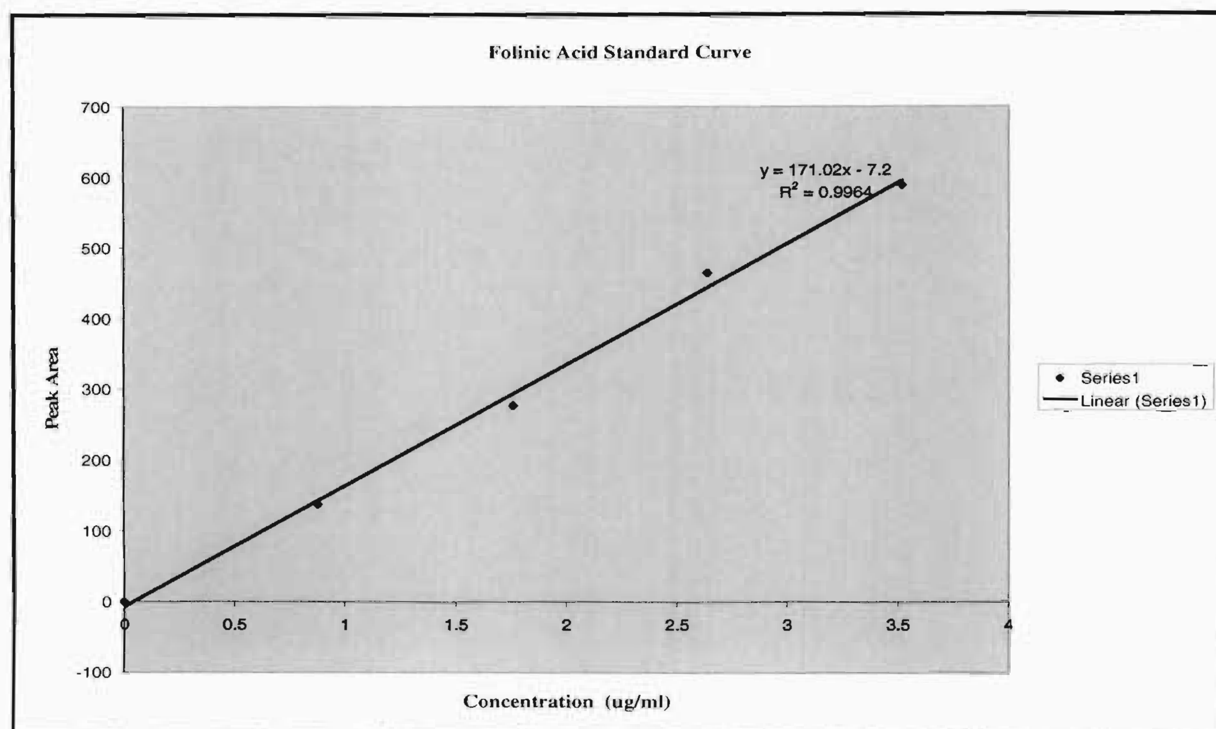
Standard curve for determining folinic acid concentration in maize meal using HPLC

APPENDIX 3



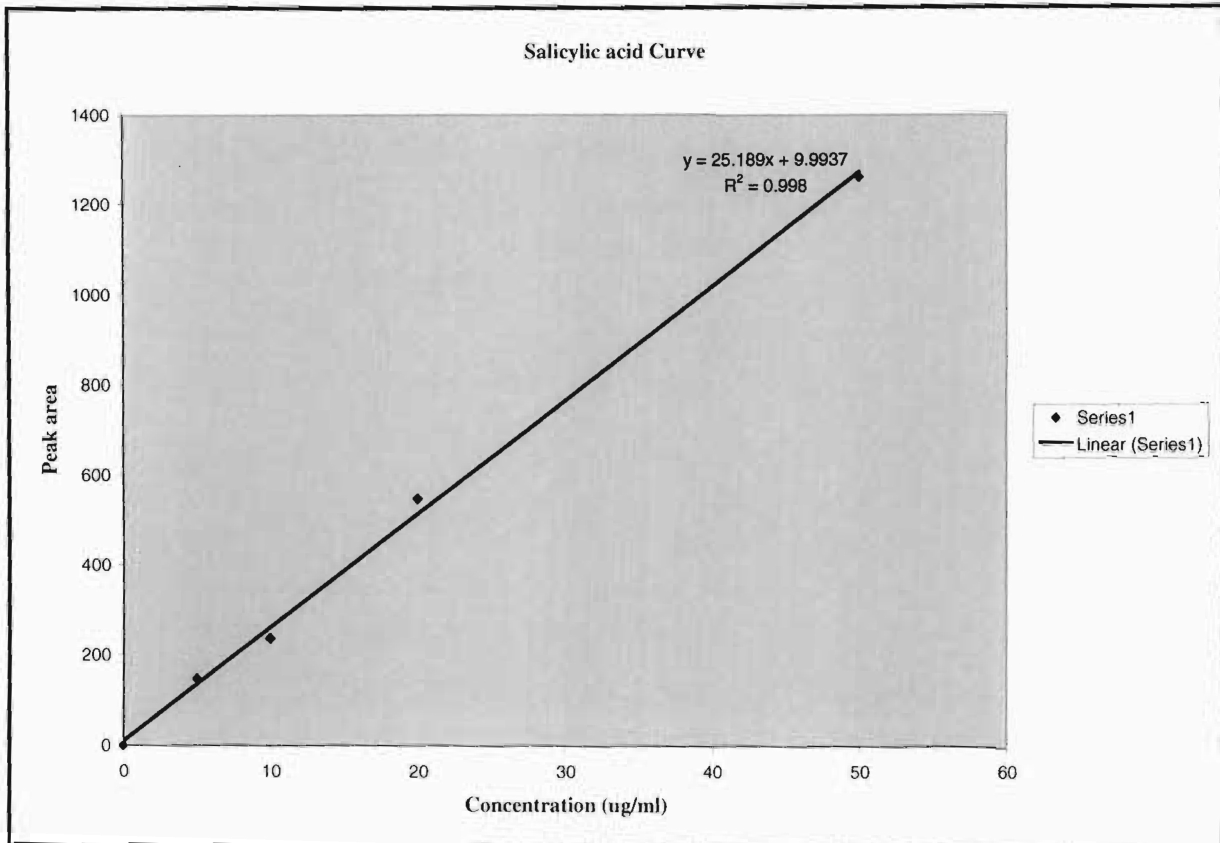
Standard curve for determining folic acid concentration in human serum using HPLC. Created from standard solutions of folic acid using isocratic elution.

APPENDIX 4



Standard curve for determining folinic acid concentration in human serum using HPLC. Created from standard solutions of folinic acid using isocratic elution.

APPENDIX 5



Standard curve for determining salicylic acid concentration (internal standard) in human serum using HPLC