CALCIUM L-5-METHYLTETRAHYDROFOLATE

First draft prepared by

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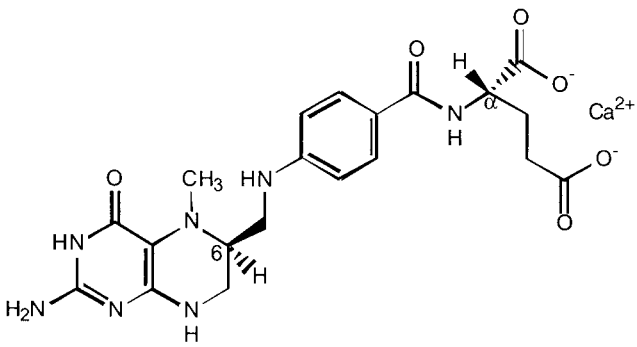
1. EXPLANATION

Calcium L-5-methyltetrahydrofolate is a synthetic derivative of naturally occurring L-5-methyltetrahydrofolic acid, which contains a reduced and methylated pteridine ring system (Figure 1). This compound has not been evaluated previously by the Committee.

Calcium L-5-methyltetrahydrofolate is structurally analogous to the reduced form of folic acid (pteroyl-L-glutamic acid), which is the nutritionally active form. The form of naturally occurring reduced folate found predominantly in food is a polyglutamyl folic acid (Scott, 2001). L-5-Methyltetrahydrofolate is a co-factor for key enzymatic reactions for the transfer and processing of the one-carbon units needed for re-methylation of homocysteine to methionine to serve as the methyl donor for numerous methyltransferases, which methylate a range of biological substrates (lipids, proteins, myelin, dopamine). It also serves as a carbon donor in the pathway leading to nucleotide synthesis, supporting the biosynthesis of DNA.

Figure 1. Structure of L-5-methyltetrahydrofolic acid, calcium salt



The safety of folic acid was evaluated by the European Commission Scientific Committee for Food, which established a tolerable upper intake level of folate at 1 mg per adult per day on the basis of the need to avoid masking vitamin B12 deficiency (Scientific Committee for Food, 1993). The same tolerable upper intake level for folate was established by the United States Institute of Medicine (1998) and the FAO/WHO consultation on human vitamin and mineral requirements (FAO/WHO, 2001). The Scientific Panel on Food Additives, Flavourings, Processing Aids and Materials in Contact with Food of the European Food Safety Authority concluded that the safety of use of calcium L-5-methyltetrahydrofolate as a source of folate in foods for specific nutritional uses, food supplements and foods intended for the general population, with a tolerable upper intake level of 1 mg per adult per day, is not a concern (European Food Safety Authority, 2004).

For calcium, a tolerable upper intake level of 2.5 g was established by the Scientific Committee for Food (2003) and the Institute of Medicine (1997). A tolerable upper intake of 3 g was established by the FAO/WHO Consultation on human vitamin and mineral requirements (FAO/WHO, 2001).

At the request of a Member State, the Committee was asked to evaluate the safety of calcium L-5-methyltetrahydrofolate as an alternative to folic acid in food fortification and supplementation.

2. BIOLOGICAL DATA

2.1 Biochemical aspects

2.1.1 *Absorption, distribution and excretion*

The absorption and distribution of calcium L-5-methyltetrahydrofolate and related folate derivatives were studied in controlled clinical trials with volunteers. One study in vitro was also considered. No information was available on absorption, distribution, metabolism or excretion in animals. In a small-intestinal model consisting

of glass compartments, L-5-methyltetrahydrofolate was more readily absorbed than synthetic folic acid from milk containing folate-binding proteins (Verwei et al., 2003).

L-5-Methyltetrahydrofolate (monoglutamate) is the only form of folate that appears normally in the plasma and is internalized by cells for use. The studies also showed that the main folate transport mechanisms involve integral plasma membrane proteins, reduced folate carrier and folate receptors, which mediate cellular uptake of reduced folate by binding with high affinity and specificity (Brzezinska et al., 2000).

In a controlled trial with ^{14}C-folic acid (pteroylpolyglutamate), 13 healthy men and women were given daily oral doses of the labelled compound, and the appearance of folic acid in plasma and its excretion in urine and faeces were monitored for ≥ 40 days. A multi-compartment model was used to fit the data. Significant distribution of metabolites of folate in the body was found, and folate oxidation and catabolic products were identified in faeces and urine. The results indicate that, after ingestion, folic acid is eliminated in faeces (38% as pteroylmonoglutamate and its oxidation products) and urine (56% as *para*-acetomidobenzoylglutamate and 5.7% as intact pteroylmonoglutamate). The bioavailability of synthetic folic acid after oral intake was estimated to be 90–95% (Lin et al., 2004).

The bioavailability of calcium L-5-methyltetrahydrofolate and pteroylpolyglutamate was assessed in a randomized, double-blind, four-period cross-over study of 21 healthy women given a single oral dose of folic acid (400 µg) or equimolar L-5-methyltetrahydrofolate with or without prior loading with folic acid (1 mg/day for 10 days). The plasma folate concentration was measured by immuno assay in fasted subjects and every hour for 8 h after intake of the test material. The area under the curve (AUC) of concentration–time was calculated for L-5-methyltetrahydrofolate without pre-loading (AUC ratio, 156%; 90% confidence interval, 137–177%) and for folic acid with pre-loading (AUC ratio, 143; 90% confidence interval, 124–164%). The bioavailability of L-5-methyltetrahydrofolate was slightly higher at the start of the study but not at the end of the supplementation period. Overall, the bioavailability of L-5-methyltetrahydrofolate was similar to that of pteroylpolyglutamate (Prinz-Langenohl et al., 2003).

In a study with 104 healthy women given calcium L-5-methyltetrahydrofolate and pteroylpolyglutamate orally in equimolar concentrations (~ 100 µg/per day), blood plasma and erythrocyte folate concentrations were measured at 4-week intervals for 24 weeks. Folate indices were measured by a microbiological assay. Similar increases in blood folate concentrations were found during and at the end of the study in both treatment groups over those in the placebo control. A steady state of saturation had not been achieved by 24 weeks (Venn et al., 2002).

A study with 180 healthy men and women to quantify the bioavailability of naturally occurring folates from food indicated that folates contain polyglutamyl conjugates and require intestinal hydrolysis catalysed by g-glutamyl carboxypeptidase for absorption of the corresponding monoglutamyl folate derivative. The bioavailability was compared with that of monoglutamyl folic acid (262 nmol per day) after 12 weeks. The bioavailability of polyglutamyl folic acid was 66% that of the monoglutamyl form on the basis of serum and erythrocyte folate concentrations. The authors concluded that the polyglutamate side-chain of folates reduces bioavailability (Melse-Boonstra et al., 2004).

In a controlled intervention study with 96 healthy men, the bioavailability of folate from naturally occurring polyglutamyl folate in food was compared with that of synthetic monoglutamyl folic acid (supplement tablet, 200 μg each) by blood sampling and analysis of serum folate and homocysteine levels. Spinach and yeast containing polyglutamate:monoglutamate folate at 50:50% and 100:0%, respectively, served as natural sources of folate from food, and tablets containing synthetic folic acid were given for 30 days. A significant increase in serum folate and lowering of homocysteine were demonstrated in men ingesting synthetic folic acid but not in those eating spinach or yeast. The authors concluded that the bioavailability of folate was 30% from spinach and 59% from yeast relative to synthetic folic acid (Hannon-Fletcher et al., 2004).

Ten healthy men and women were given a single oral dose (~ 570 nmol) of [^{13}C6]pteroylglutamic acid ([^{13}C6]folic acid) or ([^{13}C6]5-methyltetrahydrofolic acid after fasting. Kinetic monitoring of labelled 5-methyltetrahydrofolic acid in plasma by liquid chromatography–mass spectrometry for 8 h indicated that the rate of relative bioavailability of labelled folates was slower after dosing with folic acid than with 5-methyltetrahydrofolic acid (Wright et al., 2003).

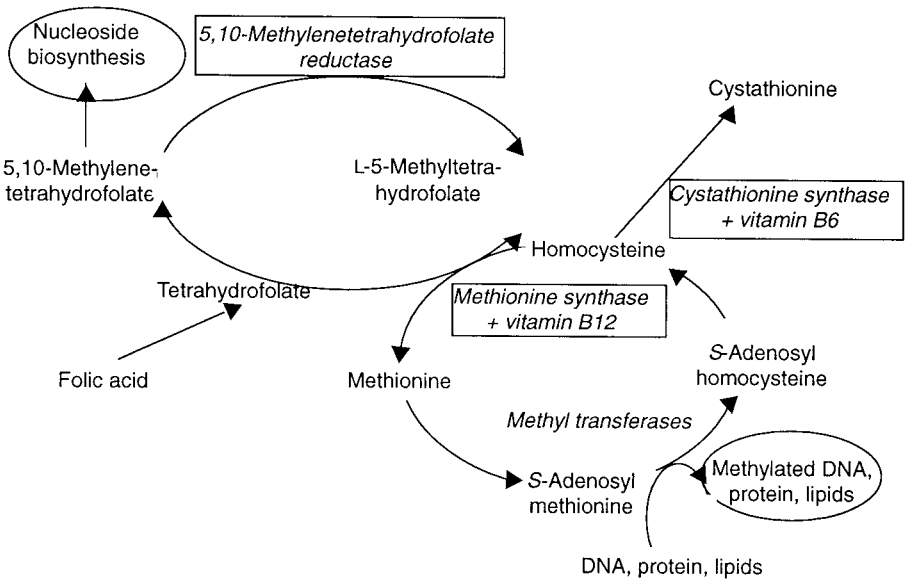
A slower rate and pattern of plasma response to folic acid may be a consequence of slow mucosal transfer of moderately high doses of folic acid to the hepatic portal vein, where significant folic acid uptake (< 70%) has been documented (Gregory, 2001; Kok et al., 2004). Low doses of radiolabelled folic acid and 5-methyltetrahydrofolic acid had similar short-term distribution, metabolism and kinetics in vivo. Therefore, differences in the bioavailability of moderately high doses (several hundred micrograms) of monoglutamyl folate species are probably due to hepatic uptake, enterohepatic circulation, tissue distribution and urinary reabsorption (Gregory, 2001). Further causes of differences in bioavailability might be reflected by rate-limiting kinetics for the metabolic conversion of folic acid to 5-methyltetrahydrofolic acid, as single doses of folic acid of several hundred micrograms exceeded the metabolic capacity for reduction and methylation (Lucock et al., 1989; Kelly et al., 1997).

2.1.2 Biotransformation

The biochemical pathways for the biotransformation of known bioactive folates are complex, involving a series of enzymatic reactions and cofactors required for formation of products essential in the normal maintenance of various physiological processes and genetic events. A simplified version of the metabolic pathways involving L-5-methyltetrahydrofolate was available (Figure 2).

In aqueous media, calcium L-5-methyltetrahydrofolate dissociates readily and completely into two components, calcium ion and L-5-methyltetrahydrofolate. L-5-Methyltetrahydrofolate derived from calcium L-5-methyltetrahydrofolate is therefore structurally identical to naturally occurring L-5-methyltetrahydrofolate, and its biotransformation would follow the same pathways (Venn et al., 2002). Absorbed folates are metabolized in intestinal mucosal cells to L-5-methyltetrahydrofolate; however, at intakes > 200–300 μg, the capacity of the human intestinal mucosa to reduce and methylate folate to L-5-methyltetrahydrofolate is limited, and unaltered folic acid appeared in circulating blood (Lucock et al., 1989; Kelly et al., 1997).

Figure 2. Metabolic scheme involving L-5-methyltetrahydrofolate



2.2 *Toxicological studies*

2.2.1 Acute toxicity

The acute oral toxicity of calcium L-5-methyltetrahydrofolate was studied in rats according to GLP and OECD Guidelines 423. Calcium L-5-methyltetrahydrofolate (purity, 97%), calcium D,L-5-methyltetrahydrofolate (racemic mixture; purity, 96.2%), calcium D-5-methyltetrahydrofolate (purity, 97.2%), *S*-triazine oxidation product of L-5-methyltetrahydrofolate (purity, 97.5%) and a hydrolysis product of L-5-methyltetrahydrofolate (purity, 98.6%) were administered at a dose of 2000 g/kg bw by gavage to groups of three fasted 7–8-week-old Hsd Cpb:Wu strain rats of each sex. Animals received food after 4 h of treatment, were left for a 15-day observation period and then killed for necropsy and examination of tissues and organs. All animals gained weight normally and survived to the end of the study. No adverse effects were observed on gross examination of organs (Heusener & von Eberstein, 1998a,b,c,d,e).

2.2.2 Short-term studies of toxicity

Rats

Groups of 10 male and 10 female Wistar rats were given calcium L-5-methyltetrahydrofolate by gavage at single daily dose of 0, 25, 100 or 400 mg/kg bw for 13 weeks. The study was performed according to GLP regulations (Switzerland), OECD testing guidelines 408 and Directive 96/54/EC, B.26. Food consumption, body weights, opthalmologicval end-points, locomotor activity and grip strength were

measured during the treatment period. A satellite group of five rats of each sex at 0 and 400 mg/kg bw per day were treated for 13 weeks and then allowed to recover for 4 weeks without treatment. After the treatments and recovery period, haematological and biochemical parameters were measured in all animals before sacrifice. None of the animals died from treatment-related effects. Organ weights were recorded, and histological samples were taken from 10 organs from all groups. Animals gained weight normally and showed no adverse effects or other changes during the observation period. Significantly lower hepatic enzyme activities (lactate dehydrogenase, aspartate aminotransferase) were found in males at the highest dose, but no other treatment-related changes were observed. The authors concluded that the lowered serum liver enzyme activity was not toxicologically relevant. The NOEL was 400 mg/kg bw per day, the highest dose tested (Hamann et al., 2001).

2.2.4 Genotoxicity

The results of studies of the genotoxicity of calcium L-5-methyltetrahydrofolate and its main impurities and oxidation products conducted according to GLP are shown in Table 1.

2.2.5 Reproductive toxicity

Rats

In a study of embryotoxicity and teratogenicity, groups of presumed pregnant Wistar rats were given L-5-methyltetrahydrofolate by gavage at a dose of 0, 100, 300 or 1000 mg/kg bw per day on days 5–19 of gestation. The rats were examined for clinical signs, body weight and food and water consumption at regular intervals from day 0 to day 20. On gestation day 20, the animals were killed and the fetuses were removed and examined for macroscopic malformations. Half the fetuses from each litter were examined for skeletal malformations and the other half for organ malformations as evidence of developmental toxicity. Maternal body-weight gain and food consumption were comparable in treated groups and controls; water intake was increased among animals at the high dose. There were no treatment-related clinical findings, and none of the animals died during the study. In each group, 22–24 rats were found to be pregnant with viable fetuses. The number of live fetuses, percent resorptions, average fetal body weight and sex ratio were not affected by treatment. Gross examination of dams did not reveal changes due to treatment. Examination of fetuses for external, visceral and skeletal malformations did not reveal fetotoxic, embryotoxic or teratogenic effects (Schubert et al., 2003). The study was conducted in compliance with the principles of GLP according to Annex 1 of the German Chemicals Act and the principles of GLP of the European Union.

2.3 Observations in humans

2.3.1 Studies on masking of vitamin B12 deficiency

The European Union Scientific Committee for Food (2000) established a tolerable upper intake of folate of 1 mg per adult per day in order to avoid masking vitamin B12 deficiency. The report described studies of vitamin B12 deficiency in humans and the clinical features and masking effects of folic acid on the diagnosis

Table 1. Results of assays for genotoxicity with calcium L-5-methyltetrahydrofolate and some of its impurities

End-point	Test system	Test substance	Concentration or dose	Results	Referencea
In vitro					
Reverse mutation	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537 and E. coli WP2 uvr ApKM101	Calcium L-5-methyltetrahydrofolate Calcium DL-5-methyltetrahydrofolate Calcium D-5-methyltetrahydrofolate *S*-Triazine oxidation product of L-5-methyltetrahydrofolate Hydrolysis product of L-5-methyltetrahydrofolate (96.2% pure)	5–5000 µg/plate	Negative	Utesch (1999a–e)
Gene mutation	tk locus in mouse lymphoma L5178Y cells	Calcium L-5-methyltetrahydrofolate (97.2% pure)	5–5000 µg/ml	Negative[a]	Utesch (2000a)
In vivo					
DNA synthesis and repair	Male Wistar rat (hepatocytes)	Calcium L-5-methyltetrahydrofolate (99% pure)	800 and 2000 mg/kg bw	Negative	Howe (2002)
Micronucleus formation	Male Wistar rat (bone marrow)	Calcium L-5-methyltetrahydrofolate	2000 mg/kg bw	Negative	Utesch (2000b)

S9, 9000 x *g* supernatant from rat liver
Studies performed according to ICH Guidelines, European Commission Directive, OECD Guidelines and the requirements of the Labor Ministry of Japan, notification dated 13 June 1979 and notification No. 1-24 of the Pharmaceutical Affairs Bureau, Ministry of Health and Welfare of Japan, dated 11 September 1989
[a] In presence of S9; toxic at 1580, 2810, 5000 µg/ml (relative growth, 13% and 4%); weakly mutagenic at two highest concentrations.

of megaloblastic anaemia in vitamin B12-deficient patients. Reversible megaloblastic anaemia and irreversible neuropathy are signs of vitamin B12 deficiency and are a result of reduced activity of methionine synthase, the enzyme that catalyses the vitamin B12-dependent conversion of 5-methyltetrahydrofolic acid to tetrahydrofolate, which leads to DNA synthesis and red blood cell formation (Figure 2). Low levels of tetrahydrofolate can therefore play a pivotal role in development of the haematological signs of vitamin B12 deficiency. The neurological effects stemming from reduced activity of methionine synthase in vitamin B12 deficiency are due to blocking of the methylation cycle (conversion of homocysteine to methinone), resulting in trapping ('folate trap') of 5-methyltetrahydrofolic acid substrate. The reduced methylation leads to neuropathy, as methyl groups are needed for methylation of myelin basic protein. Folic acid can be introduced to correct deficiencies in tetrahydrofolate levels, DNA biosynthesis and therefore the haematological effects of vitamin B12 deficiency; however, the neurological effects progress because the folic acid does not affect methionine synthase, which is responsible for the conversion of homocysteine to methinone, the pathway ultimately leading to the provision of methyl groups to methyltransferases for methylation of myelin basic protein (Hasselwander et al., 2000; Scott 2001).

A woman with tropical sprue who was deficient in folic acid and vitamin B12 was given 100 µg of D,L-5-methyltetrahydrofolate orally for 10 days. Treatment did not result in increased serum folate activity or result in clinically significant improvement. After administration of 1 µg of vitamin B12, an improvement in haematological response was observed. Thus, L-5-methyltetrahydrofolate was not metabolically active against the haematological signs of vitamin B12 deficiency and therefore did not mask the reported anaemia (Gutstein et al., 1973).

2.3.2 Studies of gene–nutrient interactions with folate intake

(a) Studies with variant methylenetetrahydrofolate reductase

5,10-Methylenetetrahydrofolate reductase is a key enzyme in folate metabolism, as it reduces 5,10-methylenetetrahydrofolate to L-5-methyltetrahydrofolate (Figure 2). Therefore, it plays a role in provision of the methyl groups required for DNA, by providing a substrate for re-methylation of homocysteine to methionine. It is also linked to production of the nucleotides essential for DNA synthesis.

There is a common polymorphism in the general population, which encodes for the 5,10-methylenetetrahydrofolate reductase gene, resulting in a point missense mutation (677C→T), which produces a thermolabile form of the enzyme (Hasselwander et al., 2000). The variant has variable penetration in different ethnic populations, estimated to be 10–15% in the United Kingdom, 20–30% in some Italian populations and only a few percent in Afro-Americans (Schneider et al., 1998). Persons expressing the mutation (CT or TT genotype) have decreased enzymatic activity (~ 34% of normal) in association with higher plasma homocysteine levels and lower folate intake than persons with wild-type (CC genotype) expression (de Bree et al., 2003; Meleady et al., 2003); they may also be at increased risk for cardiovascular disease and neural tube defects (Frosst et al., 1995; van der Put et al., 1995). The presence of a T allele appears to lower the plasma folate concentration (de Bree et al., 2003). No intervention studies were available to assess gene–nutrient interactions with regard to effects of administration of calcium L-5-methyltetrahydrofolate on plasma folate and homocysteine in this population group.

A case–control study of the association between dietary intake of folate, vitamin B12 and vitamin B6 on the risk for colorectal adenomas in persons homozygous (TT) for the 5,10-methylenetetrahydrofolate reductase mutation showed that TT persons with low folate intake were at a two- to threefold greater risk for adenomas than TT persons with high folate intake (Ulrich et al., 1999).

(b) Studies with variant thymidylate synthase

In a study of male and female patients with adenomatous polyps (510 cases and 604 polyp-free controls), data on folate intake were collected from a dietary questionnaire, and the patients were genotyped for a thymidylate synthase 28-base-pair repeat polymorphism in the *cis*-acting enhancer element. The aim of the study was to assess the affect of two polymorphisms in the thymidylate synthase gene on risk for colorectal adenomas by estimation of multivariate-adjusted odds ratios (ORs). Thymidylate synthase is a key enzyme in catalysing conversion of dUMP to dTMP; it is therefore required for DNA synthesis and repair. Double repeats are found less commonly than triple repeats, and 2.6-fold less thymidylate synthase is expressed with the double repeat than with the triple repeat. Homozygous triple-repeat individuals (3rpt/3rpt) with greater thymidylate synthase expression and high folate intake (> 440 µg/day) had a statistically significant, twofold decrease in the risk for colorectal adenomas (OR, 1.0; reference, low–medium folate intake versus 0.5 (0.3–0.9), high intake). In persons genotyped as homozygous double repeat (2rpt/2rpt), however, a high folate intake (> 440 µg per day) was associated with a statistically significant, 1.5-fold increase in risk (OR, 0.6, 0.4–0.9 versus 0.9, 0.5–1.5; $p = 0.03$). When the folate intake was low (< 440 µg per day), persons with the 2rpt/2rpt variant had a statistically significantly lower risk than those with the 3rpt/3rpt variant (OR, 1.0 versus 0.5 (0.3–0.9), high intake). The ORs for persons with 2rpt/3rpt and 2rpt/2rpt compared with persons with 3prt/3rpt were 0.8 (0.6–1.2) and 0.9 (0.6–1.3), respectively. Similar trends were observed for vitamin B12 intake (Ulrich et al., 2002). Although there are a number of common inherited polymorphisms (5,10-methylene-tetrahydrofolate reductase and thymidylate synthase, but also methionine synthase, cystathionine β-synthase, folylpolyglutamate carboxypeptidase), each intimately involved in folate metabolism, it is not known to what extent these genetic variants contribute to overall folate status and disease risk in the general population.

2.3.3 Studies of effects on plasma homocysteine

Epidemiological studies provide evidence of an association between plasma homocysteine levels and vascular disease (Loehrer et al., 1996). An important aspect in consideration of such observational studies is the association of folate status with decreased plasma homocysteine levels (Clarke, 1998). Three intervention studies were available to evaluate changes in plasma homocysteine concentrations in persons ingesting calcium L-5-methyltetrahydrofolate.

In one study, 167 healthy male and female volunteers were given 100 µg folic acid and equimolar concentration of calcium L-5-methyltetrahydrofolate or placebo for 24 weeks. Plasma folate indices and homocysteine concentrations in response to treatments were analysed at 8, 16 and 24 weeks of treatment. The mean homocysteine concentration was 14.6% lower in the group given folic acid and 9.3% and lower in that given calcium L-5-methyltetrahydrofolate than in controls (Venn et al., 2002).

In a study of similar design, 144 healthy women were given daily supplements of 400 µg folic acid and equimolar (416 µg) and half-dose (208 µg) treatments with calcium L-5-methyltetrahydrofolate. Increases were observed in plasma folate and decreases in plasma homocysteine levels by 15% in the group given folic acid, 19% in the group given the half-dose of calcium L-5-methyltetrahydrofolate and 19% in that given equimolar treatment (Lamers et al., 2003).

When 200 healthy adults were given folic acid, vitamin B12 or calcium L-5-methyltetrahydrofolate at doses up to 950 µg per person per day for 10 weeks, significantly elevated plasma folate levels were found in response to folate treatment. L-5-Methyltetrahydrofolate and folic acid significantly decreased the plasma concentration of homocysteine to a similar extent. These results indicate that calcium L-5-methyltetrahydrofolate and synthetic folic acid have similar effects with regard to lowering plasma homocysteine (Malinow, 2003).

Elevated plasma homocysteine is not a specific indicator of inadequate intake of folate, as it can be caused by dietary insufficiency of vitamin B12, vitamin B6 or riboflavin. Moreover, it is not known whether the effect of lowering blood homocysteine concentrations by oral administration of folate lowers the risk for cardiovascular disease.

2.3.4 Studies on tolerance

Studies on tolerance to calcium L-5-methyltetrahydrofolate and racemic calcium D,L-5-methyltetrahydrofolate were conducted in patients on haemodialysis or with psychiatric disorders. The patients received 15–17 mg/day for up to 6 months. No specific adverse effects were reported (Godfrey et al., 1990; Perna et al., 1997; Bostom et al., 2000). Although these controlled clinical studies were conducted under medical supervision, the Committee concluded that the test conditions lacked relevance for a safety evaluation of the proposed conditions of dietary intake of calcium L-5-methyltetrahydrofolate.

3. DIETARY EXPOSURE

3.1 Use levels

Calcium L-5-methyltetrahydrofolate is intended for use as an alternative to folic acid in dietary supplements, foods for particular nutritional purposes and regular foods. According to the producer, folic acid and calcium L-5-methyltetrahydrofolate can be used interchangeably, and the latter would be used in the same types of foods and at the same levels as folic acid. Thus, the intended uses and use levels (expressed as folic acid) are the same: food supplements provide 400 µg/day; meal replacements provide 200 µg per meal; starch-based fortified foods containing 1.5–3 µg/kg dry food (corresponding to about 400 µg/kg of prepared food) provide 2 µg per serving for bread and 60 µg per serving for noodles, pasta and rice; milk-type products containing 300 µg/l provide about 60 µg per serving.

3.2 Consumption of foods and supplements

Dietary folates can be natural or synthetic. Most natural dietary folates are reduced folates, i.e. derivatives of tetrahydrofolate, including 5-methyltetrahydrofolate.

Folic acid, a synthetic folate, is largely used in food supplements and in food fortification.

The aim of this assessment was to quantify potential exposure to L-5-methyltetrahydrofolate and to calcium that would derive from use of calcium L-5-methyltetrahydrofolate in fortified foods and supplements. It was assumed that this compound would be used as a substitute for synthetic folic acid in the same products and at the same levels. Assessments of folic acid are therefore reported.

3.2.1 Intake of folates from foods and supplements

Fortification of some foods with folates is mandatory in a number of countries (Table 2). The current levels of fortification vary from 40 to 240 µg/100 g in cereal-based products and at 160 µg/100 g in milk products in one country. In 2004, mandatory fortification of certain foods was proposed by the Australia New Zealand Food Standards Agency (2004), but has not yet been enforced.

Data on exposure to folate have been published in three countries with a history of food fortification with folic acid: Ireland, the United Kingdom and the USA.

Ireland

In Ireland, the Committee of the Food Safety Authority of Ireland (2003) suggested mandatory folic acid fortification of flour at 200 µg/100 g in food products as consumed, but the programme is not yet effective. Over-the-counter supplements are available in Ireland, containing 12.5–500 µg of folic acid, either as a single nutrient or as part of a multi-vitamin and mineral supplement

Intake of folate was assessed on the basis of a food survey involving 1379 adults aged 18–64 years: the North/South Ireland Food Consumption Survey. Food intake was determined from a 7-day food record (Irish Universities Nutrition Alliance, 2001). The results are shown in Table 3. According to this survey, only 2% of women aged 18–35 years and 5% of women aged 36–50 years achieve the recommended folate intake of 600 µg/day for women of reproductive age. All the women who followed the recommendation took folate-containing supplements. For women aged 18–50 years who took supplemental folate (14%), the mean intake of folate was 480 µg (233 µg from food and 248 µg from supplements). Overall, the consumption of dietary supplements increased the average intake of folates from food sources alone from 332 µg to 319 µg for men and from 260 µg to 225 µg for women.

The main source of folate is potatoes, which provide 17% of the mean daily intake. Vegetables contribute 12%; breads, 12%; breakfast cereals, 11%; alcoholic beverages, 8%; and non-alcoholic beverages, 6%. The contribution of folate supplements to the mean daily intake is 5%. The highest percentile of intake from all sources (662 µg at 97.5th) is that of men.

United Kingdom

The United Kingdom has adopted a voluntary approach to fortification of foods with folic acid since 1980 (Food Safety Authority, 2002a). Bread and breakfast cereals are the main foods that manufacturers have fortified with folic acid, and 80–90% of breakfast cereals consumed are estimated to be fortified, generally at 125–200 µg/100 g but some brands at a substantially higher level (333 µg/100 g).

Table 2. Countries in which folic acid fortification is mandatory

Region and country	Year mandatory fortification introduced	Foods fortified with folic acid	Level of fortification (μg/100 g)
Africa			
Malawi	2002	Maize flour	200
South Africa		Maize meal	190
		Wheat: flour white, brown	130
		Bread: white, brown	70
Zambia		Enriched maize meal	240
Middle East			
Saudi Arabia	2000	Enriched wheat, enriched treated flour	150
North America			
Canada	1998	Flour (white, enriched, enriched white) Enriched bread, pasta, pre-cooked rice	150
Mexico	2002	Wheat flour, corn flour	180, 130
USA	1998	Enriched cereal grain product including enriched: wheat flour, bread, corn grits, corn meal, farina, rice, macaroni products	140
Central and South America and Caribbean			
Argentina	2002	Wheat flour	220
Bolivia	1996	Wheat flour	150
Chile	1997	Wheat flour	200–240
Colombia	1996	Wheat flour	150
Costa Rica	2002	Wheat flour, corn flour, rice, milk	180, 130, 180, 160
Dominican Republic	2003	Wheat flour	180
Ecuador	1996	Wheat flour	60
El Salvador, Guatemala	2002	Wheat flour, corn flour	180, 130
Honduras	2002	Wheat flour, corn flour	180, 130
Nicaragua	1998	Wheat flour, corn flour	40–80, 40–80
Panama	2002	Wheat flour, corn flour	180, 130
Paraguay	2002	Wheat flour, corn flour	180, 130
	1998	Wheat flour	300
South East Asia			
Indonesia	Unknown	Enriched wheat flour	200

Modified from Australia New Zealand Food Standards Agency (2004)

Fortification of bread, to a level of about 120 μg/100 g, is less widespread (Department of Health, 2000). Some low-fat spreads have been fortified to a level of 200 μg per 20-g portion. Folic acid supplements are sold in supermarkets, other retail outlets and pharmacies, with maximum daily doses of up to 500 μg folic acid (Food Safety Authority, 2002a).

The intake of folates was assessed on the basis of the National Diet and Nutrition Survey performed in 2000–1 among adults aged 19–64 years (Food Safety Authority, 2003). Overall, the consumption of dietary supplements increased the average intake of folates from food sources alone from 344 μg to 359 μg for men and from 251 μg to 292 μg for women. The contribution of supplements was most

Table 3. Folate intake (μg) from all sources and from food sources (excluding supplements) in Ireland among persons aged 18–64 years

Source	Men (n = 662)	Women (n = 717)
All sources		
Mean	332	260
SD	128	144
Median	309	225
Percentile		
5th	164	126
95th	576	532
97.5th	662	638
Food		
Mean	319	225
SD	117	77
Median	300	212
Percentile		
5th	162	123
95th	516	368
97.5th	595	418

From Irish Universities Nutrition Alliance (2001); SD, standard deviation

marked for women aged 50–64 years, with an increase over that from food sources alone of 34% (from 268 μg to 359 μg). No estimates were available for high percentile intake of folic acid in the United Kingdom.

The main source of folates in the diets of respondents were cereals and cereal products, which provided 33% of the mean daily intake, with just under half, 15%, from breakfast cereals. Other major sources were vegetables, potatoes and savoury snacks and drinks, including beer and lager.

In 2002, the Food Standard Agency (2002b) discussed mandatory fortification of flour with folic acid and assessed the potential impact of varying the fortification rate on the intakes of population groups. In particular, they examined the potential risk that high intakes of folic acid would mask vitamin B12 deficiency in older persons. According to Clarke et al. (2004), this deficiency affects about 5% of persons aged 65–74 years and > 10% of persons aged 75 years or older. On this basis, the Food Safety Authority decided not to recommend mandatory fortification.

USA

Intake of folate was assessed on the basis of the Continuing Survey of Food Intakes by Individuals, conducted in 1996 on 5188 persons of all ages (United States Department of Agriculture, 1997). The mean folate intakes are presented in Table 4.

In the Boston Nutritional Status Survey on use of folic acid supplements by elderly men and women, the median intake of folate from supplements was 400 μg/day; the 95th percentile intake was 2400 μg/day for men and 1000 μg/day for women (Institute of Medicine, 1998).

Addition of folic acid to all enriched cereal-grain foods (which are supplemented with iron, thiamin, riboflavin and niacin) was mandated by the Food and

Table 4. Folate intake in the USA, 1 day

Sex	Age group (years)	Folate intake (μg)
Male and female	≤ 5	188
Male	6–11	263
	12–19	292
	≥ 20	303
Female	6–11	232
	12–19	247
	≥ 20	228
All persons		256

Modified from United States Department of Agriculture (1997)

Drug Administration (1996) and initiated in 1998. The amount of folic acid added to flour, rice, breads, rolls and buns, pasta, corn grits, cornmeal, farina, macaroni and noodle products is 95–309 μg/100 g of product. This range of fortification was selected on the basis of a target level of 140 μg/100 g of the cereal-grain product, to increase typical folate intake by about 100 μg/day, with a minimal intake of > 1 mg/day. Lewis et al. (1999) estimated the total folate intakes of various population groups, including children, on the basis of the results of national food consumption surveys, with corrections to reflect the required levels of folic acid to be added to foods. Their estimates, which are based on theoretical, not measured, values, suggested that 15–25% of children between the ages of 1 and 8 years could have intakes of folic acid that surpass the tolerable upper intake levels of 300 μg/day for 1–3-year-olds and 400 μg/day for 4–8-year-olds, established on the basis of body weight (Institute of Medicine, 1998; Scientific Committee for Food, 2000; FAO/WHO, 2001). The intakes exceeded predictions by an average of almost 200 μg of folate per day across all sectors of the community, including the target group of women of reproductive age (Choumenkovitch et al., 2002; Quinlivan & Gregory, 2003).

Folate was measured by a microbiological assay with trienzyme digestion in 150 enriched cereal-grain products and other products fortified with folic acid and available on the market in the USA (Rader et al., 2000). For each product, the measured amount of total folate was compared with the amounts declared on the label and the amounts of folic acid required by regulation. In a considerable number of the food products analysed, the measured amount of folate was appreciably higher than the folic acid levels required by regulation.

The Framingham Offspring cohort study showed that the prevalence of older persons who did not use supplements and who consumed less than the estimated average requirement of folate (defined as 320 μg folate equivalent per day) decreased from 49% before mandated folic acid fortification to 7% afterwards. The intake was > 1 mg only for persons who regularly consumed B-vitamin supplements containing folic acid and products fortified with folic acid. The proportion of persons who exceeded this limit rose from 1.3% before fortification to 11.3% afterwards (Choumenkovitch et al., 2002).

A study of 1573 mainly African–American women and men, who are more susceptible to pernicious anaemia than other ethnic groups showed that the proportion who had poor vitamin B12 status without anaemia did not change significantly between the pre-fortification period (39%) and after full implementation

of mandatory fortification (38%). The authors concluded that mandatory fortification did not increase the prevalence of masking of vitamin B12 deficiency (Mills et al., 2003). The introduction of mandatory fortification was found to increase the number of persons who were at risk for masking of vitamin B12 deficiency, but the value remained below 1%, and no actual cases of masking were reported in the USA.

Other countries

The average folate intake in Europe is about 300 µg/day for men and 250 µg for women (de Bree et al., 1997). High intake levels (97.5th percentile) of folate from dietary sources of around 500 µg/day have been reported. Data from the Second Dutch National Food Consumption Survey on supplement use indicated that the mean folic acid intake from supplements among users was 100 µg, with a 97.5th percentile intake of 400 µg and a maximum intake of 800 µg (Ronda et al., 1996). In Germany, the median intake of folate from natural sources was about 250 µg in adults; only a minority of the population took vitamin supplements regularly, but, among those who did, supplementation accounted for about 60% of total intake (Gonzalez-Gross et al., 2002).

3.2.2 Intake of calcium from calcium L-5-methyltetrahydrofolate

The specifications for purity provided by the producer suggest that the percentage of calcium in calcium L-5-methyltetrahydrofolate is 7.0–8.5%. Intake of calcium from calcium L-5-methyltetrahydrofolate would amount to 0.08 mg per adult per day if the intake of this compound provided 1 mg of folate per day, the tolerable upper intake level for folic acid. This intake of calcium is insignificant in comparison with the tolerable upper intake levels for calcium set by the European Union Scientific Committee for Food (2003), the United States Institute of Medicine (1997) and the FAO/WHO Consultation on Human Vitamin and Mineral Requirements (FAO/WHO, 2001), i.e. 2.5– 3 g per person per day.

4. COMMENTS

Toxicological data

Studies in humans indicate that L-5-methyltetrahydrofolic acid is the only form of folate normally taken up by cells and appearing in plasma, and that cellular uptake is mediated by a reduced folate carrier and folate receptors, which are integral plasma membrane proteins. At exposure to folate of > 200–300 µg/day per person, the metabolic capacity of the human intestinal mucosa for folic acid begins to be exceeded, resulting in small amounts of unaltered folic acid in circulating blood.

In humans given ^{3}H- and ^{14}C-folic acid orally, the bioavailability of synthetic folic acid was estimated to be 90–95%. A study of the absorption of calcium L-5-methyltetrahydrofolate indicated that it dissociates in aqueous media into Ca^{2+} and L-5-methyltetrahydrofolic acid. After absorption, the latter enters the circulation directly, becoming indistinguishable from other absorbed and metabolized natural folates or from L-5-methyltetrahydrofolate formed from synthetic folic acid. The bioavailability of calcium L-5-methyltetrahydrofolate and synthetic folic acid (400 µg/day per person as folate) was compared in a randomized, double-blind,

cross-over study of 21 healthy women. The bioavailability of the two compounds was found to be similar. In a 24-week placebo-controlled study in women, the appearance of folate derived from equimolar concentrations of calcium L-5-methyltetrahydrofolate and folic acid was compared in plasma and erythrocytes by a microbiological assay: similar values were found for the two supplements.

A comparison of the bioavailability of naturally occurring folate from food and synthetic folic acid in humans showed significant differences, with a lower level of bioavailability from natural folate than from synthetic folic acid. Another study found that synthetic folic acid appeared in human plasma more slowly than natural folate. The Committee noted that the differences in bioavailability might reflect rate-limiting kinetics for the metabolic conversion of folic acid to L-5-methyltetrahydrofolic acid, as low doses of radiolabelled compounds showed similar short-term distribution, metabolism and kinetics in vivo. Moderately high doses (several hundred micrograms) of folic acid are likely to result in significant hepatic uptake, enterohepatic circulation, tissue distribution and urinary reabsorption.

Calcium L-5-methyltetrahydrofolate was not acutely toxic to rats after a single oral dose (LD_{50} > 2000 mg/kg bw): no gross changes in organs were observed at necropsy, and all animals gained weight and survived until end of the 15-day observation period. In a short-term study of toxicity in male and female Wistar rats given calcium L-5-methyltetrahydrofolate orally for 13 weeks, no adverse effects were seen. The NOEL was 400 mg/kg bw per day, the highest dose tested. A study of embryotoxicity and teratogenicity in Wistar rats given the compound showed no effects up to the highest dose tested (1000 mg/kg bw). The results of a battery of assays for genotoxicity in vitro and in vivo did not indicate any genotoxic potential.

No long-term studies of toxicity or carcinogenicity were submitted; however, the Committee noted that, given the well-characterized metabolism and nutritional function and the known fate of naturally occurring reduced L-5-methyltetrahydrofolic acid as an essential vitamin in humans, such studies were not required.

The Committee took note of a case report in which L-5-methyltetrahydrofolic acid did not mask the clinical features of vitamin B12 deficiency. Vitamin B12 is essential for the activity of methionine synthase, which converts homocysteine to methionine, with L-5-methyltetrahydrofolic acid as a co-factor. Recycling of homocysteine back to methionine is part of the methylation cycle necessary for methyltransferases, which methylate a wide range of substrates, such as hormones, lipids and proteins, including neural myelin basic protein. In vitamin B12 deficiency, the recycling of homocysteine back to methionine diminishes with the level of methionine synthase activity, resulting in neuropathy. Owing to the diminished activity of the enzyme, administration of L-5-methyltetrahydrofolic acid has no effect on the methylation cycle. Likewise, administration of synthetic folic acid has no effect on the methylation cycle because it is not a substrate of the enzyme. The pernicious anaemia arising from vitamin B12 deficiency is corrected by synthetic folic acid because it replenishes the supply of tetrahydrofolate and thereby restores the metabolic pathway leading to DNA biosynthesis and red blood cell formation. Folic acid does not restore methylation reactions via methionine synthase, so that neuropathy can progress in the absence of pernicious anaemia (masking). L-5-Methyltetrahydrofolic acid is not expected to correct the pernicious anaemia caused by vitamin B12 deficiency because diminished methionine synthase activity leads to failure to convert L-5-methyltetrahydrofolic acid to tetrahydrofolate, the pathway leading to DNA biosynthesis and red blood cell formation. No data were available to

determine whether long-term administration of L-5-methyltetrahydrofolic acid would not mask vitamin B12 deficiency.

Epidemiological studies provided evidence that high plasma homocysteine levels are a risk factor for cardiovascular disease. A meta-analysis of clinical trials showed that 0.5–5 mg of folic acid could reduce blood homocysteine concentrations by 25–33%. In three intervention studies, lasting up to 24 weeks, groups of healthy persons were given folic acid, vitamin B12 or calcium L-5-methyltetrahydrofolate at a dose of ≤ 950 µg per person per day. Significantly elevated plasma folate levels were found in response to folate treatment, accompanied by significantly lower levels of plasma homocysteine, ranging from 9% to 19%. Calcium L-5-methyltetrahydrofolate and synthetic folic acid had similar effects.

Methylenetetrahydrofolate reductase is a key enzyme in folate metabolism, converting methylenetetrahydrofolate to L-5-methyltetrahydrofolic acid. Persons homozygous for a mutation in the gene encoding 5-methyltetrahydrofolic acid reductase have decreased specific enzymatic activity (~ 34% of normal), lower plasma folate levels and higher plasma homocysteine concentrations than persons who express the wild-type gene. The prevalence of this mutant genotype is related to ethnic group, but elevated homocysteine levels are not a specific indicator of inadequate intake of folate.

In a series of studies with healthy persons who had not been not genotyped for 5-methyltetrahydrofolic acid reductase activity, L-5-methyltetrahydrofolic acid was found to be as effective as folic acid in lowering plasma homocysteine, at doses as low as 400 µg/day as folate.

In a case–control study of the risk for colorectal adenoma associated with two polymorphisms in thymidylate synthase, a key enzyme in folate metabolism downstream of L-5-methyltetrahydrofolic acid, an intake of folic acid > 440 µg/day was associated with a 1.5-fold increase in risk for colorectal adenomas in polymorphic individuals with a double-repeat in the enhancer region of the thymidylate synthase gene and an estimated threefold decreased risk in individuals with a more common triple repeat. The Committee noted the existence of several common inherited polymorphisms in folate-metabolizing enzymes; however, the influence and human health significance of such gene–nutrient interactions on overall folate status is unclear.

No controlled studies on human tolerance to calcium L-5-methyltetrahydrofolate were submitted to the Committee. Circumstantial evidence for high tolerance to the compound and to calcium DL-5-methyltetrahydrofolate was provided by studies in which oral doses of 15–17 mg/day for up to 6 months were given to haemodialysis or psychiatric patients. Although no toxic effects were reported, the scope and design of the studies were inadequate to contribute to a safety evaluation.

Assessment of dietary exposure

Both in Europe and the USA, the average folate intake from food sources is about 300 µg/day for men and 250 µg/day for women. Assessments of exposure to folate were available from three countries with a history of supplementation and food fortification with folic acid. It was assumed that calcium L-5-methyltetrahydrofolate would be substituted for synthetic folic acid in the same products and at the same levels. Supplementation leads to increases in the average intake of folate in the adult population of 15–90 µg/day (Ireland and the United Kingdom), and

mandatory fortification of foods could increase the average intake of folate by about 200 µg/day (USA). Overall intake from natural foods, fortified foods and supplements could reach 1 mg or more per day for some segments of the adult population.

The calcium provided by 1 mg of calcium L-5-methyltetrahydrofolate amounts to 0.08 mg per adult per day which is insignificant in comparison with the tolerable upper intake levels for calcium.

5. EVALUATION

The Committee concluded that, in humans, the bioavailability of calcium L-5-methyltetrahydrofolate is similar to that of folic acid and that synthetic calcium L-5-methyltetrahydrofolate has the same metabolic fate as other absorbed natural folates. The Committee evaluated the intended use of calcium L-5-methyltetrahydrofolate as a substitute for folic acid but did not evaluate the safety of folate fortification and supplementation. The Committee had no concern about the safety of the proposed use of calcium L-5-methyltetra-hydrofolate in dry crystalline or microencapsulated form as an alternative to folic acid in dietary supplements, foods for special dietary uses and other foods.

In view of a number of common inherited polymorphisms in folate metabolism, the Committee recommended that the health effects of folates be evaluated further when there is better understanding of the role of relevant genetic polymorphisms in the population.

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