The online version of this article is located at http://www.minervamedica.it

Minerva Ginecologica 2016 April;68:99-105

ORIGINAL ARTICLE

Enhanced oral bioavailability of a novel folate salt: comparison with folic acid and a calcium folate salt in a pharmacokinetic study in rats

Niccolò MIRAGLIA [1]*, Marco AGOSTINETTO [1], Davide BIANCHI [1], Ermanno VALOTI [2]

[1]Gnosis S.p.A., Desio, Monza Brianza, Italy; [2]Department of Pharmaceutical Sciences, University of Milan, Milan, Italy

*Corresponding author: Niccolò Miraglia, Gnosis S.p.A, Via Lavoratori Autobianchi 1, 20832, Desio (MB), Italy.
E-mail: niccolo.miraglia@gnosis-bio.com

ABSTRACT

BACKGROUND: Folates play an important role to prevent neurological disorders in embryo development and in cardiovascular diseases. Folate supplementation is suggested, particularly in females of childbearing age, for the prevention of embryonal NTDs during pregnancy. Folic acid and reduced folate ((6S)5-MTHF) are currently used in supplementation. The aim of this study was to compare the bioavailability of Quatrefolic®, a novel patented (6S)5-MTHF glucosamine salt, with (6S)5-MTHF calcium salt and folic acid in Sprague Dawley rats.

METHODS: Fifty-four to fifty-five-day old male Sprague-Dawley rats were divided in 3 treatment groups, each comprising 6 animals, receiving folic acid, (6S)5-MTHF calcium salt or Quatrefolic® at the dose of 70 µg/kg of (6S)5-MTHF equivalents in a single oral administration. Folates were determined in plasma with a HPLC method employing fluorimetric detection. (6S)5-MTHF level was chosen as a convenient end point to evaluate folate absorption. The main pharmacokinetic parameters were calculated (C_{max}, t_{max}, AUC).

RESULTS: Quatrefolic® administration produced a plasmatic (6S)5-MTHF concentration peak (C_{max}: 879.6±330.3 ng/mL) 1.8 times higher than (6S)5-MTHF Ca salt (486.8±184.1 ng/mL), and 3.1 times higher than folic acid supplementation (281.5±135.7 ng/mL), while t_{max} values were similar for the three folate forms. Quatrefolic® supplementation showed AUC_{8h} (1123.9 ng/mL · h) 9.7 times higher than folic acid (114.7 ng/mL · h) and 1.12 times higher than (6S)5-MTHF Ca salt (997.6 ng/mL · h).

CONCLUSIONS: Quatrefolic® has demonstrated an enhanced oral bioavailability in comparison to other reduced folates and to folic acid in rats.

(*Cite this article as:* Miraglia N, Agostinetto M, Bianchi D, Valoti E. Enhanced oral bioavailability of a novel folate salt: comparison with folic acid and a calcium folate salt in a pharmacokinetic study in rats. Minerva Ginecol 2016;68:99-105)

Key words: Neural tube defects - Folic acid - Pharmacokinetics.

Folates occur naturally in eukaryotes and are essential cofactors involved in the enzymatic reactions in metabolism and metabolic regulation of amino acids and nucleic acids. Low levels of folates in blood are associated to neurological disorders and to cardiovascular diseases. A low-folate status can cause megaloblastic anaemia and can increase the risk of neural tube defects (NTDs) in embryos. NTDs are a group of conditions, caused by a failure of the neural tube to close, in which an opening in the spinal cord or brain remains in early human development resulting in spina bifida and anencephaly, the most frequent diagnostic categories in NTDs. In the third week of pregnancy, during the embryo phase of gastrulation, specialized cells on the dorsal side of the embryo begin to form the neural tube. When

the neural tube does not close completely, an NTD develops. NTDs are one of the most common birth defects, affecting over 300,000 births each year worldwide. Spina bifida affects approximately 0.1% of births in the US, 0.5% in Europe and 1.1% in China.

Folate intake in ordinary western diet is quite poor and folate supplementation is strongly suggested, particularly in females of childbearing age. Folic acid supplementation is widely used as a source of folates for the prevention of embryonal NTDs during pregnancy. Its effectiveness has been confirmed by population studies in different countries (Canada and USA) before and after food fortification.[1, 2] Folic acid or folate supplementation is also important for lowering plasma homocysteine levels;[3, 4] a high plasma homocysteine level is associated with arterial plaque formation and is an important risk factor for cardiovascular diseases (CVD). FDA has established a reference daily folate intake of 400 µg for adults and, particularly, of 600 µg for pregnant women. Many countries adopt food fortification in order to enhance folate plasma level in the population.

Folic acid does not occur naturally, it is a synthetic form of folate; it is stable and easily absorbed but is not directly endowed of biological activity. Natural folates are present in the diet even if their amount is not sufficient to reach the suggested intake. Vegetables and fruits are particularly reach of folates among foods. The most abundant natural folates are (6S)5-methyl tetrahydrofolate ((6S)5-MTHF) and 10-formyl tetrahydrofolate. Folic acid is reduced during absorption, by mucosal cells, to tetrahydrofolate (THF) and then converted to (6S)5-MTHF by the enzyme methylenetetrahydrofolate reductase (MTHFR). (6S)5-MTHF is the active form of folate at the cellular level and is the form found in circulation; it is transported across membranes into tissues and is the only form of folate able to cross the blood-brain barrier. From a molecular biology point of view, (6S)5-MTHF plays a role as methyl group donor, in the so called one-carbon metabolism, being involved in important cell functions like DNA synthesis and methylation of homocysteine to methionine.

Both (6S)5-MTHF and folic acid are absorbed at the small intestine mucosal level by an active carrier-mediated transport.[5, 6] A passive diffusional absorption also occurs in the same digestive tract and in the distal part of the intestine. Folic acid is rapidly converted in (6S)5-MTHF at the mucosal level.

The use of folic acid has some disadvantage. The enzymatic conversion of folic acid into (6S)5-MTHF is a saturable process and the capacity of mucosal cells to convert folic acid is limited. Thus, the excessive intake is absorbed as unaltered folic acid appearing in blood as is. This unmodified folic acid can be incorporated in tissues and be reduced in a vitamin B_{12} independent pathway to THF so masking a vitamin B_{12} deficiency. If not correctly diagnosed, vitamin B_{12} deficiency can result in irreversible neuropathy. Furthermore, between 5 and 15% of the world population are homozygous for a genetic variant of MTHFR (TT mutation), being unable to convert folic acid. Finally, bioavailability of folic acid is lower than that of (6S)5-MTHF.[7]

These disadvantages can be overcome by supplementation with (6S)5-MTHF instead of folic acid. (6S)5-MTHF calcium salts have proved its efficacy both in animals [8] and in humans [9] and many products based on (6S)5-MTHF are available on the market. (6S)5-MTHF calcium salt is stable but is characterized by a poor solubility in water. Quatrefolic® is a novel, patented glucosamine salt of (6S)5-MTHF, developed by Gnosis (WO/2009/103334). It is a glucosamine salt characterized by a long lasting stability and a peculiarly high solubility in water. These features confer to Quatrefolic® a potential improved bioavailability in oral use, and for this reason it has been tested in a pharmacokinetic study in the rat. In this study a comparative pharmacokinetic study in Sprague Dawley rats is described in which folic acid and the two different (6S)5-MTHF salts have been administered in single dose. The aim of this study was to compare the bioavailability of Quatrefolic® with that of a (6S)5-MTHF calcium salt

and with folic acid. This study was conceived as preparatory to a clinical study in healthy volunteers.

Materials and methods

Reagents

Folic acid, (6S)5-MTHF Ca, (6S)5-MTHF glucosamine (Quatrefolic®) used for rats administration were produced by Gnosis. (6S)5-MTHF Ca used as the analytical standard was produced by Gnosis and characterized as a primary reference standard (HPLC, NMR, mass spectrometry). Ascorbic acid was purchased from Fluka (Buchs, SG, Switzerland), ortophosphoric acid from Carlo Erba Reagents (Cornaredo, MI, Italy) and reagent grade acetonitrile from Sigma Aldrich (St. Louis, MO, USA).

Animals

Male Sprague-Dawley rats, 54-56-day old, weighing 170/200 g were purchased from Harlan Italy s.r.l. (San Pietro al Natisone, UD, Italy). Sprague-Dawley rats were chosen because are recognized as the species and the strain of election and accepted by regulatory authorities. The study was carried out at the Research Toxicology Center (RTC, Pomezia, Rome, Italy), a facility acting in compliance with the principles of Good Laboratory Practice (GLP) of the OECD. The study was carried out in compliance with the requirements of Commission Directive 86/609/EEC and harmonized in Decreto Legislativo No. 116 of 27 January 1992 concerning the protection of animals used for experimental and scientific purposes. The aspects of the protocol concerning animal welfare were approved by the RTC's Ethical Committee.

Animals were housed in a limited access rodent facility with controlled temperature and relative humidity of 22±2 °C and 55±15% respectively. Actual conditions were monitored and recorded. Approximately 15 to 20 air changes per hour were assured and the rooms were lit by artificial light for 12 h each day. The animals were housed up to 3 in a clear polycarbonate cage measuring 59x38.5x20 cm, with a stainless steel mesh lid and floor. Each cage tray hold absorbent paper which was inspected and changed at least 3 times a week. Drinking water was supplied ad libitum to each cage via water bottles. A commercially available laboratory rodent diet (4 RF 21) (Mucedola S.r.l., Settimo Milanese, Milan, Italy) was offered ad libitum throughout the study.

On the day of allocation all animals were weighed. Rats were allocated to the groups by computerized stratified randomization to give approximately equal initial group mean body weights.

Treatment

Rats were divided in 3 treatment groups, each comprising 6 male rats/group. Group I received folic acid, group II (6S)5-MTHF calcium salt and group III (6S)5-MTHF glucosamine salt (Quatrefolic®) (Table I). Rats were administered orally, by capsule, with an amount equivalent to 70 µg/kg of (6S)5-MTHF in a single oral administration. The dose was chosen on the basis of previous human pharmacokinetic studies and was calculated in order to have a similar dose pro-kg in rats [10] and was administered to each animal on the basis of the body weight recorded on the day of dosing. One capsule was administered to each animal in a single administration. The actual amount of folic acid or (6S)5-MTHF in the capsule was measured by HPLC before administration.

TABLE I.—*Experimental scheme for the pharmacokinetic study in Sprague Dawley rats.*

Group	Supplementation (70 µg/kg b.w. (6S)5-MTHF equiv.)	Bleeding times (hours)
I	Folic acid	pre-dose, 0.5, 1, 2, 4, 8
II	5-MTHF Ca salt	pre-dose, 0.5, 1, 2, 4, 8
III	Quatrefolic®	pre-dose, 0.5, 1, 2, 4, 8

All animals were checked in the morning after dosing and again in the afternoon for signs of reaction to treatment.

Blood samples were withdrawn from the tail vein before and 0.5, 1, 2, 4 and 8 hours after administration. Each animal was bled three times. The sampling points at pre-dose, 1 and 4 hours were drawn from 3 animals for each group, the sampling points at 0.5, 2 and 8 hours were drawn from the other 3 animals for each group, in order not to exceed with blood sampling according to GLP principles; for this reason no complete individual concentration profiles are available, but only mean values were considered to construct profiles. Blood samples of at least 0.8 ml each were drawn from the tail vein and transferred into polypropylene tubes containing lithium heparin anticoagulant until centrifugation. Samples were centrifuged at 3000 rpm (about 1760 x g) for 10 minutes in a microcentrifuge Eppendorf model 5430 (Eppendorf, Hamburg, Germany). After centrifugation the plasma was separated in 2 aliquots and stored at -80 °C pending analysis.

Folate measurements

Folates were determined with a HPLC method employing fluorimetric detection as previously described.[11] Prior to analysis, plasma samples were incubated with 10 mg/ml ascorbic acid solution (pH 4.5) under stirring for 1 hour at 25 °C in order to allow the plasma carboxypeptidase to convert the folate polyglutamates to monogultamates; at the end of incubation, 60% perchloric acid was added in order to inactivate carboxypeptidase and samples were kept at -20 °C. Samples were thawed and centrifuged in the microcentrifuge (13000 rpm for 10 min) at 4 °C to remove precipitated proteins. A 30 mg/mL ascorbic acid solution was added to supernatant. Samples were analyzed in HPLC Agilent 1100 series (Agilent, Santa Clara, CA, USA) in isocratic conditions, using a C18 column (ACE C18, 5 µm, 250x4.6 mm), kept at 25 °C, with a flow of 0.8 mL/min and using ortho-phosphoric acid solution pH 2.3 as mobile phase added with 8% acetonitrile, as an organic modifier as previously described. The HPLC was equipped with a fluorimetric detector Agilent 1200 Infinity using an excitation wavelength of 295 nm and an emission wavelength of 360 nm.

Pharmacokinetic parameters

(6S)5-MTHF plasma concentration was determined, reading peak absorbance against a standard curve obtained injecting in HPLC standard (6S)5-MTHF Ca at different concentration levels. A pharmacokinetic profile was obtained for the three products using mean concentration values at each sampling point. The area under the curve (AUC), C_{max} and t_{max}, were calculated as main pharmacokinetic parameters. Plasmatic (6S)5-MTHF level was chosen as a convenient end point to evaluate folate absorption being this the active form found in plasma. AUC, the parameter related to the total amount of absorbed compound, was calculated with the trapezoidal rule of Gibaldi and Perrier (1982). C_{max} is the maximum concentration reached in plasma, while t_{max} is the time to reach C_{max}. This last parameter is correlated to the absorption rate. All these parameters were calculated from the concentration profile measured.

Statistical analysis

ANOVA test was used for statistical analysis using Statgraphics program.

Results

(6S)5-MTHF plasma concentration for the three groups are reported in Table II. The values reported are the mean (6S)5-MTHF concentration values±standard deviation, determined at each point. Basal folate concentration resulted quite high. This is due to the presence of folate in the standard diet for laboratory rodents. Nonetheless, the three treatments produced an appreciable increase in folate level in plasma. An absorption profile was determined on the basis of (6S)5-MTHF levels (Figure 1). The plasmatic (6S)5-MTHF peak in the group of rats receiving folic acid supplementation was lower than

TABLE II.—*(6S)5-MTHF plasma levels in rats following different folate oral administrations.*

Group	Supplementation	time (hours)					
		0	0.5	1	2	4	8
I	Folic acid	168.4	228.2	281.5	163.0	167.2	182.3
	SD	33.2	16.3	135.7	82.9	49.7	55.5
II	5-MTHF Ca salt	165.9	437.2	486.8	300.0	257.6	238.4
	SD	89.4	30.1	184.1	115.6	94.7	48.4
III	Quatrefolic®	143.3	531.5	879.6*	315.7	159.5	178.5
	SD	17.7	298.2	330.3	55.3	22.6	71.9

* Statistically significant at ANOVA test ($P < 0.05$) - group III *vs*. group I

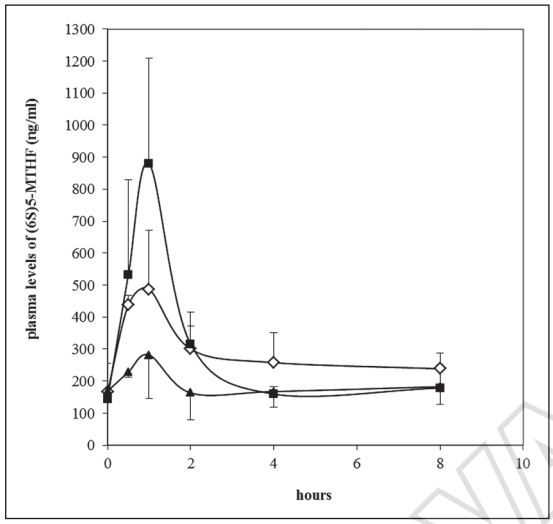

Figure 1.—Plasma concentration (ng/ml) of (6S)5-MTHF in rats receiving 70 µg/kg body weight (6S)5-MTHF equivalents of folic acid (full triangles), (6S)5-MTHF Ca salt (empty rhombs) or Quatrefolic® (full squares) in single dose.

in the groups receiving a reduced folate supplementation; in folic acid supplementation group, (6S)5-MTHF concentration rapidly returned to basal level within two hours from administration. (6S)5-MTHF level measured in the plasma of the rats that received (6S)5-MTHF Ca salt supplementation was higher than the folic acid group, with a value about 1.7 times higher (486.8±184.1 ng/mL) (6S)5-MTHF concentration in plasma, after (6S)5-MTHF Ca salt administration, resisted for a longer time showing elevated values until four hours from administration and remaining higher than basal level even at 8 hours from administration (238.4±48.4 ng/mL). Supplementation with Quatrefolic® produced a (6S)5-MTHF concentration peak of 879.6±330.3 ng/mL. The peak was reached one hour after Quatrefolic® administration, reaching the highest plasma concentration observed among treatments. Quatrefolic® related (6S)5-MTHF level returned to baseline values more rapidly than for calcium salt, showing concentration close to the basal level after four hours from administration.

Comparing (6S)5-MTHF salts, Quatrefolic® administration produced a maximum (6S)5-MTHF plasma increase, 1.8 times higher than (6S)5-MTHF Ca salt being their peak values respectively 879.6±330.3 ng/mL and 486.8±184.1 ng/mL. Quatrefolic® related peak concentration (Table II) resulted more than three times higher than the value related to folic acid administration (281.5±135.7 ng/mL) showing a statistically significant difference to the ANOVA test ($P<0.05$). Concerning the time to reach the concentration peak (t_{max}), no differences were seen among treatments; t_{max} values were quite similar (Table III). This seems to indicate that speed of absorption is not affected by supplementation with the different folate forms, while the total amount absorbed is.

AUC values were also calculated from plasma (6S)5-MTHF concentration values. AUCs were calculated from the start of treatment and

TABLE III.—*Main pharmacokinetic parameters evaluated*

Parameter	5-MTHF Ca	Quatrefolic®	Folic acid
C_{max} (ng/ml)	486.8	879.6	281.5
t_{max} (hours)	1.0	1.0	1.0
AUC_{8h} (ng/ml h)	997.6	1123.9	114.7

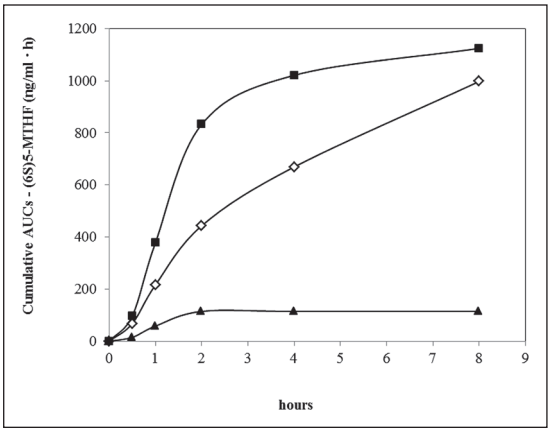

Figure 2.—Cumulative AUC values (ng/mL · h) in rats receiving 70 µg/kg body weight (6S)5-MTHF equivalents of folic acid (full triangles), (6S)5-MTHF Ca salt (empty rhombs) or Quatrefolic® (full squares) in single dose.

the final point for measure (8 hours). To better evaluate the folate concentration attributable to supplementation, the average basal (6S)5-MTHF value of each group was subtracted from the concentration measured at the different points. Figure 2 shows the cumulative folate absorbed upon administration of the different forms of folate, expressed as AUC value versus time. Quatrefolic® supplementation shows the better total accumulation of (6S)5-MTHF in plasma with a final AUC_{8h} of 1123.9 ng/mL · h, a value 9.7 times higher than folic acid (114.7 ng/mL · h) and also 1.12 times higher than (6S)5-MTHF Ca salt (997.6 ng/mL · h) (Table III). The curve of (6S)5-MTHF raising in circulation are quite different for the three forms of folate (Figure 2).

Discussion

The purpose of the present investigation was to compare the bioavailability of Quatrefolic®, a patented folate new salt of glucosamine (WO/2009/103334) in rats following oral administration in comparison with either folic acid or (6S)5-MTHF Ca salt supplementation. A single oral dose of 70 µg/kg of folate was administered to rats fed with a standard diet; all folates supplementations were able to increase (6S)5-MTHF plasma concentration. (6S)5-MTHF is the main and nearly the only form of circulating folate in the blood stream. Even in folic acid supplementation, folic acid is enzymatically converted in (6S)5-MTHF by the mucosal cells even if this conversion is sometimes incomplete. (6S)5-MTHF concentration was monitored in order to evaluate the efficacy of the different folate supplementations, being this species the active folate found in plasma.

For this purpose 6 male rats were administered with a single dose of folate and bled at different time points within 8 hours from administration (Table I).

The two groups receiving reduced folates, namely (6S)5-MTHF glucosamine salt (Quatrefolic®) and calcium salt both showed a better pharmacokinetic profile if compared to folic acid supplementation even if only Quatrefolic® showed a statistically significant difference; this result confirm previous studies both in animals or in humans.[10] Quatrefolic® showed to have a better pharmacokinetic profile even if compared to (6S)5-MTHF Ca salt supplementation. The generation of a higher concentration peak in plasma after the administration of a (6S)5-MTHF salt rather than folic acid was already been observed.[7] (6S)5-MTHF showed a better pharmacokinetics either at physiological or over-physiological doses.[7, 10] In this study, Quatrefolic® supplementation resulted in a plasmatic (6S)5-MTHF concentration peak higher than (6S)5-MTHF Ca salt supplementation, even if not statistically significant (Figure 1, Table II). A better bioavailability of Quatrefolic® results comparing C_{max} values, being this parameter 1.8 times higher for Quatrefolic® than for (6S)5-MTHF Ca salt treatment, and up to about 3.1 times higher than folic acid (Table II). The reason for that is probably related to the higher water solubility of Quatrefolic® in comparison with (6S)5-MTHF Ca salt. The solubility of the (6S)5-MTHF Ca salt, as well as that of folic acid, is known to be a concern, and is a drawback in the use of folates for food supplementation. Even if simple diffusion is present in (6S)5-MTHF intestinal absorption, the main intestinal absorption route is an active transport mediated by a carrier mainly occurring at the small intestine mucosal level. The fact that a more soluble salt of a reduced folate is able

to produce a better mucosal absorption, seems to indicate that the transport system is not saturated at the dose used in this study. Being this dose higher than the usual recommended dose for folate supplementation, this indicates that a better solubility may reflect in a global better bioavailability of folate supplementation. Supplementation with (6S)5-MTHF glucosamine salt (Quatrefolic®) is potentially able not only to overcome the main disadvantages of folic acid supplementation, like vitamin B_{12} deficiency masking and the inefficacy in the presence of the homozygous state of the mutated MTHFR (TT mutation), but is also able to overcome the drawback of the current supplementation with (6S)5-MTHF Ca salt, which is characterized by a poor solubility in aqueous environment. The pharmacokinetic data of this study show that (6S)5-MTHF disappears from plasma more rapidly in Quatrefolic® than in (6S)5-MTHF Ca salt supplementation, returning to basal levels in a shorter time. This may indicate a more rapid uptake and utilization by tissues, or a more rapid elimination for Quatrefolic® or else, a prolonged intestinal absorption for Ca salt. Anyway, AUC_{8h}, the parameter related to quantitative folate absorption, results to be 1.12 times higher (13%) in Quatrefolic® treatment than in (6S)5-MTHF Ca salt treatment and up to 10 times higher than in folic acid treatment (Table II, Figure 2). This is a clear indication of a higher overall intestinal absorption of Quatrefolic® *versus* other folates.

Conclusions

The pharmacokinetic study in rats has demonstrated an enhanced oral bioavailability for Quatrefolic®, a novel salt of reduced folate, when compared to other reduced folates or to folic acid, the common precursor of (6S)5-MTHF. This seems to confirm the hypothesis that the higher water solubility of Quatrefolic® produces a higher intestinal absorption. This positive result observed in rats enabled us to further investigate this aspect of Quatrefolic® in a clinical study involving healthy volunteers.

References

1. De Wals P, Tairou F, Van Allen MI, Uh SH, Lowry RB, Sibbald B et al. Reduction in Neural-Tube Defects after Folic Acid Fortification in Canada. N Engl J Med 2007;357:135-42.
2. Honein MA, Paulozzi LJ, Mathews TJ, Erickson JD, Wong LYC. Impact of folic acid fortification of the us food supply on the occurrence of neural tube defects. JAMA 2001;285:2981-6.
3. Lamers Y, Prinz-Langenohl R, Moser R, Pietrzik K. Supplementation with [6S]-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. Am J Clin Nutr 2004;79:473-8.
4. Zappacosta B, Mastroiacovo P, Persichilli S, Pounis G, Ruggeri S, Minucci A, *et al.* Homocysteine lowering by folate-rich diet or pharmacological supplementations in subjects with moderate hyperhomocysteinemia. Nutrients 2013;5:1531-43.
5. Visentin M, Diop-Bove N, Zhao R, Goldman ID. The intestinal absorption of folates. Annu Rev Physiol 2014;76:251-74.
6. Said HM, Nguyen TT, Dyer DL, Cowan KH, Rubin SA. Intestinal folate transport: identification of a cDNA involved in folate transport and the functional expression and distribution of its mRNA. Biochim Biophys Acta 1996;1281:164-72.
7. Prinz-Langenohl R, Brämswig S, Tobolski1 O, Smulders YM, Smith DEC, Finglas PM, *et al.* [6S]-5-methyltetrahydrofolate increases plasma folate more effectively than folic acid in women with the homozygous or wild-type 677C,T polymorphism of methylenetetrahydrofolate reductase. Br J Pharmacol 2009;158:2014-21.
8. Perez-Conesa D, Haro-Vicente JF, Braquehais FR, Ros G. [6S]-5-Methyltetrahydrofolate enhances folate status in rats fed growing-up milk. Eur J Nutr 2009;48:365-71.
9. Lamers Y, Prinz-Langenohl R, Bramswig S, Pietrzik K. Red blood cell folate concentrations increase more after supplementation with [6S]-5-methyltetrahydrofolate than with folic acid in women of childbearing age. Am J Clin Nutr 2006;84:156-61.
10. Willems FF, Boers GHJ, Blom HJ, Aengevaeren WRM, Verheugt FWA. Pharmacokinetic study on the utilisation of 5-methyltetrahydrofolate and folic acid in patients with coronary artery disease. British J of Pharmacol 2004;141:825-30.
11. Leeming RJ, Pollock A, Melville LJ, Hamon CGB. Measurement of 5-methyltetrahydrofolic acid in man by high-performance liquid chromatography. Metabolism 1990;39:902-4.

Aknowledgments.—We wish to thank the Management of Research Toxicology Centre S.p.A. (RTC), Pomezia, Italy, for managing and treating the animals in this study.

Conflicts of interest.—Niccolò Miraglia, Marco Agostinetto and Davide Bianchi are employees of Gnosis S.p.A., Italy, which produces Quatrefolic®. Ermanno Valoti is Professor at the University of Milano, Italy. The authors declare that there is no conflict of interest with any material discussed in this manuscript. D. Bianchi and E. Valoti are co-inventors on a patent on folate compositions and their use.

Manuscript accepted: February 15, 2016. - Manuscript received: February 9, 2016.