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Folates as antioxidants

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Abstract

Folic acid (FA) and its physiological reduced forms: 7,8-dihydrofolate (DHF), 5,6,7,8-tetrahydrofolate (THF) and 5-methyltetrahydrofolate (5-MTHF) were studied for their antioxidant activities using trolox equivalent antioxidant capacity (TEAC) assay with $ABTS^{\bullet+}$ radical cation, 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay and ferric reducing antioxidant power (FRAP) assay. Moreover, literature data on peroxynitrite (PON) scavenging activity and inhibition of lipid peroxidation (LPO) by folates are included. It was found that reduced forms of FA have approximately 3.5–7.5 fold, 12–16 fold and 44–71 fold higher activities than those of FA in the DPPH, TEAC and FRAP assays, respectively. Their antioxidant activities are comparable to those of vitamin C and E, which are commonly accepted as the effective water and lipid soluble antioxidants. Although natural folates cannot be really considered as food antioxidants, they may act as effective antioxidants in vivo. Their activities may become important in view of nutritional supplementation and fortification of food with FA.

Keywords: Folates; Vitamins; Antioxidant activity

1. Introduction

In a previous paper (Gliszczyńska-Świgło, 2006), antioxidant activities of folic acid, thiamine, pyridoxine, pyridoxal and pyridoxamine in the trolox equivalent antioxidant capacity (TEAC) and the ferric reducing antioxidant power (FRAP) assays were reported as a starting point in more detailed study concerning antioxidant activities of water soluble vitamins. From among all vitamins tested only folic acid (FA) showed antioxidant activity in both assays.

FA (pteroyl-L-glutamic acid, folate, vitamin B9) belongs to the class of compounds denoted as folates. It is made up of a 2-amino-4-hydroxypteridine (purine and pyrazine parts fused together to give pterin moiety) that is linked to *p*-aminobenzoic acid coupled to the L-glutamic acid via its α-amino group (Fig. 1). In FA, the pterin moiety is fully oxidized – it exists as a fully double bonded conjugated system (Gregory, 1996). FA is in vivo reduced to 7,8-dihydrofolate (DHF), in which one double bond of the pterin ring system is reduced. DHF is subsequently reduced to 5,6,7,8-tertahydrofolate (THF), which is enzymatically converted into 5-methyltetrahydrofolate (5-MTHF); in both structures two double bonds of the pterin ring system are reduced (Fig. 1). Reduced forms of FA are cofactors in the transfer and utilisation of one-carbon groups; they donate one-carbon group in biosynthesis of purine, pyrimidine and DNA and play a key role in the regeneration of methionine (Stanger, 2002).

A few literature data suggest that folates may act as antioxidants. It was shown that FA can efficiently scavenge such free radicals as $CCl_3O_2^{\bullet}$, $N_3^{\bullet}$, $SO_4^{\bullet-}$, $Br_2^{\bullet-}$, $^{\bullet}OH$ and $O^{\bullet-}$ (Joshi, Adhikari, Patro, Chattopadhyay, & Mukherjee, 2001). Its physiological reduced forms are peroxynitrite scavengers and inhibitors of lipid peroxidation (Nakano, Higgins, & Powers, 2001; Rezk, Haenen, van derVijgh, & Bast, 2003). Activity of FA against the radical-mediated oxidative damage in human whole blood was also reported (Stocker, Lesgards, Vidal, Chalier, & Prost, 2003).

The present study was undertaken to determine the antioxidant activities of FA, DHF, THF and 5-MTHF in the

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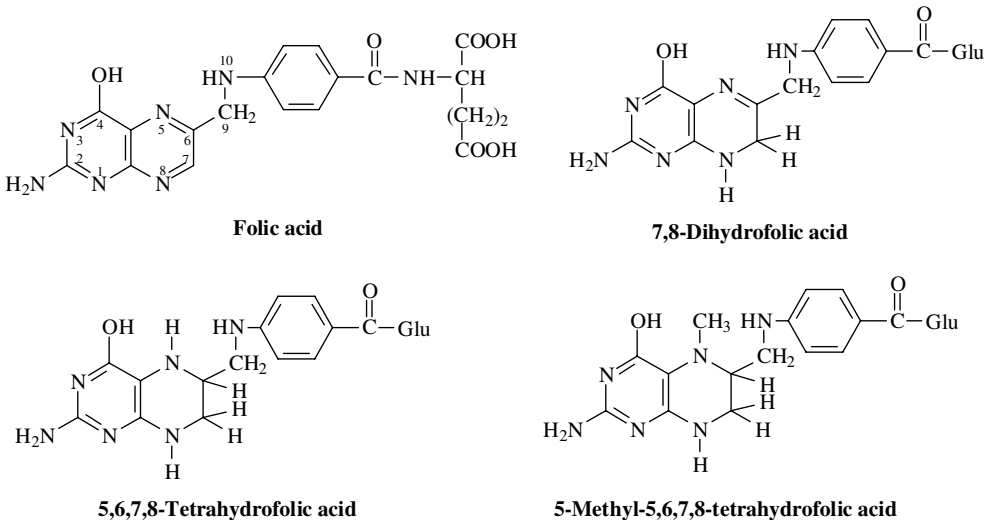

Fig. 1. Chemical structures and atom numbering system of folates under study.

TEAC, DPPH and FRAP assays. Their activities could become important in vivo in view of the use of FA for food fortification and as a nutritional supplement.

2. Materials and methods

2.1. Materials

Folic acid (pteroyl-L-glutamic acid), 5-methyltetrahydrofolic acid disodium salt (5-methyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid), dihydrofolic acid (7,8-dihydropteroyl-L-glutamic acid), microperoxidase-8 (MP8), 2,4,6-tripyridyl-s-triazine (TPTZ) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO, USA). ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and tetrahydrofolic acid (5,6,7,8-tetrahydropteroyl-L-glutamic acid) were from Fluka (Buchs, Switzerland). Trolox® and α-tocopherol (vitamin E) were from Aldrich (Steinheim, Germany) and ascorbic acid (vitamin C) was from Merck (Darmstadt, Germany).

2.2. Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant activities of vitamins were measured by the trolox equivalent antioxidant capacity (TEAC) assay, performed essentially as described by Miller, Rice-Evans, Davies, Gopinathan, & Milner (1993) & Rice-Evans & Miller (1994) with some modifications introduced by Tyrakowska et al. (1999). The TEAC value is based on the ability of the antioxidant to scavenge the blue-green coloured $ABTS^{\cdot+}$ radical cation relative to the $ABTS^{\cdot+}$ radical cation scavenging ability of the water-soluble vitamin E analogue, Trolox® (Miller et al., 1993; Rice-Evans & Miller, 1994). In the present study, microperoxidase-8 (MP8) instead of metmyoglobin, was used to generate the $ABTS^{\cdot+}$ radical cation in PBS (potassium phosphate buffered saline) pH 7.4. MP8 (final concentration 0.2 μM) and ABTS (final concentration 3.0 mM) in PBS were mixed and the reaction was initiated by the addition of hydrogen peroxide (final concentration 0.1 mM). The mixture was incubated at 30 °C for 1 h. The $ABTS^{\cdot+}$ radical cation solution thus obtained was diluted with PBS (v/v) to give an absorbance of about 0.8 at 734 nm. Vitamins and Trolox® were added as 1% (v/v) solutions of 100 times concentrated stock solutions in DMSO (FA, DHF, THF), methanol (5-MTHF, vitamin E, Trolox®) or water (vitamin C) to give the final concentration required (Tyrakowska et al., 1999). For each experiment, a solvent blank was run. The decrease in absorbance caused by vitamins, measured at 6 min, reflected the $ABTS^{\cdot+}$ radical cation scavenging capacity and was plotted against the concentration of the antioxidant. The TEAC value represents the ratio of the slope of the linear plot for scavenging of $ABTS^{\cdot+}$ radical cation by the compound tested to the slope of the plot for $ABTS^{\cdot+}$ radical cation scavenging by the water-soluble vitamin E analogue, Trolox®, used as an antioxidant standard.

2.3. The ferric reducing antioxidant power (FRAP) assay

FRAP assay was carried out by the method of Benzie & Strain (1996) with minor modification. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}-TPTZ). Vitamins were added as 1% (v/v) solutions of 100 times concentrated stock solutions to 10 mM ferric-TPTZ reagent and the increase in absorbance at 593 nm was measured at 8 min. $FeSO_4 \cdot 7H_2O$ was used as a standard. The concentrations of vitamins and $FeSO_4 \cdot 7H_2O$ were chosen to give an absorbance value not higher than 1. The FRAP value represents the ratio of the slope of the linear plot for reducing Fe^{3+}-TPTZ reagent by the vitamin to the slope of this plot for $FeSO_4$.

2.4. Radical-scavenging activity against DPPH·

The antioxidant activities of vitamins were also measured, using 2,2-diphenyl-1-picrylhydrazyl (DPPH·) as a free radical. Vitamins were added as 1% (v/v) solutions of 100 times concentrated stock solutions to 100 μM DPPH· in ethanol. The reaction mixture was incubated in the dark at room temperature for 30 min, and the decrease in absorbance was measured at 517 nm. The corresponding solvent blank readings were also taken and the remaining DPPH· was calculated. The radical-scavenging activity was expressed in terms of IC_{50} value (the concentration of the sample required to scavenge 50% of DPPH·) calculated by a linear regression analysis.

2.5. Statistical analysis of data

Data are presented as means ± SD of at least triplicate experiments. Analysis of variance was performed on the data obtained. Significance of differences between means was determined by least significant differences (LSD) at $P < 0.05$.

3. Results and discussion

A few literature data suggest that folates may act as antioxidants (Joshi et al., 2001; Nakano et al., 2001; Rezk et al., 2003; Stocker et al., 2003). In the present study, folic acid and its reduced forms were chosen as model compounds to study their antioxidant capacities in the TEAC, DPPH and FRAP assays.

Table 1 presents the results of the antioxidant activities, expressed as the TEAC and FRAP values, and IC_{50} (DPPH assay), of folates under study. For comparison, Table 1 also presents the literature data on the activities of FA and its reduced forms measured by peroxynitrite (PON) scavenging and inhibition of lipid peroxidation (LPO) (Rezk et al., 2003). It was found that all folic acid derivatives tested were able to scavenge $ABTS^{\cdot+}$ radical cation, DPPH· radical, and to reduce Fe^{3+} to Fe^{2+} in the FRAP assay. The lowest TEAC and FRAP values and the highest IC_{50} value in the DPPH assay were obtained for FA, indicating it as the least potent antioxidant among folates tested. Reduction of FA to DHF significantly increases $ABTS^{\cdot+}$ radical cation and DPPH·-scavenging activity as well as ability to reduce Fe^{3+} in the FRAP assay. Further reduction of DHF to THF results in decrease in the TEAC and the FRAP values indicating the decrease in free radical-scavenging activities of THF as compared to DHF. In contrast, the DPPH·-scavenging activity of THF compared to that of DHF was almost two-fold higher (IC_{50} values were 59.1 μM and 107 μM, respectively). Methylation of THF to 5-MTHF does not influence the TEAC value but causes further decease in FRAP value. The DPPH·-scavenging activities of 5-MTHF and THF were comparable. Results obtained by Rezk et al. (2003) indicate 5-MTHF to be the most effective peroxynitrite (PON) scavenger and THF to be the most effective inhibitor of lipid peroxidation (LPO) (Table 1). Altogether, the results obtained and literature data show that the fully reduced folates, namely THF and 5-MTHF, are the most active forms in scavenging the DPPH· and PON. THF is also the most effective inhibitor of LPO whereas DHF is most effective in scavenging $ABTS^{\cdot+}$ radical cation and it was found to be the best reductant as expressed by the FRAP value.

FA is vitamin of great interest and of great clinical value. It has ability to lower the plasma homocysteine level, which is one of the known risk factors for cardiovascular disease. Therefore, due to its homocysteine-lowering potential FA is considered to be potentially protective against cardiovascular disease (Boushey, Beresford, Omenn, & Motulsky, 1995; Nygard et al., 1997). It was, however, suggested that folate may have a direct antioxidant role in vivo, which is independent of any indirect effects through lowering of homocysteine levels (Nakano et al., 2001). Epidemiological studies have shown that FA supplementation can significantly reduce, not only the risk of cardiovascular, but also hematological diseases (Lindenbaum & Nath, 1980; Verhaar, Stroes, & Rabelink, 2002), neurological and neuropsychiatric disorders (Alpert & Fava, 2003; Manzoor & Runcie, 1976), neural tube defects (Daly, Kirke, Molloy, Weir, & Scott, 1995; Olney & Mulinare, 2002) and different forms of cancer (Akoglu, Faust, Milovic, & Stein, 2001; Duthie, Narayanan, Brand, Pirie, & Grant, 2002; Giovannucci, 2002; Zhang et al., 1999). It was proposed that the presumed protective effects of FA in the pathogenesis of these degenerative diseases could be associated with its antioxidant activity (Joshi et al., 2001; Nakano et al., 2001).

Table 1
Antioxidant activities of folic acid and its reduced forms as compared to ascorbic acid and α-tocopherol

Compound	TEAC	FRAP	DPPH IC_{50} [μM]	PON[1] IC_{50} [μM]	LPO[1] IC_{50} [μM]
FA	0.06 ± 0.01[2]	0.04 ± 0.01[2]	374 ± 30.7	>100	>500
DHF	0.98 ± 0.01[b]	2.85 ± 0.19	107 ± 6.4	2.4 ± 0.3	>500
THF	0.73 ± 0.04[a]	2.43 ± 0.13	59.1 ± 5.7[a]	1.5 ± 0.2	189 ± 26
5-MTHF	0.77 ± 0.04[a]	1.75 ± 0.06	50.5 ± 4.3[a]	0.9 ± 0.1	>500
Ascorbic acid	0.99 ± 0.05[b]	1.98 ± 0.05[b]	21.8 ± 1.6	–	–
α-Tocopherol	0.97 ± 0.03[b]	1.95 ± 0.20[b]	18.2 ± 1.1	–	–

Means with the same letters within the column are not significantly different at $P < 0.05$.
[1] Rezk et al. (2003).
[2] Gliszczyńska-Świgło (2006).

Because mammals lack the necessary enzymes to synthesize folates de novo (Friedrich, 1988), their presence depends entirely on folates in the diet. Food folates exist primarily as polyglutamates. Good source of folates are green leafy vegetables, legumes, mushrooms and liver. FA, is monoglutamate and, because of its stability, is the sole form of folate added to foods such as infant formulas and breakfast cereals, it is also used as vitamin pills. The data presented in this paper as well as literature data (Joshi et al., 2001; Nakano et al., 2001; Rezk et al., 2003) show that FA due to its poor protective effect against free radicals and lipid peroxidation is not really relevant as an antioxidant. However, its reduced physiological forms, although not considered as food antioxidants, they may act as effective free radical scavengers and inhibitors of LPO in vivo. Their free radical scavenging activities expressed as the TEAC and the FRAP values are comparable with those of vitamin C and E (Table 1), which are commonly accepted as the effective natural water- and lipid-soluble antioxidants, respectively. The activities of reduced folates in the DPPH assay are only 2–6 fold lower than those of vitamin C and E. Moreover, their TEAC values are comparable to those of natural antioxidants such as some polyphenols e.g. soybean isoflavones (genistein, genistin) and citrus flavanones (naringin, narirutin, hesperidin) (Rice-Evans, Miller, Bolwell, Bramley, & Pridham, 1995; Rice-Evans, Miller, & Paganga, 1996) but bioavailability of folates is much higher than bioavailability of plant polyphenols.

It is of importance to stress that in vitro assays for determination of the antioxidant activity of compounds under study do not reflect in vivo conditions because they do not take into account presence of other compounds, which may interact with a chemical under study and therefore influence its antioxidant activity. Moreover, the relevance of potent antioxidants in biological systems may be strongly related to their bioavailability and metabolism. Thus the data obtained by the use of in vitro methods can not be extrapolated to in vivo situations, even if these methods use physiological radicals or pH. Nevertheless, the application of a few in vitro methods to determine antioxidant activity of a chemical may give a comprehensive view of its potential antioxidant activity in vivo.

Altogether, the results presented show that antioxidant activities of physiological forms of folic acid could become important in vivo especially in view of nutritional supplementation and fortification of food with folic acid.

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