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VITAMIN B6 AND ITS METABOLITE REVEAL RADICAL SCAVENGING ACTIVITY AT BASIC pH

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Key words: vitamin B6, pH-dependent radical-scavenging activity, TEAC value, DFT calculations

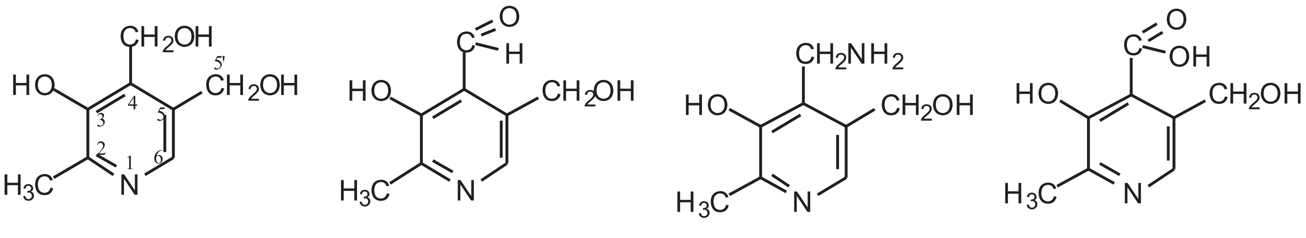
In the present study, the pH-dependent radical-scavenging activities, in the TEAC (Trolox equivalent antioxidant capacity) assay, of pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) (vitamin B6 forms) and their main metabolite 4-pyridoxic acid (PA) was determined. For all compounds, the increase in the TEAC values with increasing pH of the medium from value 7 to 9 was observed. The order of radical-scavenging activity was found as follows: PN>PM>PL>PA and it was confirmed by their oxidative potentials measured using cyclic and square-wave voltammetry. Quantitative relationship between experimental radical-scavenging activity and calculated bond dissociation energy values (BDE) was found suggesting hydrogen atom donation as the main mechanism of radical-scavenging activity of vitamin B6 and PA. The results of the present study suggest that vitamin B6 may contribute to the total antioxidant capacity of antioxidants present in human body especially at basic conditions.

INTRODUCTION

The term "vitamin B6" refers to the group of 2-methyl-3--hydroxy-5-hydroxymethylpyridines having the vitamin activity of pyridoxine (PN). The various compounds of vitamin B6 differ the one-carbon substituent at the 4-position which are an alcohol for pyridoxine (PN), an aldehyde for pyridoxal (PL) and an amine for pyridoxamine (PM) (Figure 1). These forms can be phosphorylated at the 5'-hydroxymethyl group giving pyridoxine-5'-phosphate (PNP), pyridoxal-5'-phosphate (PLP) and pyridoxamine-5'-phosphate (PMP) [Gregory, 1996]. All these six forms are biologically active and are the major forms of vitamin B6 found in mammalian tissues and body fluids [Friedrich, 1988]. They are metabolized in the body mainly to biologically inactive 4-pyridoxic acid (4-PA) (Figure 1) and excreted in the urine. PLP and to a lesser extent PMP are coenzymes in more than 100 enzymatic reactions involved in the metabolism of amino acids, carbohydrates, neurotransmitters and lipids. It is also essential for red blood cell metabolism. The nervous and immune systems need vitamin B6 to function efficiently, and it is also needed for the conversion of tryptophan to niacin [Shibata *et al.*, 1995].

Vitamin B6 is found in a wide variety of foods including fortified cereals, beans, meat, poultry, fish, and some fruits and vegetables. PM and PL forms predominate in meat but PN, generally in the glucosylated form, is the main B6 compound in fruit, vegetables and grains. PN (as the HCl salt) is the form of vitamin B6 used for fortification of food and in nutritional supplements.

Recently, it was reported [Gliszczyńska-Świgło, 2006a] that vitamin B6 forms (PN, PL, PM) may scavenge $ABTS^{\cdot+}$ radical cation, although their radical-scavenging activity at pH 7.4 after 6 min of incubation with $ABTS^{\cdot+}$ radical cation was very low. However, it was also observed that they act as radical-scavengers relatively slowly and the reaction could be described by pseudo-first order kinetics. The radical-scavenging activities of vitamin B6 forms after 30 min of incubation with $ABTS^{\cdot+}$ radical cation were about 4.4-5.4-fold higher than those measured after 6 min. Activity of pyridoxine, against the radical-mediated oxidative damage in human



Pyridoxine (PN) Pyridoxal (PL Pyridoxamine (PM) 4-Pyridoxic acid (PA)

FIGURE 1. The structures of pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and 4-pyridoxic acid (PA).

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whole blood was also reported [Stocker *et al.*, 2003]. However, in these studies, the influence of pH of the surrounding medium on the antioxidant activity of vitamin B6 was not taken into account.

The aim of the present study was to determine the effect of pH on the radical-scavenging activity of pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM) and pyridoxic acid (PA). Moreover, some DFT (density functional theory) calculations were performed to try to explain the observed increase in the radical-scavenging activity of these compounds with increasing pH of the surrounding medium.

MATERIALS AND METHODS

Pyridoxine hydrochloride, pyridoxamine, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and microperoxidase-8 (MP-8) were purchased from Sigma-Aldrich (Steinheim, Germany). Pyridoxal hydrochloride was from Serva (Heidelberg, New York). 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) was obtained from Roche (Mannheim, Germany). Hydrogen peroxide (30%) was purchased from Merck (Darmstadt, Germany).

METHODS

Trolox Equivalent Antioxidant Capacity (TEAC) assay. The TEAC assay is based on the ability of the antioxidant to scavenge the blue-green colored $ABTS^{\cdot +}$ radical cation relative to the $ABTS^{\cdot +}$ radical cation scavenging ability of the water-soluble vitamin E analogue, Trolox. The radical-scavenging activities of vitamin B6 forms and PA were measured by the modified TEAC assay performed essentially as described previously [Miller *et al.*, 1993] with some modifications concerning usage of microperoxidase-8 (MP-8) instead of metmyoglobin to generate the $ABTS^{\cdot +}$ radical cation [Tyrakowska *et al.*, 1999]. The major advantage of the modified TEAC assay is that it permits studies of the radical-scavenging activity over a wide pH range (2–10). The generation of $ABTS^{\cdot +}$ radical cation and preparation of its solutions of different pH was described previously [Gliszczyńska-Świgło *et al.*, 2006b].

Voltammetry measurements. Electrochemical measurements were carried out using μAutolab running with GPES (General-Purpose Electrochemical System) version 4.9 software (Eco-Chemie, Utrecht, The Netherlands). A three-electrode system in 2 mL volume was used. The working electrode was a glassy carbon mini-electrode of 3 mm diameter; Ag/AgCl (3 mol/L KCl) was used as a reference electrode and a platinum wire as a counter electrode. All potentials were measured at room temperature versus Ag/AgCl electrode. After each measurement, the glassy carbon working electrode was polished with diamond paste. Cyclic voltammograms were performed at scan rate 100 $mV \cdot s^{-1}$. Square wave voltammetry conditions were: frequency of 50 Hz, amplitude of 20 mV and potential step of 2 mV (effective scan rate was 100 $mV(s^{-1})$. Voltammetric scans were recorded in the potential range from 0.2 to 1.4 V.

Quantum mechanical calculations. First, geometry optimization of molecules studied was performed using B3LYP(6-31G(d,p) basis set. In order to calculate some thermochemical parameters, such as phenolic OH bond dissociation energy (BDE) and ionization potentials (IP) values, the compounds were studied in various protonation/deprotonation states as well as in one-electron oxidized state. All thermochemical data reported in this work were computed in „single-point" step with more extended B3LYP(6-311+G(3d,3p) basis set using optimized structures. All theoretical results are expressed in kcal/mol and refer to so-called „gas-phase" calculation. More details on calculation procedure can be found in our previous paper [Borkowski *et al.* 2005]. All calculations were performed using Gaussian 98 computational package (Gaussian Inc., Pittsburg, PA, USA).

RESULTS AND DISCUSSION

In the present study, the effect of pH on the radical-scavenging activity of PA, PL, PM and PA was studied. Figure 2 shows the pH-dependent changes in the TEAC values of PN, PL, PM and PA. For all compounds, the increase in the TEAC value with increasing pH from 7 to 9 is observed. This effect can be important from biological point of view because the absorption of vitamin B6 occurs primarily in the upper (proximal) jejunum [Friedrich, 1988] where alkaline conditions exist. Alkaline pH exists also in the large intestine (pH 8), the pancreas (pH 7–8.7) and in the duodenum (pH 8.3–9.3) [Grzymisławski, 2000]. The order of observed radical-scavenging activity in the whole pH range is as follows: PN>PM>PL>PA. The oxidative and/or the half-peak reduction potentials have been ascribed as a suitable parameter for representing the radical-scavenging activity of antioxidants. In the present study, irreversible oxidation of vitamin B6 forms and PA was observed (results not shown). From Figure 2, the TEAC values at pH 7.5 were derived and compared with oxidative potentials (Ea) measured using cyclic (CV) and square-wave voltammetry (SWV) (Table 1). Qualitative relationship between the TEAC and Ea values was found (Figure

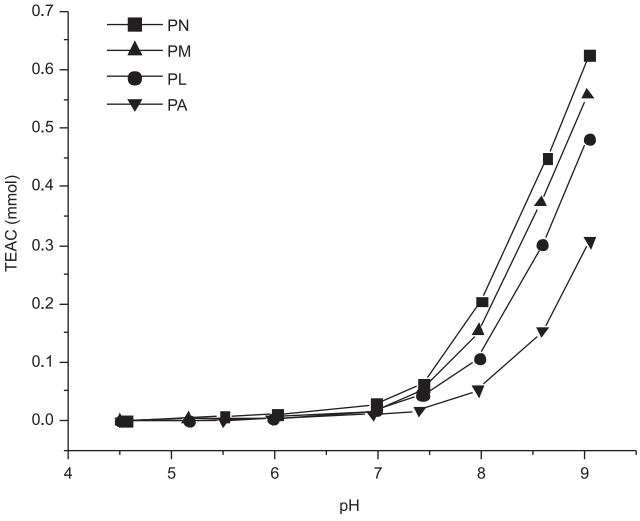

FIGURE 2. pH-dependent TEAC profiles of pyridoxin (PN), pyridoxal (PL), pyridoxamine (PM) and pyridoxic acid (PA).

TABLE 1. Results of experimental and theoretical studies for vitamin B6 and its metabolite.

Compound	TEAC pH 7.5 (mmol/L)	Ea[1] pH 7.5 (V)	BDE(N) (kcal/mol)	BDE(zw) (kcal/mol)	IP(N) (kcal/mol)	IP(A) (kcal/mol)	IP(zw) (kcal/mol)
PN	0.063	0.736 (0.715)	88.5	79.1	181.6	65.5	176.8
PL	0.043	0.807 (0.778)	94.9	83.3	192.3	76.3	178.6
PM	0.055	0.781 (0.746)	89.8	79.6	172.4	59.5	166.9
PA[2]	0.017	0.937 (0.920)	102.8	87.1	190.0	84.8	187.2

[1] – in parenthesis: Ea measured using square wave voltammetry; [2] – termochemical parameters calculated for carboxylic anion of PA

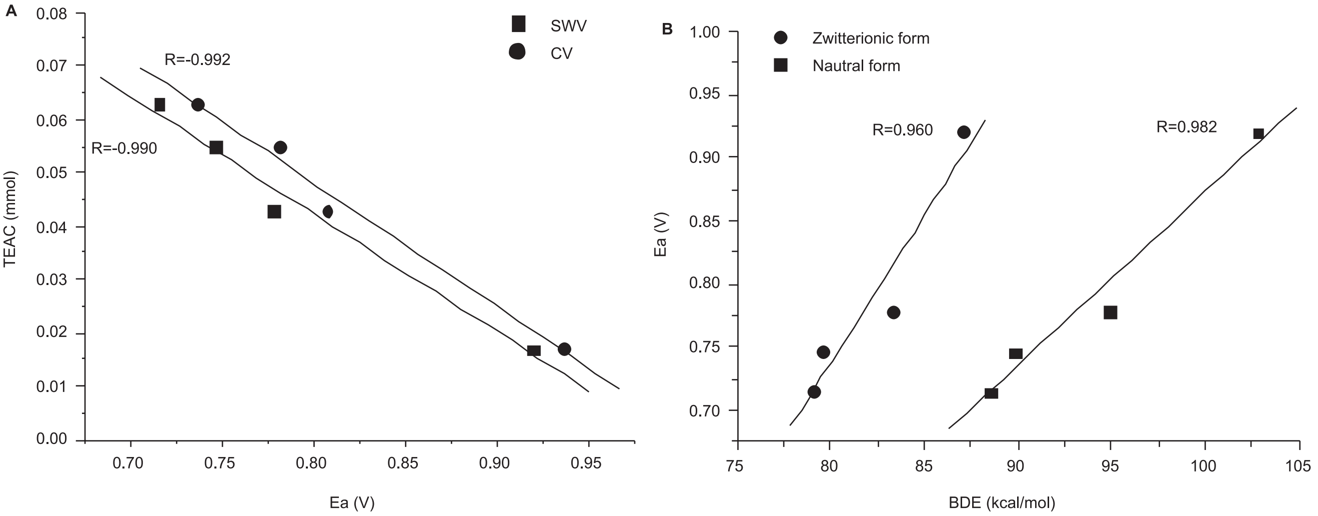

FIGURE 3. Correlation between (A) the TEAC and Ea values and (B) Ea and BDE values of vitamin B6 forms and PA.

3A) which confirms the order of radical-scavenging activity of vitamin B6 compounds.

Table 1 also presents the calculated thermochemical parameters for neutral (N), anionic (A) and zwitterionic (Zw) forms of the compounds under investigation including phenolic OH bond dissociation energy (BDE) values, representing the ease of hydrogen atom donation and ionization potential (IP) values, representing the ease of electron donation by the molecule studied. The pyridine system of vitamin B6 molecules mainly exists in zwitterionic form at neutral pH due to the basic character of pyridinium N1 (pKa about 8-9) and the acidic nature of the 3OH (pKa about 3.2-5.0) [Gregory, 1996]. The net charge distribution in vitamin B6 molecules varies markedly as a function of pH. As can be seen in Table 1, the BDE values calculated for zwitterionic form of B6 (BDE(Zw)) are much lower (by about 9-12 kcal/mol) than corresponding values calculated for neutral species (BDE(N)). For PA the difference between BDE(Zw) and BDE(N) is predicted to be even higher (by about 16 kcal/mol). Significant negative correlations between the TEAC value measured at pH 7-9 and the BDE values of neutral (Figure 4A) and zwitterionic (Figure 4B) forms were found. The correlation coefficients obtained for relationship between the Ea value measured using SWV (as an example) and BDE values of neutral form was 0.982; for zwitterionic form 0.960 (Figure 3B). No significant correlation was obtained between the TEAC or Ea values and IP values of neutral, anionic and zwitterionic forms suggesting the hydrogen atom donation, not electron donation, as possible explanation of the increased radical-scavenging activity of vitamin B6 derivatives at basic pH. Hydrogen-donation mechanism is supported by theoretically calculated BDE values for both neutral and zwitterionic forms. The BDE values are similar for PN and PM as a result of similar electron-donating effect of alcoholic OH and NH_2 groups. In PL molecule, the aldehyde group is substituted directly to aromatic ring. Taking into account strong electron withdrawing nature of C=O group it is obvious that PL is poorer hydrogen atom donor than PN and PM. PA turned out to be the poorest $ABTS^{\cdot +}$ radical cation scavenger in our study. In fact, the deprotonated carboxyl COOH group gives some extra electron density to aromatic system and it can potentially decrease the BDE value of neighboring phenolic OH group. However, strong H-bonding between OH group and COO^- group may be the reason for relatively weak radical-scavenging activity observed for PA.

The results obtained in the present study by using TEAC assay, cyclic and square-wave voltammetry and theoretical calculations suggest that, in some degree, vitamin B6 may contribute to the total antioxidant capacity of antioxidants present in human body especially at basic conditions.

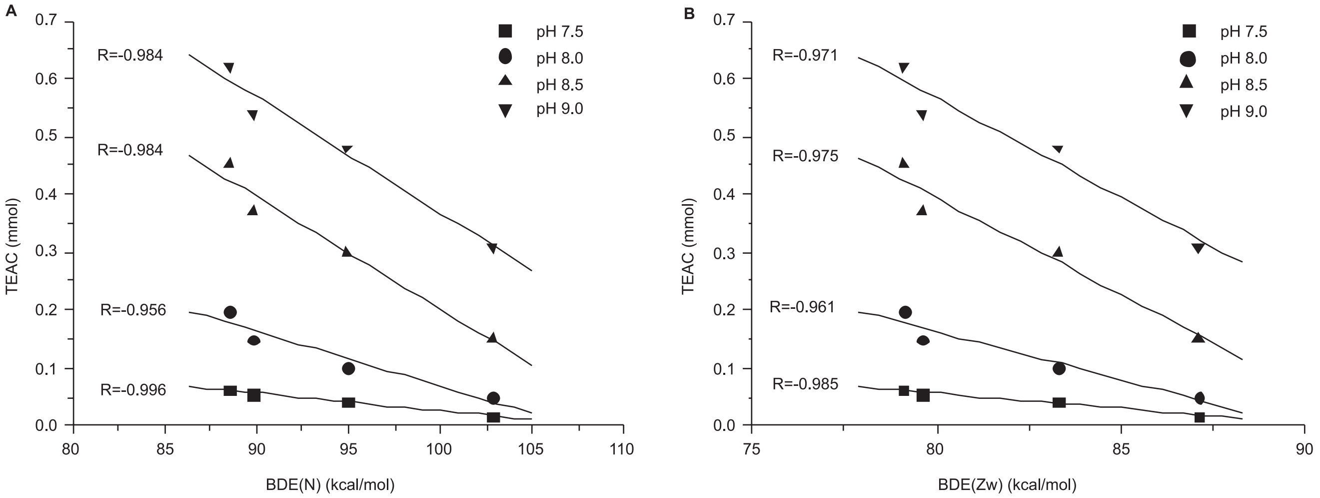

FIGURE 4. Correlation between the TEAC and (A) BDE(N) and (B) BDE(Zw) forms of vitamin B6 forms and PA.

Although, the radical-scavenging activity of all B6 forms is lower in comparison with vitamin C or with many other water-soluble natural antioxidants such as flavonoids, the observed reactions of vitamin B6 with free radicals indicate that this important vitamin can be easily destroyed by reactive oxygen species present in organism. It is worth to mention that according to Tadera *et al.* [1988] hydroxyl radicals generated during degradation of ascorbic acid can directly attack the C6 position of PN and form the 6-hydroxypyridoxine which totally lacks vitamin B6 activity.

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REFERENCES

1. Borkowski T., Szymusiak H., Gliszczyńska-Świgło A., Rietjens I.M.C.M., Tyrakowska B., Radical scavenging capacity of wine anthocyanins is strongly pH-dependent. J. Agric. Food Chem., 2005, 53, 5526-5534.
2. Friedrich W., Vitamins. 1988, Walter de Gruyter, Berlin, New York, pp. 545-618.
3. Gliszczyńska-Świgło A., Antioxidant activity of water soluble vitamins in the TEAC (Trolox Equivalent Antioxidant Capacity) and the Ferric Reducing Antioxidant Power (FRAP) assays. Food Chem., 2006a, 96, 131-136.
4. Gliszczyńska-Świgło A., Szymusiak H., Malinowska P., Betanin, the main pigment of red beet: molecular origin of its exceptionally high free radical-scavenging activity. Food Addit. Contam., 2006b, 23, 1079-1087.
5. Gregory III J.F., Vitamins. 1996, *in*: Food Chemistry, (ed. Fennema O.R.). Marcel Dekker, New York, Basel, Hong Kong, pp. 580-589.
6. Grzymisławski M., Physique of systems related to food bioavailability. 2000, in: Human Nutrition. Principals of Nutritional Science (in Polish), (eds. Gawędzki, J., Hryniewiecki, L.). PWN, Warszawa, Poland, pp. 56–72.
7. Miller N.J., Rice-Evans C.A., Davies M.J., Gopinathan V., Milner A., A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonate. Clin. Sci., 1993, 84, 407-412.
8. Shibata K., Mushiage M., Kondo T., Hayakawa T., Tsuge H., Effects of vitamin B6 deficiency on the conversion ratio of tryptophan to niacin. Biosci. Biotechnol. Biochem., 1995; 59, 2060-2063.
9. Stocker P., Lesgards J-F., Vidal N., Chalier F., Prost M., ESR study of a biological assay on whole blood: antioxidant efficiency of various vitamins. Bioch. Biophy. Acta, 2003, 1621, 1-8.
10. Tadera K., Arima M., Yagi F., Participation of hydroxyl radical in hydroxylation of pyridoxine by ascorbic acid. Agric. Biol. Chem., 1988, 52, 2359-2360.
11. Tyrakowska B., Soffers A.E.M.F., Szymusiak H., Boeren S., Boersma M.G., Lemańska K., Vervoort J., Rietjens I.M.C.M., TEAC antioxidant activity of 4-hydroxybenzoates. Free Radic. Biol. Med., 1999, 27, 1427-1436.

WITAMINA B6 I JEJ METABOLIT WYKAZUJĄ AKTYWNOŚĆ PRZECIWRODNIKOWĄ W ŚRODOWISKU ZASADOWYM

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Zbadano wpływ pH na aktywność przeciwrodnikową PN, PL, PM i PA, mierzoną w teście TEAC (trolox equivalent antioxidant capacity). Dla wszystkich badanych związków zaobserwowano wzrost wartości TEAC w zakresie pH 7-9. Aktywność przeciwrodnikowa malała w kolejności PN>PM>PL>PA, co zostało potwierdzone poprzez wartości potencjałów utleniania wyznaczone metodami woltamperometrii cyklicznej i fali prostokątnej. Stwierdzono istotną współzależność pomiędzy doświadczalnie wyznaczonymi wartościami aktywności przeciwrodnikowej lub potencjałami utleniania a teoretycznie obliczonymi wartościami energii dysocjacji grupy OH (BDE), co sugeruje, że obniżenie energii odszczepiania atomu wodoru z grupy (fenolowej) OH witaminy B6 i kwasu pirydoksynowego może wyjaśniać wzrost aktywności przeciwrodnikowej tych związków, obserwowany w środowisku zasadowym.