The Effect of Stobadine on the Copper-Induced Peroxidation of Egg Yolk Phosphatidylcholine in Multilamellar Liposomes

J. Gallová and S. Szalayová

Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Bratislava, Slovakia

Abstract. Stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido-[4,3b]-indole, is a pyridoindole derivative with antioxidant, antiarrhytmic, neuroprotective, local anesthetic, α-adrenolytic, antihistaminic, myorelaxant and other pharmacodynamic effects. The antioxidant properties were tested in a model system of egg yolk phosphatidylcholine (EYPC) multilamellar liposomes. The lipoperoxidation was induced by adding Cu^{2+} ions and *tert*-butylhydroperoxide. The course of EYPC peroxidation was monitored spectrophotometrically for conjugated diene formation. We found that stobadine prolonged the lag phase and decreased the rate of peroxidation during the lag phase in a dose-dependent manner. Surprisingly, an increase in the rate of peroxidation was observed at low stobadine concentration in the propagation phase. The possible cause of prooxidative action of stobadine is discussed.

Key words: Egg yolk phosphatidylcholine — Liposomes — Stobadine — Peroxidation — UV-VIS spectrophotometry

Introduction

Reactive oxygen species (ROS) in specified amounts play an important role in normal metabolic and signalling processes (Hensley et al. 2000; Hensley and Floyd 2002). However, a large amount of data available in the literature indicates that excessive production of ROS, known as oxidative stress, participate in numerous human pathological conditions (Halliwell and Gutteridge 1991; Cerutti 1994; Reddy and Yao 1996; Dabrowski et al. 1999; Dhalla et al. 1999; Varadarajan et al. 2000; Bailey 2003; Jenner 2003). Therefore, there is a high interest in studying the effects of compounds able to stop or to slow down the harmful action of ROS in biological tissues. Stobadine, a pyridoindole derivative, has been synthesized in a search of new antiarrhytmic drugs by Štolc et al. (1983). As reviewed by Horáková et al.

Correspondence to: Jana Gallová, Department of Physical Chemistry of Drugs, Faculty of Pharmacy, Comenius University, Odbojárov 10, 832 32 Bratislava 3, Slovakia
E-mail: jana.gallova@fpharm.uniba.sk

(1994, 1998), stobadine exerts antioxidative, cardioprotective, neuroprotective, antihistamine, antiulcerous, and other effects. Stobadine scavenges effectively some of ROS, e.g. hydroxyl, peroxyl and alkoxyl radicals and is an effective quencher of singlet oxygen (Ondriaš et al. 1989; Staško et al. 1990; Štefek and Beneš 1991; Kagan et al. 1993). As reported by Kagan et al. (1993), stobadine is not efficient scavenger of superoxide radicals. Stobadine partitions in a two-phase octanol-water system with the partition coefficient $\log P = 0.57 \pm 0.03$ (Kagan et al. 1993). Similar value of $\log P = 0.86$ was determined by Bezáková et al. (1993). Several groups studied the mechanism of stobadine antioxidant activity. Steenken et al. (1992) reported on the formation of an indolic nitrogen-centered radical of stobadine in reaction with reactive free radicals. Mišík et al. (1999) suggested that a nitroxide radical is formed after reaction of nitrogen-centered radical of stobadine with oxygen-centered species.

The antioxidant action of stobadine was proved using different model and biological membranes, for example egg yolk phosphatidylcholine (EYPC) liposomes (Ondriaš et al. 1989; Staško et al. 1990) and rat liver microsomes (Horáková et al. 1992; Kagan et al. 1993). It was found that stobadine inhibited the lipoperoxidation in EYPC liposomes more effectively than the known antioxidant BHT and calcium channel blockers nifedipine and verapamil (Ondriaš et al. 1989). Stobadine is an efficient inhibitor of lipid peroxidation in phosphatidylcholine liposomes and in rat liver microsomes but is not effective in tocopherol-deficient microsomes (Horáková et al. 1992).

Although the ability of stobadine to inhibit lipoperoxidation in phosphatidylcholine liposomes has already been demonstrated, we considered it worthwhile to study this effect in more detail. The aim of the present paper was to investigate the antioxidant effect of stobadine in different phases of lipid peroxidation in dependence on its concentration. A model system represented by multilamellar liposomes of EYPC was used. Radical products of the reaction between Cu^{2+} ions and *tert*-butylhydroperoxide initiated the peroxidation. The ability of stobadine to extend the lag phase of lipoperoxidation and to decrease the rate of peroxidation was used as a measure of stobadine antioxidant activity.

Materials and Methods

Stobadine, (−)-*cis*-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1*H*-pyrido[4,3b]-indole, was a kind gift from Dr. Štolc (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia). EYPC was isolated and purified according to Singleton et al. (1965). Methanol of analytical grade was from Mikrochem (Bratislava, Slovakia) and *tert*-butylhydroperoxide was purchased from Sigma Chemical Co. (St. Louis, MO, USA). NaCl and $CuCl_2 \cdot 2H_2O$ were from Lachema (Brno, Czech Republic) and were of analytical grade.

Stobadine and EYPC dissolved in methanol were mixed. The solvent was then evaporated under a stream of gaseous nitrogen followed by evacuation in a vacuum chamber using a two-stage rotary oil vacuum pump. The dry EYPC-stobadine

mixture was hydrated with aqueous solution of NaCl (150 mmol/l). Multilamellar liposomes were prepared by three freezing-thawing cycles, followed by vortexing (TK 3S, TechnoKartel, Italy) the suspension and short sonicating it for 1 min in a bath sonicator RK 100H Sonorex Super (Bandelin Electronic, Germany).

The peroxidation of EYPC was started by adding freshly prepared aqueous solutions of $CuCl_2$ and *tert*-butylhydroperoxide to the lipid suspension. The final concentration of EYPC in reaction mixture was 28.6 mmol/l. The peroxidation of EYPC was conducted at room temperature in glass tubes sealed with Parafilm M (American National Can, Grenwich, USA). The samples were regularly opened and aerated when small volumes of the final dispersion were taken for analysis.

The course of EYPC peroxidation was monitored using the diode array UV-VIS spectrophotometer HP 8452 A (Hewlett Packard, USA). The relative extent of EYPC peroxidation was measured by the estimation of conjugated dienes according to Klein (1970).

Results and Discussion

The UV-VIS absorption spectrum of EYPC displays a marked absorption band in the 200–220 nm region, characteristic for the absorption of carbonyl groups of EYPC (Klein 1970). The intensity of this band is not influenced by EYPC peroxidation. During Cu^{2+}-induced peroxidation, an absorption peak appears in the EYPC spectra with a maximum around 234 nm. Its intensity increases with the time of incubation (t) of EYPC multilamellar liposomes with $CuCl_2$ and *tert*-butylhydroperoxide. The absorption at this wavelength is typical of conjugated dienes (Klein 1970; Puhl et al. 1994). The structure of conjugated dienes is inherent for lipid hydroperoxides which are the early products of lipid peroxidation. To characterise the degree of EYPC peroxidation, the oxidation index $OI = A_{234}/A_{210}$ is used where A_{234} and A_{210} are absorbancies at 234 and 210 nm, respectively (Klein 1970; Salvová 1997).

The results of a typical experiment displaying the effect of stobadine on the EYPC peroxidation are shown in the Fig. 1. The peroxidation of EYPC in multilamellar liposomes was initiated by adding aqueous solution of *tert*-butylhydroperoxide (final concentration 0.178 mol/l) and $CuCl_2$ (final concentration 0.1 mmol/l). The *OI versus t* profile can be divided into two consecutive time phases, lag phase and propagation phase. The third, decomposition phase, typical for chain reaction, was not reached within the duration of the experiment.

Experiment in the Fig. 1 was repeated with another batch of EYPC and a bit different final concentrations of *tert*-butylhydroperoxide (0.155 mol/l) and $CuCl_2$ (0.6 mmol/l). The coarse of EYPC peroxidation is in the Fig. 2. In this experiment the lag phase was too short to be detected in the control sample. On the other hand, the lag phase was not finished within 6 h at the maximal stobadine/EYPC molar ratio (0.013 mol/mol). It can be concluded that *OI versus t* profile is sensitive to various experimental conditions, such as *tert*-butylhydroperoxide and $CuCl_2$ concentrations, lipid composition, initial lipid peroxidation, temperature, etc.

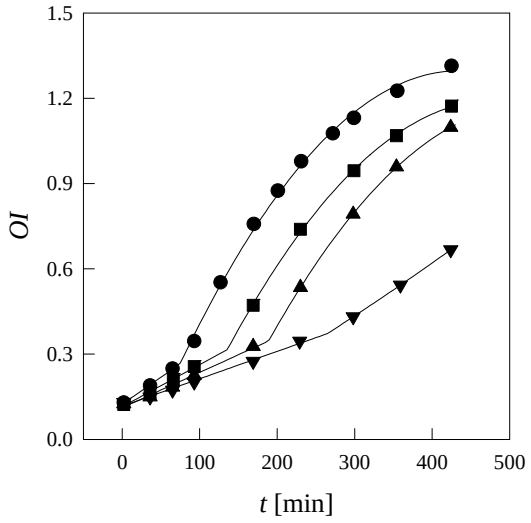

Figure 1. The dependence of the oxidation index, $OI = A_{234}/A_{210}$, on the time of EYPC peroxidation for different stobadine/EYPC molar ratios: 0 (●), 1 : 700 (■), 1 : 350 (▲), 1 : 175 (▼). Concentrations in the reaction mixture: 28.6 mmol/l EYPC, 0.1 mmol/l $CuCl_2$, 0.178 mol/l *tert*-butylhydroperoxide.

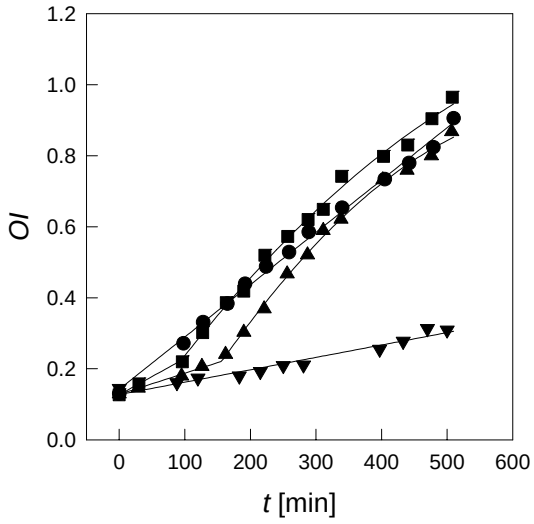

Figure 2. The dependence of the oxidation index, $OI = A_{234}/A_{210}$, on the time of EYPC peroxidation for different stobadine/EYPC molar ratios: 0 (●), 1 : 541 (■), 1 : 361 (▲), 1 : 77 (▼). Concentrations in the reaction mixture: 28.6 mmol/l EYPC, 0.6 mmol/l $CuCl_2$, 0.155 mol/l *tert*-butylhydroperoxide.

It is evident from Figs. 1 and 2 that stobadine inhibits the Cu^{2+}-induced peroxidation of EYPC. Similar inhibition effect of stobadine was observed in EYPC autoperoxidation (Ondriaš et al. 1989; Staško et al. 1990; Mišík et al. 1991) and in phosphatidylcholine and microsomal lipid peroxidation induced by $FeSO_4$ (Horáková et al. 1992).

As can be seen in Figs. 1 and 2, a linear dependence, $OI = at + b$, fits well the

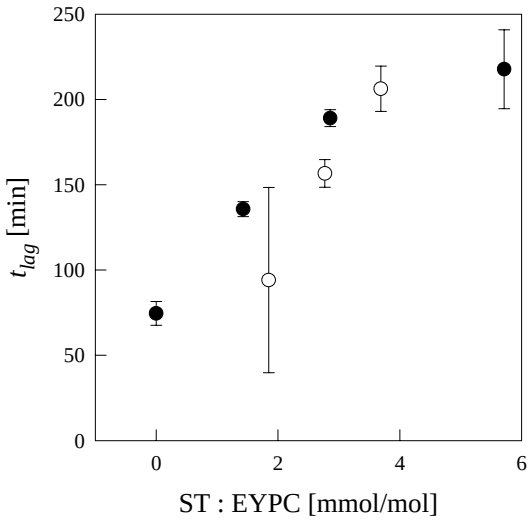

Figure 3. The dependence of the length of lag phase (t_{lag}), on the ST/EYPC molar ratio. Full and empty symbols represent results from experiment in Figs. 1 and 2, respectively. The standard errors are depicted by vertical bars. ST, stobadine.

experimental points during the lag phase while the approximation by a parabolic dependence, $OI = ct^2 + dt + e$, is more appropriate for the propagation phase. The efficiency of an antioxidant is usually evaluated by its effect on the length of the lag phase. We determined the length of the lag phase (t_{lag}) as an x-coordinate of the intersection of these linear and parabolic dependencies. It is easy to show that in the lag phase, where $t < t_{\mathrm{lag}}$,

$$OI = at + b \tag{1}$$

and in the propagation phase, where $t > t_{\mathrm{lag}}$,

$$OI = ct^2 + dt - c(t_{\mathrm{lag}})^2 + (a - d)t_{\mathrm{lag}} + b \tag{2}$$

To obtain an analytical form of the $OI = f(t)$ in the whole studied time interval, the function ABS(x) was utilized:

$$\begin{aligned} OI = {} & (at + b)[1 - (t - t_{\mathrm{lag}})/ABS(t - t_{\mathrm{lag}})]0.5 + \\ & + [ct^2 + dt - c(t_{\mathrm{lag}})^2 + (a - d)t_{\mathrm{lag}} + b] \\ & [1 + (t - t_{\mathrm{lag}})/ABS(t - t_{\mathrm{lag}})]0.5 \end{aligned} \tag{3}$$

The length of the lag phase was evaluated from experimental data in Figs. 1 and 2 using Eq. (3). The dependence of the length of lag phase on the stobadine/EYPC molar ratio is shown in the Fig. 3. As expected, stobadine prolongs the lag phase of peroxidation. Dose-dependent prolongation of the lag phase by stobadine was also observed in Cu^{2+}-initiated peroxidation of low density lipoproteins (Horáková et al. 1996) and Fe^{3+}-induced lipid peroxidation in microsomes (Horáková et al. 1992).

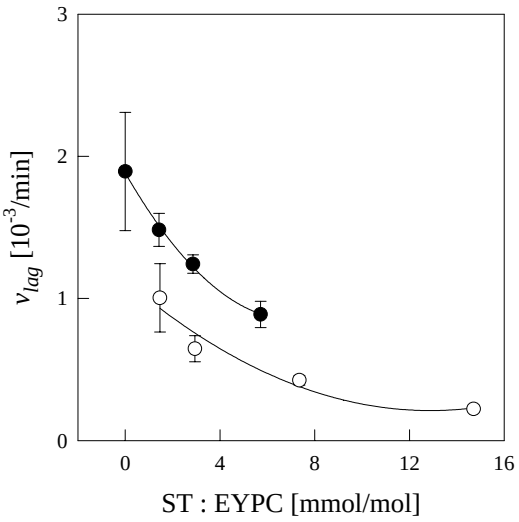

Figure 4. The dependence of the rate of EYPC peroxidation during lag phase (v_{lag}) on the ST/EYPC molar ratio. Full and empty symbols represent results from experiment in Figs. 1 and 2, respectively. The standard errors are depicted by vertical bars. ST, stobandine.

The rate of peroxidation can be defined as a rate of the increase of lipid hydroperoxide concentration (Yoshida and Niki 1992) which is proportional to the $\mathrm{d}(OI)/\mathrm{d}t$. The rate of peroxidation in the lag phase (v_{lag}) is therefore determined as a slope a of the linear dependence $OI = f(t)$ (Eqs. 1 and 3). As can be seen in Fig. 4, v_{lag} decreases with increasing stobadine/EYPC molar ratio.

The values of t_{lag} and v_{lag} are determined with relatively large experimental error for samples with low stobadine concentration. This results from the fact that only few experimental points were obtained during the short lag phase.

It is evident that the OI in the samples containing low stobadine concentrations increases as fast as in the control sample without stobadine (Fig. 1) or even faster (Fig. 2) when the process of EYPC peroxidation reaches the propagation phase. The rate of lipid peroxidation in the propagation phase was determined likewise as in the lag phase, $v_{\mathrm{prop}} = \mathrm{d}(OI)/\mathrm{d}t = 2ct + d$. Because typical values of $c \approx 10^{-6}$ are much less than $d \approx 10^{-3}$, we suppose, that parameter d characterizes properly the rate of EYPC peroxidation, at least in the beginning of the propagation phase. The dependence of the rate of EYPC peroxidation in the propagation phase on the stobadine/EYPC molar ratio is depicted in the Fig. 5. It can be seen that at molar ratio stobadine/EYPC less than ≈0.0037 (stobadine/EYPC $= 1:270$ mol), v_{prop} is comparable or even higher than in the absence of stobadine.

The influence of stobadine on the rate of lipid peroxidation was not systematically studied in the literature. It can be seen qualitatively from the Fig. 1 in Horáková et al. (1996) that increasing concentration of stobadine caused a delay in the conjugated dienes increase in the lag phase in the Cu^{2+}-initiated peroxidation of low density lipoproteins. Therein, a maximal rate in the propagation phase was

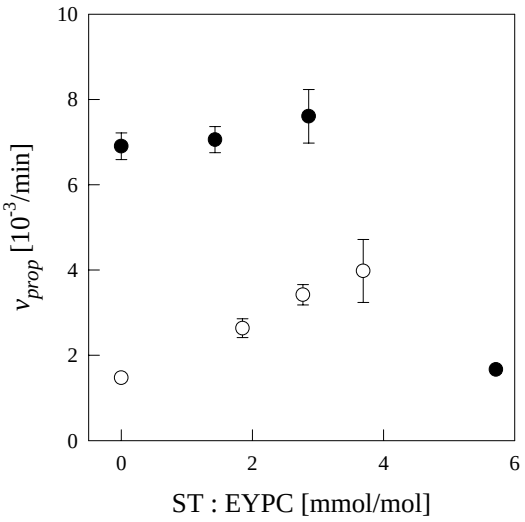

Figure 5. The dependence of the rate of EYPC peroxidation during propagation phase (v_{prop}) on the ST/EYPC molar ratio. Full and empty symbols represent results from experiment in the Figs. 1 and 2, respectively. The standard errors are depicted by vertical bars. ST, stobandine.

also evaluated. It was found that this maximal rate of peroxidation decreased with increasing stobadine concentration.

Prooxidant and antioxidant properties were also observed for ascorbate in the presence of transition metal ions as rewieved in Buettner and Jurkiewicz (1996). In general, at low concentrations, ascorbate is prone to be a prooxidant, and at high concentrations, it tends to be an antioxidant. This "crossover" effect was rationalized by Buettner and Jurkiewicz (1996) as follows. Because the one-electron reduction potential of ascorbate monoanion (282 mV) is more positive than those of Fe^{3+} and Cu^{2+}, it is able to reduce these metal ions to Fe^{2+} and Cu^{+}. Copper-induced peroxidation is initiated by following chemical reactions

$$\mathrm{Cu^{2+} + ROOH \xrightarrow{k_1} Cu^{+} + ROO^{\bullet} + H^{+}}$$
$$\mathrm{Cu^{+} + ROOH \xrightarrow{k_2} Cu^{2+} + RO^{\bullet} + HO^{-}}$$

where ROOH is any hydroperoxide, in our case *terc*-butylhydroperoxide or lipid hydroperoxide and k_1, k_2 are rate constants. Because $k_2 \gg k_1$ (Yoshida and Niki 1992) and ascorbate enhances the concentration of Cu^{+} at the expense of Cu^{2+}, it acts as a prooxidant. On the other hand, the one-electron reduction potential of ascorbate is lower compared to reactive oxygen radicals, including alkylperoxy radical and alkoxyl radical (Buettner and Jurkiewicz 1996). Therefore ascorbate is willing to donate an electron to these radicals, which is the basis of its antioxidant action. The ascorbate concentration at which the "crossover" effect appears, depends on the relative concentrations of ascorbate and metal ions.

One-electron reduction potential of stobadine is 0.580 V (Steenken et al. 1992). Similar as for ascorbate, this value lies between reduction potentials of aqueous

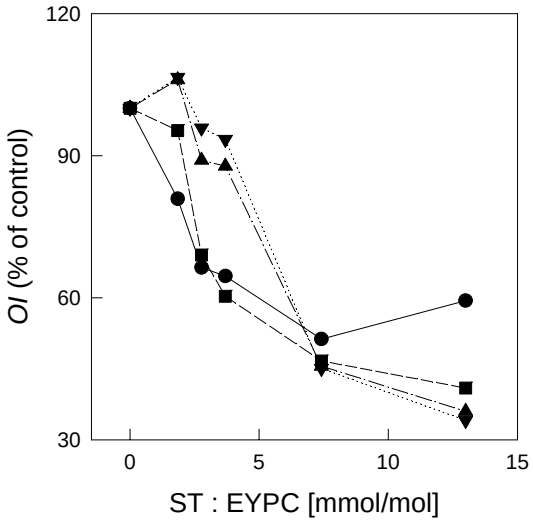

Figure 6. The dependence of the oxidation index, determined as % of control sample, on the ST/EYPC molar ratio in different intervals of EYPC peroxidation: 100 min (—●—), 190 min (- -■- -), 290 min (-·- ▲ -·-), 510 min (. . . ▼ . . .). The experimental conditions were as described in Fig. 2. ST, stobandine.

metal ions and alkylperoxyl and alkoxyl radical. Thus, we suppose that the reason, why stobadine can act as antioxidant as well as prooxidant in the presence of transition metal ions, is the same as for ascorbate.

Fig. 6 shows the effectiveness of different stobadine concentration to decrease the oxidation index in different stages of EYPC peroxidation. It is seen that during the whole process of peroxidation, the molar ratio around stobadine/EYPC = 0.0074 (stobadine/EYPC = 1 : 135 mol) is needed to decrease oxidation index to 50 % of the control sample. However, this value is dependent on the Cu^{2+} and *tert*-butylhydroperoxide concentrations.

In conclusion, stobadine inhibited markedly the Cu^{2+}-induced peroxidation of EYPC in multilamellar liposomes during the lag phase. The lag phase was prolonged and the rate of conjugated dienes formation was reduced in a dose-dependent manner. In the propagation phase, an increase in the rate of peroxidation was observed at low stobadine concentration. The prooxidative action of stobadine is probably connected with the ability of stobadine to reduce Cu^{2+} to Cu^{+}.

Acknowledgements. The autors would like to thank Dr. S. Štolc (Institute of Experimental Pharmacology, Slovak Academy of Sciences, Bratislava, Slovakia) for providing of stobadine, Dr. F. Kundračík (Faculty of Mathematics, Physics and Informatics, Comenius University, Bratislava, Slovakia) for discussion about analytical form of the $OI = f(t)$ function and Prof. P. Balgavý (Faculty of Pharmacy, Comenius University, Bratislava, Slovakia) for thorough review of this manuscript. This study was supported by the Slovak Ministry of Education, grant No. 1/9313/02 to J. G.

References

Bailey S. M. (2003): A review of the role of reactive oxygen and nitrogen species in alcohol-induced mitochondrial dysfunction. Free Radic. Res. **37**, 585—596

Bezáková Z., Blešová M., Bachratá M. (1993): Stobadinium dichloride – a study of physicochemical properties and stability. Farm. Obz. **62**, 247—252 (in Slovak)

Buettner G. R., Jurkiewicz B. A. (1996): Catalytic metals, ascorbate and free radicals: combination to avoid. Radiat. Res. **145**, 532—541

Cerutti P. A. (1994): Oxy-radicals and Cancer. Lancet **344**, 862—863

Dabrowski A., Konturek S. J., Konturek J. W., Gabryelewicz A. (1999): Role of oxidative stress in the pathogenesis of caerulein-induced acute pancreatitis. Eur. J. Pharmacol. **377**, 1—11

Dhalla N. S., Golfman L., Takeda S., Takeda N., Nagano M. (1999): Evidence for the role of oxidative stress in acute ischemic heart disease: a brief review. Can. J. Cardiol. **15**, 587—593

Halliwell B., Gutteridge J. M. C. (1991): Free Radicals in Biology and Medicine. Claredon Press, Oxford

Hensley K., Floyd R. A. (2002): Reactive oxygen species and protein oxidation in aging: A look back, a look ahead. Arch. Biochem. Biophys. **397**, 377—383

Hensley K., Robinson K. A., Gabbita S. P., Salsman S., Floyd R. A. (2000): Reactive oxygen species, cell signaling, and cell injury. Free Radic. Biol. Med. **28**, 1456—1462

Horáková L., Štolc S. (1998): Antioxidant and pharmacodynamic effects of pyridoindole stobadine. Gen. Pharmacol. **30**, 627—638

Horáková L., Briviba K., Sies H. (1992): Antioxidant activity of the pyridoindole stobadine in liposomal and microsomal lipid peroxidation. Chem. Biol. Interact. **83**, 85—93

Horáková L., Sies H., Steenken S. (1994): Antioxidant action of stobadine. Methods Enzymol. **234**, 572—580

Horáková L., Giessauf A., Raber G., Esterbauer H. (1996): Effect of stobadine on Cu^{2+}-mediated oxidation of low-density lipoprotein. Biochem. Pharmacol. **51**, 1277—1282

Jenner P. (2003): Oxidative stress in Parkinson's disease. Ann. Neurol. **53**, 26—36

Kagan V. E., Tsuchiya M., Serbinova E., Packer L., Sies H. (1993): Interaction of the pyridoindole stobadine with peroxyl, superoxide and chromanoxyl radicals. Biochem. Pharmacol. **45**, 393—400

Klein R. A. (1970): The detection of oxidation in liposome preparations. Biochim. Biophys. Acta **210**, 486—489

Mišík V., Ondriaš K., Staško A. (1999): EPR spectroscopy of free radical intermediates of antiarrhythmic-antihypoxic drug stobadine, a pyridoindole derivative. Life Sci. **65**, 1879—1881

Mišík V., Švajdlenka E., Filípek J., Gergeľ D., Ondriaš K. (1991): Inhibition of lipid peroxidation of lecithin liposomes kept in a pH- stat system near neutral pH. Free Radic. Res. Commun. **15**, 159—165

Ondriaš K., Mišík V., Gergeľ D., Staško A. (1989): Lipid peroxidation of phosphatidylcholine liposomes depressed by the calcium channel blockers nifedipine and verapamil and by the antiarrhythmic-antihypoxic drug stobadine. Biochim. Biophys. Acta **1003**, 238—245

Puhl H., Waeg G., Esterbauer H. (1994): Methods to determine oxidation of low-density lipoproteins. Methods Enzymol. **233**, 425—441

Reddy R. D., Yao J. K. (1996): Free radical pathology in schizophrenia: a review. Prostaglandins Leukot. Essent. Fatty Acids **55**, 33—43

Salvová H. (1997): The oxidation in models of low density lipoprotein studied by UV-VIS spectrophotometry (diploma thesis), Faculty of Pharmacy, Comenius University, Bratislava, Slovakia

Singleton W. S., Gray M. S., Brown M. L., White J. L. (1965): Chromatographicaly homogenous lecithin from egg phospholipids. J. Am. Oil Chem. Soc. **42**, 53—56

Staško A., Ondriaš K., Mišík V., Szöczová H., Gergeľ D. (1990): Stobadine – a novel scavenger of free-radicals. Chem. Pap. **44**, 493—500

Steenken S., Sundquist A. R., Jovanovic S. V., Crockett R., Sies H. (1992): Antioxidant activity of the pyridoindole stobadine – pulse radiolytic characterization of one-electron-oxidized stobadine and quenching of singlet molecular oxygen. Chem. Res. Toxicol. **5**, 355—360

Štefek M., Beneš L. (1991): Pyridoindole stobadine is a potent scavenger of hydroxyl radicals. FEBS Lett. **294**, 264—266

Štolc S., Bauer V., Beneš L., Tichý L. (1983): Medicine with antiarrhytmic and antihypoxic activity and its method of preparation. Patents: CS 229 067, SWED. 8204693-9, BELG. 894148, SWISS 651 754, BRD P-323 1088, SPAIN 553 017, JAP. 151 4040

Varadarajan S., Yatin S., Aksenova M., Butterfield D. A. (2000): Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. J. Struct. Biol. **130**, 184—208

Yoshida Y., Niki E. (1992): Oxidation of phosphatidylcholine liposomes in aqueous dispersions induced by copper and iron. Bull. Chem. Soc. Jpn. **65**, 1849—1854

Final version accepted: April 23, 2004