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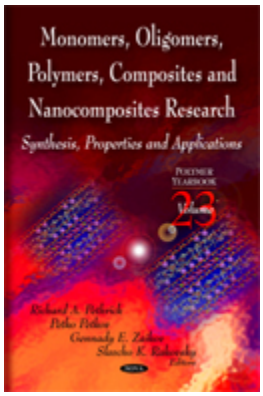

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Chapter 7

HYALURONAN DEGRADATION BY REACTIVE OXYGEN SPECIES: SCAVENGING EFFECT OF THE HEXAHYDROPYRIDOINDOLE STOBADINE AND TWO OF ITS DERIVATIVES

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ABSTRACT

The antioxidative effect of stobadine and two of its derivatives, SMe1EC2·HCl and SM1M3EC2·HCl, on the kinetics of degradation of high-molar-mass hyaluronan (HA) was tested in the oxidative system comprising Cu(II) *plus* ascorbic acid. Addition of stobadine at the initiation step of HA degradation resulted in a significant inhibition of degradation of the biopolymer. However, the protective effect of stobadine indicated dose-independent action. For SMe1EC2·HCl derivative, a significant protective effect against $AOO^{\bullet}$ and/or $AO^{\bullet}$ macro-radicals with a significant dose-dependency was observed. The stobadine derivative SM1M3EC2·HCl is a slight inhibitor at high concentrations when introduced in the oxidative system applied. At lower concentrations, this derivative significantly potentiated HA decay. When this derivative was applied one hour after the reaction onset, the action of different concentrations showed inverse actions in contrast to those found when SM1M3EC2·HCl was applied at the reaction initiation step.

Cyclic voltammetric and EPR spin trapping studies of the stobadine derivatives SMe1EC2·HCl and SM1M3EC2·HCl indicated formation of different oxidation products strongly depending on the pyridoindole substitution and on the solvent used. Oxidation

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products probably contributed to the antioxidant and radical scavenging capacity of these compounds. Standard ABTS and DPPH assays were used for the determination of total antioxidant capacity of the pyridoindole samples investigated. The compound SM1M3EC2·HCl exhibited the best hydrogen/electron donating antioxidant action with significantly higher antioxidant activity compared to stobadine. All stobadine derivatives tested exhibited higher radical scavenging activity compared to Trolox. For methoxy-substituted derivatives the EPR tests showed unusual kinetics and significant elimination of hydroxyl radicals formed in the reaction mixture.

Keywords: Free radicals, Hyaluronan degradation, Stobadine, Rotational viscometry, Stobadine derivatives, ABTS assay, DPPH assay, Cyclic voltammetry, EPR spin trapping method

INTRODUCTION

One of the intensively investigated potential drugs with substantial antioxidant action is the synthetic hexahydropyridoindole stobadine {cis-(-)-2,3,4,4a,5,9b-hexahydro-2,8-dimethyl-1-*H*-pyrido[4,3-b]indole}. Numerous studies have been focused on its antioxidant properties [1-6]. Stobadine was reported to be effective in the prevention or treatment of metabolic, cardiovascular, renal, neural, and hepatic diseases, and of disorders associated with diabetes mellitus [7-13]. In particular, there are several literature reports that confirm that stobadine suppresses lipid peroxidation and protein impairment under conditions of oxidative stress [8,9]. Various mechanisms of its action have been suggested, as scavenging of hydroxyl, peroxyl, and alkoxyl radicals, singlet oxygen quenching, repair of the oxidized amino acids, and prevention of the oxidation of -SH groups by one-electron donation [14]. The redox as well as radical scavenging properties of stobadine and its derivatives are, therefore, of high interest. However, there is but a small number of reports in the literature dealing with the comparison of the redox processes of stobadine and its radical scavenging activity. Only few reports have referred to the electrochemical oxidation of stobadine and its derivatives [14-16]. Our preliminary results both from cyclovoltammetric and antioxidant studies indicate a strong influence of stobadine substitution on redox behavior as well as on antioxidant action.

Some new indole-derived neuroprotective drugs based on the stobadine structure have been synthesized recently [17,18]. The aim was to develop derivatives with improved profiles of pharmacodynamics and toxicity. *In vitro* the best antioxidant activity was observed for the stobadine derivative 4a,9b-(cis)-2-ethoxycarbonyl-6,8-dimethyl-1,2,3,4,4a,9b-hexahydro-1*H*-pyrido[4,3-b]indolium chloride (SM1M3EC2·HCl), while the best radical scavenging capacity was observed for the compound 4a,9b-(cis)-2-ethoxycarbonyl-8-methoxy-1,2,3,4,4a,9b-hexahydro-1*H*-pyrido[4,3-b]indolinium chloride (SMe1EC2·HCl). *In vivo*, in a mice model of head trauma, some of the new stobadine derivatives administered immediately after the trauma, significantly improved sensomotoric outcome in the animals assessed one hour later. Irreversible impairment of neurotransmission resulting from hypoxia was significantly reduced in the presence of SMe1EC2·HCl. Both neuroprotective and antioxidant effects of the compound closely resembled those of stobadine, melatonin, 21-aminosteroids, α-phenyl-tert-butylnitrone and others, all well-established antioxidants, except that the range of effective concentrations was by one to two orders lower in the case of SMe1EC2·HCl.

Moreover, the acute toxicity of some of these new pyridoindoles was diminished when compared to stobadine.

Polyelectrolyte hyaluronic acid (HA) is an important component in synovial fluid forming highly viscoelastic solutions at a concentration range of approx. 2.0–4.0 mg/mL. Hyaluronan and its derivatives have been intensively studied as vehicles for the controlled delivery of a variety of drugs [19]. At higher concentrations, HA solutions exhibit the phenomenon of non-Newtonian flow behavior due to the formation of a microheterogeneous network [20]. In the presence of a reductant along with transition metal ions, in particular copper ions, HA degradation is derived from metal-mediated production of reactive free, mostly hydroxyl radicals. Under aerobic conditions, the system of ascorbate and copper(II) ions provides hydrogen peroxide, which turns into $^{\bullet}OH$ radicals by a Fenton-like reaction [21,22]. Recently, we advantageously combined viscometry measurements with EPR spectrometry in studies of pro- and anti-oxidative effects of an anti-rheumatoid drug, D-penicillamine (D-PN), or the effects of the presence of Mn(II) ions on the kinetics of high-molar-mass hyaluronan degradation, applying *Weissberger's* system comprising ascorbic acid *plus* cupric ions. Electron paramagnetic resonance spectroscopy was used to identify the generated free radicals [23,24].

The present paper is investigating the role of the pyridoindole stobadine and of its two derivatives in the time- and concentration-dependent degradation of high-molar-mass hyaluronan initiated by *Weissberger's* system, on applying rotational viscometry. Additionally, electrochemical, antioxidant and radical scavenging properties of selected stobadine derivatives were determined. Standard ABTS and DPPH assays were used for determination of total antioxidant capacity of samples. The total antioxidant capacity of the stobadine derivatives was also compared with their radical scavenging capacity, evaluated by using the EPR/spin-trapping method.

EXPERIMENTAL

Biopolymers

The high-molar-mass hyaluronan sample P9710-2A used (M_w = 808.7 kDa, M_w/M_n = 1.63, R_g in solution = 110.0 nm, content of the transition metals: 4 ppm Cu and 13 ppm Fe) was donated by Lifecore Biomedical Inc., Chaska, MN, U.S.A.

Scheme 1. Structures of the hexahydropyridoindoles investigated.

Chemicals

The analytical purity grade NaCl and $CuCl_2 \cdot 2H_2O$ were purchased from Slavus Ltd., Bratislava, Slovakia; ethanol (spectroscopy grade) from Mikrochem, Pezinok, Slovakia; L-ascorbic acid, dimethyl sulfoxide (DMSO), $K_2S_2O_8$ from Merck KGaA, Darmstadt, Germany; 5,5-dimethylpyrroline-N-oxide (DMPO), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) from Sigma-Aldrich, St. Louis, MO; tetrabutylammonium perchlorate (TBAP), $LiClO_4$, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) diammonium salt (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) from Fluka, Buchs, Switzerland. Stobadine and stobadine derivatives SMe1EC2·HCl, SM1M3EC2·HCl (Scheme 1) were synthesized at the Institute of Experimental Pharmacology and Toxicology, SAS. Redistilled deionized highquality grade water, with conductivity of ≤0.055 μS/cm, was produced by using the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

Preparation of Stock and Working Solutions

The working solutions of the HA samples (2.5 mg/mL) were prepared overnight in the dark at room temperature in 0.15 M aqueous NaCl in two steps: first, 4.0 mL of the solvent was added in the morning, then after six hours, the solvent in the volume of 3.85, 3.70, or 3.40 mL was added. The stock solutions of ascorbic acid (16.0 mM), stobadine (16.0 mM), SMe1EC2·HCl (16.0 mM), SM1M3EC2·HCl (16.0 mM), $CuCl_2$ (16.0 mM, diluted to a 160 μM solution) were also prepared in 0.15 M aqueous NaCl.

Study of Uninhibited/inhibited Hyaluronan Degradation

Antioxidative effects of stobadine and two stobadine derivatives, SMe1EC2·HCl and SM1M3EC2·HCl, on the kinetics of degradation of a high-molar mass HA sample were tested in the oxidative system comprising Cu(II) *plus* ascorbic acid by adding the substance either before the reaction onset or after one hour. The experimental set (fig. 1, Panels A, B, C) was carried out by adding the substances at the beginning of HA degradation: HA solution was stirred for 30 seconds prior to each processing. The solution of 50.0 μL of $CuCl_2$ (160 μM) solution was added and stirred for 30 seconds and left to stand for seven minutes and 30 seconds at room temperature. Stobadine or its two derivatives in the volume of 50, 200 or 500 μL (16.0 mM) were added to the solution examined and stirred again for 30 seconds. Finally, 50.0 μL of ascorbic acid (16.0 mM) was added and stirred for 30 seconds. The concentrations of stobadine and of its two derivatives in the degradative system tested were thus 100, 400 and 1000 μM, respectively. The results of the settings were compared with the reference experiments in the absence of the substances tested.

Rotational Viscometry

The resulting reaction mixture (8.0 mL) was transferred into the Teflon® cup reservoir of the Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). Recording of the viscometer output parameters started two minutes after the experiment onset. The changes of dynamic viscosity of the system were measured at 25.0 ± 0.1 °C in 3-min intervals for up to five hours. The viscometer Teflon® spindle rotated at 180 rpm, i.e., at the shear rate equaling 237.6 s^{-1}.

Cyclic Voltammetry

All cyclovoltammetric experiments were performed with HEKA PG 284 (Germany) potentiostat under argon using a standard three-electrode arrangement of a platinum wire (in DMSO) or a glassy carbon rod (in water) as working electrodes, a platinum coil as counter electrode, and a saturated calomel electrode (SCE) as reference electrode. The scan rate 100 $mV \cdot s^{-1}$ was used in cyclic voltammetry experiments and pulse amplitude 50 mV, pulse width 50 ms and pulse step 5 mV were used for square wave voltammetry measurements.

Determination of Antioxidant Capacity

Standard ABTS assay was used for the determination of total antioxidant capacity of samples. The total antioxidant capacity of the stobadine derivatives was also compared with the radical scavenging capacity evaluated, using the EPR/spin-trapping method. Antioxidant activity was measured by a modified methods of Re et al. and Arts et al. [25,26]. To prepare a solution of the ABTS cation radical, an aqueous solution of $K_2S_2O_8$ (3.3 mg in 5 mL distilled water) was added to 17.2 mg ABTS and the resulting solution was stored 14 hours in the dark. The final dark-green radical solution in the volume of 1 mL was then diluted with 60 mL of distilled water and used in the ABTS tests: 100 □L of 0.1 mM sample in 10% ethanol was added to 2 mL of $ABTS^{+\bullet}$ solution in 1 cm UV-cell and vigorously mixed. The decrease of the absorbance at 730 nm was observed for 10 minutes, using the UV-Vis-NIR Shimadzu 3600 spectrometer (Japan). The kinetics was recorded in the spectrometer with simultaneous detection of the absorbance at 1075 nm to correct the potential baseline distortions. The difference in the absorbance in the 10^{th} minute relative to the reference experiment (100 □L of 10% ethanol instead of the sample), □A, and the calibration curve of Trolox (Sigma-Aldrich, Germany) were used to calculate the Trolox equivalents (TEAC – Trolox Equivalent of Antioxidant Capacity).

Antioxidant activity determination using the free DPPH radical was similar to the ABTS assay. The amount of 200 □L of 0.1 mM sample solution in 10% ethanol was mixed with 2 mL of 0.1 mM DPPH in ethanol and the decrease of absorbance at 516 nm was observed for 10 minutes. The difference in the absorbance in the 10^{th} minute relative to the reference experiment and the Trolox calibration curve were used to express the obtained results in TEAC.

EPR/spin Trapping Method

In EPR experiments with Bruker EMX spectrometer (Bruker, Germany), the thermal decomposition of $K_2S_2O_8$ at 333 K was used as a source of reactive radicals. The amount of generated radicals was monitored in air saturated samples employing 5,5-dimethylpyrroline-N-oxide spin trap (DMPO, Sigma-Aldrich, Germany). Aqueous solutions of samples in the volume of 200 □L and the concentration of 0.5 mM, 25 □L 200 mM DMPO in DMSO and 25 □L 10 mM $K_2S_2O_8$ in H_2O were used in the reaction mixture. The time course of EPR spectra of the DMPO spin-adducts was recorded in 1-min intervals for 20 min at 60 °C (each spectrum was an accumulation of 3 scans). The EPR intensity (double integral) recorded for the sample solutions were compared with the reference measurement using 0.5 mM Trolox as antioxidant standard.

RESULTS AND DISCUSSION

Estimation of the antioxidant activity in *in vitro* tests is often dependent on the testing system. Specificity and sensitivity of one method does not lead to complete assessment of total antioxidant capacity in samples investigated, and a combination of several tests and methods is desired to obtain reliable results. The data from several independent methods are therefore compared in this study, including (i) rotational viscometry; (ii) cyclic voltammetry in DMSO as well as aqueous solutions; (iii) EPR/spin-trapping study based on thermal decomposition of $K_2S_2O_8$ covering the estimation of the ability of samples to scavenge reactive radicals; and (iv) estimation of Trolox equivalent antioxidant capacity (TEAC) by monitoring ABTS cation radical and DPPH with UV-Vis spectroscopy, delivering information about the hydrogen/electron donating antioxidant action.

Rotational Viscometry

Figure 1 shows the kinetics of dynamic viscosity (η) of the HA solution to which the two main components, i.e., Cu(II) *plus* ascorbate (0.1 μM *plus* 100 μM), had been admixed. The initial value of η = 9.93 mPa·s decreased continuously in time, and after five hours, it reaches 6.30 mPa·s (curves coded 0 in all Panels A, B, and C in fig. 1). Evidently, the addition of stobadine at the initiation step of HA degradation resulted in a significant inhibition of degradation of this biopolymer lasting approx. one hour (red curves coded 1). However, it is also evident that the protective effect of stobadine does not indicate any dose-dependent action. All three concentrations assayed, i.e., 100, 400, and 1000 μM, led to equivocal dependences, which after a period of total inhibition did not indicate any protection of HA against degradation as shown by the slope of curves (red curves coded 1, fig. 1) virtually parallel with that valid for the curve coded 0, i.e., in the absence of stobadine.

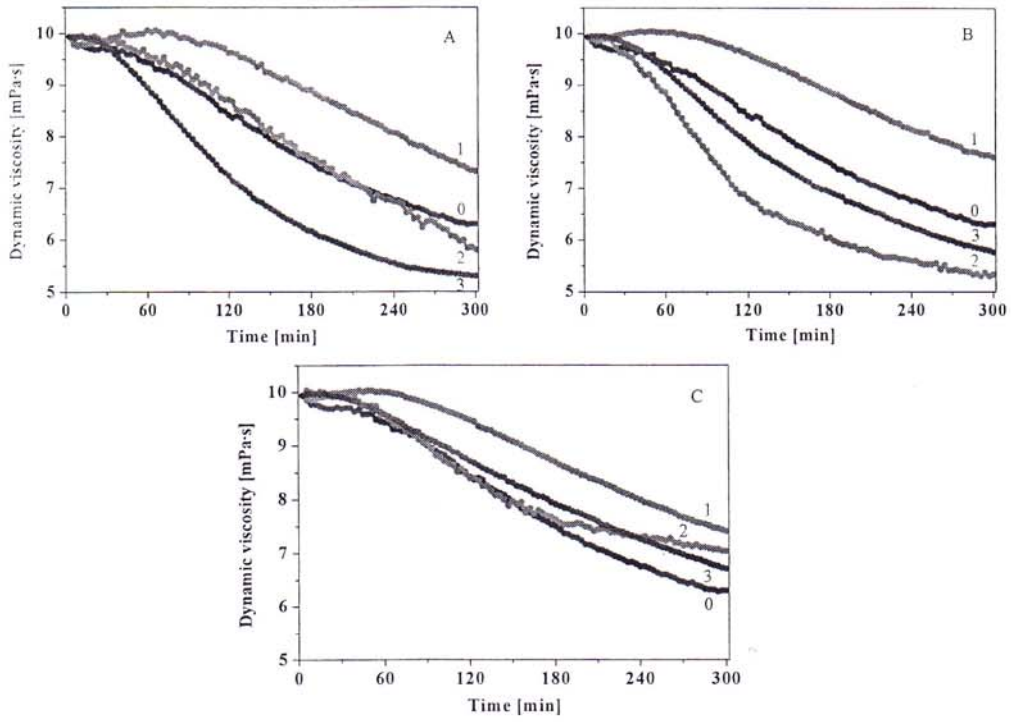

Figure 1. Time-dependent HA P9710-2A degradation in the presence of 0.1 μM $CuCl_2$ *plus* 100 μM ascorbic acid (0) and the effect of stobadine.
(1), SMe1EC2·HCl (2) or SM1M3EC2·HCl (3) (concentration 100 μM - Panel A, concentration 400 μM - Panel B, concentration 1000.
μM - Panel C) added at the reaction onset.

The above observation stimulated our further experiments where stobadine was applied into the reaction mixture just one hour after the start of the degradation process, i.e., when the propagation phase was already prevailing. Contrary to the experiments represented in Figure 1, where the generation of $^{\bullet}OH$ radicals should be a dominating process, further (cf. fig. 2) the effect of stobadine against characteristic radicals formed in the propagation phase (peroxyl and/or alkoxyl hyaluronan macro-radicals) was assayed. As seen, stobadine is really effective in this phase of reaction scavenging ROS, however, after a period lasting somewhat longer than one hour, no further protection of the HA macromolecule degradation was recorded. Moreover, the slopes of the curves (red) were practically paralleled with the slope valid for the reaction system with no addition of stodadine. As in experiments represented in Figure 1, again no dose-dependency of stobadine was marked. Moreover, it should be pointed out that stobadine indicated a slight dose-dependency in protection against HA degradation, however, only when the concentration of Cu(II) = 1.0 μM, i.e., when it was far too high to model physiological and/or slight acute phase of pathophysiological catabolism of HA within synovial fluid. The results decisively indicate that stobadine is an effective scavenger, yet its protective effect was relevant only in the highest concentration equaling to 1000 μM, either added at the reaction onset or one hour after the reaction onset (results not shown).

The stobadine derivative SM1M3EC2·HCl is a slight inhibitor of HA degradation when applied to our oxidative system, as can be deduced from the results represented in Figure 1, Panel C, blue curve. While in the concentration of 1000 μM this derivative indicated a minor inhibitory effect, the lower concentrations, i.e., 400 μM and especially that of 100 μM, significantly potentiated the decay of HA. When this derivative was applied one hour after the reaction onset, its addition in different concentrations led to actions inverse to those revealed when SM1M3EC2·HCl was applied at the reaction initiation step. Namely, SM1M3EC2·HCl in the concentration of 100 μM indicated relevant scavenging of ROS (we propose the domination of $AOO^{\bullet}$ and $AO^{\bullet}$ macro-radicals in this phase of reaction), while in the 400 μM concentration it was practically ineffective, and in the highest concentration tested, i.e., 1000 μM, it indicated HA degradation.

The SMe1EC2·HCl derivative, on the other hand, demonstrated a significant protective effect against $AOO^{\bullet}$ and/or $AO^{\bullet}$ macro-radicals, and this action was dose-dependent. The lowest concentration applied, i.e., 100 μM, was however ineffective, yet the concentrations of 400 μM and especially that of 1000 μM were practically identically effective with that demonstrated by the parent stobadine molecule.

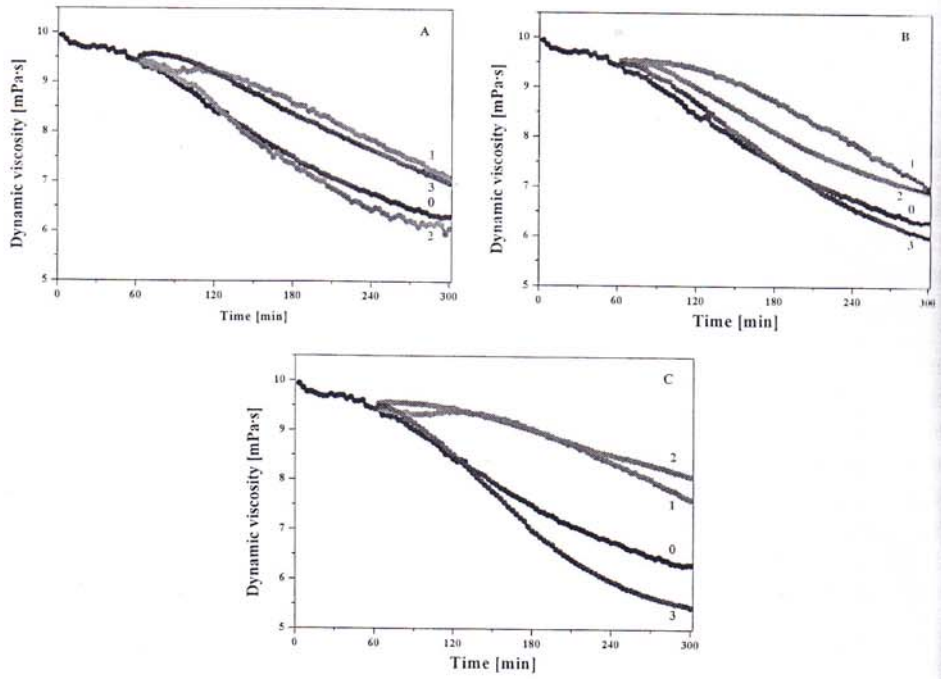

Figure 2. Time-dependent HA P9710-2A degradation in the presence of 0.1 μM $CuCl_2$ *plus* 100 μM ascorbic acid (0) and the effect of stobadine.
(1), SMe1EC2·HCl (2) or SM1M3EC2·HCl (3) (concentration 100 μM - Panel A, concentration 400 μM - Panel B, concentration 1000
μM - Panel C) added 1 h after the reaction onset.

In the further part of this study, experiments were carried out in order to explain the results shown in Figures 1 and 2 in detail. Cyclic voltammetry, EPR spectroscopy, ABTS and DPPH tests were selected for this purpose.

Cyclic Voltammetry

The electrochemical studies were focused on three representative derivatives applied in viscometry studies described above. The first molecule is stobadine as a reference sample. The substances SMe1EC2·HCl and SM1M3EC2·HCl represent hexahydropyridoindole stobadine derivatives with the $-CO_2CH_2CH_3$ group instead of the methyl group in stobadine. Additionally, there is a methoxy group on the phenyl ring of the SMe1EC2·HCl structure instead of a methyl group in stobadine. The SM1M3EC2·HCl structure has an additional methyl group on the phenyl ring compared to stobadine (see structural formulae in Scheme 1). Figure 3 shows representative cyclic voltammograms obtained in the oxidation of stobadine, SMe1EC2·HCl and SM1M3EC2·HCl in DMSO containing 0.1 M TBAP as a supporting electrolyte using a platinum working electrode as well as in aqueous solution containing 0.1 M $LiClO_4$ using a glassy carbon rod working electrode.

As seen in Figure 3, remarkable differences in redox behavior of stobadine derivatives were observed by changing the non-aqueous solvent (DMSO) representing low proton donating solvent to highly proton donating aqueous solutions. Generally, in DMSO/TBAP solutions a complex redox behavior was observed in the anodic part. Rich cyclovoltammetric responses with several oxidation peaks were found indicating the complexity of stobadine oxidation in non-aqueous media. The number of new redox couples and the corresponding oxidation potentials strongly depend on the indole substitution.

Even more complex redox behavior in the potential region from 0 V to 1 V vs. SCE was observed in aqueous solutions compared to DMSO. In all cyclic voltammograms in water solutions, several consecutive products were found in the reverse scans for the SMe1EC2·HCl sample. One of them can be again oxidized in a reversible step. The new redox peaks originate from the oxidation products formed at higher potentials as shown in Figure 4.

To confirm this fact, the cyclovoltammetric scan was started at -0.4 V vs. SCE and was increased only at the beginning of the first oxidation peak (0.3 V vs. SCE). No new redox peaks were observed in this region during potential cycling (results not shown). However, after shifting the oxidation potential to the region of the first oxidation peak or even behind this peak, a new reversible redox couple was clearly observable around 0.1 V vs. SCE (fig. 4A). This new product was also confirmed by square wave voltammetry at the same experimental conditions (fig. 4B). This demonstrates that the consecutive products originate from oxidation steps at higher potentials. The reduction of these products at low potentials and their reversible electrochemical behavior indicate that they might serve as more effective antioxidants in comparison to the parent compound. Due to the differences in the reaction mechanisms in different media, deviations in the relationship between electrochemical redox potentials and the antioxidant and radical scavenging capacity of the pyridoindoles investigated are to be expected

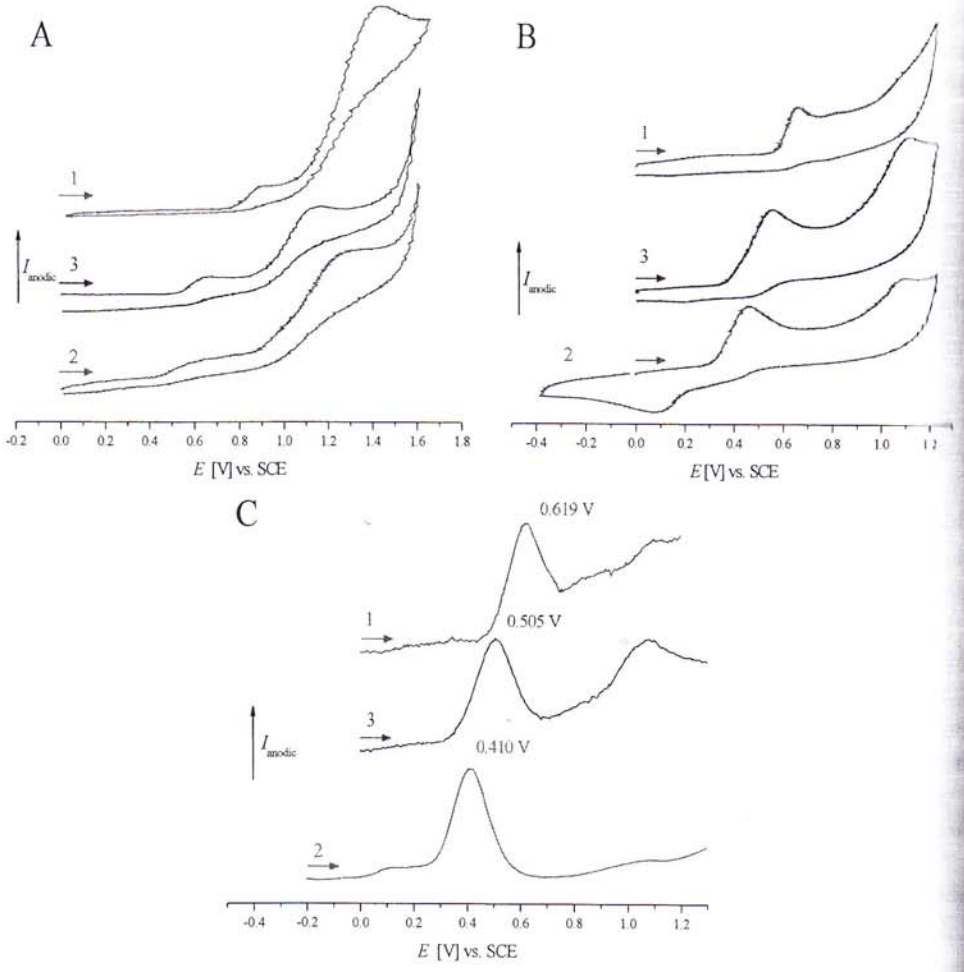

Figure 3. Cyclic voltammograms and square wave voltammograms obtained in the oxidation of pyridoindole stobadine samples investigated (1), SMe1EC2·HCl (2) and SM1M3EC2·HCl (3) obtained in A) freshly prepared 0.1 mM solutions in DMSO containing 0.1 M TBAP as supporting electrolyte and using Pt wire working electrode; B) cyclic voltammograms and C) square wave voltammograms of freshly prepared 0.5 mM solutions in H_2O containing 0.1 M $LiClO_4$ using glassy carbon rod working electrode.

Antioxidant and Radical Scavenging Assays

Standard ABTS and DPPH assays were used for the determination of total antioxidant capacity of samples expressed in TEAC. The thermal decomposition of $K_2S_2O_8$ in water solutions at 333 K was used as a source of reactive hydroxyl radicals. As seen in Figure 5, the best hydrogen/electron donating antioxidant action is exhibited by the sample SM1M3EC2·HCl. The lowest antioxidant activity was observed for stobadine in both ABTS and DPPH tests.

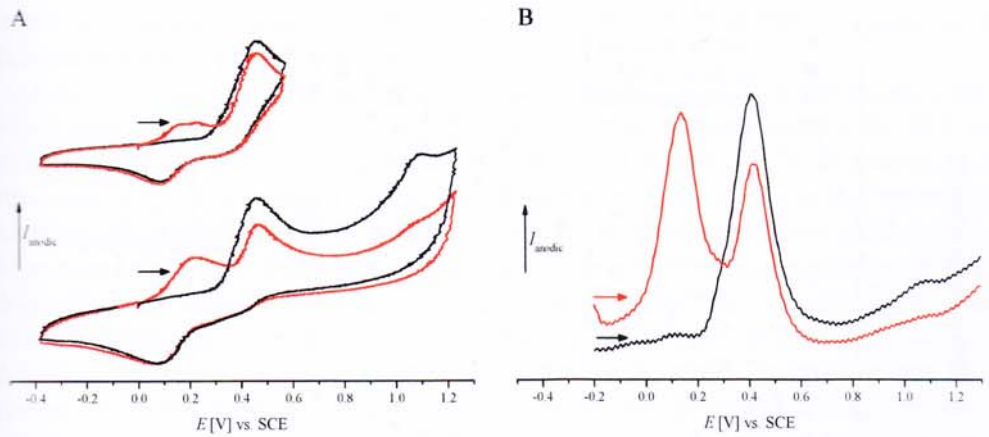

Figure 4. A) Cyclic voltammograms obtained in the oxidation of SMe1EC2·HCl in H_2O containing 0.1 M $LiClO_4$, using glassy carbon rod working electrode. B) Results from square-wave voltammetry (black lines – 1st scan, red lines – 2nd scan).

A completely different behavior was observed in the EPR test for SMe type structures in water solutions, as illustrated for SMe1EC2·HCl in Figure 6. An unusual kinetic curve shows an increased elimination of hydroxyl radicals formed in the reaction mixture. This confirms a special role of the methoxy group on the benzene ring in stobadine-derived indoles, already indicated in the cyclovoltammetric experiments, concerning antioxidant properties of these compounds.

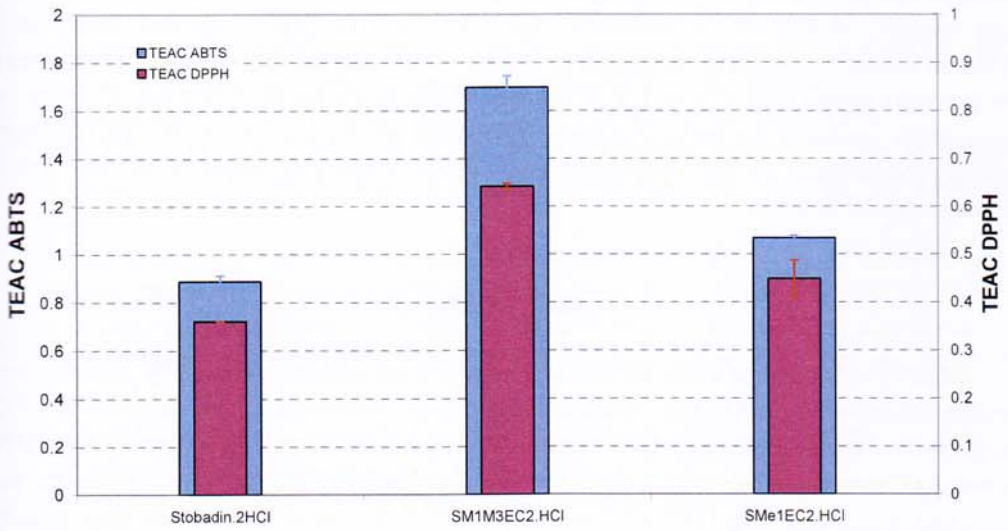

Figure 5. Trolox Equivalent of Antioxidant Capacity (TEAC) of the investigated pyridoindoles determined by ABTS and DPPH tests in aqueous solutions.

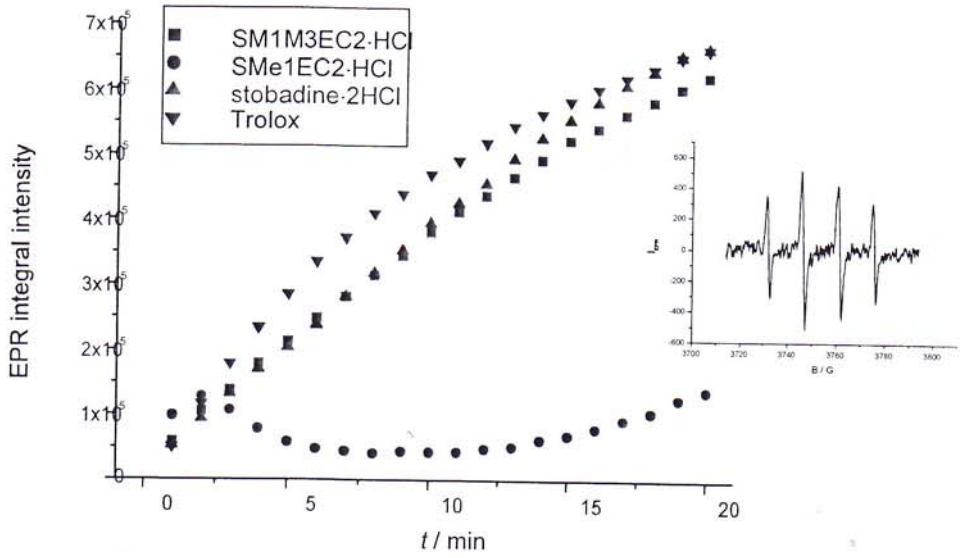

Figure 6. Time course of integral EPR intensities of •OH-DMPO spin adducts measured on indole derivatives and Trolox in EPR/spin-trapping test (Inset: representative EPR spectrum of •OH-DMPO spin adduct observed).

The samples investigated can be divided into two groups based on their redox as well as radical scavenging behavior in aqueous solutions. Stobadine and SM1M3EC2·HCl exhibited higher oxidation potentials both for the parent molecules and of the consecutive reaction products compared to the second group (SMe type derivatives). Cyclic voltammetric and EPR spin trapping studies of the stobadine derivative SMe1EC2·HCl indicated formation of different oxidation products compared to stobadine. A complex redox behavior observed in the potential region from 0 V to 1 V in aqueous solutions shows the formation of one or two consecutive products for these structures. Oxidation products of SMe1EC2·HCl probably strongly contributed to the antioxidant and radical scavenging capacity of this type of hexahydropyridoindoles.

CONCLUSIONS

Addition of stobadine at the initiation step of HA degradation initiated by *Weissberger's* system {ascorbate *plus* Cu(II)} resulted in significant inhibition of the degradation of the biopolymer. For the SMe1EC2·HCl derivative a significant protective effect against AOO• and/or AO• macro-radicals with a significant dose-dependency was observed. When the new stobadine derivatives SMe1EC2·HCl and SM1M3EC2·HCl were applied one hour after the reaction onset, the action of different concentrations showed inverse actions in contrast to the actions revealed when these derivatives were applied at the reaction initiation step.

Standard ABTS and DPPH assays were used for determination of total antioxidant capacity of samples. The compound SM1M3EC2·HCl exhibited the best hydrogen/electron donating antioxidant action with remarkably higher antioxidant activity compared to stobadine. This correlates well with previous SAR studies, suggesting that it is the sum of

aromatic substitution constants and hydration energy that are important in enhancing the radical scavenging properties of new stobadine derivatives.

All stobadine derivatives tested exhibited higher radical scavenging activity comparing to the most used antioxidant standard Trolox. For methoxysubstituted derivatives, the EPR tests showed unusual kinetics and a significant elimination of hydroxyl radicals formed in the reaction mixture. This confirms a special role of the methoxy substituent on the benzene ring concerning the exceptional ability of these derivatives to scavenge reactive radical species. Similar phenomena were found in previous studies where thermal decomposition of AAPH (2,2'-azobis(2-amidinopropane) hydrochloride) as a source of reactive radicals was used [27,28]. Thus deviations in the relationship between electrochemical redox potentials and antioxidant and radical scavenging capacity of the pyridoindoles investigated are to be expected due to the differences in the reaction mechanisms in different media. While the electrochemical redox potentials of secondary oxidation products can be clearly separated from those of the parent compounds, when measuring antioxidant properties by various tests an overall activity of the parent compound and of its secondary oxidation products is found.

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