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High-molecular-weight hyaluronan—a valuable tool in testing the antioxidative activity of amphiphilic drugs stobadine and vinpocetine

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Abstract

The antioxidative activity of stobadine and vinpocetine was studied in vitro by measuring their inhibition effect on the depolymerization of the high-molecular-weight hyaluronan by hydroxyl radicals. The radicals were generated by the $Cu^{2+}-H_2O_2$ system. Hyaluronan depolymerization was monitored by means of size exclusion chromatography. The antioxidative activity of stobadine and vinpocetine was compared to that of D-mannitol. A 50% inhibition of hyaluronan depolymerization was reached at stobadine and vinpocetine concentrations of 1.7×10^{-6} and 3.0×10^{-7} mol l^{-1}, respectively, while a D-mannitol level of 2.6×10^{-3} mol l^{-1} was needed to achieve the same inhibitory effect. © 1997 Elsevier Science B.V.

Keywords: Radical depolymerization of hyaluronan; Antioxidant; Stobadine; Vinpocetine; D-mannitol

1. Introduction

Reactive oxygen species (ROS), such as the superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$), are formed in cells as a consequence of normal aerobic respiration [1]. These reactants are responsible for many deleterious effects including irreversible damage of some macrobiomolecules, observed in inflammatory joint diseases [2].

Hyaluronan—2-acetamido-2-deoxy-D-glucano-D-glucuronan [3]—a major biopolymer of synovial fluid, seems to be depolymerized by direct oxidative cleavage of the glycosidic bond(s) in the macromolecule. Hyaluronan attacked by ROS, and especially by the $^{\cdot}OH$ radicals, yield several intermediates and end-products [4] found in increased concentrations in the synovial fluid and serum of rheumatic patients [5,6]. In vitro protection of hyaluronan against radical depolymerization was achieved by some drugs—mostly belonging to antioxidants and/or free-radical scavengers [7,8].

Stobadine (Scheme 1) is a cardioprotective drug with antihypoxic and anti-arrhythmic effects [9,10]. This drug belongs to the group of effective

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scavengers of free-oxygen-radicals protecting both synthetic and natural lipids against their spontaneous and hydrogen peroxide-induced peroxidation [11,12]. In some in vitro studies stobadine was found to have a stronger antioxidant effect than e.g. butylated hydroxytoluene or vitamin E [13]. The spin trapping technique proved stobadine to be a potent scavenger of the $^{\cdot}OH$ and $ROO^{\cdot}$ radicals [14].

Vinpocetine (Scheme 2), a cerebral vasodilatory agent derived from the alkaloid vincamine [15,16], was used in the treatment of acute and chronic hypoxic and ischemic central nervous disorders [17,18]. During acute ischemia and reperfusion this drug was shown to have a beneficial effect on cerebral circulation and cell function [19,20]. It decreases brain vascular resistance, blood viscosity, platelet aggregation, and it increases red blood cell fluidity [21]. Although the mechanism of vinpocetine action is not fully understood, its hydroxyl radical scavenging ability was demonstrated [22].

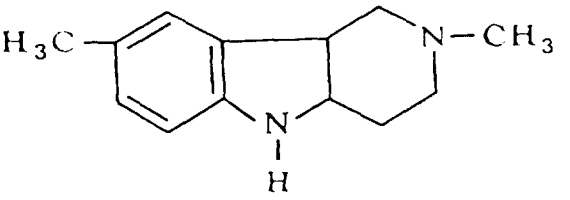
In this study we investigated the antioxidative activity of stobadine and vinpocetine on high-molecular-weight hyaluronan depolymerization by free radicals produced in the $Cu^{2+}-H_2O_2$ system. Hyaluronan depolymerization was monitored by size exclusion chromatography (SEC) [23].

2. Materials and methods

2.1. Drugs, biopolymers, chemicals

Vinpocetine, known as Cavinton®, was from Gedeon Richter (Budapest, Hungary). Stobadine was from the Institute of Experimental Pharmacology, Slovak Academy of Sciences (Bratislava, Slovak Republic).

Sodium hyaluronate, a sample with the molecular weight (M_w-average) 1.0×10^6 Da was prepared from rooster combs [24]. Sodium hyaluronate reference materials, with M_w-averages of 3.0×10^6, 1.6×10^6, 8.1×10^5, 4.9×10^5 and 3.9×10^5 Da, used for SEC device calibration, were kindly provided by Dr Ove Wik, Pharmacia Fine Chemicals (Uppsala, Sweden).

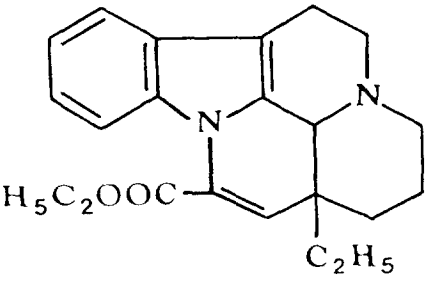


Scheme 1. Stobadine

NaH_2PO_4 and Na_2HPO_4 of p.a. grade were purchased from Serva FeinBiochemica (Heidelberg, Germany). D-mannitol (M 4125) was supplied by Sigma (St. Louis, MO). $CuSO_4$ and NaCl of p.a. grade as well as concentrated H_2O_2 solution (32%, w/w) were from Lachema (Brno, Czech Republic). Millipore Q-quality water (Millipore, Bedford, MA) was used for the preparation of buffers and the SEC eluent.

2.2. Procedure

The aqueous solution of high-molecular-weight hyaluronan ($M_w = 1.0 \times 10^6$ Da; 0.1 mg ml^{-1}), $CuSO_4$ (5.5×10^{-6} mol l^{-1}) and NaCl (0.15 mol l^{-1}) was preincubated over night at 37 ± 0.5°C. Depolymerization of the biopolymer was started by adding a given amount of concentrated hydrogen peroxide (Fig. 1). When hyaluronan depolymerization was to be inhibited, the drug to be tested was added to the reaction vessel immediately before addition of the H_2O_2 solution. At individual time intervals (Figs. 1 and 2a–c) an aliquot volume (10 μl) withdrawn from the reaction vessel was mixed with 90 μl of the SEC eluent and the final solution, filtered through a 0.45 μm



Scheme 2. Vinpocetine

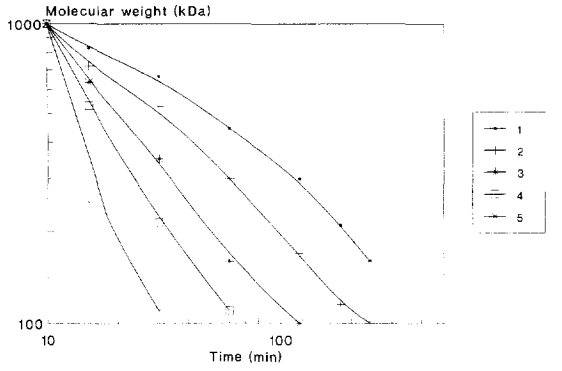


Fig. 1. Time course of hyaluronan depolymerization by the $Cu^{2+}-H_2O_2$ system containing 5.5×10^{-6} mol l^{-1} $CuSO_4$ and different amounts of H_2O_2: (1) 1.3×10^{-4}, (2) 2.6×10^{-4}, (3) 5.2×10^{-4}, (4) 1.04×10^{-3} and (5) 2.6×10^{-3} mol l^{-1}.

disposable sample clarification unit (Millipore Corporation, Bedford, MA), was then analyzed by SEC.

2.3. Chromatographic apparatus and separation conditions

The high-performance liquid chromatographic device comprised the System Gold 126 Programmable Solvent Module equipped with a 166 Programmable Detector Module set to 206 nm (Beckman Instruments, San Ramon, CA). The stainless-steel column (250 mm × 8 mm i.d.) used was filled with 'purpose made' aminopropyl-modified silica gel SG-10-6000-NH_2 particles of 9 μm with 600 nm pore size. (The 'gigaporous' silica used were prepared and kindly supplied by Dr I. Novák (Polymer Institute, Slovak Academy of Sciences, SK-84236 Bratislava, Slovak Republic) (I. Novák, Patent Pending)). The flow-rate of the phosphate buffer eluent (50 mmol l^{-1}, pH 9.5) was 0.4 ml min^{-1}. The sample injection volume was 20 μl. The analyte concentration of 0.01%, w/v, was applied so as to eliminate the concentration effects which often occur when analyzing high-molecular-weight hyaluronans. All chromatographic separations were performed at 40 ± 0.5°C [23]. At the calibration, the elution volume of the chromatographic record peak of the reference hyaluronan sample was plotted against the

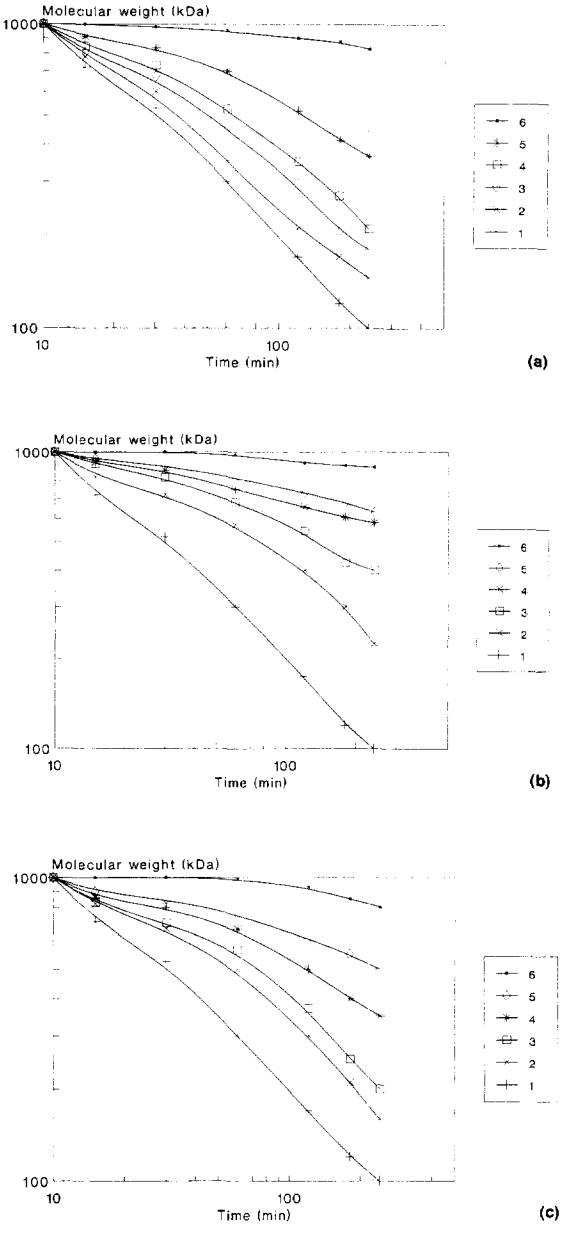


Fig. 2. Free-radical scavenging effect of stobadine (a), vinpocetine (b), and of D-mannitol (c) on hyaluronan depolymerization. Stobadine concentrations: (1) 0.0, (2) 1.3×10^{-7}, (3) 2.6×10^{-7}, (4) 6.3×10^{-7}, (5) 1.3×10^{-6} and (6) 2.6×10^{-6} mol l^{-1}. Vinpocetine concentrations: (1) 0.0, (2) 9.5×10^{-8}, (3) 1.9×10^{-7}, (4) 4.7×10^{-7}, (5) 9.5×10^{-7} and (6) 1.9×10^{-6} mol l^{-1}. D-mannitol concentrations: (1) 0.0, (2) 1.7×10^{-4}, (3) 3.3×10^{-4}, (4) 1.7×10^{-3}, (5) 3.3×10^{-3} and (6) 6.6×10^{-3} mol l^{-1}.

molecular weight of the standard material. Similarly, on evaluating the molecular weights of the depolymerized hyaluronans, the position of the chromatographic curve peak was taken to estimate the sample (apparent) molecular weight (M_r) [25].

3. Results and discussion

Simple in vitro systems used for testing the antioxidative efficacy of a compound commonly contain three basic components, i.e. a source generating ROS, the antioxidant to be tested, and an appropriate marker indicating the course of the reaction [26].

In our study hydrogen peroxide was chosen as the source of ROS since it decomposes in the presence of ions of copper (a transition metal) yielding the hydroxyl radical ($^{\cdot}OH$). Since the presence of H_2O_2 as well as ions of copper [27] was demonstrated in the synovial fluid in inflammatory diseases of the joints, the ROS generating source chosen may be considered to have a qualitative composition closely related to the given specific pathophysiological situation.

The choice of the high-molecular-weight hyaluronan as a marker of the course of the reaction involving ROS can be substantiated from various aspects. Most importantly, high-molecular-weight hyaluronan in a 'healthy' joint is known to fulfill certain lubricating functions. On the other hand, hyaluronan with a significantly lower molecular weight found in the inflamed joint can fulfill this function only to a limited extent. Due to the high sensitivity of the hyaluronan polymer chain to the action of ROS and especially to the depolymerization action of the hydroxyl radicals [4], resulting in a reduction of hyaluronan molecular weight, this biopolymer is one of the highly appropriate markers of damage of macrobiomolecules [7,8,28–30]. This was also confirmed by our experimental results shown in Fig. 1. As evident after addition of H_2O_2 the sample of high-molecular-weight hyaluronan ($M_w = 1.0 \times 10^6$ Da) is extensively depolymerized and the extent and the rate of this depolymerization is proportional to the H_2O_2 level within the concentration range assayed. Under the experimental conditions tested, the value of M_r is lowered by one order of magnitude, reaching, within 1–2 h, approximately 10% of the initial value. In the system without addition of hydrogen peroxide, however, the stability of the high-molecular-weight hyaluronan was high (not shown).

Of the systems tested, composition No. 2 (Fig. 1) was selected as the reference composition for the subsequent experiments. The parameter studied was the M_r value, or rather its change depending on the type and amount of the added antioxidant whose effectiveness was tested (Fig. 2a–c). The results show that all the substances tested, i.e. stobadine, vinpocetine and D-mannitol, exhibited a significant inhibitory effect on the process of high-molecular-weight hyaluronan depolymerization induced by hydroxyl radicals. The dependence of the percentage of inhibition

$$100 \times (M_{ri} - M_{runi})/(M_r - M_{runi})$$

on the concentration of the antioxidant tested in the system is shown in Fig. 3; M_{ri} and M_{runi} are molecular weights of the hyaluronan sample at 120 min following onset of the reaction in the presence of the inhibitor (M_{ri}) or in its absence (M_{runi}), with the molecular weight of the native sample being $M_r = M_w = 1.0 \times 10^6$ Da. The results given in Fig. 3 show that the inhibitory dependences exhibit typical S-shaped curves, and the concentration value on the abscissa corre-

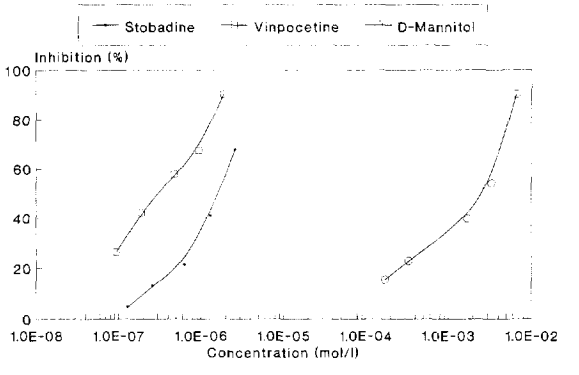


Fig. 3. The percentage of inhibition of the hyaluronan depolymerization attained after 120 min reaction time by applying various amounts of stobadine, vinpocetine, and D-mannitol to the reaction vessel.

sponding to 50% inhibition represents the so-called IC_{50}, i.e. the parameter to be determined. Comparison of the IC_{50} values clearly shows the high efficacy of both the antioxidants tested. Their IC_{50} values of 1.7×10^{-6} mol l^{-1} for stobadine and 3.0×10^{-7} mol l^{-1} for vinpocetine are by 3–4 orders lower than the IC_{50} value of D-mannitol, which was used as the reference antioxidant/scavenger of hydroxyl radicals.

The molecular interpretation of the high-molecular-weight hyaluronan depolymerization reaction brought on by $^{\cdot}OH$ radicals appears to be evident. On attacking the backbone of the polymer, the hydroxyl radical breaks up the macromolecular strand [4], thus reducing the molecular weight of the biopolymer. When an antioxidant is present in the system the decreased yield of the $^{\cdot}OH$ radicals versus biopolymer reaction results in inhibited depolymerization.

At present, the stochiometry of the process defies exact evaluation since the width of the distribution of molecular weights of the hyaluronan sample keeps gradually increasing due to its depolymerization [31]. The value $\bar{M}_n$, i.e. the number average of the molecular weight or the number average of the polymerization degree $\bar{P}_n$, should be determined as the most exact molecular-weight parameter. The optimal solution appears to be the application of HPLC/SEC by using an on-line absolute detector of molecular weights [32].

In Conclusion we can state that on determining the M_r changes by the HPLC/SEC method, the system of high-molecular-weight hyaluronan with Cu^{2+} and H_2O_2 used in our study appeared to be more than adequate for testing the antioxidative (free radical scavenging) properties of water-soluble low-molecular-weight substances. Both the prospective drugs studied displayed a high capacity to inhibit the hydroxyl radical induced depolymerization reaction of the high-molecular-weight hyaluronan.

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