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Inhibitory effects of different antioxidants on hyaluronan depolymerization

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Abstract—Hyaluronan (HA) was depolymerized by hydroxyl radicals generated from hydrogen peroxide and cupric ions. Inhibition of HA degradation by four well-known antioxidants was investigated, as HA can scavenge reactive oxygen species (ROS). Change in hyaluronan molecular weight was observed by size-exclusion chromatography. Inhibition of HA degradation was estimated from the retention times observed.

It was found that HA degradation was inhibited in a clearly concentration-dependent manner by mannitol, thiourea and vinpocetine. Propofol also inhibited the depolymerization, but its concentration-dependent effect was not so clear. The antioxidant concentrations at which HA degradation was decreased by 50% were 42 μM for thiourea; 1.35 μM for vinpocetine; and 0.39 μM for propofol. A concentration of 26.51 mM of mannitol was needed to attain the same inhibitory effect. Although many factors are involved in a therapeutic response, the results obtained in this study support the idea that HA may be protected from ROS attack by the concomitant use of well-known antioxidants.
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Keywords: Hyaluronan; Antioxidant; Reactive oxygen species; Mannitol; Thiourea; Vinpocetine; Propofol

1. Introduction

Hyaluronan (HA) is a high molecular weight non-sulfated glycosaminoglycan composed of strictly alternating sequences of 3-linked 2-acetamido-2-deoxy-β-D-glucopyranose and 4-linked β-D-glucuronic acid residues. It is produced by hyalocytes of the synovial membrane[1] and is present basically in synovial fluid, vitreous humor and skin.[2–6] Substantial evidence shows that reactive oxygen species (ROS) are responsible for HA degradation in inflammatory diseases, such as osteoarthritis, rheumatoid arthritis or chronic hepatitis.[7]

HA involvement in activation and modulation of the inflammatory response includes its antioxidant scavenging activity towards ROS, such as hydroxyl radical species (${}^{\bullet}OH$). The formation of ${}^{\bullet}OH$ can occur in several ways. However, by far the most important in vivo mechanism is mediated by hydrogen peroxide.[8] The formation of ${}^{\bullet}OH$ involves the presence of metallic ions found in the inflamed joint, and occurs *via* a Fenton reaction or an analogous reaction (Fe^{2+} in the Fenton reaction or Cu^{2+} in the analogous reaction).[9]

In vitro protection of HA against radical depolymerization has been achieved by some drugs, mostly antioxidants and/or free-radical scavengers.[10] Among them, mannitol is a polyol, which develops an antioxidant activity by scavenging ROS.[11,12] Several studies have evidenced the role of mannitol in the protection of HA against ROS. In such studies, it was observed that HA maintains its properties and avoids degradation.[10,12,13] Thiourea has also been widely used in the in vitro and in vivo prevention of metal-mediated biological damage by the hydroxyl radical.[14,15] Its role in preventing HA degradation by ${}^{\bullet}OH$ and peroxynitrite ($ONOO^-$) has been demonstrated by a lower decrease in HA molecular

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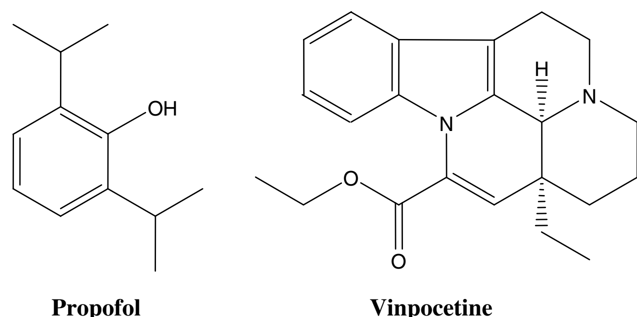

Chart 1. Chemical structures of propofol and vinpocetine.

weight.[16] Propofol (2,6-diisopropylphenol, Chart 1) is a widely used intravenous anaesthetic agent that induces and maintains anaesthesia in surgical procedures.[17] Its function as a lipid antioxidant has been demonstrated, as has its capacity to protect HA against degradation by scavenging ROS. In some studies, HA and propofol solutions were subjected to oxidative attack. A very clear inhibition of HA depolymerization was revealed.[18,19] Vinpocetine (eburnamenine-14-carboxylic acid ethyl ester, Chart 1) is a poorly water-soluble vincamine derivative, used in the treatment of disorders arising from cerebrovascular and cerebral degenerative diseases.[20,21] Its role in the treatment of the Alzheimer's disease or other neurodegenerative disorders, in which oxidative stress has been shown to be involved, has been demonstrated.[22] Although the mechanism of its action is not fully understood, its ROS scavenging ability has been pointed out using in vitro models of oxidative stress. In such models, the vincamine derivative plays a crucial role in developing preventive and protective effects.[10,23] These effects clearly protect HA from degradation under oxidative conditions.[10]

Both compounds have antioxidant properties under oxidative conditions. Such properties involve ROS scavenging ability and ROS consumption in reactions with these species, leading to the formation of harmless radicals[24] and protecting HA from depolymerization.

To investigate whether HA depolymerization can be inhibited by the antioxidants described above and whether their roles are concentration-dependent, we performed a HA degradation study involving the effects of a ·OH flux on a commercially available HA solution. We tested the inhibitory effects of different concentrations of mannitol, thiourea, propofol and vinpocetine on HA degradation.

2. Experimental

2.1. Materials

Sodium hyaluronate (average molecular weight of 1200 kDa) was commercially available (Ostenil® provided by TRB Chemedica, Lot CF0910AHA) and obtained by bacterial fermentation. The commercial Ostenil® soln is ready for injection and contains 10 mg sodium hyaluronate, sodium chloride, disodium and monosodium phosphates and water, which implies isotonicity and stabilization for clinical use. It is administered intra-articularly in the treatment of osteoarthritis.

To estimate the samples' molecular weight, a calibration curve (Fig. 1) has been drawn with sodium hyaluronate samples of known molecular weight (51, 112.2, 419.7, 940, 1500 and 1760 kDa) provided by Lifecore Biomedical (Chaska, MN, USA) and issued from a microbial process.

Sodium chloride, EDTA, mannitol, disodium and monosodium phosphates were supplied by Panreac Quimica S.A. (Madrid, Spain). Hydrogen peroxide (35%), copper sulfate, thiourea and vinpocetine were supplied by Sigma–Aldrich (Steinheim, Germany). Propofol was supplied by Fresenius Kabi Deutschland GmbH. MeOH and all of the analytical grade reagents used were purchased from E. Merck (Madrid, Spain).

2.2. Preparation of solutions

The Ostenil® soln was diluted to a final concentration of 0.5 mg/mL. EDTA was prepared at a concentration of 220 μM and $CuSO_4$ was used at a concentration of 1 mM. All of these were prepared in saline soln.

2.3. Preparation of inhibitors

The reagents were chosen because of their antioxidant and/or scavenger roles, as described in Section 1.

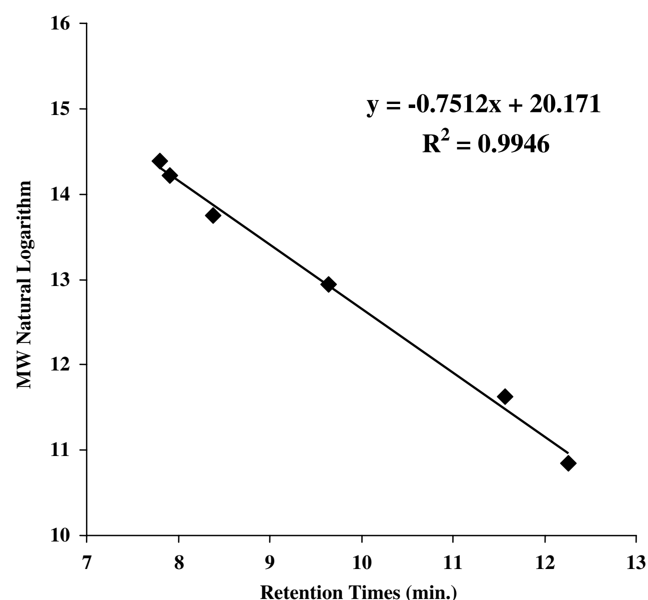

Figure 1. MW calibration curve. Molecular weight natural logarithm of six HA solutions between 51 and 1760 kDa are shown as a function of the retention times obtained by size-exclusion chromatography.

Mannitol solns in saline were tested at final sample concentrations between 15 and 200 mM, and thiourea solns between 0.1 μM and 1 mM. The commercially available propofol soln was dissolved in 50 mM phosphate buffer and adjusted to pH 5.5. It was used at final sample concentrations between 0.1 μM and 1 mM. We prepared a stock soln of vinpocetine in MeOH at a concentration of 100 μg/mL to make the vinpocetine soluble.[20] Dilutions were made in 50 mM phosphate buffer and adjusted to pH 5.5. Final sample concentrations between 0.1 and 10 μM were used.

It should be noted that the concentration of mannitol used was much higher than that used for the other compounds because lower concentrations of mannitol tested (0.1 μM to 1 mM) did not exert any inhibitory effect on HA degradation.

2.4. HA degradation and inhibition

The following parameters were studied to test the inhibition of HA depolymerization by different antioxidants: the sample's (apparent) molecular weight was estimated from a linear regression equation obtained from hyaluronates of known molecular weight, and the percentage of HA inhibition, or rather the change depending on the type and final concentration of the added antioxidant. This percentage was calculated using Eq. 1

$$100\left(1-\frac{\%\mathrm{DI}}{\%D}\right) \quad (1)$$

in which %DI is the percentage of HA degradation at 120 min after the onset of the reaction in the presence of the inhibitor; and $\%D$ is the percentage in the absence of the inhibitor. Degradation percentages were calculated from the retention times obtained by chromatographic analysis. Retention times obtained in the degradation reaction in the presence of scavengers were compared with retention times in the absence of scavengers.

Samples were prepared by mixing 800 μL HA (Ostenil® soln 0.5 mg/mL) with 100 μL EDTA 220 μM and 70 μL $CuSO_4$ 1 mM. Samples were incubated for 20–24 h at 37 °C. To inhibit the degradation of HA, the inhibitor (100 μL) was added to the samples after the incubation. A vol of 30.6 μL of hydrogen peroxide stock soln was then added immediately to initiate HA degradation, obtaining a H_2O_2 final sample concentration of 0.2 M. After the addition of H_2O_2 and at different times between 0 and 120 min, the samples were analysed by size-exclusion high performance liquid chromatography. The retention times obtained at 120 min after the onset of the reaction are the values that we used to estimate the degradation percentages explained above.

Control reaction mixtures containing no inhibitors and compensated by the addition of 100 μL of saline soln were analysed to compare the change in the samples' molecular weight in the absence and in the presence of inhibitors. HA solns alone were also used to examine any loss of molecular weight during incubation at 37 °C.

EDTA was added to the reaction vessel to obtain the chelation of Cu^{2+}. This alters the cation's redox potential, thereby making its oxidation by H_2O_2 more favourable and yielding ˙OH more rapidly.[25,26] Cupric sulfate was used as HA degradation has been shown to proceed best in the presence of a reducing agent such as bivalent cations like Cu^{2+}.[27] When H_2O_2 is added to the reaction, Cu^{2+} act as catalysts in the degradation of HA, yielding ˙OH by a reaction that is analogous to the Fenton reaction[28]

$$Cu^{2+} + H_2O_2 \rightarrow Cu^{3+} + {}^{\cdot}OH + OH^-$$

The reaction of ROS, such as ˙OH, with HA has been widely examined by other authors in order to determine the kinetic properties, the site(s) of attack and the mechanism(s) of chain cleavage. Such studies have indirectly analysed either the intermediate radicals or the reaction products. These studies emphasized that among ROS, ˙OH degrades HA in the most efficient way. Moreover, they showed that the main targets of oxidative degradation are the D-glucuronic acid residues of the HA disaccharide unit,[7,29] and the glycosidic link, which appears to be particularly susceptible to ROS damage.[27,29]

2.5. Chromatographic conditions

Samples were analysed by size-exclusion high performance liquid chromatography. This was used to measure the molecular weight and the percentage of HA degradation, during both degradative and inhibitory conditions, as a function of the retention time. Molecular weights and degradation percentages of hyaluronate samples were estimated from the retention times obtained by HPLC (Waters Ltd., Watford, UK), using a size-exclusion Ultrahydrogel™ 2000 column with a molecular weight range between 50 and 7000 kDa (Waters) and absorbance at 200 nm. The mobile phase was 50 mM phosphate buffer, adjusted to pH 5.5. The flow rate was 0.8 mL/min and all measurements were performed in duplicate at 45 °C.

The determinations were carried out at different times between 0 and 120 min after the addition of H_2O_2.

3. Results and discussion

We needed both a source of ROS and an appropriate antioxidant to test in vitro the influence of different antioxidants in protecting HA from oxidative damage. As described above, HA degradation was performed by a pseudo-Fenton reaction, which involved H_2O_2 and Cu^{2+}. This ionic environment is extremely close to the

pathophysiological situation, which is found in inflammatory joint diseases.[10]

Regarding molecular weight, we observed that the higher the inhibitory concentration, the lower the degradation of the HA. That is, HA's molecular weight decreased less as the antioxidant concentrations increased, at least for mannitol, thiourea and vinpocetine. In the case of propofol, the molecular weight of the degradation products after 120 min of ROS attack did not undergo such a clearly concentration-dependent change, when propofol was used at final concentrations of between 0.1 μM and 1 mM.

Inhibition of HA degradation as a function of each antioxidant concentration is depicted in Figures 2–5. It clearly shows the concentration-dependent effect of the antioxidants tested. Inhibition increased up to the value at which the reaction kinetics reached the saturation point. This enabled us to estimate the inhibitor concentration at which the inhibition of the HA degradation is 50%. It should be noted that this 50% inhibition (IC_{50}) refers to the total HA degradation obtained with a final H_2O_2 concentration of 0.2 M and no inhibitor (39%).

These results indicate that the IC_{50} for mannitol, thiourea, propofol and vinpocetine are 26.51 mM, 42, 0.39 and 1.35 μM, respectively. The inhibition percentages at which the reaction kinetic reaches its maximum are 34% for mannitol (Fig. 2), 27% for thiourea (Fig. 3), 10% for propofol (Fig. 4) and 48% for vinpocetine (Fig. 5). The low IC_{50} values (Fig. 6) confirm the antioxidant role of the products chosen to inhibit HA depolymerization. A comparison of the IC_{50} values clearly shows the efficacy of these antioxidants in protecting HA from $^{\bullet}OH$ in vitro degradation. This is particularly true in the case of thiourea, propofol and vinpocetine, whose IC_{50} are 3 orders lower than the IC_{50} value for mannitol. We compared the chemical

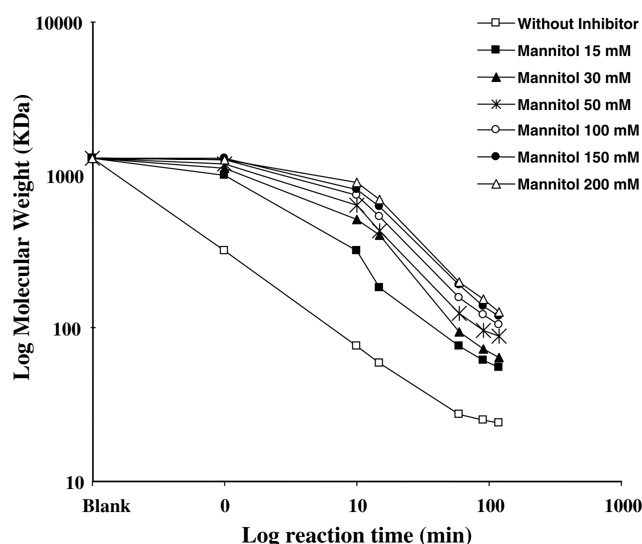

Figure 2. Logarithm of molecular weight as a function of the logarithm of the reaction time in the degradation of HA by Cu^{2+}/H_2O_2, with mannitol as antioxidant. Molecular weights were calculated from a linear regression equation, obtained from HA solutions of known molecular weight. Reaction time refers to the time elapsed after the addition of H_2O_2 to the reaction mixtures. Each data point represents the mean of two separate experiments.

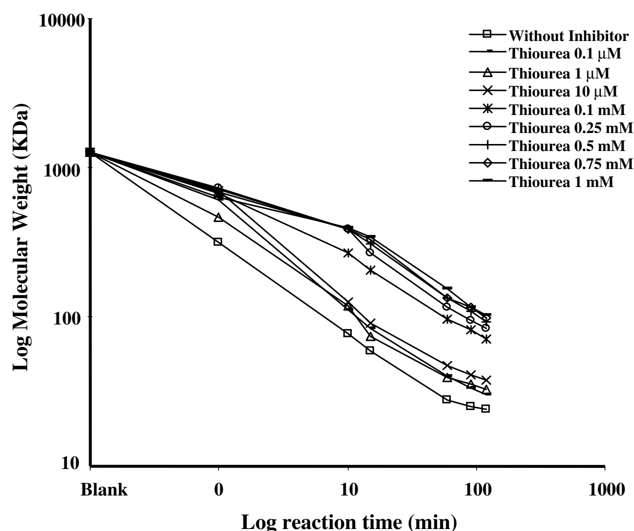

Figure 3. Logarithm of molecular weight as a function of the logarithm of the reaction time in the degradation of HA by Cu^{2+}/H_2O_2, with thiourea as an antioxidant. Molecular weights were calculated from a linear regression equation, obtained from HA solutions of known molecular weight. Reaction time refers to the time elapsed after the addition of H_2O_2 to the reaction mixtures. Each data point represents the mean of two separate experiments.

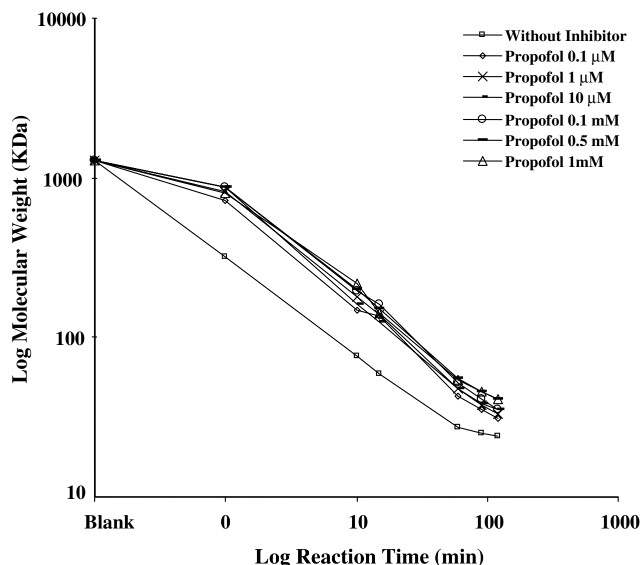

Figure 4. Logarithm of molecular weight as a function of the logarithm of the reaction time in the degradation of HA by Cu^{2+}/H_2O_2, with propofol as antioxidant. Molecular weights were calculated from a linear regression equation, obtained from HA solutions of known molecular weight. Reaction time refers to the time elapsed after the addition of H_2O_2 to the reaction mixtures. Each data point represents the mean of two separate experiments.

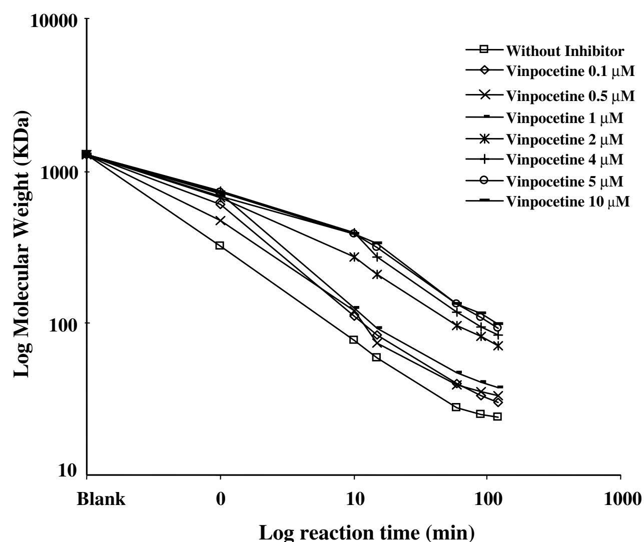

Figure 5. Logarithm of molecular weight as a function of the logarithm of the reaction time in the degradation of HA by Cu^{2+}/H_2O_2, with vinpocetine as antioxidant. Molecular weights were calculated from a linear regression equation, obtained from HA solutions of known molecular weight. Reaction time refers to the time elapsed after the addition of H_2O_2 to the reaction mixtures. Each data point represents the mean of two separate experiments.

structures of these products to find a relation between their scavenger ability. Propofol and vinpocetine, whose IC_{50} are the lowest, have benzene rings. Therefore, they can be included in the group of aromatic compounds. Such compounds are known to contribute significantly to the antioxidant capacity. Vinpocetine antioxidant activity is clearly concentration-dependent and its percentage of maximum inhibition is the highest (48%). The ability of vinpocetine, a rare example of indole derivatives, to scavenge ·OH radicals with an efficacy approaching that of vitamin E has been demonstrated.[30,31] Herraiz and Galisteo[32,33] showed that indoles in general, and not exclusively a few remarkable indoles such as melatonin, may constitute a structural family of radical scavengers (electron donors). These data suggests that this vincamine derivative is probably the most effective of the scavengers tested in this study in protecting HA from its degradation. In contrast, thiourea has a thiol group in its structure, which is critical in its inhibitory effect against copper-induced oxidation.[15]

It could be stated that ·OH degradation of HA macromolecular structure decreased its molecular weight. However, when an antioxidant is present in the system, there is a lower yield of ·OH than that given by biopolymer reaction, resulting in inhibited depolymerization.[10] This inhibitory effect differed as a function of the chemical structure of the antioxidant tested.

Other authors have developed protocols to determine the protective role that these antioxidants play in HA degradation. Their results depend to a great extent on the different ROS used and their sources, molecular weight and the composition of the HA solution due to its use (clinical or non-clinical), etc.

Orviský et al.[10] studied the depolymerization of HA (a non-commercial product) of 10^6 Da. They obtained about 90% degradation by performing a pseudo-Fenton reaction (Cu^{2+}–H_2O_2). The addition of different concentrations of mannitol and vinpocetine allowed them to obtain an IC_{50} of 2 mM for mannitol and 0.3 μM for vinpocetine, approximately. These values are an order lower than ours, and the kinetics of inhibition were very different. However, our results are in accordance with theirs in terms of the higher antioxidant effect of vinpocetine than mannitol. The differences between their study and ours may be related to the different percentage of degradation obtained and the HA solutions tested. The product we tested (Ostenil®) appears to be more stable possibly due to its defined molecular weight and concentration and that it has been stabilized for clinical use.

Oláh et al.[30] studied the scavenging capability of some antioxidants, such as mannitol and vinpocetine, against ·OH produced by a Fenton reaction. They showed that mannitol and vinpocetine act against ·OH in a concentration-dependent manner. The IC_{50} values were 12.8 mM for mannitol and 0.2 mM for vinpocetine (Fig. 6). This in vitro study suggests that vinpocetine has a higher antioxidant capacity than mannitol. In addition, it indicates that ·OH may attack the aromatic part of vinpocetine, resulting in hydroxylated products. Their results are not really comparable to ours as their study did not involve HA. However, the studies concur in that vinpocetine had a higher antioxidant activity than mannitol, and in the fact that ·OH may attack the polycyclic aromatic ring structure of vinpocetine.

Li et al.[16] performed the degradation of HA with an approximate molecular weight of 2×10^6 Da. ROS production was obtained in different ways: one of these was a pseudo-Fenton reaction (Cu^{2+}–H_2O_2), yielding ·OH. They compared the inhibitory effect on HA degradation by different scavengers, such as mannitol and thiourea. HA remained intact (not degraded) when thiourea was added at a concentration of 20 mM. However, in the presence of mannitol at this concentration, this resulted in the molecular weight of HA decreasing by half. Although these results are not comparable to ours as our solution was more stable, the studies concur in that thiourea has a higher protective antioxidant effect than mannitol.

Kvam et al.[19] performed the degradation of HA of 10^6 Da molecular weight generating ·OH by a Fenton reaction ($FeSO_4 + EDTA + H_2O_2$) and by the xanthine oxidase-hypoxanthine reaction. In both cases, HA degradation reached 90% and the addition of higher concentrations of propofol decreased this percentage to 10% at a 1.6 mM concentration of propofol. Their IC_{50} was about 0.4 mM. This value is clearly higher than

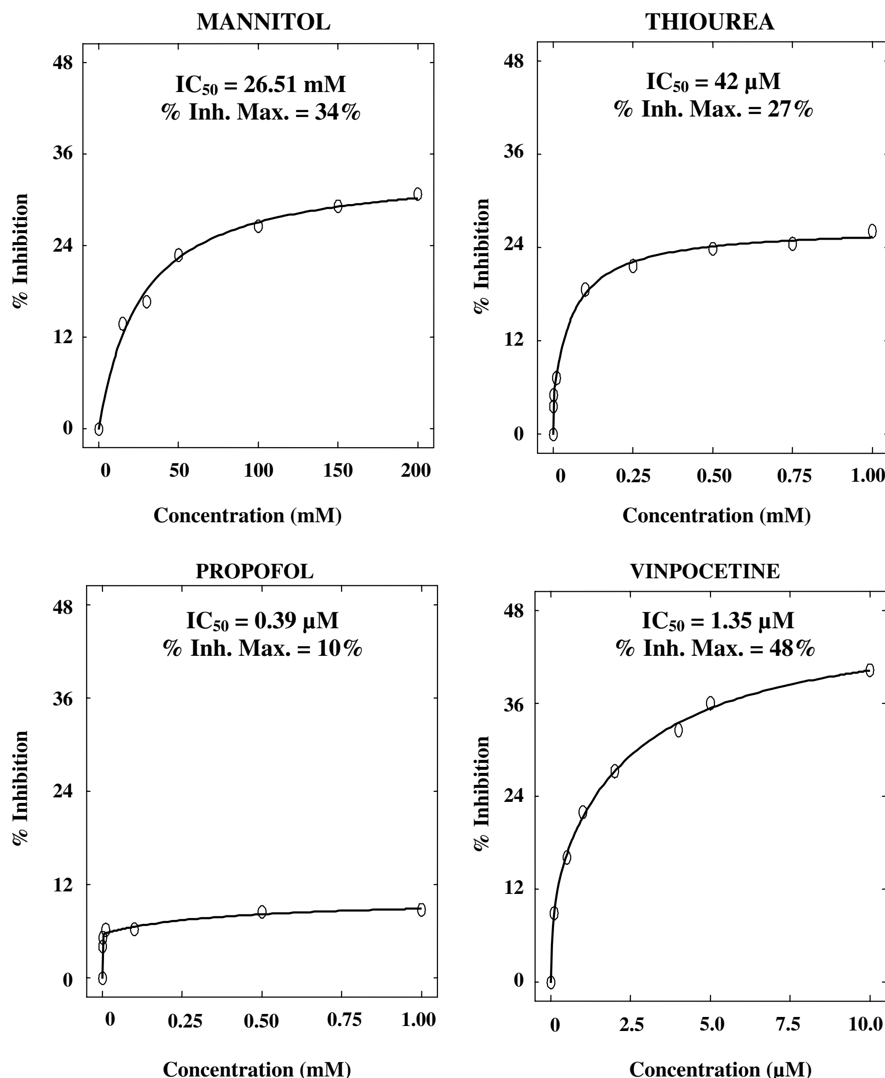

Figure 6. Percentage inhibition of HA degradation as a function of the concentration of each antioxidant examined. IC_{50} (concentration of the inhibitor, which resulted in a 50% inhibition of HA depolymerization) and maximum inhibition percentages for each antioxidant are shown. Each data point represents the mean of two separate experiments.

ours (0.39 µM), and indicates the effective antioxidative role of propofol in protecting HA against oxidative damage by the system Cu^{2+}–H_2O_2. While it is not certain whether the antioxidants propofol, vinpocetine and thiourea can be used with HA to prevent or decrease HA degradation in clinical use due to their potential systemic effects, mannitol has been used in combination with HA (Visiol, TRB Chemedica AG, Germany) and has demonstrated significant antioxidant properties against ROS.[13]

The developed method is easy, reliable, sensitive and highly reproducible, and the results are in agreement with the studies described. In all of them, inhibition of the degradation increased with an increase in the concentration of the antioxidant tested. However, as mentioned above, the results are not fully comparable, as our study was the only one to use a commercial solution of HA, such as Ostenil®, which is stabilized for clinical use as described above.

Acknowledgements

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