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Influence of tiopronin, captopril and levamisole therapeutics on the oxidative degradation of hyaluronan

Katarína Valachová [a], Mária Baňasová [a], Dominika Topol'ská [a], Vlasta Sasinková [b], Ivo Juránek [a], Maurice N. Collins [c,*], Ladislav Šoltés [a]

[a] Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Slovakia
[b] Institute of Chemistry, Slovak Academy of Sciences, Slovakia
[c] Stokes Laboratories, University of Limerick, Ireland

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ABSTRACT

The ability to protect hyaluronic acid (HA) from oxidative degradation by cupric ions and ascorbate (production of $^{\bullet}$OH and peroxy-type radicals) during acute phase joint inflammation has been investigated using the following drugs: tiopronin, captopril, and levamisole. Radical scavenging activity, *i.e.* the propensity for donation of electrons was assessed for the drugs by ABTS and DPPH assays. The kinetics of HA degradation have been measured in the presence of each drug using rotational viscometry. The results of ABTS and DPPH assays show the highest radical scavenging activity for captopril, followed by tiopronin. For levamisole, no effect was observed. Captopril and tiopronin prevented HA degradation induced by $^{\bullet}$OH radicals in a similar manner, while tiopronin was more effective in scavenging peroxy-type radicals. On the other hand, levamisole was shown to be a pro-oxidant. Recovered HA fragments were characterized using FT-IR analysis, the incorporation of a sulphur atom from captopril and tiopronin but not from levamisole into the HA molecule was demonstrated.

1. Introduction

Hyaluronan (hyaluronic acid, HA, Fig. 1a), a linear polysaccharide composed of repeating disaccharide units composed of β-D-glucosamine and β-D-glucuronic acid residues linked by (1 → 3) and (1 → 4) glycosidic bonds, has been used to study its oxidative degradation *in vitro*. In vertebrates high-molar-mass HA forms viscoelastic solutions (Collins & Birkinshaw, 2013a). HA at high molecular weight *in vivo* exhibits antiangiogenic, anti-inflammatory and immunosuppressive properties (Girish & Kemparaju, 2007; Šoltés et al., 2007). However, lower molecular weight hyaluronan demonstrates pro-inflammatory, angiogenic, and immunostimulative activities (Rychlý et al., 2006). HA is a material of increasing importance to biomaterials science and is finding applications in diverse areas ranging from tissue culture scaffolds, which has been reviewed recently (Collins & Birkinshaw, 2013b), to cosmetic materials (Vrentzos, Liapakis, Englander, & Paschalis, 2014) and cancer therapy (Shen et al., 2014). Its properties, both physical and biochemical, in solution or hydrogel form, are extremely attractive for various technologies concerned with body repair (Collins & Birkinshaw, 2013b).

HA, both *in vivo* and *in vitro*, is degraded by hyaluronidase, and/or by reactive oxygen/nitrogen species (ROS/RNS) (Bystrický, Alföldi, Machová, Steiner, & Šoltés, 2001; Stankovská et al., 2004). Studies have shown that oxidative HA degradation can be influenced by mono- and di-thiol compounds such as cysteine, cysteamine, *N*-acetylcysteine, dithioerythritol, dithiothreitol and more efficiently by glutathione along with bucillamine (Baňasová et al., 2012, 2014; Hrabárová, Valachová, Juránek, & Šoltés, 2012; Hrabárová, Valachová, Juránek, & Šoltés, 2012; Tamer, Valachova, & Soltes, 2014; Valachová et al., 2010, 2011).

Captopril and tiopronin contain a SH-group, Fig. 1b and c, which is a well-known donor of both H$^{\bullet}$ and electrons, however H$^{\bullet}$ and electron donor properties of these drugs have not been investigated to date. Captopril, 1-[(2S)-3-mercapto-2-methylpropionyl]-L-proline, has been shown to reduce anti-inflammatory properties and has been used to treat hypertension (Odaka & Mizuoki, 2000). While tiopronin, *N*-(2-mercaptopropionyl)-glycine, has been used for the treatment of rheumatoid arthritis (Mordini, Guidoni, Maestrini, Buonavia, & Lavagni, 1989). Levamisole, (6S)-6-phenyl-2,3,5,6-tetrahydroimidazo[2,1-b][1,3]thiazole, is known

Abbreviations: ABTS, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid; HA, hyaluronan; DPPH, 2,2-diphenyl-1-picrylhydrazyl; SF, synovial fluid; WBOS, Weissberger's biogenic oxidative system.

* Corresponding author at: Stokes Institute, University of Limerick, Ireland.
E-mail address: Maurice.Collins@ul.ie (M.N. Collins).

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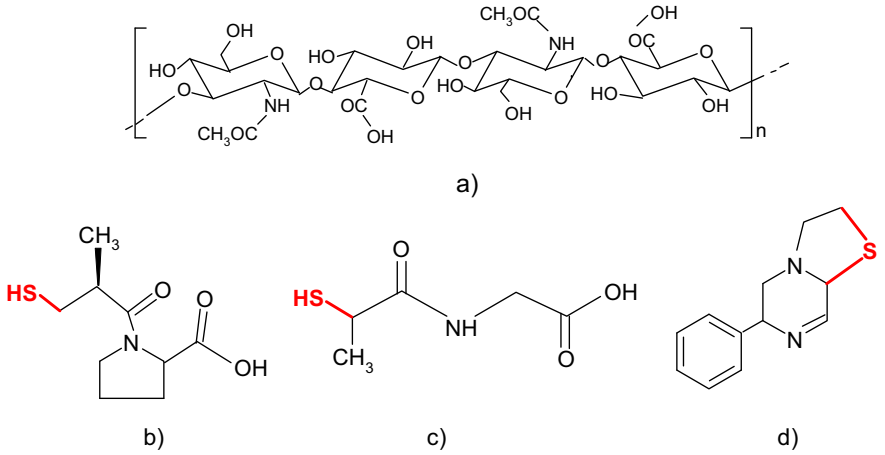


Fig. 1. Chemical structure of hyaluronan (a), captopril (b), tiopronin (c) and levamisole (d).

for its antirheumatic and anticancer properties (Mutch & Hutson, 1991). Levamisole (Fig. 1d) contains sulphur but not the —SH group.

The current study determines the electron donor properties of the drugs by ABTS and DPPH assays, whilst the kinetics of oxidative degradation of HA in the presence of the drugs is investigated by rotational viscometry. The degraded HA is characterized using FT-IR analysis to demonstrate the potential incorporation of the drugs into the HA molecule.

2. Materials and methods

2.1. Materials

HA (M_w = 970.4 kDa) was purchased from Lifecore Biomedical Inc., Chaska, MN, USA. $CuCl_2{\cdot}2H_2O$ and NaCl (analytical purity grade) were purchased from Slavus Ltd., Bratislava, Slovakia; ascorbic acid and potassium persulfate from Merck KGaA, Darmstadt, Germany; 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) from Fluka, Steinheim, Germany; 2,2-diphenyl-1-picrylhydrazyl (DPPH); tiopronin and levamisole hydrochloride from Sigma-Aldrich, Steinheim, Germany; captopril from Calbiochem, a brand of EMD Chemicals Inc., an affiliate of Merck KGaA, Darmstadt, Germany, methanol and ethanol was purchased from Mikrochem, Pezinok, Slovakia. Deionised high-purity grade water, with conductivity of ≤0.055 μS/cm, was produced by using the TKA water purification system (Water Purification Systems GmbH, Niederelbert, Germany).

2.2. Preparation of stock and working solutions

The hyaluronan samples (20 mg) were dissolved in 0.15 M aqueous NaCl solution for 24 h in the dark to prevent photodegradation (Lapčík, Omelka, Kuběna, Galatík, & Kellő, 1990, Lapčík, Chabreček, & Staško, 1991).

HA sample solutions were prepared in two steps: first, 4.0 ml of 0.15 mM NaCl was added to HA to swell and after 6 h, 3.9 or 3.85 ml of 0.15 M NaCl was added, when working in the absence and presence of the drugs, respectively. Solutions of ascorbic acid, drugs (16 mM) and cupric chloride (160 μM solution) as well as their dilutions were made in 0.15 M aqueous NaCl.

2.3. ABTS and DPPH assays – determination of IC_{50} values

The first step of standard ABTS assay was preparation of the aqueous solution of $ABTS^{\bullet+}$ cation radical. The $ABTS^{\bullet+}$ is prepared 24 h before the measurements at room temperature as follows: ABTS aqueous stock solution (7 mM) was mixed with $K_2S_2O_8$ aqueous solution (2.45 mM) in equivolume ratio. The next day, 1 ml of the resulting solution was diluted with distilled water to the final volume of 60 ml (Cheng, Moore, & Yu, 2006; Hrabárová, Valachová, Rapta, & Šoltés, 2010; Re et al., 1999). The aqueous reagent in the volume of 250 μl was added to 2.5 μl of the aqueous solutions of tiopronin, captopril and levamisole. The concentration of substances ranged from 0.078 to 20 mM. Absorbance (734 nm) of samples was recorded after 6 min, when $ABTS^{\bullet+}$ reacted completely with each drug.

At first, in the DPPH assay $DPPH^{\bullet}$ radical was prepared as follows: 2,2-diphenyl-1-picrylhydrazyl (1.1 mg) was dissolved in 50 ml of distilled methanol to generate $DPPH^{\bullet}$. The $DPPH^{\bullet}$ solution in the volume of 225 μl was added to 25 μl of the methanol solution of tiopronin, captopril and levamisole. The concentration of substances ranged from 0.078 to 20 mM. Absorbance (517 nm) of samples was recorded after 30 min.

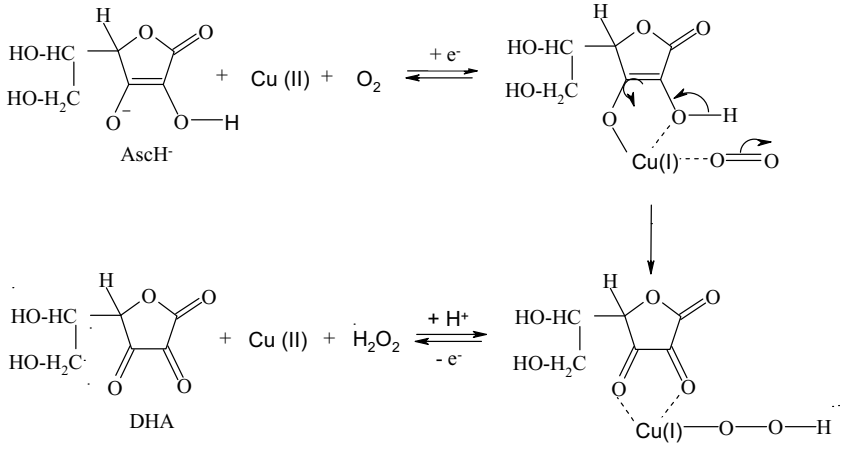
In both assays the measurements were performed in the triplicate in 96-well Greiner UV-Star microplates (Greiner-Bio-One GmbH, Germany) by using the Tecan Infinite M 200 reader (Tecan AG, Austria).

2.4. ABTS and DPPH assays – kinetics of scavenging $ABTS^{\bullet+}$ and $DPPH^{\bullet}$

The $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ were prepared as mentioned above. The stock solution of each drug at concentrations 2, 1, 0.5 and 0.25 mM in the volume of 50 μl was added to 2 ml of the $ABTS^{\bullet+}$ or $DPPH^{\bullet}$ solution. Kinetics of scavenging $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ was performed in triplicate at the wavelength 730 and 517 nm, respectively. The solutions of captopril, tiopronin and levamisole were measured during 30, 15 and 10 min, respectively. Besides aqueous solutions of glutathione used in both methods, aqueous and methanolic solutions of captopril, tiopronin and levamisole were used for the ABTS and DPPH assay, respectively.

2.5. Rotational viscometry

Degradation of high-molar-mass HA was induced *in vitro* by Weissberger's biogenic oxidative system (WBOS) comprising compounds in the respective physiological concentrations, see Fig. 2, tangibly 100 μM ascorbate *plus* 1 μM $CuCl_2$, applied under aerobic conditions. The procedure was as follows: 50 μl of $CuCl_2$ (160 μM) was added to the HA solution (7.90 ml). After stirring for 30 s, the reaction mixture was left to stand for 7.5 min at room temperature, 50 μl of ascorbic acid (16 mM) was added, and the mixture was



$$H_2O_2 + Cu(I)\text{ --- complex} \rightarrow {}^{\bullet}OH + HO^{-} + Cu(II)\text{ --- complex}$$

Fig. 2. Chemistry of Weissberger's biogenic oxidative system (adapted from Hrabárová, 2012c).

stirred again for 30 s. The resulting 8 ml of reaction mixture containing HA was transferred into the Teflon® cup reservoir of a Brookfield LVDV-II+PRO digital rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, USA), and changes in dynamic viscosity of HA were recorded at 25.0 ± 0.1 °C in 3 min intervals for 5 h. The viscometer Teflon® spindle rotated at 180 rpm, *i.e.* at a shear rate of 237.6 s^{-1} (Bañasová et al., 2012; Valachová et al., 2011).

Two experimental regimes were applied for assessing the influence of captopril, tiopronin, and levamisole on HA degradation *in vitro*. Firstly, each drug was added to the reaction mixture 30 s before the addition of ascorbic acid, which initiates oxidative degradation of HA by producing $^{\bullet}OH$ radicals. And secondly, each drug was added to the reaction mixture 1 h later, when production of peroxy-type radicals prevails.

2.6. Fourier-transform infrared spectroscopy

FT-IR spectra of the precipitated samples of the native HA, HA exposed to WBOS in the absence and presence of the drugs were measured with Nicolet 6700 (Thermo Fisher Scientific, USA) spectrometer equipped with DTGS detector and Omnic 8.0 software. The spectra were collected in the middle region from 1800 to 600 cm^{-1} at a resolution of 4 cm^{-1}, the number of scans was 128. Diamond Smart Orbit ATR accessory was used for measurement in solid state. HA was precipitated in 20 ml of 96% ethanol overnight, centrifuged for 5 min at 3000 rpm and dried in a desiccator.

3. Results and discussion

ABTS and DPPH decolorization assays were used for assessment of the electron-donor activity of compounds (Magalhaes, Segundo, Reis, & Lima, 2008). The $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radicals change according to the following reactions:

$$ABTS^{\bullet+} + e^{-} \rightarrow ABTS\,(\text{one electron reduction}) \quad (1)$$

$$DPPH^{\bullet} + e^{-} \rightarrow DPPH^{-}\,(\text{one electron reduction}) \quad (2)$$

Table 1 displays the IC_{50} values (inhibition concentration) of the drugs using ABTS and DPPH assays. For both assays glutathione, a thiol compound, was selected as a reference antioxidant. Taking captopril the IC_{50} value is 17.2 μM in the ABTS assay, which is similar in efficiency to glutathione (18.6 μM). Tiopronin displays a somewhat lower $ABTS^{\bullet+}$ scavenging activity, with an IC_{50} value

Table 1
The IC_{50} values of ABTS and DPPH decolorization assays.

Compound	ABTS IC_{50} (μM)	DPPH IC_{50} (μM)
Captopril	17.2 ± 0.4	26.1 ± 0.3
Tiopronin	29.2 ± 0.5	23.2 ± 1.7
Levamisole	Not detected	Not detected
Glutathione	18.6 ± 0.2	89.2 ± 4.1

29.2 μM. The IC_{50} of levamizole was not determinable indicating that it has poor scavenging ability.

For the DPPH assay captopril and tiopronin scavenge $DPPH^{\bullet}$ more efficiently than glutathione. Since DPPH assay runs in pure methanol, any dissociation of the glutathione —SH group is practically eliminated. Thus the reactions of the type G-SH → $G\text{-}S^{-} + H^{+}$ (dissociation) followed by the reaction GSH → $G\text{-}S^{\bullet} + e^{-} + H^{+}$ (ionization) do not occur and the IC_{50} value for glutathione is too high. Overall, the results indicate that captopril and tiopronin have the ability to donate electrons and this is attributed to the presence of thiol groups while the relatively poor performance of the levamizole drug is attributed to the lack of thiol moiety.

Fig. 3a–c illustrates the kinetics of scavenging $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ by the selected drugs. The concentrations of captopril in $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ solutions were 50, 25, 12.5 and 6.25 μM. The results in Fig. 3a, left panel demonstrated that captopril at 50 μM concentration gradually reduced $ABTS^{\bullet+}$ and after 30 min 10% of $ABTS^{\bullet+}$ remained unscavenged. For comparison, in glutathione a gradual reduction of $ABTS^{\bullet+}$ was observed, however the amount of unscavenged $ABTS^{\bullet+}$ was 20% (not shown). For captopril the scavenging rate of $DPPH^{\bullet}$ was lower than $ABTS^{\bullet}$. After 30 min 54% of $DPPH^{\bullet}$ remained (Fig. 3a, right panel). Under the same conditions $DPPH^{\bullet}$ remained unaffected by glutathione (not shown).

As shown in Fig. 3b, the kinetics of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ reduction was slower for tiopronin. The amount of unscavenged $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ was over 20%. No $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ scavenging capacity was observed in levamisole (Fig. 3c).

Results show that captopril or tiopronin (100 μM) addition inhibits HA degradation. When captopril (10 μM) is added after 1 h the rate of HA degradation in time interval 60 to ca. 130 min is unchanged and after 130 min the anti-oxidative effect begins (Fig. 4a, right panel, green curve). A similar effect was observed for tiopronin (Fig. 4b, right panel, red curve). Levamisole has little influence on HA degradation (Fig. 4c, red and green curves). Overall, tiopronin was the most effective inhibitor of HA degradation.

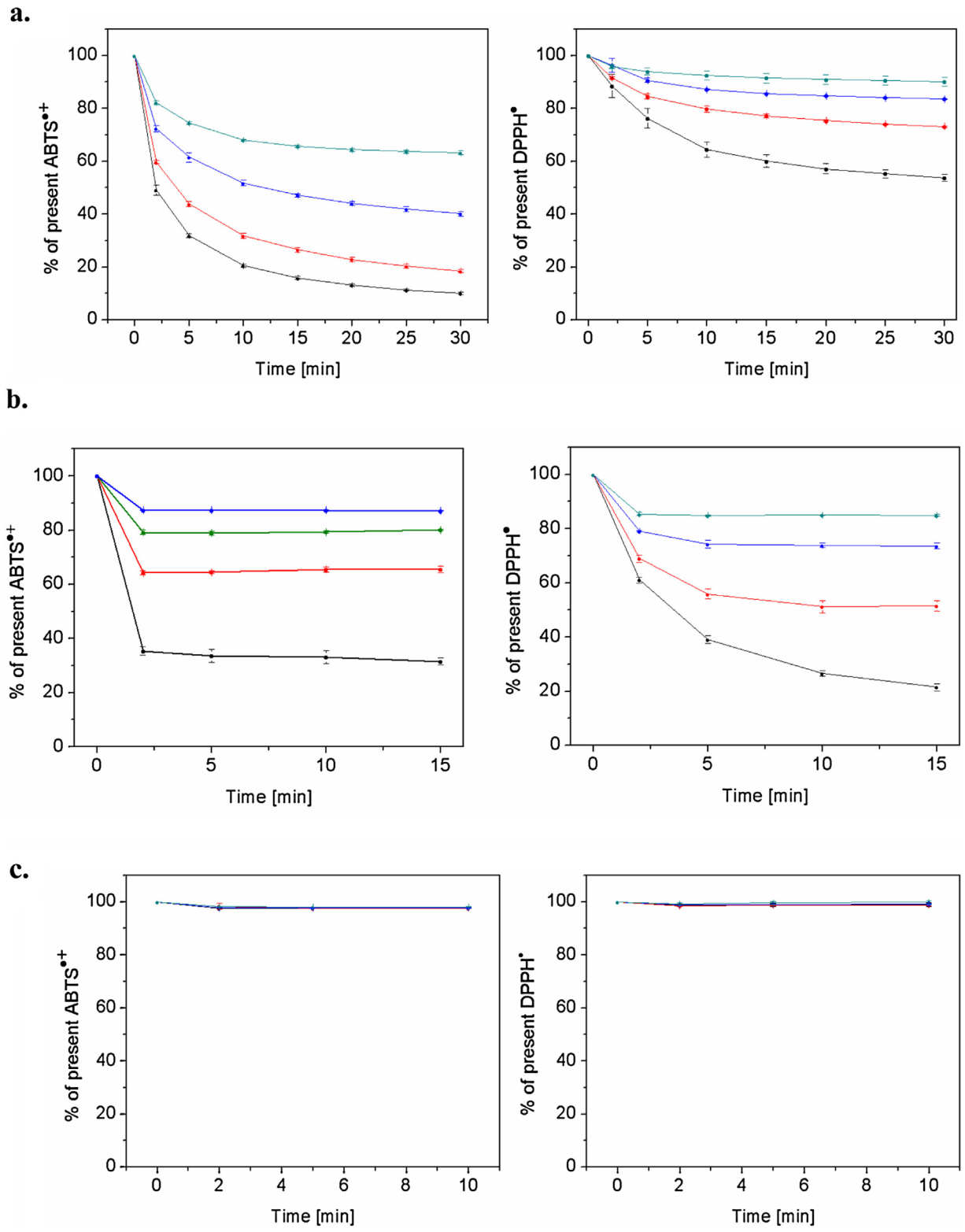


Fig. 3. Percentage of the present $ABTS^{\bullet+}$ (left panel) and $DPPH^{\bullet}$ (right panel) after addition of captopril (a), tiopronin (b), levamisole (c). The concentrations of the drugs in $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ solutions were: 50 (black), 25 (red), 12.5 (green) and 6.25 (blue) μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

It is presumed that the degradation mechanism is governed by the chemical structure of the drugs. The ease of hydrogen removal from the thiol moiety being critical as mentioned previously. For example with levamisole the sulphur atom is bonded to the stiff aromatic ring resulting in limited scope for reactivity, while captopril and tiopronin have thiol (—SH) moieties (Fig. 1b). These moieties may function both as a scavenger of hydroxyl-radicals, formed immediately after reacting Cu(II) ions with ascorbate in the HA solution, and peroxy-type radicals, which are formed 1 h later. The exposition of HA to ROS can be expressed by the reactions below.

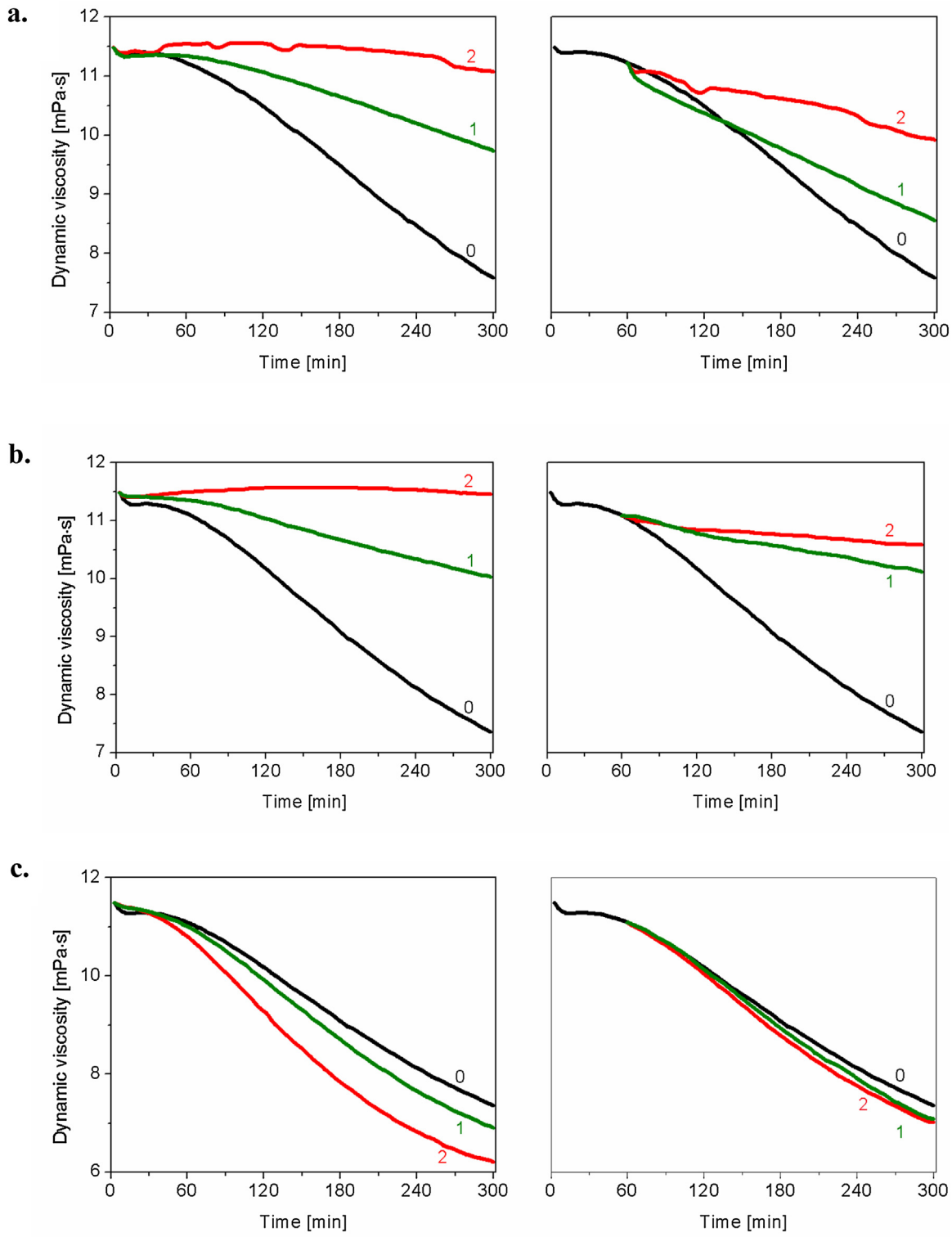


Fig. 4. Time-dependent decrease in dynamic viscosity of the HA solution exposed to the oxidative system Cu(II) ions (1 μM) and ascorbate (100 μM) (0) after the addition of captopril (a), tiopronin (b) or levamisole (c) at concentrations 10 (1) and 100 (2) μM before initiating HA degradation (left panel) and 1 h later (right panel).

Hydroxyl radicals can abstract a hydrogen radical ($H^{\bullet}$) from the HA macromolecule, which results in the formation of a *C*-macroradical, further denoted as $A^{\bullet}$.

Under aerobic conditions, the alkyl-type macroradical $A^{\bullet}$ reacts rapidly with the molecule of dioxygen to form a peroxy-type macroradical $AOO^{\bullet}$. The intermediate $AOO^{\bullet}$ macroradical may react with an adjacent HA macromolecule and thus the radical chain reaction can propagate rapidly. $AOO^{\bullet} + HA \rightarrow AOOH + A^{\bullet}$ (propagation of the radical chain reaction).

By reacting peroxy-type macroradical with the HA macromolecule, a high molecular weight hydroperoxide can be formed,

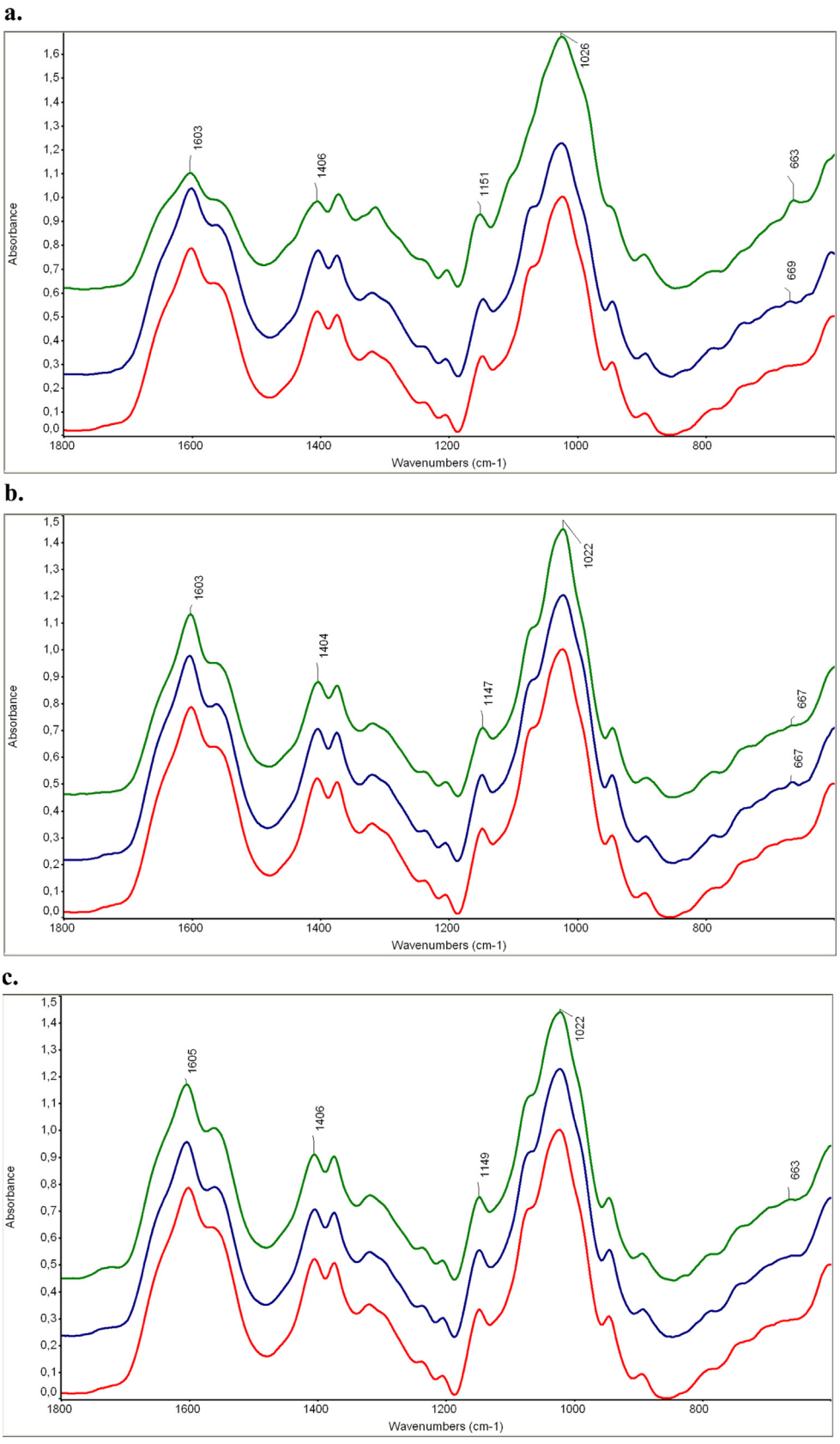


Fig. 5. The FT-IR spectrum of the native HA (green), the precipitated HA solution exposed to WBOS in the presence of 100 μM captopril (a), tiopronin (b) or levamisole (c) added before initiating HA degradation (blue) and 1 h later (red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which subsequently yields an alkoxyl type macroradical ($AO^{\bullet}$) mostly induced by Cu(I) ions

$$AOOH + Cu(I) \rightarrow AO^{\bullet} + HO^{-} + Cu(II)$$

This is a presumed intermediate of the main chain splitting, which results in fragmentation of the HA macromolecule whose solution is characterized by reduced dynamic viscosity (Valachová et al., 2013).

The thiol group may directly inhibit the propagation phase of the undesirable radical chain reaction. Moreover, the chemical structure of the compounds, are similar to the chemical structure of glutathione, indicating that these compounds may donate one $H^{\bullet}$ radical and thus to be a scavenger of $^{\bullet}OH$ radicals as follows.

$R–SH + {}^{\bullet}OH \rightarrow R–S^{\bullet} + H_2O$ thiyl-type radical formation.

Spectral data obtained from the modified HA samples *via* FT-IR (Fig. 5a–c) demonstrate changes, when compared to the native HA (green curve). Peaks are shifted towards the lower wavenumber values presumably as a consequence of modifications. Characteristic absorption bands are typical for each polysaccharide, taking into account the overlap of the absorption bands of the C—C, C—OH and C—O bonds of corresponding monosaccharide units at the region between 1200–1000 cm^{-1}. This region is sensitive to the crystallinity of the sample and also to the conformational freedom of the polymer chains. Changes of the intensity of valent vibrations for the asymmetric $\nu_{as}(COO^{-})$ and $\nu_{s}(COO^{-})$ at 1604 cm^{-1} and 1406 cm^{-1} were the most significant for captopril (Fig. 5a), less significant for tiopronin (Fig. 5b) and no changes were observed for levamisole (Fig. 5c). The highest elimination of carboxyl groups was observed for captopril (Fig. 5a).

FT-IR spectra show that thiol samples have valent vibrations ν (C—S) of a low intensity in the area 700–590 cm^{-1}. The spectra show the development of a new band at 663 cm^{-1} for captopril (Fig. 5a) and 667 cm^{-1} for tiopronin (Fig. 5b). No sulphur atom to the molecule of HA was demonstrated for levamisole (Fig. 5c). The spectra of the drugs themselves were measured (not shown) and are in accordance with the results reported in Georgia State Forensic Drug, library indices 834, 1893, 1006.

4. Conclusion

Both captopril and tiopronin, which have a similar structure to glutathione (a positive control), were reported to be effective donors of electrons, which was demonstrated by radical scavenging activity (ABTS and DPPH assay) tests. The ability of these drugs to retard HA oxidative degradation associated with acute joint inflammation has been demonstrated. These results are expected to contribute to the alleviation of trauma associated with wear in artificial joint replacements where wear debris through macrophage activity results in osteolysis (Barron, Birkinshaw, & Collins, 2015; Collins, Dalton, Leahy, & Birkinshaw, 2013). The levamisole drug was shown to be ineffective and this was attributed to its chemical structure.

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