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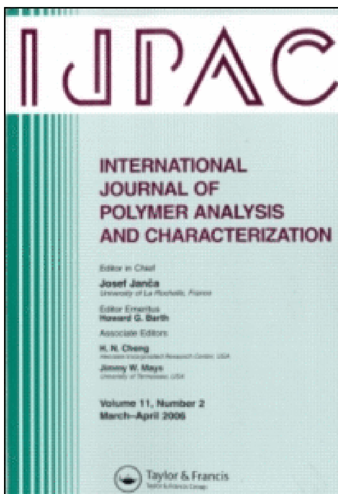

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Use of Size Exclusion Chromatography to Study the Protective Effect of Radical Scavengers on Oxygen Free-Radical-Induced Degradation of Hyaluronic Acid

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Use of Size Exclusion Chromatography to Study the Protective Effect of Radical Scavengers on Oxygen Free-Radical-Induced Degradation of Hyaluronic Acid

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A size exclusion chromatography (SEC) method has been established for the study of hyaluronic acid depolymerization induced by oxygen free radicals and of the protective effect of two widely employed anti-inflammatory agents, piroxicam and tenoxicam. Molecular weight distribution of hyaluronic acid was determined by aqueous SEC equipped with absolute online detectors, light scattering detector and a viscometer. Ten min exposure of the macromolecules to a flux of hydroxyl radicals generated by xanthine/xanthine oxidase/Fe^{2+} system caused massive depolymerization (M_w fell from $4.7 \cdot 10^5$ to $8.7 \cdot 10^4$). Both drugs exhibited a dose-dependent protective effect on hyaluronic acid degradation due to their radical scavenging properties. Tenoxicam was far more active than piroxicam in quenching highly reactive hydroxyl radicals (minimal effective concentrations 25 vs. 250 μM). This method gives a more complete analytical profile of the macromolecules with respect to conventional techniques.

KEY WORDS Hyaluronic acid, tenoxicam, piroxicam, degradation, SEC, viscometer, light scattering.

INTRODUCTION

It has been long recognized that one of the prominent features of inflammatory arthritis is a loss of viscosity of the synovial fluid due to depolymerization of its major macromolecular component, hyaluronic acid (HA). The loss of HA viscosity within the arthritic joint is accompanied by the infiltration of large numbers of polymorphonuclear leukocytes into the joint space, which release reactive oxygen species (ROS) during the respiratory burst [1–4].

HA is a high-molecular-weight, linear glycosaminoglycan (GAG) composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine, linked alternatively via $\beta(1 \rightarrow 3)$ and $\beta(1 \rightarrow 4)$ linkages. In joint inflammation, HA depolymerization is commonly ascribed to the action of oxygen-free radicals [5–7]. In a previous study [8], we have described an *in vitro* model of oxygen free radical-induced depolymerization of

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HA, which has shed some light on the kinetics of depolymerization of the macromolecule by oxy radicals and on the protective effect of some typical scavengers from natural source such as flavonoids.

The aim of the present work was to set up a size exclusion chromatography (SEC) approach for the study of the protective effect of two widely employed anti-inflammatory agents piroxicam and tenoxicam on HA depolymerization induced by hydroxyl radicals ($HO^{\bullet}$). Molecular weight distribution (MWD) of HA was determined by aqueous SEC equipped with two absolute online detectors, a light scattering detector and a viscometer, as an alternative to the conventionally used concentration detector such as a differential refractometer.

The availability of online absolute detectors greatly increases the information that can be obtained from a single SEC run. Online viscometer (SEC-VIS) allows one to obtain, via universal calibration, the MWD, intrinsic viscosity, and the coefficients K and a of the Mark-Houwink relationship. Online multi-angle laser light scattering (SEC-MALLS) yields true molecular weight without calibration and molecular size, providing that the root-mean-square radius of the macromolecules greater than 12–15 nm.

EXPERIMENTAL

Materials

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Degradation studies were carried out on native HA from cook's comb, sodium salt (purity > 95%; protein content < 0.1%) from Pentapharm (Ambrochim, Milan, Italy). The biopolymer was dissolved in 0.1M phosphate buffer, pH 7.4.

Xanthine oxidase (XO, 20 U/mL), xanthine, superoxide dismutase (SOD, 5,000 U/mg), and catalase (65,000 U/mg) were purchased from Boehringer Mannheim (Milan, Italy); piroxicam from Sigma Chemical Co. (Trimital, Milan, Italy); tenoxicam was kindly provided by Roche S.p.A. (Milan, Italy). Poly(ethylene oxide) standards were obtained from Tosoh Co. (Japan) and poly(ethylene glycol) standards were obtained from Polymer Laboratories (UK).

Experimental Protocol

The experimental protocol consisted of the following steps:

a) Characterization of the native polymer by the SEC system;

b) Depolymerization of HA by oxygen free-radicals generated in a cell-free system. The incubation mixture (final volume 5 mL) contained: 1 mg/mL HA, xanthine (0.5 mM), XO (0.0125 U/mL) and catalytic iron concentrations (75 μM $FeSO_4$/EDTA). Samples were incubated at 37°C for 10 min; the reaction was stopped by addition of a mixture of SOD (10 μg/mL) and catalase (500 U/mL);

c) Evaluation of the degree of polymerization;

d) Depolymerization in the presence of the radical scavengers and evaluation of their protective effect. Tenoxicam and piroxicam (dissolved in 0.1N Na OH) were added to the incubation mixtures before the initiation of the reaction with XO, in concentrations ranging from 0.025 to 1 mM. The protective effect (PE) on the $HO^{\bullet}$-induced depolymerization

of HA was expressed as percentage increase in weight-average molecular weight M_w (mean value of five determinations). The PE value was calculated as follows:

$$PE = \frac{M_{w_P} - M_{w_D}}{M_{w_D}} \cdot 100 \quad (1)$$

where: M_{wP} is the M_w of the protected polymer and M_{wD} is the M_w of the degraded polymer.

SEC system

The MWD of HA was obtained on a 150-GPCV (Waters, Milford, MA, USA) instrument equipped with three detectors in series: a multi-angle laser light scattering (MALLS), a viscometer (VIS), and a differential refractometer (DRI). Figure 1 shows the scheme of the SEC-VIS-MALLS system.

Light scattering Measurements were carried out on a MALLS detector Dawn DSP-F (Wyatt Technology, Santa Barbara, CA, USA) at a wavelength of 632.8 nm. MALLS detector measures the angular distribution of the scattered light at 15 fixed angles ranging, in the SEC mobile phase, from 8.1 to 171.1° with respect to the incident beam. The data acquisition and analysis software was Astra 3.02 (Wyatt). Details of the hardware and of the data analysis software have been reported elsewhere.[9,10] The calibration constant was calculated using toluene as a standard assuming a Rayleigh ratio $R_\theta = 1.402 \cdot 10^{-5}$. The normalization of the photodiodes was achieved by a narrow low-molecular-weight standard (27,000 g/mol) of poly(ethylene oxide). The refractive index increment, dn/dc = 0.153 mL/g, was determined in an interferometric refractometer Optilab 903 (Wyatt) at a wavelength of 632.8 nm in 0.1M $NaNO_3$ at 37 °C.

Viscometer The online viscometer was a single-capillary type (Waters). In a single-capillary viscometer, a differential transducer monitors the pressure drop in a stainless-steel capillary tubing (0.014 in, 6 in length). The data acquisition and analysis software were

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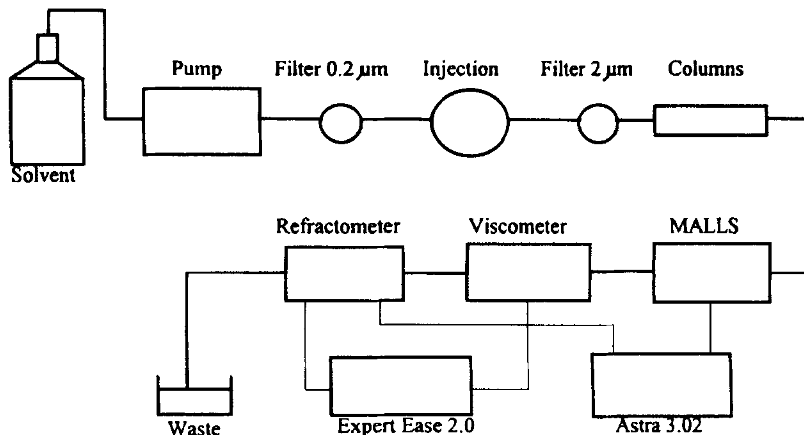

FIGURE 1 Scheme of the SEC-VIS-MALLS system

Expert-Ease 2.0 (Waters). Details of the viscometer hardware and of the data reduction software have been reported elsewhere [11,12]. Viscometry detection is based on the concept of the universal calibration [13]. The universal calibration curve was generated by twelve narrow MWD standards, poly(ethylene oxide) and poly(ethylene glycol), with molecular weight ranging from 440 to $8.5 \cdot 10^5$ g/mol.

SEC experimental conditions Analyses were performed under the following conditions:
Columns: two sets of three columns Ultrahydrogel Waters: 2000-2000-1000 Å pore size for the native polymer; and 2000-1000-250 Å pore size for the degraded polymer.
Mobile phase: 0.1M $NaNO_3$
Flow rate: 0.8 mL/min
Temperature: 37 °C
Sample concentration: 0.3 mg/mL
Injection volume: 400 μL

RESULTS AND DISCUSSION

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The *SEC* behavior of HA in its native form is strongly affected by the concentration of the polymer because of its high hydrodynamic volume due to its high molecular weight and semirigid conformation. In fact, the retention times of the peak maximum are constant at concentrations ≤ 0.3 mg/mL and tend to increase above this value.

The weight-average molecular weight M_w of the native polymer determined by the dual detector system, MALLS and the refractometer, was $4.7 \cdot 10^5$ g/mol and the polydispersity D, M_w/M_n, was 2.73. In a conventional SEC system with the refractometer detector only, using a relative calibration method with pullulan standards, we have obtained an apparent M_w value greater than $2 \cdot 10^6$ g/mol. Since the MWD determination of the biopolymer requires an absolute detector, we have used the SEC-VIS-MALLS system for the study of the kinetics of HA depolymerization and of the protective effect by the anti-inflammatory drugs.

As shown in Table I, 10 min exposure of the macromolecules to a flux of oxygen radicals generated by the xanthine/XO system, led to a marked decrease in the M_w value, from $4.7 \cdot 10^5$ to $8.7 \cdot 10^4$ g/mol. The intrinsic viscosity $[\eta]$ of the native polymer obtained from the dual detector approach, with an online viscometer and refractometer (SEC-VIS), was 10.88 dL/g (this value was confirmed by a parallel, offline set of experiments performed with a conventional Ubbelohde viscometer). After 10 min of incubation, this value drastically fell to 3.03 dL/g.

The $HO^{\bullet}$-induced depolymerization of HA was strictly time-dependent: with prolonged exposure times, the decrease in molecular weight and intrinsic viscosity became more

TABLE I
Kinetics of HA oxygen free-radical degradation.

Time	min	0	10	60	120	180
$M_w \cdot 10^{-3}$	g/mol	469.8	87.4	39.0	20.9	13.4
$[n]$	dL/g	10.88	3.03	1.64	1.02	0.73

pronounced, reaching minimal values after 180 min: $M_w = 1.34 \cdot 10^4$ g/mol; $[\eta] = 0.73$ dL/g.

Because of the high sensitivity of the biopolymer towards free-radical attack was within 10 min, this time was used for the study of the protective effects by the anti-inflammatory drugs. Figure 2 shows the effect on the molecular weight of HA exposed to HO$^•$ radicals in the presence of increasing concentrations (25 μM-1 mM) of tenoxicam and piroxicam. Although both the compounds exhibited a dose-dependent radical scavenging activity, tenoxicam was far more active than piroxicam in preventing HO$^•$-induced depolymerization of HA, with a threshold concentration at 25 μM (9.7% increase in M_w in respect to the unprotected-degraded samples: $95.8 \cdot 10^3$ vs. $87.4 \cdot 10^3$ g/mol). The percentage increases were more pronounced at higher concentrations: 21.3% ($M_w = 106.1 \cdot 10^3$ g/mol) at 50 μM; 34.7% ($M_w = 117.7 \cdot 10^3$)at 100 μM; 51.3% ($M_w = 132.2 \cdot 10^3$ g/mol) at 250 μM; 68.3% and 88.2% ($M_w = 147.1 \cdot 10^3$ and $164.5 \cdot 10^3$ g/mol) at 500 and 750 μM, respectively.

Piroxicam was totally ineffective at lower concentrations (25, 50, and 100 μM). The minimal protective response (17.7% increase in M_w, $102.9 \cdot 10^3$ vs. $87.4 \cdot 10^3$ g/mol) was achieved at 250 μM, a concentration one order of magnitude higher than that of tenoxicam. At the higher concentrations (0.5, 0.75, 1.0 mM), the radical entrapping capacity of the drug was always lower than that of tenoxicam (32.8, 70.7, and 83.2% increases, respectively). Dose-response curves gave 50% effective concentrations (EC_{50}) of 228 μM for tenoxicam and 607 μM for piroxicam.

The results of the protective effect of tenoxicam and piroxicam are summarized in Table II.

Finally, Figure 3 shows the MWD obtained from the SEC system of the degraded polymer in the absence and in the presence of 0.1 mM tenoxicam where a significant recovery in molecular weight was observed.

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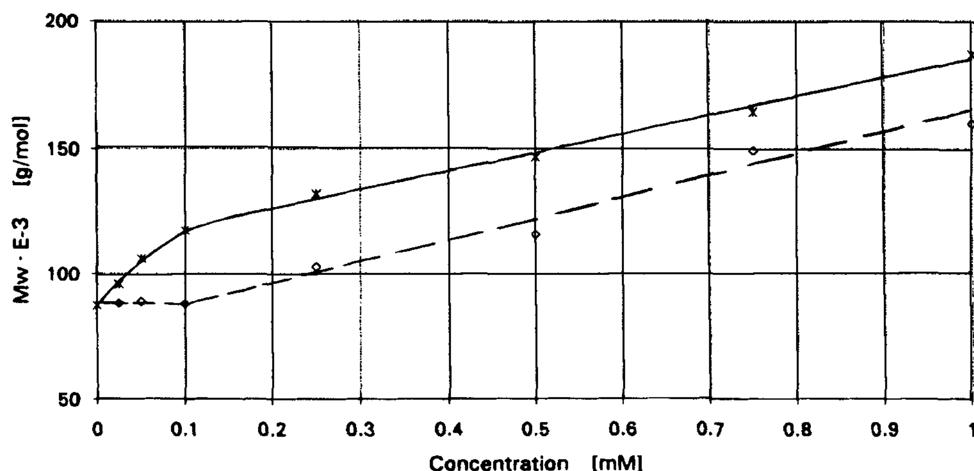

FIGURE 2 Protective effect of tenoxicam and piroxicam on HO$^•$-induced depolymerization of HA: dose-response curves; piroxicam: dashed line; tenoxicam: solid line.

TABLE II

Protective effect (PE), Equation (1), of piroxicam and tenoxicam on HO$^{\bullet}$-induced depolymerization of HA.

Concentration	mM	0.000	0.025	0.050	0.100	0.250	0.500	0.750	1.000
Piroxicam	$M_w \cdot 10^{-3}$	87.4 ±1.2%	88.2 ±1.6%	88.9 ±2.7%	87.8 ±2.4%	102.9 ±2.7%	116.1 ±2.1%	149.2 ±2.5%	160.1 ±2.2%
	PE %	—	0.9	1.7	0.4	17.7	32.8	70.7	83.2
Tenoxicam	$M_w \cdot 10^{-3}$	87.4 ±1.2%	95.8 ±2.4%	106.1 ±1.9%	117.7 ±2.0%	132.2 ±1.5%	147.1 ±2.4%	164.5 ±2.0%	184.9 ±2.0%
	PE %	—	9.7	21.3	34.7	51.3	68.3	88.2	113.9

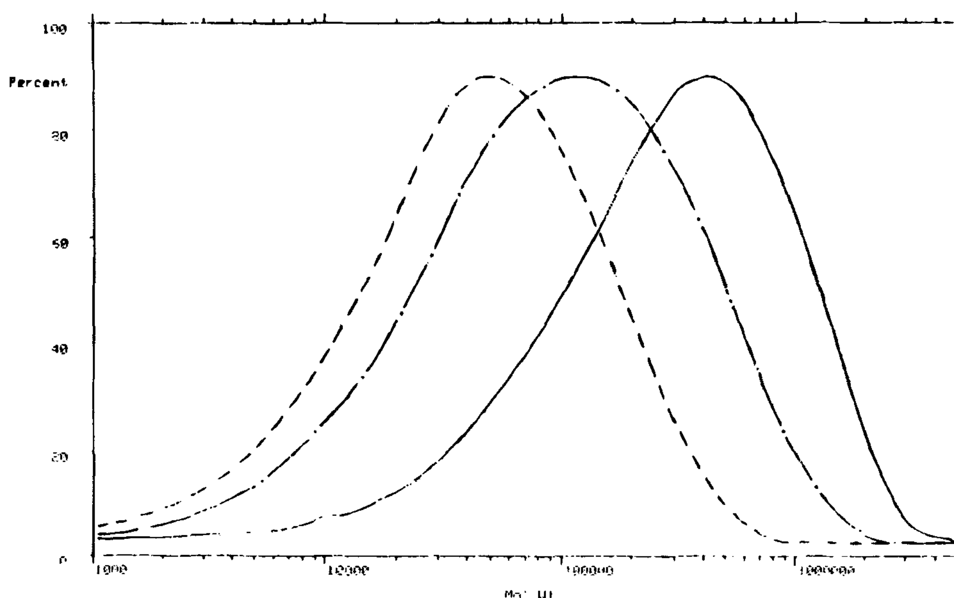

FIGURE 3 MWDs of HA obtained from the SEC-VIS-MALLS system: (_____) native polymer; (- - -) 10 min of degradation; (— · —) degradation in the presence of 0.1 mM of tenoxicam.

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CONCLUSION

It is well known that HA depolymerization by free radicals in inflamed joints and results in a marked decline in the normal viscoelastic properties of the macromolecule; the reduction in synovial fluid viscosity, in turn, exacerbates cartilage damage and increases joint instability. Hence, the extent of HA depolymerization is an important marker not only of oxidative stress linked to the inflammatory event, but also might become a valid diagnostic tool for the evaluation of the efficacy of the administered anti-inflammatory drugs.

Until now, HA characterization in inflamed joints has been carried out by separate determinations based on conventional viscometric, *SEC*, sedimentation or light-scattering techniques 4. These two last methods, which require isolation and purification of HA from synovial fluid, cannot be used as routine screening methods.

The SEC-VIS-MALLS approach described for the study of the *in vitro* depolymerization of HA and of the radical scavenging properties of two widely employed anti-inflam-

matory drugs, greatly overcomes the conventional techniques used for the characterization of HA molecular weight distribution, since it furnishes a rapid, reliable and more complete analytical profile of the macromolecule both in its native form and when subjected to oxidative degradation. It could represent the method of choice for the definition of the pathological modification of this important constituent *in vivo*, in the course of synovial inflammation.

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