

Biochemical and Pharmacokinetic Aspects of Oral Treatment with Chondroitin Sulfate

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Summary

Chondroitin sulfate (Condrosulf[®]) was characterized for structure, physicochemical properties and purity. This glycosaminoglycan has a relative molecular mass of about 14,000, a sulfate-to-carboxyl ratio of 0,95 due to the high percentage of monosulfated disaccharides (38% 6-monosulfate and 55% 4-monosulfate) and a low amount of disulfated disaccharides (1.1%) inside the polysaccharide chains. No other glycosaminoglycans were detected in the preparation.

Chondroitin sulfate was labelled by reduction with sodium ³H-borohydride and administered by oral route in the rat and dog. More than 70% of radioactivity was absorbed and found in urine and tissues. The plasma radioactivity was fractionated by size-exclusion chromatography in three fractions: radioactivity associated with high, intermediate and low molecular mass compounds. The peak value of the concentration of high molecular mass radioactivity compounds in plasma was reached after 1.6 and 2.1 h for the rat and dog, respectively. After 36 h the high molecular mass radioactivity compounds were still present in plasma of dog and rat. After 24 h radioactivity was higher in the intestine, liver, kidneys, synovial fluid and cartilage than in other tissues.

Chondroitin sulfate was orally administered to man (healthy volunteer) in a single daily dose of 0.8 g and in two daily doses of 0.4 g. The results showed that both forms of administration determined a significant increase of plasma concentration of chondroitin sulfate as compared with predose value over a full 24 h period. Elimination constant values and t_{max} (of the first administration in the case of fractionated dose) were almost the same for the two administrations.

Some biochemical parameters (number of leukocytes, proteins, sulfated glycosaminoglycans and hyaluronic acid amounts, and N-acetylglucosaminidase activity) of synovial fluid were evaluated in controls and treated osteoarthritic subjects. No variations were observed in the patient who did not receive chondroitin sulfate. Five days of chondroitin sulfate administration led to a significant increase of concentration and molecular mass of hyaluronan and a decrease of a lysosomal enzyme, N-acetylglucosaminidase. No significant differences in leukocyte count and protein content were detected.

Zusammenfassung

Biochemische und pharmakokinetische Aspekte der oralen Behandlung mit Chondroitinsulfat
Chondroitinsulfat (Condrosulf[®]) wurde auf Struktur, physikalisch-chemische Eigenschaften und Reinheit un-

tersucht. Dieses Glykosaminoglykan besitzt ein relatives Molekulargewicht von etwa 14 000, ein Sulfat/Carboxyl-Verhältnis von 0,95 aufgrund des hohen Anteils an monosulfatierten Disacchariden (38% 6-Monosulfat und 55% 4-Monosulfat) und eine geringe Menge an disulfatierten Disacchariden (1,1%) in den Polysaccharidketten. Es wurden keine anderen Glykosaminoglykane im Präparat gefunden.

Chondroitinsulfat wurde mittels Reduktion mit ³H-Natriumborhydrid radioaktiv markiert und peroral an Ratten und Hunde verabreicht. Mehr als 70% der Radioaktivität wurde absorbiert und im Harn und in den Geweben gefunden. Die Plasma-Radioaktivität wurde mittels Größenausschluß-Chromatographie in 3 Fraktionen aufgeteilt: Radioaktivität der Verbindungen mit hohem, mittlerem und tiefem Molekulargewicht. Der Maximalwert der Plasma-Radioaktivitätskonzentration der hochmolekularen Verbindungen wurde bei der Ratte nach 1,6 h und beim Hund nach 2,1 h erreicht. Nach 36 h waren die hochmolekularen radioaktiven Verbindungen im Plasma von Hund und Ratte noch immer anwesend. Nach 24 h war die Radioaktivität in Darm, Leber, Nieren, Gelenkflüssigkeit und Knorpel höher als in anderen Geweben.

Chondroitinsulfat wurde in einer einzelnen Tagesdosis von 0,8 g und in zwei Dosen von 0,4 g oral an Menschen (gesunde Probanden) verabreicht. Die Ergebnisse zeigten, daß beide Verabreichungsformen, verglichen mit dem während 24 h gemessenen Ausgangswert, zu einem signifikanten Anstieg der Chondroitinsulfat-Plasmakonzentration führten. Die Eliminationskonstanten und t_{max} (der ersten Verabreichung bei aufgeteilter Dosis) waren für beide Verabreichungen praktisch gleich.

Einige biochemische Parameter der Gelenkflüssigkeit (Leukozytenzahl, Protein, sulfatierte Glykosaminoglykane, Hyaluronsäure und N-Acetylglucosaminidase-Aktivität) wurden bei Kontrollpersonen und bei behandelten arthrotischen Patienten bestimmt. Bei den Patienten, die kein Chondroitinsulfat erhielten, wurden keine Veränderungen beobachtet. Eine fünf-tägige Chondroitinsulfat-Verabreichung führte zu einem signifikanten Anstieg der Konzentration und des Molekulargewichts der Hyaluronsäure und zu einer Abnahme eines lysosomalen Enzyms, der N-Acetylglucosaminidase. Es wurden keine signifikanten Unterschiede in der Leukozytenzahl und im Proteingehalt festgestellt.

Key words: Chondroitin sulfate, clinical studies, pharmacokinetics · Chondroprotection · Condrosulf[®] · Glycosaminoglycans

Abbreviations

HPSEC: high-performance size-exclusion chromatography
 M_r : relative molecular mass
 SAX-HPLC: Strong anion exchange - high performance liquid chromatography
 Δ Di-0S: 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-ene-pyranosyluronic acid)-D-galactose
 Δ Di-4S: 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-ene-pyranosyluronic acid)-D-galactose 4-sulfate
 Δ Di-6S: 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-ene-pyranosyluronic acid)-D-galactose 6-sulfate
 Δ Di-2,6DiS: 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-ene-pyranosyluronic acid 2-sulfate)-D-galactose 6-sulfate
 Δ Di-2,4diS: 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-ene-pyranosyluronic acid 2-sulfate)-D-galactose 4-sulfate
 Δ Di-4,6diS: 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-ene-pyranosyluronic acid)-D-galactose 4,6-disulfate
 Δ Di-2,4,6TriS: 2-acetamido-2-deoxy-3-O-(4-deoxy- α -L-threo-hex-4-ene-pyranosyluronic acid 2-sulfate)-D-galactose 4,6-disulfate)

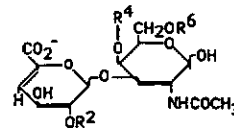
1. Introduction

The therapeutic uses of native glycosaminoglycans (hyaluronic acid, chondroitin sulfates, dermatan sulfate, heparan sulfate and heparin) as well of their low molecular mass derivatives (low molecular mass heparins, dermatan sulfate and chondroitin sulfate) and mixtures of different percentage of heteropolysaccharides have markedly increased with the knowledge of their pharmacological properties and biological functions [1, 2].

An in-depth knowledge of the metabolism of exogenous glycosaminoglycans, administered both orally and parenterally, would be particularly useful in view of their therapeutic applications. The low degree of sulfation of glycosaminoglycans allows their administration by both parenteral and oral routes [3, 4, 5]. In fact, they maintain their pharmacological properties when administered orally, unlike highly sulfated glycosaminoglycans and like heparin that is inactive as anticoagulant when administered orally [6] even if several fragments appear in the plasma [7]. Some evidence of heparin absorption by the stomach mucosa was reported in a recent paper [8], describing that the administration of 60 mg/kg of sodium heparin (or dextran sulfate) induced an antithrombotic effect in rats. Jaques et al. [8] report that heparin enters the body immediately on oral administration (2.4 and 6 min) and that this drug (or dextran sulfate) is recovered from the endothelium in large amounts and it is identified unchanged by electrophoretic technique. This gastric absorption might have been due to such a high dosage that a fraction of the drug or several of its active fractions which causes the antithrombotic effect had permeated.

Chondroitin sulfates are glycosaminoglycans composed of alternate sequences of differently sulfated residues of uronic acid (β -D-glucuronic) and α -D-N-acetyl-galactosamine linked by $\beta(1 \rightarrow 3)$ bonds [9]. The regular disaccharide sequence of chondroitin sulfate A, chondroitin-4-sulfate, is constituted by [(1 \rightarrow 4)-O-(β -D-glucopyranosyluronic acid)-(1 \rightarrow 3)-O-(2-N-acetamido-2-deoxy- β -D-galactopyranosyl-4-sulfate)]. Chondroitin sulfate C or chondroitin-6-sulfate, is mainly composed of a disaccharide unit [(1 \rightarrow 4)-O-(β -D-glucopyranosyluronic acid)-(1 \rightarrow 3)-O-(2-N-acetamido-2-deoxy- β -D-galactopyranosyl-6-sulfate)]. Disaccharides with different number and position of sulfate groups can be located, in different percentage, inside the polysaccharide chain, such as the non-sulfated or disulfated disaccharide in which two sulfate groups can be O-linked in position 2 of β -D-glucuronic acid and 6 of α -D-N-acetyl-galactosamine (disaccharide D) or in position 4 and 6 of α -D-N-acetyl-galactosamine (disaccharide E) [9] (Table 1). The hetero-

Table 1: Constituent disaccharides and physico-chemical properties of the chondroitin sulfate test preparation (T).



	R ²	R ⁴	R ⁶	T
Δ Di-0S	H	H	H	4.9
Δ Di-6S	H	H	SO ₃ ⁻	37.9
Δ Di-4S	H	SO ₃ ⁻	H	55.0
Δ Di-2,6diS	SO ₃ ⁻	H	SO ₃ ⁻	0.6
Δ Di-4,6diS	H	SO ₃ ⁻	SO ₃ ⁻	0.5
Δ Di-2,4diS	SO ₃ ⁻	SO ₃ ⁻	H	0.0
Δ Di-2,4,6TriS	SO ₃ ⁻	SO ₃ ⁻	SO ₃ ⁻	0.0
SO ₃ ⁻ /COO ⁻				0.95
Peak M _r (x 1000)				14.25

geneity of the primary structure, besides the physico-chemical properties such as the relative molecular mass and charge density, is responsible for different and more specialized biological and pharmacological functions of these glycosaminoglycans [10].

The synthesis of proteoglycans and hyaluronate has been clarified whilst their breakdown and the regulation of their turnover, in normal and pathological conditions, are less known [11, 12]. Chondroitin sulfates may be employed as chondroprotective [13] drugs with application in the therapy of tibiofibular osteoarthritis of the knee [14] and in the articular cartilage osteoarthritis by intramuscular and oral route. The oral route for chondroitin sulfates as chondroprotective drugs is very interesting, because it allows simplified use, which is more compatible with long administration periods.

The metabolic fate of orally administered exogenous chondroitin sulfate¹⁾ with defined structure and physico-chemical properties was studied in the experimental animal and in man using non-labelled and radioactive isotope labelled polysaccharide. Furthermore, we studied the plasma levels of chondroitin sulfate after repeated oral administration and the modification of cell number and biochemical parameters in synovial fluid during treatment.

2. Materials and methods

2.1. Extraction and purification of chondroitin sulfate

Bovine trachea was ground and treated with papain at 60 °C for 12 h in a reaction vessel. After heating at 100 °C for 30 min, the mixture was brought to pH 9.0 by adding 2 N NaOH. After 24 h at 40 °C, the product (brought to pH 6.0 with 2 N acetic acid) was filtered on a diatomite filter (High Performance Filter Aids from Dicalite, Los Angeles, CA, USA), and the solution containing polysaccharides was percolated through a strong anion-exchange resin column (Amberlite IRA900 in Cl⁻ form, Supelco, Bellefonte, PA, USA). Peptides and nucleic acids were eluted with 0.7-1.5 mol/l NaCl, and chondroitin sulfate was eluted with 1.6-1.8 mol/l NaCl; the recovered solution was added to 1.0-1.5 volumes of acetone. Any heparin or heparan sulfate still contaminating the preparation were degraded by nitrous acid treatment at pH 3.0 and removed by strong anion-exchange chromatography, as reported above. Any dermatan sulfate present was also removed by selective precipitation as its copper salt, as reported elsewhere [15]. Chondroitin sulfate was

¹⁾ Condrosulf[®]; manufacturer: IBSA, Institut Biochimique SA, Lugano (Switzerland).

percolated through a cation-exchange resin (Amberlite IR-120 in Na⁺ form, Supelco) and transformed into chondroitin sulfate sodium salt.

2.2. Preparation of the chondroitin sulfate fraction with a M_r of about 14,000

The native chondroitin sulfate ($M_r = 26,500$) was fractionated on a column packed with BioGel P100 (BioRad, Hercules, CA, USA; particle size range, 45–90 μm ; molecular mass range, 5,000–100,000 Dalton) and eluted with 1 mol/l NaCl. The fractions (determined by HPSEC [16]) were collected concentrated by Rotavapor and dialyzed with molecularporous dialysis membranes (Spectrapore dialysis tubing from Spectrum (Houston, TX, USA); molecular mass cut-off of 2,000) against decreasing molarity NaCl solution and then against bidistilled water; finally, they were concentrated and lyophilized. The chondroitin sulfate fraction of about $M_r = 14,000$ was further analyzed and used for experimentation.

2.3. Characterization of structure and physicochemical properties of chondroitin sulfate

2.3.1. Purity of chondroitin sulfate

The presence of other possible glycosaminoglycans (heparin, heparan sulfate and dermatan sulfate) in the preparation of chondroitin sulfate, was determined by electrophoresis on agarose-gel in barium acetate/1,2-diaminopropane [17] and cellulose polyacetate (Titan III) [18].

The presence of peptides or proteins as contaminants of chondroitin sulfate preparations was evaluated by spectrophotometric analysis according to Lowry et al. [19] and by SDS-polyacrylamide gel electrophoresis (PAGE) after complete digestion of chondroitin sulfate chains by chondroitinase ABC.

Specific optical rotation was determined at +25 °C at a concentration of 5% in bidistilled water by a polarimeter.

2.3.2. Characterization of physico-chemical properties of chondroitin sulfate

The M_r (peak molecular mass) of chondroitin sulfate was determined by high-performance size-exclusion chromatography (HPSEC) as reported elsewhere [16].

The sulfate-to-carboxyl ratio was determined by potentiometric titration [20] and by enzymatic degradation after HPLC separation of constituent disaccharides.

2.3.3. Characterization of the disaccharide pattern of chondroitin sulfate

Constituent disaccharides of chondroitin sulfate were separated and determined by strong-anion exchange (SAX)-HPLC after cleavage with chondroitinases [20]. The identification and separation of chondroitin sulfate disaccharides was performed according to the retention times of standards.

2.4. General and local toxicity

The compound was well tolerated. After oral administration, no change was observed in laboratory clinical signs, and examination of individual subjects did not show any symptom of local and general toxicity.

2.5. Preparation of ³H-chondroitin sulfate

³H-chondroitin sulfate, produced by Amersham International Laboratories, Buckinghamshire, (England), was prepared by reduction of the reducing end of chondroitin sulfate with sodium ³H-borohydride with formation of tritium labelled alditols at the reducing end of polysaccharide chains. After treatment, excess borohydride was removed by gel filtration on a Sephadex G-100 column. ³H-chondroitin sulfate had a specific activity of 12.5 mCi/mg and was devoid of low molecular mass radioactivity.

2.6. Pharmacokinetic experimental design of orally administered chondroitin sulfate in rat and dog

Wistar rats (210–260 g) and young beagle dogs (4–6 kg) of both sexes were used. The experimental animals were supplied by Morini, Polo d'Enza (Italy). The animals were housed under conditions of constant temperature and humidity (22 ± 2 °C, 55

± 5%), with a 12 h light/dark cycle (7 a.m. to 7 p.m.). A standard pellet diet of commercial rat and dog chow (Nossan GLP diet TR-M and CB-AM, Harlan Nossan, Corezzano, Milan, Italy) and tap water were given ad libitum for at least one week before using.

³H-chondroitin sulfate, conveniently added to non labelled chondroitin sulfate and dissolved in saline, was administered orally to rats at a single dose of 16 mg/kg and 90 mCi/kg. Venous blood, urine, and feces were collected at intervals after administration. Rats were sacrificed at different times and the main organs were taken and stored at –80 °C until analysis.

The labelled compound was also given to four dogs at a dose of 16 mg/kg and 22 mCi/kg. Blood, urine, and feces were collected at intervals. The compound was administered to dogs through a meat bolus.

Samples of plasma and urine were chromatographed on an Ultragel AcA 44 column (70 × 2.5 cm) equilibrated with 0.2 mol/l NaCl in 10 mmol/l pH 7 phosphate buffer. Radioactivity was measured in eluate fractions. Chondroitin sulfate and N-acetyl-galactosamine were used as reference molecular masses. In some cases, before chromatography, the samples were freeze-dried to remove volatile radioactivity (tritiated water). The radioactivity in the collected samples was measured in a liquid scintillation counter with quenching correction.

2.7. Pharmacokinetic experimental design of orally administered chondroitin sulfate in man

The pharmacokinetic study was carried out on 12 healthy volunteers, 6 males and 6 females aged 24–56 years. The means ± S.D. of age, weight and height of the healthy volunteers were 38.6 ± 11.3 years, 66.2 ± 13.5 Kg and 173.0 ± 8.7 cm, respectively. We administered a single dose of 0.8 g of sodium chondroitin sulfate at 6:00 p.m. or two doses of 0.4 g each, the first at 6:00 p.m., the second at 6:00 a.m. on the following day. There was 7 days interval between the two treatments. The volunteers did not receive other drugs.

Since food intake, physical activity, time of day and diseases may have some effect on glycosaminoglycan levels in biological fluids, some restrictions were imposed on the healthy volunteers [21, 22]. Three days before sodium chondroitin sulfate administration and during blood sample collection the intake of food containing high quantities of glycosaminoglycans was kept low for this reason, foods rich in connective tissue and sauces which may contain glycosaminoglycans were avoided. Meat deprived of visible connective tissue was restricted to 150 g/d. The quality and the quantity of food and the time of meals were about the same during these four days. They were requested to avoid physical activity during the days of blood collection. Before entering the study, the volunteers were informed in detail of the experimental protocol and the scope of the investigation. Each subject gave his informed consent to the study which was performed in accordance with the declaration of Helsinki-Venice of 1983. They were administered careful medical history, physical examination with blood pressure and ECG determination, and screened for the following laboratory parameters (differential blood count, hematocrit, hemoglobin, the transaminases SGOT and SGPT, total bilirubin, plasma electrolytes, serum glucose, alkaline phosphatase, total proteins, albumin, blood urea nitrogen (BUN), serum creatinine and urine analysis) at the beginning of the study and after each sodium chondroitin sulfate administration.

Blood samples were collected with EDTA at the following times: 0 (predose), 2, 4, 6, 12, 14, 16, 18, 24 h after the single administration of 0.8 g or after the first administration of 0.4 g. Blood was centrifuged and plasma stored at –20 °C until analysis. Blood samples were also collected one day prior to chondroitin sulfate administration at the following times of day: between 8 and 9, between 11 and 12, between 16 and 17 and between 20 and 21 o'clock. 24-h urine on the day of administration and also on the day before and after administration was collected. Urine was frozen immediately after collection without preservatives and stored at –20 °C until analysis. Before analysis, urine from each subject were thawed, and collected to form the 24-h pool, and volume was measured.

Lectinic and ionic bonds between plasma proteins and chondroitin sulfate and other acid heteropolysaccharides were broken by hydrolysis with 0.05 mol/l NaOH at 40 °C for 16 h, as suggested elsewhere [23]. After hydrolysis the pH was brought to 7.4 with HCl and the plasma was chromatographed on ion exchange Ecteola-Cellulose column (1 × 2 cm). After plasma ap-

plication, the column was washed with 0.154 mmol/l NaCl to remove proteins and low molecular mass compounds. Glycosaminoglycans were eluted with 2 mol/l NaCl and uronic acids were determined colorimetrically with *m*-phenylphenol [24]. Chondroitin sulfate was used as standard. For each volunteer, the basal values of glycosaminoglycan concentration at the different times were calculated by interpolation from data determined the day before chondroitin sulfate administration. In our controlled experimental conditions only small variations were observed in glycosaminoglycan concentrations at different times of the day. The basal glycosaminoglycan concentration obtained at different times was subtracted from the respective glycosaminoglycan concentration obtained after sodium chondroitin sulfate administration. In our experimental conditions the mean basal value of hexuronic acids is 7.8 ± 0.9 mg/ml of glycosaminoglycans. This value is very similar to reported concentrations [23, 25]. The results were indicated as plasma exogenous chondroitin sulfate (PECS).

A plasma aliquot was subjected to ultrafiltration using a Sartorius membrane with a cut-off of 5 kDa to remove proteins and heteropolysaccharides with a higher molecular mass. The ultrafiltrate was lyophilized, dissolved in 1 mol/l HCl and transferred to tubes for hydrolysis. The tubes were evacuated and vacuum sealed, and the method of Boas [26] was essentially adopted for hydrolysis. Thereafter the samples were dried and dissolved in 0.2 mol/l sodium-citrate buffer pH 2.2. Galactosamine was determined by an amino acid analyzer Multichrom Beckman (Brea, CA, USA) equipped with a 55×0.9 cm column eluted with 0.35 mol/l pH 5.25 sodium-citrate buffer [27]. Samples of galactosamine, *N*-acetylgalactosamine and chondroitin sulfate were treated like the plasma ultrafiltrate to calculate the recovery. This method allows us to determine plasma mono, oligo and polysaccharides with molecular mass lower than 5,000 which are formed during intestinal digestion of exogenous chondroitin sulfate. The plasma galactosamine concentrations determined by this method were corrected for the recovery and transformed in mg/ml of chondroitin sulfate. For each volunteer, the concentration obtained the day before treatment was subtracted from the respective value obtained at different times. The results were indicated as plasma exogenous chondroitin sulfate with molecular mass lower than 5,000 (PECS < 5 kDa). Total uronic acids were determined in 24-h urine by the above reported *m*-phenylphenol method. The uronic acid value of the 24-h urine of the day before administration (predose) was subtracted from the values of urine collected during the day of administration and during the day after administration. The resulting values are transformed in mg of chondroitin sulfate excreted during 24 h. The urinary levels of chondroitin sulfate and of low and high molecular mass compounds derived from its partial enzymatic hydrolysis were thus determined.

2.8. Calculation of pharmacokinetics parameters

Experimental data, expressed in $\mu\text{g/ml}$, relating to the plasma concentrations of each sample were utilized for non-linear regression curves according to the program ELSFIT by Sheiner L. B. (Division of Clinical Pharmacology, University of California, San Francisco, CA, USA) based on the extended least squares method. From the kinetics curves, the following parameters were calculated in compliance with the rules established by the Committee for Pharmacokinetics of American College of Pharmacology [28]: the area under curve (AUC expressed in $\mu\text{g} \cdot \text{h} \cdot \text{ml}^{-1}$), obtained by integrating the biexponential function, of which the extrapolated percentage always resulted lower than 10%; the time corresponding to the peak concentration (t_{max} expressed in h); the highest concentration in plasma (C_{max} expressed in $\mu\text{g/ml}$); half-life of apparent elimination ($t_{1/2\text{el}}$ expressed in h).

2.9. Experimental design for studying some biochemical parameters of synovial fluid in osteoarthritic patients

To evaluate the effect of repeated doses on plasma level of chondroitin sulfate, 12 osteoarthritic patients were treated with 0.8 g/day of polysaccharide in the evening (between 9:00 and 10:00 p.m.) for 30 days. The means \pm S.D. of age, weight and height of the group were 57 ± 9 years, 65 ± 10 kg and 163 ± 4 cm, respectively. Diagnoses were assigned on the basis of the criteria recommended by the American Rheumatism Association. Arthritis was defined according to the traditional four-stage evaluation (from stage 1, slight narrowing of joint space, below 50 %

to stage 4, complete loss of cartilage with marked osteophytosis and sclerotic tissue showing deformity of the edges). The study included patients affected by first and second degree arthritis. 15 days before and during the study, patients were not allowed to use other chondroprotective medications, corticosteroids, non steroids antiinflammatory drugs or undergo any physical or local therapy. Patients were allowed to use only analgesic drugs.

All patients were informed of the the experimental protocol and the scope of the investigation. Each subject gave his informed consent to the study which was performed in accordance with the declaration of Helsinki-Venice of 1983. Before treatment and on the 5th, 15th and 30th day of treatment, blood samples were collected in the morning at about 11:00 o'clock am after chondroitin sulfate administration. The samples were treated as reported before.

In 18 osteoarthritic patients with gonoarthritis who needed knee joint aspiration, the modification of some biochemical parameters of knee joint synovial fluid were investigated. The study included patients affected by first and second degree arthritis. 15 days before and during the study, patients were not allowed to use other chondroprotective medications, corticosteroids, non steroids antiinflammatory drugs or any kind of drugs including analgesic or undergo any physical or local therapy. All patients were informed of the experimental protocol and the scope of the investigation. Each subject gave his informed consent to the study which was performed in accordance with the declaration of Helsinki-Venice of 1983. The biochemical parameters were investigated before treatment and after 5 (8 patients, all females, means \pm S.D. of age, weight and height 65 ± 19 years, 65 ± 6 kg and 168 ± 7 cm, respectively) or 10 days (5 patients, 2 females and 3 males, means \pm S.D. of age, weight and height 69 ± 4 years, 62 ± 4 kg and 163 ± 5 cm, respectively) administration of 0.8 g/day of chondroitin sulfate. Five patients (3 females and 2 males, means \pm S.D. of age, weight and height 62 ± 9 years, 64 ± 5 kg and 166 ± 6 cm, respectively) received placebo instead of drug (control group). Patients were assigned randomly to each group. The synovial fluid was collected in plastic vials and small samples were removed for microscopic examination. After centrifugation the supernatant was stored at -20°C . Leukocyte counting was performed by standard methods using a haemocytometer.

Proteins were determined with Biuret method with bovine albumin as standard. Synovial fluid sulfated glycosaminoglycans were determined with dimethylmethylene blue assay [29]. Hyaluronan was measured essentially as reported by Little et al. [30]. Uronic acids and sulfated glycosaminoglycans were determined in the effluent with the methods reported above. *N*-Acetylglucosaminidase (NAG) was determined with the NAG kit by FAR (Pescantina, Verona, Italy).

3. Results

3.1. Evaluation of purity, structure and physicochemical properties of chondroitin sulfates

Electrophoretic separations of native chondroitin sulfate in agarose-gel and in Titan III did not detect other glycosaminoglycans containing uronic acids (heparin, dermatan sulfate, heparan sulfate). Less than 0.5% w/w keratan sulfate were measured in the preparation of chondroitin sulfate. Under our experimental conditions, agarose-gel electrophoresis detects 0.2% w/w of other glycosaminoglycans in the preparation of chondroitin sulfate. About 2.5% proteins or peptides were detected, and they were identified as low molecular mass compounds by PAGE.

The physico-chemical properties of chondroitin sulfate are reported in Table 1. The M_r of chondroitin sulfate is about 14,250 and the sulfate to carboxyl ratio is 0.95. This chondroitin sulfate shows a great percentage of monosulfated disaccharides in position 4 and 6 of galactosamine with an A to C ratio between the two isoforms of 1.45 (Table 1).

3.2. Metabolic fate in rat and dog

Fig. 1 reports the percentage of the radioactivity found in rat urine, feces and organs and in dog urine and feces.

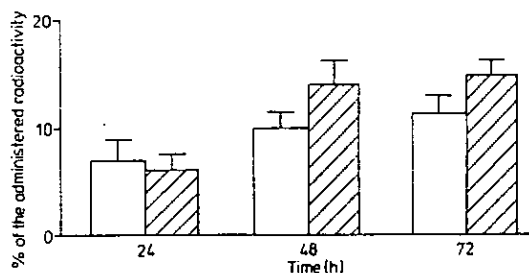
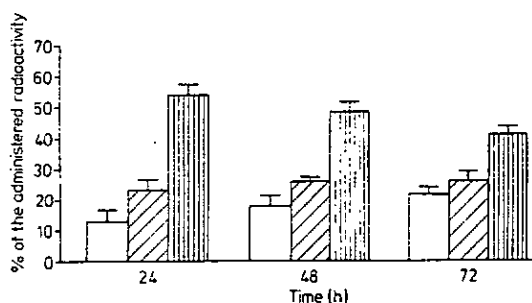


Fig. 1: Distribution of radioactivity at different times after oral administration of ^3H -chondroitin sulfate to the rat (above) and dog (below). For rat the reported values are means \pm S.D. of 10 animals. For dog the reported values are means \pm S.D. of 4 animals. \square Urine, ▨ feces, ▩ tissues.

When ^3H -chondroitin sulfate was given orally, more than 70% of radioactivity was absorbed and found in urine and tissues. Urine was the main route of excretion. In the dog, too, absorption was more than 70% of the orally given radioactivity, and urine was the main route of excretion.

After oral administration to rat the total plasma level of radioactivity rapidly rises, and already after 150 min it is about 60% of peak value (Fig. 2). A similar result was obtained in the dog. Thereafter, in both species, a slow increase of total radioactivity was observed. The peak value was reached after 14 h in the rat and after 28 h in the dog (Table 2). Actually, the course can be better described as a plateau between the 6th and the 30th h in the rat, and between the 6th and the 40th h in the dog, rather than a sharp peak. Radioactivity then slowly decreased. The plasma radioactivity peak coincided with the highest value of urinary excretion.

In urine collected during the first hours after administration and fractionated on gel filtration column, an aliquot of radioactivity had a molecular mass corresponding to that of administered chondroitin sulfate. Radioactive compounds with intermediate molecular masses, due to partial hydrolysis of chondroitin sulfate, were present, too. A large peak with the molecular mass equal or lower than that of one of the constituent monomers, N-acetylgalactosamine, and utilized as molecular mass marker was also observed. The radioactivity of the low molecular mass material progressively increased with the time after administration (Fig. 3).

The concentration of high molecular mass radioactivity compounds in plasma is quite different from that of total radioactivity. The peak value is reached after 1.6 and 2.1 h for rat and dog, respectively (Table 2). After 36 h the high molecular mass radioactivity compounds are still present in plasma of dog (Fig. 2) and rat. Fig. 3 reports the percentage of low, intermediate and high molecular mass compounds of exogenous chondroitin sulfate in rat plasma. A part of the radioactive low molecular mass material found in the urine and plasma, as well as in tissues, was volatile (tritiated water) and was removed by lyophilization. This was the aliquot which in-

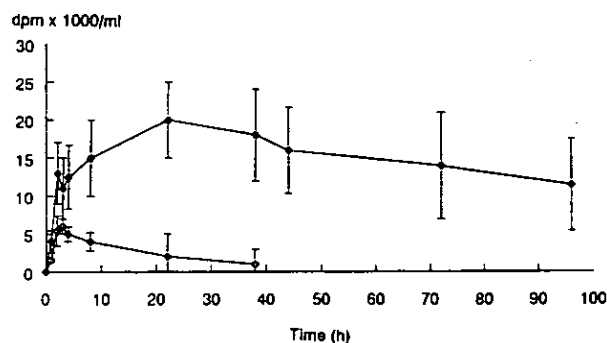


Fig. 2: Plasma levels of radioactivity (\blacklozenge total, \diamond HMM radioactivity) in the dog after oral administration of ^3H -chondroitin sulfate.

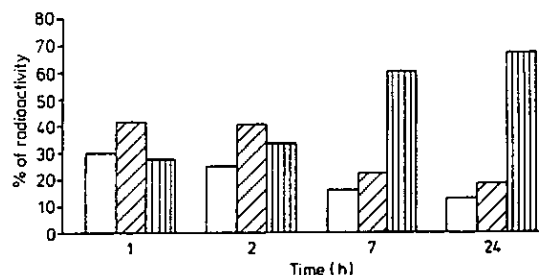


Fig. 3: Relative amount of high molecular mass (HMM, \square), intermediate molecular mass (IMM, ▨) and low molecular mass (LMM, ▩) radioactivity in plasma after 1, 2, 7 and 24 h from oral administration of ^3H -chondroitin sulfate to rat.

Table 2: Some pharmacokinetic parameters calculated from plasma total radioactivity and from plasma high molecular mass radioactivity fractionated by gel filtration after oral administration of ^3H -chondroitin sulfate to the rat and dog. The reported values are means \pm S.D.

	Rat	Dog
Total radioactivity		
t_{max} (h)	14.1 \pm 5.8	28.5 \pm 5.7
C_{max} ($\mu\text{g}/\text{ml}$)	7.1 \pm 1.2	5.9 \pm 0.7
AUC _(0-24 h) ($\mu\text{g} \cdot \text{h}/\text{ml}$)	159.8 \pm 16.3	147.0 \pm 21.4
High molecular mass radioactivity		
t_{max} (h)	1.6 \pm 0.4	2.1 \pm 0.4
C_{max} ($\mu\text{g}/\text{ml}$)	2.3 \pm 0.4	1.9 \pm 0.3
AUC _(0-24 h) ($\mu\text{g} \cdot \text{h}/\text{ml}$)	21.1 \pm 4.5	19.9 \pm 5.2

creased with the time after administration. Fig. 4 illustrates the gel filtration of plasma and synovial fluid of dog after 3 and 5 h of treatment with ^3H -chondroitin sulfate. The chromatography of the extract of rat joint cartilage after papain digestion and lyophilization is also shown in Fig. 4. Compounds with high molecular mass are present in the joint cartilage, whereas radioactivity was low in the fractions corresponding to the molecular masses of chondroitin sulfate.

Fig. 5 reports the distribution of radioactivity in some tissues after oral administration to rats. Radioactivity after 24 h was higher in the intestine, liver and kidneys, respectively, as compared to other tissues. Radioactivity was high not only in these organs involved in the breakdown and excretion of oligo- and polysaccharides but also in synovial fluid and cartilages, where radioactivity tends to accumulate. Also in the synovial fluid of the dog, radioactivity was 66.5% higher than in the plasma.

3.3. Metabolic fate in man

Fig. 6 reports plasma concentrations of chondroitin sulfate after administration of a single daily dose of 0.8 g and after administration of two daily doses of 0.4 g. No

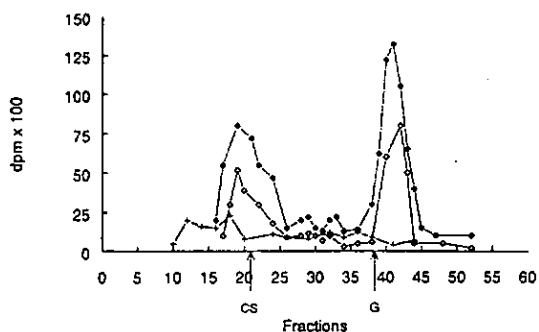


Fig. 4: Gel filtration of plasma (\diamond) and synovial fluid (\blacklozenge) of dog after 3 and 5 h of ^3H -chondroitin sulfate oral administration and gel filtration of rat joint cartilage (+) extract 24 h after oral administration. CS indicates the fraction corresponding to the native ^3H -chondroitin sulfate and G corresponding to the N-acetyl-Galactosamine.

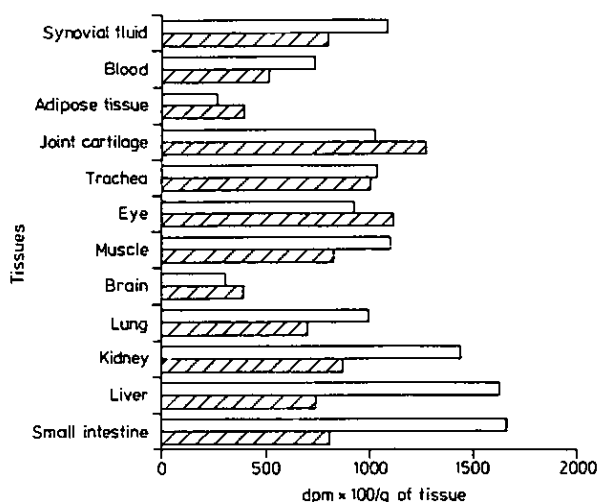


Fig. 5: Distribution of radioactivity in some tissues after oral administration of ^3H -chondroitin sulfate to the rat. \square 24 h. ▨ 48 h.

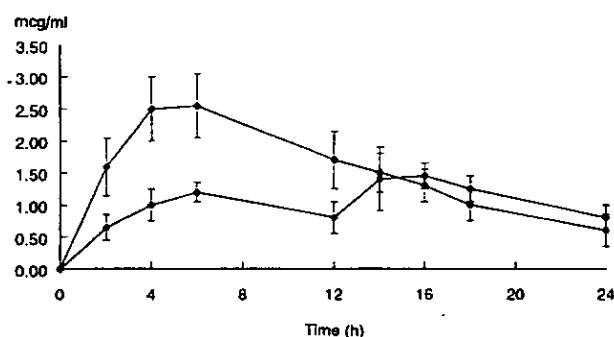


Fig. 6: Mean plasma concentration (\pm S.D.) of 0.8 g chondroitin sulfate (\blacklozenge single dose, \diamond two doses) as a function of time after oral administration of the drug in 12 healthy volunteers.

healthy volunteer showed collateral effects, neither reported symptoms of uneasiness, neither suffered illness or received any treatments. Table 3 reports the mean \pm S.D. of C_{max} , t_{max} , $t_{1/2}$ elimination, $\text{AUC}_{0-12\text{h}}$ and total AUC.

The results obtained show that both forms of administration determine a significant increase of plasma concentration of chondroitin sulfate as compared with pre-dose value over the full 24 h period. Elimination constant values and t_{max} (of the first administration in the case of fractionated dose) are almost the same for the two administrations: in fact, the observed differences are not significant. C_{max} value obtained with a single admin-

Table 3: Pharmacokinetic parameters after oral administration to healthy volunteers of 0.8 g of chondroitin sulfate in single dose or in two doses of 0.4 g each. The reported values are means \pm S.D.

	0.8 g Single dose	0.8 g Two doses
C_{max} ($\mu\text{g}/\text{ml}$)	2.6 ± 0.5	$1.2 \pm 0.2^{\text{a}}$
t_{max} (h)	5.0 ± 1.0	$5.2 \pm 1.0^{\text{a}}$
$t_{1/2}$ el (h)	10.3 ± 6.8	$10.3 \pm 2.5^{\text{b}}$
AUC_{0-12} ($\mu\text{g} \cdot \text{h}/\text{ml}$)	23.9 ± 4.2	10.6 ± 1.7
$\text{AUC}_{\text{Total}}$ ($\mu\text{g} \cdot \text{h}/\text{ml}$)	46.8 ± 10.1	37.3 ± 9.4

^{a)} Calculated on the plasma concentration value obtained after oral administration of the first dose of 0.4 g.

^{b)} Calculated on the plasma concentration value obtained after oral administration of the second dose of 0.4 g.

Table 4: Plasma exogenous chondroitin sulfate concentration in osteoarthritic patients treated with 0.8 g/d of drug in a single dose. The reported values are means \pm S.D.

Days of treatment	Exogenous chondroitin sulfate
5	1.80 ± 0.69
15	1.70 ± 0.68
30	1.89 ± 0.74

The basal concentration of plasma glycosaminoglycans is $6.90 \pm 0.8 \mu\text{g}/\text{ml}$ as hexuronic acids.

istration of 0.8 g is slightly higher than the value multiplied by two obtained with the first of two administration of 0.4 g. A difference, however moderate, was also observed in total AUC values. In fact, using the administration in two doses, we observed a total AUC value about 20% lower than the value obtained with the single dose (Table 3).

The concentration of exogenous chondroitin sulfate after 5, 15 and 30 days of administration of 0.8 g per day is reported in Table 4. It appears that the plasma level is almost constant during the month.

Note that blood samples were collected about 11 h after administration of chondroitin sulfate. A small not-significant increase was observed compared to the plasma level obtained at the same time with a single administration (Fig. 6). From the $t_{1/2}$ of elimination value and with the 24 h repeated administration used in this study it appears that the plateau is reached in 2-3 days and that only a small increase (10-20%) of the plasma level of exogenous chondroitin sulfate is expected at the steady state with respect to the plasma concentration behaviour observed with single administration:

3.4. Biochemical parameters of synovial fluid in osteoarthritic patients

Some biochemical parameters of synovial fluid are reported in Table 5. No variations were observed in the patients who did not receive chondroitin sulfate. After 5 days of chondroitin sulfate administration a significant increase of hyaluronan and a decrease of a lysosomal enzyme, N-acetylglucosaminidase, was obtained. No significant differences for leukocyte count and protein amount were detected (Table 5).

Since one of the purposes of this study was the demonstration that orally administered exogenous chondroitin sulfate reaches synovial fluid, we fractionated the synovial fluid by gel chromatography before and during chondroitin sulfate treatment. As an example, the molecular mass distribution of hyaluronan and sulfated glycosaminoglycans of a patient is reported in Fig. 7 and 8. After 5 days of treatment, the molecular mass distribu-

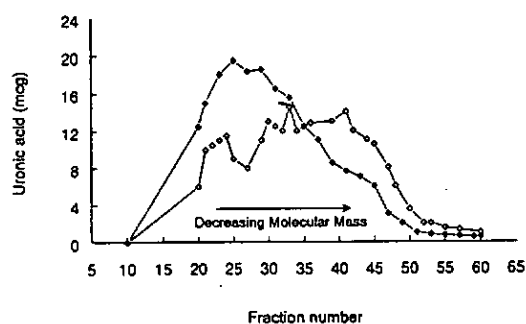


Fig. 7: Size distribution of hyaluronic acid in synovial fluid before (\diamond) and after (\blacklozenge) a 5-day treatment with 0.8 g/d chondroitin sulfate.

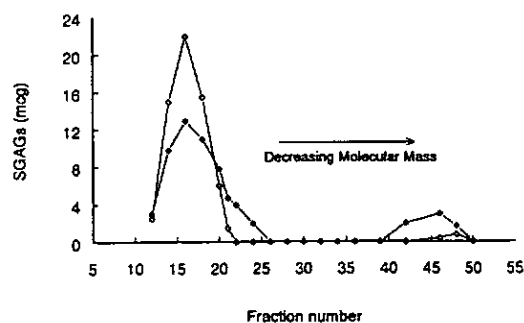


Fig. 8: Size distribution of sulfated glycosaminoglycans in synovial fluid before (\diamond) and after (\blacklozenge) a 5-day treatment with 0.8 g/d chondroitin sulfate.

Table 5: Some biochemical parameters of synovial fluid of osteoarthritic patients after 5 and 10 days of 0.8 g/d of chondroitin sulfate treatment in a single dose. The reported values are means \pm S.D.

	Leukocytes (10^9 /ml)	Proteins (mg/ml)	SGAGs (mg/ml)	HA (mg/ml)	NAG (U/l)
Treated patients					
Before treatment (n = 8)	2.3 \pm 1.6	31.9 \pm 5.9	0.20 \pm 0.08	1.7 \pm 0.6	31.2 \pm 4.4
After 5 days of treatment (n = 8)	1.8 \pm 1.6	31.4 \pm 5.6	0.20 \pm 0.09	2.0 \pm 0.6*	20.1 \pm 2.6*
Before treatment (n = 5)	2.6 \pm 1.5	30.3 \pm 4.9	0.16 \pm 0.06	1.8 \pm 0.7	41.2 \pm 6.8
After 10 days of treatment (n = 5)	2.0 \pm 1.4	29.6 \pm 1.8	0.17 \pm 0.07	2.3 \pm 0.6*	29.4 \pm 3.3*
Non-treated patients					
First synovial fluid collection (n = 5)	2.0 \pm 1.2	35.0 \pm 5.7	0.20 \pm 0.03	1.8 \pm 0.7	33.3 \pm 5.0
Second synovial fluid collection (after 5 days) (n = 5)	2.4 \pm 1.4	35.5 \pm 8.3	0.18 \pm 0.04	1.8 \pm 0.8	31.5 \pm 3.7

HA: hyaluronic acid; NAG: N-acetylglucosaminidase; SGAG: sulfated glycosaminoglycans. * $p < 0.05$.

tion of hyaluronan changes, with an increase of the high molecular mass fractions, suggesting that not only a quantitative variation but also a qualitative change of this molecule takes place during chondroitin sulfate treatment. Similar results were obtained with the other treated patients whilst the untreated subjects did not show these variations. The molecular mass of sulfated glycosaminoglycans was also changed. The high molecular mass components, markers of cartilage breakdown, decrease whereas the low molecular mass molecules increase (Fig. 8). We suggest that at least a part of the low molecular mass material present in joint synovial fluid after 5 days of treatment is exogenous chondroitin sulfate which has been shown in experimental animals to reach synovial fluid and cartilage.

4. Discussion

The absorption of sulfated glycosaminoglycans (heparin, heparan sulfate, chondroitin sulfates, dermatan sulfate) administered by oral route is a controversial question arising from the difficult to accept notion that molecules with high molecular mass and charge density may pass through gastric and intestinal mucosa. However, several experimental findings have appeared in literature on the intestinal absorption of glycosaminoglycans [3, 5, 7, 31-36]. Recently, Jaques et al. [8] showed that heparin absorption takes place at gastric as well as at intestinal levels. Considering the published experimental data, it appears that about 5 to 15% of the administered glycosaminoglycans is absorbed depending on dose, nature and physicochemical properties of the administered drugs, and on the analytical methods used to evaluate the concentration of polysaccharides and their metabolic products in biological fluids. Glycosaminoglycans with low molecular mass and charge density are preferentially absorbed [5, 7].

The pharmacokinetic parameters (peak time, peak concentration, AUC) determined on the basis of total radioactivity represent the sum of the pharmacokinetic parameters of exogenous chondroitin sulfate and of several labelled molecular species which derive from its depolymerization and catabolism. Only plasma fractionation allows to separate the different labelled molecular species and to determine their pharmacokinetic parameters. We have fractionated plasma radioactivity compounds by gel-filtration and we have determined the concentration of high, intermediate and low radioactive species as a function of time of administration. It appears that high molecular mass species have different kinetic parameters than those calculated for total administered radioactivity. In fact, the peak time of the high molecular species is observed at 1.6 and 2.1 h in the rat and dog respectively, after administration of radioactive chondroitin sulfate. This values are similar to the peak time observed in man in which only the high molecular mass species were determined. The AUC and C_{max} values are also comparable in the rat, dog and man when the higher molecular mass derivatives are considered. On the contrary, the peak time for total administered radioactivity is observed at 14.1 hours for the rat and 28.5 for the dog, respectively.

Tritiated chondroitin sulfate is labelled to the reducing carbohydrate end. The *in vivo* enzymatic degradation of the polymer precedes, step by step, from the non-reducing end toward the reducing end [37]. For this reasons, even if enzymatic or chemical fragmentation of chondroitin sulfate takes place inside the carbohydrate chains, the labelled reduced sugar at the end of the chains of the administered tritiated drug is maintained during the entire depolymerization processes. We also observed that less than 1-2% of the radioactivity of chondroitin sulfate was released by exchange during 48 hour incubation at 37 °C in plasma or saline solutions (data not shown). For these reasons, we believe that a great part of the low

molecular mass species observed in plasma is produced after the complete depolymerization of exogenous labelled chondroitin sulfate to monomers and that the tritiated water which is formed derives in a large amount from the metabolization of the reduced end sugars (e.g. xilitol, galactitol, etc.) only when these sugars are freed, since at the moment no enzymes are known to catalyze the reoxidation of the monosaccharides labelled at the reducing end of the glycosaminoglycan chains [27].

Wood et al. [38] observed that exogenous chondroitin sulfate administered by parenteral routes is depolymerized into intermediate molecular mass compounds as well to monomers and tritiated water and that the desulfation is faster than the formation of tritiated low molecular mass derivatives.

Although pinocytosis may be the main mechanism of gastrointestinal absorption of glycosaminoglycans, the observation that fractions of chondroitin sulfate with lower molecular mass and charge density are preferentially absorbed also suggests that a selective absorption mechanism occurs in the alimentary tract or that a partial desulfation and depolymerization of the compounds is possible during the pinocytotic process. In fact, no modification of the degrees of polymerization and sulfation is observed when chondroitin sulfate is maintained for 3 hours at 37 °C in human gastric juice (data not shown).

The therapeutic effects of a high molecular mass natural compound, like chondroitin sulfate, administered orally is not surprising since many other natural macromolecules have pharmacological activity also when administered orally, e.g. heparan sulfate [39], dermatan sulfate [5] and bromelase. Recently, it has been reported that also collagen type II orally administered is effective in the treatment of rheumatoid arthritis [40].

The presence of exogenous chondroitin sulfate and its depolymerized derivatives in synovial fluid is very important to explain anti-inflammatory and chondroprotective effects of this polysaccharide. In fact, it has been shown that sulfated and desulfated poly- and oligosaccharides derived from chondroitin sulfate and hyaluronan degradation have regulatory effects [41, 42, 43]. Exogenous chondroitin sulfate as well poly- and oligosaccharides may exert anti-inflammatory action on synovial cells (leukocytes) as well a regulatory activity on cartilage metabolism.

A number of clinical studies showed that chondroitin sulfate decreases pain and increases functional parameters in osteoarthritic patient [13, 14, 44-46]. Our observation give a first biochemical basis to the clinical outcomes. In fact, a few days after administration of exogenous chondroitin sulfate, some biochemical parameters of synovial fluid changes indicating that modifications take place in enzyme release and hyaluronan/glycosaminoglycan synthesis and/or degradation. Some of these effects may be related to the chondroprotective activity of exogenous chondroitin sulfate whereas others, such as the decrease of activity of lytic enzymes, may be also due to the anti-inflammatory properties of this polysaccharide, which has been shown to modify some leukocyte functions [47]. In synovial fluid, like in plasma, fractions with molecular mass higher than that of administered chondroitin sulfate were found, probably due to the binding of polysaccharide and its partially depolymerized derivatives with proteins. In fact, it has been observed that in plasma chondroitin sulfate is associated with proteins [25]. To date, we do not know if the observed quantitative variations of hyaluronan are due to its increased synthesis or decreased breakdown. In fact, chondroitin sulfate (and derivatives) stimulates hyaluronan formation [48] (besides the synthesis of proteoglycans and type II collagen [45]), and it protects hyaluronan from enzymatic degradation by inhibiting hyaluronidase activity and breakdown from free radicals.

5. References

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