

Oral absorption and bioavailability of ichthyic origin chondroitin sulfate in healthy male volunteers

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Summary

Objective: Chondroitin sulfate (CS) has proven to be a valuable therapeutic tool as a symptomatic slow-acting drug for the treatment of osteoarthritis after oral administration. The aim of this study was to assess the absorption of CS of ichthyic origin after oral administration to 20 healthy male volunteers.

Design: Ichthyic origin CS (from shark cartilage, 4 g) was orally administered to 20 healthy human volunteers, and then extracted and purified from plasma over a 48 h period. The polysaccharide absorbed by oral route was characterized and quantified by agarose-gel electrophoretic technique, and densitometric scanning. In addition, the percentage of constituent disaccharides and charge density were measured.

Results: After oral administration, ichthyic CS plasma levels increased (more than 120%) with a peak concentration at 8.7 h, with the increase reaching significance from 4 to 16 h. A significant decrease in the relative amount of non-sulfated disaccharide was measured (reaching the minimum relative percentage of 30.86±20.79% at 8 h). At the same time, 4-sulfated disaccharide increased to a maximum of 51.91±25.91% at 6 h, and 6-sulfated and disulfated disaccharides appeared in blood, reaching maximum concentrations of 15.24±16.60% at 8 h and 2.93±4.82% at 12 h, respectively. Concomitantly, the mean charge density rose from 0.40±0.14 at predose to a maximum of 0.72±0.22 and 0.72±0.21 measured 8 and 12 h after ichthyic CS administration.

Conclusions: Ichthyic CS is absorbed slowly, with a t_{\max} =8.7±4.5 h and the C_{\max} averaged 4.87±2.05 µg/ml. The differences in the absorption and bioavailability of the various CS formulations is strongly influenced by the structure and characteristics, such as molecular mass, charge density, and cluster of disulfated disaccharides, of the parental molecules.

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Key words: Chondroitin sulfate, Glycosaminoglycans, Oral route, Osteoarthritis.

Abbreviations: CS, chondroitin sulfate, ΔDi-0s, 2-acetamido-2-deoxy-3-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-D-galactose, ΔDi-4s, 2-acetamido-2-deoxy-3-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-D-galactose 4-sulfate, ΔDi-6s, 2-acetamido-2-deoxy-3-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate, ΔDi-2,6dis, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-6-O-sulfo-D-galactose, ΔDi-4,6dis, 2-acetamido-2-deoxy-3-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-4-O-sulfo, 6-O-sulfo-D-galactose, ΔDi-2,4dis, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-4-O-sulfo-D-galactose, ΔDi-2,4,6tris, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-4-O-sulfo, 6-O-sulfo-D-galactose.

Introduction

Osteoarthritis is a heterogeneous condition with various clinical expressions¹. The most common symptoms are pain and functional disability resulting from destructive changes of the osteoarthritic joint. Current treatment of osteoarthritis is not aimed at a cure but at palliative management including physical, pharmacological, and surgical approaches. Drug treatment includes analgesics, NSAIDs, and symptomatic slow-acting drugs (SYSADOA)^{2–5}. The latter class of compounds have a slow onset of action and improve osteoarthritis symptoms⁶. Some of them are administered orally and some intra-articularly. Among SYSADOA, chondroitin sulfate (CS) has proven to be a valuable therapeutic tool for the symptomatic treatment of

osteoarthritis. Several controlled trials showed its effects as a SYSADOA with application in the therapy of osteoarthritis of the knee and in articular cartilage osteoarthritis^{6–11} with very good tolerability^{12,13}. Furthermore, whether CS will be shown to have a disease-modifying effect will be seen¹⁴.

CSs are heteropolysaccharides purified from various tissues composed of alternate sequences of uronic acids (D-glucuronic or L-iduronic) and differently sulfated residues of N-acetyl-D-galactosamine linked by β(1→3) bonds^{15–17}. The regular disaccharide sequence of chondroitin sulfate A, chondroitin-4-sulfate, is constituted by [(1→4)-O-(D-glucopyranosyluronic acid)-(1→3)-O-(2-N-acetamido-2-deoxy-D-galactopyranosyl-4-sulfate)]. Chondroitin sulfate C, chondroitin-6-sulfate, is mainly composed of a disaccharide unit [(1→4)-O-(D-glucopyranosyluronic acid)-(1→3)-O-(2-N-acetamido-2-deoxy-D-galactopyranosyl-6-sulfate)]. Disaccharides with a different number and position of sulfate groups can be located, in different percentages, inside the polysaccharide chains, such as the non-sulfated or disulfated disaccharide in which two sulfate groups are O-linked in position 2 of D-glucuronic acid and 6 of

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N-acetyl-D-galactosamine (disaccharide D) or in position 4 and 6 of *N*-acetyl-D-galactosamine (disaccharide E). These heterogeneous structures, in terms of percentage of variously sulfated disaccharides, degree of sulfation, molecular mass, relative amounts of iduronic acid, and glucuronate depending on the tissue of origin, are responsible for different and more specialized functions of these glycosaminoglycans^{18–24}.

CS is mainly employed by the oral route allowing a more simple drug use compatible with long term administration^{6,10,11,25}. In a previous study²⁶, bovine CS was orally administered to 20 healthy human volunteers and extracted and purified from plasma over a 48 h period. After oral administration, CS plasma levels increased (more than 200%). Absorption of exogenous CS was also proved by the change in the composition of disaccharides in plasma after drug administration with respect to baseline.

As CS extracted from shark is one of the SYSDOA used in Europe for the treatment of osteoarthritis¹⁴, a study was performed to assess the absorption of ichthyic origin CS after oral administration to 20 healthy male volunteers, and to compare bioavailability of CS obtained from two different sources, ichthyic and bovine²⁶. This last point could be of interest to better design a more effective therapy related to the very different structural and functional properties of the two polysaccharides. In the present study, ichthyic origin CS was orally administered to 20 healthy human volunteers, and extracted and purified from plasma over a 48 h period. Polysaccharide absorbed by the oral route was characterized using the previously reported analytical approach²⁶. Pharmacokinetic parameters were determined and the percentage of constituent disaccharides and charge density were measured.

Experimental methods

SUBJECTS

The 20 healthy male volunteers participating in the study were the same volunteers reported for the previous pharmacokinetic study²⁶. The Clinical Investigator gave his approval for the participation of each subject in the study, on the basis of acceptable medical history and findings in the physical and instrumental (ECG, laboratory) examinations. Written informed consent was obtained prior to their inclusion in each study period, as per protocol.

See previous study²⁶ for the healthy volunteers details. The volunteers had the ability to comprehend the full nature and purpose of the study, to cooperate with the investigator, and to comply with the requirements of the study. They gave written informed consent.

STUDY PROTOCOL

This was an open, single centre, single dose study. Ten capsules of ichthyic origin CS 400 mg, expiry date 1/2002, were administered as a single oral dose. The active drug was constituted of ichthyic origin CS (from shark cartilage), as also confirmed by disaccharide pattern evaluation (see subsequently).

The study drugs were provided by Institut Biochimique S. A. (IBSA, CH-6915 Pambio Noranco, Lugano, Svizzera) to the Clinical Centre (Cross Research S. A. Phase I Unit, CH-6864 Arzo, Svizzera) in excess of the amount necessary for the study (25% excess). The study medications were stored in a cool, safe locked place and were dis-

pensated only by the investigator or authorised personnel. The study drug was exclusively used for the present clinical trial and was only administered to the subjects enrolled in the study. At the end of the study, all the unused supplies were returned to the sponsor, after assessment of drug accountability.

Subjects took no food or drink (apart from water) for approximately 12 h (i.e., overnight) before administration, and for up to 2 h after treatment. Starting from 24 h before drug administration, the intake of food containing high quantities of glycosaminoglycans was kept low. The previous day's dinner before each drug administration was consumed at the clinical centre. A standardised light breakfast was served at approximately 10 a.m. (2 h after drug administration), lunch at approximately 1 p.m. (after the fifth hour of sampling), and dinner at approximately 8 p.m. (after the 12th hour of sampling).

The volunteers were asked to avoid physical activity during the 3 days preceding the starting of study. The evening preceding drug administration and the start of blood sampling, volunteers were hospitalised in the Clinical Centre.

The study periods included a single oral administration at about 8 a.m. of day 1, followed by a 48 h observation period. On the last day of the study period (day 3), each volunteer underwent blood and urine tests and electrocardiogram evaluation for post-study assessment. During the study period, a single administration was performed on day 1 at 8–8:33 a.m. Each volunteer swallowed 10 capsules of the test drug (400 mg) together with 400 ml of tap water (200 ml just before and 200 ml during administration).

High doses of CS (4 g) with respect to the standard therapeutic doses¹⁴ were administered to the healthy volunteers to permit a better quantitative and qualitative evaluation of the polysaccharide absorbed.

PLASMA SAMPLES COLLECTION

Venous blood samples (15 ml) were taken from a vein of the forearm using an indwelling catheter. The cannula was rinsed after each sampling. Blood samples were collected in tubes containing citrate as anticoagulant at the following times: predose and 0.5, 1, 2, 4, 6, 8, 12, 16, 24, and 48 h after drug intake. The samples were immediately stored at 4°C, centrifuged within 1 h at 4°C for 10 min to obtain plasma and immediately divided into two 2.5 ml aliquots, transferred into prelabelled test tubes and stored frozen at –20°C until analysed.

ANALYTICS

Materials

Protease type XXI from *Streptomyces griseus* [E.C. 3.4.24.31] was from Sigma. Ecteola-cellulose (condensation product of epichlorohydrin, triethanolamine, and cellulose, cross-linked fibres; capacity of 0.3–0.4 mEq/g, particle size of 0.05–0.2 mm) was from Serva, Heidelberg, Germany. High purity agarose and barium acetate were from BioRad. 1,2-Diaminopropane and cetyltrimethylammonium bromide were from Merck, Darmstadt, Germany. Spherisorb SAX (5 µm) (trimethylammonioethyl groups Si–CH₂–CH₂–CH₂–N⁺(CH₃)₃ in Cl[–] form) was from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK. Chondroitinase ABC from *Proteus vulgaris* [E.C. 4.2.2.4] was obtained from Sigma. Non-sulfated and

variously sulfated unsaturated CS disaccharides (Δ Di-0s, Δ Di-4s, Δ Di-6s, Δ Di-2,6dis, Δ Di-4,6dis, Δ Di-2,4dis, Δ Di-2,4,6tris, see abbreviations) were obtained from Seikagaku Corporation, Tokyo, Japan. All the other reagents were of analytical grade.

Extraction and purification of plasma CS

Extraction of CS from plasma samples was performed according to the method reported elsewhere²⁶. Five hundred microlitres of protease from *S. griseus* (10 mg/ml 50 mM Tris-Cl buffer pH 8.0) was added to 1000 μ l of plasma. After incubation at 37°C for 24 h, 1000 μ l 0.1 M acetic acid and 500 μ l NaCl 3 M were added. The mixtures were boiled for 5 min, and then centrifuged at 5000g for 5 min, and 1000 μ l NaOH 0.1 M was added to the supernatants; 5 ml of acetone was then added and solutions stored at -20°C for 24 h. The precipitates were recovered by centrifugation at 5000g for 15 min and dried at 50°C for 12 h. The dried precipitates were dissolved in 1000 μ l of distilled water by prolonged mixing and, after centrifugation at 10 000g for 5 min, the supernatant was applied to a column (1 cm \times 2 cm) packed with approximately 1.5 ml of Ecteola-cellulosa, previously washed with 1 M NaOH and 1 M HCl and equilibrated with 0.05 M NaCl. After washing the resin with 10 ml of 0.05 M NaCl, 5 ml of 3 M NaCl was added. Ten millilitres of acetone was added to the eluate (5 ml) and stored at +4°C for 24 h. After centrifugation at 5000g for 15 min, the pellet was dried at 60°C for 6 h.

Agarose-gel electrophoresis

Validated agarose-gel electrophoresis was performed according to Volpi²⁶⁻²⁸. Samples were dissolved in 20 μ l distilled water and layered on 0.5% agarose-gel plate and run in 0.05 M 1,2-diaminopropane buffered to pH 9.0 with acetic acid for 3 h at 60 mA. After migration, the plate was soaked in cetyltrimethylammonium bromide (0.1% solution) for approximately 12 h, dried and then stained with toluidine blue (0.2% in ethanol-water-acetic acid, 50:49:1 v:v:v) for 30 min and destained with ethanol-water-acetic acid 50:49:1 v:v:v.

Densitometric analysis was performed with a densitometric unit composed of Macintosh IIsi computer interfaced to a Microtek Color Scanner from Microtek International Inc., Hsinchu, Taiwan. The IMAGE processing and analysis program, Version 1.41 from Jet Propulsion Lab., NASA, Florida, USA was used for densitometric analysis of agarose-gel bands.

Disaccharide composition of ichthyic CS after oral administration

Samples dissolved in 40 μ l (2-4 μ g of purified CS) of 50 mM pH 8.0 Tris-HCl buffer were incubated with 50 mU of chondroitinase ABC. The reactions were stopped after 3 h of incubation at 37°C, by boiling for 1 min. The constituent disaccharides were determined by SAX-HPLC at 232 nm²⁶. Isocratic separation was run from 0 to 5 min with 0.1 M NaCl, pH 4.00; linear gradient separation from 5 to 30 min with 100% 0.1 M NaCl, pH 4.00 to 50% 1.2 M NaCl, pH 4.00. Flow rate was 1.4 ml/min. Separation of unsaturated non-sulfated and variously sulfated disaccharides produced by the action of the bacterial lyase was performed using standards supplied by Seikagaku Kogyo Co.

The following parameters were provided after each oral administration of CS: relative percentage of each

unsaturated disaccharide calculated per 500 μ l of plasma, namely Δ Di-0s (non-sulfated disaccharide), Δ Di-6s (6-monosulfated disaccharide), Δ Di-4s (4-monosulfated disaccharide), and Δ Di-dis (disulfated disaccharides). Therefore, charge density (sulfate to disaccharide ratio) of ichthyic CS was calculated.

Molecular mass determination of ichthyic CS

Molecular mass of ichthyic CS was determined by high-performance size-exclusion chromatography (HPSEC). HPLC was from Jasco. The mobile phase was composed of 125 mM Na₂SO₄ and 2 mM NaH₂PO₄ adjusted to pH 6.0 with 0.1 N NaOH. Flow rate was 0.9 ml/min. Columns were Protein Pak 125 (Waters, 7.8 mm \times 30 cm) and Protein Pak 300 (Waters, 7.5 mm \times 30 cm) assembled in series. The retention times were plotted against the logarithm of molecular mass for standard glycosaminoglycans^{29,30}. The curve that fits the experimental data is a third grade polynomial with the formula $y(fx) = -ax^3 + bx^2 - cx + d$ reported in the Figure. The number of average molecular weight (M_n , expressing the amount of species by the number of moles), the weight average molecular weight (M_w , considering the mass of the species), and the polydispersity index (M_w/M_n) were calculated by the Jasco Borwin GPC software version 4.1 (from Jasco, Japan).

PHARMACOKINETIC PARAMETERS³¹⁻³³

The percentage increase in CS with respect to baseline value was calculated at each time point after drug administration. The following pharmacokinetic parameters were obtained from the individual values of CS plasma concentrations (μ g/ml) vs time (hours), determined up to 48 h after ichthyic CS administration, using the validated software KINETICA™ 2000 version 3.0, InnaPhase Corporation, Philadelphia, USA (KINETICA 2000 User's Manual, Innaphase Corporation, Philadelphia, USA, 1999):

C_{max} : The highest concentration value found in plasma.

t_{max} : The time from administration at which the C_{max} value is found.

AUC_{0-48} : The area under the plasma concentration vs time curve up to the last sampling time calculated by the linear-linear trapezoidal rule.

AUC_{0-24} : The area under the plasma concentration vs time curve up to the 24 h sampling time calculated by the linear-linear trapezoidal rule.

STATISTICAL ANALYSIS

The data documented in this trial and the parameters measured were compared and evaluated using classic statistics: mean, standard deviation, coefficient of variation (%), minimum and maximum values (for quantitative variables), and by frequencies (qualitative variables).

Descriptive statistics were performed by means of either SAS® version 8e for Windows® (SAS/STAT® User's Guide, Version 6, Fourth Edition, SAS Institute Inc., Cary, NC, USA, 1990) or Kinetica™.

The CS plasma concentrations, μ g/ml plasma CS for each time, measured after drug treatment, were compared to basal values (see previous study²⁶) by means of a *t*-test. The ANOVA test was then applied to compare within the group the increase/decrease at different times for treated and endogenous CS²⁶. When significant overall differences

were found, the Student–Newman–Keuls test was applied to identify significantly different means.

The above statistical comparisons of the significant pharmacokinetic parameters obtained for test vs reference treatment were made using KINETICA™.

ETHICS AND LEGAL CONSIDERATIONS

The study was performed in accordance with the relevant guidelines of the Declaration of Helsinki, 1964, as amended in Tokyo, 1975, Venice, 1983, Hong Kong, 1989, and Somerset West, 1996.

Approval of the study protocol by the relevant local (Canton Ticino) Research Ethics Committee was obtained before the start of each study period. Federal Authorities were informed about the study.

The present Clinical Trial was carried out according to the general principles of 'ICH Topic E6, CPMP/ICH/135/95', July 1996 including post Step 4 errata, status September 1997.

Results

The 20 subjects enrolled in the study were the same as in the previous clinical trial²⁶: age (years) 25.2±2.1, weight (kg) 71.1±5.6, height (cm) 178.1±4.7.

Also for ichthyic origin CS the treatment was well tolerated and did not cause relevant changes in vital signs or ECG.

The basal CS concentrations, endogenous CS, were calculated in the previous published study²⁶, and they varied during the day from a minimum average value of 1.53 µg/ml after 10 h (about 6 p.m.) to a maximum average amount of 3.37 µg/ml after 24 h (about 8 a.m.).

CHARACTERIZATION OF THE STRUCTURE AND PROPERTIES OF ICHTHYIC CS

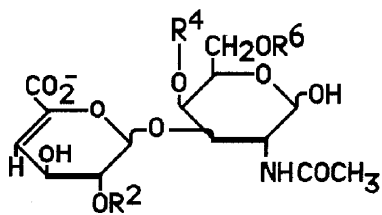
Table I summarized the disaccharide composition of ichthyic CS and its main characteristics, such as the charge density, 4-sulfated/6-sulfated ratio, and the molecular mass. Ichthyic CS is composed of a great amount of disulfated disaccharides (Fig. 1), in particular disulfated in position 2 of the uronic acid and 6 on *N*-acetylgalactosamine, with a great charge density and a low percentage of sulfate esterified in position 4 on *N*-acetylgalactosamine^{16,34–36}. Furthermore, ichthyic CS has a great M_w , approximately ranging from 40.000 to 50.000, with a low dispersity (Table I and Fig. 2) as determined by HPSEC²⁹.

QUALITATIVE AND QUANTITATIVE DETERMINATION OF CS

Agarose-gel electrophoresis permits qualitative and quantitative determination of CS after extraction from plasma (reviewed in reference 26 for method validation). CS was qualitatively evaluated by its migration time vs standard. For example, Fig. 3 illustrates agarose-gel electrophoresis of CS extracted and purified from 1 ml plasma of subject 4 at different times (from 0 to 48 h) after oral administration of 4 g of ichthyic CS.

Agarose-gel electrophoresis does not permit the differentiation of CS possessing different structure and characteristics and, as a consequence, the determination of the polysaccharide disaccharide pattern is necessary.

Table I
Percentage of unsaturated disaccharides as weight percent (for structure of disaccharides see the scheme), charge density calculated as sulfate to carboxyl ratio, and the 4-sulfated/6-sulfated ratio (4s/6s) of ichthyic CS



	R ²	R ⁴	R ⁶	CS ichthyic
ΔDi-0s	H	H	H	1.7
ΔDi-6s	H	H	SO ₃ ⁻	50.0
ΔDi-4s	H	SO ₃ ⁻	H	31.1
ΔDi-2,6dis	SO ₃ ⁻	H	SO ₃ ⁻	15.8
ΔDi-4,6dis	H	SO ₃ ⁻	SO ₃ ⁻	1.0
ΔDi-2,4dis	SO ₃ ⁻	SO ₃ ⁻	H	0.4
ΔDi-2,4,6tris	SO ₃ ⁻	SO ₃ ⁻	SO ₃ ⁻	0.0
SO ₃ ⁻ /COO ⁻				1.15
4s/6s ratio				0.49
MW _n				28 235
MW _w				44 746
Polydispersity				1.58

The number average molecular weight (MW_n), the weight average molecular weight (MW_w), and the polydispersity are reported (see Fig. 2 for HPSEC). The percentage of each unsaturated disaccharide and of the molecular mass values of the polysaccharide are averages of five different analyses.

ORAL ADMINISTRATION OF ICHTHYIC CS

After administration of ichthyic CS, plasma levels increased in almost all subjects (Fig. 4). Subjects 3 and 14 did not show an increase in CS plasma levels, whilst subject 7 showed poor absorption. Subject 11 showed a peak of maximum CS concentration at 4 h, subjects 2, 13, 16, and 18 at 6 h, subjects 1, 8, 10, 12, 17, and 19 at 8 h, subjects 4, 5, 6, and 15 at 12 h, subjects 9 and 20 at 16 h. The increase in CS plasma levels was significant from 4 to 16 h after oral administration. In particular, from 6 to 12 h after drug administration, an increase ranging from 112.50 to 126.06% was reached with respect to baseline (Fig. 4). The maximum measured plasma concentration was 4.87±2.05 µg/ml with a t_{max} of 8.7±4.5 h (Table II). After 48 h of drug administration CS plasma levels were detectable in all the subjects and averaged 2.13±1.33 µg/ml.

Under basal conditions the AUC_{0-24} averaged 49.2±16.5 µg×h/ml²⁶. In order to factor out the contribution of basal CS in the calculation of AUC, the AUC_{0-24} was calculated and it averaged 85.0±37.7 µg×h/ml for ichthyic CS (Table II). The AUC_{0-48} was 141.4±67.9 µg×h/ml after administration of CS. In order to further remove the influence of endogenous CS levels in the calculation of AUC, the area under the minimum concentration vs time curve up to 24 h sampling time was calculated for each subject and subtracted from the AUC_{0-24} . The $AUC_{(0-24)min}$ averaged 56.8±31.1 µg×h/ml after administration of ichthyic CS.

DISACCHARIDE COMPOSITION OF PLASMA ICHTHYIC CS AFTER ITS ORAL ADMINISTRATION

According to previous studies^{26,30} the disaccharide composition of plasma CS (endogenous) at predose was

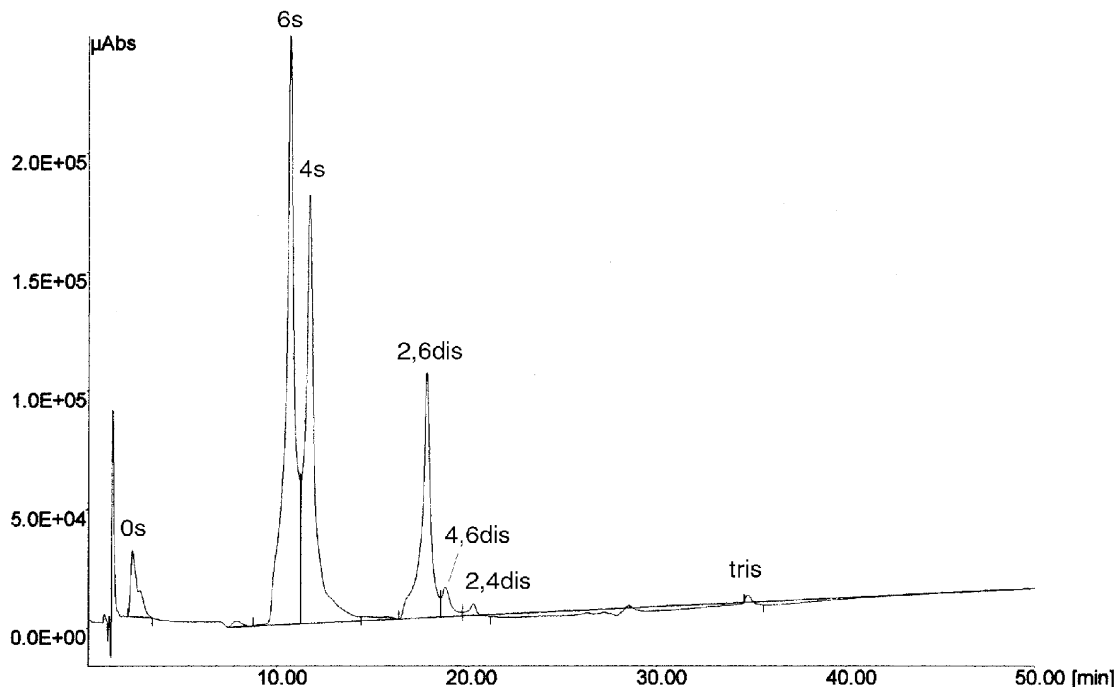


Fig. 1. SAX-HPLC chromatogram of unsaturated disaccharides of ichthyic and bovine CS. Non-sulfated (0s), 6-sulfated (6s), 4-sulfated (4s), 2,6-disulfated (2,6dis), 4,6-disulfated (4,6dis), 2,4-disulfated (2,4dis), and 2,4,6-trisulfated (tris) disaccharides. For structure see Fig. 1 and abbreviations.

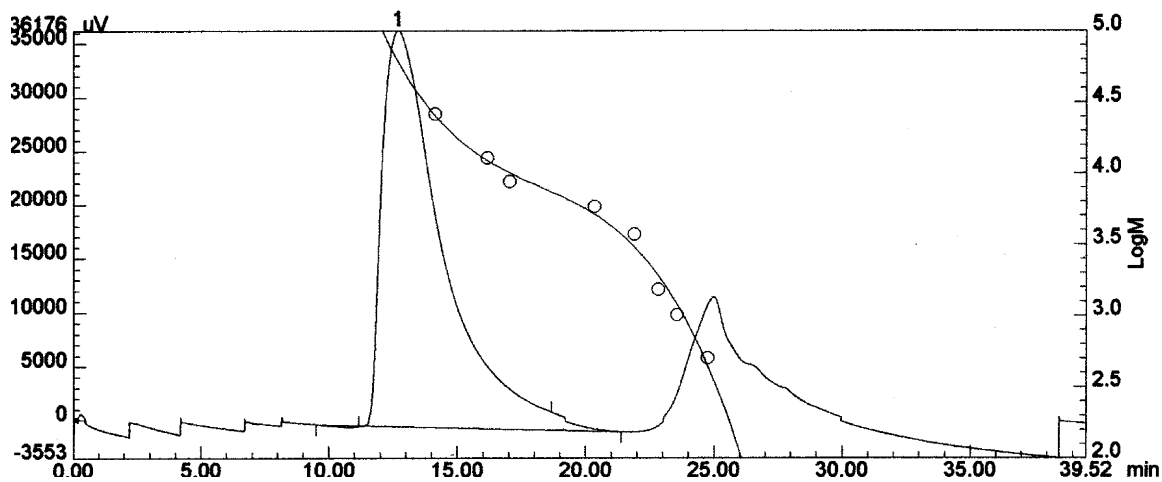


Fig. 2. HPSEC of ichthyic CS.

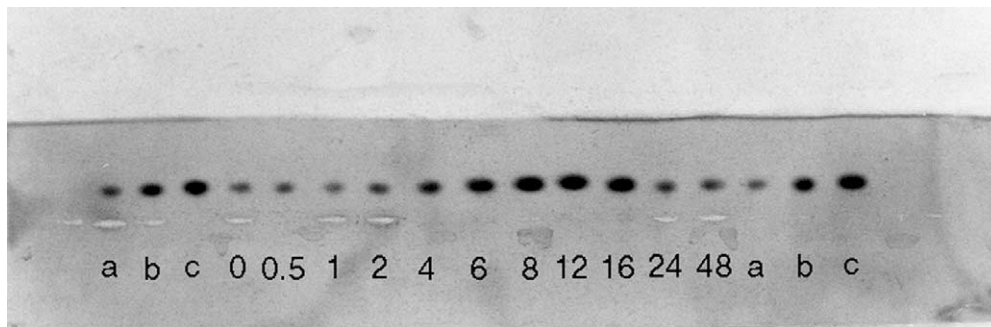


Fig. 3. Agarose-gel electrophoresis of endogenous CS (predose, 0) and polysaccharide extracted and purified from subject 4 plasma after administration of ichthyic CS at various times. (a) 1 μg CS. (b) 3 μg CS. (c) 5 μg CS. 0, Predose; 0.5, 0.5 h; 1, 1 h; 2, 2 h; 4, 4 h; 6, 6 h; 8, 8 h; 12, 12 h; 16, 16 h; 24, 24 h; and 48, 48 h.

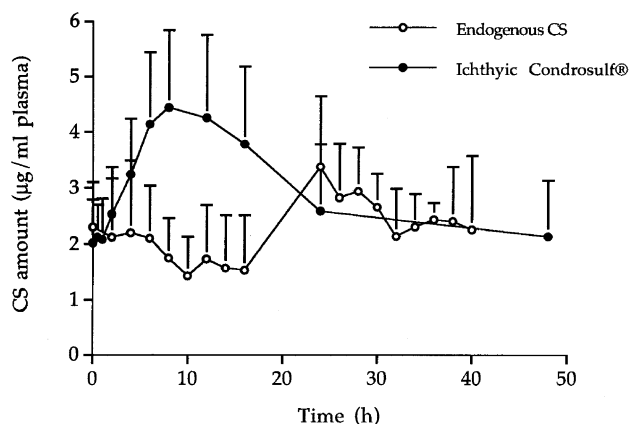


Fig. 4. Mean ($n=6$) plasma concentration of endogenous CS ($\mu\text{g/ml plasma}$)²⁶ and mean ($n=20$) plasma concentration of CS ($\mu\text{g/ml plasma}$) determined by validated agarose-gel electrophoresis and densitometric scanning at different times after administration of 4 g ichthyic CS. Endogenous CS levels were assessed in a previous study²⁶ and quantitatively determined by agarose-gel electrophoresis and densitometric scanning according to the analytical procedure illustrated above. The day time of blood samples started at 8 a.m. of day 1 (0 h in the Figure).

60.40 \pm 8.94% of non-sulfated disaccharide and 39.60 \pm 8.94% of 4-sulfated disaccharide (with a sulfate to disaccharide ratio of about 0.40) (Fig. 5).

CS of ichthyic origin has a high percentage of monosulfated disaccharides in positions 4 (31.1 \pm 5.0%) and 6 (50 \pm 5.0%) of galactosamine, about 2.0 \pm 2.0% of non-sulfated disaccharide, and about 17% of disulfated disaccharides mainly composed of disaccharide 2,6-disulfated (about 16%, Fig. 1), with a sulfate to disaccharide ratio of about 1.15 (Table I). After the administration of ichthyic CS a decrease in the relative amount of non-sulfated disaccharide was measured, reaching the minimum relative percentage of 30.86 \pm 20.79% at 8 h (Fig. 6). At the same time 4-sulfated disaccharide increased to a maximum of 51.91 \pm 25.91% at 6 h and 6-sulfated and di-sulfated disaccharides appeared in the blood CS, reaching maximum concentrations of 15.24 \pm 16.60% at 8 h and 2.93 \pm 4.82% at 12 h, respectively (Fig. 6). Concomitantly, the mean charge density rose from 0.40 \pm 0.14 at predose to a maximum of 0.72 \pm 0.22 and 0.72 \pm 0.21 measured 8 and 12 h after ichthyic CS administration (Fig. 7). After 48 h of drug administration the composition in disaccharides almost returned to basal: $\Delta\text{Di-0s}$ was 62.07 \pm 17.37%, $\Delta\text{Di-6s}$ was 2.16 \pm 6.59%, and $\Delta\text{Di-4s}$ was 35.77 \pm 16.22%. The charge density was 0.38 \pm 0.17.

Discussion

The oral absorption and bioavailability of ichthyic origin CS (from shark cartilage) was assessed after oral

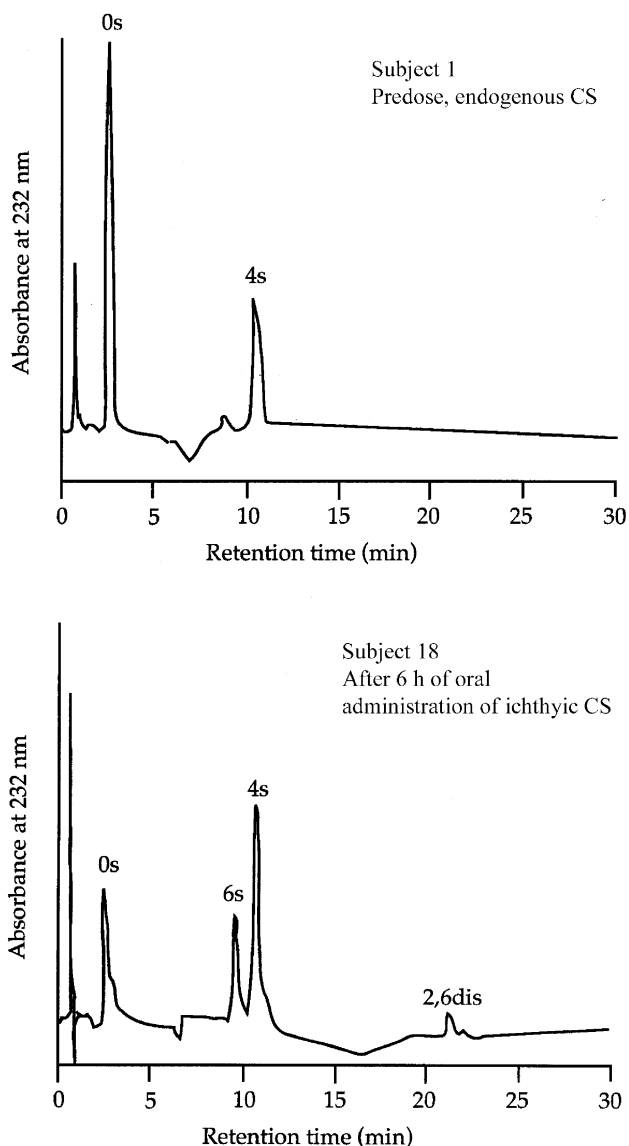


Fig. 5. SAX-HPLC chromatogram of disaccharides of endogenous CS (predose, subject 1) and after 6 h of oral administration of ichthyic CS (subject 18). 0s, non-sulfated disaccharide; 6s, 6-sulfated disaccharide; 4s, 4-sulfated disaccharide; and 2,6dis, 2,6-disulfated disaccharide.

administration to 20 healthy male volunteers using the same methodological approach for studying oral adsorption of CS from bovine²⁶. Polysaccharide absorbed by oral route was characterized and quantified by agarose-gel electrophoretic technique, and densitometric scanning. In addition, the percentage of constituent disaccharides and

Table II
Mean CS plasma pharmacokinetic parameters after administration of 4 g ichthyic CS

	C_{\max} ($\mu\text{g/ml}$)	t_{\max} (h)	AUC_{0-48} ($\mu\text{g}\times\text{h/ml}$)	AUC_{0-24} ($\mu\text{g}\times\text{h/ml}$)
Mean	4.87	8.7	141.4	85.0
SD	2.05	4.5	67.9	37.7
CV%	42.2	51.3	48.0	44.4
Min	1.5	0.0	34.8	25.2
Max	9.7	16.0	294.2	176.6
n	20	20	20	20

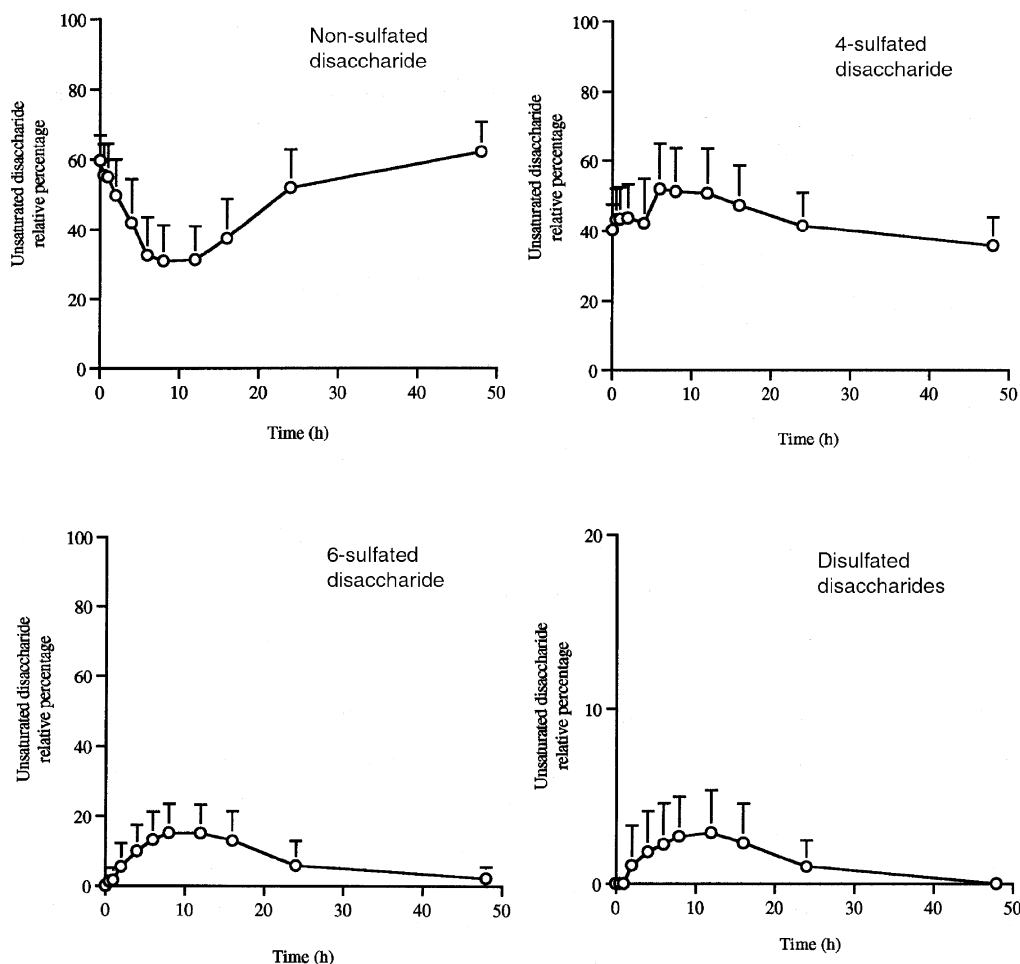


Fig. 6. The relative percentage of unsaturated disaccharides in CS determined by HPLC in 20 healthy human volunteers at different times after administration of 4 g ichthyic CS.

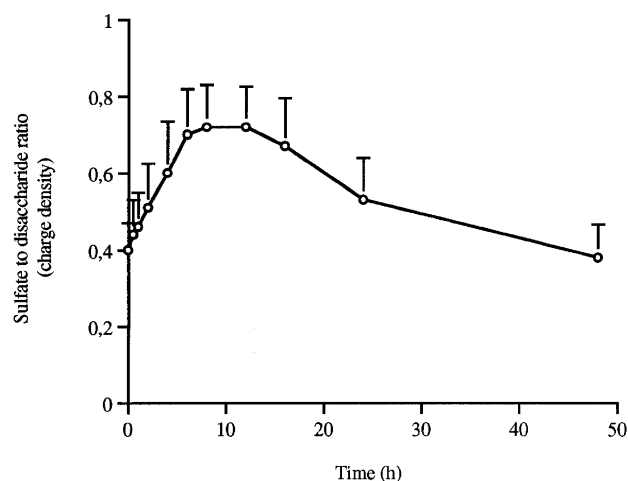


Fig. 7. Charge density (sulfate to disaccharide ratio) of CS determined in 20 healthy human volunteers at different times after administration of 4 g ichthyic CS.

charge density were measured. The combination of agarose-gel electrophoresis with the determination of unsaturated disaccharides of CS by HPLC permits us to

obtain quantitative and qualitative information (the pattern of variously sulfated disaccharides and charge density) on the modifications of plasma CS after oral administration of exogenous CS.

The absorption of exogenous ichthyic CS strongly changed the composition of plasma CS as was evident by determining unsaturated disaccharides. After drug administration a decrease of non-sulfated disaccharide and a concomitant increase of 4-sulfated disaccharide, and appearance of 6-sulfated disaccharide and disulfated disaccharides, mainly 2,6-disulfated, was evident. These structural modifications of plasma CS were evident in particular from 4 to 16 h after ichthyic CS administration concomitantly with the increase of polysaccharide plasmatic levels. The mean sulfate to disaccharide ratio also increased after CS administration, confirming the presence in human blood of a more sulfated CS species than endogenous. Furthermore, CS reaches the blood with a molecular mass greater than approximately 2000 as determined by agarose-gel electrophoresis and still possessing a low but significant amount of disulfated polysaccharide sequences.

Previous studies have demonstrated that after oral absorption the mean plasma curve of exogenous CS peaks after 3.2³⁷, 4.0³⁸, or 5.0 h³⁹. In the previous trial²⁶, bovine CS is quickly absorbed with a t_{max} of 2.4 h, while ichthyic CS is absorbed with a t_{max} of 8.7 h. On the contrary, the

extent of absorption was quite similar. These results give evidence that structure and properties of polysaccharides strongly influence their absorption and bioavailability by oral route. This could be of interest to better design a more effective therapy. In fact, a possible explanation for the difference in the absorption profile of different formulations can be found in the different molecular weights and charge densities of the two parental molecules. CS of ichthyic origin has a higher molecular mass than CS obtained from bovine tissues, thus absorption is likely to be slower. The rate of metabolic elimination is also likely to be reduced with high molecular mass molecules. This can account for the slow absorption, the low peak, and the prolonged presence of CS in the bloodstream when administered as ichthyic formulation. This was also experimentally confirmed by studying the oral absorption of partially depolymerized shark CS in men³⁸ by finding a t_{max} of 4.0 h. Moreover the presence of disulfated disaccharides in ichthyic CS increases its charge density. This can account for a stronger bond with plasma proteins, cells, and the endothelial wall from which the molecule would be slowly released into the bloodstream. In fact, glycosaminoglycans have been found to interact with several proteins in plasma, such as protease inhibitors, coagulation factors, lipoproteins and complement proteins, as well as cells and vascular endothelium^{40,41}.

This research strengthens the previous studies in man and experimental animals^{26,37-39,42-47} by confirming that molecules possessing high molecular mass and charge density can be absorbed orally. Moreover, the absorption and bioavailability of CS is strongly influenced by its structure and characteristics, such as molecular mass, charge density, and cluster of disulfated disaccharides.

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