Bioabsorbability of Nano247

(Chondroitin Sulfate Oligosaccharides)

Evidence of absorbability based on various scientific validation tests

MARUKYOU BIO FOODS Co., Ltd.

We extract chondroitin sulfate from fish cartilage and further produce chondroitin sulfate oligosaccharide by our patented micro chemical process treatment. (*1)

Since the molecular weight of natural chondroitin sulfate is as large as several hundred thousand, there was a concern that it would hardly be absorbed from the intestinal tract even if taken orally. However, nano-type chondroitin has a small weight average molecular weight of less than 3,000, and our research revealed that chondroitin sulfate oligosaccharide taken orally is absorbed from the intestinal tract and transferred into the bloodstream, which was published in an academic paper. (*2)

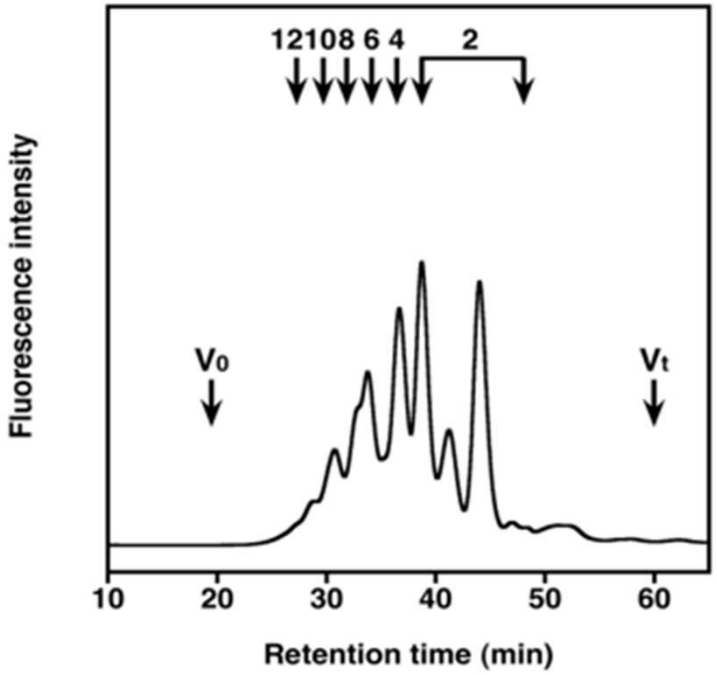
Therefore, chondroitin sulfate oligosaccharides can be expected to demonstrate excellent pharmacological effects.

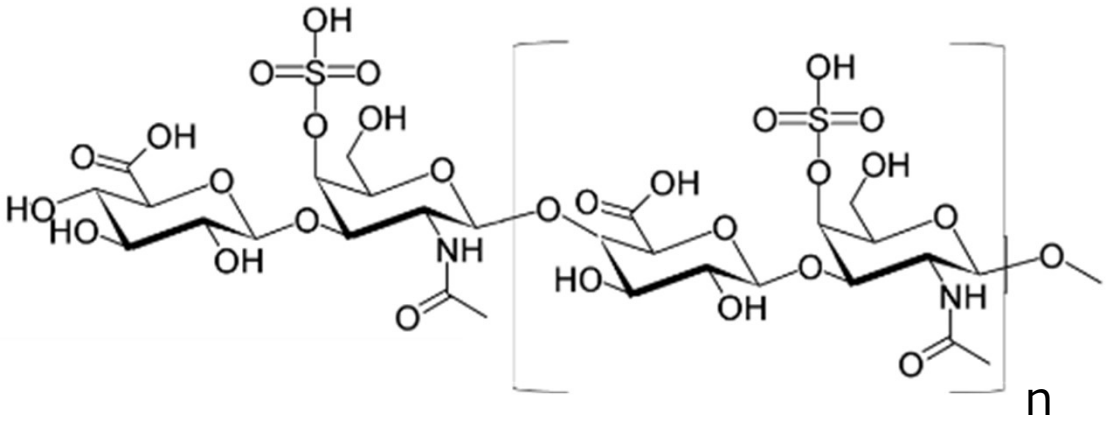
※1 Nano 247 is the trade name of the substance: chondroitin sulfate oligosaccharide, which has a weight average molecular weight of approximately 3,000 or less and a composition of about 2 to 16 chondroitin sulfate oligosaccharides.
It is a composition of about 2 to 16 chondroitin sulfate oligosaccharides with a weight average molecular weight of 3000 or less.

※2 Mizuta, et. al., Quantification of orally administered chondroitin sulfate oligosaccharides in human plasma and urine, Glycobiology, 2023;, cwad054, https://doi.org/10.1093/glycob/cwad054

Structure and composition of chondroitin sulfate oligosaccharides

- Size: Main components are oligosaccharides ranging from 2 to 12 saccharides.
- Sugar chain sequence: Having 6-sulfated chondroitin sulfate or non-sulfated chondroitin sulfate at the non-reducing terminus, Oligomers of mainly 6-sulfated chondroitin sulfate. Even and odd saccharides are present. The non-reducing end is saturated due to hydrolytic reaction.





Kawahara, et al., *Bulletin of Applied Glycoscience*, 11(2), 94–99, 2021.

Chondroitin sulfate oligosaccharides are water-soluble acidic sugars with molecular weights of 500-3000

I　Intestinal membrane permeability

Rat inverted intestinal tracts were immersed in 20 μg/mL solution of each test substance (chondroitin sulfate oligosaccharide: Mw2000, high molecular chondroitin sulfate: Mw150000) and allowed to react for 30 and 60 minutes respectively at 37° C while blowing O295% - CO25% mixed gas. After the reaction, the inner fluid permeated into the inverted intestinal tract was collected and the concentration of chondroitin sulfate was determined by the carbazole sulfate method. (Table 1)
In addition, the sugar composition was analyzed by HPLC using a size separation column.
The inverted intestinal test was conducted at the Natural Materials Research Institute (Shibuya, Tokyo), and the analysis of chondroitin sulfate was conducted at the Graduate School of Advanced Life Science, Hokkaido University (Sapporo, Hokkaido).

Table 1: Concentration of chondroitin sulfate in aqueous solution permeated into the antral intestinal tract.

elapsed time (min)		**0**	**30**	**60**
	external liquid CS (μg/mL)	internal liquid CS（μg/mL）		
CS Polymer	20,000	ND	ND	4
CS oligomer	20,000	ND	213	989

ND : Not Detected

In the chondroitin sulfate oligosaccharide test section, a peak of chondroitin sulfate was detected in the intestinal permeate over time. On the other hand, almost no chondroitin sulfate was detected in the high molecular weight chondroitin sulfate test section.

I Intestinal membrane permeability

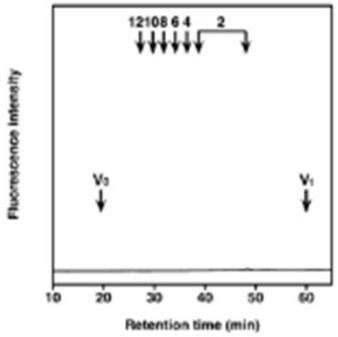
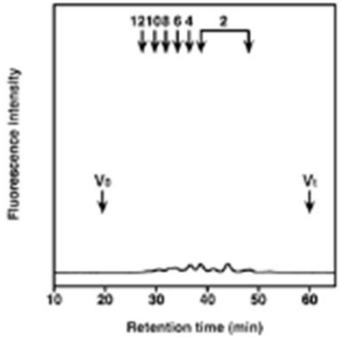
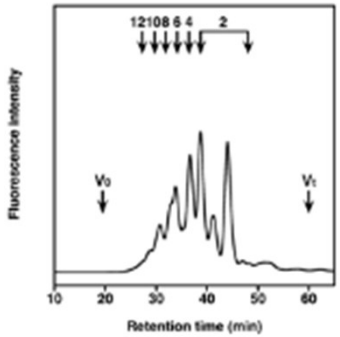
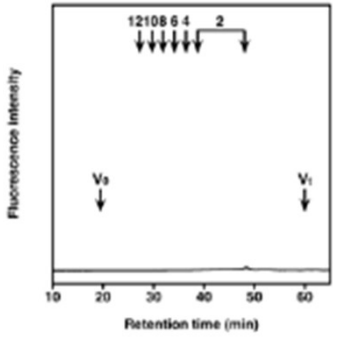
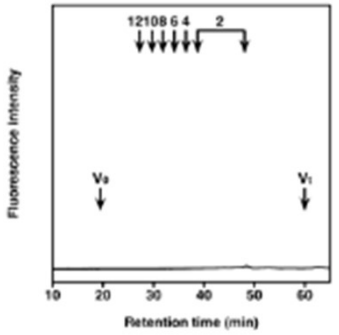
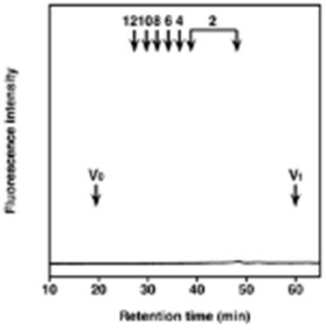
elapsed time（min）	0	30	60
chondroitin sulfate oligosaccharides			
chondroitin sulfate polysaccharide			

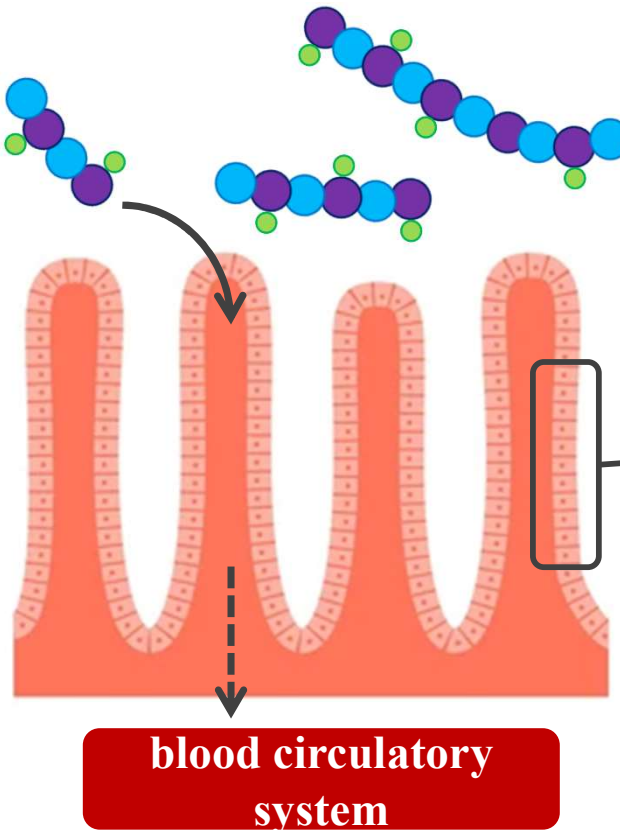
Figure 1: HPLC detection of chondroitin sulfate in aqueous solution permeated into the intestinal tract of inverted intestine.

In the chondroitin sulfate oligosaccharide test section, a peak of chondroitin sulfate oligosaccharides was obse rved over time. On the other hand, no peak was observed in the high molecular weight chondroitin test section.

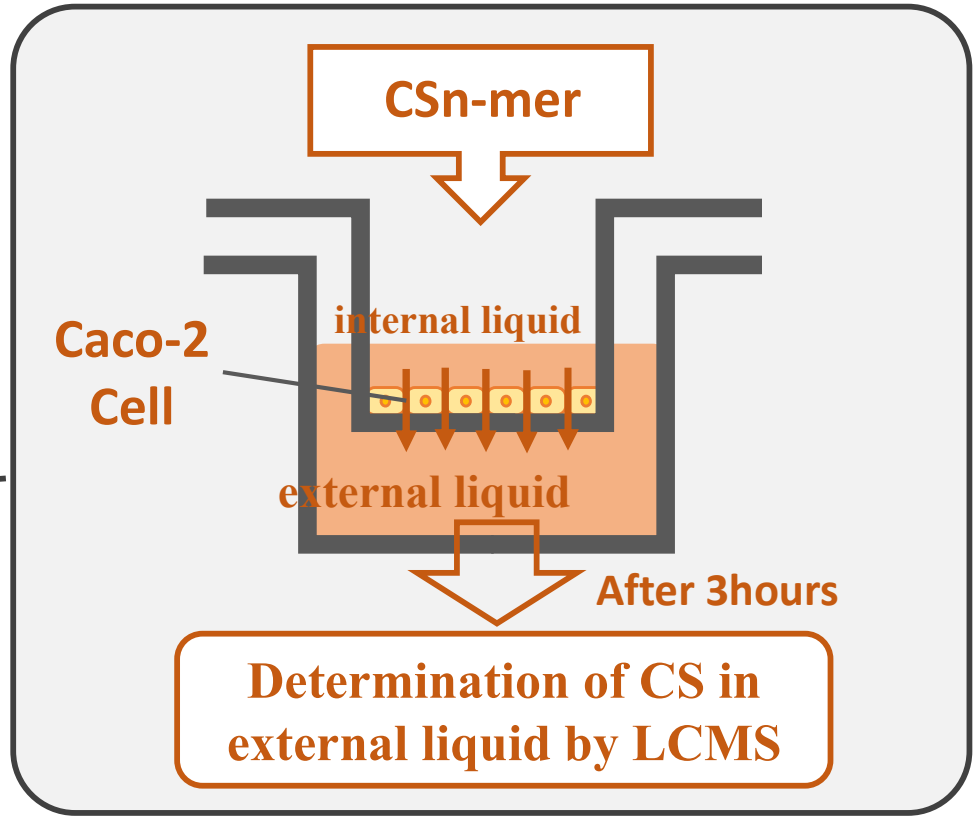
Chondroitin sulfate oligosaccharides permeate the intestinal membrane.
On the other hand, high molecular weight chondroitin sulfate does not permeate.

Ⅱ Membrane permeability test in Caco-2 cell layer

■CS absorption in small intestine



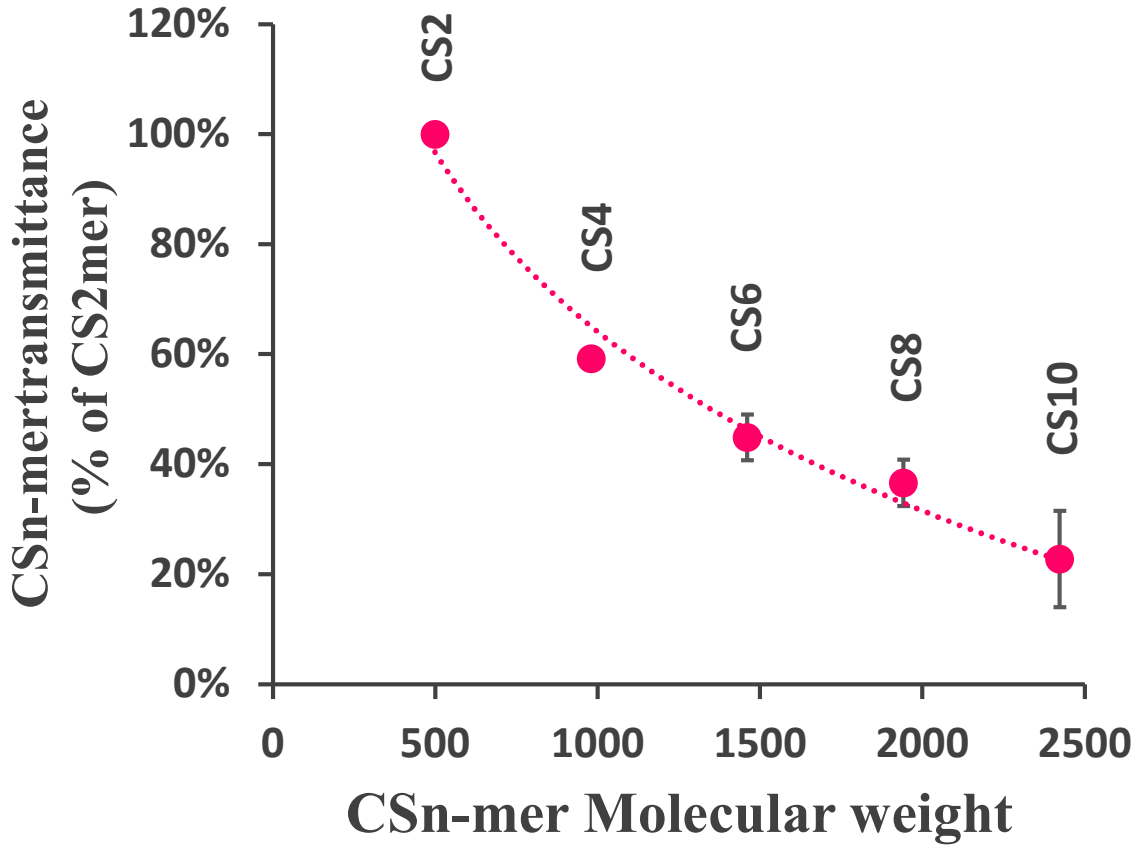
■Caco-2 small intestine absorption model



Caco-2 small intestinal absorption model reproduces the human small intestine

Ⅱ Membrane permeability test in Caco-2 cell layer

■CSn-mer transmittance

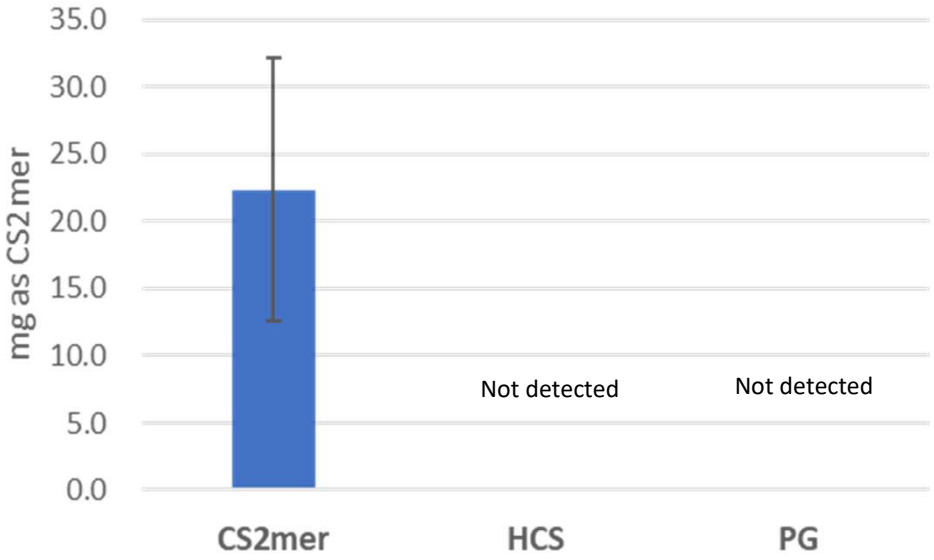


The lower the molecular weight CSn-mer, the higher the small intestinal absorption.

II Oral administration study in rats

A single oral dose of 400 mg of the test substance was administered to rats, and all urine samples from 0-24 hours and 24-48 hour s were collected to quantify the amount of the test substance contained; none was detected in the urine from 24-48 hours, so this i nformation is omitted. Quantitation was analyzed as chondroitin sulfate disaccharide, a common component.

Total amount of CS excreted in urine per rat for 24 hours after administration of test substance

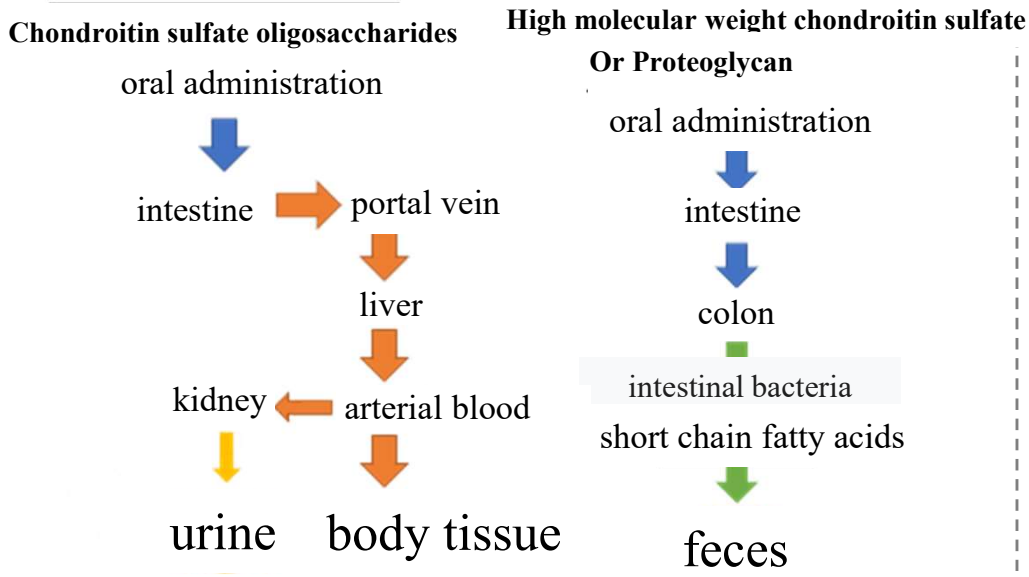


CS2mer : Chondroitin sulfate disaccharide (nano-type chondroitin component)
HCS : High molecular weight chondroitin sulfate (chondroitin sulfate released from core protein)
PG : Proteoglycan (chondroitin sulfate bound to core protein)

Consideration

Chondroitin sulfate oligosaccharides are thought to pass through the small intestinal tight junctions and are transported with water into the blood by osmotic pressure difference to the whole body, including cartilage cells.

On the other hand, high molecular sugar chains such as high molecular chondroitin sulfate and proteoglycans are not absorbed in the small intestine and reach the large intestine, where they are metabolized by intestinal bacteria to short-chain fatty acids.



In the chondroitin sulfate oligosaccharide group, chondroitin sulfate was detected in the urine.
On the other hand, it was not detected in the high molecular weight chondroitin sulfate group or the proteoglycan group.

III　Human Intervention Studies

Excerpted from the article Mizuta, et. al., Quantification of orally administered chondroitin sulfate oligosaccharides in human plasma and urine, *Glycobiology*, 2023;, cwad054

【Materials】

High molecular weight chondroitin sulfate (HMWCS) was purified according to the method of Hashiguchi et al. Chondroitin sulfate oligotetrasaccharide (4merCS) was purified according to the method of Kawahara et al.

【collecting blood】

HMWCS and 4merCS dissolved in ultrapure water, 500 mg each, were administered orally, and blood was collected in an EDTA blood collection tube before oral administration and 1, 2, 3, and 5 hours after oral administration. After blood collection, blood was allowed to stand for 15 minutes, and plasma was separated by centrifugation (2370 × G, 15 min) in a centrifuge. Plasma samples were stored frozen at -20° C.

【analysis】

One hundred μL of the plasma sample was aliquoted into a centrifugal concentration tube equipped with an ultrafiltration membrane with a fractional molecular weight of 10,000, and 100 μL of a 2 mol/L NaCl solution was added and pipetted to mix. Centrifugation (12,000 × G, 30 min) was then performed in a centrifuge, and a portion of the liquid was permeated. To approximately 40 μL of the retained solution, 100 μL of 1 mol/L NaCl solution was added and stirred by pipetting. The same procedure was repeated four times and the permeate was collected. 200 μL of the permeate was fluorescently labeled according to the method of Bigge et al. In other words, 2-aminobenzamide was induced at the reducing end of chondroitin sulfate by reductive amination reaction. HPLC (Shimadzu Corporation, Kyoto, Japan) equipped with a gel filtration column Superdex 30 Increase 10/300 GL (Cytiva (Marlborough, MA)) was used for analysis. For elution, 50 mmol/L NH4HCO3 solution was used as the mobile phase, and 100 μL sample was supplied at a flow rate of 0.5 mL/min at room temperature. Detection was performed by fluorescence at an excitation wavelength of 330 nm and detection wavelength of 420 nm. Peaks were extracted from the chromatograms obtained, and the peak areas were converted to CS plasma concentrations by correcting with a calibration curve in which plasma was supplemented with 4merCS afterwards.

【References】

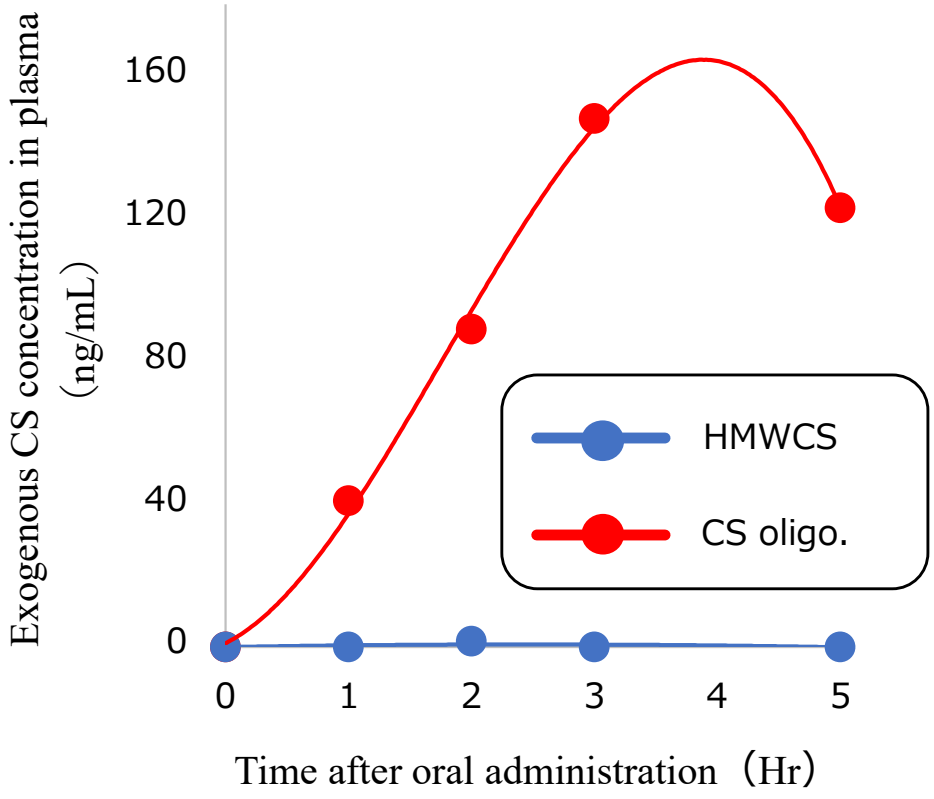
(1) Hashiguchi, et al., *Biochim. Biophys. Acta - Gen. Subj*., 1810, 406–413, (2011).
(2) Kawahara, et al., *Bulletin of Applied Glycoscience*, 11(2), 94–99, (2021).
(3) Bigge, J. C. et al., *Anal. Biochem*., 20, 229-238, (1995).

III Human Intervention Studies

High molecular weight chondroitin sulfate (HMWCS) and chondroitin sulfate oligotetrasaccharide (4merCS) were orally administered to healthy a dult males and their blood levels were examined. Five hundred mg of each was administered orally, and blood samples were taken before oral admi nistration and at 1, 2, 3, and 5 hours after administration, and the concentration of exogenous chondroitin sulfate ingested orally was determined by high-performance liquid chromatography (HPLC) using plasma separated from the blood.

Time after oral administration (hour)	Exogenous CS concentration in plasma (ng/mL)	
	4merCS oral administration	HMWCS oral administration
0	ND	ND
1	41	ND
2	89	1.6
3	148	ND
5	123	ND

ND : Not Detected



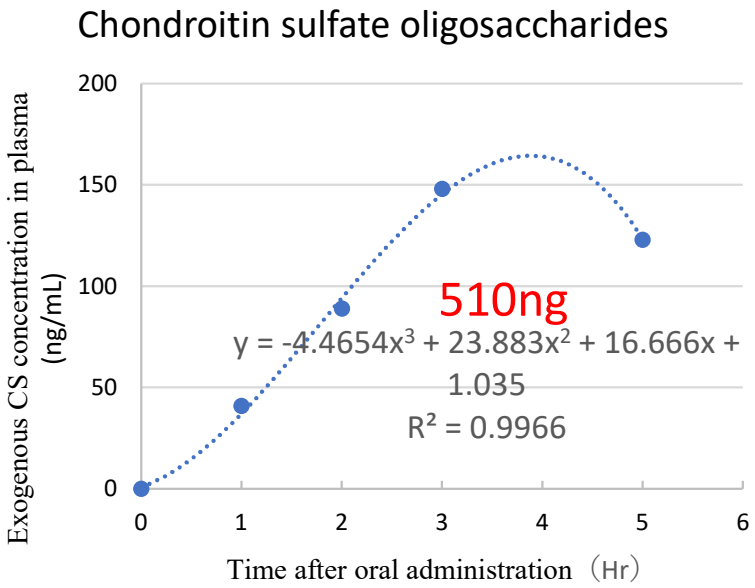
In the case of oral administration of chondroitin sulfate oligosaccharides, a temporal increase in blood chondroitin sulfate concentration with a peak at 3 hours after administration was confirmed, but in th e case of oral administration of high molecular weight chondroitin sulfate, it was hardly detected at an y time. The maximum difference in concentration detected was more than 100-fold, confirming that c hondroitin sulfate oligosaccharides are taken up into the blood by oral intake.

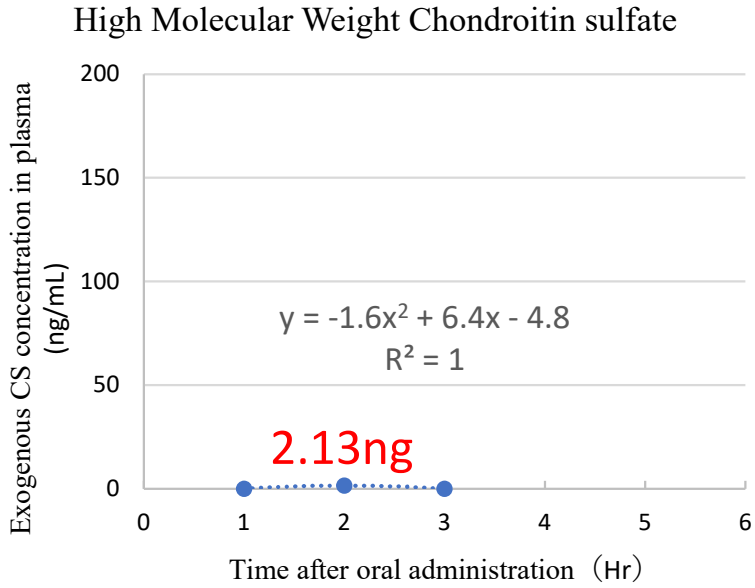
Comparison of Absorbability between Chondroitin Sulfate Oligosaccharides and High Molecular Weight Chondroitin Sulfate

（1） From the results of the inverted bowel test (p4)

Comparing the concentration of chondroitin sulfate in the internal fluid after 60 minutes, the concentration was 989 μg/mL in the chondroitin sulfate oligosaccharide test section and 4 μg/mL in the high molecular chondroitin sulfate test section. Therefore, the magnification was 247 times greater.

（2） From human intervention study results (p10)





The amount of the test substance in the blood can be obtained by integrating the area under the approximate curve of the blood concentration in each group. Comparing the period from 0 to 5 hours for which data are available, the ratio is 510:2.13, which is 239 times higher. Since the nano-type chondroitin group is thought to actually be detected after the 5th hour, the absorbed dose ratio is even higher.