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Oligosaccharide Mapping of Chondroitin Sulfate Obtained from Different Animal Sources

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SUMMARY. Oligosaccharide mapping (chondroitinase ABC enzimatic cleavage and further ion exchange chromatographic analysis of the resulting oligosaccharides) on twelve chondroitin sulfate samples isolated in our laboratory from different animal sources was performed. The same analytical methodology was applied on five comercial chondroitin sulfate samples taken as reference materials. The results were compared with bibliographic data. The usefulness of the described methodology in identifying the animal and tissular source was analyzed.

RESUMEN. "Caracterización mediante mapeo oligosacarídico de condroitín sulfato obtenido de diferentes orígenes animales". Se realizó el mapeo oligosacarídico (degradación enzimática con condroitinasa ABC y posterior análisis cromatográfico por intercambio iónico de los oligosacáridos resultantes) de doce muestras de condroitín sulfato de diversos orígenes animales obtenidas en nuestro laboratorio y de cinco muestras comerciales tomadas como sustancias de referencia. Se compararon los resultados obtenidos con la bibliografía y se analizó la utilidad de la técnica empleada con el fin de identificar el origen animal y tisular.

INTRODUCTION

Chondroitin sulfate is a glycosaminoglycan sulfate widely spread in the Animal Kingdom. It is a part of connective and structural tissues and, so, it is mainly found in soft cartilages and articular connections. Its molecule is mainly composed by the repetition of a disaccharide unit formed by N-acetyl-galactosamine and an uronic acid (glucuronic, and in a lower extension, iduronic). The union of these disaccharides conform linear polymers of 20 - 50 kDa mean molecular weight [1].

In the therapeutical field, its principal application is in the treatment of arthropaties and articular degenerative complications [1], osteoporosis treatment, hy-

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perlypidemias and the preservation of corneas for transplantation. It is indicated for human as well as for veterinary use [1, 3-5].

The sulfation of the constituent disaccharides considerably changes in quantity and position according to the species. In this sense, two different kinds of chondroitin sulfate can be distinguished; one wich is sulfated over the hydroxile group in 4-position on the galactosamine residue (chondroitin 4-sulfate or chondroitin sulfate A, CSA), and the other, sulfated in the hydroxile group in 6-position on the same moiety (chondrotin 6-sulfate or chondrotin sulfate C, CSC). Concerning the tissular source, CS from bovine tissues are mostly A-type, while chondrotin sulfate obtained from big sharks is C-type.

Although the results of the elemental analysis are quite similar for chondroitin sulfate samples obtained from different animal sources, the study of the sulfation pattern has proved to be an essential tool to find a correlation with the original species and the chromatographic profile [6-10]. The most commonly used methods are based on the polysaccharide enzymatic degradation with chondroitinase ABC, AC or AC II in order to obtain the constituents disaccharide units that are afterwards submitted to a chromatographic analysis.

At present, there are many chromatographic methodologies described: ion exchange on stirene-divinylbencene based columns [8], on amino-substituted [10] or amino-cyano-substituted [6] reverse phase columns and by ion paired chromatography [11]. In our laboratory we have adopted a chromatographic methodology already described for heparin with similar purposes [12].

The present work shows a characterization by enzymatic degradation of chondrotiin sulfate from bovine, porcine, ovine and chicken origin that, as far as we know, it has not been described until now.

MATERIALS AND METHODS

The standard disaccharide units, chondro-disaccharide kit (# 400571) and dermato/hyaluro-disaccharide kit (#400572) have been adquired from Seikagaku Kogyo Co, Tokyo, Japan. Chondroitinase ABC (*Proteus vulgaris*, # C 2905) has been adquired from Sigma Chemical Co., St. Louis, USA. Chondroitin sulfate D (shark fin, # 400676), chondroitin sulfate C (SG, shark cartilage, # 400675) and chondroitin sulfate C (SSG, shark cartilage # 400670) have been choosen as reference materials and have been purchased from Seikagaku Kogyo Co, while chondroitin sulfate A from porcine rib cartilage (# C 7571) and chondrotin sulfate C from shark cartilage (#C4384) have been purchased from Sigma Chemical Co., St. Louis, U.S.A.

The chromatographic determinations were performed on a Hewlett-Packard liquid chromatograph, mod. 1050 provided with a quaternary pump and a multiple wavelength detector. A strong anion exchange column Spherisorb 5 SAX, 250 x 4.6 (Phenomenex, CA, USA and Sigma Chemical Co.) was used.

The characterized glycosaminoglycans (see Table 1) have been obtained in our laboratory according to Roden et al. (RBCM method) [13]. The detail of the isolated molecules is the following: CS 367 C, isolated from Argentinean Sea dogfish; CS 370, isolated from bovine trachea; CS 371, isolated from Atlantic Ocean shark's total cartilage; CS 392, isolated from chicken trachea; CS 393, isolated from chicken breastbone; CS 394, isolated from the first articulation above claw in the chick-

en leg; CS 395, obtained from bovine nasal septa; 396 and CS 401, isolated from porcine trachea; CS 397, isolated from ovine trachea and CS 402 and CS 403, obtained from bovine muzzle and outer ear, respectively.

Physico-chemical characteristics, sulphur and uronic acids quantification

The determination of the sulphur content has been performed according to a classical method [14] while uronic acids have been quantified trough the colorimetric method of carbazol/sulphuric acid [15]. The molecular weight distribution has been obtained by high performance size exclusion chromatography (HPSEC). Calibration of the chromatographic system was made using a standard-house material obtained in our laboratory [1]. The molecular weight informed (M_P) corresponds to the top of the curve that describes the molecular weight distribution.

Enzymatic degradation

In order to obtain the products from the enzymatic degradation, a methodology described by Yoshida *et al.* [7] was followed. A 4 mg/ml solution of each sample and a 2 U/ml solution of chondroitinase ABC in 50 mM Tris buffer pH 7.9 were prepared. 50 µl sample and 25 µl enzyme solutions were incubated during 2 hours at 37 °C. The reaction was stopped by inmersing the tubes in a boiling water bath.

Analysis procedure

The chromatographic system was equilibrated at 40 °C and 1.2 ml.min^{-1} flow rate prior of the injection of the sample (10-20 µl). The elution program was developed according to the following scheme: 0.1 M NaCl (0-5 min), followed by a 0.1-1.2 M NaCl linear gradient (5-60 min).

Abreviations

In the present work the following terminology has been adopted (4): *ΔDi-HA,* 2-acetamyde-2-deoxy-3-O-(4-deoxy-(-L-threo-hex-4-enopyranosyluronic acid)-D-glucose; *ΔDiOS,* 2-acetamyde-2-deoxy-3-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-D-galactose; *ΔDi-6S,* 2-acetamyde-2-deoxy-3-O-(4-deoxy-(-L-threo-hex-4-enopyranosyluronic acid)-6-sulfo-D-galactose; *ΔDi-4S,* 2-acetamide-2-deoxy-3-O-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-4-O-sulfo-D-galactose; *ΔDi-diS_D,* 2-acetamyde-2-deoxy-3-O-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-6-sulfo-D-galactose; *ΔDi-diS_B,* 2-acetamyde-2-deoxy-3-O-(4-deoxy-2-O-sulfo-(-L-threo-hex-4-enopyranosyluronic acid)-4-O-sulfo-D-galactose; *ΔDi-diS_E,* 2-acetamyde-2-deoxy-3-O-(4-deoxy-(-L-threo-hex-4-enopyranosyluronic acid)-4,6-bis-O-sulfo-D-galactose; *ΔDi-triS,* 2-acetamyde-2-deoxy-3-O-(4-deoxy-2-O-sulfo-α-L-threo-hex-4-enopyranosyluronic acid)-4,6 bis-O-sulfo-D-galactose.

RESULTS

Prior to characterization by enzymatic degradation, a basic physico-chemical profile of the samples in analysis was performed. The results are summarized in Table 1:

	%S	%U	S/U(**)	Mp
CS 367 C dogfish fin	6.9	29.4	1.29	-
CS 371 shark cartilage	5.1	26.5	1.07	45000
CS shark cartilage (SG #400675)	6.6	34.8	1.04	45000
CS 370 bovine trachea	5.0	28.0	0.98	20000
CS 392 chicken trachea	5.3	29.5	0.99	18500
CS 393 chicken breastbone	5.7	33.7	0.93	19500
CS 394 chicken leg	5.1	27.6	1.03	23/24000
CS 395 bovine nasal septa	5.6	31.5	0.98	20000
CS 396 porcine trachea (*)	5.1	30.1	0.94	18400
CS 397 ovine trachea	4.6	25.5	1.00	16600
CS 401 porcine trachea	5.5	29.1	1.04	18000
CS 402 bovine outer ear (*)	4.4	27.4	0.88	15800
CS 403 bovine muzzle	5.4	28.4	1.06	17600

(*) impurified with hyaluronic acid
(**) sulfate - uronic acid molar ratio

Table 1. Sulphur (S) and Uronic acid (U) content of chondroitin sulfate samples. S/U: sulfate-uronic acid molar ratio

Since the goal was to obtain a chromatographic fingerprint for the different samples of chondroitin sulfate analyzed, a quantification of the content of each disaccharide in the polysaccharide structure was not performed; instead, a porcentual ratio for each peak in the chromatogram was obtained. In the experimental conditions choosen for the present work, peaks corresponding to Di-OS and Di-HA were not completely resolved and therefore they were consigned as an unique value. In order to verify the validity of the obtained results they were compared with previously published analysis of chondroitin sulfate samples characterized by a similar method (Table 2).

Sample	ΔDi-0S/HA		ΔDi-6S		ΔDi-4S		ΔDi-diS$_D$		ΔDi-diS$_E$		ΔDi-diS$_B$		ΔDi-triS	
	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
CS D (shark fin)	1.6	0.6	43.5	43.9	32.0	26.9	20.6	21.3	1.1	7.0	0.9		0.3	0.3
CS C (SG, shark cartilage)	1.5	1.7	73.4	72.9	17.0	15.4	8.1	9.3		0.6				
CS C (SSG, shark cartilage)	1.1		76.0		15.8		6.8		0.3					
CS (whale cartilage)	3.0	1.6	18.3	19.3	75.4	76.2	1.1	2.7	0.4	0.3	1.8			

Table 2. Comparison of disaccharide composition of chondroitin sulfate. (1) results obtained in the present work, (2) results consigned in the bibliography [7]. Values are expressed as a % of the total disaccharide content. See abbreviations in the text.

Sample	ΔDi0S/HA	ΔDi 6S	ΔDi 4S	ΔDi diS_D	ΔDi diS_E	ΔDi diS_B	ΔDi triS
CS 371 (shark cartilage)	2.5	62.3	20.2	12.6	1.4	0.7	0.3
CS 367 C (dogfish fin)	1.1	33.6	42.7	20.5	1.2	0.9	
CS 370 (bovine trachea)	3.5	37.3	58.3	0.3	0.4	0.2	
CS 402 (bovine outer ear)	3.3	17.8	77.7	0.4	0.2	0.6	
CS 403 (bovine muzzle)	3.5	17.2	77.5	1.2	0.1	0.5	
CS 395 (bovine nasal septa)	2.2	13.3	83.7	0.4	0.4	-	
CS 392 (chicken trachea)	3.1	10.0	82.3	trz	4.6	trz	
CS 393 (chicken breastbone)	3.2	15.7	79.5	0.5	0.4	0.7	
CS 394 (chicken leg)	5.1	12.9	81.1	0.2	0.3	0.4	
CS 396 (porcine trachea)	2.7	17.5	79.2	0.2	0.2	0.2	
CS 401 (porcine trachea)	3.2	17.4	78.6	0.3	0.3	0.2	
CS C Sigma (porcine rib)	3.1	28.7	66.6	0.8	0.4	0.4	
CS 397 (ovine trachea)	1.6	24.7	70.1	0.6	2.7	0.3	

Table 3. Analysis by enzymatic degradation of chondroitin sulfate from diverse animal tissues. Values are expressed as a % of the total disaccharide content. See abbreviations in the text.

The different chondroitin sulfate samples mentioned above were analyzed applying the same working methodology; the results are mentioned in Table 3.

As it can be observed, the differences among the products analyzed, although subtle in some cases, are notorious. The standard errors for the integration of the different peaks are correlated with the ratio that each one represents in the total amount. However, the coefficient of variation for the most important peaks does not exceed 10% (see Tables 4 and 5).

Sample	ΔDi-0S/HA	ΔDi-6S	ΔDi-4S
CS 370 (bovine trachea)	3.2	36.7	58.9
	3.5	37.3	58.3
	3.3	36.5	57.8
	3.6	37.7	57.4
Mean	3.4	37.05	58.1
Standard deviation (σ_{n-1})	0.18	0.55	0.65
Coefficient of variation	5.4%	1.5%	1.1%

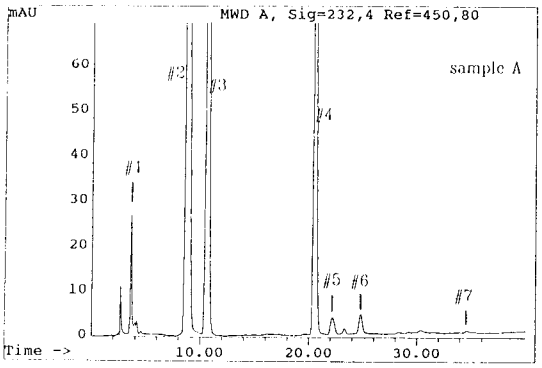
Table 4. Precision test on the results of integration with the main peaks of four (4) determinations taken at random, over a Chondroitin Sulfate sample isolated from bovine trachea. Values are expressed as a % of the total disaccharide content. See abbreviations in the text.

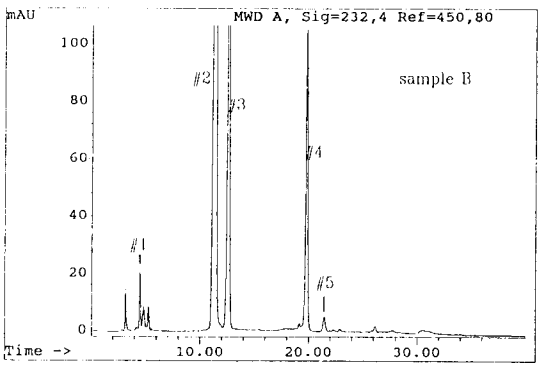
Sample	ΔDi-diSD	ΔDi-6SE	ΔDi-4SB	ΔDi-triS
CS C Sigma (shark cartilage)	15.7	2.7	0.4	0
	15.2	2.5	0.6	0.6
	15.3	2.3	0.6	0.6
Mean	15.4	2.5	0.53	0.4
Standard deviation ((n-1)	0.26	0.2	0.11	0.35
Coefficient of variation	1.7%	8%	21.6%	87%

Table 5. Precision test on the results of integration with the smaller peaks of three (3) determination taken at random, over a Chondroitin Sulfate sample isolated from shark cartilage.

CONCLUSIONS

A chromatographic analysis of the enzymatic degradation products (oligosaccharidic mapping with chondroitinase ABC) of chondroitin sulfate samples obtained in our laboratory from different tissular and animal origins has been performed. The results of the determinations were compared with similar analysis performed on commercial samples taken as reference.





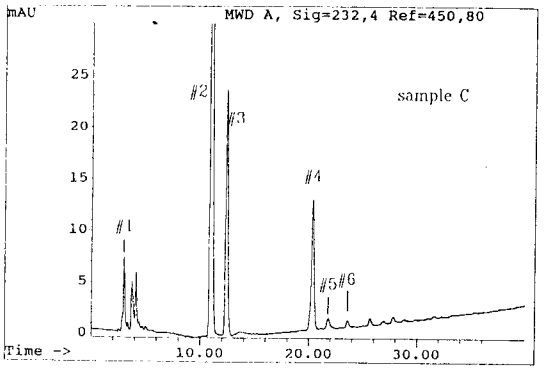


Figure 1. chromatogram comparison for three different shark CS samples; A, CS D; B, CS C (SSG); C, CS 371.

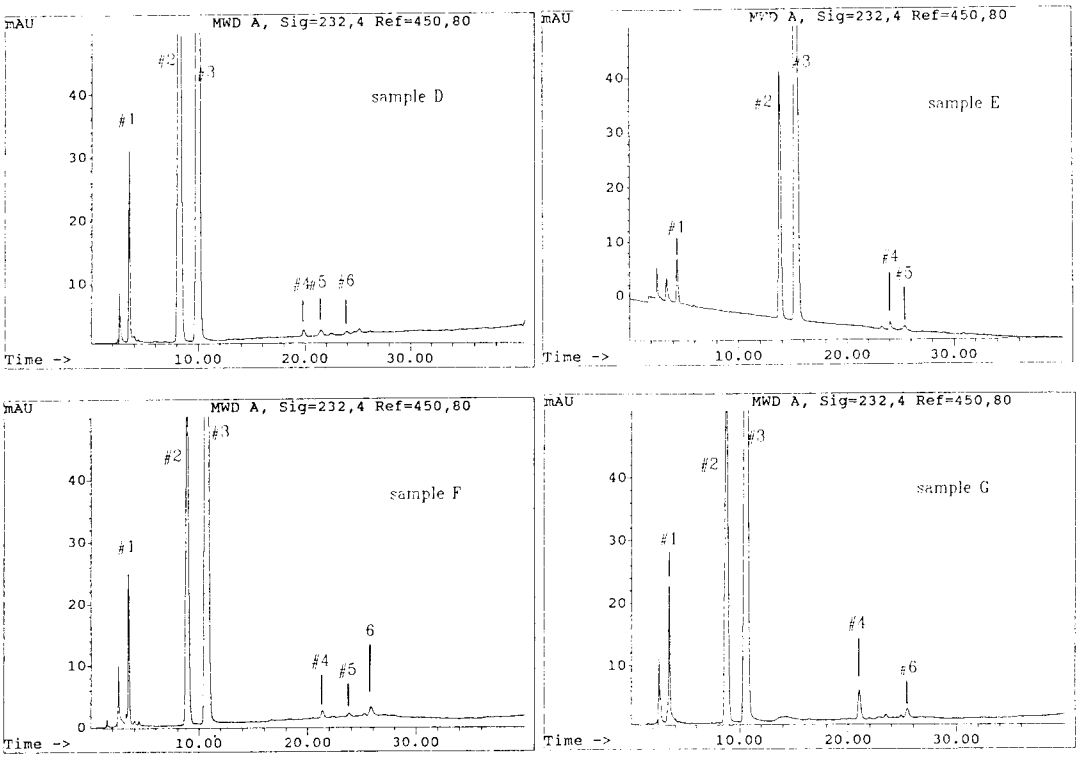


Figure 2. Comparison among chondroitin sulfate sample isolated from different bovine tissues extracts; D, CS 370; E, CS 395; F, CS 402; G, CS 403.

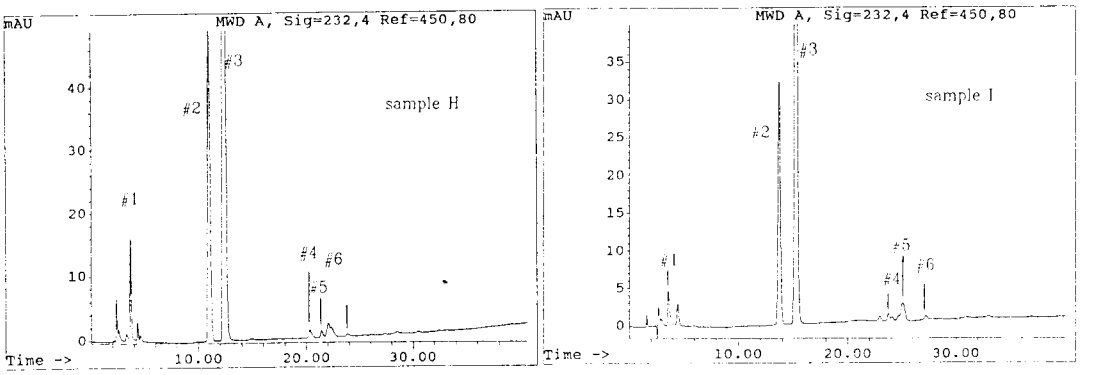


Figure 3. Chromatographic profile for two chicken chondroitin sulfate samples; H, CS 393; I, CS 397

The tabulated results indicate the existence of a particular chromatographic pattern for each sample in study, with some similitudes and important differences. Samples obtained from bovine or ovine trachea, from porcine breastbone and different samples obtained from shark cartilage, can be easily distinguished by the use of the mentioned methodology. However, the chondroitin sulfates obtained from chicken trachea, bovine outer ear and muzzle, and porcine trachea, show chromatographic profiles with a degree of similarity that difficul the identification.

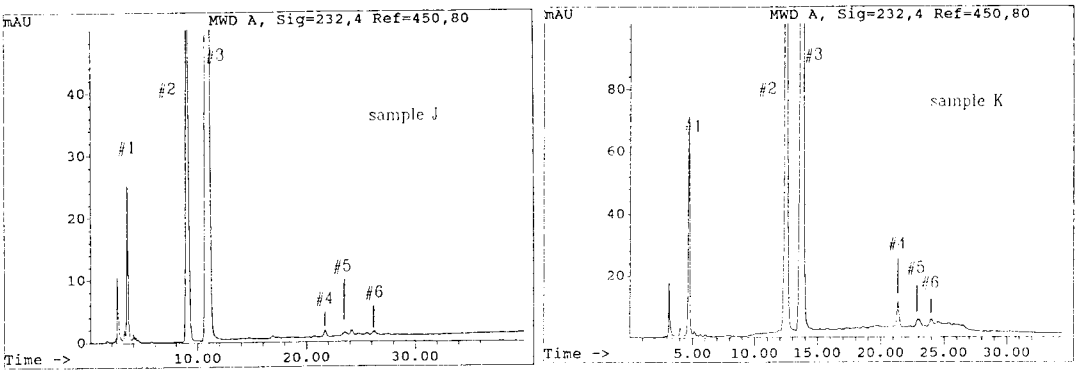


Figure 4. chromatographic profile for two porcine chondroitin sulfate samples; J, CS 401; K, Sigma pig rib CS.

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