



ELSEVIER

Journal of Chromatography B, 685 (1996) 27–34

JOURNAL OF  
CHROMATOGRAPHY B:  
BIOMEDICAL APPLICATIONS

# Purification of heparin, dermatan sulfate and chondroitin sulfate from mixtures by sequential precipitation with various organic solvents

Nicola Volpi

*Department of "Biologia Animale", University of Modena, Via Berengario 14, 41100 Modena, Italy*

Received 28 December 1995; revised 29 February 1996; accepted 21 March 1996

## Abstract

Heparin, dermatan sulfate and chondroitin sulfate in mixtures were fractionated by sequential precipitation with methanol, ethanol and propanol. The recovered fractions from 0.1 to 2.0 volumes of various solvents were analyzed by agarose-gel electrophoresis and densitometric analysis. Heparins with different relative percentages of slow-moving and fast-moving components were precipitated from 0.5 to 0.7 volumes of methanol, and in this range of volumes, the amount of slow-moving component of heparin decreases and that of the fast-moving species increases. From 0.8 to 1.6 volumes of methanol, mixtures with different percentages of the fast-moving component, dermatan sulfate and chondroitin sulfate are precipitated. Heparin was precipitated from mixtures in the range of 0.1 to 0.4 volumes of ethanol, and from 0.5 to 0.8 volumes mixtures with different relative percentages of dermatan sulfate and chondroitin sulfate were precipitated. From 1.0 to 2.0 volumes of ethanol, high purity (about 100%) chondroitin sulfate can be precipitated. Propanol induces the precipitation of heparin from 0.3 to 0.4 volumes, whilst dermatan sulfate with a purity greater than 85% is precipitated at 0.5 and 0.6 volumes of propanol. 100% chondroitin sulfate is obtained with volumes greater than 0.8. Heparin and chondroitin sulfate from a bovine lung extract of glycosaminoglycans were purified by sequential precipitation with ethanol. The fraction precipitated with 0.4 volumes of ethanol shows greater than 90% heparin and that recovered from 0.9 to 2.0 volumes is composed of 100% chondroitin sulfate.

*Keywords:* Heparin; Dermatan sulfate; Chondroitin sulfate

## 1. Introduction

Heparin (and heparan sulfate), chondroitin sulfate and dermatan sulfate are complex, sulfated, linear macromolecules that can be extracted and purified from different animal tissues [1]. The backbone of these sulfated glycosaminoglycans presents different uronic acids ( $\alpha$ -L-iduronic and  $\beta$ -D-glucuronic acids), hexosamines (N-acetyl- $\alpha$ -D-glucosamine, N-sulfo- $\alpha$ -D-glucosamine and N-acetyl- $\alpha$ -D-galac-

tosamine) and O-sulfate groups in varying amounts and O-linked in different positions [2]. These polysaccharides are very heterogeneous in terms of structure, relative molecular mass ( $M_r$ ), charge density and physico-chemical properties [3].

Glycosaminoglycans are extracted from animal tissues by treatment with organic solvents and/or by specific and nonspecific proteases, alkali extraction, and further purification steps by sequential precipitation with organic solvents, quaternary ammonium

ions, inorganic ions or adsorption on ion-exchange resins and gel filtration [1,3]. Although not frequently used today, sequential precipitation of glycosaminoglycans with ethanol and acetone has been very useful over the years. In particular, precipitation with increasing concentrations of ethanol in the presence of divalent metal ions, such as calcium, barium or zinc, has been widely used to separate glycosaminoglycans, most notably dermatan sulfate and chondroitin sulfate.

In a previous paper [4], purified sulfated glycosaminoglycans (heparin, dermatan sulfate and chondroitin sulfate with properties of polysaccharides used as drugs) in mixtures were fractionated by sequential precipitation with increasing volumes of acetone. This paper reports the fractionation of mixtures of glycosaminoglycans (with the same characteristics and structure as reported in the previous paper [4]) by sequential precipitation in the presence of methanol, ethanol and propanol. The aim of this research is to extend the previous study and to evaluate the behaviour of mixtures of glycosaminoglycans in the presence of different organic solvents and the possibility of obtaining pure species. The fractions obtained by precipitation with solvents were quantified by a colorimetric method and analyzed by agarose-gel electrophoresis and densitometric scanning analysis. The sequential precipitation with ethanol was applied to a bovine lung glycosaminoglycan extract to purify single species.

## 2. Experimental

### 2.1. Extraction and purification of sulfated glycosaminoglycans

Heparin from beef intestinal mucosa, dermatan sulfate from beef intestinal mucosa and chondroitin sulfate from bovine trachea were extracted and purified as previously reported [4,5]. Briefly, tissues were treated with papain and alkali, and glycosaminoglycans were purified by anion-exchange chromatography, sequential precipitation by acetone, precipitation as copper salt, and specific heparin degradation by low pH nitrous acid. Purified glycosaminoglycans were transformed into sodium salts by

a cation-exchange resin (Amberlite IR-120 in Na<sup>+</sup> form, Supelco, Bellefonte, PA, USA).

As reported in a previous paper [4], heparin has a  $M_r$  of 11 600, a sulfate-to-carboxyl ratio of 2.37 and an anticoagulant activity of 160 IU/mg. Densitometric analysis performed after agarose-gel electrophoresis demonstrated that 65% was present as the fast-moving component and 35% as the slow-moving species. Dermatan sulfate has a  $M_r$  of about 25 580, with a sulfate-to-carboxyl ratio of about 1.10. Chondroitin sulfate has a  $M_r$  of about 26 140 and a sulfate-to-carboxyl ratio of about 0.98.

### 2.2. Fractionation of glycosaminoglycans from mixtures

A 150-mg quantity of each purified glycosaminoglycan sodium salt was dissolved in 4 ml of double distilled water. NaCl (2%, 80 mg) was added to the mixtures, and the glycosaminoglycans were fractionated by increasing volumes of methanol, ethanol or propanol (from 0.1 to 2.0 volumes). For each 0.1 volume of solvent, the mixtures were allowed to equilibrate at 4°C for 24 h. Afterwards, the precipitate was collected by centrifugation at 5000 g for 10 min and dried at 60°C. Another 0.1 volume of solvent was added to the supernatants, and the procedure was repeated up to a maximum of 2.0 volumes of solvent.

### 2.3. Extraction and purification of bovine lung glycosaminoglycans

A 74-g amount of bovine lung was ground in the presence of acetone and, after centrifugation at 5000 g, the pellet was treated with chloroform–methanol (2:1, v/v). After centrifugation at 5000 g, the pellet was dried at 60°C, solubilized in distilled water and treated with papain [(E.C. 3.4.22.2), Sigma] (at 65°C for 24 h) and collagenase [(E.C. 3.4.24.3), Sigma] (at 37°C for 24 h) in a reaction vessel. After heating at 100°C for 30 min, the mixture was brought to pH 9.0 by adding 2 M NaOH. After 24 h at 40°C, the product (brought to pH 6.0 with 2 M acetic acid) was centrifuged at 5000 g for 15 min, and the pellet was washed twice with distilled water. Two volumes of acetone were added to the pooled supernatants and

stored at 4°C for 24 h. The precipitate was recovered by centrifugation at 5000 g for 15 min and dried at 60°C for 6 h. The dried precipitate was dissolved in 30 ml of distilled water by prolonged mixing. After centrifugation at 5000 g for 15 min, the supernatant was applied to a column (8.0×2.5 cm) packed with about 40 ml of Ecteola-cellulose (Serva, Heidelberg, Germany) previously washed with 1 M NaOH and 1 M HCl and equilibrated with 0.05 M NaCl. After washing the resin with two volumes of 0.05 M NaCl, 50 ml of 3 M NaCl were added. Two volumes of acetone were added to the eluate and stored at 4°C for 24 h. After centrifugation at 5000 g for 15 min, the pellet was dried at 60°C for 6 h. The dried precipitate was dissolved in 10 ml of distilled water by prolonged mixing and 1 ml of trichloroacetic acid (50%) was added. The preparation was stored at 4°C for 12 h. After centrifugation at 5000 g, the supernatant was neutralized with 10 M NaOH and four volumes of ethanol saturated with sodium acetate were added, and the preparation was stored at 4°C for 24 h. After centrifugation at 5000 g for 15 min, the pellet was dried at 60°C and 420 mg (0.57%) of material were recovered.

#### *2.4. Fractionation of bovine lung glycosaminoglycans by sequential precipitation with ethanol*

An 80- $\mu$ l volume (0.4 volumes) of ethanol was added to 200  $\mu$ l (20 mg/ml of 0.05 M NaCl) of bovine lung glycosaminoglycans and the mixture was stored at 4°C for 24 h. After centrifugation at 5000 g, the precipitate was collected and dried at 60°C whilst 80  $\mu$ l of ethanol (another 0.4 volumes) were added to the supernatant. After standing at 4°C for 24 h, the pellet was recovered by centrifugation at 5000 g and dried at 60°C; 240  $\mu$ l (another 1.2 volumes, giving a total of 2.0 volumes) of ethanol were added to the supernatant, and the mixture was stored at 4°C for 24 h. After centrifugation at 5000 g for 15 min, the pellet was recovered and dried at 60°C.

The three pellets recovered (at 0.4, 0.8 and 2.0 volumes of ethanol) were solubilized in 50  $\mu$ l of distilled water and analyzed by agarose-gel electrophoresis.

#### *2.5. Evaluation of the glycosaminoglycan composition of each fraction*

The glycosaminoglycans for each fraction obtained by increasing amounts of organic solvents were quantified by dimethylmethylene blue (DMB) assay according to Whitley et al. [6] against a calibration curve constructed with increasing amounts of chondroitin sulfate A (Sigma) (from 0.5 to 10  $\mu$ g).

The fractions recovered by increasing the solvent concentration were also analyzed by agarose-gel electrophoresis in barium acetate–1,2-diaminopropane, as reported elsewhere [4]. A Pharmacia Multiphor II (from Pharmacia LKB, Uppsala, Sweden) electrophoretic cell instrument was used. A 2–5  $\mu$ l volume of each fraction (from 2 to 10  $\mu$ g of glycosaminoglycans, as measured by the DMB assay) were loaded on the gel using a micropipet. The first run was performed in 0.04 M barium acetate buffer, pH 5.8, for 60 min at 60 mA, and the second run was in 0.05 M 1,2-diaminopropane (Catalogue number 821041 from Merck, Darmstadt, Germany) (buffered at pH 9.0 with acetic acid) for 120 min at 50 mA. After migration, the plate was soaked in cetyltrimethylammonium bromide (Catalogue number 8130 from Merck) (0.1% solution) for about 3 h, dried and then stained with toluidine blue (0.2% in ethanol–water–acetic acid, 50:49:1) for 30 min. After decoloration with ethanol–water–acetic acid (50:49:1, v/v), quantitative analysis of glycosaminoglycans was performed with a densitometer (Macintosh IIsi computer interfaced with Microtek Color Scanner from Microtek International, Hsinchu, Taiwan. Image processing and analysis program, Ver. 1.41 from Jet Propulsion Lab., NASA, FL, USA) using specific calibration curves, as reported elsewhere [4].

### **3. Results**

In the previous paper [4], mixtures with different absolute amounts of glycosaminoglycans and various relative percentages of each species were prepared and analyzed after sequential precipitation with increasing amounts of acetone. As no differences were observed for the percentage recoveries and for

the glycosaminoglycan components of each fraction for different mixtures, we analyzed the capacity of solvents to precipitate glycosaminoglycans in mixtures composed of the same relative percentages of the three species (150 mg/4 ml).

Fig. 1 illustrates the recovery in mg of glycosaminoglycans for each fraction precipitated by increasing amounts of methanol, ethanol and propanol, as evaluated by the DMB assay. As no differences were reported for the quantitative analysis of different glycosaminoglycan species by the DMB assay, we calculated the amount of polysaccharides for each fraction versus a calibration curve constructed with increasing amounts of chondroitin sulfate A. The percentage recovery of glycosaminoglycans for the mixture precipitated by methanol was 85.4%, for ethanol about 90.5% and for propanol was 78.9%.

Fig. 2 illustrates the agarose-gel separation of fast-moving and slow-moving heparin species, dermatan sulfate and chondroitin sulfate for each fraction obtained by sequential precipitation with methanol. Glycosaminoglycans, in particular chondroitin sulfate, are also present in the fraction precipitated with 2.0 volumes of methanol. In contrast, the last fraction of mixtures in which polysaccharides (chondroitin sulfate) are detected is obtained by precipitation with 1.4 volumes of ethanol (Fig. 3) and 0.9 volumes of propanol (Fig. 4), as also confirmed by the DMB assay (Fig. 1).

The percentages of single glycosaminoglycan

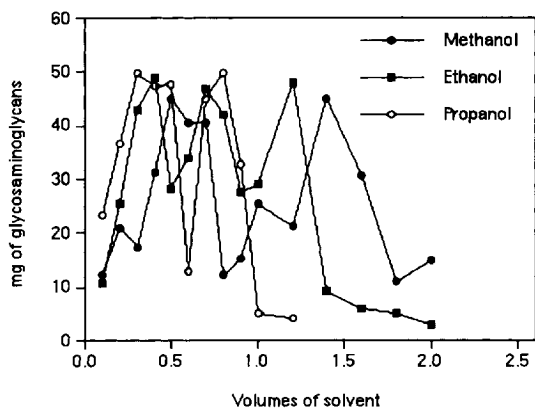


Fig. 1. Evaluation of the amount of glycosaminoglycan in each fraction obtained by sequential precipitation with methanol, ethanol and propanol, as determined by DMB assay.

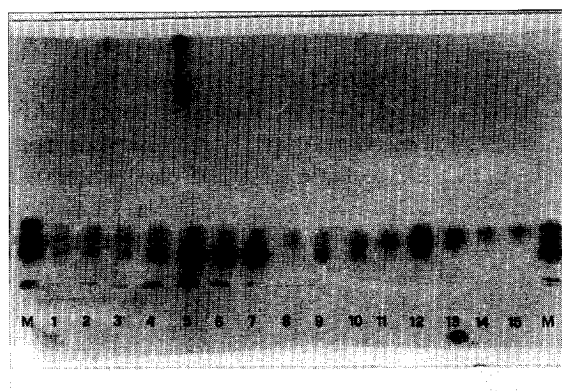


Fig. 2. Agarose-gel electrophoretic separation of fractions obtained by sequential precipitation with methanol. From left to right: M, mixture (from bottom to top: slow-moving heparin, fast-moving heparin, dermatan sulfate, chondroitin sulfate), 1, 0.1 vol; 2, 0.2 vol; 3, 0.3 vol; 4, 0.4 vol; 5, 0.5 vol; 6, 0.6 vol; 7, 0.7 vol; 8, 0.8 vol; 9, 0.9 vol; 10, 1.0 vol; 11, 1.2 vol; 12, 1.4 vol; 13, 1.6 vol; 14, 1.8 vol; 15, 2.0 vol. M, mixture. The arrow indicates the direction of electrophoretic migration.

species (fast-moving and slow-moving heparin species, dermatan sulfate and chondroitin sulfate) for each fraction measured by densitometric analysis after agarose-gel separation are reported in Table 1 (for the precipitation with methanol), in Table 2 (for ethanol) and in Table 3 (for propanol). The re-

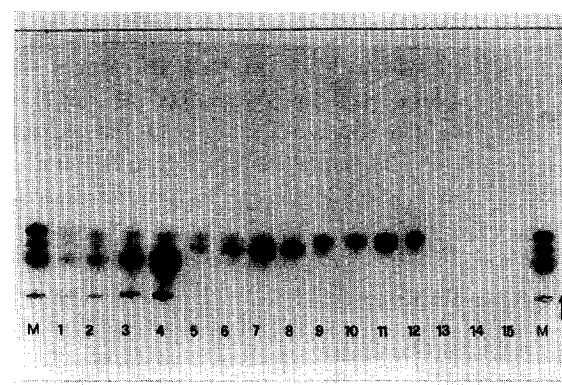


Fig. 3. Agarose-gel electrophoretic separation of fractions obtained by sequential precipitation with ethanol. From left to right: M, mixture (from bottom to top: slow-moving heparin, fast-moving heparin, dermatan sulfate, chondroitin sulfate), 1, 0.1 vol; 2, 0.2 vol; 3, 0.3 vol; 4, 0.4 vol; 5, 0.5 vol; 6, 0.6 vol; 7, 0.7 vol; 8, 0.8 vol; 9, 0.9 vol; 10, 1.0 vol; 11, 1.2 vol; 12, 1.4 vol; 13, 1.6 vol; 14, 1.8 vol; 15, 2.0 vol. M, mixture. The arrow indicates the direction of electrophoretic migration.

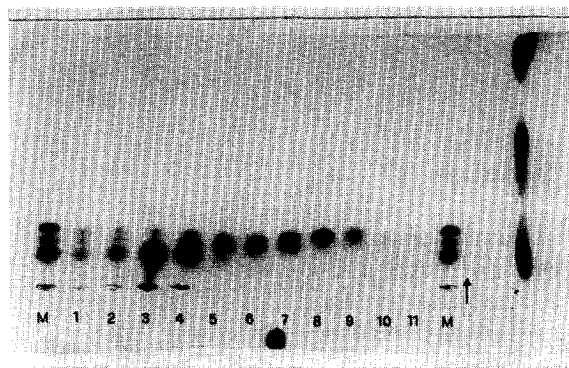


Fig. 4. Agarose-gel electrophoretic separation of fractions obtained by sequential precipitation with propanol. From left to right: M, mixture (from bottom to top: slow-moving heparin, fast-moving heparin, dermatan sulfate, chondroitin sulfate), 1, 0.1 vol; 2, 0.2 vol; 3, 0.3 vol; 4, 0.4 vol; 5, 0.5 vol; 6, 0.6 vol; 7, 0.7 vol; 8, 0.8 vol; 9, 0.9 vol; 10, 1.0 vol; 11, 1.2 vol. M, mixture. The arrow indicates the direction of electrophoretic migration.

Table 1

Recovery and percentage of mass corresponding to each glycosaminoglycan for each fraction precipitated with increasing volumes of methanol

Volume	Recovery		Percentage of mass corresponding to each glycosaminoglycan			
	mg	%	SM Hep	FM Hep	DS	CS
0.1	12.4	3.2	10.3	32.6	26.4	30.7
0.2	21.0	5.5	8.5	34.6	29.5	27.4
0.3	17.4	4.5	11.6	29.4	31.9	27.1
0.4	31.2	8.1	14.0	43.8	22.6	19.6
0.5	45.0	11.7	22.0	67.2	5.6	5.2
0.6	40.5	10.5	13.6	71.3	8.0	7.1
0.7	40.5	10.5	2.0	84.2	7.3	6.5
0.8	12.4	3.2	0.0	7.3	53.6	39.1
0.9	15.2	3.9	0.0	48.8	27.0	24.2
1.0	25.5	6.6	0.0	15.3	58.6	26.1
1.2	21.2	5.5	0.0	4.2	76.1	19.7
1.4	45.1	11.7	0.0	8.0	56.8	35.2
1.6	30.6	8.0	0.0	0.0	53.4	46.6
1.8	11.2	2.9	0.0	0.0	8.2	91.8
2.0	15.1	3.9	0.0	0.0	0.0	100

SM Hep: slow-moving heparin. FM Hep: fast-moving heparin. DS: dermatan sulfate. CS: chondroitin sulfate.

Table 2

Recovery and percentage of mass corresponding to each glycosaminoglycan for each fraction precipitated with increasing volumes of ethanol

Volume	Recovery		Percentage of mass corresponding to each glycosaminoglycan			
	mg	%	SM Hep	FM Hep	DS	CS
0.1	10.9	2.7	7.0	35.4	28.5	28.1
0.2	25.5	6.3	9.4	37.2	26.8	26.6
0.3	42.8	10.5	20.0	69.0	5.1	5.9
0.4	48.9	12.0	23.4	70.5	2.4	3.7
0.5	28.1	6.9	0.0	2.1	56.4	41.5
0.6	34.0	8.3	0.0	0.0	71.4	28.6
0.7	46.8	11.5	0.0	0.0	92.9	7.1
0.8	42.0	10.3	0.0	0.0	98.7	1.3
0.9	27.7	6.8	0.0	0.0	2.6	97.4
1.0	29.0	7.1	0.0	0.0	0.0	100.0
1.2	48.0	11.8	0.0	0.0	0.0	100.0
1.4	9.4	2.3	0.0	0.0	0.0	100.0
1.6	6.0	1.5	0.0	0.0	0.0	100.0
1.8	5.0	1.2	0.0	0.0	0.0	100.0
2.0	3.0	0.7	0.0	0.0	0.0	100.0

SM Hep: slow-moving heparin. FM Hep: fast-moving heparin. DS: dermatan sulfate. CS: chondroitin sulfate

Table 3

Recovery and percentage of mass corresponding to each glycosaminoglycan for each fraction precipitated with increasing volumes of propanol

Volume	Recovery		Percentage of mass corresponding to each glycosaminoglycan			
	mg	%	SM Hep	FM Hep	DS	CS
0.1	23.5	0.8	12.3	42.6	23.4	21.7
0.2	36.7	10.3	15.9	62.8	9.7	11.6
0.3	49.9	14.0	18.0	76.6	2.4	3.0
0.4	47.5	13.4	15.0	55.0	25.0	5.0
0.5	47.6	13.4	0.0	1.7	86.2	12.1
0.6	13.0	3.7	0.0	0.0	94.0	6.0
0.7	45.1	12.7	0.0	0.0	28.7	71.3
0.8	49.7	14.0	0.0	0.0	0.0	100.0
0.9	32.8	9.2	0.0	0.0	0.0	100.0
1.0	5.0	1.4	0.0	0.0	0.0	100.0
1.2	4.3	1.2	0.0	0.0	0.0	100.0
1.4	0.0	0.0	0.0	0.0	0.0	0.0

SM Hep: slow-moving heparin. FM Hep: fast-moving heparin. DS: dermatan sulfate. CS: chondroitin sulfate

coveries, in mg and percentage, calculated by the DMB assay for each fraction are also reported.

Bovine lung glycosaminoglycans consist of about 62% heparin (of which 38% is slow-moving and 62% is fast-moving species), 14% dermatan sulfate and 24% chondroitin sulfate, calculated by densitometric analysis after agarose-gel electrophoresis (Fig. 5). Fig. 5 also illustrates the glycosaminoglycan species in the fractions obtained by precipitation with 0.4, 0.8 and 2.0 volumes of ethanol. The fraction at 0.4 volumes is composed of about 92.0% heparin (44% slow-moving and 56% fast-moving components), 5.4% dermatan sulfate and 2.6% chondroitin sulfate. The fraction precipitated at 0.8 volumes shows about 39% fast-moving heparin species, 37%

dermatan sulfate and 24% chondroitin sulfate, whilst the fraction at 2.0 volumes consists of 100% chondroitin sulfate (Fig. 5).

#### 4. Discussion

Glycosaminoglycans are macromolecules extracted and purified from various animal tissues. They are used as drugs due to their important pharmacological activities. Heparin is an anticoagulant and antithrombotic agent [7], dermatan sulfate has anticoagulant and antithrombotic activity and does not cause bleeding, making it a promising antithrombotic drug, and chondroitin sulfate is used as a chondroprotective agent. Moreover, derivatives and mixtures of these polysaccharides are used in therapy. These macromolecules must be extracted and purified from animal tissues by different approaches.

In this paper, we report the separation of mixtures of glycosaminoglycans, generally used for pharmaceutical purposes, by sequential precipitation with different organic solvents. In a previous study, we described the separation of mixtures of the same polysaccharides by sequential precipitation with acetone. Heparin is the first glycosaminoglycan to precipitate at low percentages of acetone, and the relative amounts of slow-moving and fast-moving components of fractionated heparin depend on the percentage of solvent. Dermatan sulfate is precipitated by acetone over a narrow range (0.6–0.7 volumes), and chondroitin sulfate begins to precipitate at 0.8 volumes of acetone.

The same behaviour is obtained with methanol, ethanol and propanol. In particular, heparin is the first glycosaminoglycan species to precipitate, followed by dermatan sulfate and, lastly, by chondroitin sulfate. However, various solvents have different capacities to precipitate glycosaminoglycans. In particular, as the hydrophobic chains of the solvents increases (from methyl to propyl) there is a greater capacity to precipitate polysaccharides with lower amounts of solvent. These different properties produce glycosaminoglycan species with peculiar characteristics.

No fractionation of the polysaccharide mixture is



Fig. 5. Agarose-gel electrophoretic separation of fractions obtained by sequential precipitation of bovine lung glycosaminoglycan with ethanol. From left to right: M, mixture of glycosaminoglycans (from bottom to top: slow-moving heparin, fast-moving heparin, dermatan sulfate, chondroitin sulfate), 1, bovine lung extract (10  $\mu$ g); 2, 0.4 vol (3  $\mu$ l); 3, 0.8 vol (3  $\mu$ l); 4, 2.0 vol (6  $\mu$ l). The arrow indicates the direction of electrophoretic migration.

obtained with 0.1 to 0.4 volumes of methanol. Heparins with different relative percentages of slow-moving and fast-moving components are precipitated from 0.5 to 0.7 volumes. In fact, in this range, the amount of the slow-moving component of heparin decreases and the percentage of the fast-moving species increases (from 67% to 84%). The mixture precipitated with 0.7 volumes of methanol is composed of about 84% of the fast-moving component, and this could be useful for the production of this species of heparin. Nevertheless, a low percentage of dermatan sulfate and chondroitin sulfate can be detected in these fractions. From 0.8 to 1.6 volumes of methanol, mixtures with different percentages of the fast-moving component, dermatan sulfate and chondroitin sulfate are precipitated. No evident separation of dermatan sulfate from chondroitin sulfate was obtained. Fractions at 1.8 and 2.0 volumes consist of 90 and 100% chondroitin sulfate, respectively. In particular, sequential precipitation with methanol could be useful for purifying the fast-moving species of heparin with a purity of about 84% and in high amount (40 mg/450 mg of mixture) or to eliminate heparin and obtain mixtures that are rich in dermatan sulfate and chondroitin sulfate.

Heparin is precipitated from mixtures with 0.1 to 0.4 volumes of ethanol and, in particular, the fractions at 0.3 and 0.4 volumes are enriched with this glycosaminoglycan species (>90%). From 0.5 to 0.8 volumes, mixtures with different relative percentages of dermatan sulfate and chondroitin sulfate are precipitated. In fact, at increasing volumes of ethanol (from 0.5 to 0.8) the amount of dermatan sulfate increases and that of chondroitin sulfate decreases. Moreover, dermatan sulfate of about 95–100% purity can be obtained with 0.7–0.8 volumes of ethanol. With 1.0 to 2.0 volumes of ethanol, chondroitin sulfate with high purity (about 100%) can be precipitated. The sequential precipitation of polysaccharide species from mixtures using ethanol could be used to obtain high purity heparin (about 90%), dermatan sulfate (from 0.7 to 0.8 volumes) or chondroitin sulfate (100%) with volumes greater than 1.0. These capacities of ethanol were utilized to purify heparin and chondroitin sulfate from a bovine lung extract of glycosaminoglycans. The fraction fractionated from bovine lung extract by precipitation with 0.4 volumes of ethanol shows >90% heparin, and the

fraction recovered from 0.9 to 2.0 volumes is composed of 100% chondroitin sulfate. These results agree with the glycosaminoglycan species obtained by sequential precipitation with ethanol of mixtures composed of purified single polysaccharide species (see Fig. 3 and Table 2). Nevertheless, heparin in bovine lung is not completely precipitated by 0.4 volumes of ethanol. In fact, the fraction obtained from 0.5 to 0.8 volumes shows the presence in agarose-gel electrophoresis of about 40% of the fast-moving species. This discrepancy with the results obtained in the fractionation of mixtures of glycosaminoglycans by 0.4 volumes of ethanol could be due to the different structure (and molecular mass and charge density) of bovine lung and beef mucosa heparins [8] that could influence the precipitation of these polysaccharide species by solvents. The fraction recovered from 0.5 to 0.8 volumes is also composed of dermatan sulfate and chondroitin sulfate. However, precipitation of bovine lung extract in a narrower range of ethanol (from 0.7 to 0.8 volumes) produces a fraction composed of about 85% dermatan sulfate and 15% chondroitin sulfate (data not shown).

Propanol precipitates heparin over a range of 0.3 to 0.4 volumes, obtaining heparin with a purity of about 95% at 0.3 volumes and of 70% purity at 0.4 volumes. Dermatan sulfate with a purity greater than 85% is precipitated at 0.5 and 0.6 volumes of propanol. 100% chondroitin sulfate is obtained with volumes greater than 0.8.

Interestingly, the fractions with the greatest recovery (in milligrams) correspond to a greater purity of the single glycosaminoglycan species. In fact, precipitation with methanol produces fractions from 0.5 to 0.7 volumes with a recovery of 45.0, 40.5 and 40.5 mg, respectively, in which the relative percentage of heparin is about 85–90%. The fraction obtained at 0.4 volumes of ethanol (48.9 mg of recovery) is composed of about 90% heparin; the fractions at 0.7–0.8 volumes (46.8 and 42.0 mg) are made up of about 90% dermatan sulfate and the fraction at 1.2 volumes (48.0 mg) consists of 100% chondroitin sulfate. The fraction obtained at 0.3 volumes of propanol (49.9 mg recovered) is composed of about 95% heparin; the fraction at 0.5 volumes (47.6 mg) consists of about 86% dermatan sulfate and the fractions at 0.7–0.8 volumes (45.1

and 49.7 mg recovered, respectively) consist of 70 and 100% chondroitin sulfate, respectively.

Fractionation of glycosaminoglycan mixtures extracted from tissues and organs by sequential precipitation using a suitable solvent (methanol, ethanol, propanol or acetone) can be a useful tool, together with other separation and fractionation methods, to purify a single polyanionic species and also to obtain molecules with peculiar characteristics, such as the possibility of separating a heparin species that is rich in fast-moving or slow-moving components.

### Acknowledgments

This research was sponsored by CNR grants to N. Volpi.

### References

- [1] L. Roden, J.R. Baker, J.A. Cifonelli and M.B. Mathews, *Methods Enzymol.*, 28 (1972) 73.
- [2] F.A. Ofosu, I. Danishefsky and J. Hirsh (Editors), *Heparin and Related Polysaccharides. Structure and Activities*, Vol. 556, Ann. N.Y. Acad. Sci., New York, 1989.
- [3] D. Heinegard and Y. Sommarin, *Methods Enzymol.*, 144 (1987) 319.
- [4] N. Volpi, *Anal. Biochem.*, 218 (1994) 382.
- [5] N. Volpi, *Carbohydr. Res.*, 247 (1993) 263.
- [6] C.B. Whitley, M.D. Ridnour, K.A. Draper, C.M. Dutton and J.P. Neglia, *Clin. Chem.*, 35 (1989) 374.
- [7] D.A. Lane and U. Lindahl (Editors), *Heparin. Chemical and Biological Properties. Clinical Applications*, Edward Arnold, London, Melbourne, Auckland, 1989.
- [8] R.J. Linhardt, K.G. Rice, Y.S. Kim, D.L. Lohse, H.M. Wang and D. Loganathan, *Biochem. J.*, 254 (1988) 781.