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# Effect of structure modification of chondroitin sulfate C on its enantioselectivity to basic drugs in capillary electrophoresis

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**Abstract**

The effect of structure modification of chondroitin sulfate C on its enantioselectivity to several representative basic drugs in capillary electrophoresis was investigated. Chemical desulfation showed no remarkable decrease in selectivity, whereas depolymerization with chondroitinase ABC resulted in complete loss of selectivity. Comparison with chondroitin sulfate A indicated considerable decrease in selectivity with this isomer. The great retention of enantioselectivity in the desulfated derivative suggests that the selectivity comes from the difference of the magnitude of an interaction in the multipoint mechanism between a part of the drug molecule and a functional group in chondroitin sulfate C other than the sulfate group. The sulfate group is not considered to play a major role for chiral separation. The complete loss of selectivity by depolymerization is consistent with a general tendency of lower selectivity in smaller saccharides, and the priority of chondroitin sulfate C to chondroitin sulfate A suggests the importance of the hydroxyl group at $C_4$ in the galactosamine residue. During the course of this work we observed heavy tailing of the peaks of basic drugs in some batches of uncoated fused-silica capillaries under acidic conditions and solved this problem by doubly coating capillaries with Polybrene followed by chondroitin sulfate C. On the other hand, we demonstrated the usefulness of a special technique which uses a short, wider bore PTFE tube-attached capillary for the study of the effect of depolymerization, in order to minimize sample amount. 

*Keywords:* Enantiomer separation; Enantioselectivity; Pharmaceutical analysis; Polybrene/chondroitin sulfate C double coated capillaries; Chondroitin sulfates; Primaquine; Chloroquine; Propranolol; Doxylamine; Laudanosine; Sulfates

## 1. Introduction

It is well known that in chiral drugs, pharmaceutical activity resides mostly in one of the enantiomers, and unwanted side effects or even toxic effects are often observed for the other enantiomer [1]. From this reason the separation of enantiomers is an important subject in pharmaceutical analysis. Various analytical methods have already been developed for separation of enantiomers. Methods based on high-performance liquid chromatography (HPLC) using chiral stationary phases have been most widely used for the analysis of drug enantiomers in pharmaceutical preparations and biological fluids [2,3]. Various kinds of columns have been developed to cover a variety of chiral drugs.

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Recently, capillary electrophoresis (CE) has also received considerable attention because of its advantages of high resolution efficiency, convenient change in separation condition and extremely small volume requirements for sample and separation media [4]. A large number of examples of chiral separation by CE using a variety of chiral selectors have been reported [5–11]. Among them cyclodextrins and their derivatives are the most frequently employed [12]. Recently polysaccharides have also been found to be effective as chiral selectors in CE [4,13]. These substances have only low absorbance in the UV region, which is beneficial for high detection sensitivity. Charged polysaccharides have additionally good solubility in aqueous solutions making high concentration solutions possible. Furthermore, there are a wide range of polysaccharide species, each providing unique characteristics for chiral separation.

The first paper on chiral separation with polysaccharides by CE was published by D'Hulst and Verbeke [14] in 1992. Many ionic and neutral polysaccharides have been added thereafter to the glossary of chiral selectors for CE. Successful examples in this group include dextran, dextrin, heparin, and isomeric chondroitin sulfates [15–19]. Several new polysaccharides are still being investigated [20–22]. Nevertheless, the mechanism of chiral separation by these polysaccharides has not been fully elucidated. Chondroitin sulfates, which are negatively charged polysaccharides found in animal tissues, composed of the *N*-acetylgalactosamine and glucuronic acid residues alternately linked to each other by the β1-4 and β1-3 bonds, respectively, with one sulfate group distributed per disaccharide unit, were first used as chiral selectors for CE by Nishi and coworkers [13,17]. These isomeric polysaccharides showed wide enantioselectivity to basic drugs, and these authors speculated on the mechanism of chiral recognition based on comparison with other kinds of polysaccharides. They noted the function of the sulfate group and at the same time pointed out the significance of polymer network from the comparison with neutral polysaccharides. In order to provide further evidence, we attempted structure modification of chondroitin sulfate C, the most potent chiral selector among polysaccharides, by selective/specific methods and compared the extents of enantioselectivity to several representative drugs among the thus-prepared modifications. We present herein the resultant data and brief discussion on the mechanism of chiral separation in this system.

In the course of this study we met the following two problems: (1) Basic drugs gave heavy tailing in some batches of uncoated capillaries, and (2) depolymerization needed high cost, because a large amount of a precious enzyme (chondroitinase ABC) was required. In this paper we also describe how we could overcome these problems.

## 2. Experimental

### *2.1. Samples and chemicals*

Chloroquine diphosphate (antimalarial drug, CHL), propranolol hydrochloride (β-blocker, PRO), and doxylamine (antihistaminic drug, DOX) were obtained from Sigma (St Louis, MO, USA). Primaquine diphosphate (antimalarial drug, PRI) and laudanosine (tetanic poison, LAU) were from Aldrich (Milwaukee, WI, USA). They all were racemic compounds, and their structures are shown in Fig. 1.

Chondroitin sulfate A (sodium salt; molecular mass, 30 000–50 000) from whale cartilage and chondroitin sulfate C (sodium salt; molecular mass, 30 000–50 000; reported sulfur content, 5.5–7.0%) from bovine trachea, used as chiral selectors, were purchased from Seikagaku Kogyo (Tokyo, Japan) and Wako (Osaka, Japan), respectively. Dimethyl sulfoxide and pyridine for desulfation were from Merck (Darmstadt, Germany) and Nacalai Tesque (Kyoto, Japan), respectively. Chondroitinase ABC for depolymerization of chondroitin sulfate C and bovine albumin for stabilization of chondroitinase ABC were from Seikagaku Kogyo and Sigma, respectively. The structures of these polysaccharides and the desulfated and depolymerized products are shown in Fig. 2.

Polybrene (hexadimethrine bromide) used for capillary coating was from Aldrich. Dialysis membrane (Size 36) was purchased from Wako. All other reagents were of the highest grade commercially available. Glassware-distilled deionized water was used for preparation of running buffers and reagent solutions.

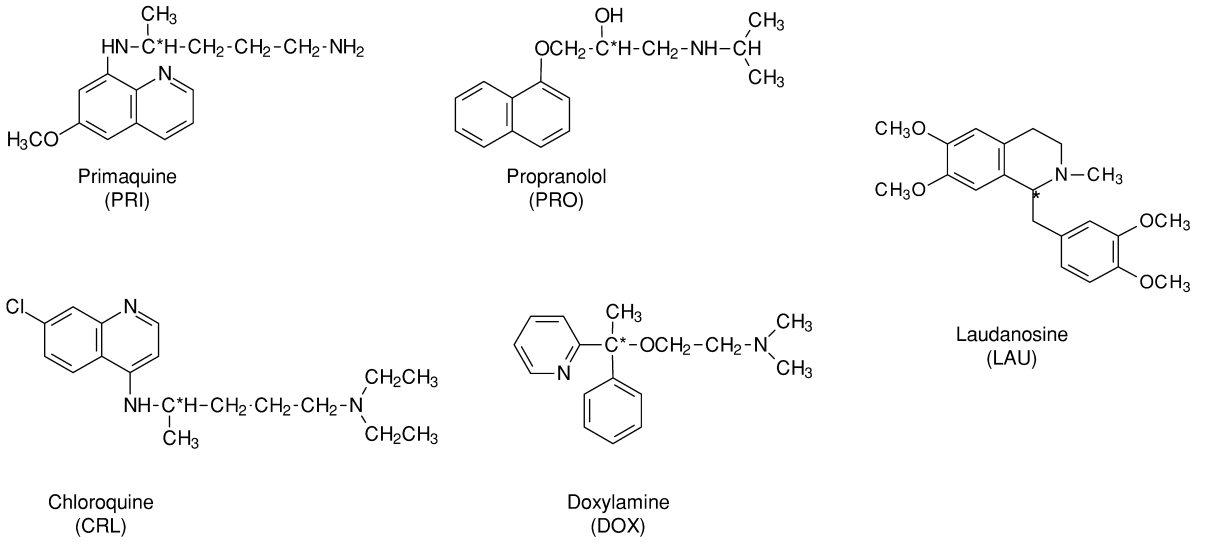


Fig. 1. Structures of the basic drugs employed.

### *2.2. Preparation of polybrene/polyacrylic acid (P/PAA) and polybrene/chondroitin sulfate C (P/CC) double-coated capillaries*

These double-coated capillaries were prepared according to the protocol described below. A capillary was rinsed with 1 *M* sodium hydroxide followed by water for 15 min each to clean the capillary and enhance the dissociation of the silanol group. A 5% (w/v) aqueous solution of Polybrene was passed through the capillary for 15 min at a constant flow rate and the capillary was allowed to stand for another 15 min for completion of coating. By this procedure the first basic layer was formed. After purging the excess Polybrene solution, a 3% (w/v) aqueous polyacrylic acid solution (for P/PAA coating) or a 3% (w/v) aqueous chondroitin sulfate C solution (for P/CC coating) was passed through the capillary for 15 min, and the capillary was allowed to stand for complete coupling of the anionic polymer to the fixed basic layer. Finally, the capillary was rinsed with water for 15 min to remove the excess acidic polymer. All these operations were performed at room temperature.

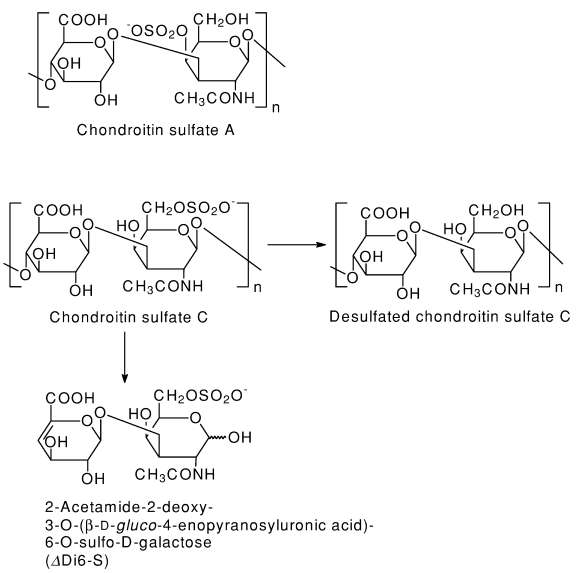


Fig. 2. Structures of chondroitin sulfate C and its modifications used for the observation of the effect of its modification on the enantioselectivity to basic drugs.

### *2.3. Desulfation of chondroitin sulfate C (for the observation of desulfation effect)*

This was carried out by heating the pyridinium salt of chondroitin sulfate C in aqueous dimethyl sulfoxide according to the literature [23,24], briefly as follows. The sodium salt of chondroitin sulfate C (1.04 g) was dissolved in 10 ml of water and the

solution was passed through a column (18 cm×8 mm I.D.) of Amberlite CG-120 (8 ml, $H^+$ form). The column was washed with water and the combined eluate and washing fluid (ca. 40 ml in total) were neutralized with pyridine and the neutralized solution lyophilized to give a white powder of the pyridinium salt of chondroitin sulfate C [23].

The pyridinium salt of chondroitin sulfate C thus obtained was dissolved in dimethyl sulfoxide (15 ml) containing water to a concentration of 10% (v/v), and the solution was heated at 80 °C for 5 h [24]. The reaction solution was cooled to room temperature and diluted with an equal volume of water, and pH was adjusted to 9.0 by adding 100 m*M* sodium hydroxide. The mixture was dialyzed for 48 h against deionized, distilled water with continuous stirring and occasional change in medium by fresh deionized, distilled water. The dialyzate was lyophilized to give a cotton-like white powder of desulfated chondroitin sulfate C. Yield, 0.81 g (theoretical amount, 0.82 g). A part of the product was dissolved in 2 *M* hydrochloric acid in a reacti-vial and heated on a boiling water bath for 5 h for sulfate estimation. The hydrolyzate was neutralized with 2 *M* sodium hydroxide and a 5% (w/v) aqueous solution of barium chloride was gradually added until no precipitation occurred. The precipitates were warmed for 2 h for maturation and filtered through a small glass filter (G 4), washed with distilled water and dried. The sulfur content calculated from the yield of barium sulfate was ca. 0.65% (w/w). The sulfur content of intact chondroitin sulfate C (reference) estimated in the same manner was 6.20% (w/w).

### *2.4. Depolymerization of chondroitin sulfate C by chondroitinase ABC (for the observation of depolymerization effect)*

This was carried out based on our previous paper [25]. Chondroitin sulfate C (1.5 mg) was dissolved in water (20 μl). To this solution were added a 250 m*M* tris(hydroxymethyl)aminomethane (Tris)–130 m*M* hydrochloric acid buffer (pH 8.0) containing sodium acetate (2.4% (w/v)) and bovine serum albumin (0.05% (w/v)) (10 μl), and an aqueous solution of chondroitinase ABC (1 U in 20 μl). The mixed solution was incubated at 37 °C for 24 h, then heated on a boiling water bath to terminate the enzyme reaction. The reaction solution was then cooled to room temperature, and pH adjusted to 2.9 by adding a 2% (w/v) phosphoric acid solution. Finally, the solution was centrifuged for 10 min and the supernatant was used as running buffer for observing the depolymerization effect. For reference, the same amount of chondroitin sulfate C was dissolved in the same volume of water, and the same volume of the same reagent solution was added as mentioned above. The identical amount of chondroitinase ABC was added, but the mixture was immediately heated on a boiling water bath to inactivate the enzyme. The procedure following this was the same as that for the depolymerization product.

### *2.5. Capillary electrophoresis*

A CAPI-3100 capillary electrophoresis system of Otsuka Electronics (Osaka, Japan) was used, which consisted of a sampling device, a power supply, a photodiode array UV detector, and a data processor. Uncoated fused-silica capillaries were obtained from the following sources. 1a (lot no. VVW04A), 1b (lot no. GFC06A) and 1c (lot no. ESY06C): Polymicro Technologies (PT, Phoenix, AZ, USA); 2: Scientific Glass Engineering (SG, Melbourne, Australia); 3: Beckman Instruments (BI, Fullerton, CA, USA). They all had inner and outer diameters of 50 and 375 μm, respectively. The capillary temperature was maintained at 20 °C. Samples were introduced by vacuum injection (0.5 kg/cm$^2$) for 0.5–4.0 s. The applied voltage was held at a constant value of 20 kV. The wavelength for detection was 259 nm (PRI), 220 nm (PRO), 256 nm (CHL), 260 nm (DOX), or 230 nm (LAU). The solvent for sample stock solution was methanol (LAU) or water (others). Stock solutions of individual racemic drugs were prepared at a concentration of ca. 1 mg/ml. Sample solutions for chiral separation were prepared by diluting the stock solutions with water to a concentration of 200 μg/ml.

Running buffer solutions containing a chiral selector were freshly prepared by dissolving either chondroitin sulfate C or a derivative in a 20 m*M* phosphate buffer having a specified pH. Since the pH value changed by this dissolution, it was then exactly adjusted to the desired value by adding 15% (w/v)

phosphoric acid or 1 *M* sodium hydroxide, and the solution was passed through a 0.45-μm filter and sonicated before use.

In the normal runs all of a capillary and anodic as well as cathodic reservoirs (capacity, ca. 2 ml each) were filled with a running buffer containing a chiral selector. In the special run for the depolymerized product, a 3-cm PTFE tube having an inner diameter of 375 μm was connected to the outlet of the analytical capillary in order to supplement the inside volume of the analytical capillary. A running buffer containing a chiral selector was filled in a P/CC double-coated capillary and this attached tube, but both reservoirs were filled with a plain buffer not containing the chiral selector.

The capillaries were rinsed with 1 *M* sodium hydroxide (for analyses using an uncoated capillary), a 3% (w/v) aqueous solution of polyacrylic acid (for analyses using a P/PAA-coated capillary) or a 3% (w/v) aqueous solution of chondroitin sulfate C (for analyses using a P/CC-coated capillary), followed by water after daily experiments were over. In addition short time conditioning using water followed by running buffer was also necessary before each run for all analyses.

## 3. Results and discussion

### *3.1. Technical problems and their solution*

#### *3.1.1. Prevention of tailing by double layer coating*

In carrying out experiments for the observation of the effects of the structure modification of chondroitin sulfate C, we met a problem of generally observed peak tailing of basic drugs in uncoated capillaries under acidic conditions. Table 1 compares the degree of peak tailing of PRI and PRO as representative drugs, by symmetry factor $S$ ($W_{0.05}/2f$, where $W_{0.05}$ and $f$ are the whole width and the front half width, respectively, of the peak at the 5% position of the peak height) in an acidic phosphate buffer with pH 2.90.

Among three lots of uncoated capillaries (1a, 1b and 1c) from PT, two lots (1a and 1b) gave seriously tailing peaks for both drugs, whereas the other lot (1c) gave relatively small *S*-values. Another two batches of uncoated capillaries from SG and BI also gave small *S*-values of ca. 4–6 for these basic drugs, but the peaks still could not be regarded symmetric. At neutral pH values, no significant tailing was observed for any batches of capillary (data not shown). At the low pH value of 2.90 as in the above cases, the drugs are protonated, whereas the dissociation of the silanol group on the capillary inner wall surface is suppressed. On the other hand, at neutral pH values, the protonation of the drugs is suppressed but the dissociation of the silanol group is facilitated. Therefore, the significant tailing at pH 2.90 is obviously related to the protonation of the drugs and the dissociation of the silanol group, but further studies will be necessary for complete understanding of this tailing phenomenon.

Table 1
*S*-values for basic drugs in different batches of uncoated capillaries

| Drug | *S*-value | | | | |
|---|---|---|---|---|---|
| | 1a | 1b | 1c | 2 | 3 |
| PRI | 12.6 | 17.1 | 4.0 | 4.1 | 6.0 |
| PRO | 15.7 | 18.2 | 5.4 | 5.1 | 5.1 |

Capillary, as above (total length, 50.0 cm each; distance from the inlet to the detector window, 37.5 cm each); running buffer, 20 m*M* phosphate buffer (pH 2.90); applied voltage, 20 kV; detection, UV absorption at 259 nm (PRI) or 220 nm (PRO).

In CE, adsorption of solutes to the capillary inner wall is often experienced. One of the strategies to prevent adsorption is coating capillaries with various polymers. Coating with linear polyacrylamide is the most popular but the procedure for this chemical coating is laborious and it is difficult to completely diminish the silanol group. In addition, the coating is not durable in strong acidic media. We examined dynamic coating with Polybrene, a strong cationic polymer, but the electroosmotic flow (EOF) was reversed and its velocity was not constant when chondroitin sulfate C, the chiral selector in question, was added to running buffer. Katayama et al. [26] reported on the stabilization of the Polybrene layer by the second coating with an acidic polymer. We attempted double coating with Polybrene followed by polyacrylic acid (P/PAA coating) and found that the P/PAA method was also useful, giving reproducible migration times for basic drugs. We further replaced the second layer by chondroitin sulfate C

Table 2
Improvement of peak tailing by double coating

| Drug | *S*-value | | | |
|---|---|---|---|---|
| | P/PAA coating 1b | P/CC coating | | |
| | | 1b | 2 | 3 |
| PRI | 3.9 | 2.6 | 3.1 | 2.9 |
| PRO | 4.7 | 3.7 | 4.3 | 4.0 |

Capillary, as above (P/PAA-coated 1b: total length, 50.0 cm; distance from the inlet to the detector window, 37.5 cm; P/CC-coated 1b, 2 and 3: total length, 80.0 cm each; distance from the inlet to the detector window, 67.5 cm). The other analytical conditions as in Table 1.

and found this method (P/CC double coating) much better in reproducibility. Table 2 shows the improvement of tailing of the basic drugs, observed when the uncoated capillaries (1b, 2 and 3) were double-coated as mentioned above.

Comparison of the data with those in Table 1 indicates that *S*-values of all basic drugs in double-coated capillaries were obviously smaller than in uncoated capillaries. In particular, the reduction was as large as 4–7 times for batch 1b. No doubt double-coated capillaries were effective for decreasing peak tailing of basic drugs. This is probably due to reduced number of the silanol groups responsible for the adsorption of the basic drugs, by the binding of Polybrene. In P/CC double-coated capillaries, chondroitin sulfate C in running buffer can repair the second anionic polymer layer in every run. Thus, a P/CC double-coated capillary could be used more than 100 times without significant changes in migration time and peak shape under the optimum conditions described in Section 3.1.2. It was also stable over a pH range of 2–12. The reproducibility of migration time was quite high; for example the relative standard deviation of the migration time of PRO was less than 1.5% for five repeated analyses. The migration times of all basic drugs became shorter in both types of double-coated capillaries than in uncoated capillaries (data not shown). This is because the velocity of EOF increased in the same direction as in uncoated capillaries.

Change of the surface of capillary inner wall was considered to give some effect on the migration of basic drugs. The surface covered with chondroitin sulfate C is considered to be more hydrophilic than untreated silanol surface because of various polar substituent groups such as the sulfate and the hydroxyl groups. Therefore, the protonated drugs will be retained on the second layer of chondroitin sulfate C by cation-exchange mechanism. So we measured the apparent mobilities of PRI as a representative basic drug in both a P/CC double-coated and an uncoated capillary from the same batch (1c) in 20 m*M* phosphate buffer (pH 2.90) not containing chondroitin sulfate C. However, the values obtained ($3.30\times10^{-8}$ and $3.21\times10^{-8}$ $m^2\ s^{-1}\ V^{-1}$, respectively) did not differ significantly from each other. This good agreement implies that there was no remarkable effect of cation exchange on the migration of basic drugs in P/CC double-coated capillaries. This is probably due to relatively small amount of chondroitin sulfate C on the inner wall compared to sample amount. As expected, there was of course no splitting of peaks due to enantiomers in this buffer not containing chondroitin sulfate C. Therefore, the chiral separation observed in chondroitin sulfate C-containing buffer was mainly due to the difference in the magnitude of interaction of the enantiomers with chondroitin sulfate C in the buffer.

Thus, the use of P/CC double-coated capillaries eliminated the problems of low symmetry and poor reproducibility of migration time in uncoated capillaries and made it possible to analyze the enantiomers without being disturbed by these problems. Therefore, we used P/CC double-coated capillaries throughout the work for chiral separation.

### *3.1.2. Optimization of the analytical conditions for chiral separation using a P/CC double-coated capillary*

Although the results of optimization for chiral separation of several other basic drugs in uncoated capillaries have already been reported [13], the optimum conditions in P/CC double-coated capillaries must be examined, because additional factors due to double coating might have an influence on chiral separation. So we carried out optimization of pH and chiral selector concentration, the most influential factors, in a P/CC double-coated capillary.

The effects of pH on the migration time and enantioselectivity were investigated in a P/CC double-coated capillary using 20 m*M* phosphate buffer containing chondroitin sulfate C (3% (w/v)) over a

pH range of 2.04–6.10. The migration times of the enantiomers of PRI and PRO decreased as the pH of running buffer increased except between pH 5.02 and 6.10. Separation factor ($\alpha$) and resolution ($R_s$) for PRI increased at first, reached the maximum values at pH 2.90, and gradually decreased above pH 2.90. The maximum values of $\alpha$ and $R_s$ at pH 2.90 were 1.030 and 2.62, respectively, indicating baseline separation at this pH. Under these conditions, the *S*-value was less than unity (ca. 0.9), presumably due to the interaction with the chiral selector flowing counter wise. The same tendency was observed for PRO under the same conditions, but the maximum values of $\alpha$ and $R_s$ at pH 2.90 (1.015 and 0.90, respectively) were not so large as those of PRI, separation being incomplete.

The effects of the concentration of chondroitin sulfate C on the migration times of basic drugs and enantioselectivity were examined in a P/CC double-coated capillary using 20 m*M* phosphate buffer containing chondroitin sulfate C at pH 2.90. In the concentration range examined (0–6.0%, w/v), the migration times of the enantiomers of PRI and PRO increased with the increase in the concentration of chondroitin sulfate C. However, the increasing rate slowed down at high concentrations of the chiral selector. Regarding $\alpha$ and $R_s$, remarkable improvement was observed when the concentration of chondroitin sulfate C changed from 0 to 3.0% (w/v). Above 3.0% (w/v), however, $\alpha$ did not increase greatly any more. With PRI and PRO, a maximum for $R_s$ was reached at 4.0% (w/v). It seems that too high concentrations of chondroitin sulfate C caused a decrease in $R_s$ value by peak broadening due to abnormal rise of electric current. Therefore, 3% (w/v) chondroitin sulfate C was better for the separation of the enantiomers of these basic drugs.

Thus, the optimum pH value was almost consistent with the values reported for other drugs [13], indicating no practical influence of capillary coating on separation of basic drugs. The effect of the concentration of chondroitin sulfate C was not inconsistent with the literature for uncoated capillaries.

### *3.1.3. Chiral separation using the minimum amount of a chiral selector*

In the normal set-up in CE, a capillary and anodic as well as cathodic reservoirs are filled with a running buffer containing an additive. Since both reservoirs have considerable capacities (normally >2 ml), a large amount of the additive is required. The capacity of a reservoir could be reduced to 0.6 ml using a narrow sample tube but the liquid level changed markedly during analysis, influencing the migration time of the analyte. In the present work for depolymerization effect it was indeed unrealistic to use the normal set-up, because such study requires a large amount of a precious enzyme (chondroitinase ABC). Therefore, we used a simplified set-up by filling only the capillary with a minimum volume of running buffer but filling both reservoirs with a plain running buffer not containing the chiral selector. This ''only capillary'' set-up was already demonstrated to be useful for the reduction of additive in our early work on carbohydrate–protein interaction [27]. First, reference experiments were performed in a P/CC double-coated capillary filled with 20 m*M* phosphate buffer (pH 2.90) containing chondroitin sulfate C (3%, w/v), using the normal set-up and the ''only capillary'' set-up. The baseline was smooth in the normal set-up (Fig. 3a) throughout the whole analysis (25 min), but an abrupt baseline change was observed at about 10 min in the ''only capillary'' set-up (Fig. 3b).

The sudden change in baseline implies that the end of the chondroitin sulfate C zone migrating to the anode passed the detection window. It indicates the replacement of chondroitin sulfate C-containing buffer by the plain buffer. It is obvious that the zone length behind the detection window was not enough to observe the overall interactions of the drugs with this chiral selector. Valtcheva et al. [28] described a partial filling technique to avoid baseline noise in the chiral separation of basic drugs in a certain kind of a protein (cellobiohydrolase I), which was performed in a protein-containing zone shorter than the distance from the inlet to the detector window. Other groups, for example, Tanaka and Terabe [29] and Fanali and Desiderio [30] extended this technique under the name of the ''partial separation zone technique'' or ''counter-flow'' technique and succeeded in chiral separation of a number of drugs in buffers containing proteins and antibiotics, respectively. In this technique the analytical conditions are not identical with those in the normal set-up, because the zone length

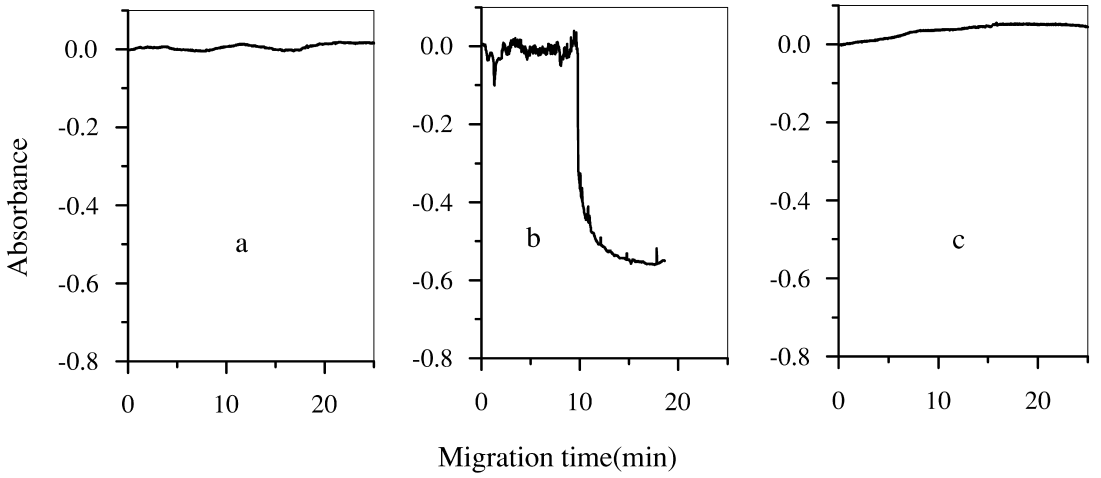


Fig. 3. Comparison of baseline drift among the ordinary (a), ''only capillary'' (b), and ''wider tube-attached'' (c) set-ups. Capillary, a P/CC double-coated capillary (a, b) (total length, 80 cm; distance from the inlet to the detector window, 67.5 cm) or a P/CC double-coated capillary (total length, 77 cm; distance from the inlet to the detector window, 67.5 cm) to which a 3-cm PTFE tube (375 μm I.D.) was connected (c); running buffer, 20 m*M* phosphate buffer (pH 2.90) containing chondroitin sulfate C to a concentration of 3% (w/v), filled in the capillary and the reservoirs (a), the capillary alone (b), or the capillary and the attached tube (c); applied voltage, 20 kV; detection, UV absorption at 200 nm.

for interaction is different from each other. However, it was important in the present study that all analyses for chondroitin sulfate C and its modifications should be done under the same conditions.

In order to provide a sufficient volume of running buffer containing a chiral selector behind the detector window, a 3-cm PTFE tube having a wider inner diameter of 375 μm (the supplemented capacity was ca. 2 μl, because the added length was ca. 2 cm) was connected to the cathodic end of a P/CC double-coated capillary. No sudden baseline change was observed at least within 25 min when this special device was used (Fig. 3c), indicating that there was enough volume of running buffer containing the chiral selector in the whole space including the capillary and the PTFE attachment. This ''wider tube-attached'' set-up is a modification of our ''only capillary'' set-up described above [27]. The migration times of the enantiomers for PRI and PRO in the ''wider tube-attached'' set-up were approximately the same as those in the normal set-up, and the separation factor as well as resolution was slightly higher. Thus, this special design having an attached PTFE tube (d) was shown to be useful to minimize the amounts of chiral selectors required (in the present work, to the nanomolar level) and to obtain the migration and resolution data under the same conditions as in the normal set-up.

### *3.2. Effect of structure modification of chondroitin sulfate C on its enantioselectivity*

Although a few groups postulated the importance of hydrophobic interaction, hydrogen bonding, dipole–dipole interaction, ionic interaction, macroscopic structure, polymer network, etc., in the enantioselectivity of polysaccharides, based on comparative studies using various kinds of carbohydrates having structures randomly different from each other [4,13], more straightforward evidence based on systematic structural variation will be necessary for a better understanding of chiral separation. Therefore, we modified a particular polysaccharide of chondroitin sulfate C, the most potent chiral selector, by selective/specific methods, and examined the effects of desulfation and depolymerization, together with sulfate placement, on its enantioselectivity using a P/CC double-coated capillary.

#### *3.2.1. Desulfation*

Since sulfated polysaccharides, such as isomeric chondroitin sulfates, heparin and dextran sulfate have

been known to commonly possess the capability of chiral separation of basic drugs, strong or weak [15–17,19], it is logical to anticipate the role of the sulfate group. In order to elucidate whether the sulfate group contributes to enantioselectivity, comparison of $\alpha$ and $R_s$ values between chondroitin sulfate C and desulfated chondroitin C will be useful. Therefore, we removed the sulfate group of chondroitin sulfate C without giving any structural change to other parts. The desulfation of chondroitin sulfate C was carried out according to the procedure described in Section 2.3. It had been confirmed that no structural changes occurred in other parts [23,24] by this procedure. The sulfur content measured by acid hydrolysis followed by gravimetric determination of the released sulfate ion as barium sulfate was as small as 0.65%, whereas intact chondroitin sulfate C gave a sulfur content of 6.20%. This result indicates that desulfation was satisfactory. Five basic drugs were then analyzed in a P/CC double-coated capillary with 20 m*M* phosphate buffer (pH 2.90) containing chondroitin sulfate C (3% (w/v)) or desulfated chondroitin sulfate C to a concentration equivalent to 3% (w/v). The results are shown in Table 3. A typical example of the separation of PRI enantiomers with desulfated chondroitin sulfate C is also given in Fig. 4b together with the electropherograms for intact chondroitin sulfate C (Fig. 4a) and other derivatives from it (Fig. 4c,d).

Table 3
Effect of desulfation on the migration times and separation of enantiomers

| Drug | Parameter | Intact chondroitin sulfate C (a) | Desulfated chondroitin sulfate C (b) |
|---|---|---|---|
| PRI | $t_1$ (min) | 15.90 | 11.36 |
| | $\alpha$ | 1.030 | 1.020 |
| | $R_s$ | 2.58 | 1.54 |
| PRO | $t_1$ (min) | 14.77 | 13.22 |
| | $\alpha$ | 1.015 | 1.013 |
| | $R_s$ | 0.90 | 0.68 |
| CHL | $t_1$ (min) | 25.39 | 21.21 |
| | $\alpha$ | 1.034 | 1.033 |
| | $R_s$ | 2.69 | 2.47 |
| DOX | $t_1$ (min) | 20.29 | 19.01 |
| | $\alpha$ | 1.012 | 1.011 |
| | $R_s$ | 0.85 | 0.84 |
| LAU | $t_1$ (min) | 19.05 | 21.82 |
| | $\alpha$ | 1.009 | 1.012 |
| | $R_s$ | 1.04 | 1.16 |

Capillary, P/CC double-coated 1b (total length, 80.0 cm, distance from the inlet to the detector window, 67.5 cm); running buffer, 20 m*M* phosphate buffer (pH 2.90) containing chondroitin sulfate C (3%, w/v) (a) or 20 m*M* phosphate buffer containing desulfated chondroitin sulfate C (equivalent to 3%, w/v) (b); applied voltage, 20 kV; detection, UV absorption at 259 nm (PRI), 220 nm (PRO), 256 nm (CHL), 260 nm (DOX) or 230 nm (LAU). $t_1$, migration time of the faster moving enantiomer.

It is observed that separation factor as well as resolution decreased to considerable extents for PRI and PRO. However, it is striking that they were almost unchanged for CHL and DOX, and even slightly increased for LAU. Desulfated chondroitin sulfate C gave shorter migration times than its precursor for all drugs except for LAU, mainly due to reduced mobility. Since longer migration times generally give higher $\alpha$ and $R_s$ values under identical conditions, the $\alpha$ and $R_s$ values will be significantly larger than the values obtained in Table 3, if they are corrected for migration time. Since the drugs showing greater difference of migration time between desulfated and intact chondroitin sulfate C gave larger difference in enantioselectivity (Table 3), it may be considered that all these drugs had enantioselectivity of the same magnitude between these two selectors.

Thus, desulfated chondroitin sulfate C still retained high enantioselectivity to basic drugs, giving baseline or partial separation for all these basic drugs. This implies that interactions other than the ionic interaction by the sulfate group play important roles in the chiral separation of basic drugs by chondroitin sulfate C, though the effect of such ionic interaction should not be ignored.

### *3.2.2. Depolymerization*

The most effective method to know whether the polymer chain length, accordingly degree of polymerization, of a polysaccharide contributes to enantioselectivity, will be to compare enantioselectivity between the polysaccharide and its sugar unit. Since chondroitinase ABC can catalyze the eliminative cleavage of the *N*-acetylhexosaminide linkage in chondroitin sulfate C, yielding exclusively a disaccharide unit with the unsaturated uronic acid residue at the non-reducing end (Fig. 2, molecular mass,

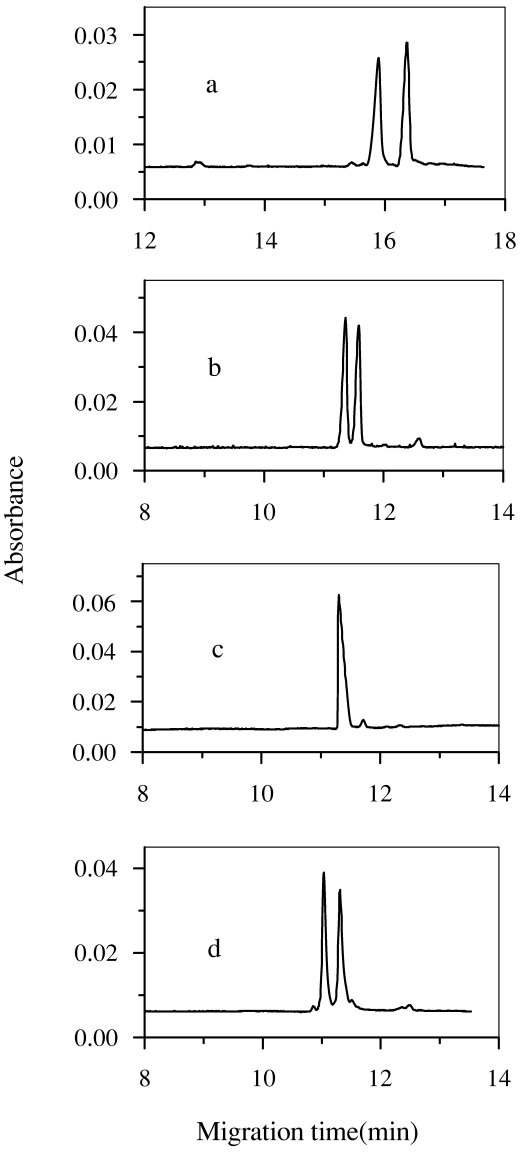


Fig. 4. Chiral separations of PRI with intact (a), desulfated (b), depolymerized (c), and isomeric (d) chondroitin sulfate C. The analytical conditions for a and b as in Table 3 (columns a and b, respectively), for c as in Table 4 (column b), for d as in Table 5 (column b).

481) without any other changes in the disaccharide structure, depolymerization of chondroitin sulfate C was performed by the protocol described in our previous paper for CE analysis of chondroitin sulfate-derived disaccharides [25]. In order to cancel out the effects of the other components in the digestion mixture, a reference was prepared from the same amount of chondroitin sulfate C and the same volume of the digestion medium. The same amount of chondroitinase ABC was also added, but the mixture was immediately heated to inactivate the enzyme. Except that chondroitin sulfate C was not depolymerized, this reference was identical in all respects with the sample of depolymerized product. Separation of the enantiomers was performed in a P/CC double-coated capillary using the ''wider tube-attached'' set-up as described in Section 3.1.3 to minimize the amount of the enzyme. Table 4 (column b) gives the results obtained using this special set-up.

The running buffer contained the depolymerized chondroitin sulfate C to a concentration equivalent to 3% (w/v) in phosphate buffer (pH 2.90). Table 4 also includes the results obtained with intact chondroitin sulfate C (3%, w/v, column a) and the digestion reference (column c). For all drugs examined, migration time decreased when analyzed in a buffer containing depolymerized chondroitin sulfate C (column b). Enantioselectivity was retained naturally in the reference experiment (column c), but completely lost in the depolymerized chondroitin sulfate C-containing buffer (column b). A typical electropherogram for PRI is shown in Fig. 4c. It is important that no chiral separation was observed with the depolymerized product for all drugs examined.

### *3.2.3. Sulfate placement*

As shown in Fig. 2, the only structural difference between chondroitin sulfate C and chondroitin sulfate A is in the attaching position (isomer C, at $C_6$; isomer A, at $C_4$) of the sulfate group in the *N*-acetylgalactosamine residue. Comparison of enantioselectivity to basic drugs between these two isomeric chondroitin sulfates was performed in a P/CC double-coated capillary, using 20 m*M* phosphate buffer (pH 2.90) containing either of these chiral selectors (3%, w/v). As shown in Table 5, the migration times of the enantiomers obtained with isomer C were longer than those obtained with isomer A for all drugs examined, except LAU, well consistent with the results reported by Nishi and coworkers for a few of the drugs examined in the present work and also for several other drugs [13].

Table 4
Effect of depolymerization on the migration times and separation of enantiomers

| Drug | Parameter | Intact chondroitin sulfate C (a) | Depolymerized chondroitin sulfate C (b) | Reference for b (c) |
|---|---|---|---|---|
| PRI | $t_1$ (min) | 15.42 | 11.30 | 12.16 |
| | $\alpha$ | 1.052 | NS | 1.043 |
| | $R_s$ | 3.92 | NS | 3.10 |
| PRO | $t_1$ (min) | 15.46 | 13.32 | 15.88 |
| | $\alpha$ | 1.017 | NS | 1.014 |
| | $R_s$ | 0.98 | NS | 0.82 |
| CHL | $t_1$ (min) | 25.11 | 14.18 | 20.68 |
| | $\alpha$ | 1.041 | NS | 1.030 |
| | $R_s$ | 3.30 | NS | 2.26 |
| DOX | $t_1$ (min) | 20.50 | 12.23 | 17.10 |
| | $\alpha$ | 1.013 | NS | 1.010 |
| | $R_s$ | 0.88 | NS | 0.76 |
| LAU | $t_1$ (min) | 19.22 | 13.82 | 18.01 |
| | $\alpha$ | 1.011 | NS | 1.009 |
| | $R_s$ | 1.08 | NS | 1.01 |

NS, not separated. $t_1$, migration time of the faster moving enantiomer. Capillary, P/CC double-coated 1b (total length, 77.0 cm; distance from the inlet to the detector window, 67.5 cm) with a PTFE tube (375 μm I.D., 3 cm); running buffer, 20 m*M* phosphate buffer (pH 2.90) containing chondroitin sulfate C (3%, w/v) (a) or depolymerized chondroitin sulfate C to a concentration equivalent to 3% (w/v) (b). c is a reference for b, where the concentration of the enzyme (chondroitinase ABC), the substrate (chondroitin sulfate C), the reagents, reaction temperature (37 °C), and reaction time (24 h) were the same as for b, but the added enzyme was inactivated by heating immediately after addition. The analyses were done by the ''wider tube-attached'' set-up as described in Section 3.1.3. Applied voltage, 20 kV; detection, as in Table 3.

Separation factor and resolution with isomer C were also markedly larger than that with isomer A for all the drugs examined. This means that this sulfate placement, accordingly the $C_4$ position of the hydroxyl group in the galactose residue is more favorable for chiral separation. Fig. 4d shows the separation of PRI with chondroitin sulfate A, an isomer of chondroitin sulfate C.

### *3.3. Mechanism of chiral recognition*

Although the contribution of the ionic interaction by the sulfate group in sulfated polysaccharides to chiral separation was suggested by other researchers (Nishi et al. [17]; Wang et al. [22]), the observed retention of enantioselectivity in desulfated chondroitin sulfate C (Table 3) strongly suggests that a major mechanism of chiral separation other than the ionic interaction by the sulfate group may exist, as far as chondroitin sulfate C is concerned. Since the dissociation of the carboxyl group in this glycosaminoglycan will be suppressed at pH values lower than its p$K_a$ value (considered to be ca. 3 in analogy with the reported value for the carboxyl group in hyaluronic acid [31]), it is supposed that the undissociated form existed in a high-molar-mass fraction for the carboxyl group in both intact and desulfated chondroitin sulfate C under the acidic conditions employed (pH 2.90). This will be favorable for hydrogen bonding formation with the amino/imino or hydroxy/alkoxy functions in the drugs. On the other hand, the result of depolymerization effect in Table 4 indicates that at least the sulfated disaccharide shown in Fig. 2 does not cause the chiral separation of the basic drugs examined. The significance of polymer network was pointed out in the chiral separation with polysaccharides, based on the observed change in enantioselectivity with degree of polymerization [13]. Since enantioselectivity was noted even for neutral polysaccharides, hydrogen

Table 5
Effect of sulfate placement on the migration times and separation of enantiomers

| Drug | Parameter | Chondroitin sulfate C (a) | Chondroitin sulfate A (b) |
|---|---|---|---|
| PRI | $t_1$ (min) | 15.90 | 11.04 |
| | $\alpha$ | 1.030 | 1.024 |
| | $R_s$ | 2.58 | 1.76 |
| PRO | $t_1$ (min) | 14.77 | 13.07 |
| | $\alpha$ | 1.015 | 1.005 |
| | $R_s$ | 0.90 | <0.4 |
| CHL | $t_1$ (min) | 25.39 | 21.27 |
| | $\alpha$ | 1.034 | 1.025 |
| | $R_s$ | 2.69 | 2.13 |
| DOX | $t_1$ (min) | 20.29 | 18.02 |
| | $\alpha$ | 1.012 | 1.009 |
| | $R_s$ | 0.85 | 0.65 |
| LAU | $t_1$ (min) | 19.05 | 22.60 |
| | $\alpha$ | 1.009 | 1.007 |
| | $R_s$ | 1.04 | 0.62 |

Capillary, P/CC double-coated 1b (total length, 80.0 cm; distance from the inlet to the detector window, 67.5 cm); running buffer, 20 m*M* phosphate buffer (pH 2.90) containing chondroitin sulfate C (column a) or chondroitin sulfate A (column b), to a concentration of 3% (w/v); applied voltage, 20 kV. The other analytical conditions as in Table 3. $t_1$, migration time of the faster moving enantiomer.

bondings to multiple hydroxyl groups can be postulated. The idea of polymer network is similar to a cluster binding hypothesis in our earlier papers on the interaction of a carbohydrate with a lectin (a polypeptide). For example, α1-6 linked glucose oligomers (isomaltooligosaccharides) showed a tendency to bind to *Lens calinaris* agglutinin (LCA) and the affinity became stronger as the degree of polymerization increased. We found this tendency by both CE [32] and high-performance affinity chromatography [33], and thought that such affinity is based on the multipoint recognition of the cluster of hydroxyl groups in the oligosaccharide chain by the peptide groups in the lectin. In a similar sense the basic drugs in the present work will bind to not only the carboxyl group but also the hydroxyl groups in chondroitin sulfate C. Such interaction can be dynamically changeable from one hydroxyl group to another, and longer sugar chains will give more chances for binding, because they have a greater number of hydroxyl groups. This will result in higher enantioselectivity in chondroitin sulfate C than in the disaccharide thereof.

Furthermore, the difference of $\alpha$ as well as $R_s$ values between chondroitin sulfates A and C (Table 5) indicates the importance of the position of the hydroxyl group. The higher selectivity of chondroitin sulfate C means that the hydroxyl group at $C_4$ of the galactosamine residue is considered to be more accessible by the basic drugs than the hydroxyl group at $C_6$ in chondroitin sulfate A.

## 4. Conclusion

The main results obtained in this study were: (1) desulfation showed no remarkable decrease in separation factor and resolution, (2) depolymerization with chondroitinase ABC to β1-3 linked enopyranurosyl *N*-acetylgalactosamine 6-sulfate caused complete loss of enantioselectivity, and (3) chondroitin sulfate C showed more marked chiral separation than its positional isomer, chondroitin sulfate A. The great retention of selectivity after desulfation suggests that the sulfate group does not play the major role in chiral recognition. The complete loss of selectivity by depolymerization was consistent with a general tendency of smaller selectivity for smaller molecules with respect to polysaccharide selectors, and the higher selectivity of chondroitin sulfate C than that of chondroitin sulfate A agreed with reported results for other drugs and confirmed the importance of the hydroxyl group at $C_4$ in the galactosamine residue.

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