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Journal of Chromatography A, 845 (1999) 247–256

JOURNAL OF CHROMATOGRAPHY A

Semisynthetic chondroitins as chiral buffer additives in capillary electrophoresis

R. Gotti[a], V. Cavrini[a,*], V. Andrisano[a], G. Mascellani[b]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Bologna, Via Belmeloro 6, 40126 Bologna, Italy*
[b]*Opocrin S.p.A., R&D Laboratories, Corlo di Formigine, Modena, Italy*

Abstract

Chemically oversulfated galactosaminoglycans with potential as therapeutic agents (inhibitors of human leukocyte elastase) were tested as chiral selectors in capillary electrophoresis of basic racemates. The high anionic character of these compounds provides them with anodic mobility in acidic buffer; using uncoated capillaries, the enantioresolution of racemic basic drugs was obtained at pH 2.5. Dimethindene, chloroquine and chlorpheniramine were enantioresolved applying negative voltage (−15 kV) while the other analytes (propranolol, pindolol, tetrahydrozoline and cloperastine) exhibited catodic migration. The addition of organic solvents to the running buffer was evaluated in order to increase the resolution; methanol provides the best results and in general, baseline separation of the analytes was reached. The studied oversulfated mucopolysaccharide, shows the same ionic character of heparin but presents different stereochemistry and sites of sulfation. A comparison with heparin, used in the same acidic conditions, may underline the role of ionic, spatial and steric features of glycosaminoglycans in the enantiorecognition.

Keywords: Buffer composition; Chiral selectors; Enantiomer separation; Affinity electrokinetic chromatography; Electrokinetic chromatography; Chondroitins; Galactosaminoglycans; Heparin; Basic drugs

1. Introduction

Capillary electrophoresis (CE) has become one of the most used separation and quantitation technique: it provides short analysis time, low operational cost and high resolution.

One of the most important fields where CE has found successful application, is that of enantiomeric analytical separations [1–3]. Direct chiral separation method involves the use of a chiral selector, often dissolved in the background electrolyte. Cyclodextrins have been successfully applied for the ability to give inclusion-complexation mechanism of enantiorecognition [4,5].

Recently heparin [6,7] and other related ionic galactosaminogylcans (GAGs: chondroitin sulfate A, B and C) [8–12], have been employed as chiral selectors for the enantiomeric resolution of several pharmaceutical compounds in CE. When ionic polysaccharides are used, the enantioseparation of electrically neutral analytes is also possible, as in micellar electrokinetic chromatography (MEKC); since the ionic pseudophase is constituted by biological components of high molecular mass, the mode of separation was labeled as affinity electrokinetic chromatography (AEKC) [8].

The ionic character of polysaccharides as well as their molecular masses are very important features that affect the ‘self-mobility’ in the separation environment. The smaller ionic character of chondroitin compared to that of dextran sulfate and

*Corresponding author. Fax: +39-51259734.
E-mail address: vcavrini@alma.unibo.it (V. Cavrini)

0021-9673/99/$ – see front matter
PII: S0021-9673(99)00295-2

heparin, enabled the first one to achieve enantioseparations with a pH range of 2.7–6.5 [10]; furthermore, in a previous study, the potential of dermatan sulfate (chondroitin sulfate B) as chiral selector in AEKC of racemic cationic drugs was shown, obtaining successful separation in the pH range of 3–6.5 [9].

In the present work, chemically oversulfated galactosaminoglycan (OSGAG) has been employed as chiral selector in AEKC. Chemical oversulfation of chondroitins, preferentially occurs at C-6 of galactosamine residues (Fig. 1); this chemical transformation was found generally to increase the inhibitory power on human leukocyte elastase, an enzyme involved in the etiology of diseases such as emphysema, atherosclerosis, and rheumatoid arthritis [13]. The oversulfation of GAGs, gives an increase in charge density, associated with a decrease in molecular mass (M_r) (as occured in sulfation of chondroitin samples under strong acidic conditions). These modifications should make the OSGAGs useful as chiral selectors in AEKC in the usually neutral or weakly acidic conditions. Interestingly, this study showed the favourable use of OSGAGs for the enantioresolution of some selected basic drugs at pH 2.5. The effect of organic solvent methanol in the background electrolyte was also considered. When compared to heparin, used as chiral selector in the same electrophoretic conditions and on the same racemic analytes, oversulfated chondroitin exhibited, in general, enhanced enantioselectivity.

2. Experimental

2.1. Apparatus

The experiments were performed with a ^{3D}CE system (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode array detector; data acquisition processing was done by HP Vectra 486/100 XMZ computer.

All the electrophoretic separations were carried out using fused silica capillaries of 48.5 cm (40 cm to the detector)×50 μm I.D., purchased from Supelco (Milan, Italy) and operating at 15°C.

The samples were introduced hydrodynamically for 10 s (injection pressure 5 kPa) and they were monitored by UV detection at 220 nm (chiral separation of drugs) and at 200 nm (GAGs analysis). The applied voltage was held constant at +15 kV or −15 kV.

2.2. Materials

All the used mucopolysaccharides (dermatan sulfate, sodium heparin, chondroitin sulfate C and chondroitin oversulfated) were from Opocrin (Corlo, Italy). The chemical oversulfation of natural chondroitins was carried out either under rather drastic conditions with chlorosulfonic acid or under milder conditions with adducts of sulfur trioxide (SO_3) in aprotic solvents [13].

Potassium phosphate, phosphoric acid, methanol and acetone were purchased from Carlo Erba (Milan, Italy); chlorpheniramine maleate, pindolol, cloperastine, tetrahydrozoline and propranolol hydrochloride, chloroquine diphosphate, were purchased from Sigma (St. Louis, MO, USA), dimethindene maleate was a gift from Novartis (Origgio, Italy). Purified water from a TKA ROS 300 system was used to prepare buffers and standard solutions.

2.3. Procedure

Running buffer solution consists of 30 m*M* potassium phosphate adjusted to pH 2.5 with phosphoric acid; chondroitin oversulfated and heparin were dissolved at a concentration of 3% without changing the buffer pH value. The sample solutions subjected to enantioseparation were prepared at a concentration of~0.1 mg/mL in water; the sample solutions for the measure of the electrophoretic mobilities of GAGs were prepared at a concentration of 2 mg/ml in water. All the solutions (buffer and sample solutions) were filtered through 0.45 μm Millex-HV filter units (Millipore, Milford, MA, USA). The capillary was rinsed prior to each run for 3 min with the separation electrolyte.

3. Results and discussion

3.1. Oversulfated chondroitin properties

Various ionic mucopolysaccharides have been

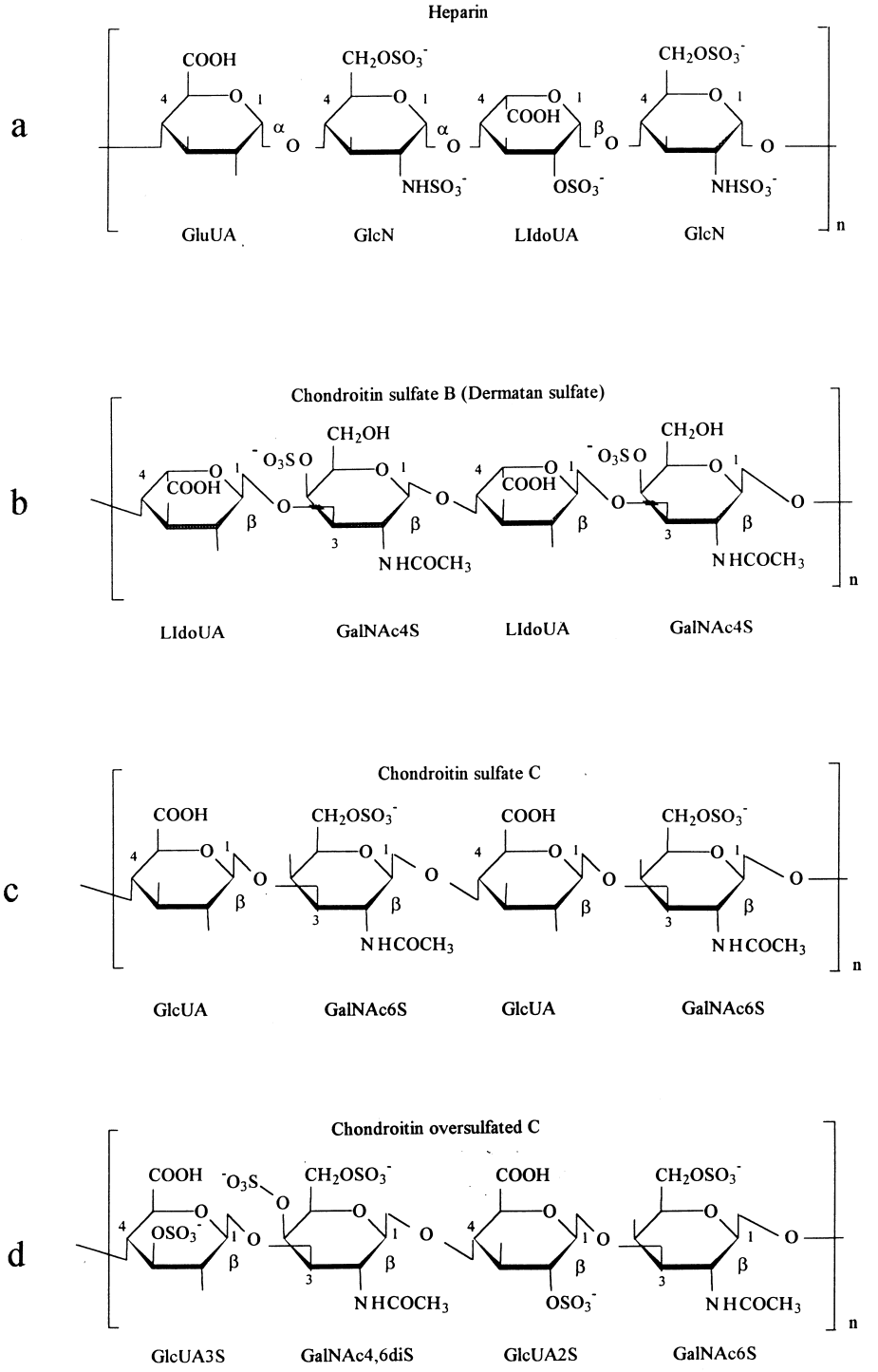

Fig. 1. Unit structure of: (a) heparin, (b) chondroitin sulfate B (dermatan sulfate), (c) chondroitin sulfate C, (d) oversulfated chondroitin.

investigated as chiral selectors in AEKC for the enantioresolution of several racemic drugs [6–12].

The molecular mass, sulfation index and effective mobility (μ_e) of the mucopolysaccharides used are reported in Table 1. The molecular mass of OSGAG was determined by GP-HPLC against molecular masses of standard compounds on a third order polynomial calibration curve. The sulfation index (SI), corresponding to SO_3H/CO_2H ratio, was determined by potentiometry with a titroprocessor, as previously described [14]. The effective electrophoretic mobility of OSGAG was measured using phosphate buffer (30 m*M* at pH 2.5) in an uncoated capillary under a constant voltage of −15 kV.

Since in such acidic conditions the carboxy groups are undissociated, heparin possesses 1.2 ionic groups (sulfate) per monosaccharide unit and the chondroitins 0.5 sulfate groups per monosaccharide unit (Fig. 1). The chemical sulfation of the chondroitin samples was carried out under acidic conditions [13] and lower M_r were found for the semisynthetic products compared to starting material (chondroitin C) (Table 1). The resulting OSGAG presents ~1.2 sulfate groups per monosaccharide unit, therefore it should have very similar electrophoretic behaviour to that of heparin, being the electrophoretic mobility of linear ionic solute inversely proportional to (molecular mass)$^{2/3}$ and directly related to the solute charge [15].

As reported [13,16], the regioselectivity of chemical sulfation of chondroitins is in the order C-6 of N-Acetylgalactosamine sulfate (GalNAc), then C-2 or C-3 of uronic acid (UA), and C-4 of GalNAc; since the used oversulfated glycosaminoglycan was obtained starting from chondroitin C, the nature of the sugar rings should keep as in Fig. 1(c) and the proposed structure for the OSGAG is given in Fig. 1(d).

In order to know the behaviour of mucopolysaccharides employed as chiral selectors in CE under the used electrophoretic conditions, the effective mobilities of heparin, chondroitins sulfate and chemically OSGAG were experimentally measured and showed in Table 1. The mobility of the electroosmotic flow was evaluated by the migration of acetone. With a detection wavelength of 200 nm and hydrodynamic injection (50 mbar, 10 s), broad peaks were obtained for each injected sample but it was possible to determine the migration of the studied mucopolysaccharides; due to the microheterogeneity in the proportion of sulfated esters, and probably in their location on the GAGs molecule as well as to their polydispersity, the evaluated mobilities were presumably an average measure of the several different single mobilities associated at each fraction defined as GAGs.

Nevertheless it was possible to understand the migration direction of each studied mucopolysaccharide and, according to the indirect method described by Stalcup [6], OSGAG and heparin exhibited similar anodic migration under the described conditions.

3.2. Oversulfated chondroitin as chiral selector

On the basis of these considerations, OSGAG should be useful chiral selector in AEKC preferably under neutral or weakly acidic conditions as described for heparin [6]. Differently, through the study of a limited series of racemic basic drugs (Fig. 2), we obtained the best enantioresolution in strong acidic background electrolyte (pH 2.5) (Fig. 3). The effective mobilities and enantioresolution values (R_s) found for the tested compounds are reported in Table 2; the concentration of chiral selector was 3% and all

Table 1
Characteristics of some glycosaminoglycans (GAGs)[a]

GAGs	Molecular mass	Sulfation index	μ_e (10^{-4} cm^2 s^{-1} V^{-1})
Chondroitin 6-sulfate	20 000–50 000	1.05	−2.88
Dermatan sulfate	15 000–30 000	1.10	−2.97
Heparin	7000–20 000	2.50	−3.92
Oversulfated chondroitin	10 000–20 000	2.66	−3.90

[a] The mobility values are determined in uncoated capillary, 50 μm×40 cm effective length; temperature, 15°C; detection 200 nm; background electrolyte: 30 m*M* phosphate buffer, pH 2.5; Voltage: −15 kV.

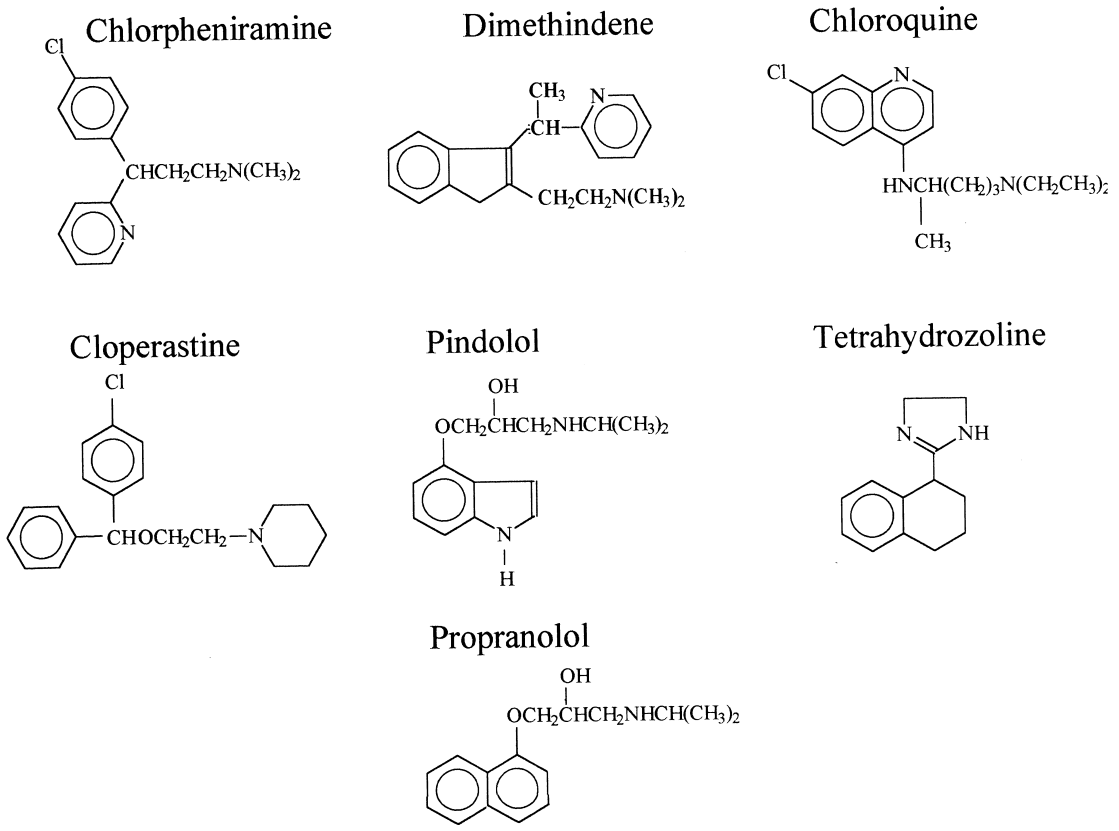

Fig. 2. Structures of the tested solutes.

the others electrophoretic conditions are described in the table's caption.

In the absence of a chiral selector, the drugs tested under the same conditions showed high catodic mobilities. Since the best enantioselectivity provided by mucopolysaccharides in CE [6–12] was obtained in the concentration range of 2–3%, a 3% solution of oversulfated chondroitin was prepared in phosphate buffer (30 m*M*, pH 2.5). As shown in Table 2, in the presence of OSGAG the migration times were generally long owing to the interactions selector-selectand; a very slow electroosmotic flow ($1.8 \cdot 10^{-5}$ $cm^2\ s^{-1}\ V^{-1}$), in fact, gives net anodic migration of the chiral selector. Interestingly, chlorpheniramine, dimethindene and chloroquine migrated in anodic direction in a very short time and exhibited good peak shapes with resolution value (R_s)≥1. The presence in their molecules of a basic quinolinic or pyridinic nitrogen makes these compounds positively charged and hence strongly attracted to the chiral selector. Tetrahydrozoline shows the strongest basicity of all the studied analytes, nevertheless it displays catodic mobility and relatively short migration time. This behaviour suggests that the role of ionic interactions cannot be considered the unique explanation to the mucopolysaccharides-binding affinity of the analytes; the spatial requirements of the chiral selector in combination with hydrogen bonding, hydrophobic interactions and the localization of the nitrogens of the analyte, must be part of the stereospecificity of the GAGs. It should be pointed out that the basic drugs examined in this study, previously were not enantioresolved when chondroitin B was used as chiral selector [9]. Therefore, this class of semisynthetic galactosaminoglycans appears to offer specific applications fields according to the sulfation index under appropriate experimental conditions.

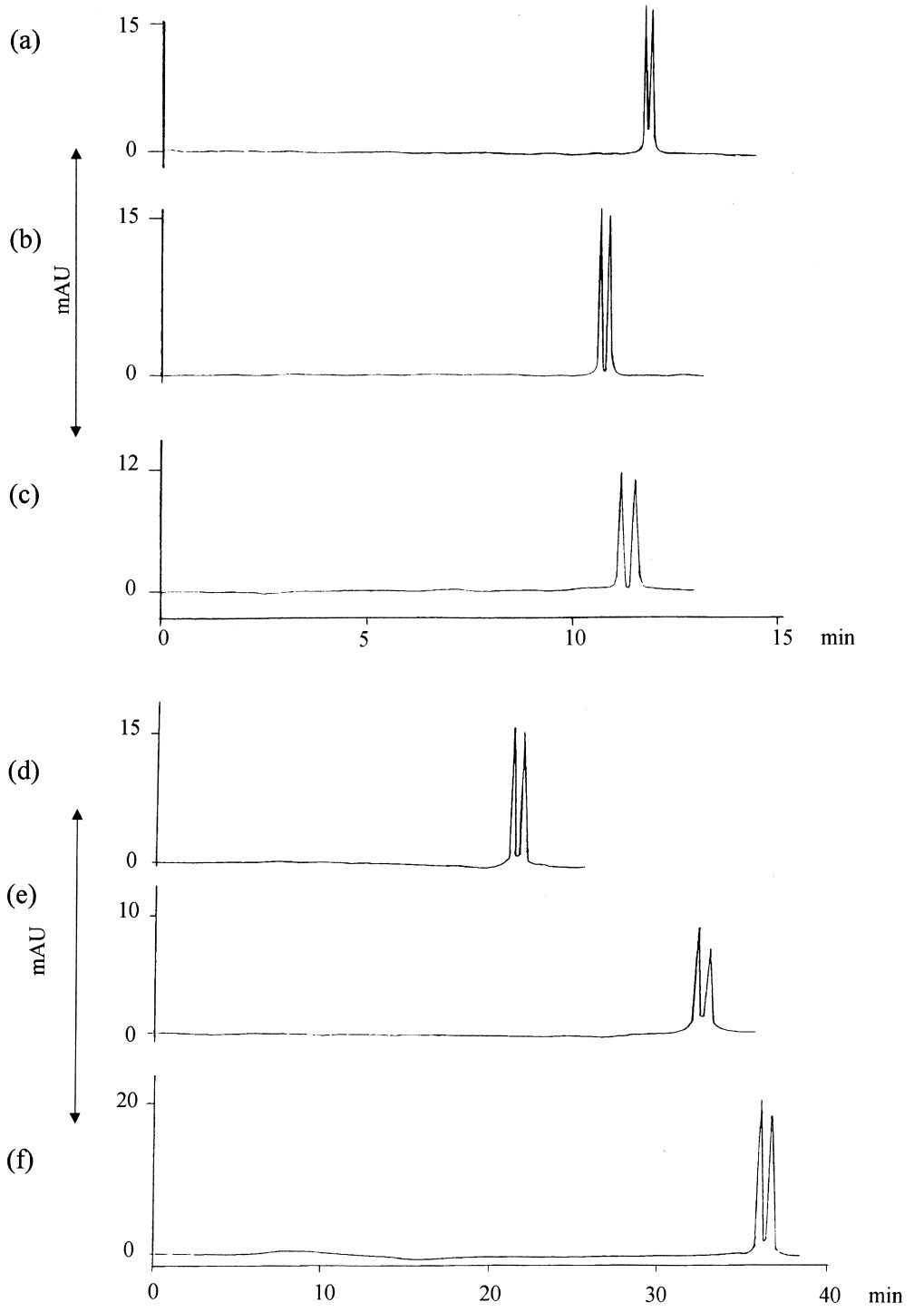

Fig. 3. Separation of enantiomers of: (a) chlorpheniramine, (b) dimethindene, (c) chloroquine, (d) tetrahydrozoline, (e) cloperastine, (f) pindolol. Conditions: 3% oversulfated chondroitin in 30 m*M* phosphate buffer (pH 2.5); uncoated capillary, 48.5 cm (40 cm effective length)×50 μm I.D.; detection wavelength, 220 nm; temperature, 15°C; injection time 10 s; applied voltage, −15 kV for (a), (b), (c) and +15 kV for (d), (e) and (f).

Table 2
Migration time (t_m)[a], effective mobility (μ_e) and enantioresolution value (R_s) of the studied basic racemates

Analyte	t_m (min)	μ_e (10^{-5} cm^2 s^{-1} V^{-1})	R_s
Propranolol	53.3	2.2	1.1
Pindolol	39.3	3.7	1.5
Tetrahydrozoline	21.8	8.1	1.6
Cloperastine	35.6	4.2	1.4
Clorpheniramine[c]	12.1	−19.8	1.2
Dimethindene[c]	10.7	−21.8	1.9
Chloroquine[c]	11.2	−21.0	1.0

[a] Migration time relative to the first peak.
[b] Conditions: uncoated capillary, 50 μm×40 cm effective length; temperature, 15°C; detection 220 nm; background electrolyte: 30 m*M* phosphate buffer containing 3% OSGAG, pH 2.5; Voltage: 15 kV.
[c] Voltage: −15 kV.

Since the mode of the enantioseparation based on the use of GAGs is defined as AEKC, the addition of organic solvent to the background electrolyte could affect the chiral recognition. Among the tested solvents, methanol provided the best results.

With 15% methanol (Table 3) an increase in the enantioresolution was observed for dimethindene and chloroquine; they exhibited a good enhancement of the resolution value (130% for chloroquine) without a significant increasing in the analysis time. Nevertheless for all the other compounds a limited improved resolution was obtained at the expense of long migration time. Unique exception was displayed with propranolol where a 15% level of methanol resulted in a significant increase of mobility with an improvement in peak shape (Fig. 4).

3.3. Comparison heparin/oversulfated chondroitin

The evaluation of the enantioselectivity of natural mucopolysaccharides in the same electrophoretic conditions employed for the OSGAG could be of interest in order to clarify the real effectiveness of the new semisynthetic glycosaminoglycan. Actually dermatan sulfate (chondroitin sulfate B) has been studied in acidic conditions (pH 3.0) [9] showing good enantioresolution values mainly on hydrophilic compounds, on the other hand chondroitin sulfate C exhibited good performance as chiral selector at both acidic (pH 2.7) and neutral (pH 6.5) conditions [10]. It seemed of interest to compare heparin and oversulfated chondroitin since they show a strong similitude in terms of molecular mass and sulfation index (Table 1). For these reasons, differences in the behaviour of the two glycosaminoglycans as chiral selector could be ascribed to their specific spatial arrangement and their ability to give hydrogen bonding.

Heparin already described as a useful chiral selector in CE [6], showed the best results in the

Table 3
Migration time and enantioresolution values for the studied basic racemates in the presence of methanol. Other conditions and symbols as in Table 2

Analyte	7% MeOH		15% MeOH	
	t_m (min)	R_s	t_m (min)	R_s
Propranolol	43.0	1.0	43.2	1.2
Pindolol	39.6	1.7	52.1	2.0
Tetrahydrozoline	20.8	1.3	29.3	1.3
Cloperastine	35.4	1.2	53.4	1.4
Chlorpheniramine[a]	16.1	1.2	18.8	1.1
Dimethindene[a]	13.7	2.1	16.8	2.6
Chloroquine[a]	14.2	1.0	19.7	2.2

[a] Voltage: −15 kV.

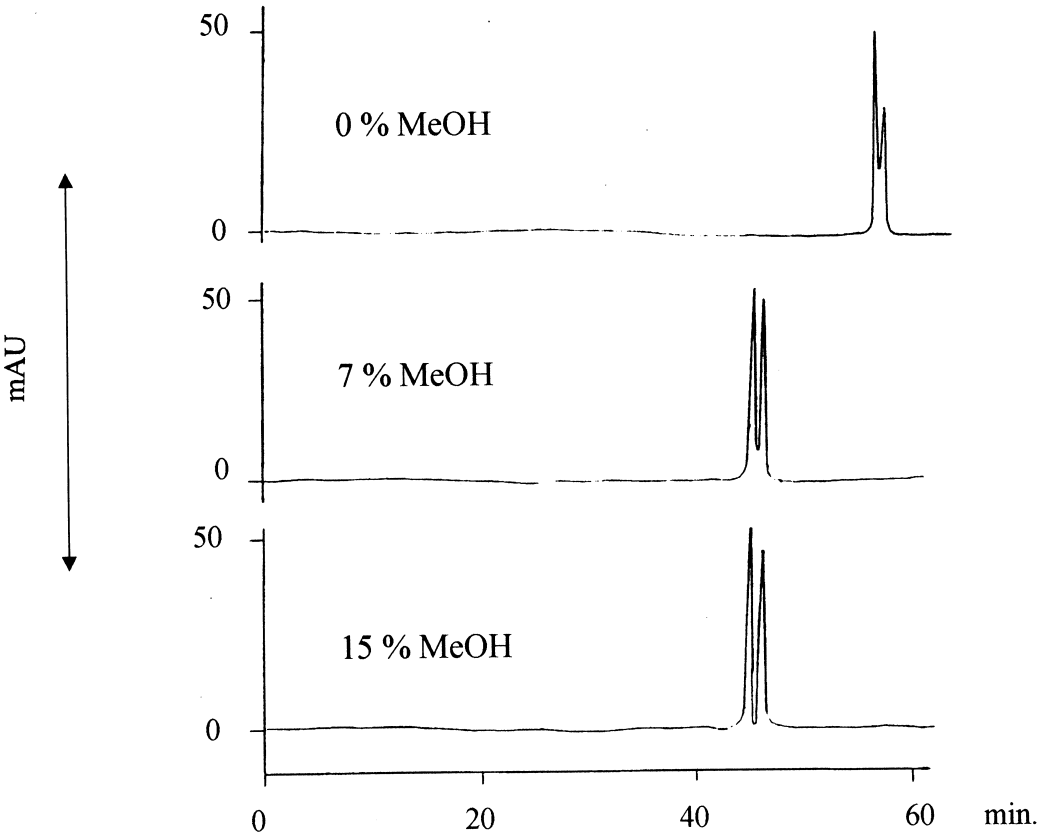

Fig. 4. Effect of methanol on enantioseparation of propranolol: conditions as in Fig. 3.

enantioresolution of racemic basic drugs under weakly acidic conditions. When heparin at 3% level was used in phosphate buffer solution at pH 2.5, an higher electroosmotic flow ($9.7 \cdot 10^{-5}$ cm^2 s^{-1} V^{-1}) was measured compared with that obtained in the presence of OSGAG and the tested compounds showed different behaviour using either heparin or OSGAG. With heparin as chiral selector, chlorpheniramine and dimethindene did not migrated within 60 min with both positive and negative applied voltage, chloroquine (anodic migration), tertahydrozoline and cloperastine (cathodic migration) exhibited a loss of resolution, while propranolol and pindolol displayed a weak improvement of separation coupled with a stronger effective mobility (migration times of the first migrating enantiomer: propranolol=15 min, pindolol=17.5 min) respect to the results obtained using OSGAG (Table 4). In order to verify the enantioselectivity of these two mucopolysaccharides toward arylossipropanolamines, other racemic β-blocker drugs were tested under the same described conditions (acebutolol, atenolol, alprenolol and timolol): no enantioresolutions were obtained using

Table 4
Effective mobility (μ_e) and enantioresolution value (R_s) of the studied basic racemates using heparin 3% as chiral selector. All the other conditions as in Table 2

Analyte	μ_e (10^{-5} cm^2 s^{-1} V^{-1})	R_s
Propranolol	3.7	1.2
Pindolol	2.7	1.3
Tetrahydrozoline	7.3	0.8
Cloperastine	2.0	<0.8
Chlorpheniramine[a]	/[b]	–[c]
Dimethindene[a]	/	–
Chloroquine[a]	−18.8	<0.8

[a] Voltage: −15 kV.
[b] /: did not migrate within 60 min.
[c] –: not determined.

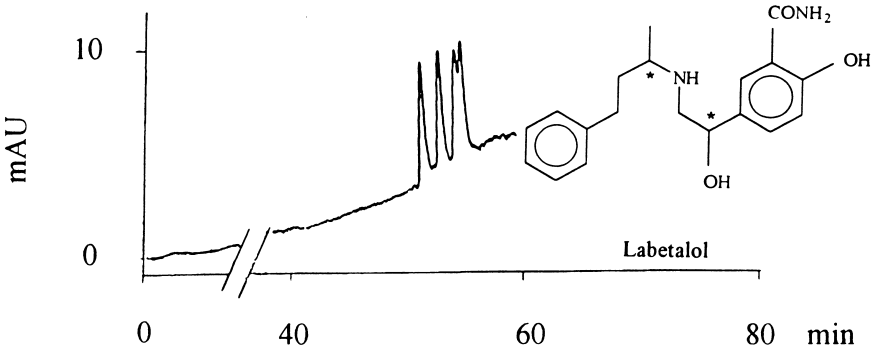

Fig. 5. Enantioseparation of labetalol using OSGAG as chiral selector. Applied voltage: 15 kV; other conditions as in Fig. 3.

both heparin and OSGAG, while the anti-α,β-adrenergic drug labetalol (with two chiral centers) was almost completely splitted into the four stereoisomers employing oversulfated chondroitin (Fig. 5).

The electrophoretic behaviour (mobility and enantioresolution) showed by the tested analytes using OSGAG and heparin as chiral selectors, reflects differences that could be related to interactions selector-selectand and to the effect of the different measured electroosmotic flows. Since the ionic character of the two studied mucopolysaccharides is comparable, the nature of the interactions between analyte and chiral selector could be ascribed mainly to hydrophobic forces, hydrogen-bonding and to the different steric effects.

4. Conclusion

Oversulfated galactosaminoglycan shows stronger ionic interactions than others used mucopolysaccharides with basic racemic drugs. When used in acidic buffer (pH 2.5) OSGAG exhibited enantioselectivity for some studied compounds. Short analysis times were achieved for basic compounds containing quinolinic or pyridinic ring (anodic migration) whereas long migration time were obtained in the enantioresolution of some tested drugs (catodic migration). When heparin was used in the same electrophoretic conditions a general loss of enantioresolution was obtained respect to the results reached with OSGAG. This limited comparative study suggested that in addition to the ionic character, the features of the saccharide residue of the ionic biopolymer could be important factors in the enantiorecognition. On the other hand the chemical oversulfation of some preparation of chondroitins was part of structure–activity relationship studies aimed to discover new potentially useful antihemocoagulative drugs. In fact the biological activity of GAGs is due to the specific and non-specific interactions with proteins and are related to the composition, sequence, charge density, molecular mass, position of the sulfate groups, type of uronic acid and conformation of the polysaccharides [17].

The effect of methanol in the running buffer on the enantioseparation was also considered using OSGAG as chiral selector: except propranolol, the use of methanol resulted in an improvement in the resolution associated to an increased migration time for the studied drugs.

Studies are in progress to better define the application field of the class of chiral selectors in capillary electrophoresis.

Acknowledgements

Thanks are due to Mr. Amedeo Luppi for his valuable technical assistance. This work was supported by MURST (Rome, Italy).

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