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Capillary electrophoresis of heparin and dermatan sulfate unsaturated disaccharides with triethylamine and acetonitrile as electrolyte additives

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Abstract

Capillary electrophoresis at constant voltage with the addition of triethylamine as electrolyte to a running buffer containing borate using fused-silica capillaries permits the complete resolution in less than 30 min of 11 standard heparin and 8 standard dermatan sulfate disaccharides, which represent degradation products of heparin and dermatan sulfate by specific lyases. Triethylamine influences the migration time of disaccharides by reducing both their electrophoretic mobility towards the anode and the electroosmotic flow towards the cathode. A modulated combination of these effects together with borate–disaccharide complex formation is responsible for separation, especially in the case of isomers which differ in the position of the sulfate groups. The addition of acetonitrile did not introduce any favourable effect in the separation of disaccharide mixtures. Under these conditions different dermatan sulfates were analysed to assess the source of the preparations.

Keywords: Capillary electrophoresis; Buffer composition; Heparin disaccharides; Dermatan sulphate disaccharides; Disaccharides

1. Introduction

Heparin (HEP) and dermatan sulfate (DES), which belong to the family of glycosaminoglycans (GAGs), are linear, highly charged polysaccharides with a molecular mass in the range 10 000–30 000.

HEP is composed of alternating 1–4-linked glucosamine (GlcN) and uronic acid (UA) residues, while DES consists of alternating 1–3-linked N-acetylgalactosamine (GalNAc) and UA. In both cases GlcN, GalNAc and UA may contain sulfate (S) groups on the hydroxyls, while the amine group of GlcN can be free, acetylated or sulfated [1].

HEP and DES exhibit a variety of biological properties which stem from their polyelectrolytic nature [2]. HEP, extracted from mast cell granules, has been demonstrated to be clinically effective as an anticoagulant [3,4]. DES has been under investigation for clinical use for several years, since it showed peculiar biological properties as an antithrombotic agent, increasing the rate of inhibition of thrombin by activation of heparin-cofactor II without the concomitant bleeding risk associated with HEP [5–8].

Due to the microheterogeneous nature of these polymers, structure determination is a

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demanding task mainly involving the study of structure–activity correlation [9–11].

A well-known strategy to analyse these molecules is to use exhaustive enzymatic degradations with heparin- and chondroitin-lyases, converting them to unsaturated disaccharides [12–14].

Even in that case, however, the similar physicochemical features of the resulting dimers and the presence of unavoidable impurities still make the analysis difficult.

Anion-exchange chromatography [12,15,16], reversed-phase ion-pairing chromatography [17] and polyacrylamide gel electrophoresis [18] have been reported to be useful in HEP and DES disaccharide mixture analysis. However, complete resolution of complex mixtures can only be obtained with anion-exchange chromatography; however, the long run-times, the dependence of the separation performance on column aging and the need to inject relatively large amounts of expensive standard disaccharides do not make this technique the most appropriate one.

Capillary electrophoresis (CE) has been introduced as a useful technique to analyse complex mixtures of inorganic ions, small organic molecules, carbohydrates, peptides and proteins [19,20]. In particular, CE is effective in the analysis of enzymic-derived dimers of GAGs with untreated fused-silica capillaries at normal and reversed polarity [21–25]. However, mixtures of HEP or DES dimers were resolved only under conditions that give long run-times (over 50 min), resulting in severe band-broadening.

With the aim to improve the performance of CE in disaccharide mixture separations, the effects of alkylamine and organic solvents, separated or combined, as electrolyte additives in the running buffer (RB) were evaluated.

2. Experimental

2.1. Apparatus

The experiments were performed with a P/ACE 2100 instrument, operated under System Gold control, data acquisition and analysis software (Beckman, Fullerton, CA, USA).

The electrophoretic separations were carried out using fused-silica capillaries of 75 and 50 μm I.D. (Beckman) and with a total length of 57 cm (50 cm to the detector), thermostatted at 25 ± 1°C. The detector was equipped with a deuterium light source with bandpass filter of 214 nm.

2.2. Materials

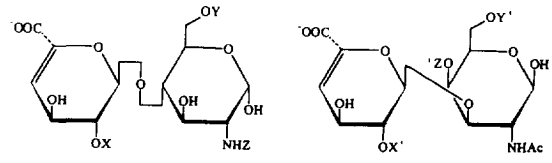
The standard disaccharides of HEP and DES used in the present work were the following. For HEP: ΔUA2S-(1→4)-D-GlcNS6S (IS), ΔUA-(1→4)-D-GlcNS6S (IIS), ΔUA2S-(1→4)-D-GlcNS (IIIS), ΔUA-(1→4)-D-GlcNS (IVS), ΔUA2S-(1→4)-D-GlcNAc6S (IA), ΔUA-(1→4)-D-GlcNAc6S (IIA), ΔUA2S-(1→4)-D-GlcNAc (IIIA), ΔUA-(1→4)-D-GlcNAc (IVA), ΔUA2S-(1→4)-D-GlcN6S (IH), UA-(1→4)-D-GlcN6S (IIH), ΔUA2S-(1→4)-D-GlcN (IIIH). For DES: ΔUA2S-(1→3)-D-GalNAc4S6S (ΔDi-triS), ΔUA-(1→3)-D-GalNAc4S6S (ΔDi-S_E), ΔUA2S-(1→3)-D-GalNAc6S (ΔDi-S_D), ΔUA2S-(1→3)-D-GalNAc4S (ΔDi-S_B), ΔUA2S-(1→3)-D-GalNAc (ΔDi-UA2S), ΔUA-(1→3)-D-GalNAc6S (ΔDi-6S), ΔUA-(1→3)-D-GalNAc4S (ΔDi-4S), ΔUA-(1→3)-D-GalNAc (ΔDi-0S). The structures are given in Fig. 1.

The HEP dimers were obtained from Sigma Chimica (Milan, Italy), while the DES dimers were purchased from Seikagaku (Tokyo, Japan).

Since the poor commercial availability of HEP and DES disaccharide standards precludes accurate weighing, equimolar solutions of each standard were prepared on the basis of amounts which gave a 0.01 absorbance at 232 nm [24], roughly corresponding to 25 μg/ml.

Porcine DES from mucosa and from skin were obtained from Opocrin (Corlo, Italy). Chondroitin ABC lyase (EC 4.2.2.4) was obtained from Sigma Chimica. Sodium tetraborate, boric acid, acetonitrile (ACN), sodium hydroxide, triethylamine (TEA) were obtained from Carlo Erba (Milan, Italy); acrylamide and Tris buffer from Bio-Rad (Milan, Italy). Water was purified with a SG Reintwasser System RS80-4UF (Barsbüttel, Germany).

Depolymerisation of porcine DES from mu-



Heparin dimers

Dermatan dimers

DIMERS	X'	Y'	Z'	Net* Charge
I S	SO_3	SO_3	SO_3	-4
II S	H	SO_3	SO_3	-3
III S	SO_3	H	H	-3
IV S	H	H	SO_3	-2
I A	SO_3	SO_3	Ac	-3
II A	H	SO_3	Ac	-2
III A	SO_3	H	Ac	-2
IV A	H	H	Ac	-1
I H	SO_3	SO_3	H	-3
II H	H	SO_3	H	-2
III H	SO_3	H	H	-2

DIMERS	X'	Y'	Z'	Net* Charge
Δ DI-Tris	SO_3	SO_3	SO_3	-4
Δ DI-S_E	H	SO_3	SO_3	-3
Δ DI-S_D	SO_3	SO_3	SO_3	-3
Δ DI-S_B	SO_3	H	SO_3	-2
Δ DI-UA2S	SO_3	H	H	-2
Δ DI-6S	H	SO_3	H	-2
Δ DI-4S	H	H	SO_3	-2
Δ DI-OS	H	H	H	-1

* at pH > 9

Fig. 1. Chemical structures of HEP and DES unsaturated disaccharides.

cosa and from skin was carried out as follows: 5 μl of 10 IU/ml of chondroitinase ABC and 30 μl of 50 m*M* Tris buffer were added to 10 μl of 10 mg/ml of DES. The mixture was kept at 37°C for 2 h, then the enzymic reaction was stopped by boiling for 1 min. The resulting mixtures were stored frozen at −20°C.

2.3. CE analysis

New capillaries were usually activated by washing with 0.5 *M* sodium hydroxide for 20 min, distilled water for 20 min, and rinsed for 30 min with RB.

CE was performed at a constant voltage of 30 kV. The samples of the standard dimers were loaded for 5 s at a pressure of $3.45 \cdot 10^3$ Pa (0.5 p.s.i.), equivalent to ca. 45 nl of injected solution [26]; mixtures of hydrolysed DES were loaded for 20 s at a pressure of $3.45 \cdot 10^3$ Pa (0.5 p.s.i.), equivalent to ca. 180 nl of injected solution.

The electroosmotic flow (EOF) was determined by measuring the migration time (t_m) of acrylamide. The values of EOF were calculated according to the following equation:

$$\mathrm{EOF} = \frac{L_D L_T}{t_m V}$$

where L_D and L_T are the length to the detector and total length of capillary, respectively, and V is the applied voltage.

A sample of 1 mg/ml of acrylamide is loaded for 5 s at a pressure of $3.45 \cdot 10^3$ Pa (0.5 p.s.i.).

Prior to each run the capillaries are conditioned for 2 min at reversed polarity and 2 min at normal polarity. At the end of each run the capillaries are washed with 1 *M* sodium hydroxide for 2 min.

In Table 1 the composition of the RBs is given. All RB solutions are filtered through 0.22-μm HA membrane filters (Millipore, Italy) and degassed by helium purging prior to use. The identity of each compound in the disaccharide mixtures is assessed by coinjection of the proper disaccharide standard.

3. Results

The effect of the addition of TEA to RBs containing borate was a reduction of the EOF depending on the TEA concentration. ACN (10%, v/v) also reduced the EOF, independently of the presence of TEA. The reductions of the

Table 1
Running buffer composition

	Composition	pH
RB-1	50 m*M* sodium borate, 10 m*M* boric acid	8.8
RB-2	50 m*M* sodium borate, 10 m*M* boric acid, 50 m*M* TEA	9.0
RB-3	50 m*M* sodium borate, 10 m*M* boric acid, 100 m*M* TEA	9.4
RB-4	50 m*M* sodium borate, 10 m*M* boric acid, 200 m*M* TEA	10.4
RB-5	50 m*M* sodium borate, 10 m*M* boric acid, 10% (v/v) ACN	8.8
RB-6	50 m*M* sodium borate, 10 m*M* boric acid, 50 m*M* TEA, 10% (v/v) ACN	9.0
RB-7	50 m*M* sodium borate, 10 m*M* boric acid, 100 m*M* TEA, 10% (v/v) ACN	9.5
RB-8	50 m*M* sodium borate, 10 m*M* boric acid, 200 m*M* TEA, 10% (v/v) ACN	10.5

EOF were more marked with the 75-μm I.D. capillary (Table 2).

In Tables 3 and 4 the migration times of HEP and DES standard disaccharides are reported, obtained with RB-1 to RB-8 in 50 μm I.D. and 75 μm I.D. capillaries, respectively.

Only with RB-4 and the 50 μm I.D. capillary were all 11 standard dimers of HEP and 8 standard dimers of DES completely resolved in less than 30 min (Figs. 2A and 2B).

The disaccharides are eluted according to their net charge, and no inversion of the migration order was detected under any of the experimental CE conditions.

Progressive increase of the TEA concentration up to 200 m*M* generally had a net effect of increasing the migration times of the compounds. More complex is the effect of ACN and TEA together; they maximize HEP and DES dimer migration times with RB-7 in the 50 μm I.D. capillary, while in the 75 μm I.D. capillary the highest migration times for HEP and DES dimers were obtained with RB-6 and RB-7, respectively.

Table 2
Values of EOF with different RBs in the 50 μm and 75 μm I.D. capillaries

	EOF ($\times 10^{-8}$ m^2 V^{-1} s^{-1})	
	50 μm I.D.	75 μm I.D.
RB-1	5.63	6.6
RB-2	5.44	6.3
RB-3	4.97	5.8
RB-4	4.79	4.9
RB-5	5.4	4.8
RB-6	4.7	4.1
RB-7	4.3	3.8
RB-8	3.4	3.4

The optimum CE performance conditions, i.e. with RB-4 and the 50 μm I.D. capillary, were used to check differences in DES from different sources. The disaccharide mixture obtained from porcine intestinal mucosa after chondroitinase ABC digestion was compared to that from porcine skin. In Fig. 3 the electropherograms of the two samples are reported; here the absence of the disaccharide ΔDI-S_E in the product extracted from skin is noticeable.

4. Discussion and conclusion

In the present work optimal CE conditions were found to completely resolve the 11 HEP and 8 DES standard disaccharides in less than 30 min.

In the choice of HEP standard dimers, we included, in addition to those used in other reports [13,23–25,27], the N-deacetylated or N-desulfated disaccharides IH, IIH, and IIIH, since these compounds could be present in enzymatically hydrolysed mixtures of natural GAGs, even if less frequently [1].

TEA has a relevant role in affording the best performance, influencing the separation mechanism and the absolute t_m values of the disaccharides.

All tested compounds have a net negative

Table 3
Migration times of HEP and DES dimers with the 50 μm I.D. capillary

Dimer	Migration time (min)[a]							
	RB-1	RB-2	RB-3	RB-4	RB-5	RB-6	RB-7	RB-8
HEP								
IVA	3.82	4.04	4.73	4.89	3.12	3.92	6.71	5.41
IIIA	5.60	5.50	6.03	7.06	3.90	5.07	9.40	7.93
IIA	5.60	5.50	6.26	7.28	4.10	5.07	9.61	8.13
IVS	5.80	5.90	6.40	7.71	5.44	5.07	10.23	8.13
IIIH	5.80	5.90	6.76	8.07	5.44	5.34	10.44	8.68
IIH	5.90	5.90	6.99	8.27	5.61	5.43	10.84	8.99
IA	9.06	8.04	9.36	10.93	8.36	7.17	17.28	12.45
IH	9.90	9.00	10.94	13.35	8.91	11.53	22.83	14.99
IIIS	10.30	9.34	11.60	13.92	9.22	11.53	24.50	15.92
IIS	10.30	9.34	11.60	14.25	9.22	11.53	24.50	16.49
IS	22.46	15.48	20.30	26.46	16.54	13.61	ne[b]	31.60
DES								
Di-0S	3.92	4.18	nr[c]	5.45	4.09	5.05	6.66	5.49
Di-4S	5.39	5.70	nr[c]	7.35	5.70	6.69	9.66	7.50
Di-6S	5.48	5.75	nr[c]	7.44	5.80	7.12	9.84	7.67
Di-UA2S	5.76	5.98	nr[c]	7.97	5.95	7.56	11.06	8.01
Di-S_E	8.35	8.40	nr[c]	11.26	8.73	11.02	17.95	11.80
Di-S_B	8.93	8.68	nr[c]	11.31	9.13	11.57	19.05	12.11
Di-S_D	9.09	8.68	nr[c]	11.68	9.34	11.57	19.68	12.31
triS	17.50	13.35	nr[c]	18.49	18.41	20.55	ne[b]	19.86

[a] Relative standard deviation (R.S.D.) in all the experiments was <5%.
[b] Not eluted.
[c] Not reported because of R.S.D. > 5%.

charge ranging from −1 to −4; therefore, at normal polarity they should migrate to the anode and be detected at the cathode because of the presence of a significant EOF.

Since adding TEA to RB-1 results in a reduction of the EOF (Table 2), probably by masking the negatively charged silanol groups of the capillary wall [28], an increase in the t_m values of the disaccharides should be expected.

The data reported in Tables 3 and 4 contradict this forecast, particularly for the highly charged disaccharides, for which a reduction in the t_m values is observed.

This behaviour could be explained by postulating the presence of an additional ion-pairing mechanism by which TEA partially neutralizes the negative charges of the disaccharides, reducing their electrophoretic mobilities to the anode.

As expected, the addition of 10% (v/v) ACN reduces the EOF [29]; however, even with increased t_m values, the selectivity of the separation of the disaccharides worsened, compared with that previously found [24].

The efficiency of CE in disaccharide analysis is particularly evident in the separation of those configurational isomers which differ only in the position of an S group, as in IIA/IIIA, IIH/IIIH and IIS/IIIS of HEP dimers and in ΔDi-4S/ΔDi-6S/ΔDi-UA2S and ΔDi-S_E/ΔDi-S_B/ΔDi-S_D in DES dimers.

It is interesting to note how the formation of anionic complexes between the disaccharide hydroxyls and borate [20,22,30] could explain, at least in part, the migration order. In the case of HEP disaccharides, the compounds IIA, IIH and IIS, which migrated later, contain unsulfated UA with respect to IIIA, IIIH and IIIS, thus permitting complex formation between a borate mole-

Table 4
Migration times of HEP and DES dimers with the 75 μm I.D. capillary

Dimer	Migration time (min)[a]							
	RB-1	RB-2	RB-3	RB-4	RB-5	RB-6	RB-7	RB-8
HEP								
IVA	3.43	3.35	3.89	nr[b]	3.16	5.31	4.91	4.55
IIIA	4.76	4.81	5.67	nr[b]	4.52	6.42	7.70	7.36
IIA	4.95	4.97	5.75	nr[b]	5.58	8.93	7.80	7.44
IVS	4.97	5.12	6.12	nr[b]	7.15	8.93	8.42	8.11
IIIH	5.15	5.35	6.12	nr[b]	7.15	9.85	8.42	8.39
IIH	5.21	5.45	6.28	nr[b]	7.61	9.85	8.65	8.45
IA	7.77	7.60	8.81	nr[b]	12.75	18.05	13.44	12.49
IH	8.41	8.49	10.40	nr[b]	14.09	18.05	16.71	15.65
IIIS	8.56	8.80	10.88	nr[b]	14.60	25.00	17.22	16.17
IIS	8.56	8.80	10.88	nr[b]	14.60	25.00	17.22	16.17
IS	16.42	15.09	21.21	nr[b]	ne[c]	ne[c]	ne[c]	ne[c]
DES								
Di-0S	4.50	3.03	4.12	4.33	4.84	4.75	5.03	5.03
Di-4S	4.80	5.50	5.68	5.83	7.35	6.84	7.12	6.80
Di-6S	4.90	5.58	5.77	5.91	7.53	7.13	7.19	6.95
Di-UA2S	5.08	5.73	6.01	6.20	7.83	7.36	7.51	7.47
Di-S_E	7.52	8.31	8.71	8.66	13.49	10.66	11.02	10.42
Di-S_B	7.94	8.53	8.93	8.80	14.88	11.01	11.38	10.83
Di-S_D	7.94	8.53	9.05	8.97	15.01	11.01	11.38	10.97
triS	16.43	14.60	14.59	13.70	ne[c]	ne[c]	20.19	18.73

[a] Relative standard deviation (R.S.D.) in all the experiments was <5%.
[b] Not reported because of R.S.D. > 5%.
[c] Not eluted.

cule and the C_2, C_3 hydroxyls of UA. This complex confers an additional, partial negative charge to the disaccharide, thus explaining the difference in migration time compared with the corresponding isomer that cannot form the same complex.

This hypothesis is indirectly confirmed in reversed-polarity CE, where in the absence of borate the migration velocity to the cathode is higher for the dimers IIIA and IIIS with respect to IIA and IIS [25,27,31].

Concerning the migration order obtained with the dimers ΔDi-6S, ΔDi-4S and ΔDi-UA2S, it was noted that ΔDi-6S and ΔDi-4S can form a five-membered ring complex with the C_2, C_3 hydroxyl groups of UA, while ΔDi-UA2S could form a six-membered ring complex with the C_4, C_6 hydroxyl groups of GalNAc.

Since the former complex should be less stable due to the five-membered ring and the destabilisation by the close, negative charge of the carboxylate group of UA, it can induce a reduced electrophoretic mobility to the anode with respect to the latter complex.

The hypothesis of borate complex formation to justify the migration order of GAG disaccharides is not supported by the compounds ΔDi-S_D, ΔDi-S_B and ΔDi-S_E; in fact, the only one which can form the borate complex, ΔDi-S_E, should move to the cathode slower than the other ones, while the opposite migration order was found.

However, in the case of ΔDi-S_D, ΔDi-S_B and

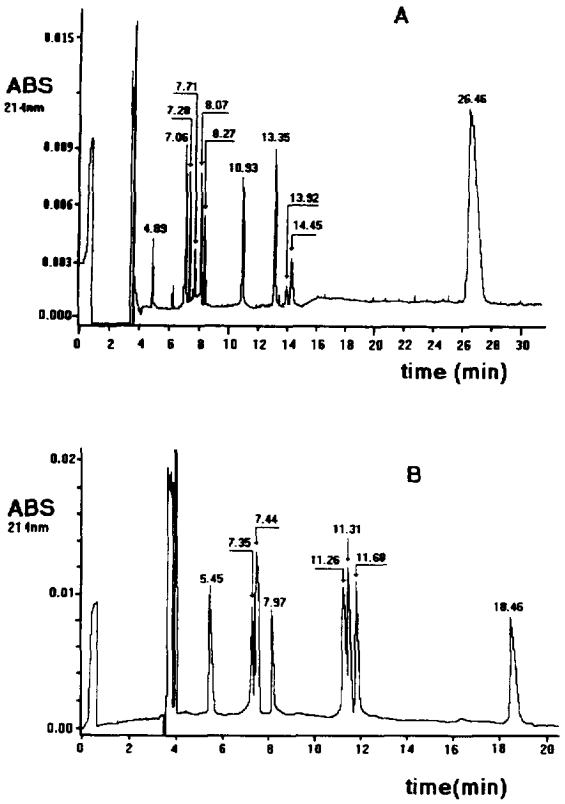


Fig. 2. Electropherograms of HEP (A) and DES (B) standard disaccharides with RB-4. CE was carried out in normal mode polarity at 30 kV, at 25 ± 1°C, with detection at 214 nm. Column size, 57 cm (50 cm effective length to the detector) × 50 μm I.D. Injection by pressurised nitrogen; 45 nl from the solutions containing around 25 μg/ml of standard dimers were loaded.

ΔDi-S_E, it can be stressed that their electrophoretic behaviour in CE seems unpredictable, since a different migration sequence under similar experimental conditions has been reported [22,25], suggesting that some variability exists, which has a decisive role in determining the electrophoretic mobility.

Finally, as shown in Fig. 3, the effectiveness of CE in analysing DES from different sources can be stressed, which is evidenced by e.g. the finding of the absence in the skin DES of the dimer ΔDi-S_E, which has been correlated with the activity of DES to potentiate heparin cofactor II activation [32].

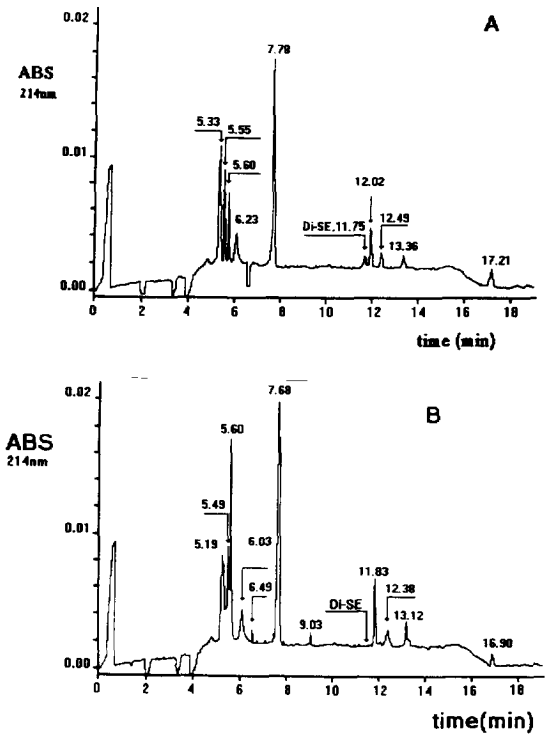


Fig. 3. Comparison of the electropherograms of DES extracted from porcine intestinal mucosa (A) and from porcine skin (B). CE was carried out as in Fig. 2 except that 180 nl of chondroitinase ABC-hydrolysed DES solutions (10 mg/ml) from porcine mucosa and porcine skin were loaded.

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