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Coupling high-performance size exclusion and ion chromatography for the analysis of low-molecular-mass heparin

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Abstract

A column-switching method, coupling high-performance size exclusion chromatography (HPSEC) and ion chromatography (IC), was developed for the analysis of glycosaminoglycans. Since the requirements of the recommended mobile phases for each system are considerably different, mobile phase composition and transfer conditions were studied in order to enable characterization of both the molecular mass averages and the free anion contents in the sample by coupling HPSEC and IC systems. Electrolyte solutions of ionic strength higher than 0.1–0.2 *M* are usually used with derivatized silica-based TSK-GSW (Supelco) columns in order to avoid extra-SEC effects. In this work, an HPSEC eluent of low ion strength provided mass averages comparable to those obtained in more concentrated eluents, and enabled the transfer of elution fractions to the IC system. Switching-intervals were studied to select the optimum transfer fractions for the determination of sulphate and phosphate. Low detection limits, 50 ng and 100 ng, good repeatability values (relative standard deviation, %R.S.D.), 1.56% and 3.37%, and long-term precision values (%R.S.D.), 2.42% and 3.50% of the coupled method, were obtained for sulphate and phosphate respectively. The method was applied to the analysis of a low-molecular-mass heparin.
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1. Introduction

Heparin, a widely used anticoagulant agent, is a polydisperse heterogeneous sulphated glycosaminoglycan, which is biosynthesised as a proteoglycan consisting of a small core protein to which polysaccharide side-chains are attached [1]. Heparin consists of sequences of the disaccharide units of uronic acid (β-D-glucuronic or α-L-iduronic acid) and β-D-glucosamine residues linked by α(1→4) bonds [2]. The glucosamine unit can be either N-acetylated or N-sulphated. These disaccharide units can be O-sulphated at C-6 and/or C-3 of glucosamine and also at C-2 of acid residues [3,4]. Two aspects contribute to the heterogeneity of heparin chains: the variety in the glycosaminoglycan chain length, which is responsible for the wide molecular mass range (3000–40 000), and the identity of their anionic substituents [2,3,5].

It is known that molecular mass distribution and degree of sulphation are closely related to the biological properties of heparin [6]. Several studies show that low-molecular-mass heparins (LMMHs), whose molecular mass values range from 2 to 8 kDa, have a better ratio of anticoagulant/bleeding effects [7,8]. LMMHs present other advantages, such as

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better bioavailability, longer biological half-life and less side-effects [8–10]. LMMHs are obtained from unfractionated heparin by different methods [11], such as fractional precipitation, gel filtration [12], chemical [9,13] and enzymatic [14] depolymerization yielding heterogeneous preparations. Some of these methods, especially those involving the use of strong reagents, may produce LMMHs with lower sulphation degrees, due to the loss of linked sulphate [11].

Therefore, both characterisation of the molecular mass distribution of LMMH samples and determination of the loss of sulphate substituents are particularly important [6]. Mass characterization of LMMHs has usually been carried out by different techniques, such as high-performance size-exclusion chromatography (HPSEC) [15–25], viscosimetry and ultracentrifugation [21]. Calibration functions required for the calculation of molecular mass averages ($\bar{M}_w$, $\bar{M}_n$, $\bar{M}_z$) by HPSEC can be obtained by different calibration methods [26,27]. Calibration with monodisperse standards is not usually feasible because heparin standards with narrow molecular mass distributions are not frequently available [28]. For that reason, different attempts have been made to solve this problem, such as the use of non-identical standards [16,18] and coupling to low angle laser light scattering [25]. In this study we used a broad standard calibration method for the calibration of the HPSEC system. This requires the use of an LMMH standard whose molecular mass distribution should be previously determined [26,27,29]. Calibration functions were obtained from the known molecular mass distribution and a chromatogram of the standard acquired in the experimental conditions.

Some authors have reported the determination of total sulphate in carbohydrate samples by colorimetric methods after previous hydrolysis [30] or pyrolysis [31,32]. Grotjan et al. determined total and free sulphate contents by ion chromatography (IC) [33]. However, direct injection of heparin samples into the anion-exchange column would dramatically reduce the life of the columns. Coupling HPSEC to IC for the simultaneous determination of both molecular mass distributions and contaminant anions would be particularly convenient, since the use of a column-switching system reduces the total analysis time and the cost of both determinations, which is particularly important for routine quality controls. HPSEC has frequently been coupled to other chromatographic systems as a first chromatographic step to clean up the sample by size separation [34–36]. HPSEC coupled systems to other chromatographic columns have been applied to biological samples with the aim of removing undesired size-fractions [37–41]. However, no analytical information such as molecular mass averages has been obtained from the HPSEC step. Column-switching techniques involve the selective transfer of fractions from a primary column to one of the secondary column, for which reason the primary eluent has to be compatible to some extent with the secondary chromatographic system. Therefore, coupling of HPSEC and non-suppressed IC is particularly difficult, because aqueous HPSEC eluents are commonly high ionic strength solutions [42–44], which are not indicated for transfer to IC columns. In this work optimum mobile phase composition, and transfer fractions for the simultaneous determination of molecular mass averages and free sulphate and phosphate, were studied and the quality parameters of the method were established. The optimized procedure was applied to the analysis of an LMMH sample.

2. Experimental

2.1. Materials

A low-molecular-mass heparin standard with broad mass distribution (molecular mass average $\bar{M}_w$: 4053 and polydispersity 1.30) and a noncommercial LMMH sample were kindly provided by Bioibérica (Palafolls, Barcelona, Spain). Both heparins were obtained from porcine intestinal mucosa. The molecular mass distribution of the standard had previously been obtained by calibration with monodisperse heparin standard characterized by viscosimetry. Working solutions at 0.4% (w/v) in purified water were prepared by dilution of 4% (w/v) solutions.

Sodium nitrate, potassium sulphate and potassium dihydrogenphosphate were obtained from Carlo Erba (Milan, Italy). A 1000 $mg \cdot l^{-1}$ stock solution of each anion was prepared and used for further dilutions. Water purified using a Culligan (Barcelona, Spain) water-purification system and filtered through a 0.45-

μm membrane filter was used for all solutions. Sodium chloride, sodium gluconate (97%), boric acid, glycerine (87%) and acetonitrile [high-performance liquid chromatography (HPLC) grade] were obtained from Merck (Darmstadt, Germany). Sodium tetraborate was obtained from Carlo Erba.

2.2. Instruments

Two LKB (Bromma, Sweden) Model 2150 pumps and two Rheodyne (Cotati, CA, USA) valves – a Model 7125 injection valve (100 μl loop) and a Model 7010 switching-valve (500 μl loop) – with a Microbeam (Barcelona, Spain) pneumatic controller were used. Switching times and valve positions were programmed with an LKB (Bromma) Model 2152 controller. The eluate from the HPSEC column was detected by either a Knauer DR differential refractometer (Bad Homburg, Germany) or an Applied Biosystem (Foster City, CA, USA) Model 757 UV–Vis detector. A Metrohm (Herisau, Switzerland) Model 690 conductivity detector was used for the determination of inorganic anions.

HPSEC data handling was performed with the option "Gel Permeation Chromatography Software", Model 2900 GPC, rev. 5.0 (Perkin Elmer, Norwalk, CT, USA). Ion chromatograms were registered in a Linear 1200 Series Model 1201 register (Linear Instruments, Reno, NV, USA).

2.3. Chromatographic conditions

Different sodium chloride concentrations from 10 m*M* up to 100 m*M* were used as mobile phase for the characterization of molecular mass distributions in HPSEC. The column was an HPSEC column, Progel-TSK G2000SW (300×7.5 mm I.D.; particle size 10 μm) (Supelco, Bellefonte, PA, USA). Flow-rate was adjusted to 0.5 $ml \cdot min^{-1}$.

Non-suppressed IC with conductivity detection was performed with a borate–gluconate buffer solution, which consisted of 0.80 m*M* tetraborate, 3.5 m*M* boric acid, 0.80 m*M* gluconate, (pH 8.5), 0.25% (v/v) glycerine and 10% (v/v) acetonitrile. A Waters IC Pak anion-exchange column (50×4.6 mm I.D.; particle size 10 μm) packed with a methacrylate-based anion-exchanger of 30 $\mu eq \cdot g^{-1}$ capacity, and an IC Pak A Anion precolumn were used (Milford, MA, USA). Flow-rate was 1.0 $ml \cdot min^{-1}$. All eluents were prepared daily, filtered and degassed.

2.4. Column-switching system

The flow diagram of the column-switching system is shown in Fig. 1. In the first step (Step 1), the sodium chloride mobile phase (pump A) eluted the heparin injected through the HPSEC column and was directed to the differential refractometer. When the anions eluted, the switching valve was rotated and the analyte effluent from the HPSEC column was

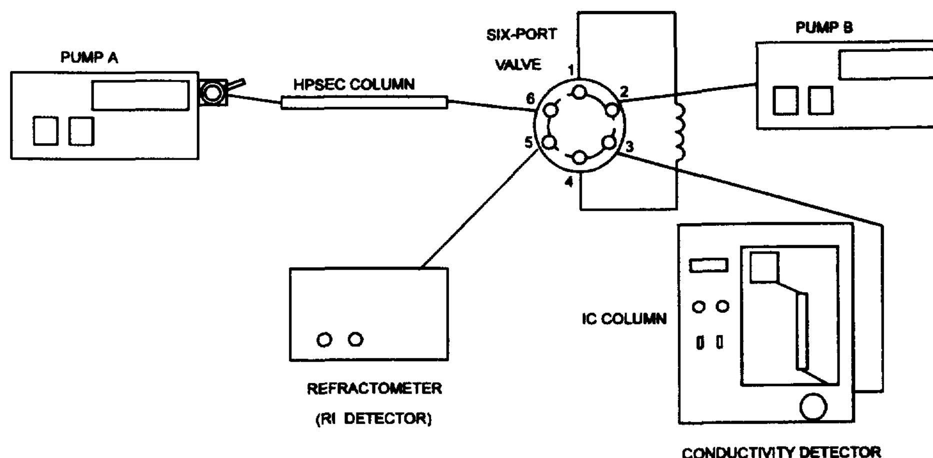

Fig. 1. Column-switching system: Step 1 (straight line), HPSEC analysis and IC equilibration; Step 2 (dotted line), anion collection; Step 3 (straight line), IC analysis.

collected in the 500 μl loop (Step 2); then the valve was rotated back and the IC mobile phase (pump B) started to elute the fraction collected from the HPSEC column (Step 3).

3. Results and discussion

3.1. HPSEC mobile phase optimization and calibration

Coupling HPSEC and IC systems by transferring elution fractions from the HPSEC column to the IC column required the optimization of both mobile phase conditions. In HPSEC a decrease in the electrolyte concentration was necessary to enable the transfer of elution fractions to the IC system. The most frequently used eluents in HPSEC are electrolyte solutions, commonly sodium chloride, acetate or sulphate, of 0.1–0.2 *M* ionic strength values to avoid the presence of non-SEC effects [42,43]. However, if relatively large volumes of these high ionic strength eluents were transferred to the IC column, the exchange capacity of the column would be exceeded.

In this work, sodium chloride solutions with concentrations ranging from 100 m*M* to 7 m*M* were used as HPSEC eluents in order to optimize the coupling conditions. Elution parameters and separation of inorganic anions from LMMH peaks, as well as the influence of electrolyte concentration of the mobile phase in the molecular mass averages obtained using eluents of different sodium chloride concentrations were studied. The influence of a decrease in the electrolyte concentration on the elution times of the LMMH and nitrate is shown in Table 1. Chromatograms were acquired by UV–Vis detection at 220 nm. Nitrate peak was used as a marker of anion elution to evaluate the separation of the glycosaminoglycans and the anion peak. Elution times of glycosaminoglycans and nitrate decreased as did the ionic strength of the eluent. These results showed evidence of the presence of electrostatic interactions between glycosaminoglycans and the negative sites of the ionised silanol groups, which affected the HPSEC mechanism [16,22,43].

Separation between LMMH and nitrate was also influenced by changes in the mobile phase composition and was related to the electrostatic interactions between the ions, the negative sites of the silanol groups and the polyelectrolytes ionizable groups [16,22,42–44]. The results given in Table 1 show that the decrease in sodium chloride concentration from 100 to 25 m*M* mainly affected LMMH elution times, while nitrate elution was more influenced by the decrease from 25 to 7 m*M*. The different behaviour of the polysaccharides and the nitrate ion can be explained by the effect of the ionic strength on the conformation of the heparin [16,18,28], which is important at sodium chloride concentrations higher than 20 m*M*. As a consequence, the best separation was achieved when mobile phases 20–25 m*M* sodium chloride were used. The decrease in the ionic strength of the eluent also affected the symmetry of the LMMH peak, as can be observed in Table 1. Asymmetry factors of the LMMH peak in different mobile phases were calculated at 10% of the LMMH peak height using refractometric (RI) detection. The results show that a significant increase in the asymmetry factor occurred at 7 m*M* NaCl, so concentrations higher than 10 m*M* are recommended to avoid distortion of peak symmetry.

Table 1
Elution times of LMMH and nitrate, and asymmetry factor of LMMH using different HPSEC mobile phases

Mobile phase	t_{LMMH} (min)	$t_{NO_3^-}$ (min)	Δt (min)	As^a_{LMMH}
100 m*M* NaCl	18.2	21.2	3.0	1.11
75 m*M* NaCl	17.2	21.2	4.0	1.12
50 m*M* NaCl	14.9	20.2	5.3	1.20
25 m*M* NaCl	12.9	19.4	6.5	1.28
20 m*M* NaCl	12.6	18.6	6.0	1.30
15 m*M* NaCl	11.7	16.6	4.9	1.33
10 m*M* NaCl	10.9	15.4	4.5	1.36
7 m*M* NaCl	10.8	15.0	4.2	2.90

[a] Asymmetry factor (from RI chromatograms).

Changes in peak shape and elution times may affect the molecular mass averages if the behaviour of the standards used is not similar to that of the sample. In order to avoid the possible effect of these changes on the mass averages, calibration in different mobile phases was carried out with an LMMH broad molecular mass distribution standard. Broad standard calibrations were performed using 10, 25, 50, 75 and 100 m*M* sodium chloride eluents. In all

mobile phases the best fit was provided by a third-degree polynomium. These calibration functions were applied to the calculation of molecular mass averages of the LMMH sample in the five studied mobile phases. The values of the different averages and repeatability, as the relative standard deviation (%R.S.D.) of six replicates carried out on the same day, are shown in Table 2. z-Average molecular mass ($\bar{M}_z$) is the molecular mass average whose values in the five studied eluents differ most. This might be due to the greater influence of the fraction with high molecular mass values on the expression of this average. These fractions eluted in the initial part of the LMMH peak, which seems to be more influenced by changes in the electrolyte concentration of the HPSEC eluent due to the combination of two important effects: size exclusion and ionic exclusion. Variance analysis was applied to the mass averages and showed that the values obtained in the five mobile phases with different electrolyte concentrations did not differ significantly with 95% probability. As a conclusion of these studies, a 10 mM sodium chloride solution was chosen as the most adequate eluent for further coupling of HPSEC to IC because it provided good separation between LMMH and anions peaks, satisfactory molecular mass average values, and the ionic strength is low enough to enable the transference of elution fractions to the IC system.

Long-term precision of the molecular mass averages was obtained by analysing four replicates per day on three different days. Mean values and %R.S.D. values using 10 mM sodium chloride are given in Table 3. $C\bar{M}_z$ is the molecular mass average that presented least reproducible values, which is in agreement with those of repeatability.

Table 3
Long-term precision of LMMH molecular mass averages[a] and peak mass obtained using a 10 mM sodium chloride eluent ($n=3$ days, 4 replicates/day)

	Mean ($n=3$ days)	% R.S.D.
$\bar{M}_w$	4281	4.97
$\bar{M}_n$	3490	6.51
$\bar{M}_z$	5288	10.72
M peak mass	3666	6.35

[a] $\bar{M}_w$, weight-average molecular mass; $\bar{M}_n$, number-average molecular mass; $\bar{M}_z$, z-average molecular mass.

3.2. Optimization of switching times and IC

The IC mobile phase was a borate–gluconate eluent, widely used for the determination of inorganic anions. A less concentrated solution than the usual eluent was needed in order to improve the separation between chloride and nitrate when large volumes of high chloride concentration solution were injected. In order to optimize eluate concentration, 500 μl of an anion solution with a concentration similar to that of the transference using a 10 mM sodium chloride eluent, 355 $\mu g \cdot ml^{-1}$ chloride, 10 $\mu g \cdot ml^{-1}$ nitrate, sulphate and dihydrogenphosphate, was injected in the column-switching system. Borate–gluconate (1:1) 0.80 mM gave enough resolution between the anions.

When coupling two HPLC systems it is convenient to introduce into the secondary column an amount of the analyte high enough to enable its determination. In this particular case nitrate, which was taken as a marker of anions elution, elutes in an overall volume about 1–2 ml in the HPSEC system. In order to prevent losses in column efficiency and a decrease in the ion-exchange capacity of the IC

Table 2
Mean and %R.S.D. molecular mass averages[a] LMMH obtained in different HPSEC mobile phases ($n=6$)

	NaCl concentration (mM)				
	10	25	50	75	100
$\bar{M}_w$ (R.S.D.%)	4192 (3.1)	4353 (5.9)	4397 (3.7)	4134 (3.7)	4011 (6.5)
$\bar{M}_n$ (R.S.D.%)	3376 (3.8)	3583 (7.3)	3218 (7.4)	3455 (8.1)	2989 (10.8)
$\bar{M}_z$ (R.S.D.%)	5009 (13.3)	5567 (7.4)	5893 (17.2)	5662 (13.4)	5405 (5.6)
Polydispersity ($\bar{M}_w/\bar{M}_n$)	1.24	1.21	1.36	1.20	1.34
M peak mass (R.S.D.%)	3601 (5.4)	3834 (5.0)	3630 (7.7)	3793 (10.4)	3794 (8.9)

[a] $\bar{M}_w$, weight-average molecular mass; $\bar{M}_n$, number-average molecular mass; $\bar{M}_z$, z-average molecular mass.

column due to the injection of large volumes of high chloride concentration, a volume of 500 μl was used.

Switching times for the collection of 500 μl transfer fractions were determined using the elution of nitrate in the HPSEC column at 220 nm. In order to enhance the reproducibility of the localisation of switching times, avoiding the influence of variation in nitrate elution time, interval times relative to nitrate were used to optimise the transfer. The initial and final times were obtained by adding ±0.5 min to the central time of the intervals, which was calculated as the product of the relative central times and nitrate retention time. A solution of 20 $\mu g \cdot ml^{-1}$ sodium nitrate and sulphate and 50 $\mu g \cdot ml^{-1}$ sodium dihydrogenphosphate was injected in the coupled system previously described, and elution fractions from the HPSEC column were collected at different switching time intervals and transferred to the IC column in order to localize the optimum transfer fractions for the determination of sulphate and phosphate. As an example, the IC chromatograms of two consecutive series of switching-time intervals are shown in Fig. 2(a,b). The influence of the first chromatographic system is very important, as can be observed from the presence of the anions in different elution fractions. Sulphate and phosphate are mainly detected in fractions that elute before the elution time of nitrate. Data presented in Table 4 show that sulphate is detected in the early elution fractions, while phosphate starts to elute from the HPSEC column in fractions closer to the elution time of nitrate. This might be related to the extent of the ion exclusion effect in the elution time of anions in the HPSEC silica-based column, which is more pronounced for the double-charged sulphate ion.

Repeatability, long-term precision, and limit of detection (LOD) for sulphate and phosphate were obtained in three transfer fractions. The results are presented in Table 5. Repeatability was expressed by the %R.S.D. of the peak areas of ten replicates, injected on the same day, of an anion solution which was 20 $\mu g \cdot ml^{-1}$ sodium sulphate and 50 $\mu g \cdot ml^{-1}$ sodium dihydrogenphosphate. Values between 3.4% and 9.1% were obtained for phosphate. Sulphate gave better values: 1.56%, in the interval $0.853t_{NO_3^-} \pm 0.5$ min, which provided its maximum area. Long-term precision for each switching-time interval was determined from the areas of ten replicates of the anion solution injected on three days. Detection limits based on a signal-to-noise ratio of 3:1 were calculated for each anion. The best detection limits were 50 ng for sulphate and 100 ng for phosphate. These values are similar to those obtained in an IC column-switching system using direct transfer technique with two IC Pak Anion columns [45].

From these results, two different switching-time intervals were selected to determine either sulphate or phosphate in their corresponding elution fractions from the HPSEC column. These intervals were $0.853t_{NO_3^-} \pm 0.5$ min for sulphate and $0.920t_{NO_3^-} \pm 0.5$ min for phosphate determination. Linear relationships between peak area and injection amount were found for sulphate and phosphate in their optimum intervals. Working linear ranges for sulphate and phosphate in their corresponding intervals were 0.5–47.5 $\mu g \cdot ml^{-1}$ and 1–50 $\mu g \cdot ml^{-1}$, respectively. Simultaneous determination of both anions can be performed by transfer of the $0.887t_{NO_3^-} \pm 0.5$ min fraction, although it involves an increase in detection limits for both anions.

3.3. Application

The optimized experimental conditions for the column-switching system were applied to the HPSEC and IC analysis of an LMMH sample. A sample containing 0.4% (w/v) LMMH was injected in the coupled system. Molecular mass averages were coincident with those obtained in the off-line HPSEC, which are shown in Table 3. The first approach of the anions determination was the transfer of the intermediate elution interval, $0.887t_{NO_3^-} \pm 0.5$ min, which gave a signal for sulphate but not for phosphate. Therefore, two elution fractions from the HPSEC column corresponding to $0.853t_{NO_3^-} \pm 0.5$ min and $0.920t_{NO_3^-} \pm 0.5$ min elution intervals were transferred to the IC system to determine sulphate and phosphate contents in their respective intervals which provided best detection limits. Quantification was carried out by standard addition method in both transfer fractions. Five samples containing 0.4% (w/v) LMMH were spiked with different volumes of sulphate and phosphate standard solutions to obtain an addition interval

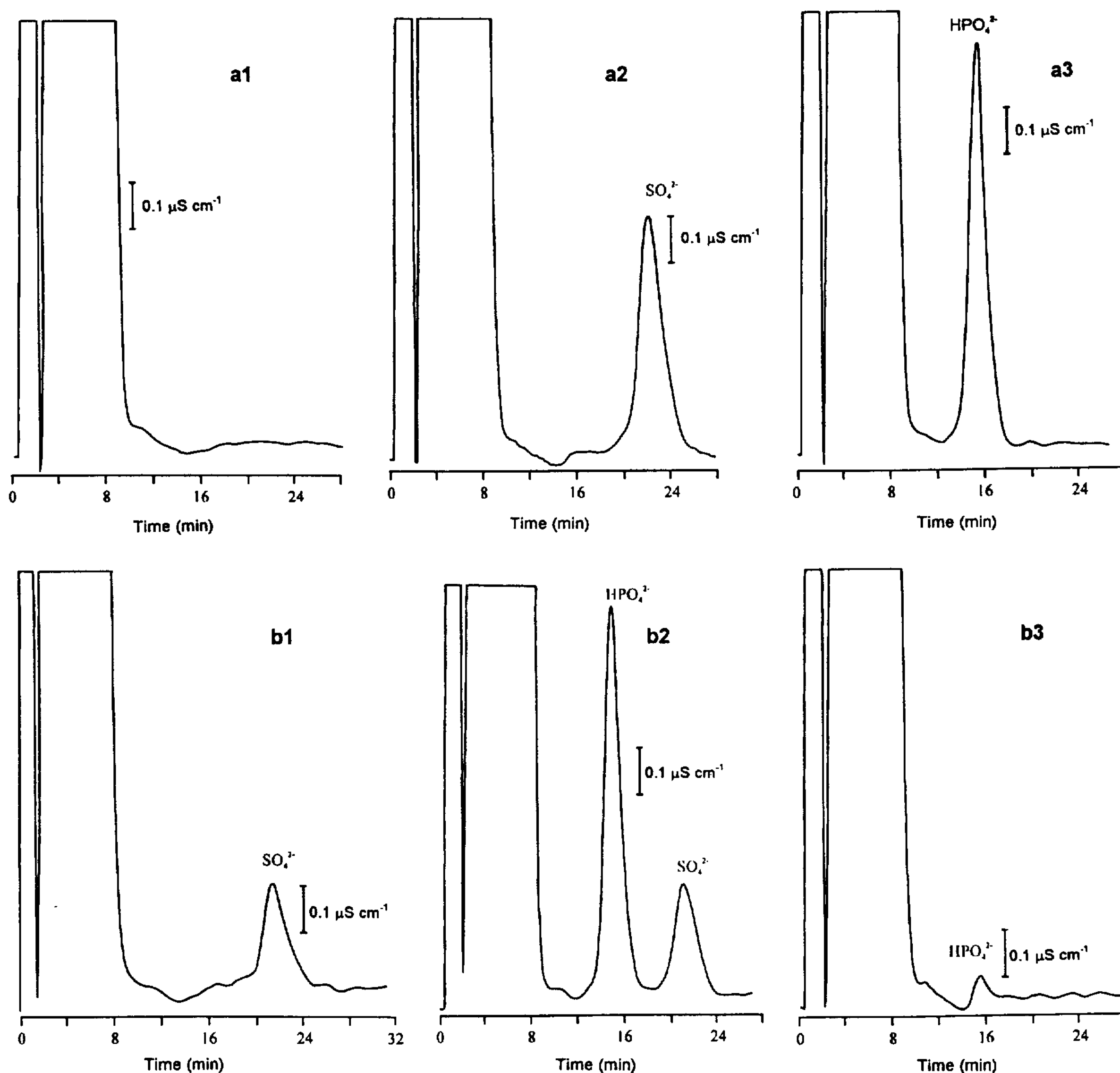

Fig. 2. (a) IC chromatograms of three consecutive transfer intervals. Experimental conditions are given in text. Intervals A: $0.787t_{NO_3^-}\pm0.5$ (Interval a1); $0.853t_{NO_3^-}\pm0.5$ (Interval a2); $0.920t_{NO_3^-}\pm0.5$ (Interval a3). (b) IC chromatograms of three consecutive transfer intervals. Experimental conditions are given in the text. Intervals B: $0.820t_{NO_3^-}\pm0.5$ (Interval b1); $0.887t_{NO_3^-}\pm0.5$ (Interval b2); $0.953t_{NO_3^-}\pm0.5$ (Interval b3).

ranging from 2.5–25 $\mu g \cdot ml^{-1}$ and 6–50 $\mu g \cdot ml^{-1}$ for sulphate and phosphate, respectively. Fig. 3 shows the IC chromatograms obtained by transfer of the $0.853t_{NO_3^-}\pm0.5$ min fraction of both a spiked and non-spiked LMMH sample. Free sulphate concentration in LMMH was 0.56%, and the %R.S.D. obtained in three analysis was 2.77%. Phosphate was not detected in the LMMH sample. Consequently, free phosphate concentration in the LMMH injected solution is considered to be lower than the LOD, which is equivalent to a 250 $\mu g \cdot g^{-1}$ concentration in the LMMH sample.

Table 4
Peak areas in transfer fractions which correspond to different switching-time intervals

	Relative switching-time intervals (min)		HPO_4^{2-} area (cm^2)	SO_4^{2-} area (cm^2)
a1	$0.787t_{NO_3^-}\pm0.5$		–	–
b1		$0.820t_{NO_3^-}\pm0.5$	–	4.1
a2	$0.853t_{NO_3^-}\pm0.5$		1.5	10.4
b2		$0.887t_{NO_3^-}\pm0.5$	12.4	4.8
a3	$0.920t_{NO_3^-}\pm0.5$		12.9	–
b3		$0.953t_{NO_3^-}\pm0.5$	0.7	–

Injection in HPSEC system: 50 $\mu g \cdot ml^{-1}$ $H_2PO_4^-$, 20 $\mu g \cdot ml^{-1}$ SO_4^{2-}.

4. Conclusions

A coupled HPSEC-IC system proved to be useful for the analysis of both molecular mass averages and free sulphate and phosphate contents of an LMMH sample. The occurrence of these contaminant anions is mainly due to the use of inorganic salts during heparin production process. Coupling of HPSEC and IC was achieved by reducing the concentration of the sodium chloride HPSEC eluent to 10 m*M*. This allowed sulphate and phosphate determination, however, acetate and chloride, whose determination as contaminant anions would be also convenient, could not be determined with the experimental conditions because of the relatively high chloride concentration of the optimized HPSEC eluent. Molecular mass averages obtained did not differ significantly from those determined using eluents with higher ionic strength. In this particular case, due to the ion-exclusion observed in the HPSEC column, a separation of sulphate and phosphate occurred. As a consequence they were mostly collected at different switching intervals. Nevertheless, an adequate interval for the simultaneous determination of both anions was established. Detection limits smaller than 100 ng, good repeatability (better than 3.4%), and long-term precision values (better than 3.5%), were obtained. To show the applicability of the method sulphate and phosphate determination and also molecular mass averages were carried out for an LMMH sample. The results obtained showed that coupling of HPSEC and IC would be successfully applied to the analysis of other polymeric compounds using a HPSEC column with an adequate

Table 5
Repeatability, long-term precision and limit of detection (LOD) obtained in each transfer interval

	$0.853t_{NO_3^-}\pm0.5$ min	$0.887t_{NO_3^-}\pm0.5$ min	$0.920t_{NO_3^-}\pm0.5$ min
Repeatability			
HPO_4^{2-}	4.67%	3.37%	9.10%
SO_4^{2-}	1.56%	15.50%	–
Long-term precision			
HPO_4^{2-}	18.06%	3.50%	–
SO_4^{2-}	2.42%	19.40%	–
LOD[a]			
HPO_4^{2-}	250 ng	150 ng	100 ng
SO_4^{2-}	50 ng	200 ng	>5000 ng

[a] Injection volume in HPSEC system: 100 μl.

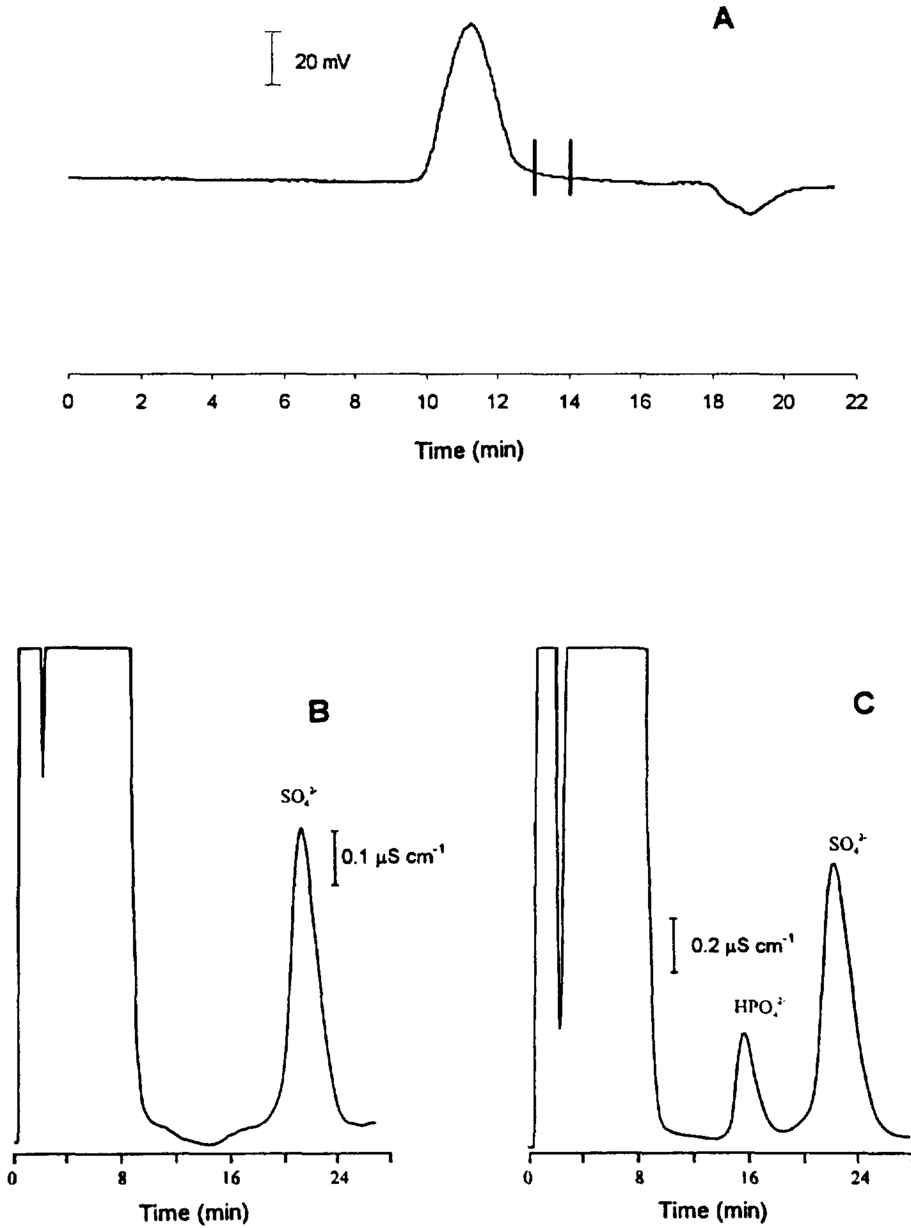

Fig. 3. (A) HPSEC chromatogram of the LMMH sample and (B) IC chromatogram of the transfer fraction. (C) IC chromatogram of the transfer fraction of a spiked LMMH sample (50 $\mu g \cdot ml^{-1}$ $H_2PO_4^-$ and 20 $\mu g \cdot ml^{-1}$ SO_4^{2-}). Chromatographic conditions: HPSEC system, Progel-TSK-G2000SW column, 10 m*M* sodium chloride, injection volume 100 μl, refractometric detection; IC system, IC-Pak Waters anion column, 0.80 m*M* borate–gluconate (1:1), 10% acetonitrile, 0.04% glycerine. Elution interval transferred to IC: $0.853t_{NO_3^-} \pm 0.5$ min.

exclusion limit. Studies are being carried out with other glycosaminoglycans such as chondroitin sulphate and non-fractionated heparins.

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