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Analytical characterization of heparin by capillary zone electrophoresis with conductivity detection and polymeric buffer additives

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Abstract

A capillary zone electrophoresis (CZE) method for the analytical characterization of intact (high-molecular-weight) heparin was developed. For the first time, a hydrodynamically closed CZE separation system with conductivity detector was used for the separation, detection and quantitation of this highly sulfated, linear polysaccharide. Glycine (25 mM) adjusted to pH 9.0 by bis–Tris–propane served as the running electrolyte system. Polymeric additives, polyvinylpyrrolidone (PVP), dextran (DEX), were used to improve the separation selectivity as they strongly retarded the heparin macromolecule while they did not practically influence comigrating inorganic anions. The proposed electrophoretic method was successfully validated. It was convenient for the sensitive, simple, rapid and reproducible assay of heparin in raw materials and isotonic saline. Here, the use of the conductivity detector was advantageous as it allowed heparin to be analyzed without a sample pretreatment. The CZE method should be an alternative to the pharmacopoeial conventional gel electrophoresis having used in the quality control of heparin so far. In addition, it should be convenient to quantitative estimation of heparin present in a preparation used, e.g., as the chiral selector in CE separations.
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Keywords: Heparin; Capillary zone electrophoresis; Conductivity detection; Polymers; Dextran; Polyvinylpyrrolidone; Raw material; Saline

1. Introduction

Heparin is a naturally occurring linear glycosaminoglycan (GAG), polydisperse with regard to molecular weight (M_r 5000–40,000; average M_r 14,000) as well as sites and degree of sulfation [1]. The polymer consists of repeating 1 → 4 linked D-glucosamine and uronic acid residues (Fig. 1) [2]. Commercial heparin is prepared from bovine lung and porcine intestinal mucosa and is in use as an anticoagulant [3]. Many times, heparin was used as a chiral selector for capillary zone electrophoresis (CZE) when a variety of drugs and compounds of pharmaceutical interest were analyzed [4]. Any raw material, intended to use in pharmaceutical industry or analytical research, has to be proved. A preliminary characterization of GAG in the preparation consists in verifying of GAG identity and quantity analyzing the intact polymer. On the other hand, monosaccharide analysis can be effective method of identifying GAG types and also to determine the cross-contamination between GAGs in raw materials and provides more detailed information related to structural carbohydrate characterization [5,6].

It was reviewed [7] that intact heparin can be analyzed by a variety of spectral, chromatographic and electrophoretic methods. Since electrophoresis is basically an analytical method for ions, heparin, a highly charged polyanion, is often the direct object of this method. The gradient polyacrylamide gel electrophoresis is useful in determining molecular weight [8]. However, it suffers from a lack of resolution particularly of different oligosaccharides having identical size. In this field, capillary electrophoresis (CE) has been approved for its high sensitivity and excellent resolving power. CE analysis of intact GAGs may provide useful information on the amount, the molecular size, polydispersity

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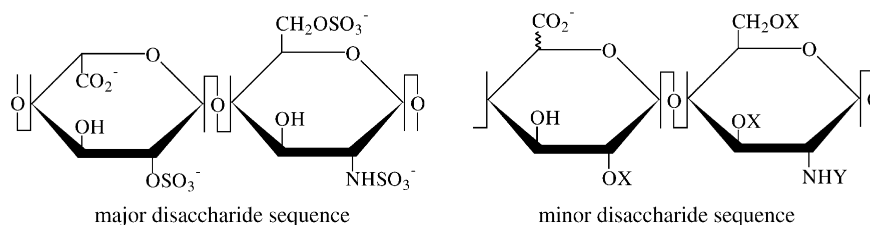

Fig. 1. Chemical structure of heparin (X=H or SO_3^-; Y=H, SO_3^- or Ac).

and the charge density of the chain [9–11] and it has been used to control the purity of isolated GAG species [6]. CE at low pH can easily be used to separate and characterize intact GAGs in respect of the average number of sulfates present per repeating unit. As for a more detailed characterization, CE has been widely applied to the disaccharide compositional analysis of GAGs [12–14], requiring a sample pretreatment (enzymatic depolymerization, derivatization) and various means of detection (direct and indirect UV detection, detection as a metal complex, MS detection) [15].

This work was aimed at elaborating a CZE method suitable for the determination of intact heparin present in raw materials and isotonic saline. For reasons discussed elsewhere [16], we favored the CZE separations in a hydrodynamically closed separation compartment. In such case, CZE experiments are carried out in a wide bore capillary tube with a porous membrane serving as a hydrodynamic barrier to prevent a flow of the solution in the separation compartment due to a pressure difference between the electrode vessels. The analytical advantages of using columns of larger i.d. include mainly enhanced sample load capacity and significantly reduced contributions of electromigration dispersion. It may be useful, especially, when the ratio of the analytes is high, e.g., when the sample contains matrix constituents at high concentrations (e.g., inorganic ions in saline, body fluids). In addition, the use of such analytical approach was expected to enhance repeatability/reproducibility of separations as a result of elimination of the hydrodynamic flow of the separation solution. Malá et al. [17] reported an analysis of heparin-like pharmaceutical by CZE and isotachophoresis, using similar separation compartment. In general, intact, underivatized GAGs have not been successfully analyzed by CE because of the absence of a chromophore. In our work, a conductivity detection was chosen as an alternative to the indirect UV detection or the direct one, based on the formation of a copper (II) complex, which have been used for intact heparin [15,18]. Minimum specific requirements concerning the composition of the carrier electrolyte system favored conductivity detection over photometric. In this study, various polymeric buffer additives at their various concentrations were tested in order to change the separation selectivity. The linear water-soluble polymers were expected to be effective in separations of molecules of different sizes and nature (inorganic ions, heparin), detectable by universal detection, due to the sieving effects and association interactions.

2. Experimental

2.1. Instrumentation

A CS Isotachophoretic Analyzer (Villa-Labeco, Spišská Nová Ves, Slovak Republic) was used in a single-column configuration of the separation unit. The separation unit consisted of the following modules: (i) a CZE injection valve with a 100 nl internal sample loop (Villa-Labeco); (ii) a column provided with a 300 μm i.d. (650 μm o.d.) capillary tube made of fluorinated ethylene–propylene copolymer (FEP) of 210 mm total length (160 mm to the conductivity detector); (iii) a counter-electrode compartment with a hydrodynamically (cellophane membrane) closed connecting channel to the separation compartment (Villa-Labeco).

The CZE column was provided with an on-column conductivity detector (Villa-Labeco). The signal from the detector was led to a PC via a Unilab data acquisition unit (Villa-Labeco). ITP Pro32 Win software (version 1.0) obtained from KasComp (Bratislava, Slovak Republic) was used for data acquisition and processing.

Prior to the use, the capillary was not particularly treated to suppress an electroosmotic flow (EOF). A dynamic coating of the capillary wall by means of a 0.2% methylhydroxyethylcellulose (m-HEC) in background electrolyte solutions served for this purpose [19]. CZE analyses were carried out in anionic regime of the separation with direct injections of the samples. The experiments were performed in constant current mode [16]. The driving current was 70 μA. The temperature was 20 °C.

2.2. Chemicals and samples

The carrier electrolyte solution was prepared from chemicals obtained from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland) in water demineralized by a Rowapure-Ultrapure water purification system (Premier, Phoenix, AZ, USA). Linear water-soluble polymers, polyvinylpyrrolidone 750,000 (PVP), m-HEC 30,000 and dextran 70,000 (DEX) were obtained from Serva (Heidelberg, Germany) and Biotika (Slovenská L'upča, Slovakia), respectively. All chemicals used were of analytical grade or additionally purified by the usual methods. The solutions of the electrolytes were filtered before use through disposable membrane filters (a 1.2 μm pore size) purchased from Sigma (St. Louis, MO, USA).

Model samples (sodium salts of inorganic acids) were purchased from Lachema (Brno, Czech Republic). A test substance of heparin sodium (HP) was obtained from Bel Novamann (Bratislava, Slovakia) as a powder of pharmaceutical grade. Heparin sodium BRP (HP*) was a reference solution obtained as an injection.

2.3. Procedures for sample and standard solution preparations

2.3.1. Standard solutions

A concentration of the standard stock solution of HP* was 5.0 mg/ml. The standard stock solutions of the inorganic anions (chloride, sulfate), including internal standard (phosphate), were prepared at a 10^{-1} mol/l concentration. Working solutions were made by further appropriate dilutions in demineralized water (for the resulting concentrations, see legends to the electropherograms). All of the solutions were stored at −8 °C.

2.3.2. Calibration graph

Appropriate amounts of the stock solution (HP*) were diluted with demineralized water yielding concentrations of 100, 200, 300, 400 and 500 μg/ml. Six replicate injections of each were made.

2.3.3. Substance

Twenty-five milligrams of the powder was weighed accurately into a 10 ml volumetric flask and dissolved in demineralized water. The flask was placed in ultrasonic water bath for 5 min before completion to the mark with the water. An appropriate amount of this stock solution was mixed with the internal standard and/or chloride stock solutions, diluted with demineralized water (for the resulting concentrations, see legends to the electropherograms) and directly injected into the CE equipment. The solutions were stored in a freezer at −8 °C.

2.3.4. Recovery test

2.5 mg of the powder was weighed accurately into a 10 ml volumetric flask, dissolved in 5 ml of demineralized water and appropriate amounts of HP* and internal standard were added. The flask was placed in ultrasonic water bath for 5 min before completion to the mark with the water. Resulting concentrations of HP and internal standard were ∼250 μg/ml and 2×10^{-4} mol/l, respectively, while that one of HP* varied as follows: 50, 100, 150, 200 and 250 μg/ml. Six replicate injections of each sample were made.

3. Results and discussion

3.1. Method optimization in free solution without physical network

CZE in a hydrodynamically closed separation system with suppressed electroosmotic flow was used to analyze heparin.

Table 1
Electrolyte systems

Parameter	ES 1	ES 2	ES 3
Solvent	Water	Water	Water
Carrier anion	Glycine	Glycine	Glycine
Concentration (mmol/l)	25	25	25
Counter ion	BTP	BTP	BTP
pH	9.0	9.0	9.0
EOF suppressor	m-HEC	m-HEC	m-HEC
Concentration (%, w/v)	0.1	0.1	0.1
Complexing/sieving additive	–	PVP	DEX
Concentration (%, w/v)	–	0.5–2.0	1.0–10.0

In this work, suitable electrophoretic conditions for the migrants were achieved using glycine and bis–Tris–propane {1,3-bis[Tris(hydroxymethyl)methylamino]propane), BTP} buffer ions (Table 1), which ensured low enough conductivity of the buffer in order to eliminate the thermal dispersion (induced by Joule heat) that increases with internal diameter of the capillary (here, a 300 μm i.d. capillary was used). A concentration of the buffer ions, pH and the applied current were optimized with respect to buffer capacity, analysis time, separation efficiency and resolution. A 25 mmol/l glycine running electrolyte adjusted at pH 9.0 and a 70 μA stabilized current was chosen for further experiments as optimal (see very lower traces at Fig. 2a and b). The peak breadth observed in the figure is believed to be primarily ascribable to the polydispersity and/or sequence heterogeneity of heparin being analyzed.

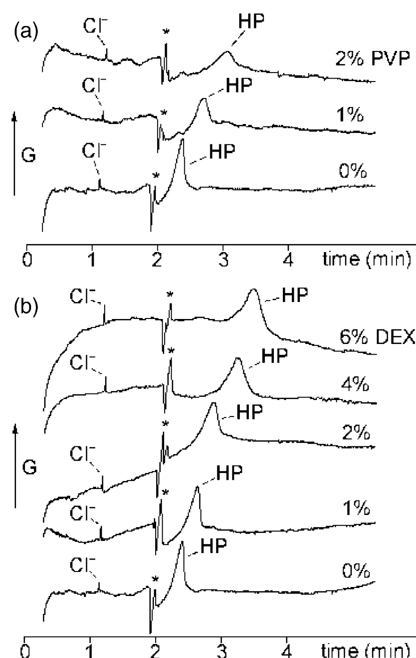

Fig. 2. CZE separations of heparin in electrolyte systems containing different polymers at the different concentrations (Table 1). The separations were carried out in (a) ES 1, (b) ES 3; the very lower traces in (a) and (b) were obtained using ES 1 and served as references. The concentration of the analyte in the injected sample was 250 μg/ml. The driving current was stabilized at 70 μA and the corresponding driving voltage was 7.2 kV. Peak assignments: HP, heparin; Cl^-, chloride; asterisk (*), migration position of sulfate. PVP, polyvinylpyrrolidone; DEX, dextran.

Table 2
Precision data for repeat injections of heparin sodium BRP (n = 6, c =250 μg/ml)

Factor	Repeatability[a], R.S.D. (%)			Reproducibility[b], R.S.D. (%)		
	ES 1	ES 2[c]	ES 3[c]	ES 1	ES 2[c]	ES 3[c]
Migration time	1.38	1.51	1.29	1.75	1.83	1.61
Relative migration time[d]	1.22	1.35	0.94	1.33	1.58	1.08
Peak area	–	1.87	1.53	–	2.21	1.95
Peak-area ratio[d]	–	1.76	1.31	–	2.05	1.67

[a] Run-to-run precision.
[b] Day-to-day precision (two weeks between the series).
[c] Concentration of the polymer was 2% (w/v).
[d] Relative migration and relative response data were obtained using phosphate as an internal standard.

3.2. Separation in the presence of polymers as physical networks

As can be seen in Fig. 2 (very lower traces), under the separation conditions without a complexing additive heparin migrated close by the migration position of sulfate. In such case, a higher concentration of sulfate in the sample can prevent the separation of heparin. With regard to molecular weight of the analyte and its interferent (sulfate), it seemed reasonable to use a polymer as a buffer additive to affect their electrophoretic mobilities. The influence of PVP and DEX was studied.

In the presence of physical networks consisting of linear polymers, the selectivity of the system was enhanced. However, there were some differences observed between the separations with polymers differing by the molecular weights and the monomeric structure. Electropherograms a and b in Fig. 2 show that PVP more strongly as DEX, at the same concentrations, retarded heparin. It could be attributed to some extent to a higher viscosity of PVP buffer. However, even at the same migration times of heparin (at different concentrations of the polymers), less intensive and a bit broadened heparin peak was obtained in PVP system. This suggests that PVP provided stronger interactions for heparin as DEX did.

The effect of increasing polymer concentrations in the electrolyte solution on the resolutions is well marked (Fig. 2). A general increase in migration time observed upon increasing the polymer concentration can be attributed to some extent to the change in viscosity of the solution due to the addition of the polymers. Of utmost importance, however, was the continuous increase in the resolution of heparin upon increasing the concentrations of the polymers. According to our expectation, the effects were not equally strong for all ions detected as the compounds were of different structure. High-molecular-weight heparin was strongly retarded and the resolution significantly increased while low-molecular-weight inorganic anions were slightly influenced (due to viscosity) and the resolutions remained practically unaffected as the concentration of the polymeric additive increased. It could be explained by the fact that a higher concentration of the polymer increased noncovalent and/or steric interactions with heparin while these effects were not relevant for inorganic anions. In this way, it was possible to separate heparin from sulfates effectively. Moreover, this approach aided to verifying heparin identity more confidently.

3.3. Validation

The validation aspects assessed included performance parameters such as sensitivity, linearity, precision (repeatability and reproducibility) and accuracy (recovery). Electrolyte system no. 3 (Table 1) was used to obtain validation data. Here, optimized concentration of DEX was 2% (w/v). These conditions (type and concentration of the polymer) were favored with respect to the highest efficiency of the separation maintaining a sufficient resolution of heparin from the interferent sulfate. Heparin sodium BRP reference solution (HP*) was examined in validation procedure.

Using on-capillary contact conductivity detector, limit of detection (estimated as 3σ) of heparin was 40 μg/ml (4×10^{-9} g) while limit of quantitation (estimated as 10σ) was 133 μg/ml. The linearity of detector response (peak area) for heparin was assessed over the range 100–500 μg/ml. This represents interval suitable for evaluation of heparin in various commercial preparations. The straight line equation was $y = 106 + 4781x$ (x, mg/ml) and corresponding correlation coefficient $k = 0.9984$. Use of the internal standard (phosphate, 2×10^{-4} mol/l) slightly improved correlation coefficient (∼0.02%) reducing scatter of points due to random error.

Precision was assessed with or without internal standard at a 250 μg/ml concentration of heparin. In our study, phosphate served as the internal reference. Six repeated injections of heparin and phosphate gave the data present in Table 2 (run-to-run and day-to-day precision). These results

Table 3
Identification and quantitation of heparin in raw materials[a]

Parameter	1	2	3	4	5
Relative content[b] (%)	94.8	92.6	97.8	94.4	96.1
R.S.D. (%), n = 6	1.64	1.59	1.67	1.71	1.65
Ratio of migration times[b]	0.98	0.99	1.02	0.99	1.00
R.S.D. (%), n = 6	1.37	1.44	1.31	1.39	1.42

[a] The separations of heparin in five different batches of raw heparin materials were carried out in the ES 3 (Table 1); concentration of the polymer was 2% (w/v).
[b] Relative data were calculated using heparin BRP as a reference.

clearly indicated that CZE separations in hydrodynamically (membrane) closed separation system provided highly reproducible migration data.

3.4. Application

CZE in the hydrodynamically closed separation system and separation conditions validated (ES 3, 2%, w/v, DEX) were applied to the analysis of raw heparin materials dissolved both in demineralized water and isotonic saline. A practical use of the proposed method is obvious from the results present in Table 3. Here, data from the separations were obtained with a good precision. Quantitative estimations of heparin, migrating under optimized separating conditions, brought consistent results (see heparin contents found out in five preparations tested). The recovery test showed a reasonable accuracy of the method. The recovery, determined by adding known amounts of HP* reference (Section 2) to the samples at the beginning of the sample preparation process, was in the range of 96.4–102.8%. The mean absolute recovery of heparin from the sample (batch no. 1) was found to be 97.8%. The mean ratio of the migration time of tested HP (batch no. 1) and reference HP* was 0.98 (n = 6) using the proposed CZE method. The results were compared and they were in a good agreement with those obtained using gel electrophoresis (performed according to the European pharmacopoeia [20]). Here, the confirmation of identity of heparin was based on a comparison of the mobility of the principal band in the electropherogram obtained with the test solution (HP) with that obtained with the reference solution (HP*). The mean ratio of the mobilities was 0.97 (n = 6). Hence, it can be concluded that the proposed CZE method is convenient to reliable identification and quantitation of heparin in raw materials.

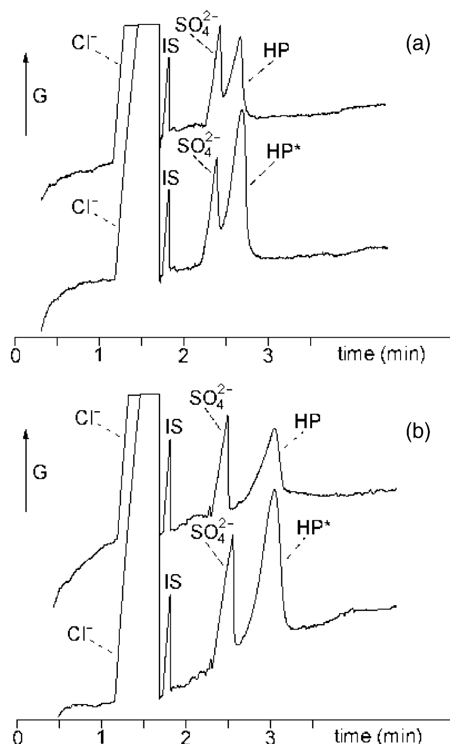

Fig. 3. Electropherograms from the determination of heparin content in raw material dissolved in isotonic saline. The separations were carried out in (a) ES 1, (b) ES 3; concentration of the polymer was 2% (w/v). The lower traces, serving as references, were obtained in the separations of heparin BRP solution at a 250 μg/ml concentration while the upper traces belong to examined heparin (125 μg/ml of the substance). Concentrations of inorganic migrants were $\sim 10^{-2}$ mol/l (chloride), 2×10^{-4} mol/l (phosphate), $\sim 10^{-3}$ mol/l (sulfate). The driving current was stabilized at 70 μA and the corresponding driving voltage was 7.2 kV. Peak assignments: HP, heparin; HP*, heparin BRP reference; Cl^-, chloride; SO_4^{2-}, sulfate; IS, internal standard (phosphate).

Heparin is commonly used as a clinical anticoagulant and often it is present in infusion solutions. With regard to a practical use of this GAG, raw heparin materials were dissolved in isotonic saline. Electropherograms from the determination of heparin content in such preparations are in Fig. 3. As discussed above, a higher concentration of sulfate in the sample can prevent the separation of heparin because of their close migration position. Indeed, only a partial resolution of heparin from the interferent sulfate was achieved when sulfate present at a 10^{-3} mol/l concentration (Fig. 3a). Under optimized separation conditions, all migrants (major as well as minor) were well resolved (Fig. 3b) so that heparin could be determined confidently. From the results obtained, it is obvious that the CZE method with conductivity detection and polymeric buffer additive (at a proper concentration) is convenient to reliable identification and quantitation of heparin in commercial preparations including inorganic anions as macroconstituents (e.g., infusions).

4. Conclusion

This work outlined a significant potential of CZE working in hydrodynamically (membrane) closed separation system, employing polymeric buffer additives for providing analytical characterizations of intact heparin in raw materials. In general, a concentration and type of a polymeric buffer additive should be optimized with regard to an amount and kind of sample matrix constituents comigrating. The detection approach used is advantageous in heparin analysis as it allowed the analyte to be analyzed without a sample pretreatment (derivatization). Moreover, minimum specific requirements concerning the composition of the carrier electrolyte system (chromophores, methal ions) favored conductivity detection over photometric. The capillary of a larger i.d. (300 μm) employed provided favorable conditions in term of a sample load capacity. It was useful to determination of heparin at high concentrations of sample matrix constituents (here, inorganic ions in saline). In addition, membrane-closed capillary provided high precision of the separations as a result of elimination of the hydrodynamic flow of the separation solution.

Successful validation was achieved including suitable assessments of sensitivity, linearity, precision (run-to-run, day-to-day) and accuracy (recovery). It is concluded that the reported operating conditions are suitable for the routine assay of heparin.

CZE in a hydrodynamically closed separation system, used for the analysis of heparin for the first time, should be convenient also for complex biological sample applications, as it can be easily on-line combinable with the purification CE modes (e.g., isotachophoresis).

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