DETERMINATION OF INTACT HEPARIN BY CAPILLARY ELECTROPHORESIS WITH CONTACTLESS CONDUCTIVITY DETECTION IN BACKGROUND ELECTROLYTES CONTAINING HYDROPHILIC POLYMERS

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Intact heparin was characterized and determined in model samples, in infusion solutions and in blood plasma by capillary electrophoresis (CE) with contactless conductivity detection. The CE separation of polydisperse heparin took place in open silica capillaries, in electrolytes containing a polymer (hydroxyethyl)cellulose, poly(ethylene glycol) or dextran. The best separation of heparin from excess inorganic ions present in real samples was attained in a background electrolyte consisting of 0.8 M acetic acid and 1% (w/v) dextran (100 kDa). The limit of detection (LOD) was 1.3 μmol l^{-1}. This electrolyte was used in determination of heparin in blood plasma and in infusion solutions.

Keywords: Capillary zone electrophoresis; Contactless conductivity detection; Heparin; Human plasma; Polymers; Polysaccharides, Sulfates.

The polysaccharide heparin is a biologically active substance with anticoagulation effects and thus is widely employed in medical practice[1,2]. Heparin is administered to patients parenterally when there is a danger of blood clotting. It is also commonly used in vessels for blood samples. Heparin does not affect the level of free calcium ions, in contrast to other anticoagulation agents (EDTA, citrate and oxalate). It is irreplaceable in the instruments for external circulation, e.g., in renal dialysis. Heparin is obtained for medicinal purposes by isolation from bovine and porcine intestine mucose or from lungs.

Heparin is a (glycosamino)glycan; its chemical structure is quite complex[3]. In the linear chain of heparin disaccharide units alternate, which are formed by glucuronic acid and glucosamine and which are further N- and

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O-sulfated and also N-acetylated (Fig. 1). The native heparin isolated from biological materials exhibits a high degree of polydispersity in molecular weight (the number of disaccharide units) and in the charge (the number of SO_3^- groups). The molecular weight of native heparin ranges from 6 to 40 kDa, on average 12 kDa, and the average number of unit negative charges equals 75. This high polydispersity of heparin strongly complicates its determination.

The heparin concentrations in blood plasma are low[4,5] varying from 1 to 50 μg ml^{-1}. Standard clinical procedures for monitoring the anticoagulation activity of heparin are based on measurement of the activated clotting time (ACT) or the activated partial thromboplastin time (aPTT); a colorimetric test for heparin (chromogenic antifactor Xa assay) is also used[6,7]. In addition to the biological methods, a number of chemical approaches have also been developed, e.g., flow-injection analysis[8], an ion-selective[9] and a membrane electrode utilizing complexation with protamine[10], a piezoelectric quartz crystal sensor[11] and spectrofluorimetric methods[12].

Among separation methods, two-dimensional slab gel electrophoresis and size exclusion or ion exchange HPLC are primarily used for study of heparin[13–15]. Useful information on the degree of polydispersity and the amount and purity of intact native heparins is also obtained using capillary electrophoresis in capillaries filled with a polyacrylamide gel[14,16–18]. The use of these capillaries for routine analyses is problematic because of easy degradation of the gels and their limited lifetime. The original attempts at CE determinations of heparin and analogous substances were carried out in aqueous solutions, using non-standard plastic capillaries stoppered with porous membranes[19] or short silica capillaries, 30 cm long, with inner wall coated with polyacrylamide[20]. In this work, a standard CE separation of intact heparin using common open silica capillaries and adding a polymer to the background electrolyte (BGE) solution was tested. Polymers in BGEs strongly interacted with heparin and enable its separation from inorganic ions. The work with polymer solutions of low viscosities is easy, compared

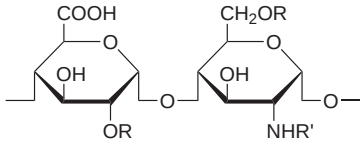

FIG. 1
Structure of a heparin disaccharide: R = SO_3^- (major) or H (minor); R′ = SO_3^- (major) or acetyl (minor)

with work with capillaries filled with polyacrylamide gels. Using the pressure system of the electrophoretic instrument, the capillary is readily filled up with a polymer solution which is then easy to wash out of the capillary at the end of measurement. Moreover, modification of the BGE with the polymer modifier is simple in this case. A capacitively coupled contactless conductivity detector (C^4D)[21,22] instead of contact conductivity measurement[19] was used to detect heparin, which does not absorb electromagnetic radiation in the UV/VIS spectral range[20]. Conductivity detection is a universal technique permitting to attain the LOD values 10^0 μmol l^{-1} for inorganic and organic analytes using BGEs of optimized compositions, which is fully comparable with the values obtained with standard photometric detectors[23]. We investigated the potentials of CE/C^4D for the study of heparin interactions with solutions of various polymers as well as for the heparin determination in blood plasma and in solutions of high salinities. C^4D is especially suitable for detection of substances in these complex matrices because the electrodes are not in direct contact with the test solution.

EXPERIMENTAL

CE Instrumentation

The CE measurements were carried out using an $HP^{3D}CE$ system (Agilent Technologies, Waldbronn, Germany) equipped with a built-in C^4D and controlled by ChemStation CE software. The C^4D was developed and constructed at the Department of Physical Chemistry, Faculty of Science, Charles University in Prague[26]. The detector was placed in the cartridge for the separation column at a distance of 13 cm from its end. A fused-silica capillary covered with a protective polyimide layer (80 cm total length, 67 cm to C^4D, 75 μm i.d. × 375 μm o.d., from Composite Metal Services, U.K.) was used at a controlled temperature of 25 °C. Before its first use and on changes of the BGE, the capillary was conditioned by washing with 0.1 M NaOH (20 min), then with deionized water (20 min), and finally filled up with a BGE. Between each two CE runs, the capillary was washed successively with 0.1 M NaOH (2 min), deionized water (2 min) and the given BGE (4 min). The experimental conditions for the CE separations (sample injection, separation voltage and the BGE composition) are specified in descriptions of the individual experiments.

Chemicals

All the chemicals used were of analytical grade purity. The heparin sodium salt from bovine intestinal mucose (151 U mg^{-1}), (hydroxyethyl)cellulose (HEC), NaCl, $Na_2SO_4 \cdot 10H_2O$ were purchased from Sigma. Acetic acid (AcOH), dextran from *Leuconostoc ssp.* (100 kDa), poly(ethylene glycol) (PEG; 300 Da), poly(ethylene glycol) (8000 Da) and acetonitrile (ACN) were obtained from Fluka. The stock solutions, a 2% (w/v) (Mass of solute (in grams) dissolved in a final volume of 100 ml of solution.) dextran (100 kDa) and a 5% (w/v) PEG (8000 Da), were prepared by dissolving the appropriate amounts of solid substances in hot water;

they were used at room temperature for the BGE preparation. Deionized Milli-Q water (Millipore, Bedford, U.S.A.) was used to prepare the BGE and stock solution of heparin (1 mg ml^{-1}), which were stored in a refrigerator at 4 °C. pH was measured using a laboratory pH meter (pMX 3000, Wissenschaftliche technische Werkstätten, Germany).

Preparation of Human Plasma and Infusion Solution Samples

Arterial blood was collected from 4 healthy volunteers (4 men) into vessels containing an anticoagulant solution of EDTA. Blood samples were centrifuged at 2000 *g* for 10 min to remove the blood corpuscles; the resulting plasma was dosed into 0.5 ml microtubes (Eppendorf) and stored at –80 °C. Before use, the plasma samples were defrosted and then filtered by centrifugal filter units (Centricon YM 50, Millipore) at 5000 *g* for 10 min; in this way, the plasma is freed of high-molecular-weight proteins which would adsorb on the silica capillary walls and thus irreversibly damage the capillary. The filtrate was injected directly into the separation capillary.

An infusion solution sample with the declared heparin content 10 U ml^{-1} was obtained from the Anesthesiological Department of the Faculty Hospital at Královské Vinohrady (Prague) and was diluted with water at a 1:1 ratio prior to analysis.

RESULTS AND DISCUSSION

Separation of Intact Heparin in Solutions with Added HEC

CE separation of intact heparin. To electrophoretically separate intact heparin, the BGE containing acetic acid (AcOH) with an addition of (hydroxyethyl)cellulose (HEC) was first used. AcOH previously yielded good results in CE/C^4D determinations of amino acids, amines and peptides, which are separated as the cations in the positive mode[23,27–29]. The HEC added to the BGE strongly suppresses the electroosmotic flow (EOF) and effectively prevents adsorption of the analyte molecules on the silica capillary wall. The determination of heparin in AcOH/HEC was carried out in the negative mode used for anion separations. The AcOH concentration was 1.7 mol l^{-1}, with various additions of HEC: 0.1, 0.4 and 0.8% (w/v); pH 2.15. In the absence of HEC, an EOF was observed, directed from the detection to the injection end of the capillary, and thus this BGE was impossible to use in CE of anions.

It can be seen on the electropherogram in Fig. 2 that the heparin mobility decreases with increasing HEC concentration whereas the mobilities of the Cl^- and SO_4^{2-} inorganic ions, present as impurities in the heparin standard, remain unchanged. With increasing HEC concentration, the intensity of the interaction between the heparin macromolecules and the HEC molecules increases causing an increase in the migration time, whereas the migration rate of low-molecular-weight substances is unaffected by the HEC

presence. The heparin peak height rapidly decreases with increasing HEC concentration, due to a greater dispersion of the heparin zone and also due to smaller sample amounts injected to the capillary, caused by an increase in the BGE viscosity and thus by a decrease in the hydrodynamically injected sample volume. It can be seen from Fig. 2 (trace A) that the sample of intact heparin contains three main fractions corresponding to the three peaks on the CE/C^4D recording.

To enhance the sensitivity of the CE/C^4D determination, acetonitrile was added to the heparin sample, up to the overall water/ACN ratio of 1:1. The presence of ACN improves the efficiency of CE separations of low-molecular-weight substances[29–31] and its favourable effect has also been shown for heparin (Fig. 3). The peak height and the estimate of the peak width at a half-height ($w_{1/2}$) for the main heparin fraction in the absence of ACN amounted to (0.9 ± 0.1) mV and (39.1 ± 1.0) s, respectively, for 0.1 mg ml^{-1} standard solution of heparin at the 2000 mbar s injection. On an addition of ACN, the peak height increased by almost 70% to (1.5 ± 0.1) mV and the $w_{1/2}$ value decreased to (29.3 ± 0.9) s.

The presence of ACN in the sample decreases the conductivity of the sample zone and thus the intensity of the electric field within the sample zone is higher than that in the surrounding BGE on application of the separation electric field. The analyte ions are faster in leaving the sample zone

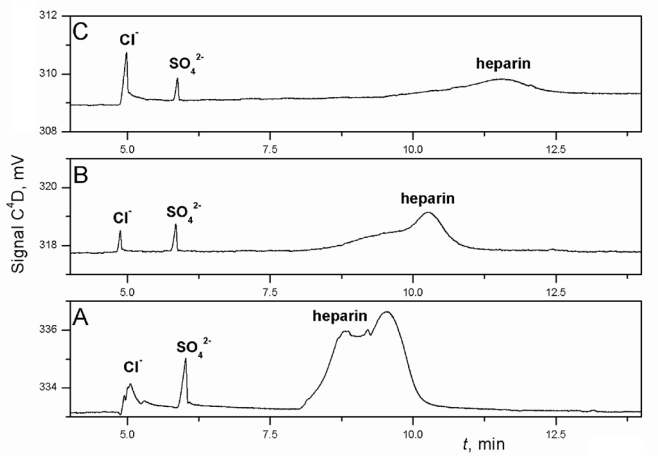

FIG. 2
CE/C^4D separation of a standard solution of heparin with a concentration of 1 mg ml^{-1} in the BGE of 1.7 M AcOH (pH 2.15) with varying HEC contents [in % (w/v)]: A 0.1, B 0.4, C 0.8. Hydrodynamic injection at 1000 mbar s; –20 kV/–20 μA

and the zone is sharpened – this is the field amplified stacking[32]. On the other hand, the lower electric field intensity within the surrounding BGE leads to an increase in the heparin migration time.

Separations in samples with high concentrations of chloride ions. Samples of heparin in blood plasma and in infusion solutions are marked by high NaCl

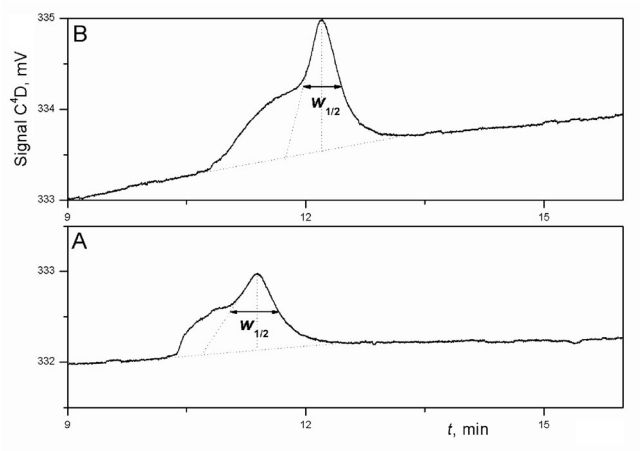

FIG. 3

CE/C^4D separation of a standard heparin solution with a concentration of 0.1 mg ml^{-1} dissolved in: A H_2O, B a mixture of H_2O/ACN 1:1. BGE: 1.7 M AcOH and 0.1 % (w/v) HEC. Hydrodynamic injection at 2000 mbar s; –20 kV/–20 μA

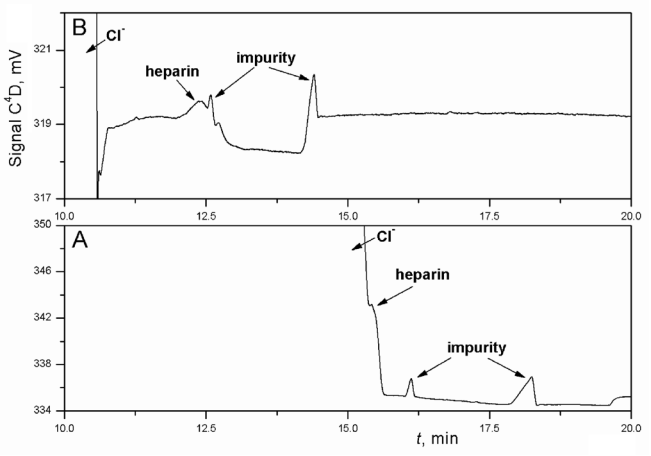

FIG. 4

CE/C^4D separation of heparin (0.3 mg ml^{-1}) at the NaCl concentration of 100 mmol l^{-1} in the BGE of 1.7 M AcOH and HEC [in % (w/v)]: A 0.1, B 0.4. Hydrodynamic injection at 1000 mbar s; –20 kV/–20 μA

contents (ca. 100 to 150 mM NaCl). Therefore, it is necessary to find conditions under which the heparin peak is sufficiently separated from the substantially larger peak of chloride ions. Figure 4 depicts an electropherogram of a heparin sample in a 100 mM NaCl solution. At a HEC concentration of 0.1% (w/v) in the BGE, heparin is not completely separated from the chloride peak and is located on its descending branch. An increase in the HEC concentration to 0.4% (w/v) slows down heparin migration and the peaks are thus separated. However, an improvement of the separation is associated with a decrease in the sensitivity of the determination of 60% compared with that attained in the solution with 0.1% (w/v) HEC (see Fig. 2B). It can be seen in Fig. 4 (electropherogram B) that the heparin peak coincides with a peak of an impurity that could not be identified. The favourable effect of added ACN on analytical parameters of the determination cannot be utilized with samples containing excess NaCl. When the concentration of NaCl in the mixture ACN/water (1:1) is 100 mmol l^{-1}, NaCl is precipitated and so is heparin, which was confirmed by the evaluation of the C^4D response to the heparin concentration. Therefore, ACN was not added to heparin samples with high NaCl contents.

Separation of Intact Heparin in Solutions Containing Poly(ethylene glycol) and Dextran

CE separation of intact heparin. The CE separation and characterization of intact heparin was further studied in BGE solutions containing entangled polymers[33]. The linear poly(ethylene glycol) with molecular weights of 300 and 8000 Da and the branched dextran (100 kDa) were tested. Solutions of these polymers have already been used for separations of polymers, in particular proteins, and initial attempts at separation of heparin have also been reported[19]. The polymer concentrations in 1.7 M AcOH in the present work were selected to equal the overlap threshold values[34], i.e., the values at which the macromolecules begin to entangle in solution. The appropriate overlap threshold values for the given polymers were taken from the literature[35]: PEG (300 Da) 10% (w/v), PEG (8000 Da) 2.5% (w/v), dextran (100 kDa) 1% (w/v).

It follows from Fig. 5 that the interaction of heparin molecules with a polymer solution depends both on the polymer type and on its molecular weight. In the BGE consisting of 1.7 M AcOH and 10% (w/v) PEG (300 Da), longer migration times were obtained for heparin macromolecules and for low-molecular inorganic ions (Cl^- and SO_4^{2-}) contained in intact heparin samples. This is caused by the fact that PEG of 300 Da does not sufficiently

suppress the EOF which opposes the anion migration. Solutions of entangled polymers with higher molecular weights (PEG 8000 Da and dextran 100 kDa) suppress the EOF with the same efficiency as does HEC. In a solution of 2.5% (w/v) PEG (8000 Da) in 1.7 M AcOH, three unpronounced maxima can be seen on the heparin peak corresponding to the individual fractions, whereas in a 1% (w/v) solution of dextran (100 kDa) in 1.7 M AcOH, the heparin peak is sharp with a single maximum. The sensitivity of the CE/C^4D determination is highest in this BGE.

The effect of the AcOH concentration was studied in the BGE containing 1% (w/v) dextran, using AcOH concentrations of 0.5, 0.8 and 1.7 mol l^{-1}. The increase in the heparin migration time is caused by changes in the heparin dissociation (Fig. 6). At lower AcOH concentrations, i.e., at higher pH values, the COOH groups in heparin are dissociated to a higher degree and thus heparin migration is faster. The character of the heparin peak also changes with changing AcOH concentration: maxima appear at lower AcOH concentrations, little pronounced at 0.8 M AcOH and quite sharp at AcOH concentration of 0.5 mol l^{-1} (Fig. 6C). The area of the wide peak on the electropherogram in Fig. 6B is 750 mV s and equals the sum of the two sharp peaks and the main peak on the Fig. 6C (753 mV s). Since the migration times of the sharp maxima are shorter than that of the main peak, it can be assumed that the sharp maxima correspond to fractions of intact

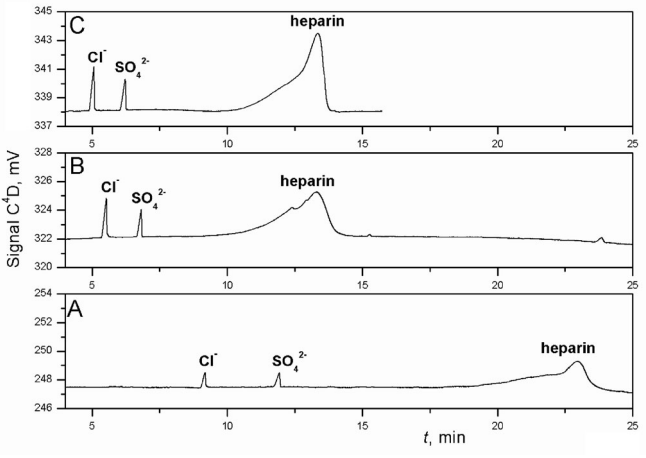

FIG. 5
CE/C^4D separation of a standard heparin solution (1.0 mg ml^{-1}) in the BGE of 1.7 M AcOH (pH 2.15) with various entangled polymers added [in % (w/v)]: A 10 PEG (300 Da), B 2.5 PEG (8000 Da), C 1 dextran (100 kDa). Hydrodynamic injection at 1000 mbar s; –20 kV/–20 μA

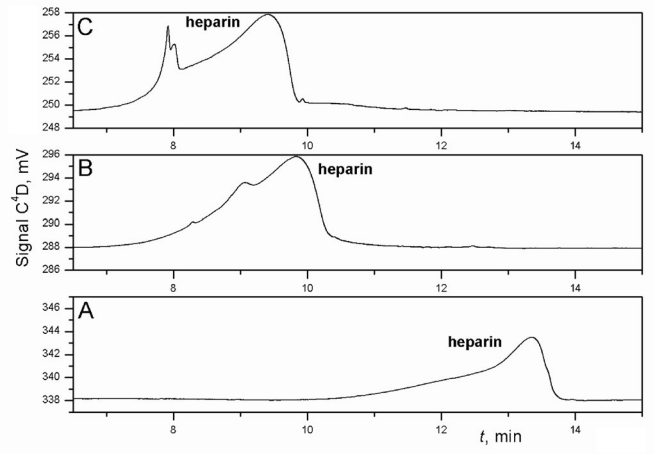

FIG. 6
CE/C^4D separation of a standard heparin solution (1 mg ml^{-1}) in the BGE containing 1% (w/v) dextran (100 kDa) and various AcOH concentrations (in mol l^{-1}): A 1.7 (pH 2.15), B 0.8 (pH 2.3), C 0.5 (pH 2.4). Hydrodynamic injection at 1000 mbar s; –20 kV/–20 μA

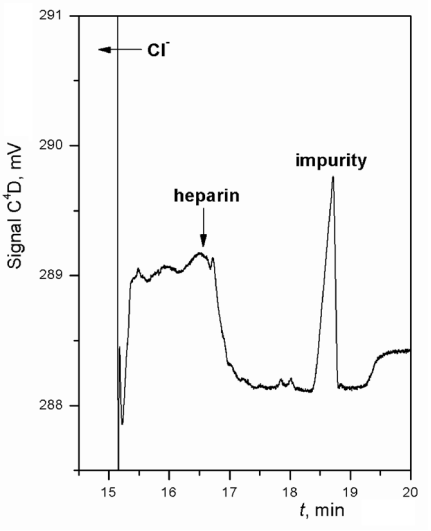

FIG. 7
CE/C^4D separation of heparin (0.1 mg ml^{-1}) at the NaCl concentration of 100 mmol l^{-1} in the BGE of 0.8 M AcOH (pH 2.3) and 1% (w/v) dextran (100 kDa). Hydrodynamic injection at 1000 mbar s; –20 kV/–20 μA

heparin whose molecular weights are lower than the average molecular weight.

Separation at High Concentrations of Chloride Ions

In the BGE consisting of 0.8 M AcOH and 1% (w/v) dextran, heparin can be separated from excess Cl^- ions up to their concentration of 100 mmol l^{-1}. It follows from the performed studies that this solution is best suited for the determination of heparin in blood plasma and in infusion solutions containing high NaCl concentrations (Fig. 7). The sensitivity of the CE/C^4D determination of heparin at the NaCl concentration of 100 mmol l^{-1} was determined from the dependence of the peak area on the concentration (Table I). The LOD was determined from the calibration dependence using the formula LOD = 3 × standard error/slope. The LOD value obtained for heparin, 1.3 μmol l^{-1}, is fully comparable with the LOD values for CE/C^4D determinations of small inorganic ions for which the C^4D was originally developed. Moreover, the LOD for heparin is negatively affected by the polydispersity of the polymer and would be lower for the pure polymer. The LOD value, 16 μg ml^{-1}, obtained by the developed CE/C^4D procedure, is better than the LOD reported in the literature[19] (Table II).

TABLE I
The calibration parameters for CE/C^4D determination of heparin in a model sample and in blood plasma[a]

Parameter	Model sample[b]	Plasma
Concentration range tested, mg ml^{-1}	0.025–1.0	0.1–0.5
Linear dynamic range, mg ml^{-1}	0.025–1.0	0.2–0.5
Slope (sensitivity), mV s ml mg^{-1}	403.1 (2.6)	414.4 (4.2)
Intercept, mV s	–1.0 (1.2)	–10.3 (1.3)
Standard error, mV s	2.15	0.600
Coefficient of correlation	0.9999	0.9999
LOD, μg ml^{-1}	16.0	100.0
LOD[c], μmol l^{-1}	1.3	8.3

[a] BGE: 0.8 M AcOH and 1% (w/v) dextran; the concentrations measured in triplicate, evaluating the peak areas. Standard deviations are given in parentheses. [b] In 100 mM NaCl. [c] Average molecular weight of intact heparin, 12 kDa.

The separation of heparin from excess chloride ions under experimental conditions common in CE is the principal advantage of the present study in comparison with the previous work[19]; the basic differences are summarized in Table II from which the simplicity of the proposed method is evident.

TABLE II
Comparison of experimental conditions for CE determination of heparin in previous work and current study

Parameter	This paper	Previous work[19]
Capillary	fused silica, 80 cm, 75 μm ID	fluorinated ethene–propene copolymer, 21 cm, 300 μm ID, hydrodynamically separated from ground electrode electrolyte with membrane
BGE	0.8 M AcOH and 1% (w/v) dextran (pH 2.3)	25 mM glycine/bis-tris-propane, 0.2% (w/v) (hydroxyethyl)methylcellulose and 2% (w/v) dextran (pH 9.0)
Injection	hydrodynamic, 1000 mbar s	injection valve, 100 nl
Detection	contactless conductivity, capillary can be easily exchanged as necessary	contact conductivity, electrodes are integral part of the capillary
LOD	16 μg ml^{-1}	40 μg ml^{-1}

Analysis of Real Samples

The method developed was tested in determinations of heparin in blood plasma and in the infusion solutions used to prevent thrombosis during operations. No heparin peak can be observed on the electropherogram of filtered plasma (Fig. 8A). On the first addition of 0.1 mg ml^{-1} heparin, its peak has an area of 5.0 mV s; the area after the addition of the same amount increases to 20.5 mV s (Figs 8B and 8C). The concentration dependence is linear in the range 0.2–0.5 mg ml^{-1} and the sensitivity is close to that obtained for an infusion solution which is substantially simpler than the plasma (Table I). The nonlinear behaviour at low concentrations can be explained by firm binding of a part of heparin to a blood plasma component. Heparin in the plasma appears free only after saturation of the binding sites and only then can be determined.

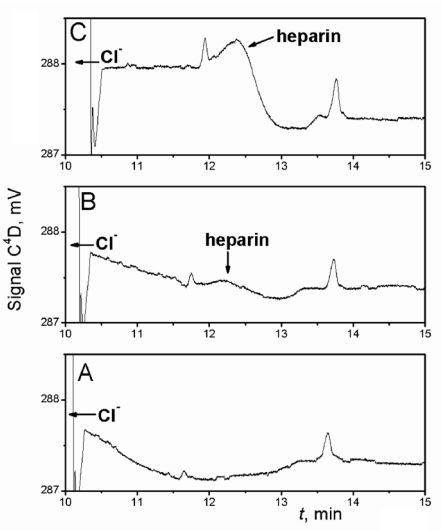

FIG. 8

CE/C^{4}D determination of heparin in blood plasma. Heparin added (in mg ml^{-1}): A 0, B 0.1, C 0.2. BGE: 0.8 M AcOH and 1% (w/v) dextran. Hydrodynamic injection at 1000 mbar s; –20 kV/–20 μA

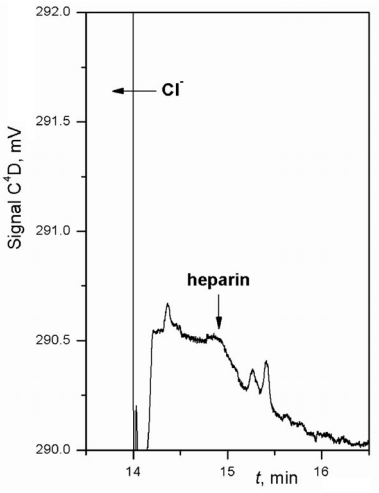

FIG. 9

CE/C^{4}D determination of heparin in the infusion solution used during operations, with the declared heparin concentration 10 U ml^{-1} (ca. 65 μg ml^{-1}). The infusion solution was diluted with water (1:1) prior to analysis. BGE: 0.8 M AcOH and 1% (w/v) dextran. Hydrodynamic injection at 1000 mbar s; –20 kV/–20 μA

The procedure is also applicable to determination of heparin in the infusion solution used in anesthesia (Fig. 9). The determined concentration, (42 ± 4) μg ml^{-1}, is lower than that declared, 10 U ml^{-1} (ca. 65 μg ml^{-1}). This difference may be caused by the fact that the heparin concentration in the infusion solution is close to the LOD.

CONCLUSIONS

Hydrophilic polymers, HEC, PEG and dextran, were added to the BGE solution for CE characterization of polydisperse heparin. The polymer dissolved in the BGE interacts with heparin and decreases the velocity of its migration. Sharp maxima can be observed on CE/C^4D electropherograms of standard heparin in the optimized BGE, corresponding to the predominating fractions of the polydisperse mixture. The C^4D technique is advantageous for detection of heparin which does not absorb radiation in the UV/VIS range. The LOD value, 1.3 μmol l^{-1}, obtained for heparin is comparable with the LOD values for small inorganic ions. Heparin is separated from excess chloride ions in the BGE consisting of 0.8 M AcOH and 1% (w/v) dextran. This BGE was tested for determination of heparin in blood plasma. Heparin can only be observed on the electropherograms at higher concentrations (exceeding ca. 0.1 mg ml^{-1}) because its certain part is apparently bound to plasma components. Therefore, the procedure developed is better applicable to determinations of heparin in infusion solutions and to purity control of pharmaceutical preparations.

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