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Short communication

One-dimensional cellulose acetate plate electrophoresis—A feasible method for analysis of dermatan sulfate and other glycosaminoglycan impurities in pharmaceutical heparin

Rainer Domanig, Wolfgang Jöbstl, Silvia Gruber, Thomas Freudemann*

IPC Analytical & Technical Process Support Labs, Sandoz GmbH, Biotechnical Production Plant Schaftenau, Biochemiestraße 10, 6336 Langkampfen, Austria

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ABSTRACT

A cellulose acetate plate electrophoresis method for analysis of pharmaceutical heparin and its potential glycosaminoglycan impurities, e.g. dermatan sulfate, chondroitin sulfate and oversulfated chondroitin sulfate, is presented. Heparin is chemically degraded by application of nitrous acid and residual glycosaminoglycans are electrophoretically separated thereafter. After staining using Alcian blue 8GS, these glycosaminoglycan impurities can be quantified by means of comparison to a dermatan sulfate standard. Results of a validation study of this analytical method are shown, demonstrating its feasibility for routine use in analytical quality control labs under GMP conditions.

1. Introduction

Pharmaceutical grade heparin [1,2] in Europe and the US is usually derived from intestinal pig mucosa [3]. It consists of a mixture of glycosaminoglycan (GAG) carbohydrate polymers. Typical impurities in pharmaceutical heparin (HP) may therefore include other GAGs, in particular dermatan sulfate (DS) and chondroitin sulfate A&C (CA/CC) [4].

Heparin is one of the oldest drugs still in clinical use [5] due to its anticoagulative activity. It is degraded when taken orally and therefore has to be administered parenterally. In special medical circumstances, high doses of heparin have to be injected [6]. Thus, it is vital for pharmaceutical companies as well as for independent quality control laboratories to be able to control its purity by reliable analytical methods.

In February 2008, FDA published a warning concerning severe adverse effects in patients who received bolus injections of heparin sodium for injection and recommended recalls of certain heparin lots [7]. A new impurity was found in heparin and identified as a chemically modified, i.e. oversulfated chondroitin sulfate (OSCS) [8]. OSCS is suspected to be the cause for clinical adverse effects [9]. OSCS is not detected by common analytical methods [1,2], for instance assays of anticoagulative activities or size exclusion chromatography methods. Therefore, additional analytical tools for reliable quality control of pharmaceutical heparin are in urgent demand.

Two analytical methods which proved suitable for detection of the new impurity found in heparin were recently published by FDA [7]: ^{1}H NMR and capillary electrophoresis (CE) [10].

In the present paper we demonstrate an additional method for detection and quantification of glycosaminoglycan impurities in heparin. This method is based on electrophoretic separation of glycosaminoglycans using one-dimensional cellulose acetate electrophoresis. A review on this topic was published by Volpi and Maccari [11].

A method first described by Cappelletti et al. [12–14] and modified by Hopwood and Harrison [15] was refined in our lab in order to suit the requirements necessary for pharmaceutical quality control of heparin under GMP-conditions and validated thereafter. We elaborated a reliable and simple test method which can be used for routine quality control of GAG impurities in heparin. In addition, only inexpensive analytical equipment is necessary in contrast to the ^{1}H NMR- or CE-method, respectively. The method enables the separation and detection of various glycosaminoglycan impurities which may be present in heparin, e.g. chondroitin sulfates A&C, dermatan sulfate and the new impurity OSCS. This cellulose acetate plate electrophoresis method can be performed as a limit test as well as for quantitation of glycosaminoglycan impurities in heparin.

* Corresponding author. Tel.: +43 5372 6996 5282; fax: +43 5372 5010.
E-mail address: thomas.freudemann@sandoz.com (T. Freudemann).

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2. Materials and methods

2.1. Glycosaminoglycans

CA (from bovine trachea and CC from shark cartilage were purchased from Sigma–Aldrich (Vienna, Austria). DS (from porcine intestinal mucosa) and GAG-mixture was purchased from Calbiochem (Merck Biosciences, Nottingham, UK). All heparin probes are industrial samples.

2.2. Electrophoretic apparatus

The arrangement of the electrophoretic apparatus (electrophoretic chamber: Amersham Biosciences Multiphor II; (Amersham, GE Healthscare Bio-Sciences, Uppsala, Sweden) was published by Hopwood and Harrison [15].

The device is cooled to 4 °C prior to the electrophoresis procedure.

2.3. Sample and cellulose acetate plate preparation

1. All solutions (samples and buffers) are filtered using a 0.45 μm membrane filter prior to use.
2. 150 mg heparin sample is dissolved in 5 ml water (30 μg/μl). DS reference solutions 4.5%, 2.5% and 0.5% or 1.35 μg/μl, 0.75 μg/μl and 0.15 μg/μl, respectively are prepared using DS reference substance.

2.4. Nitrosation

1. The cellulose acetate plate (CAP) (Helena Titan III 76 mm × 94 mm (Helena Laboratories, Beaumont, USA)) is equilibrated for 20 min in 0.5 M $NaNO_2$/0.01 M NaOH.
2. 1 μl of the sample solutions each are loaded to the start of the moist CAP using a 8-channel multi-applicator (Hamilton, Bonaduz, Switzerland).
3. Immediately after loading the CAP is immersed in 0.5 M $NaNO_2$/0.01 M NaOH for 2 min.
4. The CAP is blotted using blotting paper (Pharmacia, GE Healthscare Life-Sciences, Uppsala, Sweden) and placed in 1 M HCl (caution-development of nitrous gases!) for 2 min.
5. The CAP is blotted again and equilibrated in 0.1 M Barium acetate, pH 5.0, for 2 min.

2.5. Electrophoresis

1. CAP is blotted, one drop of *n*-decane and a polyester plate (Pharmacia, GE Healthscare Life-Sciences, Uppsala, Sweden) with the hydrophobic side facing downwards put on the CAP, letting ends uncovered for attachment of current bridges. A few drops of *n*-decane are allowed to drip onto the cooling plate, then the covered CAP is placed onto it and any air bubbles are smoothed out.
2. The current bridges are placed on the uncovered ends of the CAP, the precooled (4 °C) stack of glass plates is laid on top and the apparatus is closed.
3. A current of 230 V (60 V h) is applied for 20 min.
4. The CAP is blotted and immersed in cooled buffer 0.1 M Barium acetate, pH 5.0/15% (V/V) isopropanol for 2 min.
5. The procedure is repeated as described starting in item 1.
6. A current of 130 V (220 V h) is applied for 75 min.

2.6. Staining/destaining

1. The CAP is briefly immersed in 3.5% (V/V) aqueous isopropanol solution and rinsed with water.
2. The CAP is stained for approx. 3–4 min by shaking gently in dye solution (0.1% (m/V) Alcian blue 8 GS in 1% (V/V) acetic acid, stirred for at least 30 min at room temperature and filtered through a fluted filter). The CAP is rinsed immediately with water thereafter.
3. The CAP is immersed in 200 ml of 5% (V/V) aqueous acetic acid solution and agitated gently on the shaker for approx. 10 min; the acetic acid solution is changed and the process is repeated three times.

2.7. System suitability test criteria

The following system suitability test criteria must be met for valid results:

1. The 0.5% dermatan sulfate standard spot must be clearly visible on the CAP while moist otherwise the analysis needs to be repeated with a longer staining period.
2. The spots from the tested sample must be clearly separated from any heparin residue bands.
3. No non-removable colour spikes (e.g. foreign particles, artefacts) are found on the spots to be evaluated.
4. The quantification of the control sample must result in a recovery of ±10% of the theoretical dermatan sulfate content.

2.8. Evaluation

The densitometric evaluation of the destained, moist CAP is performed using a suitable scanner and an imaging software, which measures the optical density of the dyed spots. The heparin residue spots are not taken into account in the evaluation.

The relative amount (percentage) of dermatan sulfate and any other individual glycosaminoglycan impurity in the heparin sample is calculated by means of a linear 3-point calibration curve, which was calculated from the densitometric signals (peak areas) of the dermatan sulfate standard spots.

Other glycosaminoglycan reference standards, e.g. OSCS, may be used as well if available.

3. Results and discussion

3.1. Specificity

A typical example of a CAP obtained after the regular electrophoretic separation is illustrated in Fig. 1.

Heparin is degraded by application of nitrous acid. Only minor artifacts of non nitrous acid degradable heparin may be visible on the CAP in some cases. However, these artifacts do not interfere with stained GAG spots of chondroitin sulfate, dermatan sulfate or the new impurity. If visible, they are of weak intensity and migrate faster than dermatan sulfate, but slower than chondroitin sulfate. OSCS does not exhibit any electrophoretic mobility under the experimental conditions and remains at the origin of the electrophoresis plate.

Dermatan sulfate and other possible impurities (chondroitin sulfate A&C, OSCS) are clearly separated from each other, only chondroitin sulfate A and chondroitin sulfate B could not be separated; specific quantification of OSCS, dermatan sulfate and chondroitin A&C is thus possible.

Analytical results received by the cellulose acetate plate electrophoresis method were compared with data obtained by the methods published by FDA [7] (^{1}H NMR and CE [10]). These experiments indicate that the impurity depicted in Fig. 1 is identical with the new impurity found by FDA [7] and recently identified as oversulfated chondroitin sulfate [8].

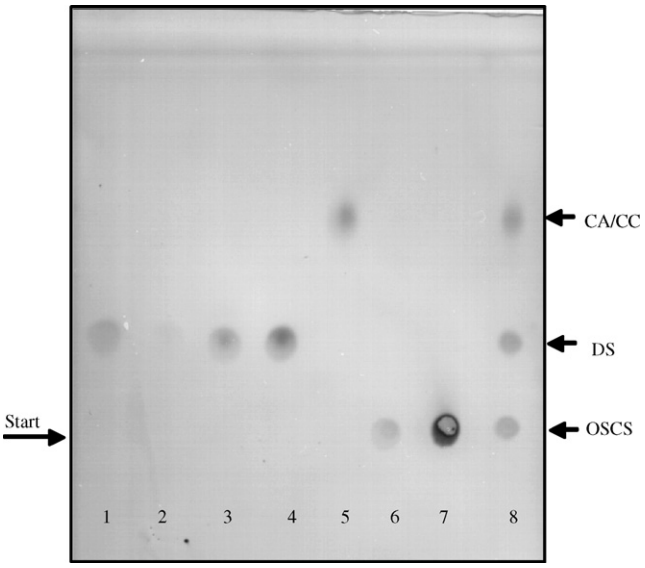

Fig. 1. Exemplary electrophoretic separation for demonstration of specificity of the analytical method as described in Section 2. (1) Control heparin sample (with known dermatan sulfate content of 2.0%); (2) Dermatan sulfate standard 0.5%; (3) Dermatan sulfate standard 2.5%; (4) Dermatan sulfate standard 4.5%; (5) Chondroitin sulfate A&C 2.0%; (6) Heparin sample containing 2.0% OSCS; (7) Heparin sample containing 10.0% OSCS; (8) Mixture: heparin sample with OSCS 2.0% + dermatan sulfate 2.0% + chondroitin sulfate A&C 2.0%.

In Fig. 2, a typical CAP of a modified electrophoretic method is depicted. If the nitrosation step of the analytical procedure is omitted, heparin is not degraded by nitrous acid on the CAP. After electrophoretic separation and staining/destaining carried out as usual, heparin fractions of different electrophoretic mobilities can be distinguished on the CAP, in particular slow- and fast-moving heparin. Slow-moving heparin is electrophoretically immobile under the experimental conditions and remains at the start of the cellulose acetate plate. Spots of minor GAG impurities in pharmaceutical heparin may overlap with heparin bands under these conditions, preventing their detection.

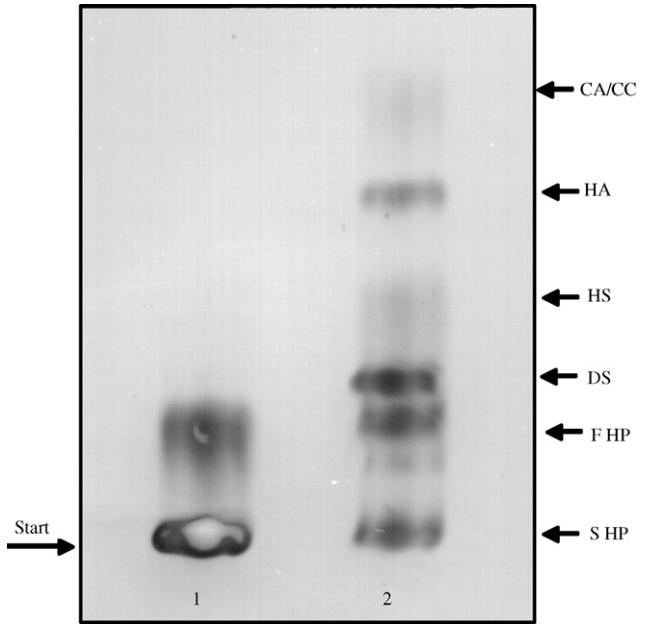

Fig. 2. Typical cellulose acetate plate electrophoresis without nitrous acid degradation step; instead, the CAP is equilibrated in 0.1 M Barium acetate, pH: 5.0 for at least 12 h. (1) Pure pharmaceutical heparin (25 μl; 7.5 μg/μl) exhibiting slow- and fast moving fractions; (2) GAG-mixture (25 μl; 7.5 μg/μl) consisting of 13% chondroitin sulfate A&C (CA/CC), 5% hyaluronic acid (HA), 25% heparan sulfate (HS), 24% dermatan sulfate (DS), 19% fast-moving (F HP) and 14% slow-moving heparin (S HP).

For quantification purposes, an internal calibration on the CAP using one or more standard spots has to be performed, because the amount of sample loaded on each CAP may vary due to the equilibration and blotting procedure. In addition, the staining/destaining step may lead to varying signal intensities when different CAPs or Alcian blue 8GS-lots are compared.

A typical example of an optical densitometric evaluation is depicted in Fig. 3.

3.2. Linearity

Dermatan sulfate is a GAG-impurity frequently present in heparin preparations, thus a linear three-point DS-standard calibration curve was chosen in order to give reliable quantification results for DS. Results of a linearity study of the examined analytical range of 0.4–4.8% were: correlation coefficient of 0.9997; *y*-axis intercept 7.049; slope 806.6; residual sum of square 6499. Thus, requirements for linearity were fulfilled for the examined range.

GAG spots on CAP must show optical densities in the range of the internal calibration curve for valid results. In case of sample 7) in Fig. 1, OSCS is too concentrated (approx. 10%) and thus the experiment had to be repeated with lower concentrations in order to deliver valid quantitative results.

3.3. Precision

3.3.1. Repeatability

After 8 repeats of the whole analytical procedure by one analyst at day 1 a coefficient of variation of 1.823% relative was observed.

3.3.2. Intermediate precision

8 repeats of the electrophoresis were carried out by analyst 2 on day 2. Together with the results obtained on day 1 (repeatability), a coefficient of variation of 2.610% relative was observed.

3.4. LOD/LOQ

To determine the limit of detection (LOD) and limit of quantification (LOQ) of dermatan sulfate, a dilution series from 0.1% to 0.8% DS in heparin (quantification concentration) was applied to the CA plate and evaluated.

After visual evaluation of the peaks, a detection limit of 0.4% and a quantification limit of 0.5% dermatan sulfate in heparin sodium were established. In Fig. 4, a densitometric plot at the LOQ (0.5% DS in heparin) is depicted.

3.5. Accuracy

The accuracy was determined by calculating the recovery rates at three different concentration levels (4 determinations each of 3 mixed samples on 3 different CA plates).

Mixed samples were prepared with 1.0%, 2.0% and 3.0% dermatan sulfate in heparin (30 μg of heparin per μl) and then evaluated against the 0.5%, 2.5% and 4.5% dermatan sulfate standards.

Mean recovery rate observed was 93.7%; 95% confidence interval of the mean was found to be ±7.14%.

3.6. Limit test

In the case that no quantification of GAG impurities in heparin is required, the analytical methodology may be used as a limit test.

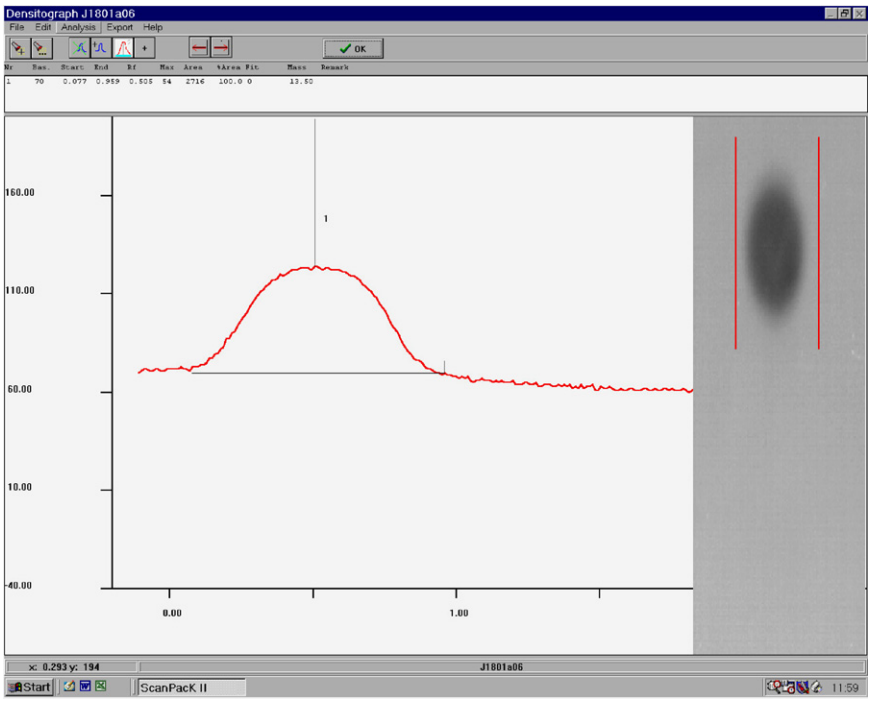

Fig. 3. Typical example of densitometric evaluation of dermatan sulfate standard spot dyed with Alcian blue 8 GS on the electrophoresis plate.

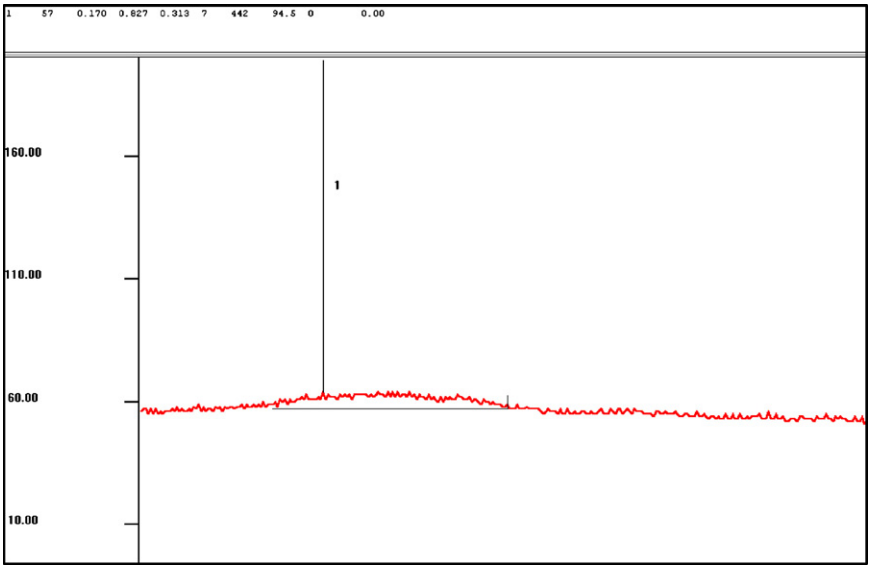

Fig. 4. Limit of quantification: Densitometric plot of 0.5% dermatan sulfate in heparin separated by CAP electrophoresis method.

In this case, only a 0.5% dermatan sulfate standard spot is applied on the electrophoretic plate, the analytical procedure is carried out as usual, and the probes are compared visually with the resulting standard spot. Thus no device for evaluation of optical density is necessary, and two more analytical samples can be placed on the CAP, raising the throughput of the method.

4. Conclusions

The method described in this paper is a modification of electrophoretic methods previously described by Cappelletti et al. [12–14] and by Hopwood and Harrison [15] and refined in our lab in order to deliver reliable results for quality control of pharmaceutical heparin under GMP conditions. Typical glycosaminoglycan impurities of heparin -chondroitin sulfate and dermatan sulfate- can be specifically separated, detected and, if desired, quantified by means of calibrated optical densitometry. In addition, an impurity which was found recently in medical heparin [7] and identified as oversulfated chondroitin sulfate [8,9] can be detected and quantified as well.

The validity of this method has been shown to fulfill pharmaceutical industry (GMP) standards.

Unlike all other recommended analytical methods [7] for detection of the new impurity in heparin, ^{1}H NMR and CE [10], this cellulose acetate plate electrophoretic method requires only inexpensive analytical equipment, which is affordable by any quality control lab, especially if quantification of the impurities is not necessary and thus only a simplified limit test method is needed.

Therefore we recommend this simple and reliable method as an additional tool for quality control of pharmaceutical heparin to be used by health authorities and in the pharmaceutical industry.

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