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Short communication

Alternative method for determination of contaminated heparin using chiral recognition

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ABSTRACT

Since 2008 a significant amount of work has focused on the development of methods to analyze contaminated heparin. This work focuses on utilizing heparin's ability to serve as a chiral selector as a means for determining contamination. Specifically, the effect of contamination on the separation of pheniramine and chloroquine enantiomers was explored. Separations were conducted using heparin contaminated with chondroitin sulfate at varying levels. For each pair of enantiomers, electrophoretic mobility and resolution were calculated. For pheniramine enantiomers, an increase in contamination leads to a decrease in the electrophoretic mobility and resolution. A linear relationship between contamination level and electrophoretic mobility of the pheniramine enantiomers was observed for the entire contamination range. A linear relationship was also found between contamination level and resolution of the enantiomers between 0 and 70 percent contamination. For the separation of chloroquine enantiomers, it was found that at low levels of contamination, the resolution of enantiomers was increased due to the secondary interaction between the chloroquine enantiomers and the chondroitin sulfate. Results of this study illustrate the potential of using chiral recognition as a means to determine heparin contamination as well as the improvement of the chiral resolution of chloroquine with the additional of low levels of chondroitin sulfate A.

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1. Introduction

Heparin, a naturally occurring linear glycosaminoglycan, has been used extensively as an anticoagulant since 1935 [1]. Between November 2007 and January 2008, 113 patients displayed adverse reactions to heparin. Further investigation revealed that the heparin had been contaminated with over-sulfated chondroitin sulfate (OSCS) [2]. These initial reports lead to a world-wide investigation of heparin manufacturing. Since the initial recall of heparin, the FDA estimates that over 200 deaths have occurred in the United States and heparin samples containing OSCS have been found in at least 12 countries worldwide [3].

Heparin is a complex biopolymer and, due to its extensive sulfonation, is one of the most anionic biopolymers currently known [4]. Because heparin cannot be synthesized directly and must be extracted from various animal tissues, the basic subunit of heparin can vary depending on the source (e.g., porcine, bovine, ovine). Due to the way heparin is manufactured, a number of impurities occur naturally, the most common being dermatan sulfate (DS) which is present in pharmaceutical grade heparins at a range of 1–7%. With the recent deaths related to heparin contamination, significant research has been conducted over the last five years to examine the effect these contaminates have in a clinical setting [5–7] and to create new methods for the detection and quantification of both naturally occurring impurities as well as contaminants such as OSCS [8,9]. For example, high performance liquid chromatography (HPLC) with ultra-violet absorption [10–13], mass spectrometry [14,15], and circular dichroism [16,17] have been investigated for this. Capillary electrophoresis (CE) has also been used for the determination of heparin purity [13,18–23]. This technique is particularly attractive due to its use of small amounts of sample, short analysis time, and minimal waste. In addition to these two separation techniques, a wide range of other analytical techniques have been developed to investigate heparin contamination including nuclear magnetic resonance [13,24,25], gel electrophoresis [24,26], potentiometric techniques [27,28], colorimetric and fluorescence assays [29,30] and other biochemical techniques [31,32].

Beyond the use of heparin as an anti-coagulant, its utility as a chiral selector using CE has also been explored [33,34]. Initial studies by Stalcup et al. [35] explored the use of heparin as a chiral

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selector for enantiomers of antimalarial and antihistamine compounds such as chloroquine and pheniramine. While the exact mechanism for chiral recognition by heparin remains unknown, it was postulated that chiral recognition was based on a combination of ionic, hydrogen bonding, and hydrophobic interactions. Chloroquine, with a +3 charge at the operating pH had one of the highest charge densities of all the compounds tested and therefore had the strongest interaction with the heparin as illustrated by its long retention times compared to other compounds tested. In contrast, pheniramine with its +2 charge at the operating pH, was found to have weaker interaction with heparin. However, baseline resolution of the pheniramine enantiomers was still observed [35]. Several subsequent reports explored the effects of pH, buffer concentration, and heparin concentration on the resolution of these compounds [36–44].

This work investigates the use of heparin's role as a chiral selector as a potential means for determining heparin contamination. Specifically the effect of contaminated heparin on the electrophoretic mobility and the resolution of pairs of enantiomers was evaluated. Chloroquine and pheniramine were chosen for this work in order to evaluate enantiomers with both a stronger (chloroquine) and weaker (pheniramine) interaction with heparin.

2. Materials and methods

2.1. Reagents and solutions

Heparin sodium salt from porcine intestinal mucosa (avg. $M_W \sim 10{,}000$ Da) was obtained from Alfa Aesar (Ward Hill, MA). Chloroquine was purchased from the United States Pharmacopeia (Rockville, MD). Nitromethane was obtained from Aldrich Chemicals (Milwaukee, WI). All other chemicals were from Sigma Chemical Co. (St. Louis MO). All reagents were used without further purification.

2.2. Instrumentation

All CE experiments were run on a Beckman–Coulter (Fullerton, CA) P/ACE MDQ CE system equipped with a UV detector and controlled by a PC using 32 Karat Software (v. 8.0). Bare fused-silica capillary (50 μm i.d.) was obtained from Polymicro Technologies (Phoenix, AZ) and prepared to the following dimensions: 60.2 cm total length, 50.0 cm effective length.

The capillary electrophoresis buffers consisted of 100 mM NaH_2PO_4 and used heparin (4.0%) as the chiral selector. The buffer pH was adjusted following the addition of heparin. Contaminated heparin was prepared by substitution of chondroitin sulfate A at varying contamination levels. All CE solutions were degassed and filtered through a 0.2-μm filter (Nalge Nunc International Corp., Rochester, NY) before use. Stock solutions (5.0 mM) of the chiral analytes were prepared by dissolving an appropriate amount in distilled water. Working samples of these compounds were then prepared by dilution with a 1:10 buffer–water mixture to a final concentration of 0.5 mM. Nitromethane was used as the neutral marker for all samples. Prior to use, capillaries were rinsed with run buffer for 2 min at 10 psi. This rinsing procedure was also used between subsequent injections. All chiral samples were injected hydrodynamically into the CE system at 0.5 psi for five seconds. Separations were performed under cathodic detection with an applied voltage of 20 kV (for pheniramine separations) or 30 kV (for chloroquine separations). Detection occurred at 214 nm and all experiments were performed in triplicate at 25° C.

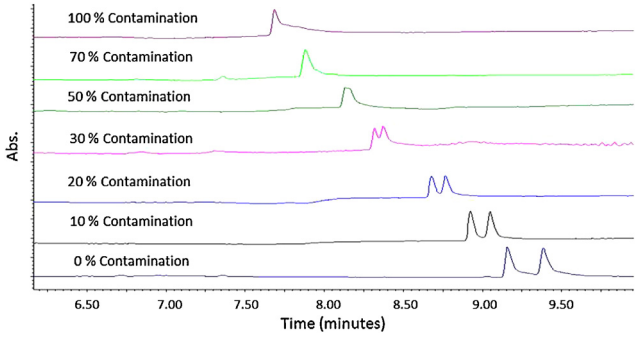

Fig. 1. *Effect of contamination on the separation of pheniramine enantiomers.* Electropherograms illustrating the effect of increasing contamination on the resolution and electrophoretic mobility of pheniramine enantiomers. Separation conditions as indicated in the experimental section.

3. Results

3.1. Chiral separations using heparin

Based on previous reports optimum conditions for chiral resolution utilize a 100 mM phosphate buffer with 4% heparin as the chiral selector at pH 4.5 (for pheniramine) and 5.3 (for chloroquine) [44]. To explore the relationship between contamination and the mobility and resolution of chloroquine and pheniramine enantiomers a series of buffers were prepared at contamination levels ranging from 0–100% with chondroitin sulfate A used as the model contaminant.

3.1.1. Effect of contamination on separation of pheniramine enantiomers

Fig. 1 shows representative electropherograms of pheniramine enantiomers at several different contamination levels. As can be seen in this figure, the resolution between the two enantiomers of pheniramine decreases with increasing contamination. As expected, at 100% contamination, i.e., 0% heparin, no separation of the two enantiomers can be observed. Specifically, a linear relationship is observed between resolution and contamination at levels ranging from 0–70% contamination ($R^2 = 0.94$) with resolution dropping to zero at contamination levels greater than 70%. In terms of electrophoretic mobility, as can be seen in Fig. 1, the mobility of the pheniramine enantiomers increases with increasing contamination levels. A linear relationship is observed between mobility and contamination across the entire range of contamination ($R^2 = 0.97$). The changes in resolution and mobility are directly related to the decreasing amounts of heparin in the system. As less heparin, which serves as the chiral selector, is available to interact with the pheniramine enantiomers the resolution between the enantiomers disappears. In addition, the electrophoretic mobility increases with decreasing amounts of heparin as less heparin is present to interact with the pheniramine and slow its migration through the capillary.

Check samples were prepared at 25% and 75% contamination levels and analyzed ($n = 3$). Using the relationship between contamination and resolution these samples were determined to be 24.3% (±1.3%) and 60.1% (±0.1). Using the relationship between concentration and mobility the check samples were found to be 22.5% (±4.5%) and 69.5% (±0.4%). As expected the mobility was found to be more accurate at higher contamination concentration levels compared to resolution. Both resolution and mobility are comparable at low levels of contamination.

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