A CAPILLARY ELECTROPHORETIC METHOD FOR FINGERPRINTING LOW MOLECULAR WEIGHT HEPARINS[#]

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Running Title: Fingerprinting Low Molecular Weight Heparins

[#]Patent application on the use of this method is in process.

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Abstract

Clinically used low molecular weight heparins are anticoagulants of choice and are phenomenally complex mixtures of millions of distinct natural and unnatural polymeric sequences. The FDA recommends that each LMWH be considered as an independent drug with its own activity profile placing significant importance on the biophysical characterization of each intact LMWHs. We report a robust protocol for fingerprinting these pharmaceutical agents. Capillary electrophoresis of three LMWHs, enoxaparin, tinzaparin and a Sigma preparation, under reverse polarity conditions in the presence of selected linear alkyl polyamines gives an electrophoretic pattern that is characteristic of the nature of the starting material. The buffers that best provided optimal resolution without compromising sensitivity and speed of analysis were 50 mM sodium phosphate, pH 2.3, and 100 mM ammonium formate, pH 3.5. Resolution was strongly dependent on the structure of polyamine with pentaethylenehexamine being most effective for enoxaparin and Sigma LMWH. In contrast, tinzaparin could be best resolved with tetraethylenepentamine. Cyclic polyamines were ineffective. Resolution was also dependent on the concentration of resolving agents and displayed a narrow window that provides optimal resolution. These features suggest a strong structural origin of the fingerprint pattern. Overall, the simple protocol will find special use in assessing LMWH quality and batch-to-batch variability.

Keywords

Capillary electrophoresis, low molecular weight heparins, biophysical analysis, batch-to-batch variation

Introduction

Heparin (H[@]) and low molecular weight heparins (LMWHs) are the two most common agents used to treat and prevent thrombotic disorders. Both are linear polymers composed of alternating 1→4-linked uronic acid and glucosamine residues that are variably sulfated, which generates phenomenal microheterogeneity. Clinically used heparin, appropriately named unfractionated heparin (UFH), is obtained from animal mucosa. UFH is a mixture of millions of chemical species differing from each other in size and chemical constitution.[1,2] LMWHs are about 3-fold smaller (M_R ~5000, Fig. 1) than UFH and are produced by chemical or enzymatic depolymerization, or chromatographic separation of the natural product.[1] Thus, LMWHs also comprise of millions of distinct polymeric sequences. In fact, they may contain additional non-native structures arising from the method of depolymerization.

Three LMWHs are currently approved by the USFDA including enoxaparin, tinzaparin, and dalteparin. New LMWHs have also been introduced in other markets including Brazil, India, China, and others. There is a possibility that generic LMWHs may be introduced in the US, although it will be difficult to define 'generic LMWH' considering the problems of polydispersity, microheterogeneity and method of preparation. Additionally, different standards of regulation in foreign markets may lead to introduction of LMWHs that have considerably different compositions and pharmacological profile.[3]

LMWHs suffer from several adverse effects, among which the major concern is enhanced bleeding risk. The majority of adverse reactions arise from the presence of a large number of sulfate and carboxylate groups along the polymer chain that induce interaction with practically

[@] Abbreviations used: AMAC, 2-aminoacridone; CE, capillary electrophoresis; GPC, gel permeation chromatography; H, heparin; LC-MS, liquid chromatography – mass spectrometry; LMWH, low molecular weight heparins; M_R, average molecular weight; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; UFH, unfractionated heparin; USFDA, United States Food and Drug Administration;

any protein that carries a cationic domain.[4] A conservative estimate puts the number of heparin-binding proteins in our body at more than 100. Yet, difference in LMWH composition induces different levels of interactions with proteins. Recently, the structural heterogeneity of LMWHs has led the FDA to recommend that each LMWH be considered as an independent drug with its own anticoagulant profile.[5,6]

A large number of biophysical techniques have used to characterize heparins. Gel electrophoresis, especially PAGE, has been developed to analyze heparin polydispersity,[7-10] while chromatography, e.g., SEC or GPC, has been developed to assess the M_R and oligomeric composition.[8,10-16] Other chromatographic techniques, e.g., reverse-phase, ion-pairing and strong anion exchange, have been used to prepare heparin oligosaccharides as well as perform oligosaccharide compositional analysis.[17-24] NMR spectroscopy has also been used to assess saccharide composition and sulfation pattern,[25-28] while being especially useful for identifying certain non-native structures. Recently, tandem LC-MS has been exploited to perform sequence analysis on relatively purified preparations of oligosaccharide.[29-34]

A technique that has gained widespread acceptance in LMWH and heparin oligosaccharides analysis is capillary electrophoresis (CE). The earliest application of CE for disaccharide compositional analysis of LMWHs[35,36] has now been modified to protocols with much better sensitivity and resolving power.[37,38] A significant improvement in sensitivity of CE detection has been pre-column labeling with chromophores or fluorophores.[38-41] Further advances in CE applicability have been the development of a tandem CE–MS system for elucidating structural information.[42] Unfortunately, these powerful approaches work on essentially pure oligosaccharides, enzymatically depolymerized samples, or mixtures of smaller heparin chains. Additionally, none of the methods is particularly suitable for assessing LMWH

preparations on a routine basis. More importantly, absence of a rapid and simple biophysical protocol for monitoring product quality is a major impediment for identifying LMWHs complications. In this work, we present an extremely simple, but robust, CE-based method to fingerprint intact LMWHs that is especially useful for assessing product identity, quality, and batch-to-batch variability.

Experimental Procedures

Chemicals and Electrophoresis Supplies — Enoxaparin (Lovenox^{TM}, 40 mg syringes, Lot # 094480 and 094422) and tinzaparin (Innohep^{TM}, 2 mL, 20,000U vial, Lot # DB1586) were purchased from Medical College of Virginia Pharmacy Services, Richmond, VA. 2-Aminoacridone (AMAC) and sodium cyanoborohydride were purchased from Sigma (St. Louis, MO). Linear and cyclic polyamines were from either Aldrich (St. Louis, MO) or Acros (Geel, Belgium) All other reagents/chemicals were analytical grade and purchased from either Fisher (Fair Lawn, NJ) or Sigma-Aldrich (St. Louis, MO). Fused silica capillaries were from Beckman-Coulter (Fullerton, CA).

AMAC-labeling of LMWHs — The labeling of oligosaccharides at the reducing terminus with 2-aminoacridone has been described extensively in the literature.[39-41] An essentially equivalent protocol was followed herein. Briefly, clinically available enoxaparin and tinzaparin were dialyzed extensively against deionized water (MWCO 500) to eliminate excipients and lyophilized to obtain a solid. Sigma LMWH (ID# HR-3400) was obtained in solid form and used as such. Solid LMWH (10–15 mg) and sodium cyanoborohydride (25 mg) were dissolved in 560 μL of deionized water and mixed with a solution of AMAC (4 mg) dissolved in 158 μL of 85% (v/v) acetic acid:DMSO. The mixture was allowed to incubate at 37 ^{O}C for 16 hours, then

dialyzed against deionized water to remove free, unreacted AMAC, and lyophilized. The solid so obtained was dissolved in deionized water containing 10% DMSO (v/v) at 10 mg/mL and stored at -78 ^{O}C until use.

Capillary Electrophoresis of LMWHs — CE was performed using a 75 μm fused silica capillary (40 cm to the detector window) installed in a Beckman-Coulter P/ACE MDQ capillary electrophoresis system. A fresh capillary was activated using 5 min flushes each of 1 M NaOH, deionized water, 1 M H_3PO_4, and deionized water in sequence, while between each runs the flush time was reduced to 30 sec with a final run buffer flush of 2 min. The stock solution of a LMWH was diluted nearly 10-fold with 10% DMSO/water for injection into the capillary. CE run buffers studied included 50 mM sodium phosphate buffer, pH 2.3; 100 mM ammonium formate buffer, pH 3.5, 50 mM sodium citrate, pH 2.8, each containing 10% DMSO and appropriate resolving agent at the desired concentration. Run buffers were degassed before start of experiments. Every CE run was performed with fresh 1 mL buffer vials. The temperature of the capillary was maintained at 15 ^{O}C and the run current was held constant at -75 μA. AMAC-labeled LMWH was injected for 10–15 sec at 1 PSI giving a total injection amount of 100–150 ng and an injection volume of ~ 5–10 % of the total capillary volume. Electrophoresis was monitored at 254 nm with a data collection rate of 4 Hz.

Results and Discussion

Linear alkyl polyamines resolve electrophoretic profile of LMWHs — Analysis of unfragmented, intact LMWHs is challenging because of polydispersity and microheterogeneity, which are major impediments to resolution despite the power of CE. Typically, a wide peak is observed for intact LMWH samples in normal as well as reverse polarity implying that the

mixture of the millions of species cannot be resolved.[43-45] Recently, Ramasamy et al.[46] and Patel et al.[47] attempted to fingerprint LMWH using a bare fused silica capillary under reverse polarity conditions. Both groups reported an essentially broad LMWH peak consisting of few shoulders in the peak front. To devise a more robust method for assessing product identity and quality, we reasoned that the presence of certain polycationic agents, which modify the effective charge density of the highly sulfated polymeric chains in a structure-dependent manner, will generate a characteristic fingerprint pattern in CE of intact LMWHs.

Two clinically used LMWHs, enoxaparin (Lovenox^{TM}) and tinzaparin (Innohep^{TM}), and one LMWH from Sigma were chosen. To aid detection, each LMWH was reductively coupled with 2-aminoacridone (AMAC) on the reducing end. CE of AMAC-labeled tinzaparin in 50 mM sodium phosphate buffer containing 10 % DMSO at pH 2.3[#] gave an unsymmetrical broad peak between 16 and 24 min (Fig. 2), supporting previous results on other LMWHs.[46,47] However, in the presence of 200 μM tetraethylenepentamine (4EP), a linear molecule containing five basic nitrogens separated by ethylene groups, the broad peak showed much longer migration time and displayed multiple components. Some of the components, especially in the peak front, were baseline resolved (Fig. 2). Among the several buffers investigated, 50 mM sodium phosphate (pH 2.3) and 100 mM ammonium formate (pH 3.5) provided optimal resolution without compromising sensitivity and speed of analysis (not shown). These results suggest that interaction with 4EP, which assumes polycationic nature in strongly acidic conditions, dramatically alters the electrophoretic mobility of LMWH chains. More importantly, the multiple peaks observed suggest that structurally different LMWH chains are affected to different extents.

[#] Our use of pH 2.3 is equivalent to the use of pH 2.5 by other workers in the field. A lower pH implies further reduction in electroosmotic flow, which typically aids resolution of these polysaccharide – polyamine complexes by pure electrophoresis.

Fingerprinting pattern depends on the structure of the resolving agent — To assess whether the structure of resolving agent affects the resolution of LMWHs, we screened several cyclic and linear polyamines (Fig. 3). We reasoned that the cyclic amines would present a dense cationic scaffold for possible interaction with closely knit polyanionic domains in LMWHs, while the linear amines would favor recognition of longer cationic domains. Also, linear polyamines containing either two-, three- or four-carbon spacers between nitrogen atoms (Fig. 3) were investigated to assess recognition of saccharide domains with different charge densities.

Figure 4 shows the electrophoretic profile of enoxaparin in 50 mM sodium phosphate buffer, pH 2.3, in the presence of 125 μM concentration of either SPM, 4EP or 5EH. As the number of nitrogen atoms increase (SPM < 4EP < 5EH), the resolving agent is able to interact with enoxaparin better resulting in slower migration times. Sigma LMWH behaves in a similar manner. In contrast, tinzaparin displays meaningful resolution only with 4EP. SPM did not resolve even at high concentrations, while 5EH spread the electropherogram over a wide range without any resolution (not shown). This suggests that structural domains in the chains of the two groups of LMWHs are different.

Although both 3ET and SPD (Fig. 3) contain four basic nitrogens, the former weakly resolved enoxaparin, while SPD was virtually ineffective at concentrations as high as 500 μM. Cyclic polyamines 3AN and 4AD were also completely ineffective. Likewise, polybrene, a longer cationic polymer, was also not effective (not shown). This suggests that fingerprinting is not a general property of all polyamines. More importantly, optimal distribution of basic nitrogens and chain length is necessary for good fingerprinting pattern.

The fingerprint resolution is highly sensitive to not only the structure of the resolving agent, but also its concentration. More specifically, the resolving agents, e.g., SPM and 4EP,

displayed a narrow range of concentration that gave best resolution (not shown). This suggests that the fingerprint pattern is characteristic with respect to both the LMWH and the resolving agent.

It is important to note that several peaks are baseline resolved in the fingerprint pattern with 5EH, especially in the region of 22 to 30 min (Fig. 4). Screening 5EH concentrations higher than 125 μM resulted in considerably enhanced signal spread, which reduced peak intensities to nearly undetectable levels (not shown). It is likely that use of laser-induced fluorescence will result in enhanced sensitivity of LMWH detection to enable a fully baseline-resolved fingerprint pattern.

Different LMWHs display different fingerprint pattern — To assess whether fingerprint pattern is characteristic of individual LMWHs, we compared CE runs of enoxaparin, tinzaparin and Sigma LMWH in the presence of 50 μM 4EP at pH 2.3 (Fig. 5). Each LMWH shows a characteristic fingerprint pattern defined primarily by the extent of interaction with the resolving agent. Whereas enoxaparin displays prominent peaks at 25 and 30 min, Sigma LMWH is devoid of the pattern at ~30 min. In contrast, both these patterns are absent in tinzaparin. Also, tinzaparin displays much lower resolution than enoxaparin and Sigma LMWH. Equivalent results were observed for other resolving agents including SPM and 5EH (not shown). These fingerprint patterns are highly reproducible with an intra-day variation of less than 5% (Fig. 6A). The variability in migration time was investigated in more detail for several resolving agents (see Table S1 in Supplementary Information). It was observed that the protocol yields an average migration time variability of 21 sec, which suggests the possibility of automated comparative analysis. With respect to inter-day reproducibility, a variation of approximately 5–10% in migration time and peak height was noted (Fig. 6B). The electrophoretic response displayed

good linearity over a wide range concentration with a measured limit of detection and quantitation of 140±23 and 290±47 μg/mL, respectively (see Fig. S1 and Table S2 in Supplementary Information). These limits are not as good as expected and it is likely that the use of higher sensitivity chromo- or fluorophores or laser-induced detection may improve sensitivity. Overall, the results indicate that fingerprinting pattern, especially with multiple resolving agents, and the stability of electropherograms could greatly help identify and quantitate individual LMWHs.

Fingerprinting is useful in batch-to-batch variability analysis — A key aspect in the use of LMWHs as anticoagulants is the necessity to maintain consistency between different preparations. To assess whether our fingerprinting protocol can identify batch-to-batch variability, we studied two lots of enoxaparin, # 94480 and #09422. Fingerprinting of both lots using 150 μM 4EP in 50 mM sodium phosphate buffer, pH 2.3, containing 10% DMSO showed a fairly consistent profile indicating that the two lots are essentially identical. However, small differences in component pattern between the two lots are clearly noticeable. For example, the 22 and 25 min regions show new components suggesting a discernible difference in composition, while the component pattern is reversed for the region at 27 min (Fig. 7). One can predict that our simple protocol can be expected to rapidly identify small and large compositional differences between lots, and thereby be especially useful in batch-to-batch analysis.

Conclusions and Significance

Our results show that LMWHs can be readily fingerprinted using a simple capillary electrophoretic protocol. The resolution in the presence of polyamines occurs because of recognition of the heparin fine structure resulting in the modification of overall charge density of the chains, which alters the electrophoretic mobility resulting in differential migration profiles.

Our data show that the interaction of LMWH – polyamine is both structure- and concentration-dependent. Thus, the electrophoretic resolution appears to be a function of the affinity of the polyamine for heparin chains.

The protocol uses readily available chemicals, is rapid, and is highly reproducible in producing distinctive fingerprint patterns. It can be exploited for identifying intact LMWHs, monitoring product quality and for checking batch to batch variability. Although the resolution achieved using a single resolving agent is sufficient, the power of fingerprinting can probably be expanded using a mixture of resolving agents. This is especially important considering that a number of LMWHs are being rapidly introduced in the world market.[3]

Agent 5EH was found to be especially good at resolving enoxaparin and Sigma LMWH into several baseline-resolved peaks. It is likely that full baseline-resolution will become possible with selected modifications to the protocol, e.g., use of laser-induced fluorescence. This will enable detailed sequence analysis of nearly all LMWH chains through tandem CE-MS/MS approaches. A major advantage of the MS-based analysis is the possibility of identifying the proportion of LMWH chains containing the high-affinity pentasaccharide sequence, which governs anticoagulant activity *in vivo*.[1] Likewise, it is likely that the CE-MS/MS approach will become useful in deciphering heparin structure – activity relationships in areas other than coagulation.

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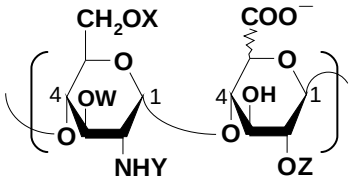
Figure Legends

1. **Natural sequences present in UFH and LMWH.** The 1→4-linked linear polysaccharides are composed of 2-sulfated or acetylated β-D-glucosamines (substituent Y) and, β-D-glucuronic or α-L-iduronic acid residues. *O*-Sulfate groups may be present at the 2-position of iduronic acid (substituent Z), or 6- (substituent X) and 3- (substituent W) positions of glucosamines. On average, UFH and LMWH chains are 50 and 15 monosaccharides long.
2. **Linear alkyl polyamines resolve LMWH in CE to generate a fingerprint profile.** Capillary electrophoresis of AMAC-labeled tinzaparin was performed at -75 μA in 50 mM sodium phosphate buffer, pH 2.3, containing 10% (v/v) DMSO in the presence (black trace) or absence (grey trace) of 200 μM 4EP, a resolving agent (see Fig. 3 for structures).
3. **Structures of linear and cyclic polyamines screened for fingerprinting LMWHs.**
4. **LMWH resolution depends on the structure of the resolving agent.** CE profiles of AMAC-labeled enoxaparin in the presence of 5EH (black bold trace), or 4EP (black thin trace), or SPM (grey bold trace), each at 125 μM. Electrophoresis was performed at -75 μA in 50 mM sodium phosphate buffer, pH 2.3, containing 10% (v/v) DMSO. Peaks marked '*x*' are sudden disturbances due to bubble formation (probably arising from heat generated during the run).

5. **Fingerprint pattern is characteristic of individual LMWHs.** CE profiles of AMAC-labeled LMWHs, tinzaparin (black bold trace), enoxaparin (black thin trace), Sigma (grey bold trace), in the presence of 50 μM 4EP at -75 μA in 50 mM sodium phosphate buffer, pH 2.3, containing 10% (v/v) DMSO. Peaks marked 'x' are sudden disturbances due to bubble formation during the capillary run.
6. **Intra-day (A) and inter-day (B) reproducibility of LMWH fingerprints**. Three consecutive runs of AMAC-labeled Sigma LMWH at -75 μA in 50 mM sodium phosphate buffer, pH 2.3, containing 10% (v/v) DMSO in the presence of 200 μM SPM. B) AMAC-labeled Lovenox (B) resolved using 75 μM 5EH on two different days. The run buffer used in these experiments was 50 mM sodium phosphate buffer, pH 2.3, containing 10% DMSO. Peaks marked 'x' are sudden disturbances due to bubble formation during the electrophoretic run.
7. **Analysis of batch-to-batch variability of LMWHs.** Two lots of AMAC-labeled Lovenox, #94480 and #09422, were resolved using 150 μM 4EP in 50 mM sodium phosphate buffer, pH 2.3, containing 10% DMSO. Note the difference in component pattern between the two lots in the 22, 25 and 27 min regions (marked in the bottom figure). Red arrows at 22 and 25 min show new components present in the lot. In contrast, the component pattern is reversed for the region at 27 min. Peaks marked 'x' are sudden disturbances due to bubble formation during the electrophoretic run.



X = $-SO_3^-$ or –H; Y = $-SO_3^-$ or $-COCH_3$
Z = $-SO_3^-$ or –H; W = $-SO_3^-$ or –H

UFH = $\mathbf{M_R}$ **~15,000** **LMWH** = $\mathbf{M_R}$ **~5,000**

Figure 1

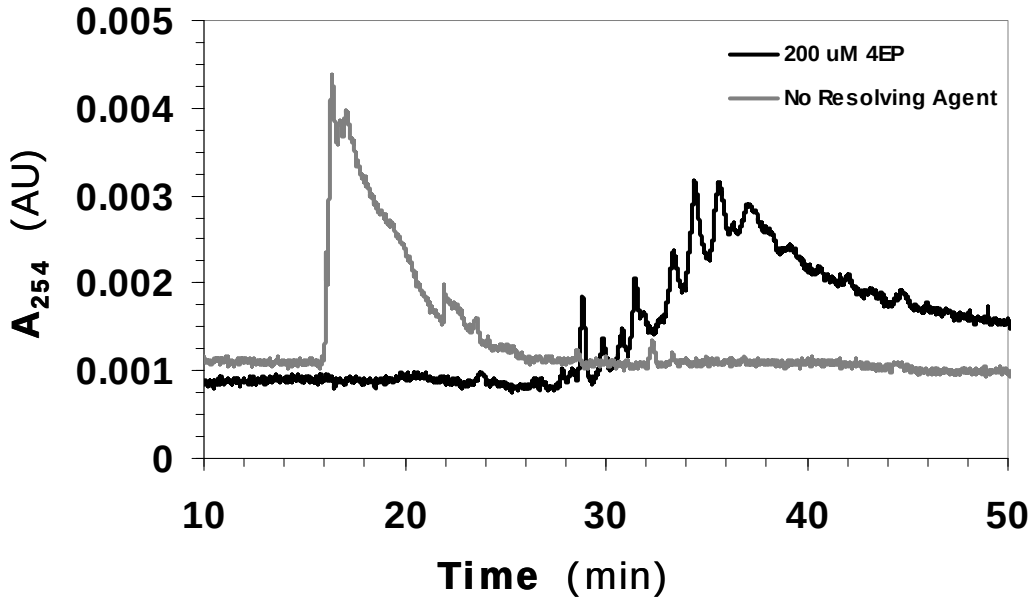

0.005
0.004
0.003
0.002
0.001
0
A254 (AU)
200 uM 4EP
No Resolving Agent
10
20
30
40
50
Time (min)

Figure 2

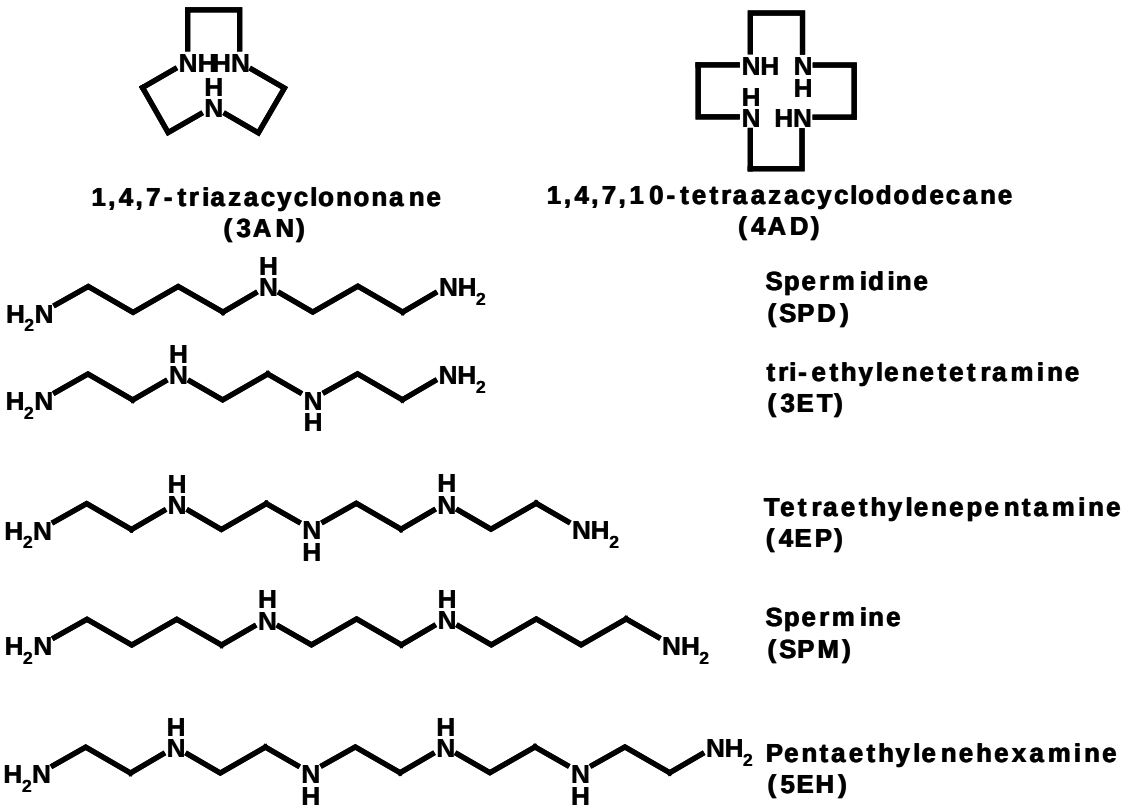

1,4,7-triazacyclononane
(3AN)
1,4,7,10-tetraazacyclododecane
(4AD)
Spermidine
(SPD)
tri-ethylenetetramine
(3ET)
Tetraethylenepentamine
(4EP)
Spermine
(SPM)
Pentaethylenehexamine
(5EH)

Figure 3

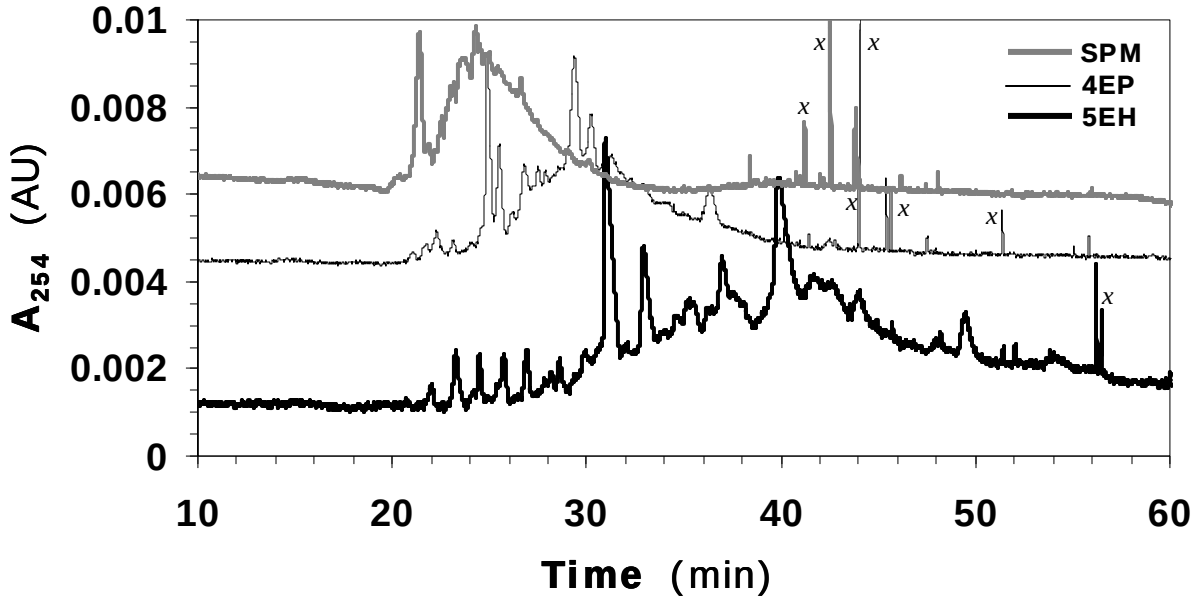

0.01
0.008
0.006
0.004
0.002
0
A254 (AU)
10
20
30
40
50
60
Time (min)
SPM
4EP
5EH
x
x
x
x
x
x
x

Figure 4

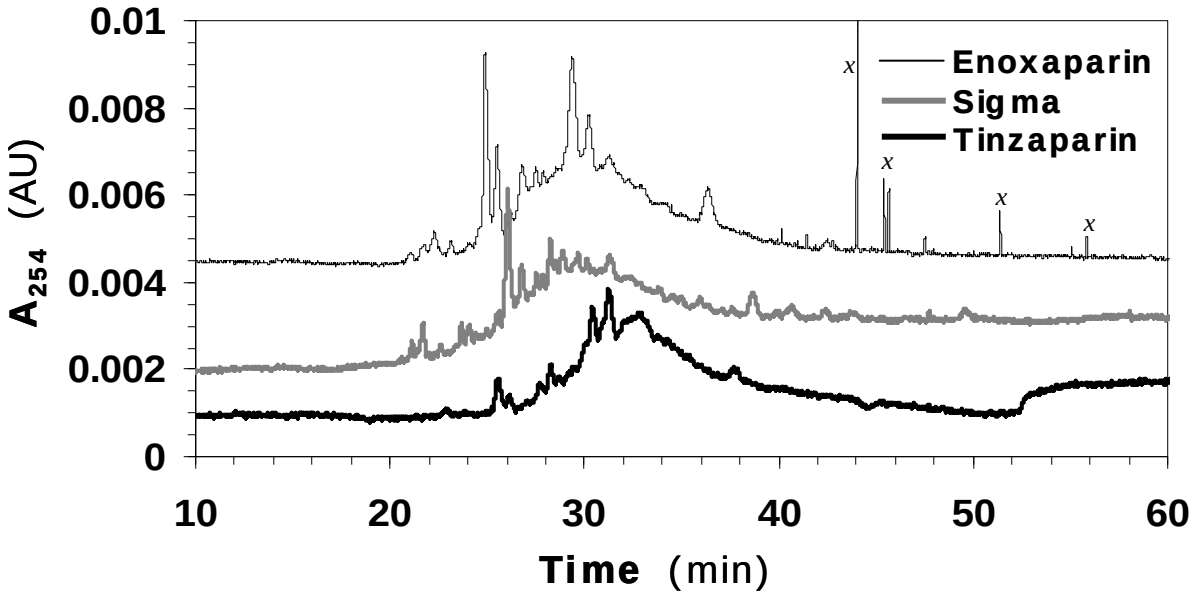

0.01
0.008
0.006
0.004
0.002
0
A254 (AU)
10
20
30
40
50
60
Time (min)
Enoxaparin
Sigma
Tinzaparin
x
x
x
x

Figure 5

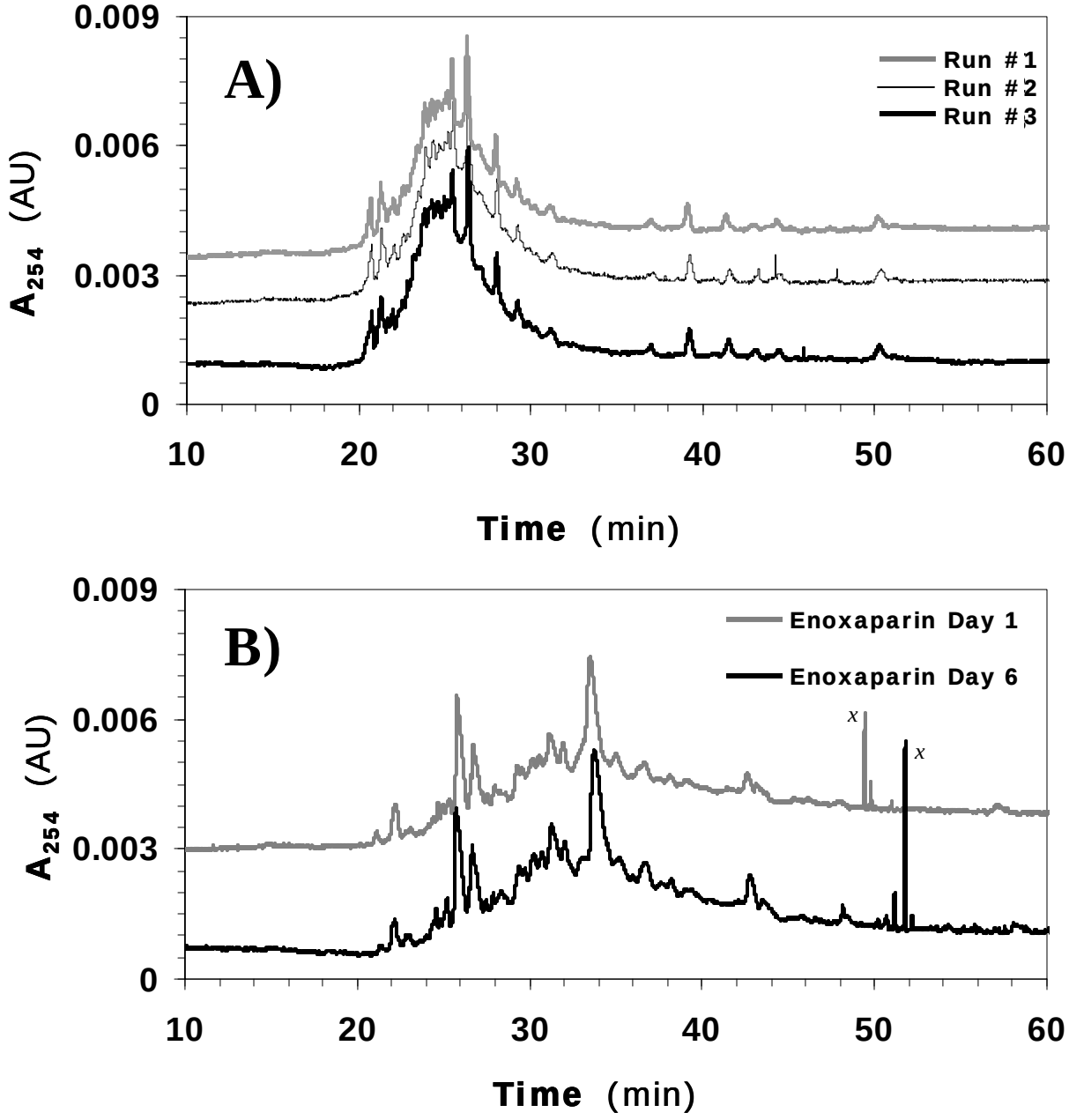

A)
Run #1
Run #2
Run #3
0.009
0.006
0.003
0
A254 (AU)
10
20
30
40
50
60
Time (min)
B)
Enoxaparin Day 1
Enoxaparin Day 6
x
x
0.009
0.006
0.003
0
A254 (AU)
10
20
30
40
50
60
Time (min)

Figure 6

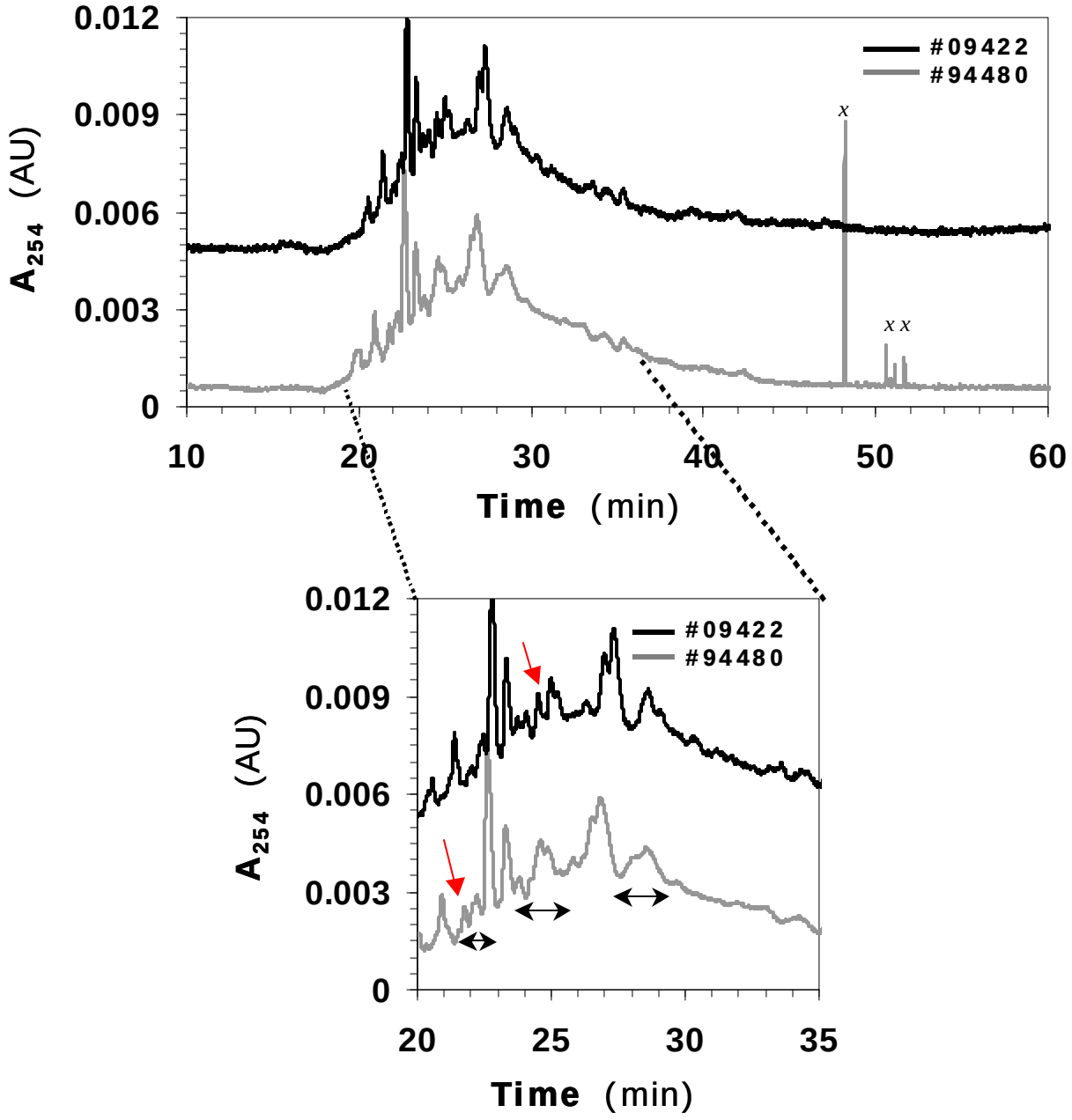

0.012
0.009
0.006
0.003
0
A254 (AU)
#09422
#94480
x
x x
10
20
30
40
50
60
Time (min)
0.012
0.009
0.006
0.003
0
A254 (AU)
#09422
#94480
20
25
30
35
Time (min)

Figure 7