Enoxaparin: Physicochemical Investigations into the Effects of Freezing and Heating

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DECLARATION

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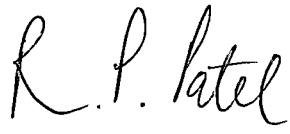

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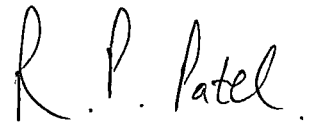

(Rahul P Patel)

CONTENTS

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LIST OF ABBREVIATIONS

ACT	Activated clotting time
AFIIa	Antifactor IIa
AFXa	Antifactor Xa
APTT	Activated partial thromboplastin time
AT	Antithrombin
BCA	Bicinchoninic acid
CE	Capillary electrophoresis
Da	Dalton
DAD	Diode array detection
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
ELSD	Evaporative light scattering detection
EOF	Electroosmotic flow
EP-LMWHS	European low-molecular-weight heparin standard
GAGs	Glycosaminoglycans
HPLC	High-performance liquid chromatography
HP-SEC	High-performance size exclusion chromatography
IC	Ion chromatography
IU	International unit
LMWH	Low-molecular-weight heparin
MS	Mass spectrometry
MALL	Multi-angle laser light scattering
PAGE	Polyacrylamide gel electrophoresis

1,2-PD	1,2-propanediol
pNA	*Para*-nitroaniline
RP-HPLC	Reversed-phase high-performance liquid chromatography
RSD	Relative standard deviation
SAX	Strong anion exchange chromatography
SD	Standard deviation
SEM	Scanning electron microscopy
TBA	Tributyl amine
TBAH	Tetrabutyl ammonium hydroxide
TCT	Thrombin clotting time
TEA	Triethyl amine
Tg	Glass transition temperature
UFH	Unfractionated heparin
UV	Ultraviolet

SUMMARY

Introduction

Low-molecular-weight heparins (LMWHs) are modified heparin fractions with a molecular weight range of 2 000 to 8 000 Da prepared by chemical or enzymatic depolymerisation of unfractionated heparin (UFH). Although UFH was the standard anticoagulant, LMWHs constitute an effective alternative antithrombotic therapy to UFH, and they have more favourable pharmacokinetic profiles and several clinical advantages. LMWHs present a special set of difficulties for chemical and structural analysis because they are highly negatively charged, structurally complex, and polydisperse in nature. Various LMWHs are prepared by different processes and show dissimilarity in physical, chemical and biological properties. Lack of versatile and efficient analytical techniques makes characterisation and stability analysis of various LMWHs difficult. An earlier study of enoxaparin stabilities showed that the antifactor (AFXa) activity decreased upon freezing and showed an unusual pattern of change upon heating.

Objectives

The main aim of the study was to investigate the mechanisms behind the observed activity changes of enoxaparin upon storage at elevated and reduced temperatures, with the potential goal of improving the stability of LMWHs. Secondary objectives were to develop new analytical techniques in order to accomplish the above mentioned aim.

Analytical methods development

A low-volume microtitre plate assay was developed for the determination of AFXa activity of enoxaparin. This method was validated against a standard method and equivalent results were obtained. A simple, selective and accurate capillary electrophoresis (CE) method was developed with a superior resolution than previously reported CE methods for the separation and identification of various LMWHs and UFH. The developed CE method was successfully applied to demonstrate batch-to-batch variations in enoxaparin. An efficient ion-interaction reversed-phase high performance liquid chromatography

(ion-interaction RP-HPLC) method with diode array detection was developed. Resolution of various LMWHs was superior to any of the previously reported analytical techniques. A novel application of ion-interaction RP-HPLC coupled to an evaporative light scattering detection (ELSD) system was also developed.

Freezing study

Enoxaparin solutions were frozen and thawed under different conditions and the AFXa activity was determined. Freezing adversely affected the AFXa activity of enoxaparin solution. Physical investigations of enoxaparin solution suggested that formation of ice crystals or glassy state transitions were not responsible for the loss in activity. Chemical investigations of enoxaparin solution showed that the loss of AFXa activity did not involve the loss of N-sulfate groups or breakdown of glycosidic bonds. Freezing-induced loss of AFXa activity could be reduced by the inclusion of dimethyl sulfoxide (DMSO), by dilution with water and by controlling the freezing and thawing rates. The activity loss could be partially reversed by sonication and sonication was more effective in the presence of DMSO. The loss in AFXa activity was found by high performance size exclusion chromatography (HP-SEC) to be primarily due to aggregation.

Dilution study

Commercially prepared undiluted enoxaparin or enoxaparin diluted with sterile water or sterile 4% glucose was aseptically transferred into plastic syringes or glass vials. Samples were kept at 4 °C, -12 °C or -80 °C for up to 31 days. The AFXa activity of stored solutions was determined after 0, 7, 14 and 31 days. The AFXa activity of the diluted samples was compared with the AFXa activity of undiluted enoxaparin sodium solution stored for the same time periods at 4 °C. Enoxaparin sodium diluted with 4% glucose retained greater than 99% of its initial AFXa activity at 4 °C after 31 days. Enoxaparin sodium diluted with water lost almost 10% of its original activity after 31 days at 4 °C and lost more than 10% of its activity after freezing at -12 °C or -80 °C. Storage in glass or plastic containers made no difference to the loss in the activity.

Heating study

Enoxaparin samples were kept at 70 °C for up to 576 hours. Enoxaparin activity decreased to 74% of its initial AFXa activity after 8 hours at 70 °C followed by a rapid increase in the activity after 12 hours to 94% and then a gradual decrease in the AFXa activity. The chemical changes to enoxaparin which account for the AFXa activity changes following thermal degradation were studied. Enoxaparin was heated at 70 °C for up to 24 days in the presence and absence of various concentrations of oxygen. Samples were collected at regular time intervals and AFXa activity, free sulfate groups, free amino groups and reducing capacity were determined. Samples stressed at 0, 8 and 12 hours were fractionated by HP-SEC. The fractions were collected and analysed by ion-interaction RP-HPLC and for AFXa activity and sulfate concentration. Enoxaparin thermal degradation resulted in the loss of sulfation, particularly N-sulfate groups, and the breakdown of glycosidic linkages confirmed by reducing capacity assay and CE analysis.

The initial decrease (at 8 hours) and subsequent increase (at 12 hours) of enoxaparin AFXa activity was found to be unrelated to oxygen content. No differences between the 0 hours and the 8 hours samples were observed by HP-SEC. Ion-interaction RP-HPLC analysis of 0 hours and 8 hours treated fractions (collected by HP-SEC) clearly showed changes in some of the 8 hours treated fractions. Ion-chromatography (IC) and AFXa activity analyses of the fractions showed loss of sulfate groups and a corresponding decrease in the AFXa activity. Only some of the fractions lost sulfate and AFXa activity. Other fractions appeared to be more resistant to thermally-induced desulfation and retained their AFXa activity. HP-SEC of the 12 hours treated sample showed the presence of extra peaks which were confirmed by ion-interaction RP-HPLC. The increased activity after 12 hours at 70 °C was found to be because of the fragmentation of large oligosaccharides to smaller oligosaccharides, as confirmed by AFXa activity analysis and increased in the number of reducing ends.

Conclusion

CE and ion-interaction RP-HPLC methods were developed and successfully applied to investigate the mechanisms involved behind the loss in AFXa activity of enoxaparin under various storage temperatures and conditions. The observed loss in AFXa activity was consistent with an aggregation hypothesis. Aggregation was reversible by sonication and sonication was more effective in the presence of DMSO. Controlling the freezing or thawing conditions, dilution with water or addition of a small percentage of DMSO ameliorated the loss of enoxaparin AFXa activity. Dilution of enoxaparin with 4% glucose offers a potential method for the preparation of stable paediatric diluted doses of enoxaparin. Chemical and AFXa activity analysis following the heating of enoxaparin at 70 °C clearly distinguished thermally stable fractions from the thermally labile. The generation of new active fragments was found after heating with higher AFXa activity. The thermally stable fractions of enoxaparin offer the potential for new LMWH formulations with greater stability and shelf life.

CHAPTER 1

General Introduction

1.1 HEPARIN

Heparin, a clinical anticoagulant, has been one of the most effective and widely used drugs of the 20th century [1]. Heparin was discovered in the year 1916, by Jay McLean working with William Howell, and first used clinically by Clarence Crafoord. Heparin was identified as an anionic polysaccharide containing uronic acid residues [1] (Figure 1.1). However, early studies showed that heparin also contains O-sulphate ester and N-sulfated glucosamine residues. Heparin consists of a group of glycosaminoglycans (GAGs), which are produced in mast cells and consist of long chains of alternating saccharide residues of uronic acid and glucosamine [2]: Over the past two decades, the structure of the pentasaccharide sequence that binds to the antithrombin (AT) site resulting in anticoagulant activity, has been discovered. Together with structure elucidation of heparin has come an improved understanding of its conformation [3-5] and interaction with various proteins [6-8].

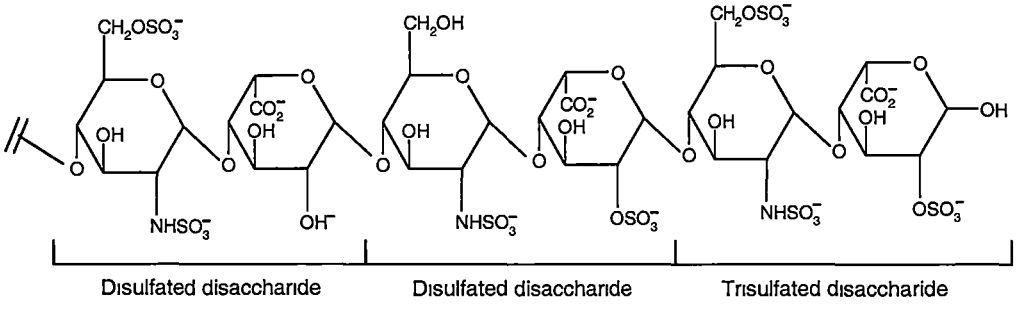

Figure 1.1 The chemical structure of a portion of a heparin chain showing trisulfated and disulfated disaccharides. Heparin chains contain approximately 75% trisulfated and 25% disulfated disaccharides.

1.2 HEPARIN: STRUCTURE AND BIOLOGICAL ACTIVITES

Heparin is prepared by extraction from animal tissues including porcine intestine, bovine lung and ovine intestine. Heparin, like other natural polysaccharides, contains a large number of chains having different molecular weights (is polydisperse) [9,10]. Heparin chains (with an average molecular weight of 12000 Da) are primarily N-sulfated but also contain on an average a single N-acetyl group per chain [1]. Heparin is composed of a trisulfated disaccharide repeating unit (Figure 1.1), but also contains a large number of disulfated disaccharide units [8,11,12]. These additional disaccharides units add complexity to the heparin structure and are important for AT binding, which is essential for heparin's anticoagulant activity. The key structural unit of heparin is a unique pentasaccharide sequence (Figure 1.2). This sequence consists of three D-glucosamine and two uronic acid residues. The central D-glucosamine

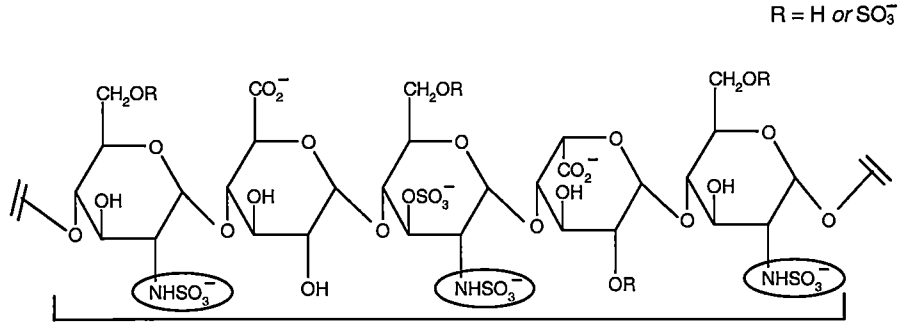

Figure 1.2 The pentasaccharide sequence of heparin, whose binding to AT is important for anticoagulant activity.

residue contains a unique 3-O-sulfate moiety that is rare outside of this sequence. Sulfate groups on the D-glucosamines, encircled in the figure, are found to be critical for retaining high anticoagulant activity. Elimination of any one of them results in a dramatic reduction in the anticoagulant activity. Removal of the unique 3-O-sulfate group results in complete loss of the anticoagulant activity [14-16]. Not all heparin

chains contain an AT pentasaccharide binding site. Only 20-50% of the polysaccharide chains in heparin contain the AT binding site [1]. Some high molecular weight chains contain more than a single AT binding site and hence show a high level of anticoagulant activity [13].

Heparin has a molecular weight ranging from 5 000 to 40 000 Dalton (Da) with an average of 10 000 to 15 000 [17]. Heparin's major biological activity is its anticoagulant activity. It is used to prevent clot formation in extracorporeal therapy, such as kidney dialysis or following surgery [18]. Heparin has a number of other biological activities associated with its ability to bind to a large number of important proteins. Heparin stimulates the release of lipoprotein lipase from the endothelium and activates it, which contributes to its anti-atherosclerotic activity by decreasing the concentration of atherogenic lipoproteins [19]. Heparin also decreases atherogenesis by inhibiting smooth muscle proliferation that follows damage to the endothelium [20]. Heparin is an antiviral agent having anti-human immunodeficiency virus activity [21]. Heparin has a role in the regulation of new blood vessel formation and this activity may give heparin a role in processes where new blood vessel growth occurs, including wound healing, tumour growth, ovulation and fetal development [22,23]. Heparin binds to many enzymes and other proteins activating, inhibiting or protecting their activities [22,24].

1.3 PREPARATION OF COMMERCIAL HEPARIN

Preparation of pharmaceutical grade heparin has changed with time as the principle tissue source has changed from dog or beef liver to beef lung and finally to porcine intestine [25]. Commercial preparation of heparin mainly involves five basic

steps: the preparation of tissue, extraction of heparin from tissue, recovery of raw heparin, purification of heparin, and recovery of purified heparin [25].

The preparation of tissue begins with the collection of the appropriate animal organ tissue. The second step involves hydrolysis by proteolytic enzyme at alkaline pH, followed by filtration or screening of digested tissue to remove any large particles, yielding a deeply coloured solution containing peptides and nucleic acids. After this, the proteolytic enzyme is inactivated by heating the filtrate at 90 °C for 15 minutes. Anion exchange resin is used to adsorb the heparin. After complete adsorption of heparin, the resin is washed and the adsorbed heparin is eluted by sodium chloride solution. The concentrated crude heparin is normally filtered, precipitated and vacuum dried. Purification of heparin is performed by dissolution of the crude heparin in purified water followed by filtration at low pH to remove residual proteins and oxidation at alkaline pH to decolorize and depyrogenate the material. Cation exchange chromatography and ethanol precipitation are performed to remove extraneous cations and to reduce levels of nucleotides respectively. Recovery of purified heparin is performed by precipitation. The final product is either vacuum dried or redissolved in purified water, followed by various filtration steps.

1.4 PHARMACOKINETICS OF HEPARIN

Absorption of heparin is poor after oral administration since the heparin polysaccharide chain is degraded in gastric acid. Heparin must therefore be administered intravenously or subcutaneously [26]. Heparin should not be given intramuscularly because of unpredictable absorption rates, local bleeding, irritation and danger of haematoma formation [27]. There is evidence that heparin administered by intermittent intravenous injection is associated with more bleeding than when it is

administered by a continuous intravenous route. Following injection, heparin binds to endogenous plasma proteins, such as histidine-rich glycoprotein, polymeric vitronectin, platelet factor, fibronectin and von Willebrand factor. Due to binding with proteins, less heparin is available to interact with AT and the anticoagulant activity of the heparin is reduced [28]. When heparin is given in therapeutic doses, close laboratory monitoring of clotting time is required. Because of its highly acidic sulfate groups, heparin exists as the anion at physiologic pH and is usually administered as the sodium salt. Heparin is partially metabolised in the liver by heparinase to uroheparin, which has only slight antithrombin activity. Twenty to fifty percent is excreted unchanged.

1.5 LOW MOLECULAR WEIGHT HEPARINS (LMWHs)

LMWHs are known as salts of GAGs. They have a molecular weight of less than 8 000 Da. The goal of pharmaceutical scientists, before any LMWHs had been approved for human use, was to develop a product that resembled the structure of heparin in all possible aspects except molecular weight and ratio of antifactor Xa (AFXa) to antifactor IIa (AFIIa) activity [29]. LMWHs are obtained by controlled enzymatic or chemical depolymerisation processes. LMWHs are mixtures and each possesses distinctive biological properties depending on the content and structure of its components. A LMWH is characterised by its average molecular weight and polydispersity. The distribution of individual chains found in the mixture and their sequence are also important. The character of each LMWH is mainly dependent on the tissue source of the parent heparin and the method and conditions employed for its preparation [29].

LMWHs have replaced unfractionated heparin clinically to a large extent because of their desirable pharmacokinetic properties and fewer side effects [28].

LMWHs are mainly used in the treatment of venous embolism, deep vein thrombosis and unstable angina [30-36]. They are also indicated in the prophylaxis of general and orthopaedic surgery, acute spinal cord injury, multiple trauma and in patients with ischaemic stroke [37-40].

1.6 DIFFERENT METHODS FOR THE PREPARATION OF LMWHs

Treatment of heparin under acidic and neutral conditions leads to formation of small desulfated products [41]. As heparin molecules are not large enough to be shear-sensitive, physical parameters such as agitation result in no structural alteration or damage. Oxidative instability of heparin is a well known phenomenon. Various antioxidants like sodium metabisulphite and metal chelators are added to heparin at various stages to enhance its stability [41]. These observations resulted in the use of oxidative methods for the preparation of LMWHs.

The enzyme heparin lyase I (heparinase) acts on heparin through β-eliminative cleavage in a random endolytic manner [42,43]. This enzymatic reaction can be enhanced chemically by esterification of the carboxyl group of the uronic acid residue and treating the resulting heparin with base [44]. Thus, a possible second mechanism for the manufacturing of LMWHs is offered by enzymatic or chemical β-eliminative cleavage.

1.6.1 *Oxidative depolymerisation*

Oxygen-containing reagents like hydrogen peroxide or ionizing γ-irradiation (Figure 1.3) are used to break down heparin oxidatively [45-47]. These oxidative methods generate oxygen radicals which then oxidise the oxygen sensitive saccharide residues within the heparin polymer. Heparin chains contain both reducing and

non-reducing ends among which the latter are inert to hydrogen peroxide except when treated in the presence of alkali or metal catalyst. Treatment with hydrogen peroxide or ionization with γ-irradiation lead to the generation of hydroxyl radicals which will subsequently react with the sugar residues and degrade them to the 1-, 2- and 3-carbon fragments without modifying the residues on either side of the attack point. Of these two methods only hydrogen peroxide is used for the preparation of commercially available LMWHs for clinical use (Table 1.1).

Table 1.1 LMWHs, their average molecular weight and method of preparation. Adapted from Linhardt and Gunny, 1999 [29].

LMWH	Mean Molecular Weight (Da)	Preparation Method
Ardeparin sodium	6 000	Oxidative depolymerisation with H_2O_2
Certoparin sodium	5 500	Deaminative cleavage with isoamyl nitrite
Dalteparin sodium	6 000	Deaminative cleavage with nitrous acid
Enoxaparin sodium	4 500	β-eliminative cleavage of the benzyl ester of heparin by alkaline treatment
Nadroparin calcium	5 000	Deaminative cleavage with nitrous acid
Reviparin sodium	3 900	Deaminative cleavage with nitrous acid
Tinzaparin sodium	4 500	β-eliminative cleavage with heparinase

1.6.2 *Deaminative Cleavage*

Oxidative depolymerisation of heparin through deamination is also a possible way to prepare LMWHs (Figure 1.2). In these reactions, either nitrous acid or another nitrosating reagent such as isoamyl nitrate is used to make heparin N-nitrosated at the amino group of its N-sulfoglucosamine residues. The resulting N-nitrososulfamide is unstable, loses nitrogen and sulfate groups and generates a carbocation at C-2 of the saccharide residues. Subsequent ring contraction of this residue and hydrolysis of the

adjacent glycosidic bonds give a LMWH. Several LMWHs prepared through deaminative cleavage are currently used clinically (Table 1.1).

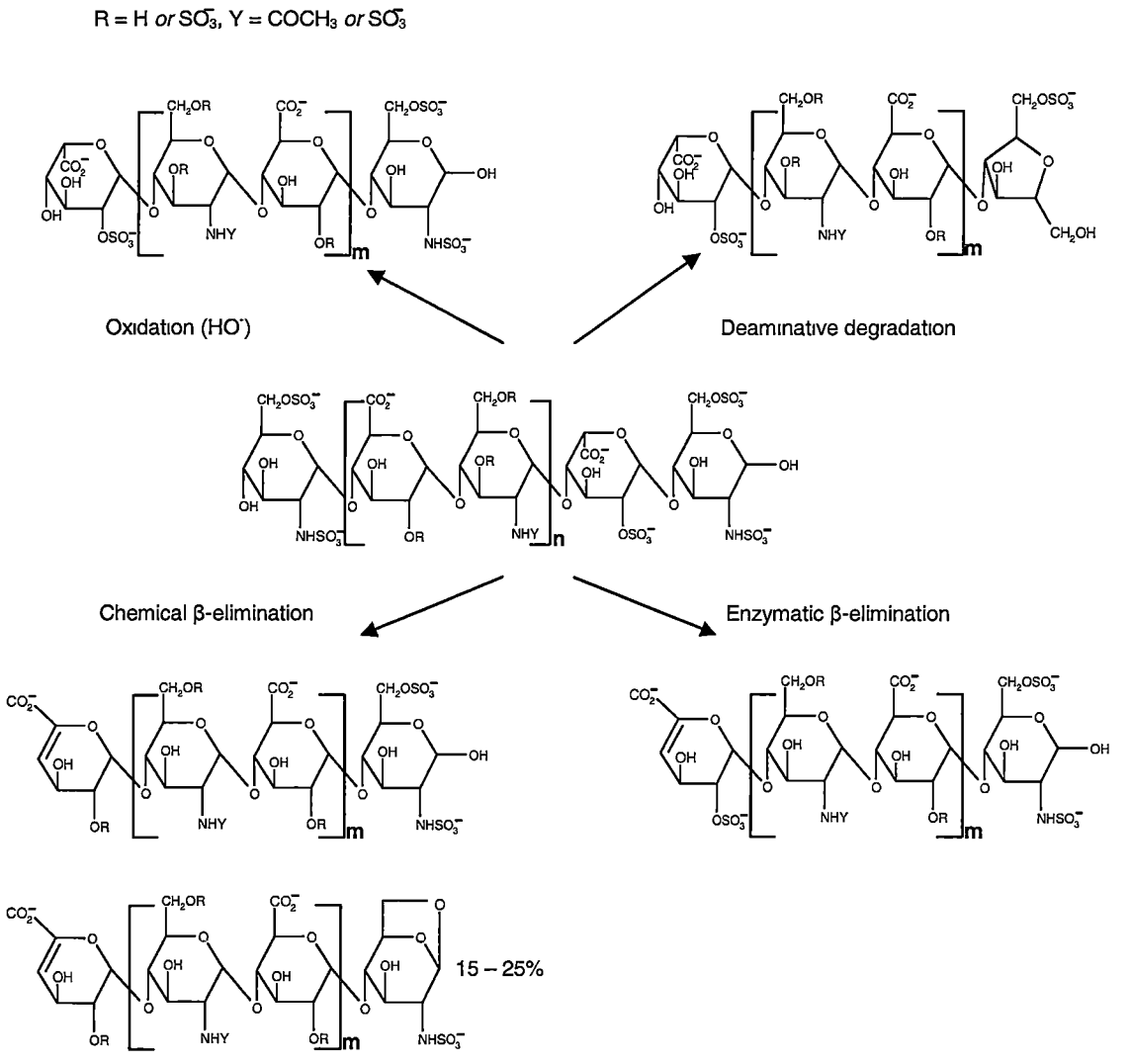

Figure 1.3 Four different depolymerisation processes to prepare commercial LMWHs. The heparin chain in the centre is reduced in length (n>m) to produce a LMWH. Adapted from Linhardt and Gunny, 1999 [29].

1.6.3 *β-eliminative Cleavage*

β-Eliminative cleavage processes, either enzymatic or chemical, are used to prepare commercially available LMWHs (Table 1.1). Depolymerization of heparin by enzymatic cleavage is carried out using heparinase [48]. The extent of reaction is

monitored by the change in absorbance associated with the formation of unsaturated residues [43,49]. Inactivation of the enzyme halts the depolymerisation process. The chemical β-elimination process involves treatment of heparin directly with base or quaternary ammonium salt [50,51]. Under this treatment β-elimination takes place, leading to a LMWH which contains an unsaturated urinate residue at its non-reducing ends. Enoxaparin is prepared by chemical β-eliminative cleavage of the benzyl ester of heparin with alkaline treatment.

1.7 STRUCTURAL DIFFERENCES BETWEEN AND WITHIN LMWHs

A total of eight LMWHs are approved for clinical use worldwide as shown in Table 1.1. All are prepared through chemical or enzymatic depolymerisation and have average molecular weights between 3 000 and 7 000 Da [9,52]. LMWHs display similar physical, chemical and biological properties. A close examination, however, suggests structural differences between these LMWHs. The average molecular weights and dispersity of different LMWHs showed marked variability determined by polyacrylamide gel electrophoresis (PAGE) and high performance size exclusion chromatography (HP-SEC) [53]. PAGE analysis of different digested LMWHs has demonstrated a variation in the presence and distribution of oligosaccharide chains of defined degree of polymerisation. Disaccharide compositional analysis of various LMWHs by PAGE has also revealed significant differences in the degree of sulfation between different LMWHs. [11,53].

Detailed study of chemically or enzymatically digested LMWHs by NMR has shown enormous differences in their structures. Preparation of LMWHs by deaminative depolymerisation (dalteparin) results in an anhydromannitol residue at the reducing end as shown in Figure 1.3. Treatment of heparin with nitrous acid to produce LMWH

results in a tetrasaccharide, containing an unusual reducing end residue that was observed following oligosaccharide analysis [54]. LMWH prepared by oxidatively using hydrogen peroxide (ardeparin) contains a higher degree of sulfation compared to LMWHs prepared by other methods. β-eliminative cleavage of heparin by heparinase causes the formation of 2-O-sulfo unsaturated uronate residues at the non-reducing end of the LMWH (tinzaparin) [29]. Chemical β-elimination is less specific, yielding both unsulfated and 2-O-sulfo unsaturated residues at the reducing end (Fig. 1.3) [29]. An average 15-25% of polysaccharide chains of LMWH prepared by chemical β-eliminative cleavage (enoxaparin) contain 1,6-anhydro groups at the reducing end.

Two LMWHs, dalteparin and nadroparin, are produced by nitrous acid treatment. Disaccharide and oligosaccharide compositional analysis showed that dalteparin and nadroparin are more similar compared to other LMWHs produced by different processes but less similar to each other than to different batches of the same respective LMWH from a single manufacturer [52,55-58]. Enoxaparin and tinzaparin, produced by chemical and enzymatic β-elimination respectively, are structurally related to each other (both having unsaturated uronate non-reducing ends) but have different physical and chemical properties [52,55-58]. LMWHs prepared by different processes are dissimilar in both their end-groups and physical and chemical properties. Studies have shown real and significant differences between and within various LMWHs based on their biological activity. The type of heparin chosen to be degraded and the degree of purification of the starting material both contribute to the final characteristics of the LMWH.

1.8 MECHANISM OF ACTION OF HEPARIN AND LMWHs

Heparin exerts its anticoagulant activity by activating AT. A unique pentasaccharide sequence binds to AT and makes a conformational change within the AT structure that accelerates its interaction with thrombin and factor Xa by 1 000 times. The major difference between LMWHs and unfractionated heparin is their relative inhibitory effect on factor Xa and thrombin [59]. Both LMWHs and unfractionated heparin can inhibit factor Xa, by binding to AT as shown in Figures 1.4 and 1.5. To inactivate thrombin, heparin must bind to AT and the enzyme, thereby forming a ternary (heparin-AT-thrombin) complex. Heparin chains are composed of more than 16 saccharide units so they can easily form the ternary complex [60].

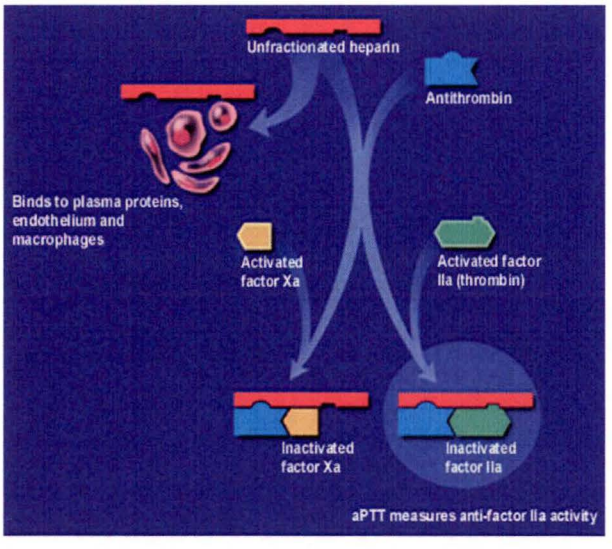

Figure 1.4 Mechanism of action of heparin. Adapted from Whiteand and Ginsberg, 2003 [60].

Unlike heparin, LMWH chains are composed of fewer than 16 saccharide units so they can not bind to both thrombin and AT to form the ternary complex and hence have an inhibitory effect only on factor Xa not on thrombin [60]. The way in which heparin and LMWHs exert an anticoagulant effect is not limited to just inhibition of factor Xa and factor IIa, there is also strong evidence that both increase the release of a

tissue factor pathway inhibitor from the endothelium, which reduces the level of blood coagulation factor.

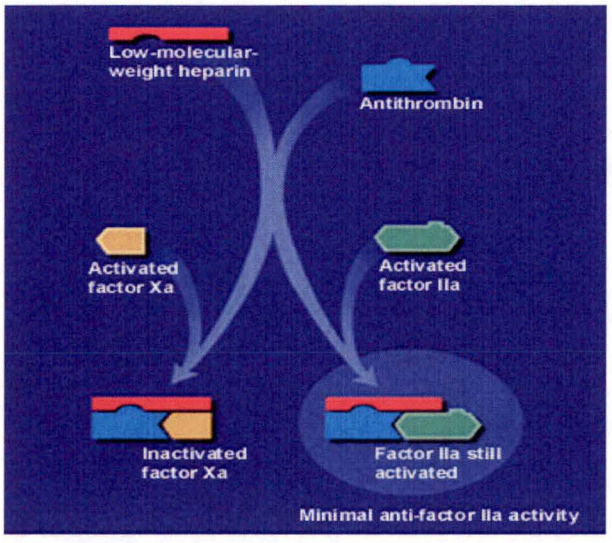

Figure 1.5 Mechanism action of LMWH. Adapted from Whiteand and Ginsberg, 2003 [60].

1.9 DETERMINATION OF THE ACTIVITY OF LMWHs

LMWHs have a higher affinity for inhibition of clotting factor Xa compared to unfractionated heparin. For this reason antifactor (AFXa) assays are always used to measure LMWH activity [61]. The activity of heparin is usually estimated by different clotting tests such as Activated Partial Thromboplastin Time (APPT), Thrombin Clotting Time (TCT) or Activated Clotting Time (ACT). These assays are non-specific and reflect the ability of heparin to interfere with several coagulation cascades. Specific assays measure the heparin or LMWH accelerated AT activity on a single coagulation enzyme, either factor Xa or factor IIa. The enzyme activity is determined either by clotting assay or by chromogenic assay [61].

1.9.1 *Clotting AFXa assay*

In 1973, Yin and coworkers introduced a clotting AFXa assay [62]. It is based on the inhibitory activity of heparin or LMWHs on factor Xa. In the starting phase of the reaction, the amount of factor Xa neutralized is directly proportional to the heparin concentration, if AT is present in excess. Residual factor Xa is then measured using a clotting technique. This assay is not suitable for the measurement of plasma AFXa activity of LMWHs because of their sensitivity toward residual antifactor IIa (AFIIa) activity [63].

1.9.2 *Chromogenic AFXa assay*

Teien and co-workers in 1976 introduced a photometric version of the AFXa clotting assay [64]. This method was further modified by addition of pure AT of known concentration to the sample, to reduce the influence of varying concentrations of AT. The principle of this modified method was the same as the clotting-based assay except that the AFXa activity was determined by using a synthetic chromogenic factor Xa substrate. In chromogenic AFXa activity determination a known amount of excess factor Xa and patient plasma are added along with excess AT. Factor Xa will be inhibited if heparin or LMWH is present in human plasma, based on the fact that heparin or LMWHs binds to AT. In the chromogenic assay peptide substrates are used. They are generally composed of 3-4 amino acids with *para*-nitroaniline (pNA) which is the chromogenic group attached to the carboxy terminal of arginine. When the synthetic substrate is cleaved by factor Xa, the yellow chromophore pNA is liberated. The colour is measured at 405 nm, either during the reaction (kinetic method) or after stopping the reaction (endpoint method). The colour intensity is inversely proportional to the amount of LMWH or heparin present. Results are reported as anticoagulant concentration in AFXa units/ml, such that high AFXa values indicate high levels of anticoagulation and

low AFXa values indicate low levels of anticoagulation. Chromogenic assays using synthetic peptide-based substrates and purified factor Xa or factor IIa offer accurate and reliable measures of anticoagulant activity [63]. Chromogenic assays are not subject to many of the interferences observed in older clotting-based assays [63].

1.10 COMPARISON OF LMWHs AND UNFRACTIONATED HEPARIN

Several studies have shown possible benefits of LMWHs over unfractionated heparin in clinical use. These advantages over heparin are justified by various desirable chemical and physical properties of LMWHs. Major drawbacks of heparin are its non-linear dose-dependent kinetics and the unpredictable effect of a standard dose [30]. Monitoring of clotting time is required for heparin. Laboratory monitoring with the LMWHs is not required because of their linear dose-dependent kinetics and they do not prolong the APTT. Affinity of LMWHs towards proteins, platelets, vascular matrix proteins, endothelial cells and macrophages is less compared with heparin because of their reduced chain length. Fewer side effects (i.e. heparin-induced thrombocytopenia, bleeding complications), higher AFXa activity and superior bioavailability make LMWHs the drug of choice over heparin for various indications [30,59,60].

1.11 ANALYSIS OF HEPARIN AND LMWHs

Heparin and LMWHs in particular have been the subject of considerable recent research into their chemical, biological and therapeutic properties. The limitation in understanding the composition of intact LMWHs or unfractionated heparin is the lack of high resolution analytical techniques. Heparin and LMWHs are linear polysaccharides, and have proven to be extremely difficult to analyse because of their high negative charge, structural complexity and high polydispersity [65-67]. These

characteristics make their separation, without further fractionation or depolymerisation prior to analysis, one of the most difficult analytical challenges.

One approach is to chemically or enzymatically digest intact heparin or LMWHs with subsequent analysis by various analytical techniques. Ion exchange chromatography, following sample digestion, can be accurately used to determine the level of sulfation or the presence of contaminating metals or anions. PAGE has been successfully used to determine molecular weight and polydispersity of digested heparin and LMWHs [68]. In recent years capillary electrophoresis (CE) has gained popularity, making disaccharide and oligosaccharide analysis extremely rapid and sensitive [54,69]. Strong anion exchange chromatography (SAX) has been the preferred analytical technique for the separation of depolymerised or fractionated heparin [70,71]. However, this method is limited by long retention times, poor separation and resolution of highly charged oligosaccharides, the inability to separate oligosaccharides containing hydrophobic protecting groups and the requirement for extensive desalting of digested oligosaccharides prior to analysis. A few applications of CE for the separation of intact LMWHs have been documented [72-74]. However, these techniques resulted in limited resolution of individual constituents of intact LMWHs. HP-SEC is the standard method for chromatographic analysis of intact LMWHs [10,74-76]. It involves the time consuming production of essential molecular weight standards. HP-SEC gives an indication of the molecular weight profiles of LMWHs and can resolve the lower mass polymers, but fails to resolve higher mass components well [77]. Although HP-SEC resolves shorter molecules, it can not distinguish between different oligosaccharides of the same or similar mass. This requires the collection of fractions from HP-SEC, and their analysis by a second method. This is a time consuming and laborious process.

It is established that LMWHs are structurally different depending on the employed method of depolymerisation or fractionation. Structural differences between various LMWHs may differentiate them by their chemical profiles or their pharmacokinetic/pharmacodynamics properties. As a result, each of these LMWHs should be identified as a separate entity. Identification of physicochemical properties of each LMWH is necessary. Particular consideration is required for the development of simple analytical methodology, capable of detecting differences between two different LMWHs or two different batches of the same LMWH without the requirement for depolymerisation before analysis.

LMWHs are commanding more and more attention in pharmaceutical and clinical fields. Each LMWH is a complex mixture of various structurally different oligosaccharides and may have different chemical or physical stability. Studies of these intact LMWHs under different storage conditions would potentially provide important information about their stability and the mechanism(s) responsible for their degradation. Accelerated decomposition study of intact LMWHs under different conditions, the stability of different oligosaccharides, analysis of degradation product(s) and investigation of possible mechanism(s) of degradation have not been described, despite the routine clinical use of LMWHs.

An earlier study of enoxaparin stability in our laboratory showed that the AFXa activity decreased upon freezing and showed an unusual pattern of change upon heating. The primary objective of this study was to investigate the mechanisms behind the observed activity changes of enoxaparin upon storage at elevated and reduced temperatures, with the potential goal of improving the stability of LMWHs. New analytical techniques including CE and ion-interaction RP-HPLC were developed and

validated for the characterisation and stability analysis of various LMWHs in order to accomplish this objective.

CHAPTER 2

Development of a Modified Low Volume Microtitre Plate Assay Method for the Determination of AFXa Activity of Enoxaparin

2.1 SUMMARY

The aim of this study was to develop and validate a modified low-volume microtitre plate assay for the determination of the AFXa activity of enoxaparin solution. The standard ACTICHROME® kit procedure for the AFXa activity determination of LMWHs was modified. This modified assay was validated in terms of linearity, inter- and intra-day accuracy and precision. This methodology is more sensitive than the standard kit method, allowing AFXa activity measurement of enoxaparin with much lower concentrations. This method is more economical as it uses smaller quantities ($1/8^{th}$) of reagents and samples. Unlike the standard kit method, this modified method allows simultaneous determinations and reduces the time required per sample analysis. This method is accurate and reproducible for routine AFXa activity determination of enoxaparin.

2.2 INTRODUCTION

The anticoagulant activity or potency of heparin or LMWH is expressed in terms of international units (IU)/ml. An IU is defined as the amount of heparin or LMWH that is required to prevent the coagulation of 1 ml of whole blood for 3 minutes. There are number of different types of assays to measure anticoagulant activity of UFH or LMWH. One measure of the anticoagulant activity is determined by the ability to inhibit factor Xa in the presence of AT. At present, the chromogenic AFXa assay is considered the gold standard method to determine AFXa activity of LMWHs as it is not subject to many of the interferences observed in older clotting assays [61]. ACTICHROME® is one of the commercially available chromogenic assay kits. It provides human AT III (lyophilized human AT III in buffer of 0.05 M

Tris-HCl, 0.175 M NaCl, 7.5 mM EDTA, pH 8.4), lyophilized bovine factor Xa (20 nKat) and lyophilized FXa substrate (4 μmoles). As shown in Figure 2.1, for the AFXa activity analysis of sample, AT III is added to the sample to form a heparin-AT III complex. The sample is then incubated with a known amount of factor Xa, and a chromogenic substrate specific for factor Xa, is added. At this stage two simultaneous reactions occur. The sample and AT III complex inhibits factor Xa and, secondly, factor Xa hydrolyses the chromogenic substrate. Hydrolysed substrate is yellow in colour and the intensity of the colour is measured by spectroscopic analysis at 405 nm.

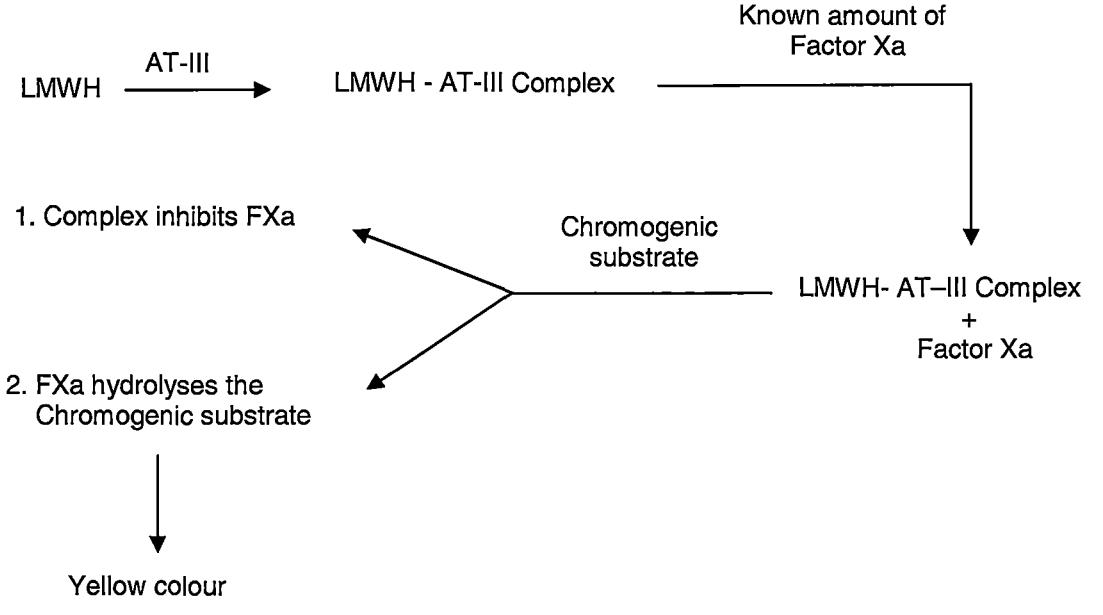

Figure 2.1 Schematic presentation of chromogenic assay to measure the AFXa activity of LMWHs.

There are several problems associated with the commercially available ACTICHROME® AFXa assay kit. It is technically difficult to perform manually as a change in the incubation temperature (between 1 °C and 3 °C), time, and volume of added reagents have affect on the binding of sample to AT III and subsequent inhibition of factor Xa. Simultaneous analysis of samples is not possible with this AFXa assay kit method. It is expensive and in total only 80 samples can be analysed from the provided reagent in a single prepacked kit. Therefore there is an important need to have a simple and economical chromogenic assay which allows the simultaneous determination of AFXa activity, requires less volume of reagents for sample analysis, and hence decreases the technical complexity and cost per sample analysis. The LMWH enoxaparin was selected as a test analyte to develop a modified low-volume mictotitre plate assay. The assay was validated in terms of linearity, reproducibility, inter- and intra-day accuracy and precision.

The objective of this study was to develop and validate a simple, faster, economical and sensitive modified low-volume mictotitre plate assay for the analysis of AFXa activity of LMWHs.

2.3 EXPERIMENTAL

2.3.1 *Materials*

The sodium salt of enoxaparin (10 000 IU/ml) was purchased from Aventis Pharma Pty Ltd. (Sydney, NSW, Australia) and used as supplied at pH 7.0. The AFXa activity kit # 832 (ACTICHROME®) was from American Diagnostica (Stamford, CT, USA). Microtitre plates were flat-bottomed polystyrene plates from Corning (Corning, NY, USA). Polyethylene capped 1.4 ml centrifuge vials were from

Eppendorf (North Ryde, NSW, Australia). All spectrophotometric measurements were made with a Bio-Rad microplate reader Model-680 (Bio-Rad Laboratories, Hercules, CA, USA). Acetic acid and sodium chloride were of analytical grade from Sigma Aldrich (Castle Hill, NSW, Australia). The multi block heater was from Rankin Biomedical Corporation (Clarkston, MI, USA).

2.3.2 *Analysis of AFXa Activity*

The activity of enoxaparin was determined using the AFXa chromogenic assay kit to measure its potentiating effect on AT III inhibition of activated factor Xa. The use of low volume microtitre plates allowed a micro-method to be developed. Enoxaparin solution (5 µl, 10 000 IU/ml) was pipetted into 2 ml capped polyethylene tubes and diluted to 1 000 µl with 995 µl of saline (0.9% w/v sodium chloride) to obtain a solution with 50 IU/ml activity. A sample (10 µl) of the 50 IU/ml enoxaparin solution was diluted to 1 000 µl with 990 µl of saline to obtain a solution with 0.5 IU/ml concentration. The 0.5 IU/ml solution (250 µl) was serially diluted with equal volumes of saline to obtain 0.25, 0.125, 0.625 and 0.0325 IU/ml concentrations. The reagents provided in the kit were diluted to 5 ml with deionised water according to the manufacturer's instructions. Two modifications were made from the standard procedure: 25 µl of each reagent and 10 µl of sample were used instead of 200 µl and 25 µl respectively, and the incubation time was extended to 10 minutes instead 5 minutes after addition of spectrozyme FXa substrate. Up to 35 samples (10 µl each) were transferred into capped polyethylene tubes and heated to 37 °C in an aluminum block heater with water placed in each heating compartment to maximise heat transfer. Each provided reagent was sequentially added to tubes using same multi dispensing pipetter at 1 second intervals to minimise the pipetting error and to

maintain identical incubation time for each sample. AT III (25 µl) was added first to the each sample. After 120 ± 2 seconds factor Xa (25 µl) was identically added and incubated for 60 ± 2 seconds. Substrate FXa was then added and each solution was mixed and incubated for 10 minutes. Stop reagent (25 µl glacial acetic acid) was then added to each tube. Vials were centrifuged at 15 000 rpm for 1 minute and contents were carefully transferred to wells of a microplate using micropipetter. The intensity of the colour, which was inversely proportional to the concentration of enoxaparin present in the sample, was read at 405 nm using a microplate reader, after shaking for 60 seconds to remove any air bubbles present. Each sample was analyzed for AFXa activity in triplicate.

2.3.3 *Linearity*

Enoxaparin solution (10 000 IU/ml) was diluted with normal saline solution to obtain 5 different concentrations (0, 0.0312, 0.0625, 0.125 and 0.25 IU/ml). These standards were analysed for AFXa activity using the low-volume micrtotitre plate method. Three independent calibration plots were constructed and fit to a linear model by least-squares regression.

2.3.4 *Reproducibility*

Reproducibility was determined by repeat analysis of 7 enoxaparin samples having the same concentration (0.0625 IU/ml) and AFXa activity was determined. Reproducibility results are expressed as % relative standard deviation (% RSD).

2.3.5 *Precision*

Intra-day precision was evaluated by AFXa determination of 5 different concentrations of enoxaparin solution (n=5) on the same day, under the same

experimental procedure and controlled conditions. The inter-day precision of the method was determined by AFXa analysis of 5 different concentrations of enoxaparin solution over four consecutive days (n=5). Results obtained by the modified microtiter plate method were compared with the standard methods using the assay performance measures provided with the kit instruction leaflet.

2.3.6 *Accuracy*

Intra- and inter-day (over four consecutive days) accuracy of the microtitre plate method were investigated with the repeat analysis of 5 different concentrations of enoxaparin solution (n=5). Mean intra- and inter-day accuracy was calculated as follows: (observed reading-actual reading)/actual reading × 100. Results are presented as % RSD.

2.4 RESULTS AND DISCUSSION

Three independent calibration plots were generated by plotting the 5 different concentrations of enoxaparin solution versus absorbance as shown in Figure 2.2. Good linearity was obtained in the 0.0325-0.25 IU/ml range compared to the 0.2-0.8 IU/ml range (standard kit method) for the determination of AFXa activity of enoxaparin solution. This result has demonstrated that this method gives linear absorbance response with a low concentration range of enoxaparin and hence is more sensitive than the standard kit method. A linear regression equation was calculated by the least squares method, and the values of the correlation coefficient was found to be $r^2 \geq 0.9884$ for the newly developed low volume microtitre plate method compared to $r^2 = 0.953$ for the standard kit method (degree of freedom is 5 for each of the compared system).

The reproducibility of the method was determined by assaying samples of enoxaparin with the same concentration (n=7) and results are shown in Table 2.1. The enoxaparin activity ranged from 1.02×10^4 to 1.18×10^4 IU/ml, with an average value of 1.07×10^4 IU/ml and an RSD of 3.16%.

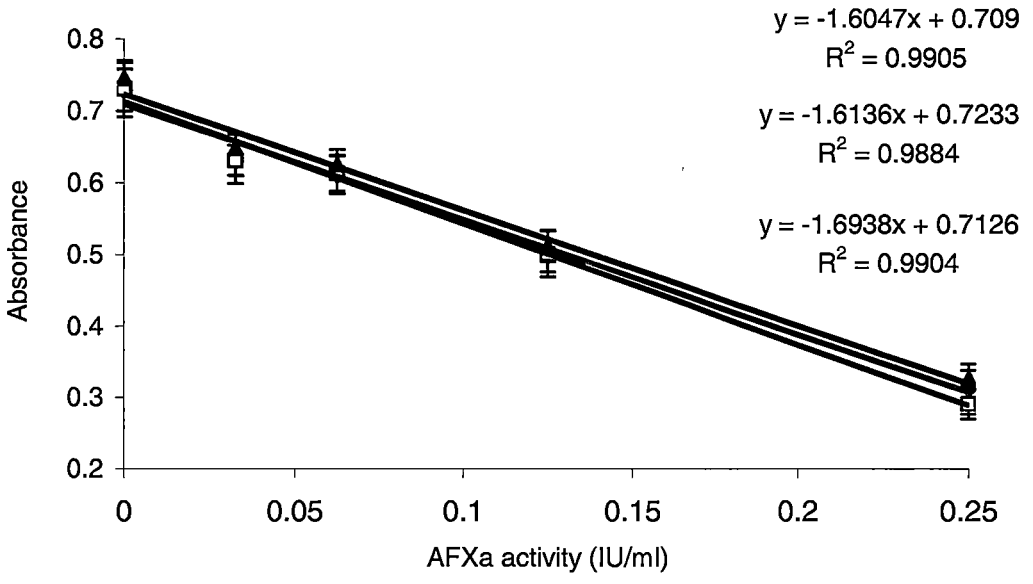

Figure 2.2 Calibration plots of the low-volume microtitre plate method for determination of AFXa activity showing absorbance (mean ± 95%confidence interval) at 405 nm vs. AFXa activity of standard enoxaparin solutions, (n=5).

Table 2.1 Reproducibility of the microtitre plate assay obtained for the determination of AFXa activity of enoxaparin sodium (0.0625 IU/ml, n=7).

Sample assay	AFXa activity (IU/ml)	Average AFXa activity (IU/ml)	%RSD
1	0.061		
2	0.063		
3	0.067		
4	0.066	0.064	4.14
5	0.061		
6	0.060		
7	0.064		

Intra- and inter-day accuracy and precision of the newly developed low-volume microtitre plate method were determined and results are presented in Table 2.2 together with the standard kit method results given by the manufacturers. Percentage RSD values obtained for both accuracy and precision were less than 5%. The microplate method developed here demonstrated low intra- and inter-assay variation, suggesting accuracy and precision comparable with the standard kit method.

Table 2.2 Assay performance of the microtitre plate method for determination of AFXa activity and comparison with the standard kit method, (n=5).

	Enoxaparin (Units/ml)	Intra-day precision (%RSD)	Intra-day accuracy (%RSD)	Inter-day precision (%RSD)	Inter-day accuracy (%RSD)
Microtitre method	0.00	1.5	1.06	2.9	0.56
	0.0325	1.4	-12.1	3.2	-1.9
	0.0650	2.9	4.8	3.6	4.7
	0.125	1.1	- 9.1	3.9	-5.6
	0.250	2.7	- 2.1	4.6	-2.5
Standard kit	0.24	2.9*	not available	6.2*	not available
	0.48	5.1*	not available	9.3*	not available

*results provided by the kit manufacturer

2.5 CONCLUSION

Extensive dilution of enoxaparin (in the order of 10^5) in the microtiter plate assay gives scope for the measurement of lower concentrations of enoxaparin or its constituents (0.0325 to 0.25 IU/ml) with appropriately adjusted dilution level compared to the standard kit method (0.2 to 0.8 IU/ml). AFXa activities analysed by chromogenic AFXa assay using the microtitre plate method developed here demonstrated low intra- and inter-assay accuracy and precision. The microplate

method allows the use of smaller quantities of reagents and samples. For analysis of each sample, the volume of sample and reagent is reduced by $1/5^{th}$ and $1/8^{th}$ and hence the cost also decreases. As many as 35 determinations could be performed simultaneously which decreases the time required for the assay compared with the standard method. This method is sensitive and possesses good linearity, precision and accuracy. The results of validation show that the developed modified low-volume microtitre plate method is accurate and can be applied to the routine AFXa activity determination of enoxaparin solution.

CHAPTER 3

A Simple Capillary Electrophoresis Method for the Rapid Separation and Determination of Intact Low-Molecular-Weight and Unfractionated Heparins

3.1 SUMMARY

A simple, selective and accurate CE method has been developed for the rapid separation and identification of various LMWHs and heparin. The developed method used a 70 cm fused silica capillary (50 µm i.d.) with a detection window 8.5 cm from the distal end. Phosphate electrolyte (pH 3.5; 50 mM), an applied voltage of -30 kV, UV detection at 230 nm and sample injection at 20 mbar for 5 seconds were used. The method was successfully applied to the European Pharmacopeia LMWH standard (EP-LMWHS), dalteparin, enoxaparin and heparin with a significant reduction in the run time and increased resolution compared with previously reported CE methods. Different CE separation profiles were obtained for various LMWHs and heparin showing significant structural diversity. The current methodology was sensitive enough to reveal minor constituent differences between two different batches of enoxaparin. This CE method also clearly showed chemical changes that occurred to LMWHs under different stress conditions. The sensitivity, selectivity and simplicity of the developed method allows its application in research or manufacturing for the identification, stability analysis, characterisation and monitoring of batch-to-batch consistency of different LMWHs and heparin.

3.2 INTRODUCTION

Over the past two decades, CE has been increasingly applied as a sensitive method of high resolving power for the analysis of complex mixtures of peptides, nucleotides and polysaccharides [78,79]. However, most of the polysaccharide work utilising CE involves the analysis of chemically or enzymatically depolymerised oligosaccharides [54,74,78-88]. Only a few applications of CE for the analysis of intact anionic polysaccharides have been documented [72,73,89-93]. Malsch et al [72], Ramasamy et al [73] and Toida et al [74] provide examples of the potential use of CE for the analysis of intact LMWHs, however these CE assays have limitations

associated with resolution and run time. Ramasamy et al. [73] used an acidic copper sulfate buffer for the separation of LMWHs that resulted in a peak with a base width of nearly 30 minutes. Malsch et al. [72] used an acidic phosphate buffer for the analysis of heparin that resulted in a peak width of nearly 5 minutes. Similarly the acidic buffer containing copper employed by Toida and Linhardt [74] for heparin analysis resulted in a peak width of about 8 minutes.

Polyanions can be separated by CE with either high or low pH buffers, using normal or reverse polarity [54,72]. GAGs are negatively charged over a wide range of pH values and hence possess a favourable electrophoretic mobility for CE, due to the large number of sulfate groups. Under acidic conditions electroosmotic flow (EOF) is almost negligible due to protonation of silanol groups on the fused silica capillary surface. Samples introduced from the cathodic end migrate towards the anode without influence from the EOF. The separation of LMWHs is directly proportional to the average number of sulfate groups present in the repeating units [86]. In an alkaline environment the intrinsic mobility of the EOF is high due to dissociated silanol groups. Samples introduced from the anode possessing lower electrophoretic mobility than the EOF are swept towards the cathode [85,94,95].

Up to the present time CE has been under-exploited for polysaccharide analysis. An extensive literature review failed to find a versatile method with high resolution and a short run time for the separation of intact LMWHs, for the evaluation of stability and batch-to-batch variation in pharmaceuticals. Particular attention must be paid to the efficiency, precision, accuracy and selectivity of any analytical method used for pharmaceutical quality control. The LMWH dalteparin was selected as a test analyte to develop a simple and rapid CE separation method. Several parameters were

investigated such as buffer concentration, pH of the background electrolyte, different capillary lengths and diameters and the amount injected. The developed CE method was validated in terms of linearity, precision, accuracy and selectivity. Furthermore, the developed CE method was applied to the characterisation of structurally different LMWHs and different batches of the same LMWH, and chemically and thermally stressed enoxaparin and dalteparin to demonstrate its potential in applications for the analysis of intact LMWHs.

The objectives of this study were to develop and validate a simple and versatile CE method to separate and identify different LMWHs and heparin without depolymerisation or fractionation prior to analysis, to detect structural or chemical changes in LMWHs at different storage temperatures and conditions, and to evaluate batch-to-batch consistency of the same LMWH.

3.3 EXPERIMENTAL

3.3.1 *Materials*

The sodium salts of dalteparin (12 500 IU/ml) and heparin were purchased from Pharmacia (Rydalmere, NSW, Australia). The sodium salt of enoxaparin (10 000 IU/ml) was purchased from Aventis Pharma (Sydney, NSW, Australia). The European Pharmacopeia low-molecular weight heparin standard (EP-LMWHS) was from Pharmacopée Européenne (Strasbourg, France). Orthophosphoric acid, sodium hydroxide, sodium chloride, hydrochloric acid and hydrogen peroxide were of analytical grade and purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Electrolytes and standard solutions were prepared from Milli-Q water and were

filtered through a 0.22 μm pore size membrane filter (Millipore, NSW, Australia) prior to use.

3.3.2 *Instrumentation*

An Agilent^{3D} capillary electrophoresis instrument (Waldronn, Germany), equipped with a deuterium UV lamp and diode array detector (190-600 nm) was used for CE investigations. Data acquisition and instrument control were carried out using Agilent Chemstation system software. Bare fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). A detection window was established through the polyimide coating at 8.5 cm from the capillary end using a butane torch. The detection wavelength and separation temperature were 230 nm and 25 °C respectively. PL-600 Lab pH Meter (MRC Limited, Holon, Israel) was used to determine the pH of the sample solution. Calibration with at least two different buffer standards was performed every time the pH meter was used. The first calibration standard used with a pH of 7.00 (neutral pH), and the second standard was selected to match the pH range in which the measurements were to be taken. The pH measurements of the calibration solutions and the samples were carried out at 25 °C.

3.3.3 *CE analysis*

Between each run, the capillary was flushed for 1 minute with electrolyte. The samples were introduced into the cathodic end of the capillary by hydrodynamic injection for 5 seconds at 20 mbar. The separation was performed in reverse polarity mode with a constant voltage of -30 kV. In order to ensure reproducible results of the CE method, the capillary was conditioned by applying a voltage of -30 kV with the electrolyte solution for 20 minutes at the start of each day. The capillary was flushed

with Milli-Q water for 5 minutes followed by electrolyte for 2 minutes prior to running samples.

3.3.4 *Electrolyte preparation*

A stock solution of phosphate electrolyte (200 mM) was prepared from phosphoric acid with a minimal concentration of 85% w/v. The pH was adjusted to 2.0 by careful addition of 1 M sodium hydroxide solution. Preparation of phosphate electrolytes of different concentrations and pH values was performed by appropriate dilution of electrolyte stock solution with Milli-Q water and adjustment of pH using 1 M sodium hydroxide solution.

3.3.5 *Method development*

The method development was performed using dalteparin solution diluted with Milli-Q water to a concentration of 5 mg/ml. Fused silica capillaries of 30, 50 or 70 cm length with 50 or 75 μm i.d. were used. Phosphate electrolyte (20, 50 or 100 mM) with a pH of 2.0, 3.5 or 5.0 was prepared from the stock solution. Analyses were run with an applied voltage of -10, -20 or -30 kV. The effect of salt on the separation parameters was determined using dalteparin (40 mg/ml) prepared with or without 20 mM sodium chloride.

3.3.6 *Assay performance*

Intra- and inter-day (over five consecutive days) precision were investigated using peak area, with repeat analysis (n=6) of 5 mg/ml dalteparin solution. The intra-day precision of different concentrations of dalteparin (10, 20 and 40 mg/ml, n=6) was also determined. Mean intra- and inter-day accuracy were calculated as follows; (observed concentration – expected concentration)/expected concentration

×100. The linearity of the method was investigated using 2.5, 5, 10, 20 and 40 mg/ml dalteparin (estimated using correlation coefficient r^2) and peak retention time were obtained on each of the 5 days.

3.3.7 *Degradation of dalteparin*

Dalteparin and enoxaparin solutions were subjected to chemical and thermal stresses in sealed N_2-filled glass ampoules, but the solutions were not degassed. Dalteparin solutions (100 mg/ml) were mixed with either 0.5 M hydrochloric acid or concentrated hydrogen peroxide and then heated at 100 °C for 30 minutes. Enoxaparin solution (100 mg/ml) was mixed with concentrated hydrogen peroxide and then heated at 100 °C for 30 minutes. A further sample of enoxaparin (100 mg/ml) was heated at 70 °C for 2 hours. CE analyses were performed on both stressed and unstressed samples for comparative purposes.

3.3.8 *Preparation of heparin and various LMWHs samples*

Heparin (5 000 IU/ml), EP-LMWHS, enoxaparin (10 000 IU/ml) and dalteparin (12 500 IU/ml) were diluted to 10 mg/ml with Milli-Q water before CE analysis. Six samples were analysed in all cases to test the reproducibility of the technique.

3.4 RESULTS AND DISCUSSION

3.4.1 *Influence of separation parameters on dalteparin analysis*

Dalteparin was selected as the test analyte because it gave a simple peak in its electropherogram, and its CE analysis has been demonstrated previously [73]. Efforts were made to improve the electrophoretic separation of intact dalteparin. Molecular

dispersion of polysaccharides is known to be influenced by the applied voltage and the distance they are required to migrate before the detection window [96]. The highest voltage tested, -30 kV, gave rapid migration with acceptable peak shape. Consequently, this voltage was chosen for further analyses. The effect of different length and inner diameter combinations of the fused silica capillary were investigated with respect to the separation efficiency that could be achieved. When using the 75 μm i.d. and 40 cm capillary, electrophoretic current was too high and current breakdown was observed, which had a negative impact on the separation. Decreasing the diameter of the capillary, increasing the length of the capillary or decreasing the ionic strength of the electrolyte has been shown to decrease the magnitude of the current generated [96]. This avoids the deleterious effects caused by boiling within the capillary. Increasing the length of the 75 μm i.d. capillary to 50 cm did not achieve better peak shape in the sample. Hence a 50 μm i.d. capillary was utilised which allowed relatively higher ionic strength electrolyte solutions to be used. This aided in sample stacking, hence improving sensitivity and reduced the magnitude of current generation compared to using the same higher electrolyte concentration in a 75 μm i.d. capillary. Hence the best separation conditions achieved were obtained using a capillary of reduced i.d. (50 μm) of greater length (70 cm) with the 50 mM phosphate electrolyte at pH 3.5.

3.4.2 *Effect of electrolyte pH on the separation efficiency*

It is established that at low pH oligosaccharides can be efficiently separated without complexation with borate. Operation at lower pH ensures that the cathodic EOF is negligible allowing migration of the analytes in the opposite direction towards the cathode. In order to maximise the electrophoretic mobility of the negatively

charged polysaccharide dalteparin, only acidic phosphate electrolytes (pH range 2 to 5) were investigated. The strength of the electrolyte was maintained at 50 mM at all the tested pH values.

The electrolyte pH was found to greatly influence the CE separation. Migration times varied across the tested pH range, with an increasing trend with increase in pH. As seen in Figure 3.1, at pH 5.0 a broad peak was

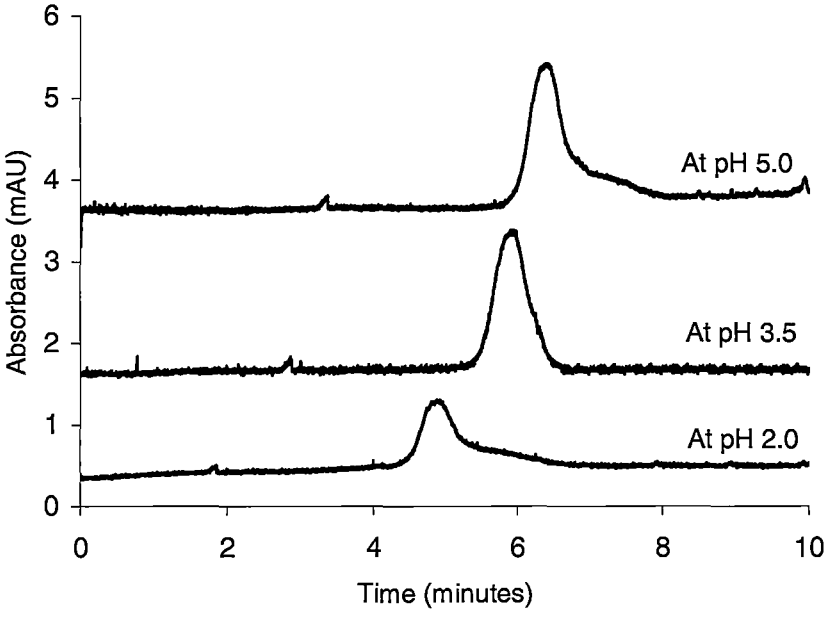

Figure 3.1 Effect of the electrolyte pH on the peak shape and migration time of the dalteparin peak (5 mg/ml): fused silica capillary of total length of 70 cm with 50 µm i.d; background electrolyte 50 mM phosphate buffer at pH specified; applied voltage of -30 kV; detection at 230 nm, injection at 20 mbar for 5 seconds.

obtained with longer migration time. This is because an increase in pH increases the EOF, whose direction is towards the inlet electrode. Consequently, the decreased net mobility of the solutes tends to increase the migration time, leading to higher dispersion of the solute in the capillary. At pH 2.0, the sample migrated earlier past the detector but with a poor peak shape. The peak height and area were also less at

pH 2.0 compared with results obtained using electrolyte of higher pH values. This could be explained by the partial degradation of dalteparin in very acidic conditions [97]. The best efficiency for dalteparin separation was achieved at pH 3.5. However, the electrolyte required replacement with freshly titrated electrolyte after every 100 minutes due to the minimal buffering capacity of phosphate at this pH value. This time interval allowed the running of 10 sample analyses without any observed change in electrophoretic separation.

3.4.3 *Influence of the electrolyte concentration*

The effect of increasing the electrolyte strength from 20-200 mM was investigated, since increasing the electrolyte strength increases beneficial sample stacking, which improves separation efficiency. Increasing the concentration of electrolyte also increases the current during electrophoresis. Potential Joule heating effects limit the electrolyte concentration used.

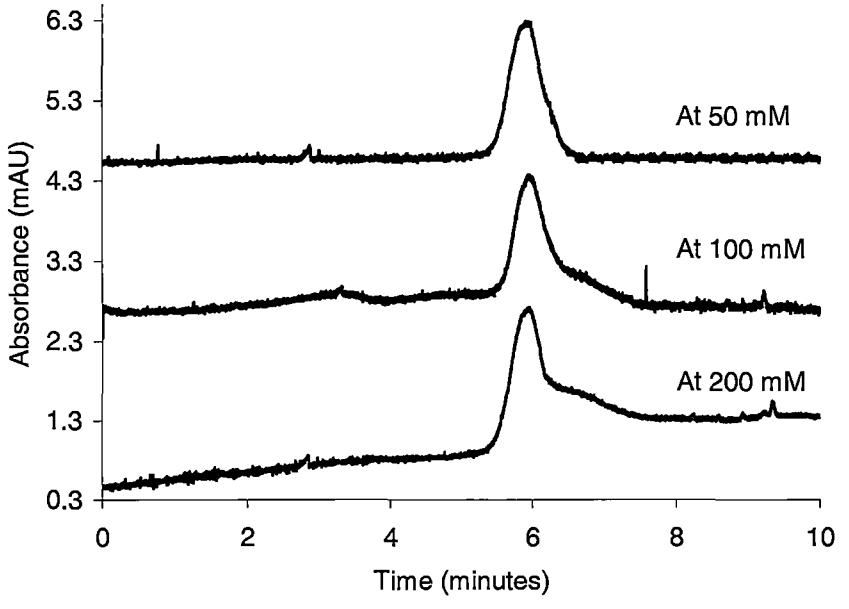

Figure 3.2 Effect of the electrolyte concentration on the peak shape of dalteparin peak (5 mg/ml). CE conditions are the same as those described in Figure 3.1 at pH 3.5.

As shown in Figure 3.2, at a concentration of 100 mM phosphate electrolyte the peak became broad with tailing and at 200 mM the dalteparin peak shape degraded dramatically. The 50 mM electrolyte gave a sharp, well resolved and represented peak with a good compromise between current and peak shape. Highly negatively charged large molecules, including polysaccharides such as heparins, tend to become more compact in higher strength solutions due to charge neutralisation that effectively decreases the intra-molecular repulsive forces from the negatively charged sulfate groups. This change in conformation also benefits electrophoretic separation, as has been observed in the CE separation of highly sulfated carrageenans [98].

3.4.4 *Effect of sodium chloride on the separation of dalteparin*

The presence of salt increases the viscosity and changes the ionic strength, hence the reproducibility of the method may be affected when analysing samples of varying salt concentrations. The presence of sodium chloride can have a strong effect on the stacking phenomenon and hence efficiency of the separation. The influence of sodium chloride on the separation of dalteparin is illustrated in Figure 3.3. It is apparent that the tested concentration of sodium chloride had virtually no effect on the CE performance and separation of dalteparin. The increase in the migration time with an increase in salt concentration was possibly because of decrease electrical field and simultaneously increased conductivity in the sample zone. However, the disturbance in the electrical field was minor because the sample volume injected was very small.

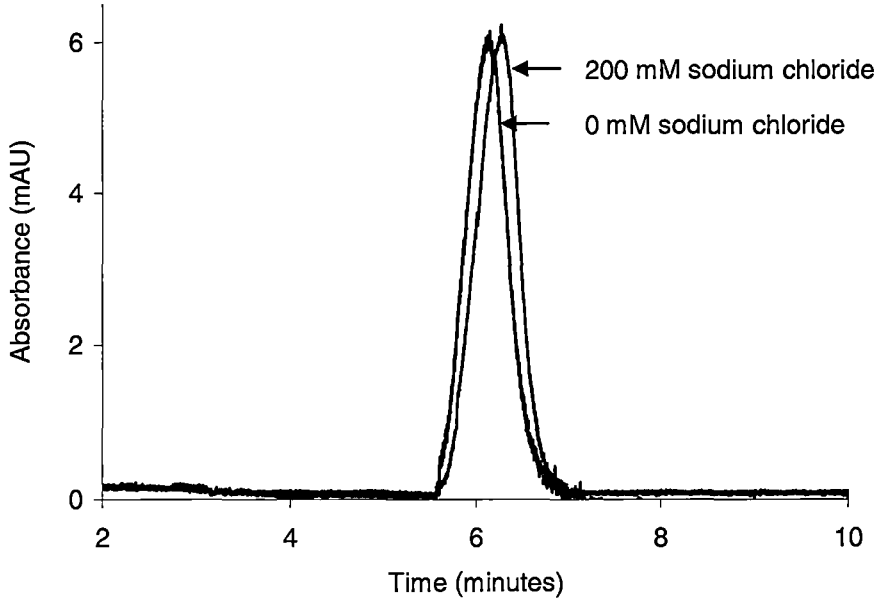

Figure 3.3 Electropherograms of dalteparin (40 mg/ml) diluted with 200 mM sodium chloride or Milli-Q water. CE conditions are the same as those described in Figure 3.2.

3.4.5 *Assay Performance*

Electropherograms of different concentrations of dalteparin are shown in Figure 3.4. The intra- and inter-day precision RSD was 3.2% (n=6) and 4.4% (n=5) respectively at the level of 5 mg/ml dalteparin. The intra- and inter-day accuracy was +4.0% and +5.5% respectively at 5 mg/ml dalteparin. The intra-day precision RSD at the level of 10, 20 and 50 mg/ml of dalteparin (n=6) was 3.8%, 4.4% and 2.3% respectively. Linearity estimated by correlation coefficient r^2 was greater than 0.997 for standard plots utilising 5 different concentrations of dalteparin (2.5, 5, 10, 20 and 50 mg/ml) over 5 days. Mean migration time of dalteparin was 5.69 minutes with an intra-day and inter-day migration time RSD of 0.57% (n=6) and 1.99% (n=24) respectively.

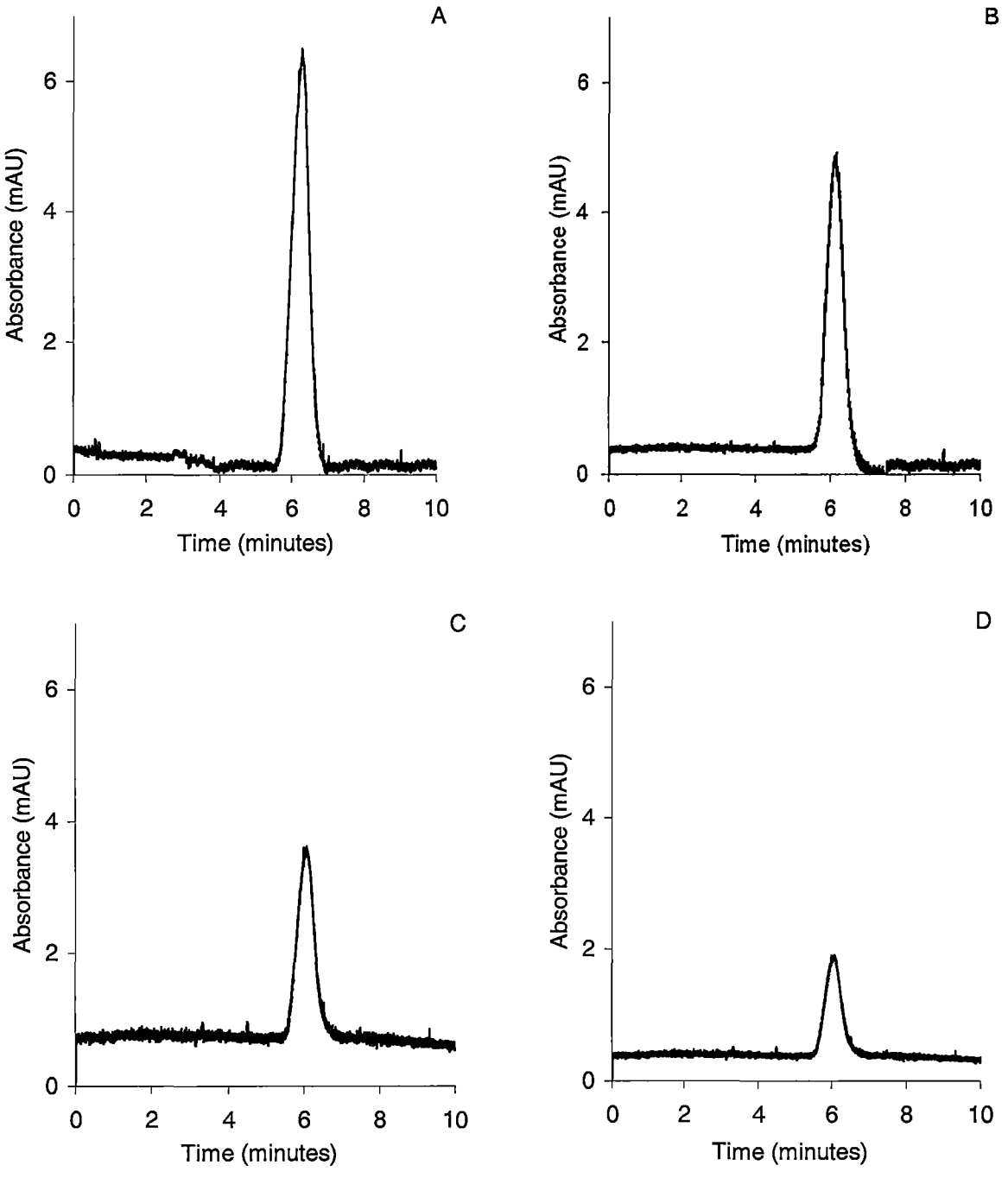

Figure 3.4 Electropherograms of dalteparin 40 mg/ml (A), 20 mg/ml (B), 10 mg/ml (C) and 5 mg/ml (D). CE conditions are the same as described in Figure 3.2.

3.4.6 *Analysis of stressed LMWHs*

The CE profiles of degraded and non-degraded dalteparin using the method developed in this work, are presented in Figure 3.5. In the electropherogram of unstressed dalteparin (Figure 3.5), the main peak migrated at 6.42 minutes. The area of the main peak was reduced by more than 80% after acid stress. Two small peaks with migration times of 6.48 and 6.68 minutes were obtained, instead of a single main dalteparin peak, after treatment with H_2O_2. With acid stressed dalteparin, a degradation product migrated past the detector after 3.2 minutes. The peak of the degradation product was increased in height and area following peroxide treatment.

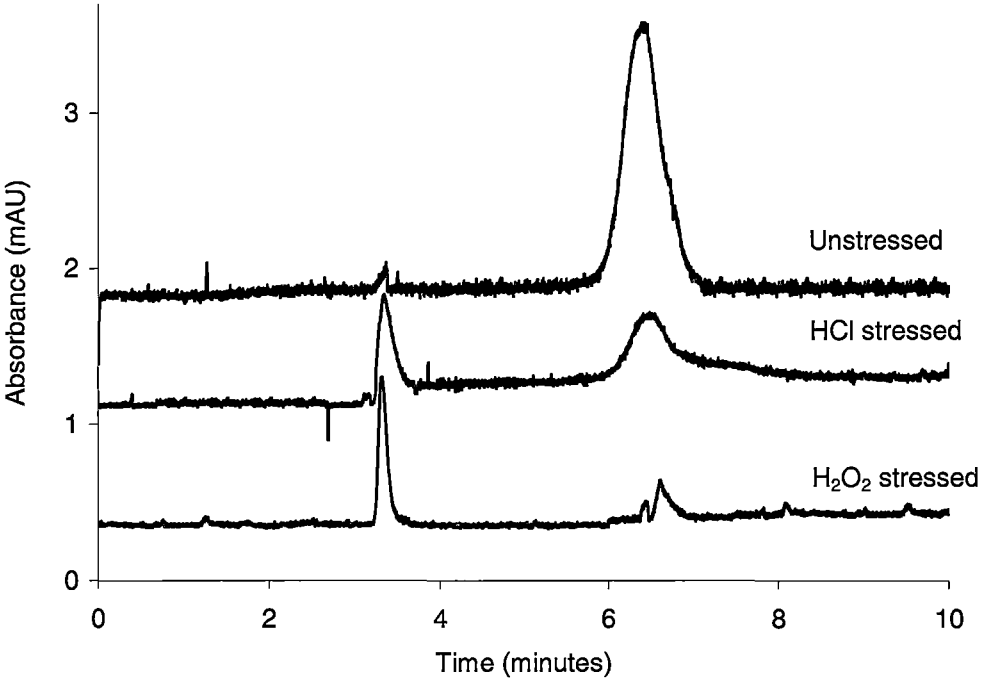

Figure 3.5 Electropherograms of dalteparin (5 mg/ml) before and after stressing under oxidative or acidic conditions. CE conditions are the same as those described for Figure 3.2.

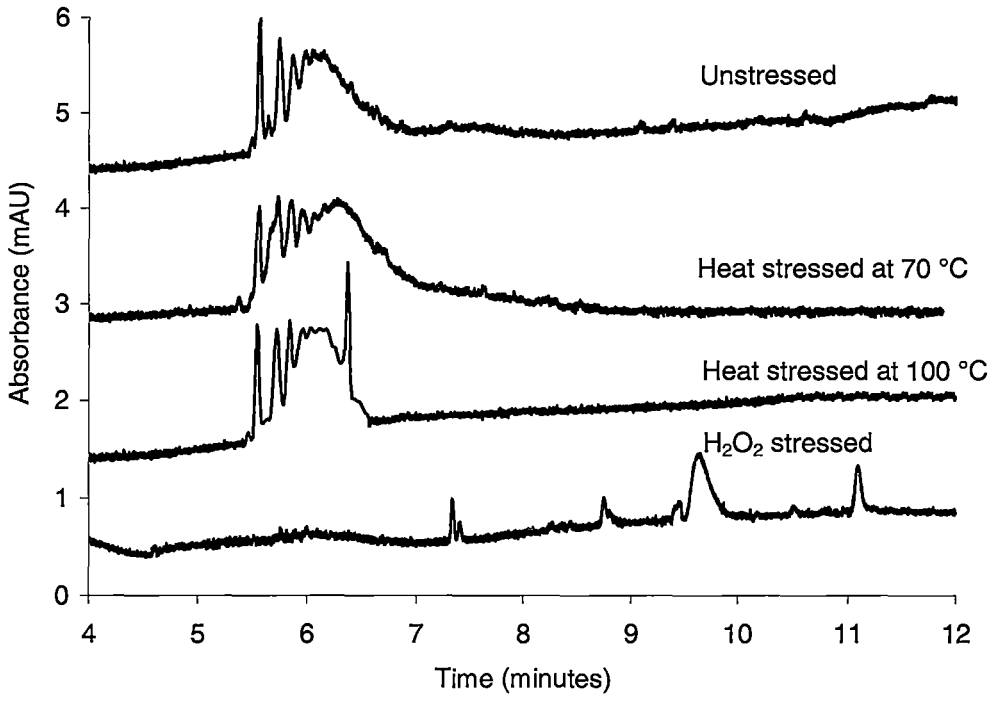

Figure 3.6 Electropherograms of enoxaparin before and after stressing at 70 °C for 2 hours or under acidic conditions (B). CE conditions are the same as those described for Figure 3.2.

The CE profiles of degraded and non-degraded enoxaparin are presented in Figure 3.6. This CE method resolved enoxaparin in to three sharp peaks followed by a broad peak as shown in Figure 3.6. These peaks migrated at 5.58, 5.75, 5.96 and 6.13 minutes respectively. It is evident from the electropherograms that changes have occurred even during relatively mild heating of enoxaparin. With the 70 °C stressed sample, an extra peak at 5.98 minutes was observed. A decrease in the peak area of the first peak (migration time 5.58 minutes) and an increase in the peak area of the second peak (migration time 5.75 minutes) were observed. With the 100 °C stressed sample a degradation product with a migration time of 6.38 minutes was observed. The H_2O_2 stressed enoxaparin sample produced a number of new peaks accompanied by complete loss of the original enoxaparin peaks. This technique offers a method that may be suitable for pharmaceutical stability studies of various LMWHs.

3.4.7 *CE analysis of different LMWHs and heparin*

This CE method resolved EP-LMWHS into three sharp peaks followed by two small broad peaks. Overlaid electropherograms of EP-LMWHS, analysed on two different days, are shown in Figure 3.7. As seen from the electropherograms this developed CE method is accurate and reproducible, as each peak of EP-LMWHS migrated at the same time with identical peak areas.

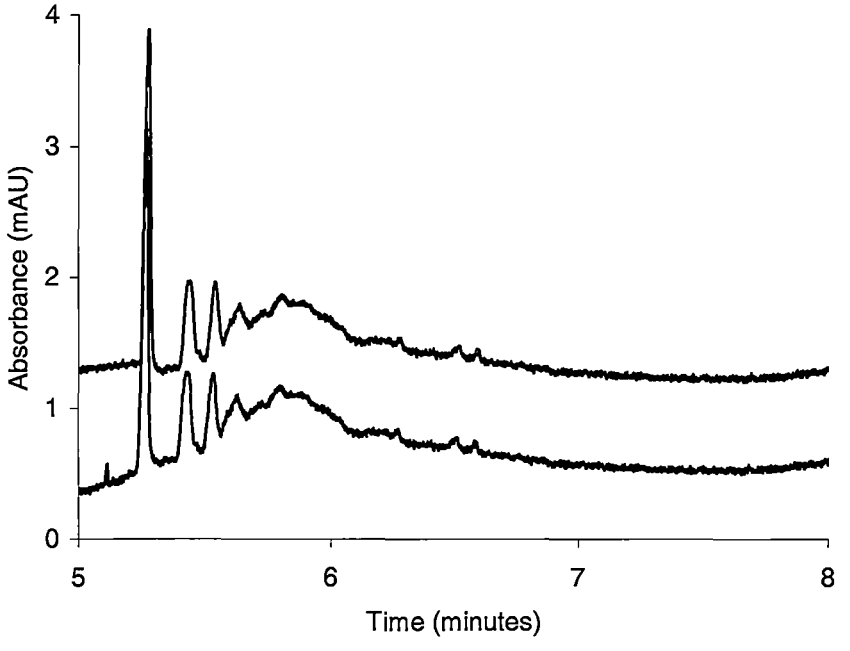

Figure 3.7 Electropherograms of EP-LMWHS (5 mg/ml). CE conditions are the same as those described for Figure 3.2.

Electropherograms of two different batches of enoxaparin are shown in Figure 3.8. This CE method was so sensitive that it clearly revealed differences in 6 different regions of the electropherograms from two different batches of enoxaparin. These compositional differences may be due to the inherent structural variability of the precursor heparin from which the LMWH was derived or due to differences in the fractionation process. As enoxaparin is used for wide ranges of different indications,

the observed compositional differences in two different batches of enoxaparin may have clinical implications. As a dose of enoxaparin varies with different indications, minor compositional differences may lead to biological or clinical variability [29]. These results demonstrate that this method would be suitable for analysis of batch-to-batch variation in pharmaceutical LMWHs or for "fingerprinting" batches of LMWHs.

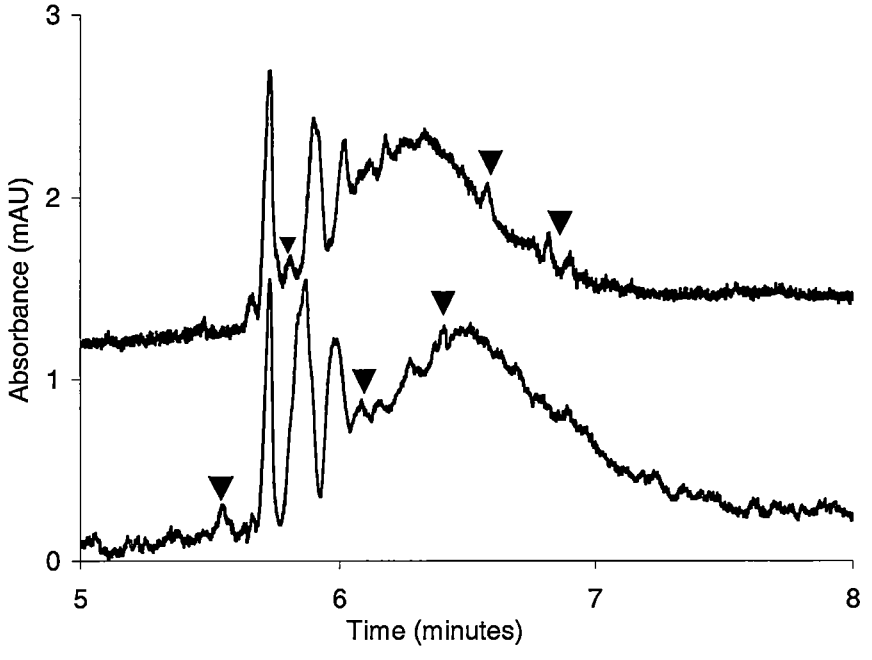

Figure 3.8 Electropherograms of two different batches of enoxaparin with differences highlighted by the arrows. CE conditions are the same as those described for Figure 3.2.

Heparin was resolved into two different peaks that migrated at 4.87 and 7.96 minutes as shown in Figure 3.9. Although heparin contains a large number of different oligosaccharides, CE result suggested that these oligosaccharides fall into two chemically different groups. CE separation of polysaccharides is based on the charge of the analyte, with higher the charge earlier the migration. Peak migrated at

5.55 minutes represents highly charged constituents with greater degree of sulfation compared to constituents migrated at 8.36 minutes.

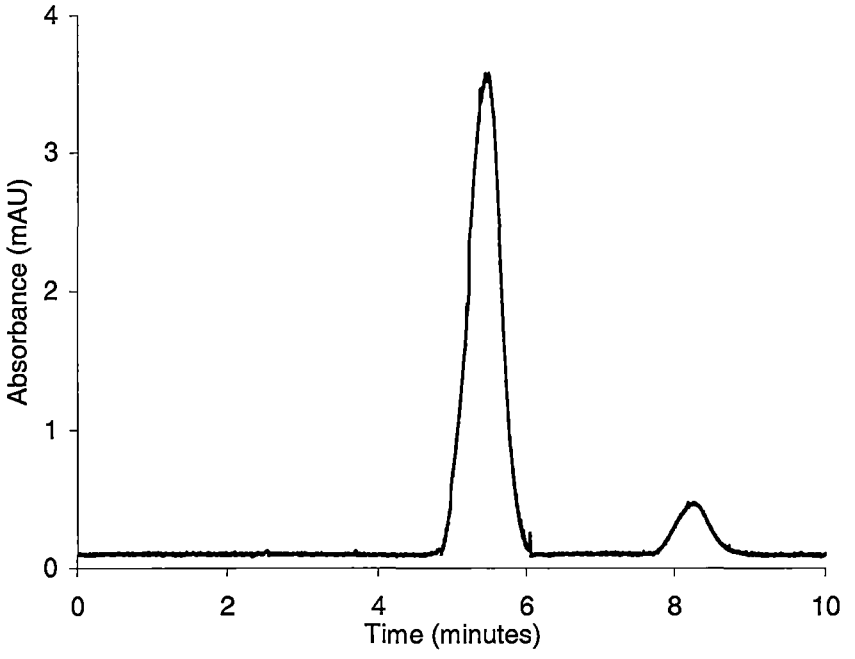

Figure 3.9 Electropherogram of heparin. CE conditions are the same as those described for Figure 3.2.

The electrophoretic profiles of various LMWHs differed from the EP-LMWHS. The CE method detected major structural differences between dalteparin and enoxaparin. The electrophoretic profile of dalteparin (Figure 3.1) was more similar to its parent compound heparin compared with enoxaparin or the EP-LMWHS. The mean migration time of the principle peak of LMWHs and heparin (n=6) were 5.23 minutes (0.96% RSD; EP-LMWHS), 5.68 minutes (0.74% RSD; enoxaparin) and 4.87 minutes (0.63% RSD; heparin). The intra-day precision RSD of the principle peak of EP-LMWHS, enoxaparin and heparin (n=6) were 3.5%, 4.0% and 3.2% respectively.

The CE method described here has several advantages over previously reported CE methods for intact LMWHS and heparin. Electrophoretic separation can be performed in less than 10 minutes for LMWHs which compares with more than 20 minutes and 40 minutes for previous CE methods [73,74]. Using the current methodology, enoxaparin or EP-LMWHS were resolved or partially resolved in to 11 or 8 different peaks compared with only 4 or 5 peaks evident in electropherograms of the same LMWHs using the method described by Ramasamy et al [73]. In contrast with previously reported methods [72-74] by the method reported here, each LMWH showed distinctive electropherogram features with narrow peak width and characteristic migration times and each LMWH could readily be distinguished from other LMWHs and heparin. This method could be applied to further characterise different LMWH constituents by comparison of results with CE analyses of oligosaccharide standards of known molecular weight. This method combined with mass spectrometry (MS) detection can allow the analysis of individual molecular components of LMWH and heparin, which is not possible following enzymatic or chemical digestion procedures prior to CE analysis [54,84-87].

3.5 CONCLUSION

This work has demonstrated the advantages of the developed CE method, with specified length and diameter of capillary, applied voltage, ionic strength and pH of background electrolyte, to achieve efficient separation of LMWH and heparin. The CE assay performance was assessed in terms of linearity, accuracy, precision, selectivity and gave acceptable performance and reproducibility. This method is simple, quick to perform and gives higher resolution than other reported CE methodologies for intact LMWHs. This versatile method was able to clearly differentiate three different LMWHs and heparin, and each giving electropherograms

with sharp peaks at consistent migration time. This method is capable of detecting compositional differences between batches of the same LMWH and able to show degradation products of LMWHs after chemical or mild thermal stress conditions. This CE method potentially offers a simple and rapid analytical technique to study of batch-to-batch variation, the characterisation and stability testing of pharmaceutical LMWH and heparin.

CHAPTER 4

An Effective Reversed-Phase Ion-Interaction High-Performance Liquid Chromatography Method for the Separation and Characterisation of Intact Low-Molecular-Weight Heparins

4.1. SUMMARY

A simple, selective, reproducible and efficient reversed-phase ion-interaction high-performance liquid chromatography (ion-interaction RP-HPLC) method with diode array detection was developed, using enoxaparin as the test analyte. Acetonitrile concentration, different ion-interaction reagents, concentration of ion-interaction reagent and pH were found to be critical for the efficient separation. It was demonstrated that, in general, elution time increased with molecular weight. Suitability of the ion-interaction RP-HPLC separation was also demonstrated with evaporative light scattering detection (ELSD). EP-LMWHS, dalteparin and enoxaparin were analysed with superior resolution and peak shape compared with results using other analytical methods for intact LMWHs. Differences between the LMWHs were demonstrated. This method clearly showed chemical changes that occurred to enoxaparin following heat stress. The ion-interaction RP-HPLC method has potential for investigation of intact LMWHs.

4.2 INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the most commonly used mode of partition chromatography. It consists of a non-polar stationary phase bonded to a solid support that is generally microparticulate silica gel and a polar mobile phase. It is used to separate non-polar and slightly polar species that are partitioned between the mobile and the stationary phases. The separation is normally performed using aqueous mobile phases containing different percentages of organic modifiers (e.g. methanol or acetonitrile) to increase the selectivity between species.

Highly negatively or positively charged analytes in RP-HPLC show little or no retention on lipophilic stationary phases when reversed phase eluent is used [99]. However, retention and subsequent separation of charged analytes on these stationary phases can be achieved by the addition of a reagent having the opposite charge to that of the analyte. The oppositely charged reagent is termed an ion-interaction reagent and this modified technique is called ion-interaction RP-HPLC. The ion-interaction reagent is a compound with a polar head group (such as ammonium or sulfate) and a non-polar tail (such as alkyl or phenyl). Separation in ion-interaction RP-HPLC depends on the stationary phase functional group, ionic strength, pH, ion-pairing reagent and the organic modifiers used in the mobile phase.

The separation mechanism involved in ion-interaction RP-HPLC is not completely understood. There are three proposed mechanisms by which ion-interaction RP-HPLC facilitates separation of negatively charged analytes [99]. According to the ion-pair mechanism, the positively charged ion-pair reagent forms ion-pairs with negatively charged analyte. The resultant neutral ion-pair can then be adsorbed onto the lipophilic stationary phase. The degree of retention of the ion-pair is dependent on the lipophilicity of the ion-interaction reagent itself. An increase in the percentage of organic modifier in the eluent decreases the interaction of the ion-pairs with the stationary phase and therefore reduces their retention. According to the dynamic ion-exchange model, the positively charged ion-interaction reagent is adsorbed on the stationary phase and imparts a charge to the stationary phase, causing it to behave as an ion-exchanger. The total concentration of ion-interaction reagent adsorbed on the stationary phase is dependent on the percentage of organic solvent, with a lower concentration of organic solvent giving a higher concentration of the

ion-interaction reagent on the stationary phase. Retention of the negatively charged analyte on the stationary phase is based on a conventional ion-exchange mechanism. The ion-interaction model proposes a combination of the two previous mechanistic models, with ion-interaction reagent forming a dynamic equilibrium between eluent and stationary phase. The adsorbed positively charged ion-interaction reagent ions are spaced evenly over the stationary phase due to repulsion effects. The adsorbed ion-interaction reagent constitutes a primary charge layer, to which is attracted a diffuse, secondary layer of oppositely charged ions. The amount of charge in both primary and secondary charged layers is dependent on the lipophilicity of the ion-interaction reagent, the ion-interaction reagent concentration and the percentage of organic solvent in the eluent. The negatively charged analyte competes for a position in the secondary charge layer and then moves into the primary layer as a result of electrostatic attraction. The presence of negatively charged analyte into this layer decreases the overall charge of primary layer, so to maintain charge balance further ion-interaction reagent enters into the primary layer. The overall result is the formation of pairs of ions (that is, negatively charged analyte and positively charged ion-interaction reagent).

Ion-interaction RP-HPLC has not been explored as an alternative analytical methodology to HP-SEC, CE or SAX for the analysis of intact LMWHs. Several applications of ion-interaction RP-HPLC have been employed but only for the analysis of smaller oligosaccharides and disaccharides from enzymatically depolymerised heparin [100,101] and LMWHs [102]. An efficient ion-interaction RP-HPLC method for the analysis of intact heparin and LMWHs would offer the ability to study individual constituents of the undigested polymer mixtures, potentially

giving important structural information relating to biological activity. This method would allow the study of specific changes brought about by enzymatically- or chemically-mediated digestion of specific heparin constituents. It would also be useful for pharmaceutical stability studies that can not be performed using currently available analytical techniques.

Literature searches (using MEDLINE, SciFinder and Google) were unable to find any reports describing a versatile and efficient analytical technique for the characterisation of various constituents of intact LMWHs without prior depolymerisation or fractionation. There is an important need to have a simple and reproducible analytical technique for the separation of intact LMWHs with high resolution. For various reasons, as described below, enoxaparin was selected as a test analyte to develop a simple ion-interaction RP-HPLC method. Enoxaparin is the first approved LMWH approved by the FDA [1]. In the treatment and prevention of venous thrombosis enoxaparin has demonstrated its effectiveness over heparin and other LMWHs [26,40,55]. Among all other LMWHs, enoxaparin is commonly used in pediatric patients. Enoxaparin is obtained by β-eliminative cleavage of unfractionated heparin resulting in a LMWH with a high polydispersity and chemical variability [20]. This presents a great analytical challenge and the potential for further study of many different constituents of enoxaparin. Several parameters were investigated such as the concentration of organic modifier, different ion-interaction reagents, concentration of ion-interaction reagent and the pH of the mobile phase. The developed ion-interaction RP-HPLC method was validated in terms of linearity, precision, accuracy and selectivity. Furthermore, this method was applied to the analysis of enoxaparin fractions from HP-SEC, various LMWH samples and a heat stressed enoxaparin

sample in order to demonstrate its potential in applications for the detailed analysis of intact LMWHs.

The primary objective of this study was to develop an effective high resolution ion-interaction RP-HPLC method to separate intact LMWHs without depolymerisation or fractionation prior to analysis that allows the chemical and structural characterisation of individual oligosaccharides in relation to their biological activity.

4.3 EXPERIMENTAL

4.3.1 *Materials*

The sodium salt of enoxaparin (10 000 IU/ml) was purchased from Aventis Pharma (Sydney, NSW, Australia). EP-LMWHS was from Pharmacopée Européenne (Strasbourg, France). Acetonitrile, ammonium acetate, sodium sulfate, hydrochloric acid, acetic acid, tetrabutylammonium hydroxide (TBAH), tributylamine (TBA) and triethylamine (TEA) were of analytical grade and purchased from Sigma Aldrich (Castle Hill, NSW, Australia).

4.3.2 *High performance liquid chromatography (HPLC) instrumentation*

The HPLC system consisted of a Prostar 230 solvent delivery module, Prostar 335 diode array detector and Prostar 410 autosampler (Varian Inc, Melbourne, VIC, Australia) and Alltech ELSD 2000ES detector (Alltech Associates, Sydney, NSW, Australia). Data acquisition and instrument control were carried out using Star Chromatography Workstation software.

4.3.3 *Ion-interaction RP-HPLC separation*

Ion-interaction RP-HPLC of enoxaparin sodium was performed on a 5 μm Varian Microsorb C_{18} column (150 × 4.6 mm) (Varian Inc, Melbourne, VIC, Australia). The mobile phase consisted of acetonitrile, water, ion-interaction reagent and ammonium acetate. Diode array detection was carried out at 230 nm. For ELSD, nebuliser temperature and drift tube temperature were set at 100 °C. Nitrogen was used as a carrier gas with a flow rate of 2.0 l/minute. The detector sensitivity was set at gain 1 with impactor off. The analyses were performed with 30 μl or 50 μl of sample injection at a flow rate of 1.0 ml/minute.

4.3.4 *Method development*

Enoxaparin was diluted with mobile phase to a concentration of 1 mg/ml. Mobile phase contained various concentrations of acetonitrile (10% to 52%), together with an ion-interaction reagent [either TBAH (10, 15 or 20 mM), TBA (15 mM) or TEA (15mM)] and ammonium acetate (10, 25 or 50 mM). The pH was adjusted to 3.0, 5.0 or 7.0 by the appropriate addition of acetic acid. The pH of the sample solutions was adjusted as described in section 3.3.2.

4.3.5 *HP-SEC separation*

HP-SEC was performed using a Shodex OHpak SB-802.5 HQ 8mm × 300 mm size exclusion column and Security Guard equipped with a GFC 2000 cartridge (Phenomenex, NSW, Australia). The mobile phase was 0.3 M sodium sulfate (pH adjusted to 5.0 with 0.1 M HCl) at a flow rate of 0.3 ml/minute. The EP-LMWHS for calibration with a number average molecular mass of 3 700 was analysed. Enoxaparin was similarly analysed and in addition 20 different fractions (each fraction of 150 μl)

eluting between 18 and 28 minutes were collected. Each collected fraction was reanalysed by HP-SEC for peak distribution confirmation and selected fractions were then reanalysed by ion-interaction RP-HPLC.

4.3.6 *Assay Performance*

Intra- and inter-day precision (over five consecutive days) were investigated using peak areas, from 5 different arbitrarily chosen peaks, with repeated analysis of 1 mg/ml enoxaparin solution. Mean intra- and inter-day accuracy was calculated as follows; (observed concentration – expected concentration)/expected concentration ×100. Mean intra- and inter-day peak retention time was also determined.

4.3.7 *Analysis of stressed enoxaparin*

Enoxaparin solution (100 mg/ml, 50 µl) was subjected to thermal stress in sealed N_2-filled 1 ml glass ampoules, but the solution was not degassed. Enoxaparin solution was heated at 70 °C for 6 hours. Ion- interaction RP-HPLC analyses were performed on both stressed and unstressed samples for comparative purposes.

4.3.8 *Preparation of various LMWHs samples*

Enoxaparin (10 000 IU/ml), EP-LMWHS and dalteparin (12 500 IU/ml) were diluted to 1 mg/ml with mobile phase before ion-interaction RP-HPLC or HP-SEC analysis. Each sample was analysed six times to test the reproducibility of the technique.

4.4 RESULTS AND DISCUSSION

4.4.1 *Effect of acetonitrile concentration*

The elution behaviour of enoxaparin was investigated under different isocratic conditions in which the concentration of acetonitrile was systematically changed over the range 52% to 30%. Ammonium acetate (50 mM) and TBAH (15 mM) concentrations at pH 7.0 were kept constant. Chromatograms of enoxaparin obtained using different concentrations of acetonitrile are shown in Figure 4.1. The highest studied concentration of acetonitrile (52%) failed to separate enoxaparin. The chromatographic resolution of enoxaparin was only slightly affected by the concentration of acetonitrile between 52% to 39%. However, a small decrease in the acetonitrile concentration from 39% to 36% had a remarkable effect on the separation of enoxaparin. Mobile phase containing 36% acetonitrile resolved enoxaparin into more than 15 different peaks compared to two peaks obtained using eluent containing 39% acetonitrile. However, with 36% acetonitrile concentration, overlapping peaks were observed and resolution was further improved by decreasing the acetonitrile concentration to 33%. Resolution of the early eluted peaks (between 1.0 and 10 minutes) with 33% acetonitrile was improved by decreasing the concentration of acetonitrile by 1.0%. The lowest studied acetonitrile concentration (30%) gave a similar resolution to that of 32% but with analysis taking more than 90 minutes, resulting in no additional chromatographic benefits. An acetonitrile concentration of 32% was selected for further method development.

The amount of ion-interaction reagent adsorbed onto the surface of the stationary phase is influenced by the concentration of organic modifiers [103]. The vastly different resolution and retention times, between mobile phase containing 39%

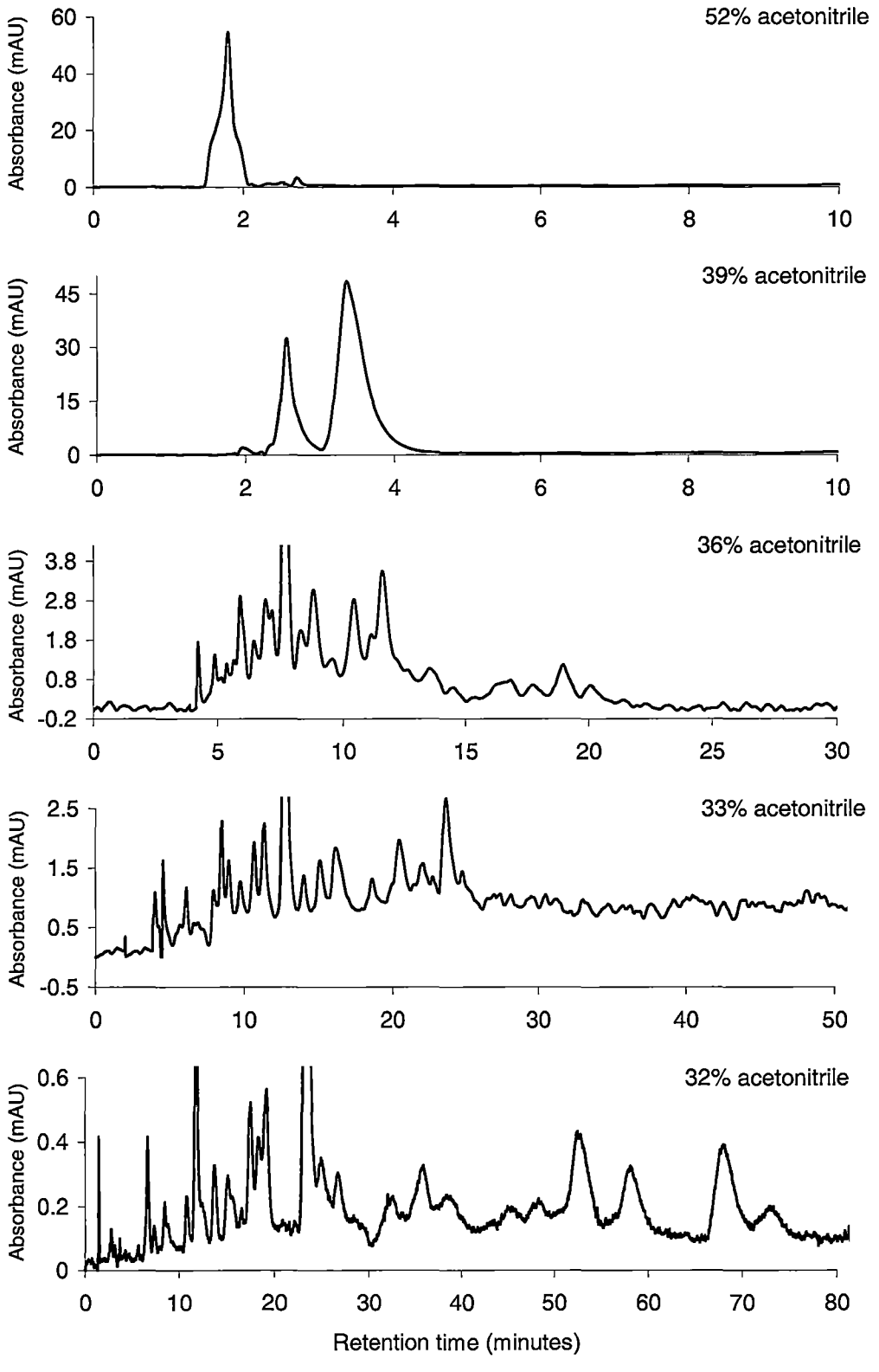

Figure 4.1 The influence of various concentrations of acetonitrile on the ion-interaction RP-HPLC separation of enoxaparin. Experimental conditions: Varian C_{18} (150 x 4.6 mm), 5 µm column; eluent containing acetonitrile-water (as specified in chromatograms); TBAH (15 mM); ammonium acetate (50 mM); pH 7.0; detection wavelength 230 nm; flow rate 1ml/minute; injection volume 30 µl.

and 36% acetonitrile, indicated a large change in the relative affinities of the mobile and stationary phases for the negatively charged polysaccharide molecules of the analyte over this acetonitrile concentration range. This change may be due to a large increase in the adsorption of the ion-interaction reagent to the stationary phase at 36% compared with 39% acetonitrile concentration. This increased adsorption of the ion-interaction reagent on the stationary phase would result in increased interaction between analyte and the stationary phase resulting in a large impact on the retention and resolution of enoxaparin polysaccharides.

4.4.2 *Effect of the ion-interaction reagent*

Enoxaparin is a highly negatively charged polysaccharide, which makes it difficult to retain and resolve on a C_{18} column in the absence of an ion-interaction reagent. Three different ion-interaction reagents, TEA, TBA and TBAH were investigated to determine their ion-interaction capacity and hence the effect on chromatographic separation. It was attempted to determine the optimal acetonitrile concentration for each of the ion-interaction reagents by steadily reducing the acetonitrile concentration until satisfactory resolution was achieved within a reasonable time frame (90 minutes). As seen from Figure 4.2, TEA failed to resolve enoxaparin at even 10% acetonitrile concentration. TBA at 20% acetonitrile concentration gave separation of early eluting peaks but did not resolve the late eluting peaks. TBAH successfully resolved both the early and late eluting peaks at 32% acetonitrile concentration making it a preferable ion-interaction reagent for the analysis of intact enoxaparin over TBA.

Alkyl chain length and the bulk structure of the ion-interaction reagent are important parameters in ion-interaction chromatographic separations [104]. The

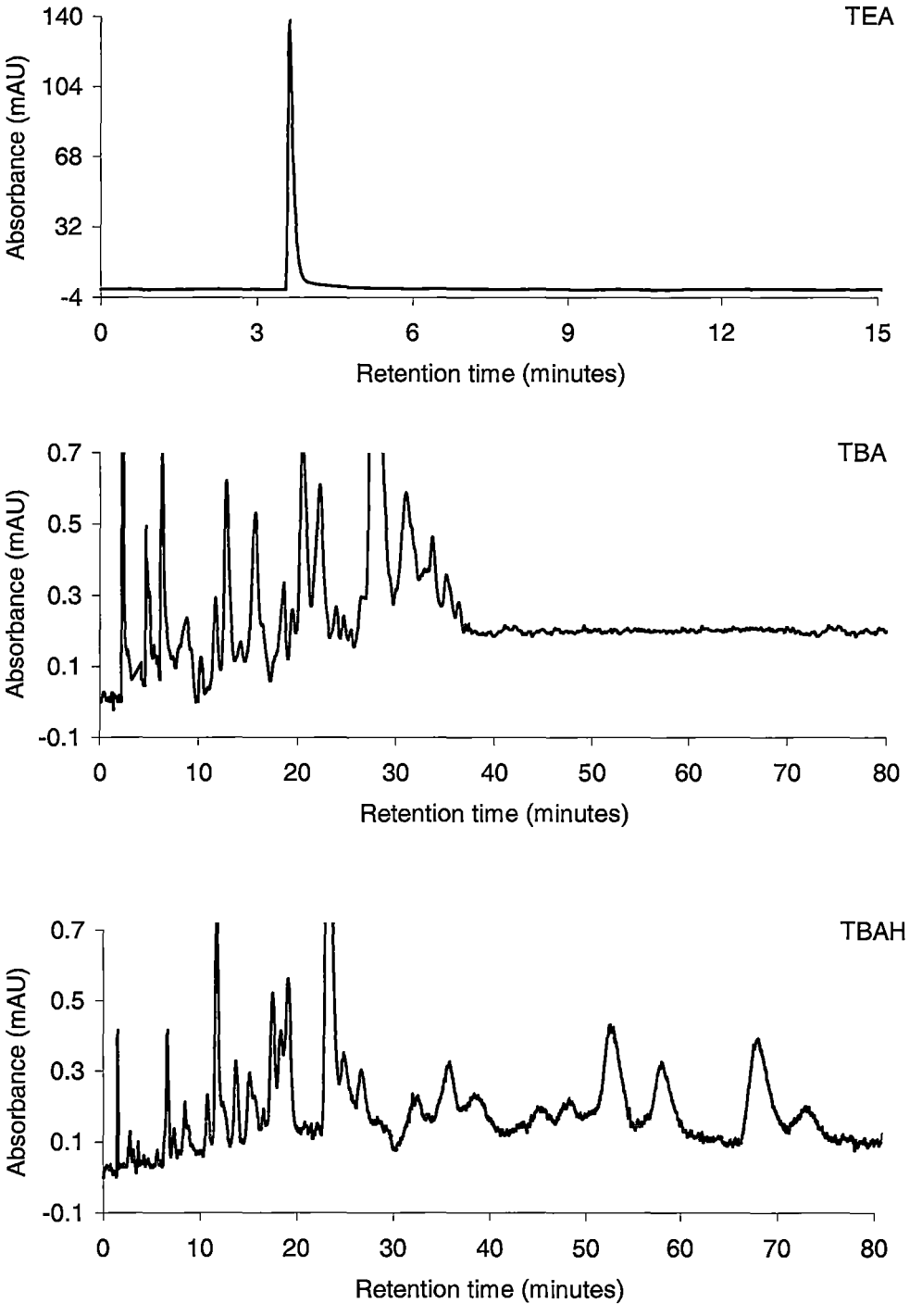

Figure 4.2 the effect of different ion-interaction reagents at 15 mM concentration on the chromatographic separation of enoxaparin. Experimental conditions are the same as those described in Figure 4.1 except for the eluent which contained 10%, 20% or 32% acetonitrile for TEA, TBA and TBAH respectively.

longer the alkyl chain and the greater the bulk structure the more hydrophobic the counter ion and the greater the retention. Acetonitrile in the eluent decreases the effective capacity of the C_{18} column at higher concentrations. The effectiveness of TBAH at higher concentrations of acetonitrile, compared with TBA and TEA, could be explained by its greater hydrophobicity.

4.4.3 *Influence of TBAH concentration on the separation parameters*

The effect of cationic ion-interaction reagent on the separation parameters was investigated in the 10 mM to 20 mM concentration range. The optimal concentration of acetonitrile for each TBAH concentration was first investigated before the comparison of chromatographic resolution was made. A remarkable TBAH concentration-dependent effect on the separation and on the peak retention time was observed. As seen from Figure 4.3. at 10 mM TBAH concentration, more than 15 different peaks were observed, however the early eluting peaks were poorly resolved and peak tailing and broadening of later-eluting peaks were observed. Enoxaparin was successfully resolved using 15 mM TBAH with satisfactory resolution and peak shape. When 20 mM TBAH was used, retention times increased and peaks were broader.

The resolving power of cationic ion-interaction reagents is solely due to their interaction with the ionisable groups of the analyte. This resolving power increases with an increase in the concentration of the ion-interaction reagent. With the current system, increased retention times limited the useful concentration of ion-interaction reagent to 15 mM; hence this concentration was chosen for further chromatographic separations.

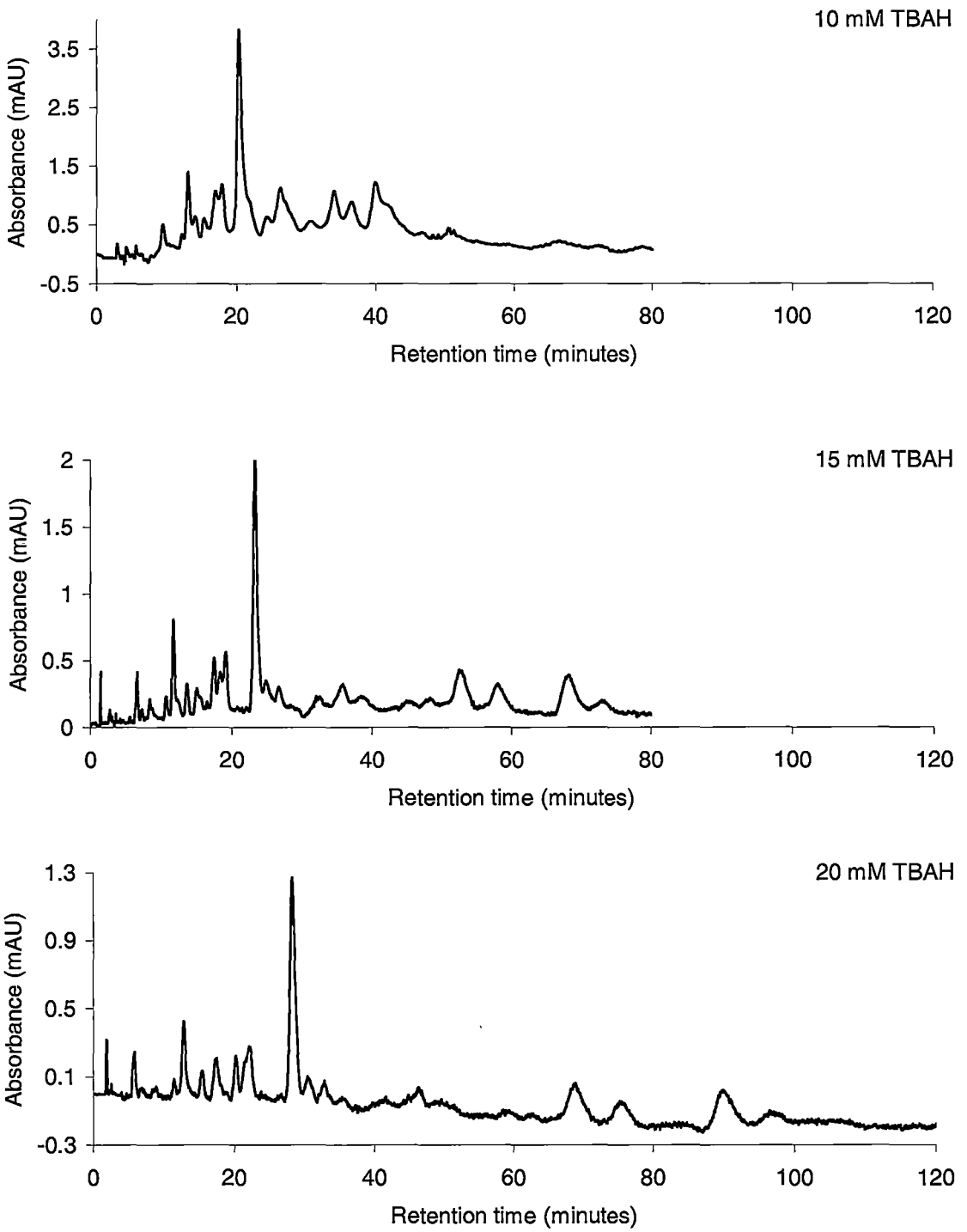

Figure 4.3 The effect of different concentrations of TBAH on the separation of enoxaparin. Experimental conditions are the same as those described in Figure 4.1 except for the eluent which contained 28%, 32% or 34% acetonitrile for 10 mM, 15 mM and 20 mM TBAH respectively, which were found to be the optimum concentration of acetonitrile for each ion-interaction reagent.

4.4.4 *Influence of pH and ammonium acetate concentration*

The mobile phase containing 32% acetonitrile, 15 mM TBAH and 50 mM ammonium acetate at different pH values ranging from 3.0 to 7.0 was investigated to study the effect of pH on the separation of enoxaparin sodium. Higher pH values enhanced the chromatographic separation of enoxaparin, especially of the late eluting peaks. The pKa values of O-sulfo, N-sulfo and carboxy groups in heparin are greater than 1.0, 1.5 and 3.0 respectively [105]. The negative charge and pKa values increase with larger polymer size [105]. Thus smaller oligosaccharides may be completely ionised at pH 5.0 but a higher pH is required for complete ionisation of larger polymers. Enhanced resolution at pH 7.0 is probably because complete ionisation of both sulfo and carboxy groups allowed their greater interaction with cations of the ion-interaction reagent.

Mobile phase containing different concentrations of ammonium acetate was investigated. Mobile phase without ammonium acetate did not provide satisfactory chromatographic resolution. As shown in Figure 4.4, increased concentration of ammonium acetate improved the separation of enoxaparin fractions and adequate resolution was achieved with the use of 50 mM ammonium acetate. Hence mobile phase consisting of acetonitrile-water (32:68), 50 mM ammonium acetate and 15 mM TBAH at pH 7.0 was selected for the assay performance investigation.

4.4.5 *Ion-interaction RP-HPLC analysis of HP-SEC enoxaparin fractions*

The HP-SEC chromatograms of EP-LMWHS and enoxaparin are shown in Figure 4.5A. HP-SEC resolved or partially resolved the EP-LMWHS into 8 different peaks (labelled *a* to *h*). The composition of the oligosaccharides corresponding to the EP-LMWHS HP-SEC peaks have previously been reported [106]. By comparison of

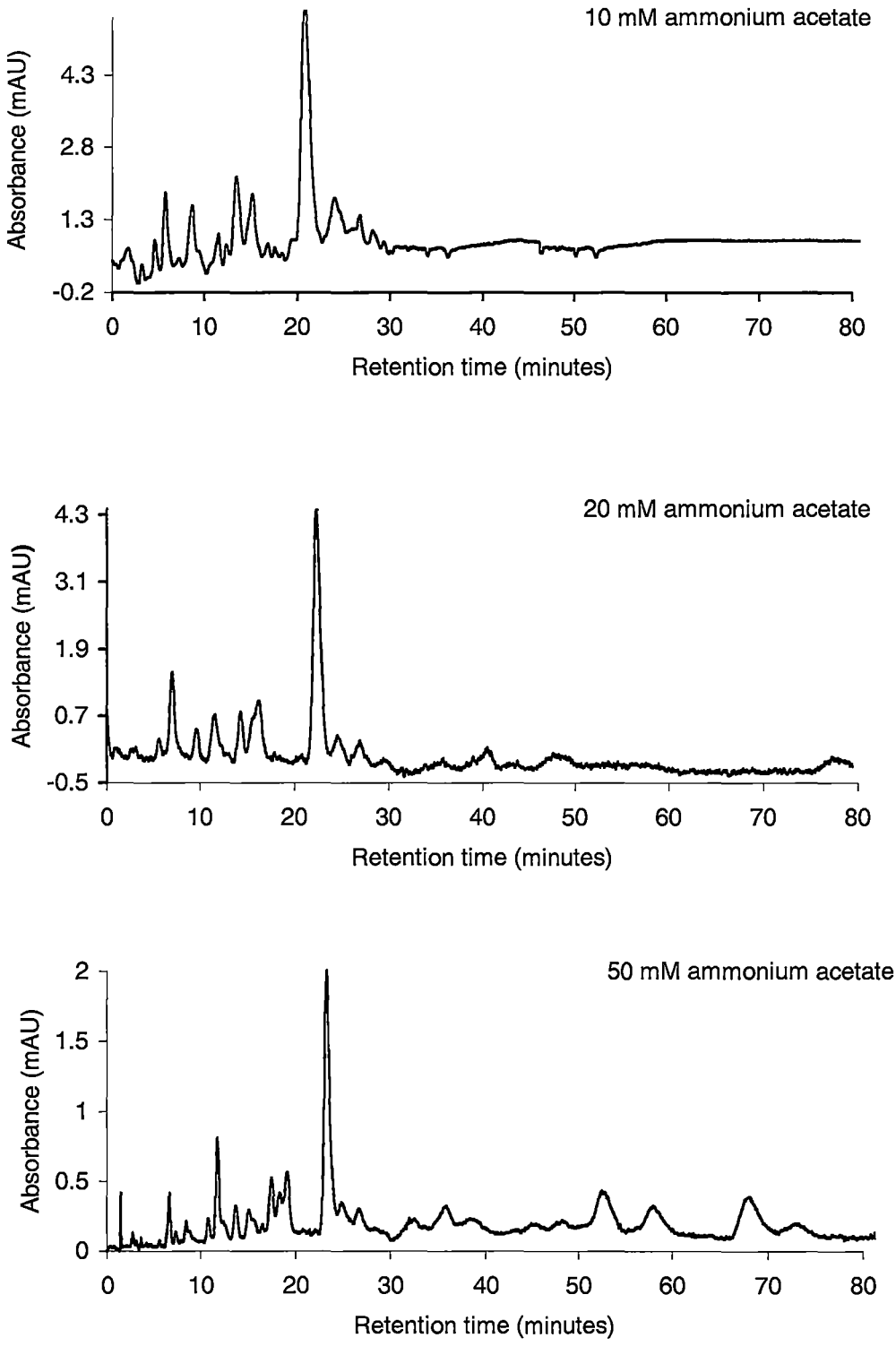

Figure 4.4 The effect of different concentrations of ammonium acetate on the separation of enoxaparin. Experimental conditions are the same as those described in Figure 4.1 with the eluent which contained 32% of actonitrile.

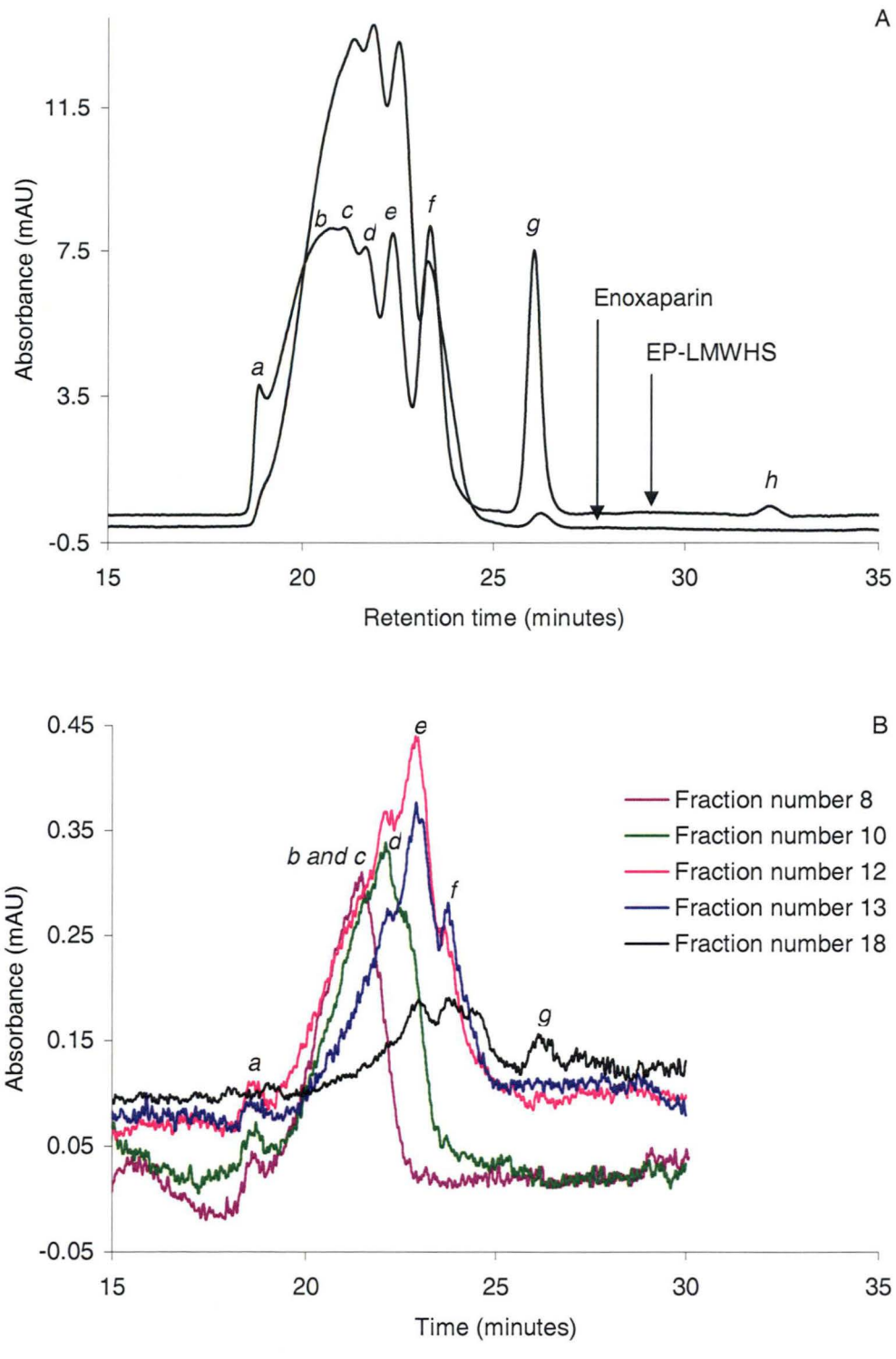

Figure 4.5 HP-SEC analysis of EP-LMWHS, enoxaparin (A) and HP-SEC collected fractions of enoxaparin (B). Peaks *b-h* represent tetradeca-, dodeca-, deca-, octa-, hexa-, tetra- and di-saccharides respectively. Experimental conditions: Shodex OHpak SB-802.5 HQ 8mm × 300 mm size exclusion column, Security Guard column equipped with a GFC 2000 cartridge, eluent containing 0.3 M sodium sulfate (pH 5.0), detection wavelength 230 nm, flow rate 0.3 ml/minute, injection volume 30 µl.

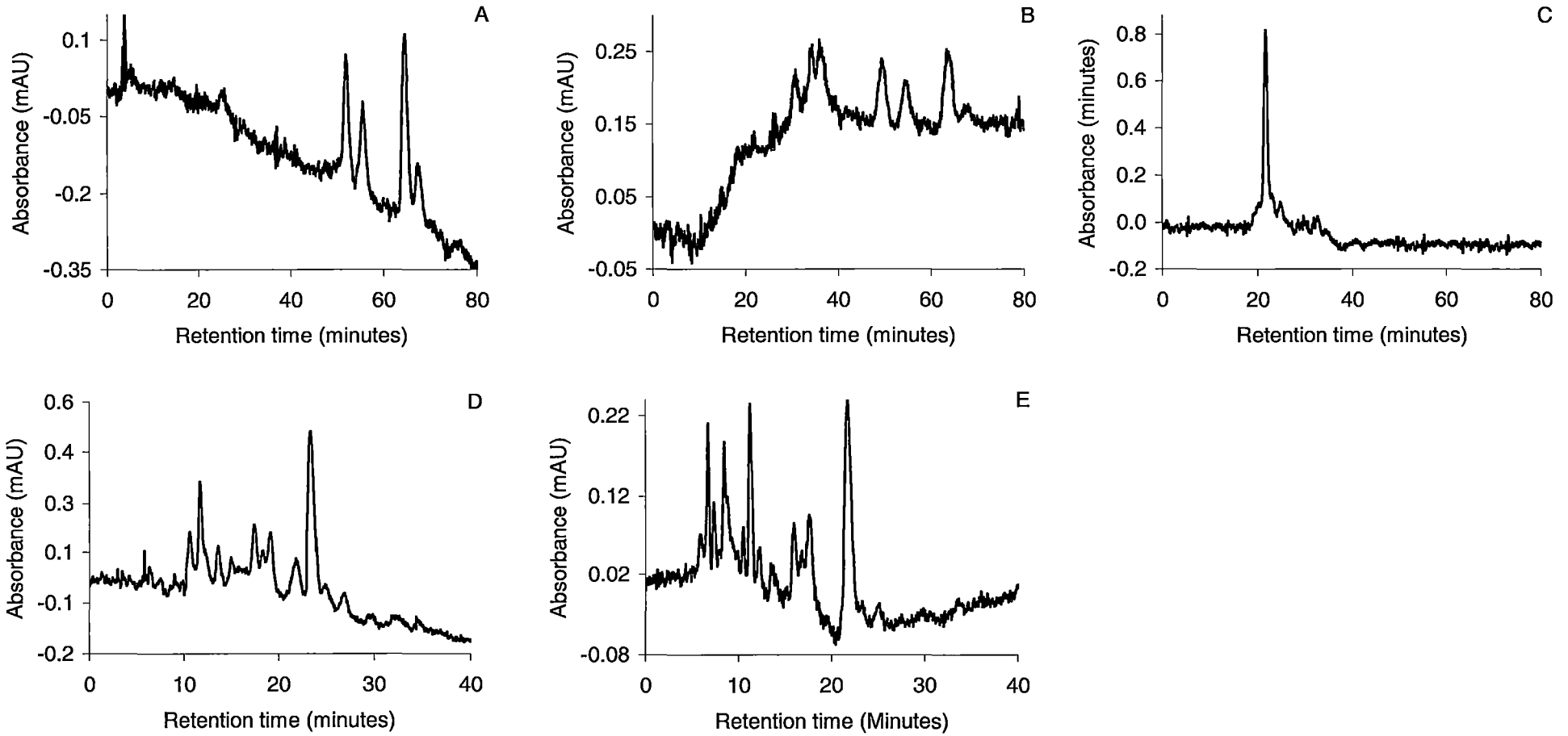

Figure 4.6 Ion-interaction RP-HPLC analysis of HP-SEC enoxaparin fractions 8 (A), 10 (B), 12 (C), 13 (D) and 18 (E), mainly composed of dodeca-, deca-, octa-, hexa- and tetra-saccharides. Ion interaction RP-HPLC was carried out under the same conditions as described in Figure 4.1 with eluent containing 32% acetonitrile.

the EP-LMWHS peaks with the enoxaparin peaks, it was possible to assign the oligosaccharide composition of each enoxaparin peak. Reanalysis by HP-SEC of 20 different fractions of enoxaparin collected using HP-SEC showed that fractions 8, 10, 12, 13 and 18 contained of dodeca-, deca-, octa- and hexa-saccharides respectively with some overlap between fractions as shown in Figure 4.5B. No additional attempts were made to purify the enoxaparin fractions prior ion- interaction RP-HPLC analysis. Ion-interaction RP-HPLC chromatograms of fractions 8, 10, 12, 13 and 18 are presented in Figure 4.6. It can be seen that the larger oligosaccharides eluted later by ion-interaction RP-HPLC. This developed method was able to resolve each group of oligosaccharides into several different peaks. As seen from Figure 4.6A, ion-interaction RP-HPLC separated fraction 8, mainly composed of dodecasaccharides, into four distinct peaks. The chromatogram of fraction 10 (Figure 4.6B) showed, in addition to the peaks observed in fraction 8, three additional peaks due to decasaccharides. Fraction 12 (Figure 4.6C) showed one sharp peak followed by two small peaks of octasaccharides. Fraction 13 (Figure 4.6D), enriched with hexasaccharides, was separated into eight different peaks. The chromatogram of fraction 18 (Figure 4.6E) containing tetrasaccharides, showed four different peaks in addition to the peaks of fraction 13. Unlike the HP-SEC technique for the analysis of intact LMWHs, this ion-interaction RP-HPLC methodology is capable of distinguishing between different oligosaccharides of similar molecular weight as well as separating smaller oligosaccharides from larger oligosaccharides. As the chemical and biological properties of different oligosaccharides vary with their molecular weight, the high resolution of this technique offers a potential method for the separation of intact oligosaccharides to enable their structural characterisation and biological activity determination. This can be achieved by preparative collection of

different LMWH fractions by ion-interaction RP-HPLC. These fractions can be analysed by ion-interaction RP-HPLC-MS and low-volume microtitre plate chromogenic assay for structural and biological activity information.

4.4.6 *Assay performance*

The assay performance was determined using 5 different arbitrarily chosen peaks within the enoxaparin chromatogram and results are shown in Table 4.1. Peak areas were obtained using peak valley (trough) baseline determinations. The intra- and inter-day precision RSD for each peak was less than 5.0% (n=6) and 6.1% (n=5) respectively at the level of 1 mg/ml enoxaparin. The intra- and inter-day accuracy RSD for each peak was less than +5.6% and +6.5% respectively at 1 mg/ml enoxaparin. Intra- and inter-day retention time RSD for each of the 5 arbitrarily selected enoxaparin peaks were less than 0.61% (n=6) and 2.17% (n=5) respectively.

4.4.7 *Ion-interaction RP-HPLC analysis using ELSD*

Enoxaparin was separated by ion-interaction RP-HPLC using a mobile phase containing 15 mM TBA, 32% acetonitrile and 50 mM ammonium acetate at pH 7.0. Flow was split immediately after the column using a T-piece to allow simultaneous detection by ELSD and UV. Chromatograms obtained by ELSD and UV are shown in Figure 4.7. Similar chromatographic signals were observed with both detectors except for an additional early ELSD signal attributable to salts. TBA allows the option of ELSD detection, whereas TBAH is not sufficiently volatile for ELSD. The TBA method could be further optimised for ELSD applications, to enhance resolution, particularly if a later, more sensitive ELSD was used. The advantage of ELSD, compared with UV, is that peak areas are not dependent on UV chromophores, for which extinction coefficients can vary by orders of magnitude, depending on the

Table 4.1 RP-HPLC assay performance determined by analysis of five enoxaparin peaks.

Peak number	Retention time	Peak Area	Precision (% RSD)		Accuracy (% RSD)		Retention time (% RSD)	
			Intra-day[a]	Inter-day[b]	Intra-day	Inter-day	Intra-day	Inter-day
1	6.39	79573	2.33	4.88	5.27	6.40	0.42	2.17
2	10.45	489277	4.91	5.36	5.58	5.92	0.60	1.39
3	19.46	1436578	4.56	5.78	3.48	4.96	0.11	0.67
4	28.58	146817	4.89	6.08	0.13	0.19	0.39	0.98
5	51.52	265300	4.06	5.90	2.26	3.20	0.30	0.53

[a] for all intra-day, n=6 [b] for all inter-day n=5

chemistry of the analyte, but are more dependent on the mass of analyte present. No ion-interaction RP-HPLC methods with ELSD detection have previously been reported for heparin or its low molecular weight derivatives. As ELSD compatible methods are also compatible with MS, this method could easily be applied for MS detection. The combination of ion-interaction RP-HPLC with mass spectrometry detection has the potential to provide both molecular weight and structural information of various LMWH oligosaccharides.

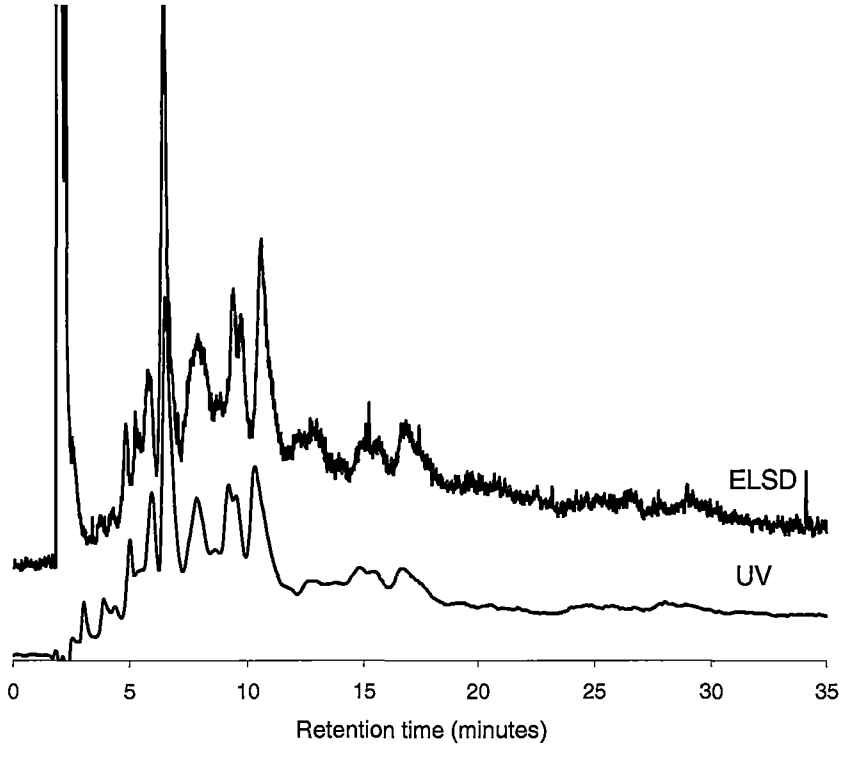

Figure 4.7 Ion-interaction RP-HPLC chromatograms of enoxaparin with simultaneous ELSD and UV detection. Experimental conditions for UV detection are the same as those described in Figure 4.1 except for the eluent, which contained 32% acetonitrile and 15 mM TBA. Details of ELSD operational conditions are given under methods.

4.4.8 *Analysis of stressed enoxaparin solution*

The ion-interaction RP-HPLC chromatograms of thermally stressed and unstressed enoxaparin samples are presented in Figure 4.8. The developed

ion-interaction RP-HPLC method has clearly demonstrated the changes that occurred in enoxaparin during heating at 70 °C for 6 hours. It is evident from Figure 4.8 that enoxaparin undergoes chemical changes, particularly after relatively mild thermal treatment, to the lower molecular weight fractions. This analytical technique offers a method that can be applied to pharmaceutical stability analysis of various intact LMWHs.

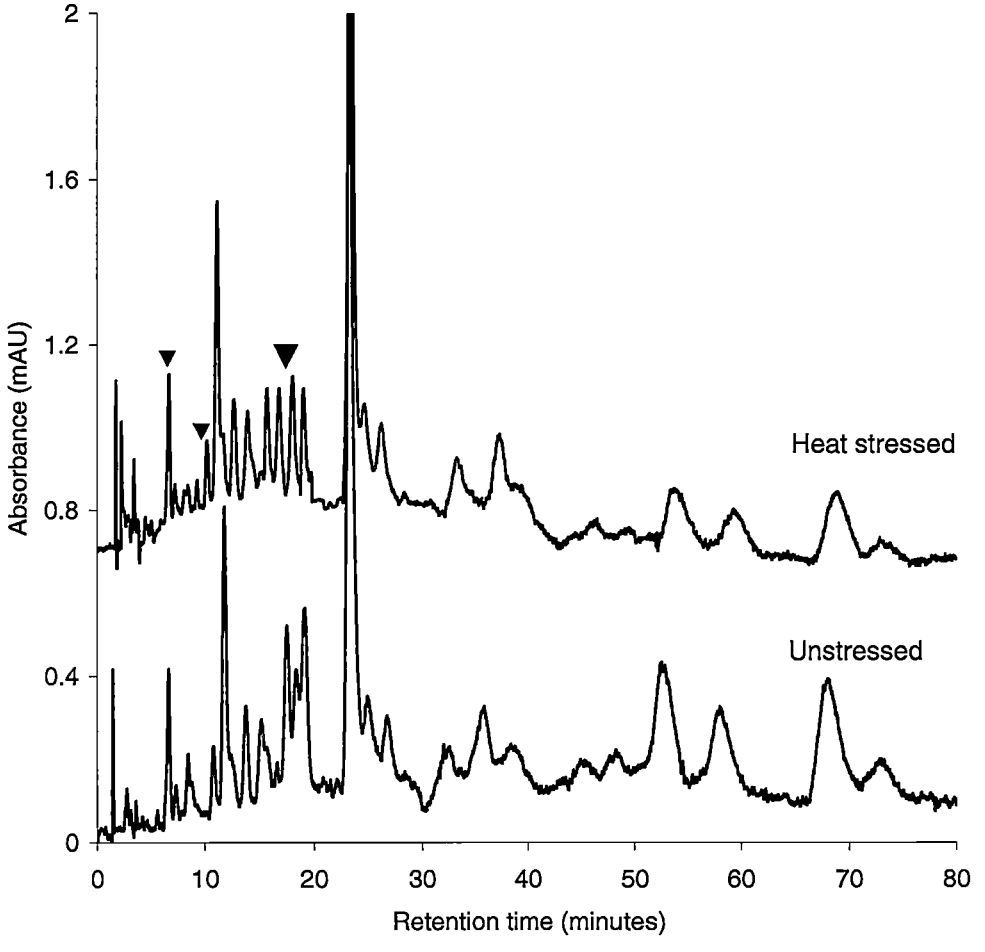

Figure 4.8 Ion-interaction RP-HPLC chromatograms of enoxaparin before and after stressing at 70 °C for 6 hours with the main differences highlighted by the arrows. Ion-interaction RP-HPLC conditions are the same as those described in Figure 4.6.

4.4.9 *Ion-interaction RP-HPLC analysis of different LMWHs*

The chromatograms of dalteparin, enoxaparin and EP-LMWHS are shown in Figure 4.9. Each LMWH showed distinctive chromatographic features with narrow peaks, good resolution and characteristic migration times and each LMWH could readily be distinguished from other LMWHs. The ion-interaction RP-HPLC method clearly demonstrated the presence of chemically distinct constituents in dalteparin (prepared by deaminative cleavage) in comparison with constituents of enoxaparin and EP-LMWHS (both prepared by β-elimination). The chromatograms of enoxaparin and EP-LMWHS show some common peaks and other peaks found in one or the other, despite sharing similar molecular weight distribution profiles by HP-SEC and a similar preparation method. Ion-interaction RP-HPLC analysis showed the presence of more disaccharides and tetrasaccharides in EP-LMWHS than in enoxaparin. High levels of these lower mass oligosaccharides are also evident by HP-SEC analysis (Figure 4.5A).

This developed method has several advantages over other analytical techniques for the separation and characterisation of intact LMWHs. It successfully resolved both lower (less than 2 000 Da) and higher (more than 5 000 Da) molecular weight oligosaccharides of intact LMWHs compared with the previously reported SAX methodology [54] that failed to resolve higher molecular weight oligosaccharides and showed broad peaks with poor resolution.

The CE procedures for the separation of intact LMWHs using different buffers have limitations associated with poor peak shape and inadequate resolution. For example, analysis of dalteparin by CE showed one broad peak with a few minor

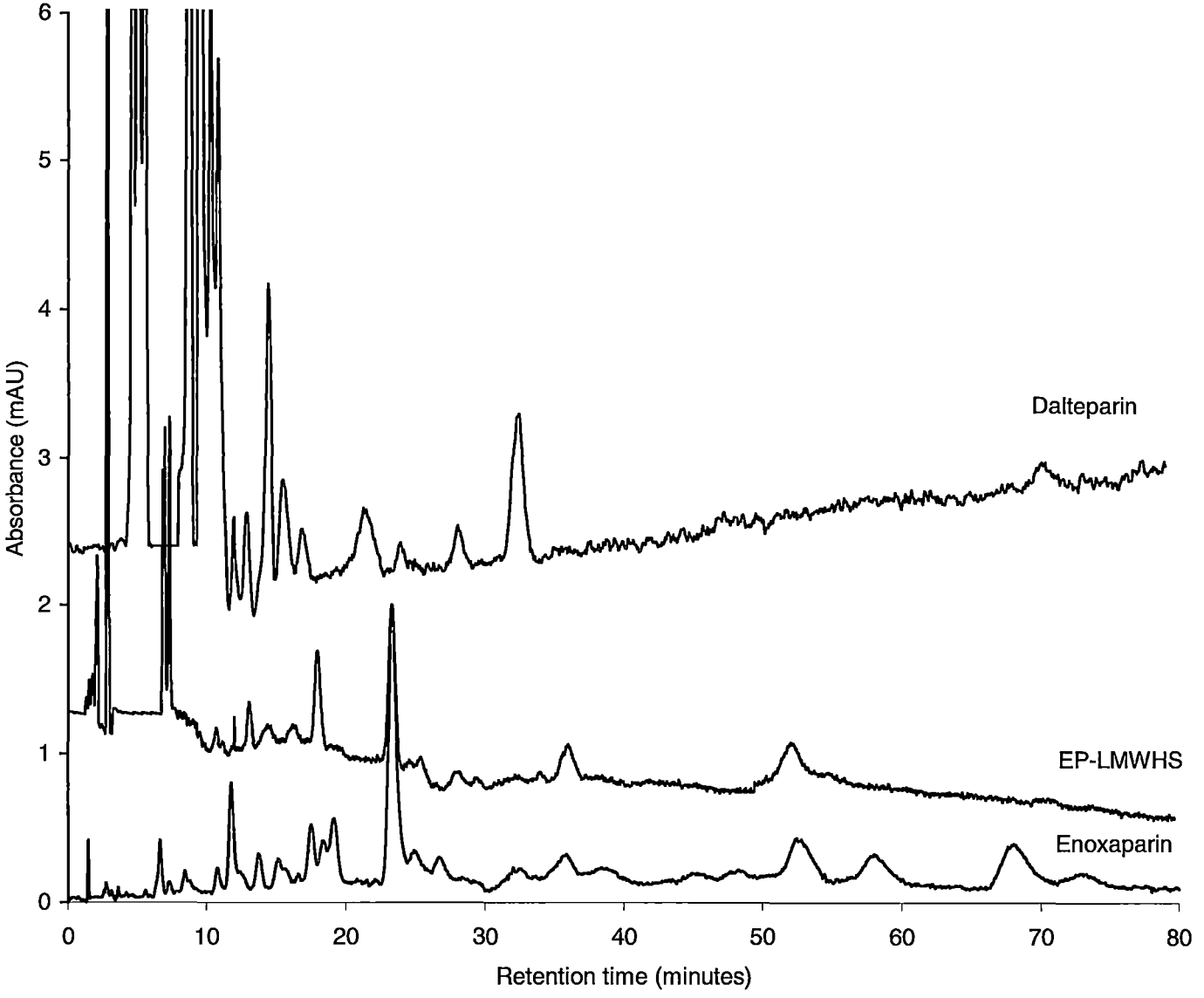

Figure 4.9 Ion-interaction RP-HPLC chromatograms of dalteparin, EP-LMWHS and enoxaparin. Experimental conditions are the same as those described in Figure 4.6.

semi-resolved peaks [73]. Most reported HP-SEC methods failed to resolve dalteparin into more than two different peaks [72,76]. The HP-SEC method demonstrated by Mulloy et al resolved dalteparin into eight different peaks [10]. PAGE analysis of dalteparin gave only a few defined bands [72]. The RP-HPLC method described here gave more than 16 well resolved peaks for dalteparin.

HPLC is available in most laboratories, the method is relatively quick and can be automated and has the potential, by careful selection of organic modifier concentration, ion-interaction reagent and its concentration, for fine tuning for specific applications, such as enhanced resolution of lower or higher mass oligosaccharides. Gradient elution techniques may also allow the development of more versatile analyses for some applications. The ELSD-compatible ion-interaction RP-HPLC method may also be suitable for detection by mass spectrometry.

4.5 CONCLUSION

An ion-interaction RP-HPLC method was developed for the separation of LMWHs using a standard 150 mm column containing 5 μm C_{18} stationary phase and an eluent containing acetonitrile-water (32:68), 15 mM TBAH and 50 mM ammonium acetate at pH 7.0. This methodology is relatively quick and gives resolution superior to previously reported methods for the analysis of intact LMWHs. The method showed differences and similarities between different LMWHs. It also showed changes resulting from heat stress of enoxaparin. The potential application of the ion-interaction RP-HPLC method coupled to ELSD was also demonstrated. Ion-interaction RP-HPLC of intact LMWHs is a powerful and versatile technique that can allow identification of LMWHs or their constituents by comparison with authenticated standards; the chemical characterisation and pharmaceutical stability

determination of various intact LMWHs; and can be applied to the investigation of changes to individual oligosaccharides present in LMWH formulations.

CHAPTER 5

Investigation of Freezing- and Thawing-Induced Biological, Chemical and Physical Changes to Enoxaparin Solution

5.1 SUMMARY

This study investigated the effect of freezing and thawing on the biological, physical and chemical properties of enoxaparin solution. Solutions were frozen and thawed under different conditions, in the presence or absence of dimethyl sufoxide (DMSO) or 1,2-propanediol (1,2-PD), and the AFXa activity was determined. Enoxaparin solution lost more than 60% of its AFXa activity when thawed rapidly after freezing at -196 °C. The loss of AFXa activity was less with higher freezing temperatures and increased with the number of freeze/thaw cycles, but was independent of the duration of freezing. Slow freezing to -196 °C with rapid thawing, or rapid freezing with slow thawing, resulted in negligible loss of AFXa activity. The loss of AFXa activity did not involve the loss of N-sulfate groups, the breakdown of glycosidic bonds, water crystal formation or the glassy state transition. Controlling the freezing or thawing conditions, dilution with water or addition of a small percentage of DMSO ameliorated the loss of enoxaparin AFXa activity. The loss in AFXa activity was found by HP-SEC to be primarily due to aggregation and was reversed by sonication in the presence of DMSO. These results may provide insight into solutions for the long-term storage of concentrated or diluted enoxaparin.

5.2 INTRODUCTION

Enoxaparin is obtained by controlled alkaline depolymerization of the benzyl ester of natural porcine heparin. This process results in the formation of lower molecular weight chains of which 15-25% contains a 1,6-anhydro-glucosamine at the reducing end. The presence of this structural feature, unique to enoxaparin among LMWHs, decreases AFXa activity minimally [107]. N-sulfo and O-sulfo groups present in the pentasaccharide sequence are important for maintaining the anticoagulant activity of heparin [6,15,108,109].

Commercial enoxaparin is available as a 100 mg/ml solution, which is stored below 25 °C and is not to be frozen. Other polysaccharides such as carrageenan, carboxymethyl cellulose and sodium alginate are added to various frozen food formulations. They are believed to protect the food product from the development of coarse texture as a result of ice recrystallization or growth during heat shock [110,111]. There has been little published on the freezing behaviour of heparin solutions or, more generally, of sulfated oligosaccharides. By comparison there is an extensive literature associated with the freezing and thawing of protein solutions, the subject of a recent review [112]. In general, for enzymes, it has been found that rapid freezing followed by rapid thawing results in the greatest retention of enzymatic activity. When an aqueous solution freezes, ice crystal formation results in concentration of the solute in the spaces between the ice crystals (freeze concentration). This can give rise to numerous consequences, including changes in pH, crystallisation of solutes, and phase separation between incompatible constituents. When no further ice formation can take place, the maximally concentrated solute forms a rigid glassy solid [112]. The more rapid the freezing the smaller the ice crystals formed and the greater the total ice crystal surface area. These results in a large ice:freeze-concentrate interfacial area. Hence, freeze/thaw treatment has the potential to induce chemical and physical changes to solutes that may be dependent on the rates of freezing and thawing and may involve freeze concentration, ice formation, glassy-state transitions or other processes.

A search of MEDLINE and SciFinder databases was conducted to determine the effect of freezing or long-term storage on the activity of LMWHs, or more generally, on heparin. The search found one study related to heparin activity at long-term storage [113]. This study reported formation of aggregates in heparin solution.

Size distribution or aggregation of various proteins compared with polysaccharides has been extensively studied using different analytical methods. These includes HP-SEC [114-118], analytical ultracentrifugation [119-121], field flow fractionation [122-123], HP-SEC with online multiple laser light scattering (MALLS) [124-126], quasi-elastic laser scattering techniques [113, 127-128], MS [129-130], electrophoresis [131], fluorescence correlation spectroscopy [132] and others [133-134].

HP-SEC is a simple, fast and convenient technique for the detection of aggregates [115-118]. The major problem associated with HP-SEC detection is the elution time of the analyte, which may change if the analyte interacts with the column matrix [135-136]. HP-SEC is not capable of distinguishing between different oligosaccharides of similar molecular mass [77]. Analytical ultracentrifugation is widely used for the determination and analysis of various aggregates, especially macromolecules. It is routinely used to study the nature of soluble protein aggregates in pharmaceutical formulations [119-120]. Application of HP-SEC in the study of pharmaceutical aggregates is sometimes restricted as it requires a mobile phase that is different and may not be compatible with the formulation [137]. Successful use of field flow fractionation has been reported in separation of protein aggregates. Unlike HP-SEC, field flow fractionation uses an empty channel instead of a column matrix to avoid interaction with the analyte [137-138]. HP-SEC - MALLS has been successfully used for the quantification of soluble aggregates. The advantage of this combination technique is that the determination of molecular weight is not dependent on the retention time of the analyte, but rather based on its refractive index [124-126, 136]. The use of quasi-elastic laser scattering or its combination with other techniques for the analysis of particle size distribution or changes in radius of gyration has been reported. This technique is quick and reliable for the analysis of large aggregated

molecules [139]. Decker et al have reported aggregation of lignin at pH below 8.5 by quasi-elastic laser scattering which was not observed using UV and refractive index detectors [140]. Various MS approaches have been employed for the accurate molecular weight analysis of different aggregates. This technique has outperformed many conventional techniques such as gel electrophoresis [129-130].

A preliminary study associated with stability testing found a significant loss in AFXa activity of frozen/thawed enoxaparin solution. Increased clinical interest in LMWHs, the intriguing behavior of frozen solutions of polysaccharides and the observed loss of AFXa activity following freezing underline the need to improve the understanding of the behavior shown by enoxaparin under different freezing conditions. By developing an understanding of the freezing process of enoxaparin solution, and the loss of anticoagulant activity, it was postulated that it may be possible to develop a method for the long-term storage of concentrated or diluted enoxaparin solutions.

The purpose of the study was to explore the effects of freezing and thawing on the AFXa activity of enoxaparin solution, to investigate freezing- and thawing-induced chemical and physical changes and to develop methods to ameliorate the freezing- and thawing-induced loss in the AFXa activity of enoxaparin.

5.3 EXPERIMENTAL

5.3.1 *Materials*

The sodium salt of enoxaparin (100 mg/ml) was purchased from Aventis Pharma (Sydney, Australia) and used as supplied at pH 7.0. The AFXa activity

kit # 832 was from American Diagnostica Inc. (Stamford, CT, USA). The bicinchoninic acid (BCA) assay kit to determine reducing capacity and fluoraldehyde assay reagent to measure free amino groups were from Pierce (Rockford, IL, USA). N-acetyl-D-glucosamine, Dimethyl sulfoxide (DMSO), 1,2-propanediol (1,2-PD), sodium sulfate, hydrochloric acid and glycine were of analytical grade and all other chemicals were of reagent grade from Sigma Aldrich (Castle Hill, NSW, Australia). Microtitre plates were flat-bottomed polystyrene plates from Corning (Corning, NY, USA). Cryogenic tubes were from InterMed (Roskilde, Denmark).

5.3.2 *Analysis of AFXa activity*

The activity of enoxaparin solution was determined using the AFXa chromogenic assay kit as described in section 2.3.2. The AFXa activity of enoxaparin samples was determined in triplicate.

5.3.3 *Freezing of enoxaparin solution at different temperatures*

Enoxaparin solution (100 mg/ml, 50 µl) was frozen at different temperatures below 0 °C. Liquid thermostat baths were produced by using a stirred solid-liquid mixture at its melting point. The solvents *n*-pentane, isobutyl alcohol, acetone and chlorobenzene were used to attain temperatures of -130 °C,-108 °C, -95 °C and -45 °C respectively. Solvent was placed in a polystyrene-insulated beaker and liquid nitrogen was added with gradual stirring to make a slurry mixture. Enoxaparin solution (10 µl) was added to a cryogenic tube, precooled in the slurry mixture, and frozen in the bath. The samples were kept in the freezing mixture for 5 minutes. Samples were similarly frozen in liquid nitrogen at -196 °C. Standard laboratory freezers were used to freeze samples at -80 °C, -26 °C and -12 °C. Cryogenic tubes containing enoxaparin solution

frozen at different temperatures were placed in a polystyrene tray and allowed to thaw at ambient temperature. The AFXa activity of these and control samples of unfrozen enoxaparin was determined.

5.3.4 *Repeated freeze/thaw cycles*

The effect of repeated freeze/thaw cycles on the AFXa activity was examined as follows. Enoxaparin solution (100 mg/ml) in a cryogenic tube was frozen at -196 °C in liquid nitrogen and held at that temperature for 5 minutes. The sample was thawed at ambient temperature. The freeze/thaw cycle was carried out repeatedly. Aliquots of enoxaparin solution (2 × 5 µl) were removed from the vial after every 5 freeze/thaw cycles, up to a total of 50 cycles.

The effect of freezing time on the AFXa activity was examined. Aliquots (10 µl) of enoxaparin solution (100 mg/ml) were transferred to cryogenic tubes and frozen in liquid nitrogen. Samples were removed from liquid nitrogen and thawed after frozen storage times of 1, 12, 24, 72, 120 and 168 hours. The AFXa activity of these and control samples of unfrozen enoxaparin was determined.

5.3.5 *Different freezing and thawing conditions*

After fast freezing, the subsequent thawing of enoxaparin solution was carried out under rapid or slow conditions. Under fast freezing/rapid thawing conditions, 10 µl aliquots of enoxaparin solution (100 mg/ml) in cryogenic tubes were frozen in liquid nitrogen for 5 minutes and allowed to thaw at room temperature in a polystyrene tray. Under fast freezing/slow thawing conditions, identical enoxaparin samples were frozen in liquid nitrogen as before and the samples were then kept in freezers, first at -80 °C for 12 hours, then at -26 °C for another 12 hours, and finally

at -12 °C for 12 hours. After this, the samples were allowed to thaw on ice at 0 °C before AFXa activity analysis. The effect of slow freezing with rapid thawing to room temperature was investigated by freezing small aliquots (10 µl) of enoxaparin solution (100 mg/ml) in cryogenic tubes at -12 °C for 12 hours. The samples were then stored in freezers at -26 °C for 12 hours and -80 °C for 12 hours and finally in liquid nitrogen for 12 hours. After this, the samples were removed from liquid nitrogen and allowed to thaw at room temperature in a polystyrene tray before AFXa activity determination together with controls consisting of unfrozen enoxaparin.

5.3.6 *Dilution of enoxaparin solution prior to freezing*

The effect of dilution prior to freezing on the AFXa activity was investigated by diluting enoxaparin solution (100 mg/ml) 2, 5, 10 and 100 times with deionized water. Aliquots of each solution (20 µl) were transferred into four cryogenic tubes. Three were frozen in liquid nitrogen and thawed after five minutes, and one was kept as a control. Samples and controls were analysed for AFXa activity.

5.3.7 *Analysis of free sulfate*

Determination of free sulfate in unfrozen and freeze/thawed enoxaparin solution was carried out by a previously described ion chromatographic method [141]. Analyses were performed with a Dionex DX-120 instrument (Dionex Corporation, Sunnyvale, CA, USA), which consisted of a GP50 gradient pump, CD25 conductivity detector and AS50 auto sampler. A Dionex IonPac AS11 column was used with a mobile phase gradient of water and KOH. Hydroxide eluent gradients were generated online using the Dionex EluGen II KOH cartridge, from 1 mM to 15 mM over 15 minutes. Detection was carried out by conductivity in the suppression mode with a

flow rate of 1 ml/minute and injection volume of 25 µl. Instrument control and data acquisition were performed using Chromeleon software. A standard plot was prepared using sodium sulfate containing 0 to 20 µg/ml of sulfate.

5.3.8 *Analysis of free amino groups*

The determination of free amino groups in unfrozen and freeze/thawed enoxaparin solution was determined by a sensitive fluoraldehyde-based assay [142]. Sample (20 µl) was mixed with deionized water (180 µl) and fluoraldehyde assay reagent (1 ml) was added and mixed well. The fluorescence of each sample was measured at 455 nm after excitation at 360 nm using a fluorescence spectrophotometer Model 1605-10S (Perkin-Elmer, Tokyo, Japan). A standard plot was prepared using glycine in the range of 0 to 600 µg/ml.

5.3.9 *Analysis of reducing capacity*

The reducing capacity of enoxaparin solution frozen at -196 °C and thawed at room temperature and unfrozen controls was determined by a previously described assay [143]. Assay reagent C was prepared by mixing 50 parts of reagent A, consisting of sodium carbonate, sodium bicarbonate, BCA detection reagent and sodium tartrate in 0.1N sodium hydroxide, with 1 part reagent B containing BCA. Sample (20 µl) was added to assay reagent C (100 µl) and mixed well for 3 minutes in a microtitre plate. The absorbance was measured at 560 nm using a Bio-Rad microplate reader Model-680 (Bio-Rad laboratories, Hercules, CA, USA). A standard plot was generated using N-acetyl-D-glucosamine in the range of 0 to 10 mg/ml.

5.3.10 *Scanning electron microscopy analysis*

Microscopic evaluation was carried out using a Philips XL20 scanning electron microscope (Philips Electron Optics, Eindhovan, Netherlands). Scanning electron microscopy (SEM) stubs were cooled to -196 °C for 5 minutes. A drop of enoxaparin solution (100 mg/ml) was placed on the stub and allowed to freeze. The stub holding the frozen sample was placed on a cold table (-5 °C) and observed by SEM. Visual comparisons were made with similarly-frozen water.

5.3.11 *Determination of glass transition temperature*

Modulated differential scanning calorimetry (DSC) was performed using a Model 2920 calorimeter (TA Instruments, Newcastle, DE, USA) to determine the glass transition temperature (Tg) of enoxaparin solution. Samples of enoxaparin solution (100 mg/ml) were cooled to -130 °C in one of three ways: rapid quenching; cooled at a rate of -5 °C/minute; cooled at a rate of -20 °C/minute. Samples were held at -130 °C for 5 minutes before heating at 10 °C/minute to 30 °C. Analyses were performed in duplicate.

5.3.12 *Effects of cryoprotectants*

Enoxaparin solution was diluted with DMSO or 1,2-PD to obtain enoxaparin stock solutions containing 5% v/v of DMSO or 1,2-PD. The stock solution was serially diluted with enoxaparin solution (100 mg/ml) to achieve concentrations of 1.0, 0.2, 0.04 and 0.008% of each cryoprotector in enoxaparin solution. Aliquots of each solution were transferred to cryogenic tubes, frozen in liquid nitrogen for 5 minutes and thawed at room temperature. Frozen and thawed samples, with corresponding controls, were analyzed for AFXa activity.

5.3.13 *Sonication of enoxaparin solution after freeze/thaw treatment*

A cryogenic tube containing 200 µl of enoxaparin solution (100 mg/ml) was frozen in liquid nitrogen and thawed at room temperature. After 5 freeze/thaw cycles, aliquots (5 µl) were diluted with 0%, 0.1% or 1% DMSO in normal saline (995 µl). Half the aliquots were sonicated in a Sanophon ultrasonic processor (Ultrasonic Industries, Tasmania, Australia) for 15 minutes and the corresponding controls were not sonicated. Solutions were analyzed for AFXa activity. Comparisons of AFXa activity between frozen samples with 0%, 0.1% and 1% DMSO were performed by one way analysis of variance (ANOVA), followed by a fishers protected least squares difference (PLSD) post hoc test for different conditions. Results were considered statistically significant with $p<0.05$.

5.3.14 *HP-SEC analysis*

HP-SEC was used to obtain comparative chromatographic profiles of samples frozen in liquid nitrogen and thawed at room temperature, before and after sonication in the presence and absence of DMSO and controls. HP-SEC analysis of samples was performed as described in section 4.3.5.

5.4 RESULTS AND DISCUSSION

5.4.1 *Effects of freezing and thawing on AFXa activity*

The loss of AFXa activity after freezing at different temperatures is shown in Figure 5.1. Loss in AFXa activity occurred when enoxaparin solution was frozen at or below -26 °C. By comparison, samples frozen at -12 °C displayed no loss of AFXa activity. Enoxaparin lost 67% and 14% of control AFXa activity after freezing at

-196 °C and -26 °C respectively. The lower the temperature of freezing the greater was the loss of AFXa activity.

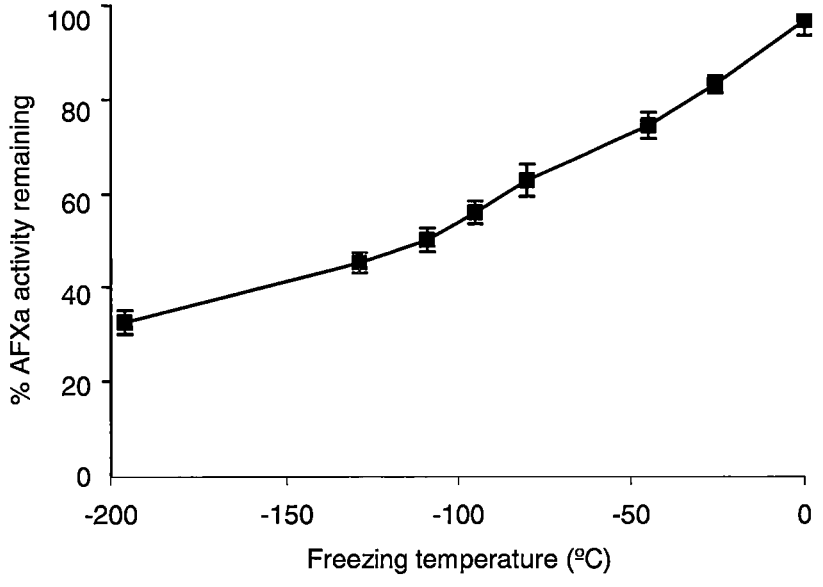

Figure 5.1 The effect of freezing at different temperatures on the AFXa activity of enoxaparin (n=6, error bars represent ± SD).

The effects of repeated freeze/thaw cycles, the duration of freezing time, and the freezing and thawing rates on AFXa activity are presented in Figure 5.2. It can be seen from Figure 5.2A that the AFXa activity decreased with increasing number of freeze/thaw cycles and that only 13% of the initial AFXa activity remained after 50 freeze/thaw cycles. Figure 5.2B shows that the duration of the time of freezing made no difference to the loss in AFXa activity. Figure 5.2C shows that fast freezing with rapid thawing resulted in enoxaparin activity losses of up to 65%. Fast freezing with slow thawing resulted in preservation of more than 98% of the initial AFXa activity even after 5 freeze/thaw cycles. Slow freezing with rapid thawing resulted in negligible loss of AFXa activity.

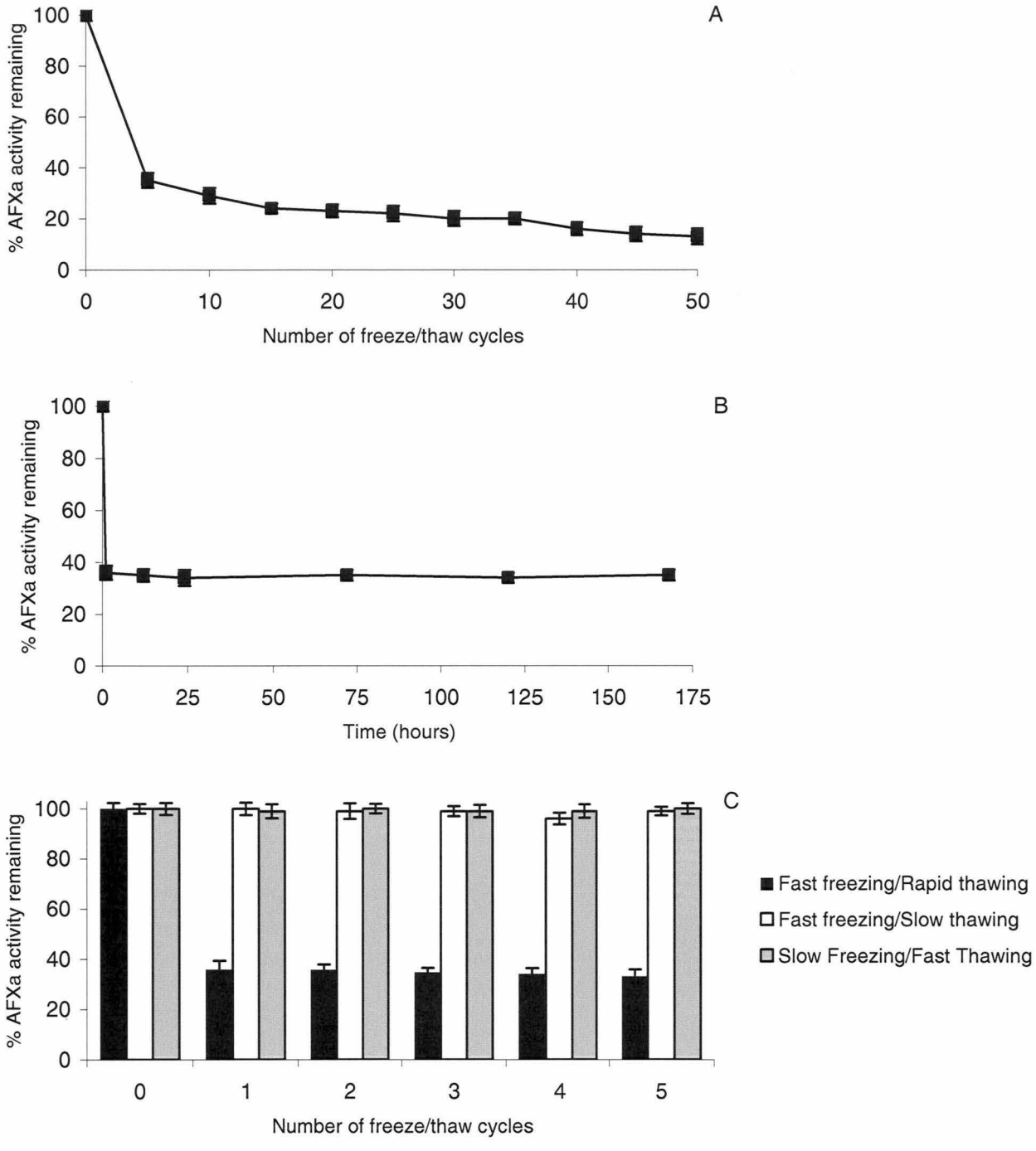

Figure 5.2 The effect on AFXa activity of: repetitive freezing (-196 °C) and thawing (room temperature) (A); increasing the duration held frozen at -196 °C before thawing (room temperature) (B); fast freezing (-196 °C) and thawing (room temperature), fast freezing (-196 °C) and slow thawing (-80 °C, -26 °C, -12 °C, 0 °C over 36 hours), slow freezing (-12 °C, -26 °C, -80 °C, -196 °C over 48 hours) with rapid thawing (room temperature) (C) (n=6, error bars represent ± SD).

It is recommended by the manufacturers that enoxaparin solution be stored below 25 °C. It is obvious from the results of this study that the freezing and subsequent thawing of enoxaparin solution can reduce its AFXa activity. The degree of activity loss was shown to be dependent on two factors, the freezing rate and the thawing rate of enoxaparin. Although freezing at lower temperatures appeared to increase the amount of AFXa activity lost, this was probably due to the greater rate of freezing rather than the exposure to lower temperatures, *per se*, since the gradual cooling of a sample to -196 °C, after freezing slowly at -12 °C, resulted in negligible loss of activity. Additionally, the length of low temperature storage time did not appear to have an effect on the degree of activity loss, over the time interval of the study.

5.4.2 *Chemical changes in frozen/thawed enoxaparin solution*

Results of analyses of free sulfate, free amino groups and reducing sugars present in unfrozen and liquid nitrogen frozen/thawed enoxaparin samples are presented in Table 5.1. A Student *t*-test showed no differences in the concentration of each analyte between frozen/thawed samples and corresponding controls. Therefore enoxaparin did not lose its anticoagulant activity by elimination of N-sulfo or O-sulfo groups [6,15,108,109] which, in the case of N-sulfo groups would give rise to free amino groups [97]. Secondly, fragmentation of polysaccharide chains leading to generation of new non-reducing and reducing ends also was not responsible for changes in AFXa activity. Changes in the reducing capacity and associated changes in AFXa activity due to modification of the 1,6-anhydro groups can also be discounted.

Table 5.1 Determination of free sulfate groups, free amino groups and reducing sugars in unfrozen and frozen/thawed enoxaparin solution. Samples were analysed in triplicate with mean and relative standard deviation (% RSD, n=3) represented.

Enoxaparin sample	Free sulfate groups (mmoles/l)		Free amino groups (mmoles/l)		Reducing ends (mmoles/l)	
	Mean	%RSD	Mean	%RSD	Mean	%RSD
Unfrozen	0.0181	1.52	0.011	3.6	0.019	5.9
Frozen/thawed	0.0189	1.75	0.013	5.7	0.022	6.9

5.4.3. *SEM analysis of frozen enoxaparin solution*

The cryo SEM images of quench-frozen enoxaparin solution and water are presented in Figure 5.3. The image of frozen water (Figure 5.3A) shows the formation of ice crystals with clearly defined margins between the crystals. By comparison there are no obvious ice crystals or crystal margins evident in the images of enoxaparin solution quench-frozen on stubs cooled to -196 °C (Figure 5.3B). It was hypothesized that since ice crystal formation in polysaccharide solutions has been shown to be dependent on freezing temperatures [144], the observed decrease in AFXa activity of frozen enoxaparin may have been related to differences in ice crystal formation. The observations from the SEM study suggested that ice crystal formation was not responsible for the observed decreases in AFXa activity, since no ice crystals were visible in the frozen enoxaparin solution. This observation led to the investigation of the frozen glassy state transition (Tg) of enoxaparin solution.

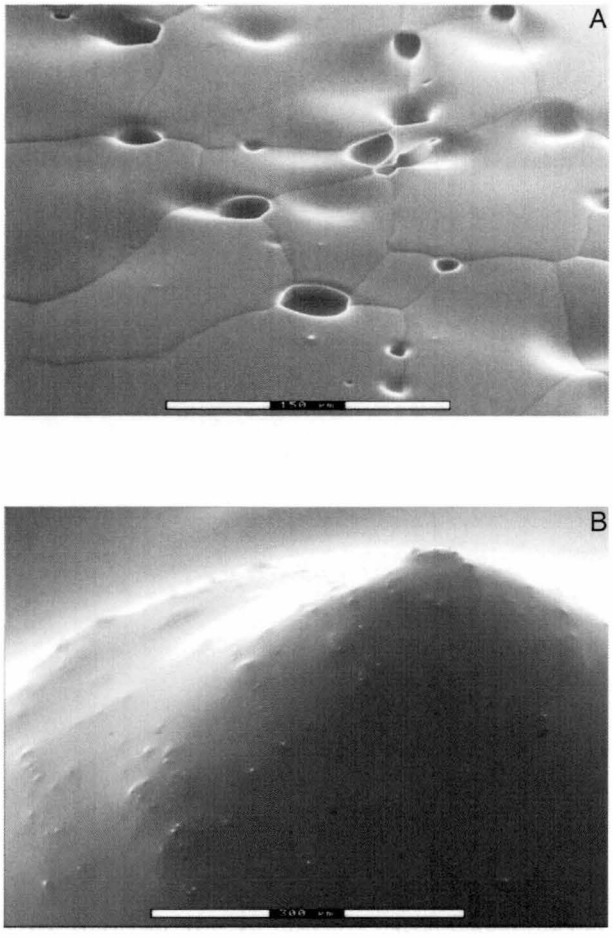

Figure 5.3 The SEM images of water (A) and enoxaparin solution (B) frozen on SEM stubs precooled in liquid nitrogen at -196 °C.

5.4.4 *Investigation of Tg of enoxaparin solution*

The DSC thermograms of enoxaparin solution are presented in Figure 5.4. The thermograms show heat flow during freezing at two different cooling rates, but not of the quench-frozen solution, and during thawing of three samples. These freezing rates were selected to understand the possible mechanism behind the observed differences in the activity loss under different freezing and thawing conditions (Figure 5.3C). The thawing thermograms through the Tg are presented in Figure 5.5. It can be seen from Figure 5.5 that Tg was similar regardless of the freezing rate. Calculated glass transition and melting parameters of quench-frozen enoxaparin solution are presented

in Table 5.2. Glass transition parameters were calculated using DSC software from the middle point within the range of temperature where the glass metastasis is almost done (Figure 5.5).The Tg occurred at -17.8 ± 0.2 °C, which has not previously been reported for enoxaparin or other forms of heparin. The DSC results show that frozen enoxaparin solution exists in a glassy rather than a crystalline state, which is consistent with the SEM observations. Given that a similar glass transition was observed in enoxaparin solutions that were frozen at different rates and that the loss in AFXa activity was minimal with slow freezing, it appears that the loss in AFXa activity was not related to the glassy state transition.

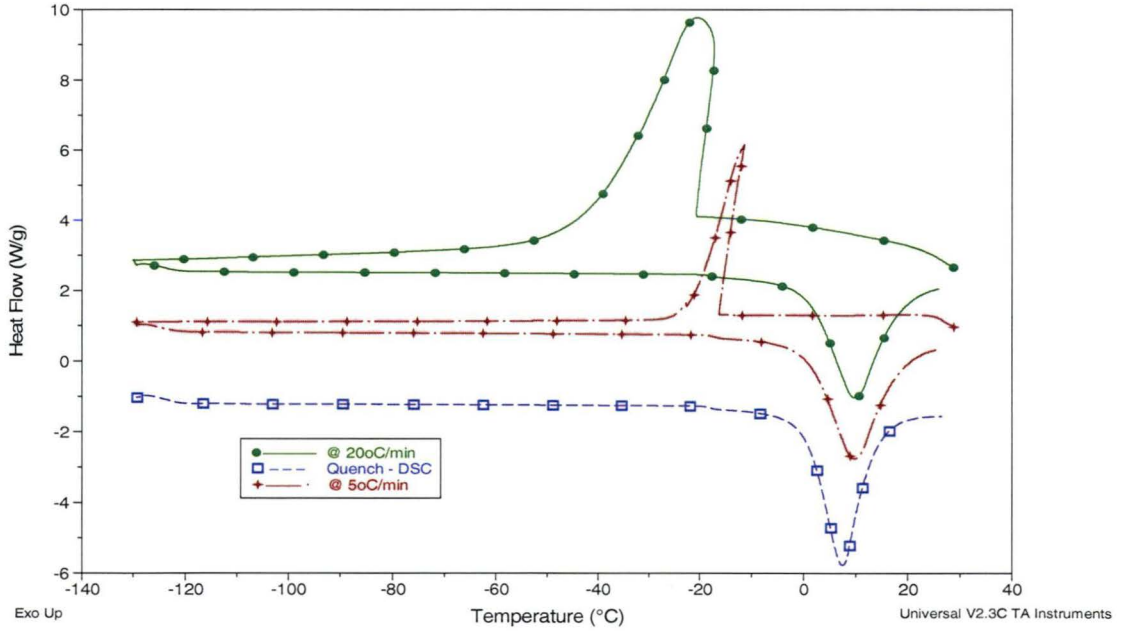

Figure 5.4 DSC thermograms of enoxaparin solution (100 mg/ml) cooled at three different rates and then warmed at 10 °C/minute.

Table 5.2 Glass transition and melting parameters of quench-frozen enoxaparin solution from the DSC thermograms. All determinations are mean ± range (n=2).

Glass transition parameters	°C
Onset temperature, T_o	-19.0 ± 0.1
Glass transition temperature, T_g	-17.8 ± 0.2
End temperature, T_e	-17.7 ± 0.0
Melting parameters	°C
Onset temperature, T_{onset}	0.4 ± 0.0
Peak temperature, T_{peak}	7.4 ± 0.2
End temperature, T_{end}	26.3 ± 0.7

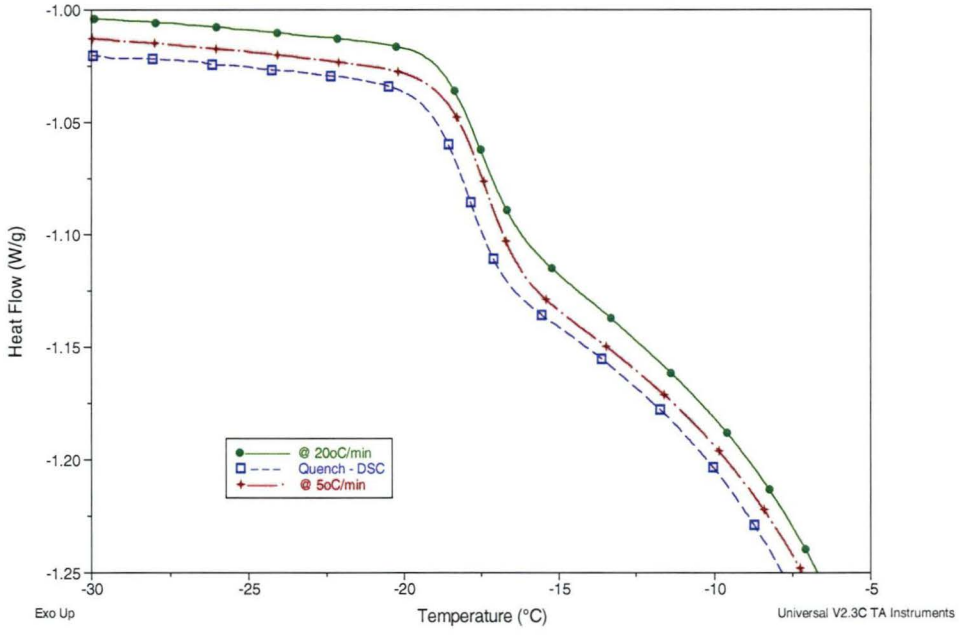

Figure 5.5 The DSC thermogram during warming of enoxaparin solution (100 mg/ml) showing the glassy state transition between -20 °C and -15 °C, for enoxaparin solutions frozen at three different rates.

5.4.5 *Role of cryoprotectants*

The effects of different concentrations of DMSO and 1,2-PD on the loss of AFXa activity of enoxaparin solution (100 mg/ml) frozen in liquid nitrogen and thawed at room temperature are shown in Figure 5.6. 1,2-PD and DMSO had

markedly different effects on the AFXa activity. 1,2-PD was ineffective in preventing AFXa activity loss when its concentration was 0.04% or less. A concentration of 5% 1,2-PD still resulted in loss of 20% of the original AFXa activity. DMSO was more effective than 1,2-PD. It prevented the loss of AFXa activity at all concentrations tested, down to 0.008% v/v. These observations suggested that the action of DMSO, in preventing the loss of AFXa activity, is not related solely to its ability to inhibit the formation of ice crystals. Although it is used routinely as a cryoprotectant [145-148], the mechanisms by which DMSO acts as such are not well understood [149]. DMSO was reported to act as a disaggregating agent towards various starches [150] and was shown to be adsorbed on to the polysaccharide chain of aeromonas gum and prevent its aggregation [151].

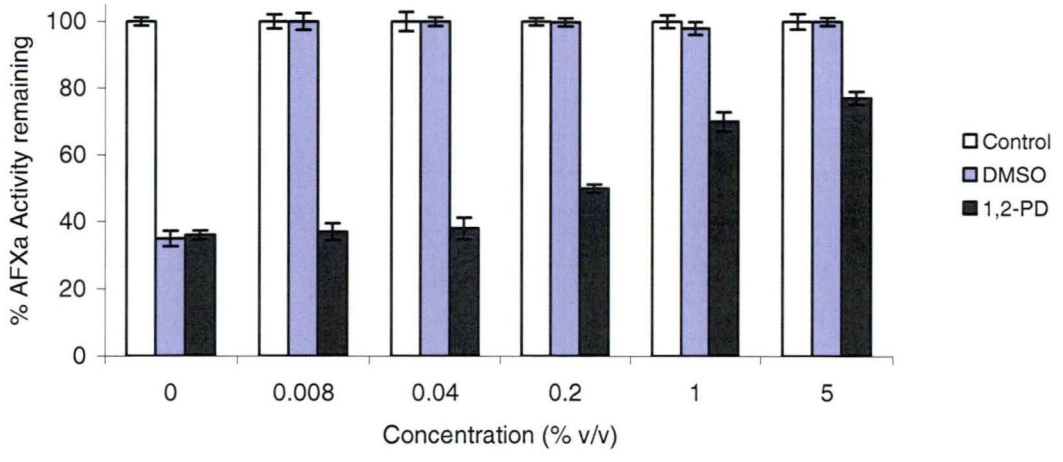

Figure 5.6 The effect of cryoprotectants at different concentrations on the loss of AFXa activity of enoxaparin solution (100 mg/ml) following freezing in liquid nitrogen and thawing at room temperature, (n=9, error bars represent ± SD).

5.4.6 *Influence of dilution prior freezing on the loss of AFXa activity*

The use of deionised water for the dilution of various LMWHs has been reported. [152-153]. The use of deionised water for the dilution of LMWH prior to the activity analysis is also recommended by the chromogenic assay kit manufacturer. Dager et al has reported no difference in the activity measurement of enoxaparin when it was diluted with water or 0.9% sodium chloride solution [152]. The loss in AFXa activity of diluted enoxaparin after freezing in liquid nitrogen and thawing at room temperature is shown in Figure 5.7. When enoxaparin was diluted with water, the loss of AFXa activity decreased after freezing with increased dilution. Loss in AFXa activity was around 66% when 100 mg/ml enoxaparin was frozen at -196°C. When it was diluted with water by a factor of two, the loss in activity was 45%. Ten-fold dilution resulted in loss of only 4% of the original activity. Polysaccharides have a strong tendency to undergo aggregation with increased concentration and vice versa [154-156]. The observed reduced loss in AFXa activity with increased dilution supports the hypothesis that freezing- and thawing-induced aggregation of enoxaparin solution may be responsible for losses in AFXa activity.

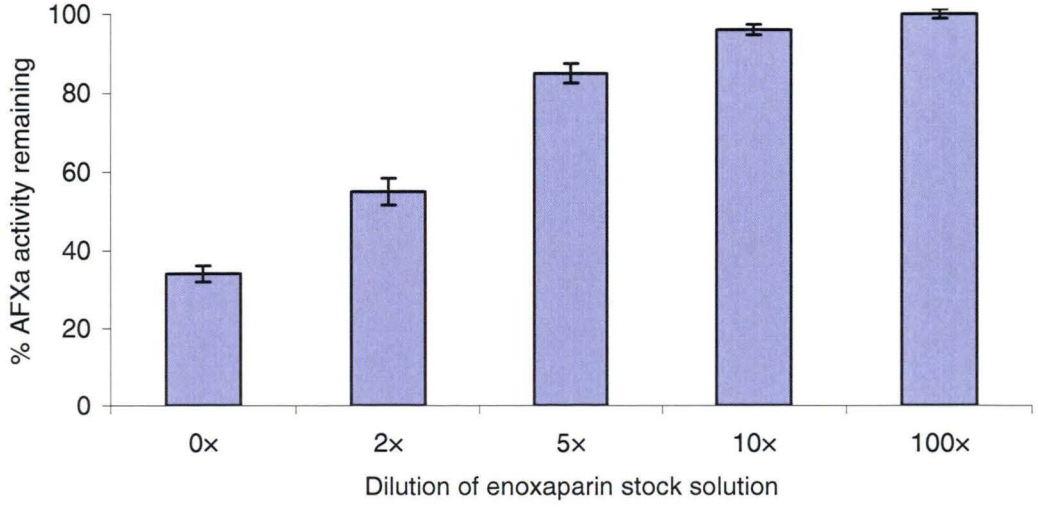

Figure 5.7 The effect of dilution with water on the loss of AFXa activity of enoxaparin stock solution (100 mg/ml) following rapid freezing in liquid nitrogen and thawing at room temperature, (n=9, error bars represent ± SD).

5.4.7 *Effect of sonication on AFXa activity with different concentration of DMSO*

The AFXa activity of enoxaparin solution (100 mg/ml) remaining after freezing in liquid nitrogen followed by thawing at room temperature and the effects of sonication in the presence of different concentrations of DMSO on AFXa activity loss are presented in Fig. 5.8. Enoxaparin solution lost more than 45% of its initial AFXa activity after freezing and thawing. Addition of DMSO after freeze/thaw made no difference to the loss in AFXa activity when sonication was not employed. Comparison of the results from the sonicated samples by ANOVA revealed a significant main effect of DMSO concentration on AFXa activity [$F(2,21)=184.2$, $p=<0.01$]. Fisher's PLSD post-hoc tests revealed differences between AFXa activities in solutions with DMSO concentrations of 0% and 0.1% ($p<0.01$), 0% and 1% ($p<0.01$), and 0.1% and 1% ($p<0.01$). The recovery of lost AFXa activity with the addition of 1% DMSO and following sonication was 82% of the control (no sonication) activity loss. Sonication as a disaggregation treatment for many polysaccharides has been reported [157,158]. Geresh et al found sonication to be a successful means of disaggregating algal sulfated polysaccharide solutions [157]. Akiyoshi and co-workers found sonication to be more effective as a disaggregation treatment for a pullulan derivative in the presence of DMSO [158]. The current sonication study demonstrated that the loss in the AFXa activity after freezing and thawing can be partially restored with sonication and that sonication more effectively restores AFXa activity in the presence of 1% DMSO. The results are consistent with freeze/thaw-induced aggregation of enoxaparin resulting in loss of its AFXa activity. Presumably this loss in activity is due to the reduced availability of active antithrombin binding sites since many of these sites would be hindered within the bulk of the aggregate, and there would also be steric effects from the aggregate bulk.

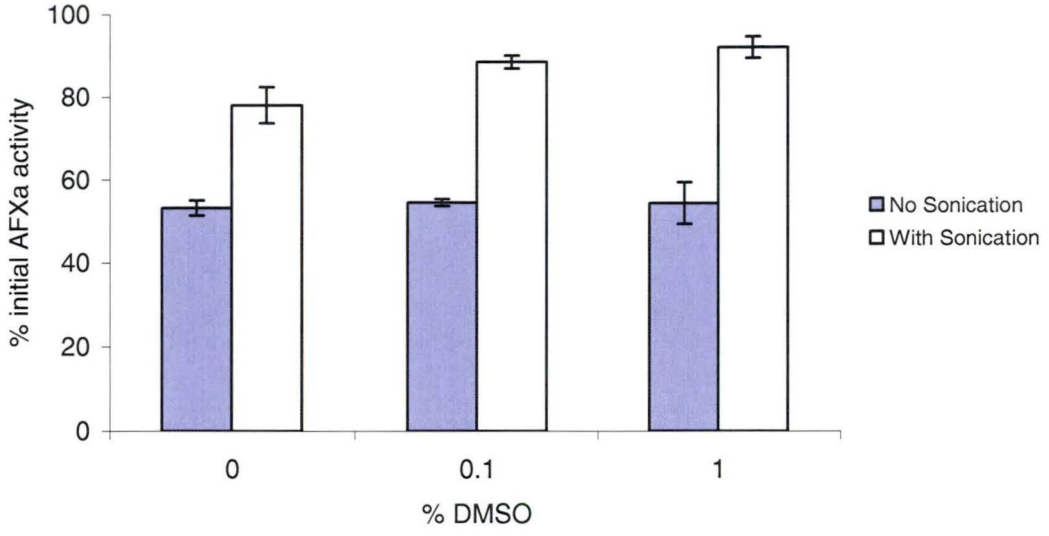

Figure 5.8 The effect of sonication on the AFXa activity of enoxaparin solution after 5 rapid (-196 °C) freeze/thaw cycles. DMSO was added to some solutions just prior to sonication. (n=15, error bars represent ± SD).

5.4.8 *HP-SEC analysis*

The HPSEC chromatograms of an unfrozen sample of enoxaparin solution, samples frozen at -196 °C and thawed at room temperature with sonication (in the presence or absence of 1% DMSO) or without sonication are given in Figure 5.9. The chromatogram of unfrozen enoxaparin solution (control, A) showed six distinct peaks that eluted over 10 minutes. The frozen and thawed enoxaparin sample (B) showed a marked reduction in the areas of peaks 3, 4 and 5 which are lower mass fragments, and there was a corresponding large increase in the areas of peaks 1 and 2 corresponding to higher mass fragments. Samples sonicated in the absence of DMSO (C) showed an increase in the peak areas of lower molecular weight constituents with little change in the areas of the early eluted peaks. The chromatogram of enoxaparin sample sonicated in the presence of 1% DMSO after freeze/thaw treatment (D) was similar to that of the unfrozen sample.

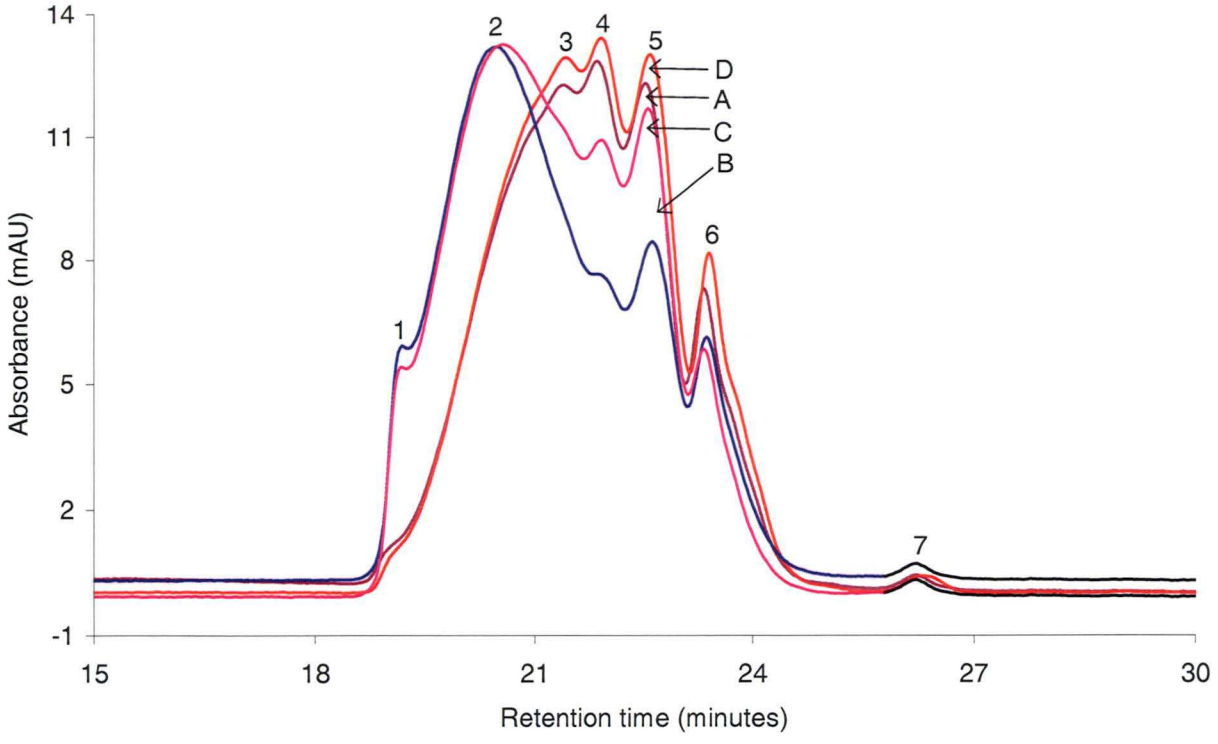

Figure 5.9 HPSEC chromatograms of enoxaparin solution (100 mg/ml) that was untreated (A), frozen rapidly (-196 °C) and thawed (room temperature) (B), frozen and thawed as for B then sonicated either without (C) or with 1% DMSO (D) (n=3). Peaks have been numbered to allow easy reference in the discussion.

HP-SEC is the widely used analytical technique to study the aggregation behavior of polysaccharides. HP-SEC separation of polysaccharides is size dependent, in which larger fragments elute earlier than the smaller ones. The early elution of aggregated polysaccharide molecules has been reported [157,158]. The HP-SEC analysis of enoxaparin solution showed the presence of high mass fractions, consistent with polysaccharide aggregates, after freezing and thawing. After sonication of freeze/thawed enoxaparin in the presence of 1% DMSO, the peak area of higher mass fragments returned to the control levels and there was a parallel increase in the peak area of lower mass fragments, consistent with disaggregation. This change also resulted in a corresponding increase in the AFXa activity (Figure 5.8).

It is known that polysaccharides are aggregated in aqueous solution on account of inter- or intra-molecular hydrogen bonding resulting from hydroxyl groups [124]. Dilution or addition of DMSO prior to freezing of enoxaparin decreased the activity loss. Xu and co-workers have reported that the driving force for the aggregation of polysaccharides increased with increasing the concentration at lower temperatures [159], consistent with the result obtained in this study. Dilution increases the distance between different constituents of enoxaparin and decreases the inter- and intra-molecular hydrogen bonding and hence aggregation. It has been shown that DMSO forms coating on the polysaccharide chains of aeromonas gum and prevents contact of the oligosaccharide chains and hence decreases the number of aggregates [151]. Based on the results obtained in this study it is suggested that DMSO has prevented aggregation by disturbing inter- or intra-molecular hydrogen bonding at lower temperatures.

HP-SEC is the established standard in pharmaceutical industries for the detection and quantification of soluble aggregates [141]. HP-SEC is considered as a sensitive, efficient and reliable technique for the determination of aggregates [113-114, 117]. The main reason behind the use of HP-SEC in this study was its easy accessibility in our laboratory. Ultracentrifugation requires expensive specialised instrument and the procedure for aggregate determination is time consuming [136]. This technique was not available for this study. Another possible alternative was the combination of HP-SEC - MALLS. The superiority of a light scattering technique is that the analysis is based on the molecular weight and concentration of solute. For a low molecular weight analyte (MW < 6000 Da) this method requires a higher concentration of solute to be injected on to the column compared with UV detection [136]. Injection of more than 1 mg/ml of enoxaparin (MW 4500 Da) in the HP-SEC

column had an adverse affect on the chromatography, with a decrease in the number of peaks detected. Column overload could potentially have prevented the applicability of this light scattering technique. Quasi-elastic laser scattering was not used in this study because of its well known drawback of low resolution. Unlike HPSEC, it is not able to resolve two constituents unless they differ in molecular mass by factor 8 [139]. Therefore it is considered as a poor technique for studying low mass molecules such as enoxaparin. A search of the literature did not show the use of MS for the analysis of intact LMWHs, therefore restricting its use in our study.

Recommended storage conditions for heparin formulations are not unambiguous. Storage recommendations given on the packaging of heparin sodium formulations from different manufacturers differ from each other [113]. It is generally understood that a particular formulation should be stored according to the manufacturer's instructions. The label for enoxaparin (Clexane) states "store below 25 °C" whereas, the EP-LMWHS (also prepared by β-eliminative cleavage) instructions state "keep at 4 °C and do not store at lower temperatures to avoid deterioration of the material". The label for dalteparin (Fragmin) states "store below 30 °C and do not freeze". It is evident from this study that freezing adversely affects AFXa activity and that enoxaparin should not be frozen. Based on the results obtained in this study, it is recommended that the storage recommendations for enoxaparin should state that the preparation should not be frozen.

5.5 CONCLUSION

This study showed that the effect of freezing and thawing on the AFXa activity of enoxaparin solution is significant. Enoxaparin lost 64% of its initial AFXa activity after freezing at -196 °C and thawing at room temperature. The loss in

activity was even higher with an increase in the number of freeze/thaw cycles. The decrease in AFXa activity was not seen when enoxaparin solution was either frozen or thawed slowly. The frozen enoxaparin solution exists in a glassy state having a Tg at -17.8 °C. The freeze/thaw effect on the AFXa activity of enoxaparin solution can be reduced by dilution with water or addition of DMSO prior to freezing. Aggregation was found to be the main cause of the lost AFXa activity after freeze/thaw treatment. Aggregation was found to be reversible by sonication in the presence of 1% DMSO. Insight into the mechanisms by which enoxaparin lost AFXa activity after freeze/thaw treatment and different ways of preventing or recovering the lost activity may potentially give rise to new methods for the long-term storage of concentrated or diluted enoxaparin solutions.

CHAPTER 6

Stability Enhancement of Diluted Enoxaparin at Paediatric Dosage Levels

6.1 SUMMARY

Diluted enoxaparin is used for the long-term treatment of paediatric patients. The stability of diluted enoxaparin over extended periods has not been demonstrated. Diluted enoxaparin prepared extemporaneously has a shelf life of only 24 hours. This has resulted in the requirement for frequent fresh preparation of diluted enoxaparin for the long-term treatment of paediatric patients. The objective of the study was to find a method to store diluted enoxaparin for 31 days without losing significant AFXa activity. Commercially-prepared undiluted enoxaparin (100 mg/ml) or enoxaparin diluted with sterile water or sterile 4% glucose (to 20 mg/ml) was aseptically transferred into plastic tuberculin syringes or glass vials. Samples were kept at 4 °C, -12 °C or -80 °C for up to 31 days. The AFXa activity of stored solutions was determined after 0, 7, 14 and 31 days. The AFXa activity of the diluted samples was compared with the AFXa activity of undiluted enoxaparin solution stored for the same time periods at 4 °C. HP-SEC was used to examine the changes to the diluted enoxaparin solutions after storage. Enoxaparin diluted with 4% glucose retained greater than 99% of its initial AFXa activity at 4 °C after 31 days. Enoxaparin diluted with water lost almost 10% of its original activity after 31 days at 4 °C and lost more than 10% of its activity after freezing at -12 °C or -80 °C. Storage in glass or plastic containers made no difference to the loss in the activity. Dilution of enoxaparin with 4% glucose and storage at 4 °C preserved more than 99% of the initial AFXa activity after 31 days, compared with a loss of 10% of the initial activity in the enoxaparin solution diluted with water and stored under the same conditions. Dilution with 4% glucose offers advantages for the preparation of stable paediatric doses of enoxaparin.

6.2 INTRODUCTION

Thrombotic incidents are not frequent in paediatric patients. However, these events can occur as secondary complications of heart diseases, prematurity, infections and cancer [160,161]. Many neonates receive prolonged intravenous infusions that require indwelling catheters. Patients with central venous infusion are at risk of catheter related thrombi [162-165]. Resolution of thrombi requires long-term LMWH

therapy [166,167]. LMWHs are the preferred anticoagulant agents, particularly in paediatric patients, over unfractionated heparin because of minimal monitoring requirements, more predictable dosage and fewer adverse effects. Among all the current existing LMWHs, enoxaparin has the majority of data supporting its paediatric use [162,168,169].

At present, the least concentrated commercial form of enoxaparin available is 100 mg/ml. Paediatric enoxaparin doses are weight-based and the commercially available preparations cannot be used to measure the small doses required by infants. Therefore, prior dilution must be made for safe and effective use. The dose of enoxaparin to achieve adult therapeutic AFXa levels in paediatric patients has been assessed [170]. The initial treatment and prophylactic doses of enoxaparin for patients aged greater than 2 months were determined to be 1.0 mg/kg and 0.5 mg/kg respectively. Infants less than 2 months of age required a greater dose per kilogram because of their larger volume of distribution or decreased plasma concentrations of AT. Treatment and prophylactic doses for these patients were determined to be 1.5 mg/kg and 0.75 mg/kg respectively. The use of diluted enoxaparin in the management and prophylaxis of thromoembolism in low-weight patients has been described [171]. Levin et al have described the advantages of using enoxaparin to support treatment at the patient's home [172].

The dilution of concentrated enoxaparin to a concentration of 20 mg/ml has been described, with an expiry time of 24 hours under refrigeration after its preparation [167]. For paediatric outpatients, a weight-based diluted dose of LMWH is required from the pharmacy or hospital every second day. Daily preparation involves high labour cost and possible drug wastage as well as necessitating frequent

visits to a pharmacy or hospital for the infant's carers. In some cases, the hospital pharmacy gives detailed instructions and teaching sessions to the parents about dilution procedures, stability and storage conditions of the drug. Hospitals provide a procedure information sheet designed as a take home reference for parents. However, dilution on a daily basis can cause considerable challenges to the pharmacy technician or patient's carers, such as accuracy in dilution, sterility, drug wastage and failure to double check [173].

A supply of commercially-prepared or repackaged sterile diluted enoxaparin solution would be convenient and potentially reduce the stress to parents or care givers of frequent visits to the pharmacy or hospital. For this approach to be practicable, preservation of the AFXa activity of the diluted preparations for the duration of outpatient use must be confirmed.

Based on the aggregation findings in the Chapter 5, it was hypothesised that the loss in activity of enoxaparin (20 mg/mL) stored at 4 °C observed by Dager et al [152] was due to aggregation and could be prevented by the addition of an agent such as DMSO. DMSO is used as a cryoprotectant [145-148] and it also acts to prevent aggregation by altering the hydrogen bonding interactions between and within molecules [151]. However, because DMSO has the potential for necrotizing and inflammatory effects upon subcutaneous injection [174], we chose safe and easily available glucose (monosaccharide) as an alternative substance to use as a modifier.

The physicochemical investigations into the stability of frozen enoxaparin led us to the study of the stability–enhancing effects of various additives. The aim of this

study was to demonstrate the enhanced stability of diluted enoxaparin over a 31 day period under different storage conditions.

6.3 EXPERIMENTAL

6.3.1 *Materials*

The sodium salt of enoxaparin (100 mg/ml) was purchased from Aventis Pharma (Sydney, NSW, Australia). Enoxaparin activity was measured using a chromogenic AFXa activity kit # 832 from American Diagnostica Inc. (Stamford, CT, USA). All spectrophotometric measurements were made with a Bio-Rad microplate reader Model-680 (Bio-Rad Laboratories, Hercules, CA, USA). All other chemicals were of reagent grade from Sigma Aldrich (Castle Hill, NSW, Australia).

6.3.2 *Preparation and AFXa activity determination of diluted enoxaparin*

Triplicate solutions of diluted enoxaparin were prepared from commercially available enoxaparin injection (100 mg/ml) and preservative-free sterile water or sterile 4% glucose solution. Concentrated enoxaparin solution (100 mg/ml) was diluted using a sterile needle and sterile plastic syringes to obtain a final concentration of 20 mg/ml. Diluted enoxaparin solutions were aseptically transferred either into 1 ml plastic tuberculin syringes or glass vials and stored at 4 °C, -12 °C or -80 °C for up to 31 days. Additionally, concentrated enoxaparin solution was drawn from its commercial syringes into 1 ml plastic syringes under sterile conditions and stored at the same temperatures and for the same durations as the diluted enoxaparin solutions.

Aliquots were removed from solutions stored in plastic containers at 0, 7, 14 and 31 days, whereas aliquots from solutions stored in glass containers were removed

after 31 days, to measure AFXa activity. The activity of enoxaparin was determined as described in section 2.3.2. A standard plot was generated on each test day by triplicate analysis of 5 different concentrations of enoxaparin (0.25, 0.125, 0.0625, 0.0312 IU/ml). Two controls with known, but different concentrations (0.25 IU/ml and 0.0312 IU/ml) of enoxaparin were tested concurrently with the test samples on each week of analysis to confirm testing accuracy. At each time point concentrated or diluted enoxaparin samples from three separate plastic syringes were analysed in duplicate.

6.3.3 *Statistical analysis*

The AFXa activity of stored enoxaparin solutions at each time point was compared with baseline AFXa activity. Mean values for the AFXa activity between initial and subsequent days were analysed by analysis of variance (factorial design by three treatments: temperature, concentration and diluent) using SAS/STAT software. Differences between baseline and subsequent time periods, with $p<0.05$, were considered statistically significant. A clinically significant loss in the activity of a pharmaceutical drug is routinely defined as a loss of greater than 10% of the initial activity.

6.3.4 *HP-SEC analysis*

HP-SEC was performed, as described in section 4.3.5, on water- and 4% glucose-diluted enoxaparin samples before and after storage at -12 °C for 31 days.

6.4 RESULTS AND DISCUSSION

The remaining AFXa activities of diluted (20 mg/ml) and undiluted (100 mg/ml) enoxaparin samples after storage for 31 days at three different temperatures are presented in Table 6.1. The recommended temperature for storage of diluted enoxaparin is 4 °C. Enoxaparin diluted with water retained more than 98% of its initial AFXa activity after 7 days at 4 °C but lost 10% of AFXa activity after 31 days. By comparison, enoxaparin diluted with 4% glucose stored at the same temperature, lost no activity after 31 days.

Storage of frozen solution at -12 °C resulted in a 15.8% loss of AFXa activity in the concentrated enoxaparin solution after 31 days. By comparison, water-diluted enoxaparin also lost 12.4 % of its initial activity. However, 4% glucose-diluted enoxaparin solution did not lose a statistically significant amount of its AFXa activity.

The freezing of concentrated or diluted (with water or 4% glucose) enoxaparin at -80 °C resulted in loss in AFXa activity after 7 days. Concentrated and water-diluted enoxaparin lost more than 18% of the initial AFXa activity after 31 days of storage. By comparison, enoxaparin diluted with 4% glucose solution preserved more than 90% of its AFXa activity.

The AFXa activity remaining after repackaging of glucose-diluted enoxaparin solution in to glass vials at three different temperatures over 31 days are also shown in Table 6.1. Storage in glass, compared with plastic, containers made no difference to the loss in AFXa activity.

Table 6.1 Comparison of % AFXa activity remaining in enoxaparin sodium solutions diluted to 20 mg/ml with 4% glucose or water, or undiluted (100 mg/ml) after storage at 4 °C, -12 °C or -80 °C for 0, 7, 14 or 31 days. Samples were prepared in triplicate and analysed in duplicate.

		% AFXa activity remaining								
		Plastic							Glass	
Diluent	Temperature (ºC)	Day 0	Day 7	% RSD	Day 14	% RSD	Day 31	% RSD	Day 31	% RSD
Glucose	4.0	100.0	99.4	1.4	98.6	1.5	99.4	1.4	99.3	2.3
Water	4.0	100.0	98.1	1.8	**93.7***	3.2	**90.0***	2.4		
Undiluted	4.0	100.0	100.0	2.0	100.0	2.5	100.0	1.1		
Glucose	-12.0	100.0	98.5	1.5	97.3	1.9	97.8	1.0	97.2	1.4
Water	-12.0	100.0	97.0	1.2	**91.2***	2.2	**87.6***	3.5		
Undiluted	-12.0	100.0	**95.1***	2.8	**88.4***	2.0	**84.2***	2.1		
Glucose	-80.0	100.0	**94.2***	1.9	**93.4***	2.3	**90.4***	2.2	**90.1***	2.9
Water	-80.0	100.0	**89.1***	3.0	**85.2***	2.6	**81.8***	2.6		
Undiluted	-80.0	100.0	**80.4***	3.7	**79.3***	3.7	**79.8***	2.7		

*$p<0.05$ compared with Day 0

The molecular distribution profiles of enoxaparin diluted with water or 4% glucose stored at -12 °C over 31 days are presented in Figure 6.1. HP-SEC resolved or partially resolved enoxaparin into six different regions. Both diluted enoxaparin samples gave an essentially identical HP-SEC profile prior to storage on Day 0. Interestingly the distribution profile of water-diluted enoxaparin differed from that for the glucose-diluted enoxaparin after storage at -12 °C for 31 days. HP-SEC of water-diluted enoxaparin showed a reduction in the peak areas of regions 3, 4, 5 and 6 and an increase in peak area 2. The distribution profile of the 4% glucose-diluted enoxaparin sample was no different from that of the samples before storage.

Dilution of enoxaparin with water resulted in a significant loss of AFXa activity after 31 days of storage at every experimental temperature. Undiluted enoxaparin lost an even greater percentage of its AFXa activity when stored as frozen solutions at -12 °C and -80 °C. It is clear from these results that the freezing of concentrated or water-diluted preparations of enoxaparin is not a suitable way to preserve AFXa activity. By comparison, enoxaparin diluted with 4% glucose maintained more than 99% of its original activity when stored for 31 days at 4 °C. When frozen at -12 °C, glucose-diluted enoxaparin lost only 2% of its AFXa activity after 31 days.

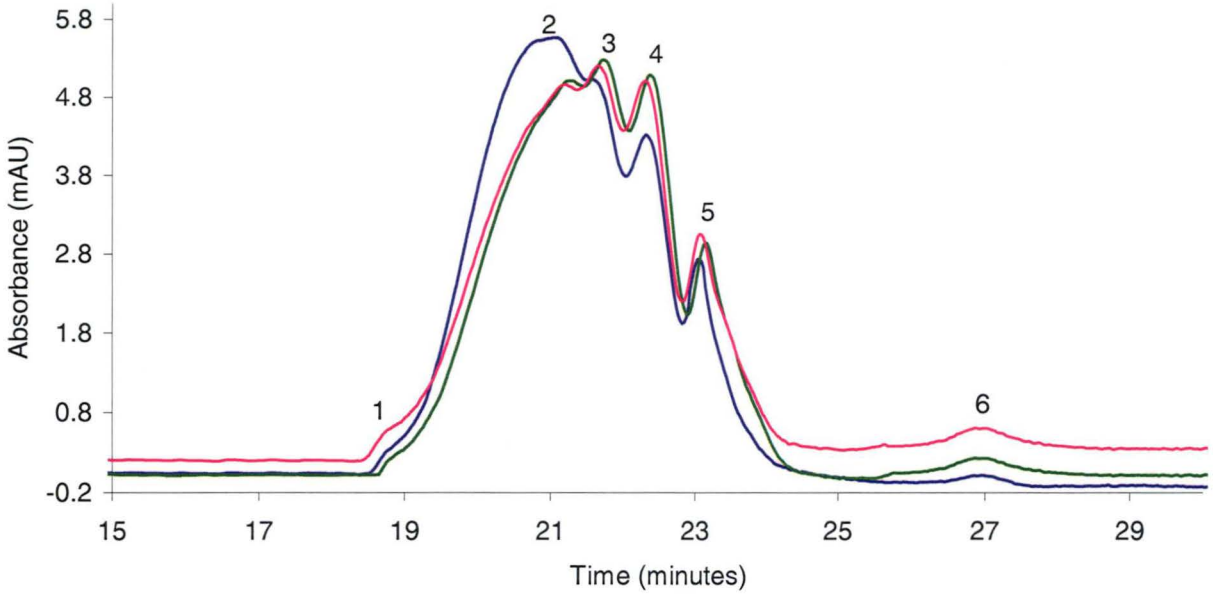

Figure 6.1 HP-SEC chromatograms of diluted enoxaparin at Day 0 (——), and 4% glucose diluted (——) and water diluted enoxaparin (——) stored at -12 °C for 31 days.

These results show that glucose acts to prevent the storage-induced loss of AFXa activity of diluted enoxaparin when stored unfrozen or frozen at moderate temperatures. At a low freezing temperature (-80 °C) glucose gave some protection but did not prevent a significant loss in AFXa activity. Diluted enoxaparin for paediatric use could be safely stored in a domestic refrigerator at 4 °C without losing AFXa activity for at least 31 days, if diluted with 4% glucose, but not if diluted with water. In this study, the measured AFXa activity loss of the water-diluted enoxaparin stored at 4 °C (10%) for 31 days was consistent with the study conducted by Dager et al [152]. They reported a 9% loss in AFXa activity of water-diluted enoxaparin after 28 days at 4 °C.

The effect of freezing on the AFXa activity of water-diluted and concentrated enoxaparin was dramatic. Evidence of aggregation can be seen in the HP-SEC chromatogram of water-diluted enoxaparin frozen at -12 °C. Aggregation resulted in

the loss of the peak areas of the lower mass fragments that eluted later from the size exclusion column. There was a corresponding increase in a higher mass fraction. The increase in this fraction and the corresponding loss of AFXa activity were shown to be reversible by sonication in Chapter 5.

Few studies of monosaccharides as cryo-protectants have been reported. [110,144,175-181]. An alternative modifier considered was DMSO, but the relative safety and ready availability of 4% glucose solution in clinical pharmacies made this modifier the first choice. Two different mechanisms by which monosaccharides act as cryo-protectants have been reported. Firstly, these cryo-protective agents prevent the rapid ice crystal growth and stabilise food products [110,144]. However, SEM and DSC results suggested formation of a glassy state rather than crystalline state in frozen enoxaparin solution. Secondly, the formation of hydrogen bonding between sugar and protein molecules minimises the aggregation and stabilises them at lower temperature [175]. Aggregation of polysaccharides at lower temperature as a result of inter- and intra-molecular hydrogen bonding has been reported. Hydrogen bonding between glucose (36 mg/ml) and oligosaccharides (20 mg/ml) in the 4% glucose diluted enoxaparin solution (20 mg/ml) may have prevented inter- or intra-molecular oligosaccharide interaction that results in aggregation. The mechanism for aggregation of oligosaccharides and larger polysaccharides has not been extensively studied. The freezing behavior and aggregation of polysaccharide mixtures are complex processes and not well understood.

A paediatric enoxaparin dosage, if diluted with 4% glucose solution, would contain 0.8 to 2.4 mg of glucose per kg body weight delivered subcutaneously. This delivery of 0.8-2.4 mg/kg of glucose compares with intravenous infusions containing

glucose for infants which deliver around 360 mg/kg/hour [182]. Typical infant blood glucose levels are around 76 mg/100 ml [182], so the subcutaneous delivery of 0.8-2.4 mg/kg of glucose would have negligible effects on blood glucose levels.

A supply of sterile 4% glucose-diluted enoxaparin for paediatric use would avoid the necessity for frequent preparation of paediatric dosages of enoxaparin resulting in numerous advantages for carers of paediatric patients. There would be savings in preparation cost, greater quality control (compared with home based dilution by non-health personnel) and less inconvenience to care givers.

Various LMWHs are different in regard to their parent unfractionated heparin source, their chemistry, production processes and their safety and efficacy [183]. The demonstrated 31 day stability of 4% glucose-diluted enoxaparin cannot be presumed for other commercially available LMWHs. Further *in vivo* study of 4% glucose-diluted enoxaparin solution delivered subcutaneously is needed in order to prove its safety in paediatric patients.

6.5. CONCLUSION

This study has shown that enoxaparin diluted with 4% glucose to a paediatric dosage concentration (20 mg/ml) can be successfully stored for a minimum of 31 days at 4 °C without significant loss of AFXa activity. Dilution with 4% glucose offers a potential method for the preparation of stable paediatric doses of enoxaparin. Further studies to determine the safety of glucose-diluted enoxaparin and its stability over a longer period of time are warranted.

CHAPTER 7

Investigation of the Effect of Heating on the Chemistry and Biological Activity of Enoxaparin

7.1 SUMMARY

The objective of this study was to investigate the effects of heating on the chemical and physical properties of enoxaparin and subsequent changes to its AFXa activity. Enoxaparin samples were heated at 70 °C for periods of up to 576 hours (24 days). Enoxaparin lost more than 25% of its initial AFXa activity after 8 hours and then regained more than 75% of the lost activity over the next 4 hours. Activity then decreased slowly, with a further 14% loss of the initial activity after 576 hours. The initial loss in activity correlated with desulfation, particularly N-desulfation, which was demonstrated by analysis of free sulfate and free amino groups. Analysis of reducing capacity demonstrated that fragmentation of oligosaccharides occurred at elevated temperature. CE analysis supported these findings. Enoxaparin was fractionated by HP-SEC and individual fractions were analysed. Early eluting fractions, containing aggregated oligosaccharides, increased in concentration following heating. There was a loss of up to 65% of sulfate from some fractions containing primarily hexa- and octa-saccharides after 8 hours corresponding to decreased AFXa activity. Fractions corresponding to low mass oligosaccharides increased in concentration and had increased AFXa activity between 8 and 12 hours. Ion-interaction RP-HPLC analysis supported these findings. Deca-, dodeca- and tetradeca-saccharides were resistant to chemical degradation when heated. Three processes, desulfation, aggregation and fragmentation occur during the heating of enoxaparin that result in the initial rapid loss, recovery and subsequent gradual loss of AFXa activity. These processes do not affect all fractions of enoxaparin to the same extent.

7.2 INTRODUCTION

Biological activity and chemical studies of pharmaceutical formulations exposed to elevated temperatures are commonly carried out to determine the stability of a drug and possible mechanisms by which the drug undergoes degradation and loss its activity. An accelerated stability study of heparin under acidic and basic conditions showed that the decomposition of heparin involved the endolytic hydrolysis of

glycosidic linkages and desulfation [97]. Stability studies of enoxaparin (10 mg/ml) have shown that more than 15% of its initial AFXa activity was lost after 10 days at 22 °C and 10% of the initial AFXa activity was lost from diluted enoxaparin (20 mg/ml) at 4 °C after 30 days [173,184]. However, these studies did not expose enoxaparin to high thermal stress and did not investigate the mechanism(s) responsible for the loss in AFXa activity. Despite its routine and extensive use, detailed stability studies of enoxaparin have not been reported and the mechanism(s) by which it loses its AFXa activity are not elucidated. A preliminary study of enoxaparin stability at elevated temperatures showed unusual patterns of change in the AFXa activity, with an initial rapid decrease in activity followed by an increase in activity, then a gradual decrease [185]. By developing an understanding of the chemical changes that occurred during heat-induced degradation of enoxaparin solution, in combination with measurement of AFXa activity, it was postulated that it may be possible to increase the understanding of the chemistry, activity and stability of various constituents of enoxaparin.

The purpose of the study was to investigate the effects of heating on the AFXa activity of enoxaparin solution, to examine heating induced chemical and physical changes to enoxaparin, and to determine the mechanism(s) by which enoxaparin solution loses its AFXa activity at elevated temperature.

7.3 EXPERIMENTAL

7.3.1 *Materials*

The sodium salt of enoxaparin (100 mg/ml) was purchased from Aventis Pharma (Sydney, NSW, Australia) and used as supplied at pH 7.0. The EP-LMWHS

was from Pharmacopée Européenne (Strasbourg, France). The AFXa activity kit # 832 was from American Diagnostica (Stamford, CT, USA). The bicinchoninic acid (BCA) assay kit to determine reducing capacity and fluoraldehyde assay reagent to measure free amino groups were from Pierce (Rockford, IL, USA). N-acetyl-D-glucosamine, ammonium acetate, sodium sulfate, sodium hydroxide, hydrochloric acid, acetic acid, orthophosphoric acid, nitric acid, hydrogen peroxide, glycine, azure A, acridine orange, TBAH and acetonitrile were of analytical grade and all other chemicals were of reagent grade from Sigma Aldrich (Castle Hill, NSW, Australia). Microtitre plates were flat-bottomed polystyrene plates from Corning (Corning, NY, USA). Ultrafiltration membranes with 1 000 Da cutoff were from Millipore (North Ryde, NSW, Australia). Electrophoresis was performed on a Criterion® precast gel (10% Tris-HCl) using a Bio-Rad power/Pac 200® power supply and Criterion® electrophoresis cell from Bio-Rad laboratories with silver staining performed using the Silver Stains Plus Kit®, all from Bio-Rad laboratories (Hercules, CA, USA).

7.3.2 *Analysis of AFXa Activity*

The activity of enoxaparin solution was determined using the ACTICHROME® AFXa chromogenic assay kit as described in section 2.3.2. The AFXa activity of each enoxaparin sample was determined in triplicate.

7.3.3 *Sample preparation for sulfate determination*

Total sulfate content of untreated enoxaparin solution (3 mg in 30 µl) and enoxaparin fractions (100 µl of each HP-SEC fraction) was determined after acid hydrolysis with a mixture of nitric acid and hydrogen peroxide as described previously [186]. Enoxaparin samples were mixed with nitric acid (1 ml or 200 µl) in a capped glass test tube or a capped 1.4 ml polyethylene centrifuge tube respectively.

Samples were heated at 80 °C overnight in a temperature controlled heating block. Hydrogen peroxide (0.2 ml or 50 µl respectively) was then added and the temperature was increased to 110 °C for 6 hours. Solutions were then neutralised using an experimentally determined volume of 1 M sodium hydroxide. The digested untreated enoxaparin was diluted with Milli-Q water to 5.0 ml in a volumetric flask and then 250 µl of this solution was further diluted to 5.0 ml. The HP-SEC fraction samples were freeze dried and dissolved in Milli-Q water (500 µl). Samples were injected into an IC system for sulfate analysis. Total sulfate determination of the digested untreated enoxaparin samples was also carried out using gravimetric and spectroscopic analyses. Determination of free sulfate in undigested enoxaparin was determined after ultrafiltration of diluted enoxaparin using a 1 000 Da cutoff filter at 15 000 rpm for 10 minutes.

7.3.4 *Gravimetric sulfate analysis*

Total sulfate content in the enoxaparin sample was determined by precipitation of sulphate ions in the acid digested solution as barium sulphate [186]. Enoxaparin solution was acid digested as described above and 10% w/v barium chloride was added. The suspension was allowed to settle for 2 hours and complete precipitation was tested by addition of barium chloride to the supernatant. The mixture was then filtered through ash-free filter paper and filtrate was placed in a previously weighed porcelain dish. The filtrate was then kept in a muffle furnace at 800 °C for an hour.

7.3.5 *Spectrophotometric sulfate analysis*

Spectrophotometric analysis for total sulfate determination was carried out using conditioning and precipitating reagents. The conditioning reagent was prepared as follows: hydrochloric acid (0.3 ml), 95% isopropyl alcohol (0.7 ml), sodium

chloride (0.5 grams) and glycerol (0.4 ml) were dissolved in deionised water (2.5 ml) and mixed well. The precipitating reagent was prepared by dissolving barium chloride crystals (225 mg) in deionised water (1 ml). The acid digested enoxaparin sample (170 µl) was transferred to a low volume microtitre plate. Conditioning reagent (20 µl) and then barium chloride solution (10 µl) were added and the plate was kept aside for 10 minutes. After 10 minutes the plate was shaken for 3 minutes and spectroscopic readings were taken at 420 nm. Sulfate stock solution was prepared by dissolving anhydrous sodium sulfate (40 mg) in distilled water (10 ml) and serial dilution was performed to achieve standard sulfate concentrations.

7.3.6 *IC Analysis of sulfate*

Determination of sulfate content was carried out by IC analysis as described in section 5.3.7.

7.3.7 *Analysis of free amino groups*

The determination of free amino groups in enoxaparin samples was determined by a fluoraldehyde-based assay as described in section 5.3.8.

7.3.8 *Analysis of reducing capacity*

The reducing capacity of enoxaparin samples was determined by a reducing capacity assay as described in 5.3.9.

7.3.9 *CE analysis*

CE analysis of enoxaparin samples was performed as described in section 3.3.2. with the use of a 70 cm fused silica capillary (50 µm i.d.) having a detection window 8.5 cm from the distal end, phosphate electrolyte (pH 3.5; 50 mM), an

applied voltage of -30 kV, UV detection at 230 nm and sample injection at 20 mbar for 5 seconds. Adjustment of pH of enoxaprin solutions was carried out as described in section 3.3.2.

7.3.10 *PAGE analysis*

Unstressed and heat stressed enoxaparin samples were subjected to PAGE. The tank buffer solution was prepared by mixing glycine (2.2% w/v) with Tris base (4.5 grams) and pH was adjusted to 8.3 with 5 N hydrochloric acid. Sample buffer solution was prepared by mixing Tris base (1.5% w/v) with 5 ml of glycerol. Bromophenol blue was then added as an indicator to the above solution and the pH was adjusted to 6.8 with 5 N hydrochloric acid. Samples were diluted with deionised water to achieve concentrations of 10 ng/ml to 100 µg/ml. These samples were again diluted with an equal volume of sample buffer solution. Unstressed (5 ng/ml to 50 µg/ml) and stressed (30 and 50 µg/ml) enoxaparin samples (10 µl) were identically loaded on to each lane of four different 12 lane gels. Electrophoresis was performed at a constant current of 40 mA.

Electrophoresis of the first gel was performed by running it for 4 hours. Subsequent staining was performed with a solution containing acridine orange (10 mg), methanol (5 ml) and deionised water (95 ml) for 1 hour. This gel was fixed for 1 hour with an aqueous solution of methanol (40% v/v) and acetic acid (10% v/v). The stained and fixed gel was then destained by washing several times with deionised water.

Electrophoresis of the second gel was performed by running the gel for 2 hours. Subsequent staining was performed with a solution containing sodium

formate (0.34% w/v) and azure A (0.1% w/v) at pH 3.5 for 1 hour. Following staining of the gel, destaining was performed with the use of a solution containing sodium acetate (0.08% w/v) at pH 5.0.

Electrophoresis of the third gel was performed by running it for 2 hours and subsequent silver staining was performed using the Bio-Rad Silver Staining Plus Kit®. Silver staining was fixed with acetic acid solution (5% v/v) for 15 minutes with subsequent washing using deionised water for 30 minutes.

Electrophoresis of the fourth gel was performed by running the gel for 30 minutes. This gel was stained with azure A, destained, then silver stained as described above.

7.3.11 *HPLC*

HP-SEC was performed using a Shodex OHpak SB-802.5 HQ 8mm × 300 mm size exclusion column and Security Guard equipped with a GFC 2000 cartridge (Phenomenex, Sydney, NSW, Australia). The mobile phase was 0.3 M sodium or 0.3 M ammonium acetate (pH adjusted to 5.0 with 0.1 M HCl). The analyses were performed with a 30 µl sample injection at a flow rate of 0.3 ml/minute.

Ion-interaction RP-HPLC of enoxaparin sodium was performed on a 5 µm Varian Microsorb C_{18} column (150 × 4.6 mm) (Varian, Melbourne, VIC, Australia). The mobile phase consisted of acetonitrile (32%), water (68%), TBAH (15 mM) and ammonium acetate (50 mM) at pH 7.0. Diode array detection was carried out at 230 nm. The analyses were performed with a 30 µl sample injection at a flow rate of 1.0 ml/minute.

7.3.12 *The effect of heat stress on AFXa activity*

Glass ampoules (1ml) were flushed with nitrogen gas for 5 minutes after addition of 50 µl of enoxaparin solution (100 mg/ml) and were rapidly flame sealed. Sealed glass ampoules with corresponding controls were kept at 70 °C in an oven or at 4 °C in a standard laboratory refrigerator [97]. Duplicate samples were removed from the oven at 0, 2, 4, 8, 12, 24, 48, 96, 182, 288 and 576 hours and were kept at 4 °C until analysis. All samples, with corresponding controls, were analysed for AFXa activity on the same day. Duplicate samples were analysed in triplicate.

7.3.13 *The effect of oxygen on the loss of AFXa activity following heat stress*

Glass vials (2 ml) containing 50 µl of enoxaparin solution (100 mg/ml) were sealed with a crimped aluminium cap containing a teflon lined septum. Enoxaparin solution was then bubbled with oxygen or high purity nitrogen for 15 minutes *via* an 18 G × 1 ½" (1.25 × 38 mm) needle inserted through the septum, with a second needle acting as an exhaust port. After removal of the needles, the septum was sealed with neutral cure silicone sealant. Further glass vials containing enoxaparin were allowed to stand uncapped on the bench for 30 minutes in order to equilibrate with room air. Glass vials, in triplicate, containing different concentrations of oxygen were kept in an oven at 70 °C for up to 24 hours and corresponding controls were kept at 4 °C. Samples were removed from the oven at 0, 2, 4, 8 and 12 hours and AFXa activity, with corresponding controls, was determined in triplicate. A paired t-test was performed to investigate any significant differences between AFXa activity of controls and samples containing different concentrations of oxygen, with $p<0.05$ considered statistically significant.

7.3.14 *Investigation of heat-induced chemical changes on enoxaparin*

Enoxaparin solution (50 µl, 100 mg/ml) in glass ampoules was heat stressed at 70 °C for up to 576 hours as described above. Each sample was analysed for free sulfate concentration, free amino groups and reducing capacity. Duplicate samples were analysed in triplicate. Selected samples were investigated chromatographically by HP-SEC and ion-interaction RP-HPLC and electrophoretically by CE and PAGE.

7.3.15 *Fractionation of enoxaparin samples and investigation of chemical and chromatographic properties of the fractions*

Enoxaparin samples stressed at 70 °C for 0, 8 or 12 hours were fractionated by HP-SEC. For each sample 20 fractions were collected over the same elution period between 18 and 28 minutes. Fractions were reanalysed for the peak distribution confirmation by HP-SEC. All the fractions were analysed for AFXa activity and sulfate content. Fractions containing higher mass components, previously shown to contain aggregated polysaccharides, were subjected to sonication for 0, 5, 20, 60 or 300 seconds, in the presence of 1% DMSO, a demonstrated disaggregation treatment [123], as described in section 5.3.13. AFXa activity of these fractions was determined before and after the disaggregation treatment. A paired t-test was used to determine any significant differences between AFXa activity of controls and disaggregated samples, with $p<0.05$ considered statistically significant.

7.4 RESULTS AND DISCUSSION

7.4.1 *AFXa activity of enoxaparin after heating*

A plot of percentage of AFXa activity remaining as a function of time at 70 °C for up to 576 hours is shown in Figure 7.1. There was a rapid decline in the AFXa activity between 0 and 8 hours, with a maximum loss of 27% of the initial AFXa activity. AFXa activity then increased after 8 hours, with AFXa activity returning to a maximum of 94% of the initial activity after 12 hours. AFXa activity decreased after 12 hours, with activity falling to 84% after 48 hours, but then decreasing only to 80% of control over the next 22 days. The oxidative instability of heparin has been reported and anti-oxidants have been added during various manufacturing stages of heparin to enhance stability [41]. It was hypothesised that the initial rapid decrease in AFXa activity may be due to oxygen dependent degradation, due to the presence of some oxygen in the ampoules containing enoxaparin. Oxygen may have been dissolved in the enoxaparin solution or may have entered the ampoule before it was sealed. When enoxaparin was heated in the presence or absence of different concentrations of oxygen there was no difference in the loss of AFXa activity as shown in Table 7.1. These results demonstrated that the initial rapid decrease in the AFXa activity was not oxygen dependant.

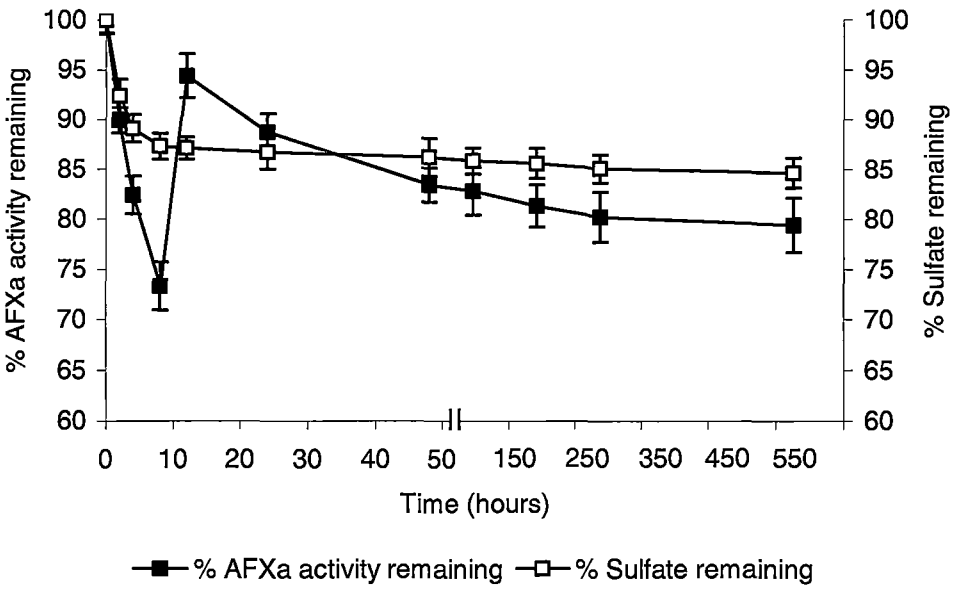

Figure 7.1 Percentage of initial AFXa activity and sulfate content remaining in enoxaparin solution kept at 70 °C for up to 576 hours.

Table 7.1 Determination of AFXa activity of enoxaparin solution stored at 70 °C in the presence or absence of different concentrations of oxygen for up to 12 hours.

Oxygen concentration (%)	Time (hours)	AFXa activity remaining (%)
0.0	0.0	100.0
	4.0	82.4
	8.0	73.3
	12.0	94.4
20.0	0.0	100.0
	4.0	81.9
	8.0	73.7
	12.0	94.9
100.0	0.0	100.0
	4.0	82.4
	8.0	72.1
	12.0	93.8

7.4.2 *Desulfation of enoxaparin*

Chromatograms of different concentrations of sulfate ions in standard sulfate solution determined by IC are presented in Figure 7.2. The calibration plot of sulfate concentration vs. peak area showed a linear range (r^2=0.9987, estimated using correlation coefficient) between 0.5 and 20 µg/ml concentarion.

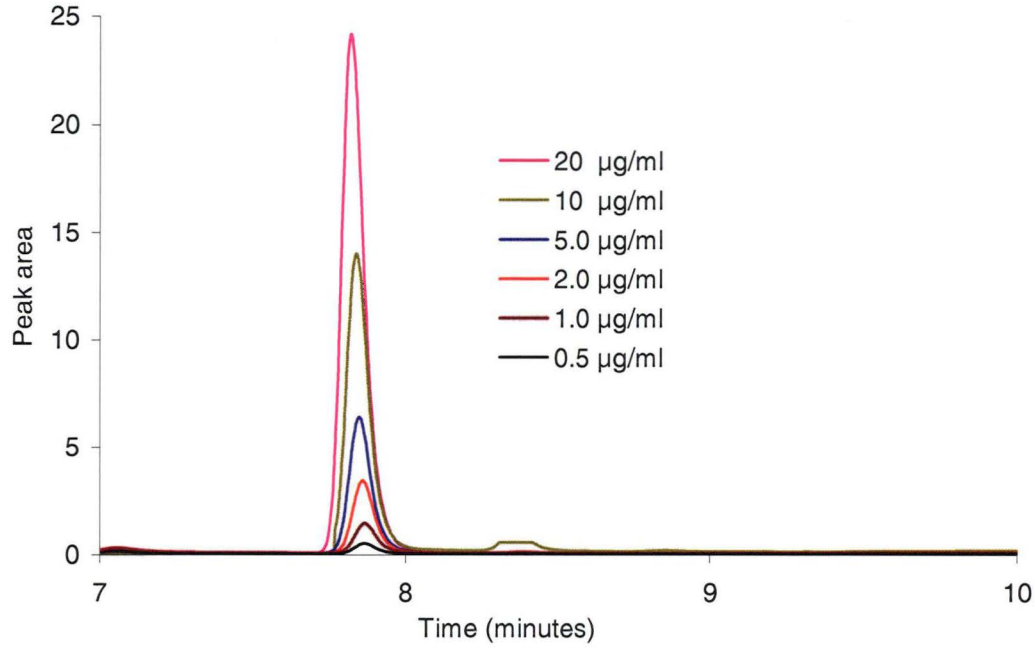

Figure 7.2 Calibration IC chromatograms of sulfate standards. IC separation was carried out by hydroxide eluent generation using Dionex EluGen II KOH cartridge from 1 mM to 15 mM over 15 minutes. A Dionex IonPac Column (AS11) was used with a mobile phase comprising water and KOH as a gradient. Detection was carried out under suppression mode with flow rate of 1ml/minute and injection volume of 25 µl.

Free sulfate in enoxaparin solution determined by IC after ultrafiltration was found to be equivalent to 0.46% w/w based on an enoxaparin concentration of 100 mg/ml. Total bonded sulfate in enoxaparin solution after acid hydrolysis, and allowing for the free sulfate, determined by IC, gravimetric and spectroscopic

analyses was 38.8% (0.59 %RSD), 37.4% (4.3 %RSD) and 36.4% w/w (5.9% RSD) respectively. Results for total bonded sulfate of enoxaparin obtained by three different methods were similar and the lowest %RSD was obtained with IC method. These values are similar to the theoretical estimation of sulfate content of enoxaparin (41.0% w/w) based on 75% trisulfation of the heparin disaccharide repeating unit from porcine mucosa, an average of 1 N-acetyl group per parent heparin molecule and the presence of 1,6-anhydro groups on the reducing end of 20% of the enoxaparin molecules. An experimental value of 34.7% w/w was determined previously for a non-commercial LMWH sample [186].

The loss of sulfate from heated enoxaparin is presented in Figure 7.1. Enoxaparin lost sulfate rapidly, with more than 12% of the bonded sulfate content lost after 8 hours at 70 °C. The rapid loss of sulfate was paralleled by a similarly rapid loss of AFXa activity. Between 8 and 576 hours of treatment enoxaparin lost only a further 2.6% of the initial bonded sulfate. Therefore, the increase in the AFXa activity of enoxaparin after 8 hours is not related to any change in sulfate levels.

The key structural unit of heparin or LMWH that confers AFXa activity is a pentasaccharide sequence consisting of three D-glucosamine and two uronic acid residues. The AFXa activity has been shown to be dependant on N- and O-sulfate groups of this pentasaccharide sequence [6,15,16]. Elimination of the sulfate groups results in a decrease in AFXa activity, but the elimination of the 3-O-sulfate group of the central D-glucosamine results in complete loss of AFXa activity.

In naturally occurring mammalian polysaccharides, the amino groups are usually N-acetylated but heparin contains a majority of N-sufated groups [97].

N-sulfate groups are more heat labile than N-acetyl groups [187] and the loss of N-sulfate groups gives rise to the formation of free amino groups. The concentration of free amino groups following the heating of enoxaparin was determined using a sensitive fluoraldehyde assay and the results are presented in Figure 7.3.

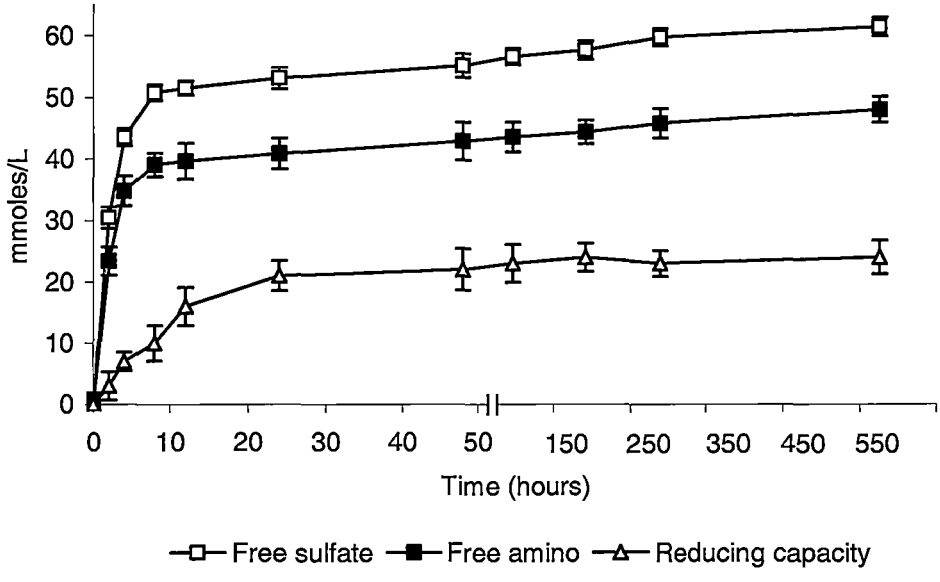

Figure 7.3 Increases in the concentrations of free sulfate, free amino groups and in the reducing capacity of enoxaparin solution kept at 70 °C for up to 576 hours.

A rapid increase in free amino concentration was observed in enoxaparin samples heated for up to 8 hours at 70 °C followed by a small increase after this time. The increase in the free amino concentration concurrently with increased free sulfate concentration indicated that there was a rapid loss of N-sulfate groups from enoxaparin. The concentration of sulfate exceeded that of free amino groups, indicating that some loss of O-sulfate also occurred. These findings suggest that the initial rapid loss of AFXa activity was due to desulfation of thermally labile constituents of enoxaparin. It is also evident that some sulfate sites or sulfates on

some enoxaparin constituents are thermally resistant to desulfation, since little desulfation occurred after 48 hours, and desulfation did not exceed 16% of the initial total bonded sulfate after 576 hours.

7.4.3 *Determination of reducing capacity*

The reducing capacity of heat stressed samples was measured after different periods of heating. The control reducing capacity was 0.019 mmoles/l and the increases in reducing capacity brought about by heating are presented in Figure 7.3. A rapid increase in the reducing capacity of enoxaparin was observed throughout the first 24 hours of heating. No substantial further increase was observed in the time period from 24 hours to 576 hours. Polysaccharides, in general, have one reducing and one non-reducing end. The observed increase in reducing capacity of the heat treated samples could represent the formation of new reducing ends through the breakdown of glycosidic linkages, the formation of more reactive reducing ends through chemical modification of the reducing sugar residues or a combination of both processes. Enoxaparin, as a consequence of β-eliminative cleavage has 1,6-anhydro groups on approximately 20% of its reducing ends. Hydrolysis of these groups could potentially result in increased reducing capacity. However, the magnitude of the increased reducing capacity was too great for it to be accounted for by chemical modification of existing reducing ends. These results indicated that fragmentation of oligosaccharides must have occurred. The cleavage of a long chain polysaccharide into two smaller chains can result in increased AFXa activity if these chains are sufficiently long to possess the pentasaccharide sequence that binds AT and is responsible for AFXa activity [188].

7.4.4 *CE analysis*

The changes in enoxaparin following exposure to elevated temperature were studied by CE analysis. Since most chemical changes were evident within 48 hours, CE analysis was performed using samples kept at 70 °C for 0, 8, 12, 24 and 48 hours. Different electropherograms were observed for each sample and are presented in Figure 7.4. A new peak with a migration time of 5.9 minutes was observed after 8 hours. This was consistent with a desulfation product that would be expected to have an increased migration time due to it carrying less charge. CE analysis of polysaccharides in reverse polarity mode has shown that lower mass constituents migrate earlier than higher mass constituents [74]. Electropherograms of enoxaparin heated for 12, 24 and 48 hours showed a shift towards a greater proportion of more mobile constituents, with shorter migration times, with an increased number of peaks detected, consistent with more low mass constituents. This observation suggested the fragmentation of larger to smaller oligosaccharides following heating. These results are consistent with the reducing capacity results and demonstrated chemical changes and fragmentation in the samples treated at elevated temperature.

7.4.5 *PAGE analysis*

PAGE was used in attempt to determine the chemical changes in enoxaparin following heating at 70 °C for 0, 8 and 12 hours. PAGE has been successfully employed for the analysis of various oligosaccharides with the use of different cationic dyes for their visualization. In this study, staining of the gel was performed with acridine orange or azure A or by silver staining. No stained bands were visible when electrophoresis was performed for 4 or 2 hours following staining with acridine orange or azure A. Initially it was thought that these cationic dyes were not sensitive

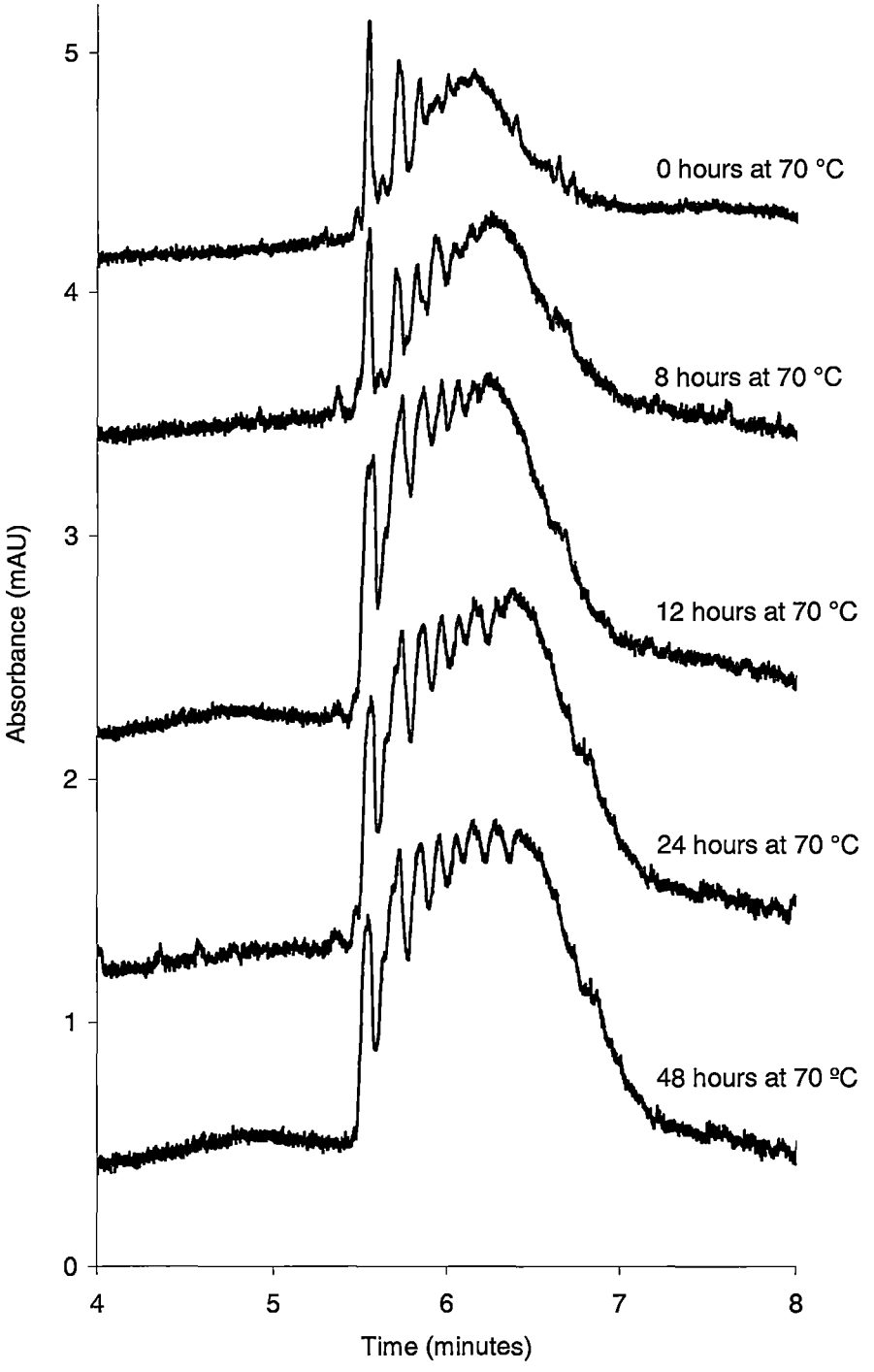

Figure 7.4 Electropherograms of enoxaprin before and after stressing at 70 °C for up to 48 hours. CE conditions are same as those described for Figure 3.2.

enough to visualise the separated bands. Therefore staining was performed by the more sensitive silver staining method. However, no band was visualised with silver staining, possibly because of the high mobility of the highly negatively charged constituents of enoxaparin. Subsequently a gel was run for 30 minutes in order to ensure retention of samples on the gel. This gel was stained with azure A followed by silver staining. A single band of enoxaparin (50 µg/ml) was clearly visible which moved at the same rate as the bromophenol blue indicator. This result suggested that staining method was successful but commercially available 10% Tris-HCl was not able to retain highly electrophoretic negatively charged constituents of enoxaparin. In a study carried out by Jandik and co-workers, PAGE was able to separate various degradation products of unfractionated heparin only after heating heparin at 100 °C for 1 000 hours [97]. The conclusion from our study was that PAGE using commercial gels was unsuitable for the analysis of enoxaparin and its degradation products.

7.4.6 *HP-SEC analysis and fractionation of heat stressed enoxaparin*

Most chemical and AFXa activity changes were evident in the samples treated for up to 12 hours (Figure 7.1 and Figure 7.2). There fore HP-SEC was performed using samples kept at 70 °C for 0, 8 and 12 hours. The HP-SEC chromatograms of EP-LMWHS and enoxaparin treated for 0, 8 and 12 hours are presented in Figure 7.5. EP-LMWHS was resolved or partially resolved into 8 different peaks (labelled as *a-h*) by HP-SEC. The approximate molecular masses of oligosaccharides in the EP-LMWHS HP-SEC peaks have previously been described [106]. These data were used to construct a partial standard plot covering the lower end of molecular mass (Figure 7.6) as described previously [189].

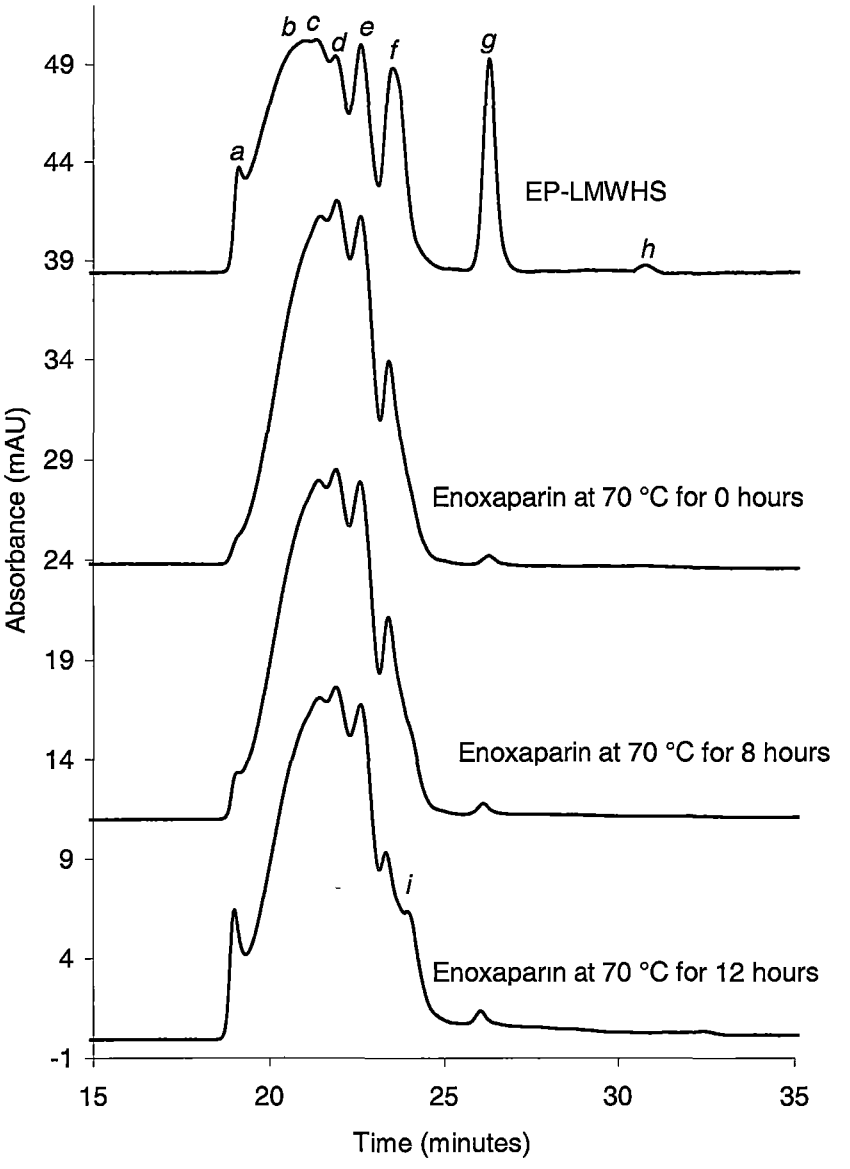

Figure 7.5 HP-SEC analysis of EP-LMWHS and enoxaparin. Enoxaparin solution was stored at 70 °C for up to 12 hours. Peaks *b, c, d, e, f, g* and *h* correspond to the tetradeca-, dodeca-, deca-, octa-, hexa-, tetra- and di-saccharides respectively.

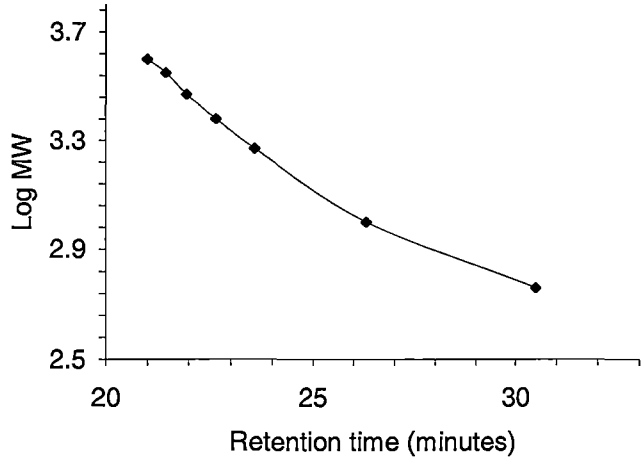

Figure 7.6 The HP-SEC calibration plot generated using a value of 600 Da per disaccharide.

Different HP-SEC chromatographic profiles were obtained for enoxaparin samples heated for 0, 8 and 12 hours, as shown in Figure 7.5. Unstressed enoxaparin was resolved or partially resolved into 7 different peaks (labelled as *a-g*) having the same retention times as the EP-LMWHS peaks. With the unstressed enoxaparin, peak *a* eluted at 19.3 minutes. The same peak was increased in area and height after 8 hours and became even higher following 12 hours of heating. Generation of an extra peak, *i*, was observed in the 12 hours treated sample at 24.0 minutes with a concomitant decrease in the area of peak *f*. The approximate molecular masses corresponding to peaks *c, d, e, f, i* and *g* of enoxaparin were estimated as 3 670, 3 070, 2 490, 1 960, 1 830 and 1 200 Da respectively. The molecular mass of peak *i* was determined from the standard plot. The other peaks were estimated by direct comparison with the identified peaks of EP-LMWHS which is also prepared by β-eliminative cleavage of parent heparin, resulting in oligosaccharides with masses similar, if not identical, to the oligosaccharides in enoxaparin.

Unstressed and stressed samples were fractionated by HP-SEC and 20 different fractions of each sample, eluted in the span of 18 minutes to 28 minutes, were collected and reanalysed by HP-SEC for the peak distribution confirmation as described in section 4.4.5 and results are as shown in Figure 7.7. The AFXa activity of HP-SEC collected fractions is shown in Figure 7.8. Fractions 1 to 4 and 16 to 20 of unstressed enoxaparin were found to be less active than the other fractions. Fractions 11 to 13 showed the highest AFXa activity. After 8 or 12 hours of heating the AFXa activity of fraction 2 was higher compared to fraction 2 from the control sample. Higher AFXa activity of fraction 2 was consistent with increased peak area of peak *a*. Heating made no difference to the AFXa activity of fractions 4 to 10 compared with control. After heating for 8 hours there was a loss in the AFXa activity of fractions 11 to 13 compared with control. Fraction 13 decreased in AFXa activity by 65%. Heating for 12 hours had the same effect on fractions 11 to 13 as heating for 8 hours.

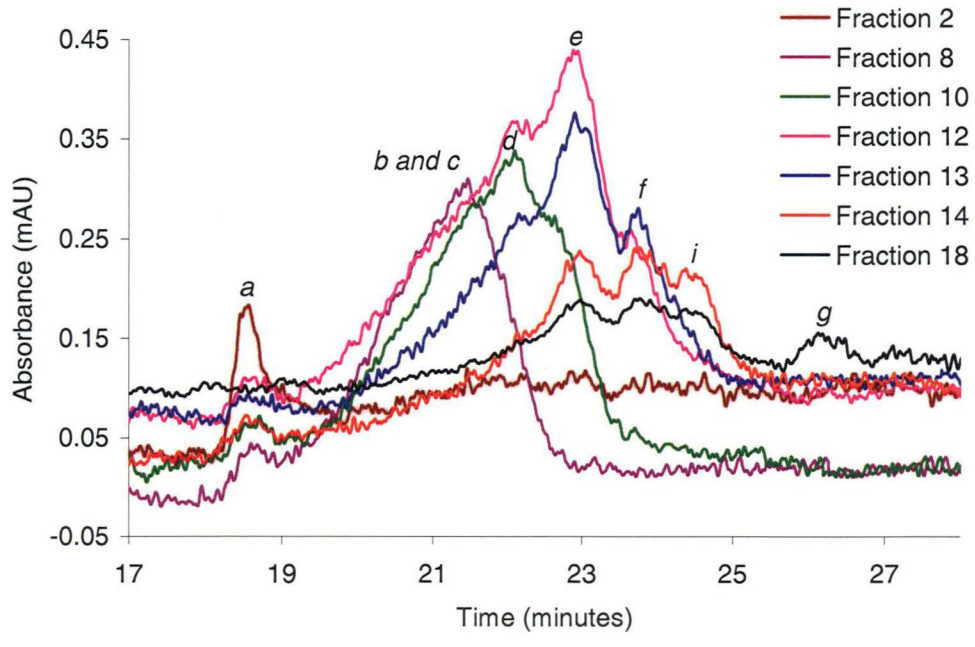

Figure 7.7 HP-SEC analysis of selected HP-SEC collected fractions of enoxaparin sample kept at 70 °C for 12 hours.

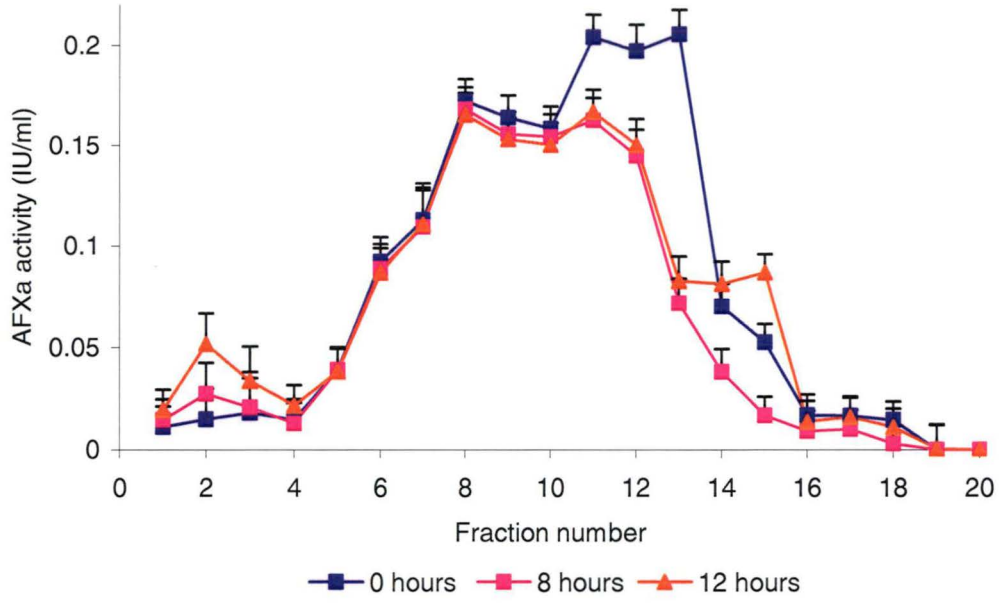

Figure 7.8 AFXa activity of HP-SEC collected fractions of enoxaparin samples kept at 70 °C for 0 or 8 or 12 hours.

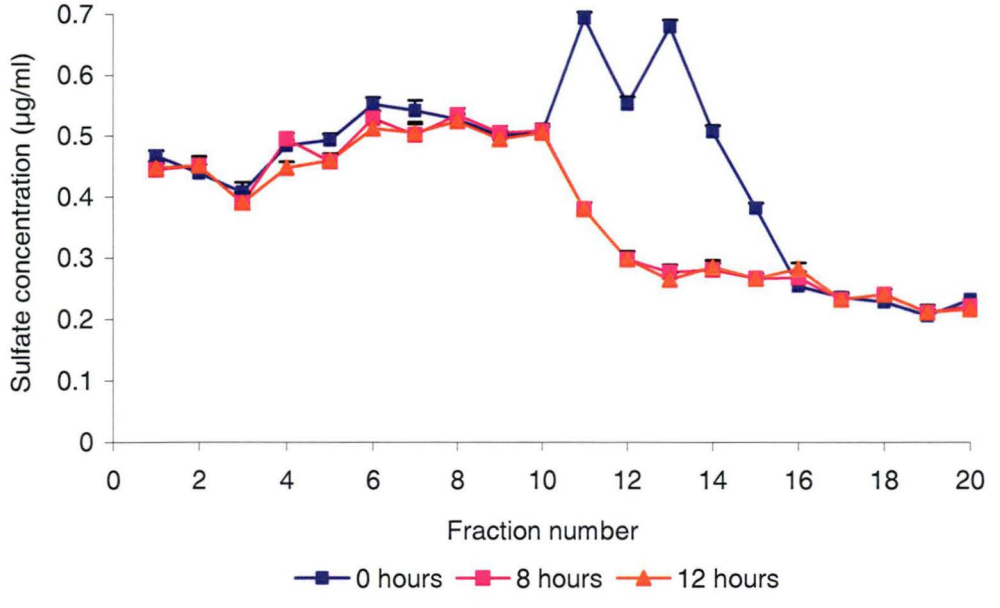

Figure 7.9 Sulfate contents of HP-SEC collected fractions of enoxaparin samples kept at 70 °C for 0 or 8 or 12 hours.

After 12 hours of heating, fractions 14 and 15 showed 114% and 418% increases in activity, respectively, compared with activity after 8 hours, corresponding to respective increases of 15% and 65% over control values. Increased AFXa activity of these fractions after 12 hours of heating was accompanied by the appearance of peak *i* in the HP-SEC chromatogram.

The results of sulfate determination of HP-SEC fractions are presented in Figure 7.9. Heating resulted in the loss of sulfate only from fractions 11 to 15 compared with control. These fractions also showed a parallel loss of AFXa activity after 8 hours of heating, but after 12 hours of heating fractions 14 and 15 (corresponding to peak *i*) recovered some AFXa activity, compared with 8 hours. These results have confirmed that there are thermally labile enoxaparin constituents that are rapidly desulfated and are responsible for the early rapid loss in the AFXa activity. Other constituents (fractions 4 to 10, corresponding to tetradeca-, dodeca- and deca-saccharides) are resistant to thermal desulfation and retained their AFXa activity. Fraction 13, corresponding to hexasaccharides, was the most thermally vulnerable fraction. It lost 65% of its initial AFXa activity and 60% sulfate loss was observed after 8 hours of heating at 70 °C.

Samples showed the most obvious chemical and biological changes following heating were selected for the ion-interaction RP-HPLC analysis. The ion-interaction RP-HPLC chromatograms of thermally stressed and unstressed enoxaparin samples are presented in Figure 7.10. Ion-interaction RP-HPLC analysis of the enoxaparin HP-SEC fractions allowed the assignment of ion-interaction RP-HPLC peaks to the equivalent HP-SEC peaks (*c-g* and *i*) as shown in Figure 7.5. No major chromatographic differences in the later eluted peaks corresponding to the HP-SEC

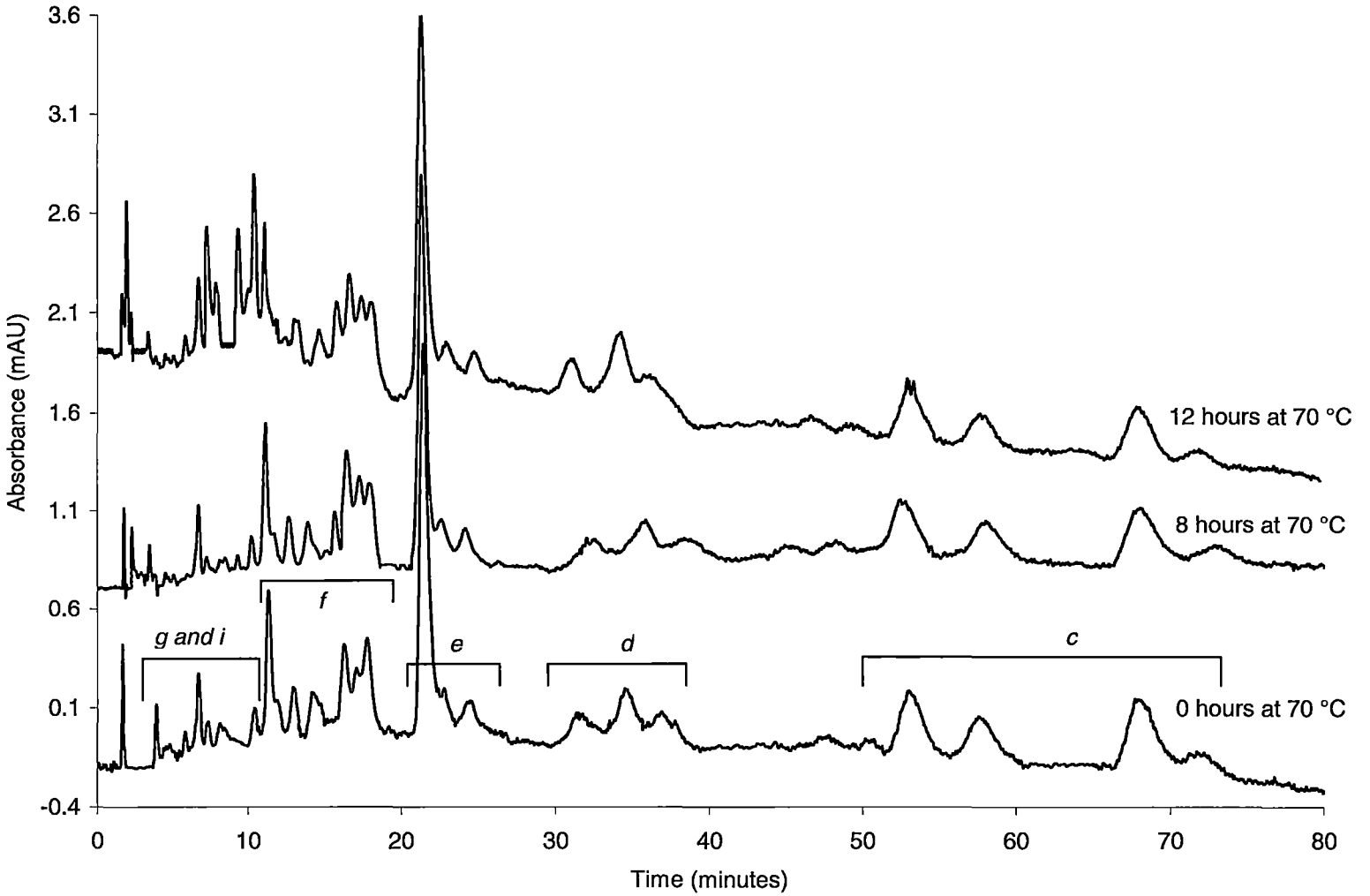

Figure 7.10 Ion-interaction RP-HPLC analysis of enoxaparin solutions kept at 70 °C for 0, 8 or 12 hours. The Ion-interaction RP-HPLC methodology is described in experimental section. Peaks *c*, *d*, *e*, *f*, *g* correspond to tetradeca- (3 670 Da), dodeca- (3 070 Da), deca- (2 490 Da), octa- (1 960 Da), hexa-saccharides (1 830 Da) respectively.

peaks *c*, *d* and *e* following thermal stress at 70 °C for 8 or 12 hours compared with control sample were observed. This observation is consistent with the HP-SEC results.

Different chromatographic profiles of thermally stressed and unstressed samples corresponding to HP-SEC peaks *f*, *g* and *i* were obtained as shown in Figure 7.11. The total peak area of the peaks corresponding to HP-SEC peak *f* in the chromatogram (eluted between 11 and 19 minutes) decreased by 30% after heating for 12 hours. The peaks corresponding to HP-SEC peaks *g* and *i* (eluted between 3.5 and 11 minutes) increased in total area by 340% after heating for 12 hours, compared with control. Some peaks were unchanged including those that eluted at 5.8 and 6.7 minutes. The peak that eluted at 3.9 minutes decreased in area by 94% and peaks that eluted at 7.4, 7.9, 9.3, 9.9 and 10.1 minutes increased in area. Peaks in the sample heated for 12 hours that eluted at 7.4, 9.3 and 9.9 minutes were not detected in the control sample. These peaks represent new compounds resulting from chemical changes brought about by heating. The increase in the area of these peaks is comparable to the increased area and AFXa activity of fractions 14 and 15, corresponding to peak *i*, following 12 hours of heating.

The AFXa activity of HP-SEC fraction 2 (corresponding to peak a) from samples heated for 0, 8 or 12 hours, was determined before and after sonication for 5, 20, 60 and 300 seconds in the presence of 1% DMSO, and the results are presented in Figure 7.12.

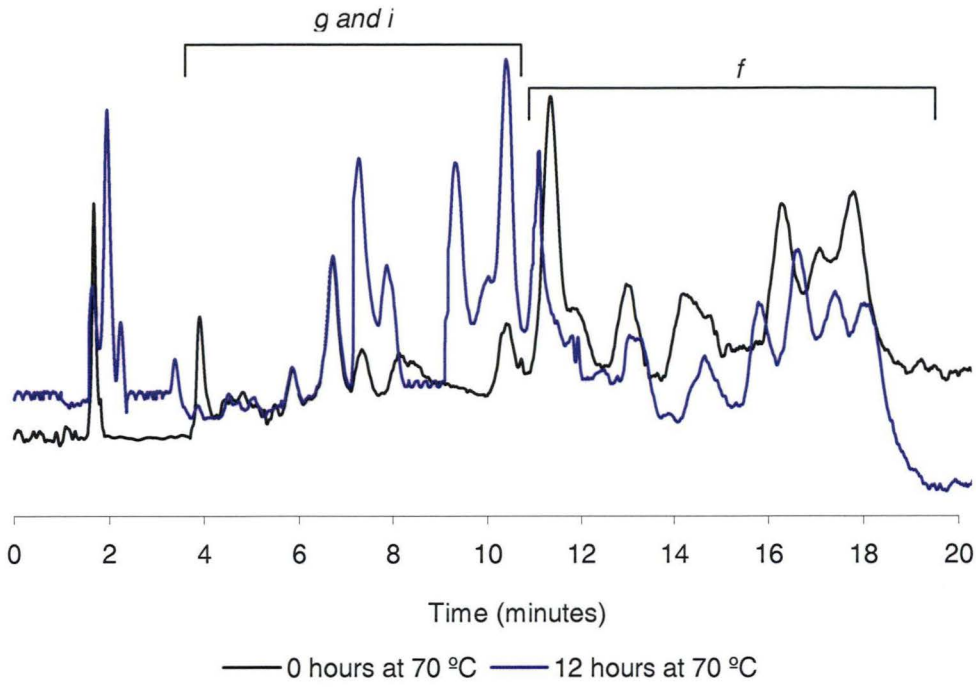

Figure 7.11 Ion-interaction RP-HPLC chromatogram of enoxaparin solution kept at 70 °C for 0 or 12 hours.

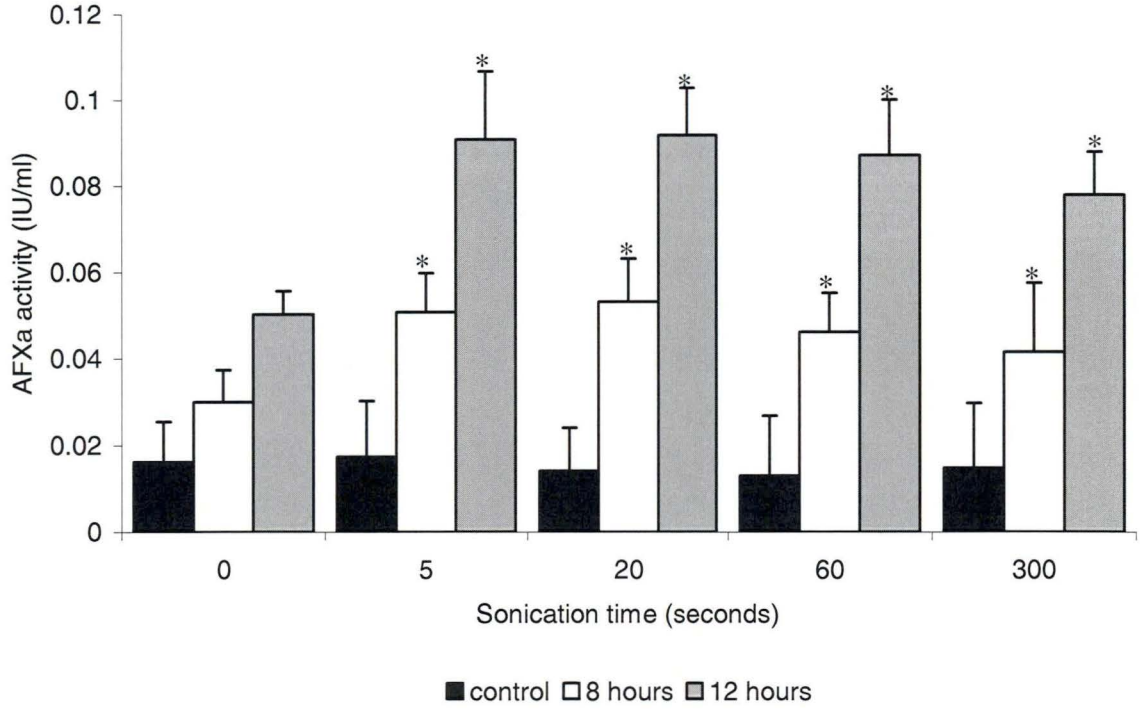

Figure 7.12 The AFXa activity of HP-SEC fraction 2 of enoxaparin samples kept for 0, 8 or 12 hours at 70 °C, before and after sonication for up to 300 seconds in the presence of 1% DMSO. *Significant difference (P≤ 0.05) from corresponding control (without sonication in the presence of 1% DMSO). Error bars indicate mean ± S.D.

Figure 7.12 demonstrates three different things. Firstly, HP-SEC fraction 2 after heating for 8 or 12 hours, had greater AFXa activity than the fraction 2 following heating for 0 hours. The aggregated fraction has a greater concentration of oligosaccharides and is expected to have higher AFXa activity. Fraction 2 after heating for 8 hours and 12 hours had 53% and 200% more AFXa activity than the control fraction (0 hours heated). HP-SEC analysis of unstressed and stressed enoxaparin showed a higher concentration of oligosaccharides in early eluted fraction 2 following heating (Figure 7.5). These results indicated aggregation of oligosaccharides following heating had occurred.

Secondly, the recovery of AFXa activity of fraction 2 of heated enoxaparin samples was less when sonication time was greater than 20 seconds. The increase in the AFXa activity of fraction 2 of 12 hours heated sample was 15% less after sonication for 300 seconds compared with sonication for 20 seconds. Longer sonication time may have altered the structure of aggregated oligosaccharides as it has been reported that sonication of polysaccharide longer than 5 seconds causes degradation of aggregated constituents [123]. A short sonication period (5 or 20 seconds) was found to be the optimal time of sonication for the disaggregation of aggregated enoxaparin constituents.

Thirdly, sonication in the presence of 1% DMSO, a treatment shown to disaggregate polysaccharides [123], increased AFXa activity of 8 and 12 hours treated fractions but not of the control fraction (without sonication). Increases in the AFXa activity following sonication (20 seconds) in fraction 2 from enoxaparin samples heated for 8 and 12 hours were 77% and 88% compared with the control fraction.

These activities represent increases over the activity of untreated fraction 2 by 230% and 470% respectively.

Decomposition studies of other LMWHs or parent heparin at elevated temperature would be required to determine whether the changes observed in this study of enoxaparin are unique to this LMWH or can be generalised to other LMWHs and unfractionated heparin. Further structural characterisation of thermally labile and thermally stable constituents and the degradation products detected in this study may allow the determination of the properties of these constituents.

7.5 CONCLUSION

This study identified three different processes that affect the AFXa activity of enoxaparin upon heating at 70 °C. A rapid loss of AFXa activity was primarily due to oxygen independent desulfation, particularly of N-sulfate groups and mostly involving hexa- and octa-saccharides. Desulfation occurred rapidly in the first 8 hours. A further, comparatively slower loss of AFXa activity was due to aggregation. Increased AFXa activity after 8 hours was due to fragmentation, as determined by reducing capacity and supported by CE, HP-SEC and ion-interaction RP-HPLC analyses. Some fractions appeared to be resistant to chemical changes brought about by heating. A preparation containing these thermally stable constituents may provide a more stable LMWH formulation.

CHAPTER 8

General Conclusion

Commercially available LMWHs are depolymerised porcine mucosal heparin preparations prepared by chemical or enzymatic digestion methods. Depolymerisation results in partial desulfation, reduced charge density and fewer AT binding sites. The depolymerisation procedure also creates LMW products about one third of the size of the standard heparin molecule. These LMW products are diverse in structure and functional properties as a result of different depolymerisation processes.

Enoxaparin is the first of the new class of LMWH and is widely used as an anticoagulant agent. Despite its routine use, the possible mechanisms by which it undergoes degradation and loss of its activity have not been previously elucidated in the literature. This thesis has advanced the understanding of physicochemical properties of enoxaparin related to both biological activity and storage conditions. This has been achieved by improvements in the existing AFXa activity determination methodology and the development of improved novel analytical techniques with good resolution.

European and British Pharmacopeial Commissions have adopted AFXa activity as a measure of biological activity of LMWHs. AFXa assays are based on the inhibition of a known amount of factor Xa, and are measured using a synthetic peptide substrate. Investigation of the changes that occur during the heating and freeze/thaw treatment of enoxaparin required a large number of AFXa activity analyses. The commercially available ACTICHROME® AFXa assay kit from American Diagnostica provides reliable and reproducible data for both heparin and

LMWHs. This AFXa assay kit method is difficult to perform manually, very expensive and extremely time consuming. The ACTICHROME® AFXa kit method was modified to a low-volume microtitre plate method. This method allowed simultaneous determination of 35 samples in less than 15 minutes compared with determination of 35 samples in 5 hours by the standard kit method. The low-volume microtitre plate method is not only faster and more sensitive but also economical as it uses fewer quantities (8 times) of reagents and samples (5 times) required for the AFXa activity analysis. This method facilitated multiple analyses of HP-SEC fractions, required to determine physicochemical properties of various oligosaccharides. This low-volume method allowed triplicate determination of AFXa activity, sulfate content and activity of disaggregated oligosaccharides of the same HP-SEC fraction. These multiple analyses would have not been possible with the ACTICHROME® AFXa kit method without preparative HP-SEC fraction collection. This method can also be successfully applied to the multiple analyses of RP-HPLC and CE fractions.

Because of its high negative charge and structural complexity, enoxaparin and other LMWHs are extremely difficult to analyse by chromatographic methods. Several analytical techniques such as SAX and CE offer adequate resolution of various oligosaccharides, prepared by time consuming and laborious fractionation or depolymerisation of enoxaparin. However, analysis after digestion does not offer a convenient methodology for stability testing of enoxaparin. This thesis presents development and validation of two novel analytical methodologies, CE and ion-interaction RP-HPLC. The CE method has demonstrated a reduction in the run time and increased resolution compared to existing CE methods. It has the potential to

show structural differences within the two different batches of the same LMWH. The novel application of ion-interaction RP-HPLC method was demonstrated with both ELSD and diode array detection. This method showed improved resolution and base line separation of different LMWHs compared to any other existing analytical techniques. These methods have the potential to indicate stability and structural diversity between different LMWHs and were successfully applied to investigate chemical and physical properties of stressed and unstressed enoxaparin.

The packaging labels for storage recommendations of three different LMWHs are different from each other. The label for enoxaparin bears "store below 25 °C". Recommended storage condition for EP-LMWHS is "store at 4 °C" whereas the packaging label for dalteparin states "do not freeze". Despite ambiguity about storage conditions, the effect of freezing and thawing on the activity of LMWHs has not been investigated. The freezing study presented here has demonstrated a severe effect of freeze/thaw treatment on the activity of enoxaparin. Based on this result, storage recommendations given on the packaging of enoxaparin formulation should state that the preparation should not be frozen. This study has established the different conditions under which AFXa activity is lost, retained and recovered. Detailed mechanistic study of enoxaparin showed aggregation as the main cause for the activity loss upon freezing and thawing. The effect of heating on the chemistry and AFXa activity of enoxaparin was comprehensively investigated. Development of novel and high resolution analytical techniques (CE and ion-interaction RP-HPLC) allowed exploration of the mechanisms involved for the rapid lost and subsequent increase in the AFXa activity of enoxaparin at elevated temperature. This study

determined three different processes, desulfation, fragmentation and aggregation which affect enoxaparin activity at elevated temperature.

HP-SEC analysis has shown that upon freezing, larger oligosaccharides (deca-, dodeca- and tetradeca-saccharides) of enoxaparin undergo rapid aggregation compared to the smaller oligosaccharides (hexa- and octa-saccharides). IC, HP-SEC, CE, ion-interaction RP-HPLC and reducing capacity analyses showed that larger oligosaccharides (deca-, dodeca- and tetradeca-saccharides) are thermally stable constituents of enoxaparin. Smaller oligosaccharides of enoxaparin such as hexa- and octa-saccharides are clinically active but rapidly undergo fragmentation and desulfation. Smaller oligosaccharides are more stable at low temperatures than larger oligosaccharides. A LMWH preparation containing low mass fractions such as hexa and octa-saccharides can provide a formulation with greater stability at low temperatures. A LMWH preparation containing thermally stable fractions (larger oligosaccharides) offers a new formulation with greater shelf life.

The stability of enoxaparin solution at paediatric dosage level is only 24 hours, which results in a requirement of fresh preparations. This study has shown that dilution of enoxaparin with 4% glucose at paediatric dosage levels can be successfully stored for a minimum of 31 days without loss in the AFXa activity. This stable formulation offers substantial advantages such as superior quality control, more convenience to care givers and lower cost of formulation.

Analysis of various LMWHs by CE, ion-interaction RP-HPLC and HP-SEC showed that these anticoagulant agents are structurally and chemically different. Observed stability and physicochemical properties of various oligosaccharides of

enoxaparin at different storage conditions cannot be generalised to other LMWHs. Similar investigation of other LMWHs at freezing and accelerated temperatures would be desirable to determine whether these LMWHs undergo identical degradation processes or they behave differently depending on their unique structural characters.

The research material presented here has shown the development and the novel application of CE and ion-interaction RP-HPLC analytical techniques for the stability analysis, chemical characterisation and batch-to-batch variation evaluation of intact LMWHs. Storage induced chemical and physical changes in enoxaparin under different conditions have been studied in detail. This study has investigated previously unknown processes which influence the AFXa activity of enoxaparin. There is now scope for the future formulation development of desirable LMWH constituents to increase shelf life, to improve therapeutic efficacy and to decrease adverse effects based on the novel analytical methods and extensive structural elucidation of enoxaparin presented in this thesis.

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