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# Some physicochemical and biological characteristics of heparin fractions prepared by gel chromatography

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## Summary

Heparins of defined molecular weights fractionated on gel chromatography in the presence of salt, have been assayed for their anticoagulant (B.P.) and anti-Xa activities, protamine neutralization and equivalent weight values as well as their abilities to release lipoprotein lipase. The anticoagulant activity and lipoprotein lipase releasing ability appears to decrease with molecular weight. In contrast the anti-Xa activity of the low molecular weight fraction showed an increase. No discernable trend was noted for equivalent weight and protamine neutralization values of the fractions.

## Introduction

Heparin is a sulphated mucopolysaccharide with unique biological effects. Its anticoagulant activity derives mainly, or completely, from its ability to bind to antithrombin III—a natural inhibitor of the serine proteases (thrombin and the activated forms of coagulation factors IX, X, XI, XII) involved in the intrinsic coagulation system. Heparin also releases several lipoprotein lipases from endothelium, adipose tissue and liver but the precise mechanism of lipase release is still unknown (Olivecrona et al., 1977).

In the present study, fractions of heparin of different molecular weight have been assessed, following intravenous injection, for their ability to release lipoprotein lipase

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in rats. In addition, the anticoagulant activity of the fractions has been assayed using the British Pharmacopoeia and the anti-Xa methods and fluorescence techniques have been employed to provide information about the physicochemical properties such as equivalent weight and protamine neutralization values of the fractions.

## Materials and Methods

Sodium heparin (1.25 g) isolated from pig intestinal mucosa was obtained from Diosynth, Morden, Surrey, U.K., and dissolved in 1 M sodium chloride (18 ml) and was chromatographed on Ultragel ACA44 to produce 5 distinct heparin fractions (Morton et al., 1981).

The anticoagulant activities of the heparin samples were determined using the British Pharmacopoeia (BP) 1973 method, and the anti-factor Xa assay (Teien et al., 1976) was measured using the synthetic substrate, S-2222 (Kabi, Stockholm). Bovine factor Xa (FXa) (Diagnostic Reagents, Thame, Oxon) (100 $\mu$l of a solution prepared by dissolving one ampoule (3 mg) of FXa in 0.8 ml of distilled water) was allowed to react for 30 s with the heparinized plasma (200 $\mu$l). Afterwards the substrate solution (200 $\mu$l of 1 mM) was added and incubated for exactly 3 min at 37°C. This reaction was terminated after 3 min by the addition of acetic acid (300 $\mu$l of 50%). Absorbances were read at 405 nm against a plasma blank. In all these assays the Third International Reference Preparation of Heparin was used as a standard.

Equivalent weight (i.e. the moles of anionic sites) determinations were performed using acridine orange titrations as previously described (Edwards et al., 1978). Protamine neutralization values were also measured using the acridine orange titration technique (Titration method B in Edwards et al., 1980) using the First International Reference Preparation of Protamine. In all these fluorescence experiments measurements were made on a Perkin Elmer MPF 43 Fluorescence Spectrophotometer using the right-angle viewing mode.

*Animal studies*

Male Lister Hooded/Ola rats, weight 390–590 g, were starved for 24 h prior to the start of the experiments but had free access to water. The rats were anaesthetized with an interperitoneal injection of sodium pentobarbitone (60 mg/kg). When under anaesthesia, the femoral vein was exposed by cutting and scraping back subcutaneous tissue. A 25 mm-gauge needle was used to inject heparin (50 units/kg rat made up to 0.5 ml with 0.9% NaCl) into the vein.

After 10 min the rats were exsanguinated, via the exposed abdominal aorta, into a glass syringe containing 1 ml of 3.13% trisodium citrate in 0.9% NaCl, to give a final blood citrate ratio of 9 : 1. The collected blood sample (10 ml) was cooled over ice for 2 min before being centrifuged at 1800 *g* for 20 min to give a platelet-poor plasma. The plasma was then carefully removed and divided equally into 2 small plastic containers. These were then stored at −20°C until tested for lipoprotein lipase (LPL) activity, using the method of Naher (1974). The LPL determinations were performed in duplicate on all plasma samples and repeated if the standard error of the two readings were > 10 $\mu$l.

The activity of the enzyme was calculated from the 5th to the 25th minute of the titration, where the rate of release of free fatty acids increases linearly with time. One unit of lipase activity, in this assay is defined as the amount of enzyme that releases $10^{-3}$ moles fatty acid/min at 25°C per litre of plasma. Control rats were injected with 0.5 ml of 0.9% NaCl and showed no detectable lipoprotein lipase activity.

## Results

Commercial heparin from a mucosal source was fractionated on gel chromatography to produce heparins of different molecular weights. The fractions denoted I–V were isolated and were shown to be in a molecular weight range of 5000–15,000

TABLE 1

PHYSICOCHEMICAL AND BIOLOGICAL CHARACTERISTICS OF HEPARIN FRACTIONS PREPARED BY GEL CHROMATOGRAPHY

| | Diosynth heparin | Fraction I | Fraction II | Fraction III | Fraction IV | Fraction V |
|---|---|---|---|---|---|---|
| Molecular weight range (daltons $\times 10^3$) | – | 12–15 | 8–12 | 8–9 | 6–8 | 5–6 |
| Equivalent weight (moles anionic sites) | 171.6 | 170.6 | 177.5 | 169.0 | 179.7 | 177.6 |
| Protamine [a] neutralization value | 110.5 (0.67) | 131.9 (0.77) | 130.1 (0.75) | 109.7 (0.67) | 89.2 (0.73) | 61.7 (0.75) |
| Anticoagulant activity (BP units/mg) | 165.0 | 171.5 | 173.5 | 163.0 | 122 | 82.0 |
| Lipoprotein [b] lipase activity and standard deviation | 202 ±25 | 129 ±20 | 225 ±44 | 175 ±12 | 102.5 ±13 | 138 ±17 |
| Enzyme activity (units/litre/mg heparin/kg rat) | 667 | 459.2 | 756.0 | 577.6 | 262.4 | 219.2 |
| Anti-factor Xa (u/mg) | 165 | 174 | 161 | 165 | 169 | 275 |
| Anti-factor Xa activity (% of unfractionated) | 100 | 98.4 | 91.0 | 92.3 | 96.1 | 158.1 |
| Xa/BP ratio | 1 | 0.99 | 1.08 | 0.99 | 1.38 | 3.35 |

[a] Number of units of heparin neutralized by 1 mg of protamine and figure in parenthesis indicates the weight of heparin (mg) that would neutralise 1 g of protamine.

[b] u/l after injection of 50 units/kg of heparin to rats.

daltons as indicated in Table 1. It was observed that the activity measured by the British Pharmacopoeia method was low for the lower molecular weight fractions compared to the unfractionated heparin, whilst the anti-Xa potency remained unchanged over the same molecular weight range, with the exception of fraction V where the anti-Xa activity increased significantly. The heparins were also characterized in terms of equivalent weight and protamine neutralization values (Table 1). Both these parameters do not appear to change significantly on a weight basis for the 5 molecular weight fractions isolated.

The LPL assay was determined with 5 animals for each heparin fraction and the animal-to-animal variation in response to the heparin injected partly accounted for the standard deviations recorded for enzyme activity. It appears that the LPL activity is in a range between 100 and 230 u/l for all the heparins injected and since the injections were on an anticoagulant unit basis, it appears that there is no apparent relationship between anticoagulant activity and the LPL-releasing activity of the heparin molecule. However, when the data is presented based on weight of heparin administered/kg rat, the lower molecular weight fractions (IV and V) have a lower activity than the higher molecular weight fractions (I, II and III).

## Discussion

The heterogeneity of heparin preparations has been a major obstacle to gaining a better understanding of its structure–function relationship. When polydisperse heparin preparations have been fractionated according to molecular weight, it has been invariably found that the anticoagulant activity of the fractions increases with the degree of polymerization of the heparin chains (Johnson and Mulloy, 1976). Laurent et al. (1978) have suggested that the increase in overall anticoagulant activity (when measured by the BP method) with molecular weight could be related to the probability of finding the active binding region of heparin to antithrombin III —a natural inhibitor of thrombin and other coagulation factors. However, some of the other interactions of heparin lack the specificity of the heparin–antithrombin III complex and activity could be related to bulk properties rather than to specific molecular sites. In this paper therefore the heparin fractions separated on a molecular weight basis by gel filtration have been evaluated for their anticoagulant and other pharmacological effects as well as their physicochemical properties.

Commercial heparin was fractionated using gel chromatography in the presence of 1 M sodium chloride. Mulloy and Johnson (1980) have shown that the fractionation of heparin by gel permeation chromatography using water as a solvent does not lead to a differentiation simply on a molecular weight basis and that a correlation between biological activity and molecular size using such fractions must be treated with caution. These same researchers have shown that the fractionation on gels using sodium salts at sufficient concentration separates the heparin on a molecular weight basis and the same principle has been used in the present work.

The relationship between molecular weight and anticoagulant activity has been extensively reported and the data presented here show a decrease in anticoagulant

activity (as measured using the BP method) with molecular weight over the range 9000–5000 daltons. This is a similar pattern to that obtained by Laurent et al. (1978). The anticoagulant activity of heparin is the sum of the anticoagulant activities of its constituent heparin chains. The removal of some of the low molecular weight low activity chains during the fractionation process leads to a slight increase in the activity of fractions I and II compared to the original commercial heparin. Previous studies on subcutaneously administered heparin fractions have shown that low molecular weight heparin induced considerably higher anti-Xa activities than high molecular weight heparin (Lane et al., 1977; Michalski et al., 1977), resulting in an increase in the ratio of anti-Xa to APTT (activated partial thromboplastin time) activities with lower molecular weight fractions.

The data presented here show a similar trend but using the BP assay instead of the APTT. The lowest molecular weight fraction (fraction V) has a Xa/BP ratio of 3.55 whilst the ratio for the other fractions is nearer unity. Fraction V could therefore, whilst retaining its ability to neutralize Factor Xa, have a decreased effect on the blood-clotting mechanism. Indeed Kakkar et al. (1982) have shown that a single injection of a low-molecular weight heparin was found to be a convenient way of preventing deep vein thrombosis in high-risk patients undergoing major abdominal surgery. However, another study (Thomas and Merton, 1982) indicated on the basis of clotting assays that there seems no reason to expect a lower incidence of haemorrhagic side-effects using a 6000 dalton low molecular weight heparin fraction.

The equivalent weight term used in this work is a measure of the charge density of heparin and during the preparation of commercial heparin many of the non-active (and often low charge density) components are removed. We have shown that a relationship exists between the equivalent weight and anticoagulant activity of heparin fractions isolated during the preparation of commercial heparin (Edwards et al., 1978). However, the data in Table 1 show that the fractionation on the basis of molecular size does not lead to any significant variation in the equivalent weight parameter. Even fractions IV and V with their reduced anticoagulant activity still retain a nearly identical equivalent weight as the unfractionated heparin. It follows therefore that a bulk property such as equivalent weight is not a reliable measure of the chemical and physical changes responsible for heparin's anticoagulant activity. The protamine neutralization values for the unfractionated and 5 fractions are also nearly identical when compared on a weight-to-weight basis. Heparin combines with protamine to form a stable complex, an interaction which finds clinical application when it becomes necessary to neutralize heparin activity in the blood. We can tentatively conclude that the protamine neutralization values of heparins do not vary significantly following fractionation on a molecular weight basis.

The occurrence in mammalian tissue of a native polysaccharide that affects LPL but lacks effect on blood coagulation may have important physiological and therapeutic implications. Heparin releases lipase from tissue sites into the circulating blood and the mechanism probably involves the formation of a heparin–lipase complex. Bengtsson et al. (1977) have found that high and low activity heparins (isolated by affinity chromatography using antithrombin III) were indistinguishable in their ability to release lipoprotein lipase, indicating that the specific structure

which binds to antithrombin III differs from that required for the interaction with lipoprotein lipase. But commercial heparins do vary in their ability to release the 3 kinds of lipase at different dose levels (Ganesan and Bass, 1976) and careful consideration must be given to the time interval following heparin injections and the half-life of the enzyme.

The dose of heparin administered, time of obtaining blood after administration and the type of heparin used varies widely among the investigators studying post-heparin lipolytic activities. In this study the dose is in a similar range used by other researchers (Bengtsson et al., 1980) and the interval of 10 min has been shown to be optimal for lipoprotein lipase release in rats. These details are more important when comparing data from two laboratories. Graham and Pomeroy (1980) have shown that lipolytic activity showed no consistent trend with molecular weight in fractions isolated from lung and mucosal heparins. In the data presented here, it appears that the LPL activity is in a range of 102–255 u/l for all the heparins injected and since the injections were based on anticoagulant activity, there appears to be no apparent relationship between anticoagulant and lipoprotein lipase releasing activity.

Given this lack of relationship, the question arises whether on a weight basis some fractions are more active in terms of lipoprotein lipase releasing ability. Heparin was administered at a dose of 50 units/kg and therefore comparison on a weight basis must take into account the weight of the rat, the anticoagulant activity of heparin as well as enzymatic activity. Given this built-in complexity, it is noted that the lower molecular weight fractions (IV and V) have a lower activity than the higher molecular weight fractions (I, II and III) although there is some variation in the values of these latter fractions. If one accepts a range of lipoprotein lipase activity for commercial and high molecular weight fraction then fractions I and II are significantly lower and the data in fact support the findings of Bengtsson et al. (1980) where evidence is presented for a decrease in lipoprotein lipase interaction with decreasing molecular weight. This indicates that distinct sequences of chemical groupings on the heparin molecule are responsible for the release of the enzymes from tissue sites. However, the present data can be interpreted in another way. It can be concluded that a linear relationship exists between chain size and ability to release lipase in a range of 5000–12,000 daltons. Heparins of different molecular weights might also vary in their ability to release the different types of lipases and that the assay of total enzyme activity 'overlooks' such interactions. Such an explanation could account for the difference in lipoprotein lipase releasing ability between fractions I and II.

In conclusion, these tests indicate that the biological activities of the low molecular weight fractions need extensive definition in order to maximize the possible pharmacological benefits of this improved drug.

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