International Journal of Cardiology 212S1 (2016) S5–S9

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Recent innovations in the structural analysis of heparin

Edwin A. Yates[a,*] and Timothy R. Rudd[a,b]

[a] Department of Biochemistry, Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool, L69 7ZB UK
[b] National Institute for Biological Standards and Controls (NIBSC), Blanche Lane, South Mimms, Hertfordshire, EN6 3QG UK

KEYWORDS

Heparin
Low molecular weight heparin
Structure-activity
Structural analysis
Heparin regulation
Heparin provenance

ABSTRACT

Heparin, the widely used anticoagulant drug, is unusual among major pharmaceutical agents being neither single chemical entity nor a defined mixture of compounds. Its composition, while conforming to approximate average disaccharide composition or sulfation levels, exhibits heterogeneity and variability depending on the source, as well as its geographical origin. Furthermore, individual polysaccharide chains, whose physico-chemical properties are extremely similar, cannot be separated with current state-of-the-art techniques, presenting a challenge to those interested in the quality control of heparin, in ensuring its provenance and safety, and those with an interest in investigating the relationships between its structure and biological activity. The review consists of two main sections: The first is the *Introduction*, comprising (i) The History, Occurrence and Use of Heparin and (ii) Approaches to Structure-Activity Relationships. The second section is *Improved Techniques for Structural Analysis*, comprising; (i) Separation and Identification, (ii) Spectroscopic Methods, (iii) Enzymatic Approaches and (iv) Other Physico-Chemical Approaches. The ~60 references cover recent technological advances in the study of heparin structural analysis, largely since 2010.

1. Introduction

1.1. History, occurrence and use

The discovery of heparin is generally attributed to McLean and Howell, although there was considerable debate, not least among themselves, concerning priority. Heparin was first commercialised in the 1920s despite the observation of side-effects arising, as we now know, from impurities. By 1933, Banting and Best had developed heparin from bovine liver and this was trialled in Canada between 1933 and 1936, culminating in its successful use in humans in 1937. During this period, Jorpes in Sweden had been developing the purification process and, by 1936, an improved heparin product was available. In the 1940s, Moloney and Taylor developed a method for mass-producing heparin, which superseded the earlier one. Subsequently, heparin was used extensively as an anticoagulant in surgery and during dialysis. Later refinement of heparin has included the introduction of low molecular weight heparin (LMWH) variants, achieved using a variety of depolymerisation techniques. LMWH was produced following the discovery that the action of heparin via antithrombin (AT) required shorter fragments than its action through thrombin (factor IIa) and, by exploiting this selectivity, favourable clinical properties could be obtained. It was later found that a short (pentasaccharide) sequence within the heparin chains provided high affinity binding to AT and high anticoagulant activity. This line of enquiry culminated in the production of a synthetic pentasaccharide, fondaparinux, which has been commercialised. We do not intend to discuss the clinical and biochemical properties of these agents here; this has been addressed elsewhere by others far better qualified to do so [1–3]. Instead, we will restrict ourselves to recent developments (largely since 2010) in the structural analysis of heparin, which is itself of importance for several reasons. First, the production quality and, indeed, the identification of particular processes employed during production (especially of LMWH) can be monitored. Second, the natural variation, as well as any potential unwanted or unintended structures, can be monitored and the overall quality followed. Additionally, the complex question of structure-activity relationships, not only between heparin and proteins of the blood clotting cascade, but also many other proteins [4,5] can be advanced. In recent years, following concerns during the 1990s about the possibility of contaminating heparin with the agents responsible for bovine encephalopathies that led to the widespread abandonment of bovine heparin as a pharmaceutical agent, commercial heparin has been obtained almost exclusively from pig intestinal mucosa, apart from some bovine material, principally in South America. However, since the contamination of the heparin supply chain with a chemically modified glycosaminoglycan (GAG) in 2008 [6], there is renewed interest in diversifying the sources of heparin to the extent that bovine sources are now being considered for re-introduction into the United States market [7,8].

Heparin is a complex mixture of polysaccharides of the GAG class, whose other members include the closely related heparan sulfate (HS), as well as chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronic acid (HA), and is one of the

* Corresponding author at: Department of Biochemistry, Institute of Integrative Biology, University of Liverpool, Crown Street, Liverpool L69 7ZB UK.
E-mail address: eayates@liv.ac.uk (E.A. Yates).

most important and widely-used pharmaceutical agents in current use. It is employed primarily in situations where the aim is to prevent thrombi forming, such as during surgery and blood dialysis. In its role as an anticoagulant, its mode of action is *via* several components of the blood-clotting cascade, principally antithrombin and thrombin (factor IIa) but, it also exerts action on several other components, including factors IX and XI, as well as many other proteins. At least some of these interactions with proteins seem to arise by virtue of the underlying structural similarity between heparin and the naturally occurring cell-surface polysaccharide components of proteoglycans, HS [9]. The principles underlying the interactions between heparin and some of these proteins, most notably AT and thrombin, have been studied extensively, employing strategies that first depolymerise the heparin into oligosaccharides, isolate the binding saccharide components and then characterise them. Using such approaches, the caveats to which have been discussed elsewhere [10], the contrasting nature of interactions between heparin and AT, or with thrombin, were revealed. This early success, which led to the production first of LMWH and then to the targeted synthesis of the pentasaccharide with high activity against AT, encouraged a similar approach to be employed for the attempted isolation of analogous high-affinity oligosaccharides active with other proteins. This strategy has almost completely failed, leading some to propose an explanation of the anomalous case of AT in terms of the experimental methods used and to suggest a general property of redundancy regarding the interactions of saccharides with proteins [11,12].

1.2. The approach to structure-activity relationships

To attempt to correlate the structure of heparin (and its oligosaccharide constituents) with protein binding and activity remains the ultimate aim of structure-function studies in this area. However, to do so it is now clear that the approach adopted above needs to be modified. There are a number of over-arching strategies available: One is to fractionate or, ideally, separate to homogeneity (using one or several chromatographic techniques) the components in a mixture and to determine their structure, then to correlate these with binding and activity data, noting the important point that binding does not necessarily equate with activity directly. From these data, it is then hoped that the factors determining activity will become apparent. A complementary approach attempts to conduct structural analyses on a mixture or a partially separated mixture, about which some detailed structural information is, nevertheless, available with the aim of providing some insight into the structural features of heparin responsible for activity.

Heparin is unusual among mainstream pharmaceutical agents in that it does not consist of a single chemical structure. In fact, the situation is even more complex and challenging than this simple statement would suggest, because heparin cannot be defined even as a mixture of defined substances. The reasons for this are several. In the first place, heparin is a polydisperse polysaccharide whose chain lengths and their distribution vary, not only in the source material as a function of individual animal and geographic location but, also as a consequence of subsequent processing. Next, the sequences present within heparin samples, while conforming to broad compositions in terms of the constituent disaccharides and gross sulfation position that allow, for example porcine mucosal heparin to be distinguished from bovine mucosal heparin [13], also vary to some extent, leading to the property of micro-heterogeneity. Third, the processing and treatment of the heparin as it is rendered into its pharmaceutical state can also induce some additional structural changes and these depend on the process involved but, can also vary between batches, even when those processes are deemed to be identical [14].

The overall challenge is therefore two-fold: *How to define 'heparin' in the first place?* Then; *how to relate the structures that comprise a heparin sample to its activity?* This second part masks further major technical obstacles which include; *the identification of the sequences present within a sample of heparin* and its usual pre-requisite; *how to separate its constituent chains* to allow that sequencing to take place. It must be stated at the outset that, with the present state of technical know-how, both of these procedures involve studying oligosaccharide fragments, rather than the full-length heparin chains and the equivalence of these approaches, especially concerning biological activity, has been questioned [10]. Much of this review will be devoted to surveying recent technical improvements and innovations that address these dual challenges or provide methods with which this might be undertaken.

It might also be informative to recall that the relationship between heparin oligosaccharide fragments and a very wide range of protein-binding events and activities have been found to exhibit a degree of redundancy, by which it is meant that several heparin oligosaccharide sequences appear able to elicit similar binding or activities. This is not to say, however, that all or even most sequences exhibit the same activity. A summary of the large body of data amassed to date would be that the determining properties seem to relate to a combination of charge distribution and conformation but, that these properties do not usually relate in any simple way (for example, through a straightforward consensus of particular sulfation positions or patterns) nor, in many cases, to overall charge density. It can be concluded that the physical characteristics of a binding or active oligosaccharide, while undoubtedly determined ultimately by their sequence and sulfation pattern, are not readily discernible experimentally with the present state of the art. Indeed, the apparent similarity in behaviour of many heparin oligosaccharides at the level of molecular interactions with proteins must serve as a warning of the difficulties that are likely to be encountered when attempting their separation on the basis of global properties such as charge, volume or cross-sectional area. Nevertheless, some notable progress has been made employing multi-dimensional approaches, many of which will be described below.

Undoubtedly a major step in the analysis of heparin (and other GAGs) that has blossomed in recent years is the application of mass spectrometry to structure determination. As stated above, this is usually dependent on the prior separation of oligosaccharides, although the application of ion-trap techniques can contribute to alleviating this requirement. Nevertheless, even these advances are not without their difficulties; the migration of sulfate groups during the ionisation process and the isomeric nature of several of the constituent disaccharides (hence of many oligosaccharides) being two significant examples. In the following, attempts to improve separation and conduct structural analysis, which are frequently constituent parts of the same research article, will be treated together.

2. Improved techniques for structural analysis

2.1. Separation and identification

Separation and quantification of heparin oligosaccharides has been achieved employing reverse phase, ion-paired high-performance liquid chromatography (HPLC) combined with electrospray quadrupole-time of flight mass spectrometry (Q-TOF), which included an amine additive and 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) to improve separation [15]. Heparan sulfate, heparin and LMWH-derived disaccharides derivatised with 2-aminoacridone (2AA) were separated using reverse-phase HPLC with fluorescence detection and electro-spray ionization mass spectrometry (ESI-MS) [16]. Disaccharides from heparin, the main tetrasaccharide bearing a 3-O-sulfate group and unusual oligosaccharides arising from LMWH were separated using reverse-phase, ion paired HPLC [17].

A substantial contribution to the analysis of GAGs, applied to heparin or with the potential to be applied to heparin, has