FEATURE ARTICLE

USP compendial methods for analysis of heparin: chromatographic determination of molecular weight distributions for heparin sodium

Barbara Mulloy · Alan Heath · Zachary Shriver · Fabian Jameison · Ali Al Hakim · Tina S. Morris · Anita Y. Szajek

Published online: 24 June 2014
© Springer-Verlag Berlin Heidelberg 2014

Abstract
Heparin is a polysaccharide product isolated from glycosaminoglycans of porcine mucosa (or occasionally other tissues and species). It is a linear non-uniform polymer consisting of alternating glucosamine and uronic acid monosaccharide residues and is highly sulfated. Heparin sodium drug product (HP) used in medicine consists of chains with molecular weight (MW) ranging from under 5,000 to over 50,000. Although HP has been used as an injectable antithrombotic medicine for more than 70 years, many aspects of its structure and purity, including its MW, have not been specified by public standards until recent years. In 2008, a number of HP lots associated with severe adverse effects, including fatalities, were found to have been contaminated with oversulfated chondroitin sulfate. This incident led to thorough revision of compendial standards worldwide. In the USA, the Food and Drug Administration (FDA) encouraged the inclusion of enhanced standards for purity and identity in the relevant monographs of the United States Pharmacopeia (USP) including acceptance criteria for MW distribution.

The findings and conclusions presented have not been formally disseminated by the FDA and should not be construed to represent any FDA determination or policy.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-014-7940-3) contains supplementary material, which is available to authorized users.

B. Mulloy (✉) · A. Heath
National Institute for Biological Standards and Control, Blanche Lane, South Mamm's, Potters Bar EN6 3QG, UK
e-mail: barbara.mulloy@kcl.ac.uk

Z. Shriver
Department of Biological Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

F. Jameison · T. S. Morris · A. Y. Szajek
Biologics and Biotechnology, The United States Pharmacopeial Convention, 12601 Twinbrook Parkway, Rockville, MD 20852-1790, USA

A. Al Hakim
Office of New Drug Quality Assessment, CDER, FDA, 10903 New Hampshire Avenue, Building 21, Room 2524, Silver Spring, MD 20993, USA

Present Address:
B. Mulloy
Institute of Pharmaceutical Science, King’s College London, Franklin-Wilkins Building, 100 Stamford Street, London SE1 9NH, UK

Abbreviations
BST Broad standard table
FDA Food and Drug Administration
HP Heparin sodium drug product
\( M_n \) Number-average molecular weight
\( M_p \) Peak molecular weight
\( M_w \) Weight-average molecular weight
MW Molecular weight
RSD Relative standard deviation
SD Standard deviation
SEC Size-exclusion chromatography
USP United States Pharmacopeia

Introduction
Heparin is a polysaccharide product isolated from glycosaminoglycans of porcine mucosa (or occasionally other tissues and species). It is a linear non-uniform polymer consisting of alternating glucosamine and uronic acid monosaccharide residues and is highly sulfated. Heparin sodium drug product (HP) used in medicine consists of chains with molecular weight (MW) ranging from under 5,000 to over 50,000.

Although HP has been used as an injectable antithrombotic medicine for more than 70 years, many aspects of its structure and purity, including its MW, have not been specified by public standards until recent years. In 2008, a number of HP lots associated with severe adverse effects, including fatalities, were found to have been contaminated with oversulfated chondroitin sulfate. This incident led to thorough revision of compendial standards worldwide. In the USA, the Food and Drug Administration (FDA) encouraged the inclusion of enhanced standards for purity and identity in the relevant monographs of the United States Pharmacopeia (USP) including acceptance criteria for MW distribution.

Heparin originates in mast cell granules, in which it is the polysaccharide part of the proteoglycan serglycin. On degranulation, heparin is released from mast cells and is broken down by endogenous heparanase to fragments, most of which are between 5 and 30 kDa in mass.
limits of acceptability should be, it is desirable to ensure that comparable results are obtained for MW determinations from different laboratories.

Heparin is not a peptide, and even the most modern mass-spectrometric methods are limited to short oligosaccharides [7]. Liquid chromatography with mass spectrometry has been used to profile heparin preparations [8-10]. The technique is capable of resolving up to about 20-mers; for larger oligomers, overlapping MW patterns prevent interpretation of the data. Thus, it is not possible at present to measure complete MW distributions for HP or for low MW heparins by this method. A further problem arises from the sequence heterogeneity of heparin. The main repeating disaccharide structure, \([-4]-\alpha-L-LydA(2SO_3^-)-(1\rightarrow4)-\alpha-D-Glc(\text{NSO}_3^-,6\text{SO}_3^-)-1]-\), accounts for more than 70% of heparin, but complexities of the biosynthetic process mean that the remainder is heterogeneous in sequence, and arranged in a way that is not predictable [11]. The severe complexity arising from variations in sequence and in polysaccharide chain length mean that MW determinations for heparin samples cannot be achieved with complete certainty by current technology. It is therefore important to introduce an element of consensus between expert laboratories both in the characterization of a calibrant material for general use in the analysis of HP and in the validation of the method.

Both HP and low MW heparins are non-uniform polymers, with MW dispersion that can be described by means of number-average and weight-average MWs (\(M_n\) and \(M_w\), defined in the Electronic supplementary material). The MW distribution of heparin can also be presented in slice tables, indicating what fractions of the polysaccharide pullulan is possible only at particular MWs match the values provided in the table specific to that calibrant. Software packages for the analysis of SEC data are available to automate this process, which can, more laboriously, be performed using a simple spreadsheet.

We report here on the development of a broad standard calibrant to be established as the USP Heparin Sodium Molecular Weight Calibrant reference standard, and of a simple SEC method for determination of MW distributions of heparin sodium. This project required two phases of international collaboration. Phase 1 involved characterization of the calibrant material in eight laboratories, and phase 2 involved 21 laboratories in an assessment of the interlaboratory reproducibility of the SEC method and in data gathering for the setting of acceptance criteria for heparin sodium MW distribution. The resulting method is to be incorporated into the 'Heparin Sodium' monograph of the USP. For the first time, a convenient calibrant is widely available so that direct comparison may be made between MW values for unfractionated heparin determined by different laboratories.

### Materials and methods

Details of the materials and methods used are given in the Electronic supplementary material. The eight participating laboratories in phase 1 of the study characterized the proposed USP Heparin Sodium Molecular Weight Calibrant reference standard by SEC with light scattering detection, using their own choice of protocol. Analytical ultracentrifugation was used to estimate the MW of a monodisperse or polydisperse sample (e.g. mass spectrometry [9], or the UV/refractive index ratio of a sample prepared by beta-elimination [18, 19]). For unfractionated heparin, the most widely implemented method is SEC with refractive index and light scattering detection [20, 21]. This method does not itself require calibration, and so is suitable for the characterization of heparin-based calibrants.

Narrow standard calibrants, not completely monodisperse but with a clearly defined peak MW (\(M_p\)), may be prepared from native heparin by fractionation. Individual laboratories have produced such standards on a small scale and characterized them by viscosity measurements [22], light scattering [23], or a combination of both [24]. The production of MW markers of this type for unfractionated heparin on a large scale is a difficult task.

A broad standard is a polydisperse sample of a polymer. One or more such standards can be used to determine the relationship between MW and retention time in a specific chromatography system if \(M_n\) and \(M_w\) are known [12]. An alternative strategy is to define for the broad standard a table, listing the proportion of the sample falling above (or below) a series of MWs. This approach to calibration of SEC columns has been used successfully for low MW heparin products [25]. The calibration curve is generated by inspection of the integrated chromatogram to find the retention time at which the proportions above and below particular MWs match the values provided in the table specific to that calibrant. Software packages for the analysis of SEC data are available to automate this process, which can, more laboriously, be performed using a simple spreadsheet.

The pur propose ref...
then used as an independent check of the calibrant's performance.

The protocol distributed to the 21 participating laboratories in phase 2 of the study is described in the Electronic supplementary material. In brief, the chromatography system used (based on a published method [2]) was as follows. A mobile phase of 0.1 M ammonium acetate and 0.02 % sodium azide in water was filtered through a 0.22-μm membrane. The chromatography columns were a TSK guard column (6 mm × 4 cm), a TSK SWXL 4000 column (7.8 mm × 30 cm) and a TSK SWXL 3000 column (7.8 mm × 30 cm) in series, at 30 °C. The flow rate was 0.6 ml/min. Refractive index detection was used, at the same temperature as the columns.

Data were collected, digitized and transferred to a workstation for analysis, using SEC specialist software or a spreadsheet capable of implementing the broad standard calibration and reporting both mean MWs and distribution slice tables. On each of four separate days, participants were asked to perform duplicate analyses of the system suitability sample, and analysis of as many samples of HP as they chose.

Results and discussion

On the basis of determination of the dry weight of the ampoule contents for the USP Heparin Sodium Molecular Weight Calibrant reference standard, as described in the Electronic supplementary material, participants in phase 1 of the study were asked to assume that each ampoule of the proposed calibrant 07/324 contained 10.0 mg.

Phase 1

The purpose of phase 1 of the study was to characterize the proposed USP Heparin Sodium Molecular Weight Calibrant reference standard by light-scattering-detected SEC in eight experienced laboratories. Each laboratory received a single HP sample for analysis, the candidate high MW calibrant. This material was a regular HP lot with particularly high polydispersity. Using the equipment, chromatography columns and variable parameters of their choice, the participants obtained results for both \( M_\text{n} \) and \( M_\text{w} \) covering a range of roughly 30 % of the maximum value, giving relative standard deviations (RSDs) of around 10 % [listed with polydispersity \( (M_\text{w}/M_\text{n}) \) values in Table 1]. The most obvious contributing factor to this wide variability was the value chosen for the parameter \( dn/dc \), the coefficient describing the relationship between the refractive index of a solution and the concentration of the solute. The values used for this parameter (listed in Table S1a) ranged between 0.141 mIL/g (laboratory 6) and 0.12 mIL/g (laboratory 7). When given the opportunity to comment on the results of phase 1 of the study, laboratory 7 recalculated some of its results using other values for \( dn/dc \) and was able to show that for the same chromatogram of the proposed calibrant, \( M_\text{w} \) ranged from 16,403 (\( dn/dc = 0.141 \text{ mIL/g} \)) to 19,306 (\( dn/dc = 0.12 \text{ mIL/g} \)). The other laboratories in the study all used values of \( dn/dc \) between 0.129 and 0.134 mIL/g, but excluding laboratory 7, the range for \( M_\text{w} \) is still high at 15,100–20,175. Therefore, other sources of variability at laboratories are clearly as influential as the \( dn/dc \) value used. One potential source of variation is the type of column used. Silica-based TSK SWXL columns were used by several participants; others used a variety of polymer-based columns (Table S1b). Some of the polymer columns used may not be optimal for chromatography of unfractionated heparin, giving chromatograms in which some material is not included in the gel and so is eluted at the void volume. On the other hand, silica columns sometimes shed silica particles into the light scattering detector. Participants were asked to provide slice table data so that a consensus broad standard table (BST) could be derived. Two participants did not provide this data set. The remaining six laboratories either sent full integrated chromatograms (laboratories 3, 5, 7 and 8) or reduced data sets (laboratories 1 and 2). A consensus BST was produced from all the data submitted as follows; at extremes of the MW range, where a value was not available, the data table was populated with 0 or 100 % as appropriate.
towards laboratories contributing several data sets, a single median value for the percentage of material below each MW point was derived for each laboratory, and then the values were combined by taking the median value of the laboratory medians. By this means a consensus MW distribution was determined reflecting contributions from all the participants (shown in Fig. 1 by round markers). When given the opportunity to comment on the results of phase I of the study, laboratory 4 submitted a MW distribution table for the proposed calibrant that was very close to the median line (grey line in Fig. 1).

Table S2 summarizes the data provided by each laboratory, and the median data set that was used to derive a BST. The data set used is shown graphically, with the median, in Fig. 1. The resulting BST is shown in Table 2.

Sedimentation velocity analytical ultracentrifugation

Analytical ultracentrifugation was undertaken to check, by a completely independent method, that measurements made using the SEC method using the proposed calibrant, with the BST from phase I, are accurate.

Values for dn/dc for all the samples were found to be in the range 0.130–0.134 mL/g. MWs for HP samples 07/334 and 97/578 are listed in Table 3 with results from SEC calibrated by the participants, except for a few results from laboratory 10, readily corrected from data provided by the participant, and laboratory 15, for which the results were recalculated using the spreadsheets provided by the participant. Some laboratories (19 and 20) presented more than one cycle of 4 days' work, and these have been treated as separate data sets, giving a total of 20 data sets in all.

Participants in phase 2 of the study readily met the system suitability requirements for $M_w$ (Fig. 2) and $M_p$ (as described in the protocol for the phase 2 study; see the Electronic supplementary material). All the laboratories met the

![Fig. 1 The molecular weight distribution of the proposed calibrant, as determined by size-exclusion chromatography with light scattering detection in phase 1 of the study, involving eight laboratories. Line styles distinguish between laboratories, the median line has round markers. Results from laboratory 4 are shown in grey.](image)

Table 2

<table>
<thead>
<tr>
<th>MW</th>
<th>Mean vt</th>
<th>SD</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>22,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>28,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>36,000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40,000</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AUC analysis

aMean vt and poly material.

b$M_w$(AU obtained integrations: 2

Phase 2

Phase 2 of the study had two aims: first, to assess the interlaboratory reproducibility of the proposed USP monograph method for MW characterization of HP; and second, to collect MW data for numerous current lots of HP so that suitable acceptance criteria could be set. For all the HP samples tested, participants submitted results for $M_w$ and in addition the percent proportion of material within several MW ranges as listed in Table S3.
null
ranged from 2 to 21. Participants involved in heparin manufacture analysed HP lots of their own, and sometimes also analysed material from other manufacturers. All of the participating laboratories, except laboratory 2, provided results for quadruplicate determinations so that the SD and RSD could be calculated. Intralaboratory reproducibility as measured using these HP samples was not analysed in detail, but the SD and RSD for \( M_w \) and the distribution slices are broadly similar to the values obtained for the system suitability sample.

Suitable acceptance criteria for the MW distribution of HP were chosen on the basis of the data provided by the participants in phase 2 of the collaborative study for heparin lots with current active Drug Master Files. Certificates of analyses were available for almost all of the samples, but those without a certificate of analysis were removed from the data set at this stage. Similarly, products from one participating laboratory were found to be on the FDA's Import Alert list, and were excluded from further consideration for that reason. Results from the single laboratory which did not complete the system suitability check were, however, included as the data provided by that participant made it clear that the intralaboratory variability for this laboratory (Table S3J) was acceptable.

![Graph](image)

**Fig. 2** Values for \( M_w \), \( M_{16000} \), and \( M_{52000} \) of the heparin sodium lots provided by participants, displayed in histogram format. Each box represents the mean of duplicate determinations in the laboratory specified by its number.

![Graph](image)

**Fig. 3** Values for \( M_w \), \( M_{16000} \), \( M_{14000} \), \( M_{14000-24000} \), and \( M_{54000} \) for the active pharmaceutical ingredient lots provided by the participants.
The parameters considered were $M_{24000}$, $M_{8000-16000}$, $M_{16000-24000}$ and $M_{24000}$. Polydispersity ($M_p/M_n$) does not yield explicit information about the proportions of material in specific MW ranges, so was not considered suitable as an acceptance criterion.

A decision was taken not to use the parameter $M_{6000}$ owing to the low interlaboratory precision of its estimations in phase 2, and its relative lack of variability between lots. In addition, low MW heparin has low potency by anti-IIa assay [26], so the proportion of such material is limited in lots of HP by the necessity to meet the potency specification in the USP monograph. By contrast, the parameter $M_{24000}$ provided a direct indication of the content of high MW material, potentially relevant to problems of side effects and contamination. This parameter was also found to be a major contributor to the variability of the MW distribution between HP lots, with a strong influence over the variability in $M_w$.

The parameters $M_{8000-16000}$ and $M_{16000-24000}$ account for most of the material in HP. Setting numerical acceptance values for these parameters was thought to be unnecessary; the specification that the value of $M_{8000-16000}$ should exceed the value of $M_{16000-24000}$ addresses, to some extent, the possible contamination of HP with compounds in a similar MW range. This specification is also intended to discourage the blending of failing HP lots to meet the MW criteria (e.g., adding low MW heparin to a very high MW lot of HP).

As the spread of $M_w$ values in the study (Fig. 3) represents a genuine difference in products, and is not an issue of experimental precision, there is no clear rationale for basing criteria on some multiple of the SD; extreme values are not statistical ‘outliers’. No data are available to link side effects to the MW distribution of HP, although it is known that heparin-induced thrombocytopenia is commoner in patients treated with HP than with low MW heparin [27].

At present, HP products and lots differ in MW profile; setting a standard prevents the range of MWs widening. All brands of HP USP share the same name and description and are likely to be blended if failing HP lots are recalled. Results could be obtained using only the SD and $M_{24000}$, similar to the variance in most product lots.

Comparison of HP USP lots was possible using the SD and $M_{24000}$ provided that a sufficient number of lots with similar SDs were selected. Such a system could be tested at this stage without a large investment, and the data collected could be used in a future study to determine if a smaller set at this stage is sufficient. We did not test the system using a small number of a specific laboratory and were therefore unable to provide results. Results could be obtained using only the SD and $M_{24000}$, similar to the variance in most product lots.


The new MW method and acceptance criteria may help avoid gross contamination with compounds differing from HP in MW distribution. Together with other orthogonal methods in the new monograph, this new measure will contribute to the safety and consistency of HP.

Acknowledgments The participants in both stages of the study are acknowledged with gratitude. They are J. Capilla and J. Glaich (Momenta Pharmaceuticals, Cambridge, MA, USA), E. K. Chess and W. Wasylenko (Baxter Healthcare, Round Lake, IL, USA), Huilong Fan (NIPCPB, Beijing, China), D. Fulchiron (Bay Bioanalytical Laboratories, Hercules, CA, USA), L. Rao and Y. Wang (Scientific Protein Laboratories, Wansauke, WI, USA), S. Bertini and G. Torri (Istituto Ronzoni, Milan, Italy), W. Jeske (Loyola University, Maywood, IL, USA), C. S. Venkatesan (Gland Pharma, Hyderabad, India), R. van Herpen (Aspen, The Netherlands), P. Tornabu (APP, Skokie, IL, USA), S. O. Herr (Leo Pharma, Ballerup, Denmark), H. Chen (Sepax Technologies, Newark, DE, USA), J. Oran (Pfizer, Franklin, OH, USA), R. Van Graaf (Celsius Laboratories, Cincinnati, OH, USA), Z. Xiang (Chengouzhi Qionghang Biopharm, Chengzhou City, China), Hao Meixia (Yantai Dongcheng Biochemicals, Yantai, China), H. Stefan-Hinrichs (Sandoz, Kundl, Austria), Li Tan (Shenzhen Haplink Pharmaceutical, Shenzhen, China), L. Liverani (Opocin, Corlo di Formigine, Italy), J. Cabalas (Bioiberica, Palafolls, Spain), Yu-Juan Song and Huilong Fan (NFIDC, Beijing, China), R. Yin (Chongqing Imperial Biochem, Chongqing, China), G. Gratzi (Boehringer Ingelheim, Benlue Laboratories, Bedford, OH, USA), and C. Martinez and P. Anger (Sanofi-Aventis, Arcamon, France). Members of the USP Unfractionated Heparin Expert Panel (E. W. Workman, E. Chess, E. Gray, F. Huihong, K. Johansen, P. Tornabu and C. Viskgov) are thanked for fruitful discussions.

References


Barbara Mulloy worked at the National Institute for Biological Standards and Control for many years, specialising in the physicochemical characterisation of biological medicines. She is now Visiting Professor at the Institute of Pharmaceutical Science, King’s College London, and has honorary appointments also at Imperial College and Royal Holloway University of London. Barbara is a member of the United States Pharmacopeia Convention’s expert panels on unfractionated heparin and low molecular weight heparin.

Alan Heath has recently retired from National Institute for Biological Standards and Control, where he worked for 26 years, and was head of the biostatistics group. Alan specializes in the statistical analysis of assays for biological medicines, and multicentre studies to establish biological and other reference materials.

Zachary Shriver is currently a research affiliate at MIT and Vice President of Research at Visterra. He received his Ph.D. degree in applied biosciences from MIT, in the area of sequencing complex polysaccharides through integrating analytical techniques and bioinformatics.
Fabian Jameison is a reference standards scientist at the United States Pharmacopeial Convention. He is responsible for the development and establishment of reference standards for compendial use. He manages a diverse portfolio of reference standards including small molecules, complex actives and biologicals.

Ali Al-Hakim joined the Food and Drug Administration in 1994 as a review chemist in the division of gastrointestinal and coagulation drug products. He became Heparin Expert in 2001 and was promoted to Chemistry, Manufacturing and Control Lead in 2005. He is currently a branch chief in the Office of New Drug Quality Assessment (Branch II, Division I).

Anita Y. Szajek is a principal scientific liaison in biologics and biotechnology in the Department of Global Science and Standards Division at the United States Pharmacopeial Convention. She is the scientific liaison for the USP Unfractionated Heparin Expert Panel, the USP Low Molecular Weight Heparins Expert Panel, the USP Therapeutic Peptides Expert Panel, and the USP Monographs-B&B I Expert Committee as well as other expert panels. Since the 2007 heparin contamination crisis, she has been working on revising the United States Pharmacopoeia ‘Heparin sodium’ monograph to include new and improved tests to control quality and impurities.

Tina Morris is Vice President, Biologies and Biotechnology, USP-NF at the United States Pharmacopeial Convention. She coordinates all standard-setting activities in the division related to biologics and biotechnology for the US-based compendia and manages the scientific staff responsible for the relevant expert committees, the development of biological reference materials, and the biologics laboratory at the United States Pharmacopeial Convention headquarters. Before she joined the United States Pharmacopeial Convention, her industrial experience included major biotechnology companies in the areas of analytical development, especially mass spectrometry, and recombinant protein characterization.