Anal Bioanal Chem (2014) 406:4815–4823
DOI 10.1007/s00216-014-7940-3

FEATURE ARTICLE

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

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Published online: 24 June 2014

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*

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.

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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 [1]. Heparin sodium drug product (HP) used in medicine consists of chains with molecular weight (MW) ranging from under 5,000 to over 50,000 [2].

Although HP has been used as an injectable antithrombotic medicine for more than 70 years [3], 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 [4]. 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 [5]. 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 [6]. It is reasonable to expect that variations in manufacturing procedures will result in corresponding differences in the MW distribution of the finished HP product. To ensure an acceptable degree of consistency between HP products, and to decide what the

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)-α-L-IdoA(2SO_3^-)-(1→4)-α-D-Glc(NSO_3^-,6SO_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 percentage of the material in question falls within a specified set of ranges. The commonest method for the determination of MW profiles of non-uniform polymers is size-exclusion chromatography (SEC), sometimes called gel permeation chromatography, in which the macromolecular sample of interest passes through a porous gel [12]. For a particular type of molecule, the retention time on a suitable gel (one from which no molecules in the sample are completely excluded or completely included) can be related to molecular size and therefore MW by fitting an empirical function such as a polynomial.

The degree of inclusion within the gel depends on the shape of a molecule, as well as its size. For example, globular proteins do not run through a gel column at the same rate as linear polysaccharides of the same MW [13]. Even within the class of linear polysaccharides, the degree of flexibility of the chain is a factor with a strong effect on SEC retention; universal calibration for heparin using well-characterized narrow fractions of the polysaccharide pullulan is possible only at very high ionic strength [14]. Heparin is an unusually rigid polysaccharide, behaving in solution as a semirigid polymer [15, 16]. It is therefore best to establish reference materials for heparin SEC using heparin itself [17].

For low MW heparin, derived from unfractionated heparin by partial depolymerization, a number of methods have been 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.

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

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 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_n and M_w covering a range of roughly 30 % of the maximum value, giving relative standard deviations (RSDs) of around 10 % [listed with polydispersity (M_w/M_n) values in Table 1]. The most obvious contributing factor to this wide variability was the value chosen for the parameter d*n*/d*c*, 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 mL/g (laboratory 6) and 0.12 mL/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 d*n*/d*c*

Table 1 Average molecular weights (MW) and polydispersity (M_w/M_n) for the proposed USP Heparin Sodium Molecular Weight Calibrant

Laboratory[a]	M_n	M_w	M_w/M_n
1 method 1 (2)	14,935	17,955	1.202
1 method 2 (2)	15,255	20,175	1.322
2 method 1 (5)	14,008	17,626	1.258
2 method 2 (5)	13,932	17,423	1.251
3 (3)	12,200	16,397	1.344
4 (5)	13,351	17,267	1.293
5 method 1 (4)	11,735	15,330	1.307
5 method 2 (4)	12,003	15,578	1.298
5 method 3 (4)	12,675	16,910	1.335
5 method 4 (4)	14,028	17,933	1.279
6 (4)		16,065	
7 method 1 (5)	15,689	19,509	1.243
7 method 2 (3)	15,532	19,318	1.244
7 method 3 (3)	15,185	19,360	1.275
7 method 4 (2)	14,873	20,023	1.346
7 method 5 (2)	16,073	20,220	1.259
7 method 6 (3)	14,370	19,880	1.383
8 (1)	11,070	15,100	1.364
Mean	13,936	17,893	1.3
SD	1,481	1,714	0.05
RSD (%)	10.6	9.6	3.7

RSD relative standard deviation, *SD* standard deviation

[a] The number of independent determinations is given in parentheses.

and was able to show that for the same chromatogram of the proposed calibrant, M_w ranged from 16,403 (d*n*/d*c* = 0.141 mL/g) to 19,306 (d*n*/d*c* = 0.12 mL/g). The other laboratories in the study all used values of d*n*/d*c* between 0.129 and 0.134 mL/g, but excluding laboratory 7, the range for M_w is still high at 15,100–20,175. Therefore, other sources of variability between laboratories are clearly as influential as the d*n*/d*c* 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. To avoid bias

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 1 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 1, are accurate.

Values for d*n*/d*c* 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 using the candidate USP Heparin Sodium Molecular Weight Calibrant reference standard. There was excellent agreement between M_w as determined by SEC and that obtained from analytical ultracentrifugation. With the analytical ultracentrifugation measurements, it was noted that there was some variation with both sample concentration and rotor speed. To further investigate the MW characteristics of the samples, multiple data sets of each sample were fit with one model simultaneously. This results in a single MW estimate for 07/334 of 14,400 and for 97/578 of 16,900. Notably, in every case, there was less than 5 % difference between M_w as calculated by SEC and that calculated by analytical ultracentrifugation.

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.

Intralaboratory and interlaboratory reproducibility as measured using the USP Heparin Sodium Identification reference standard

Seventeen laboratories submitted results for the USP Heparin Sodium Identification reference standard; these results are shown in Table S3. The values shown are those submitted 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

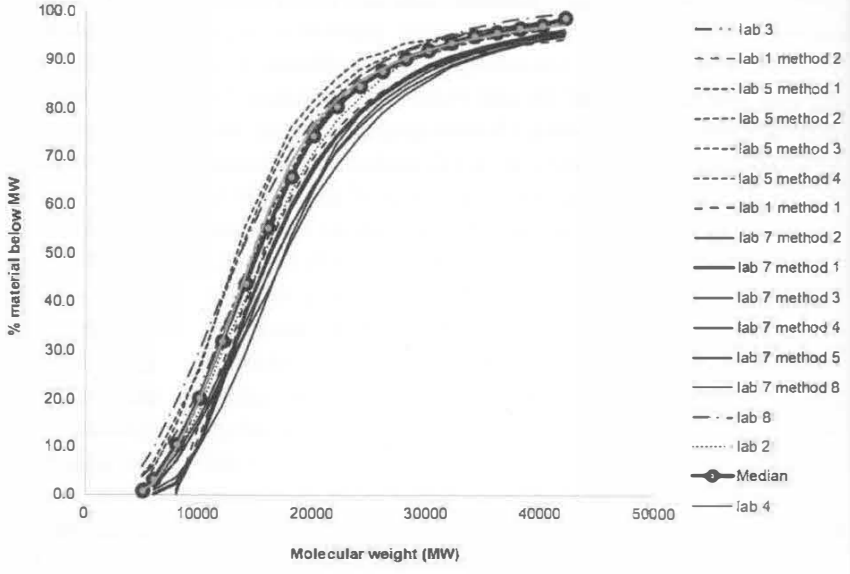

Fig. 1 The molecular weight distribution of the proposed calibrant, as determined by size-exclusion chromatography with light scattering detection in phase 1 of the study, involving eight laboratories. Line styles distinguish between laboratories; the median line has round markers. Results from laboratory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

Table 2 Median broad standard table (BST) for the proposed USP Heparin Sodium Molecular Weight Calibrant reference standard from phase 1 of the study

MW	% below MW	% above MW
6,000	3.2	96.8
8,000	10.4	89.6
10,000	19.8	80.2
12,000	31.7	68.3
14,000	43.4	56.6
6,000	55.5	44.5
18,000	66.0	34.0
20,000	74.4	25.6
22,000	80.3	19.7
24,000	84.4	15.6
26,000	87.5	12.5
28,000	90.1	9.9
32,000	93.4	6.6
36,000	95.6	4.4
40,000	97.0	3.0

requirement for M_w, and all but one laboratory met the requirement for M_p; laboratory 11 did not calculate M_p and did not submit duplicate results for each day of the study. Intralaboratory variability is summarized in Table S4. The standard deviation (SD) for M_w, as measured in individual laboratories, ranged between 22 and 272, resulting in RSD ranging between 0.2 and 1.7 % of the mean. RSDs for the distribution slices were very high in some laboratories for the percentage with M_w below 6,000 and the percentage with M_w below 8,000, but this is the result of low values for the mean, not high values for the SD, so is not a good measure of experimental precision.

Table 3 MWs of two unfractionated heparins, measured using the phase 2 protocol method using the USP Heparin Sodium Molecular Weight Calibrant with the BST from phase 1 of the study (Table 2)

Sample	M_n[a]	M_w[a]	M_w (AUC)[b]
07/334			
Mean	12,125	14,331	14,200
SD	102.4	87.7	700
RSD (%)	0.84	0.61	4.93
97/578 (5th IS)			
Mean	14,339	16,550	16,500
SD	169.0	141.7	600
RSD (%)	1.18	0.86	3.64

AUC analytical ultracentrifugation

[a] Mean values, SD, and RSD for eight independent estimations. M_n, M_w and polydispersity are as defined in the electronic supplementary material.

[b] M_w (AUC) and SD were determined by averaging the results from runs obtained at 30,000 rpm (midpoint) with three different heparin concentrations: 2, 1 and 0.5 mg/mL.

Interlaboratory variability is summarized in Table S5. The mean value for M_w was 15,944, just below the labelled value of 16,000, with SD of 98.7, resulting in an RSD of 0.6 % of the mean. The RSDs are higher for the distribution slices, especially for M_{6000} and M_{8000}, where the mean value is low. All other RSDs were less than 10 %.

The interlaboratory RSD for M_w of less than 1 % compares favourably with that obtained from phase 1 (9.6 %). The combination of simple chromatography, using defined columns and conditions with a common reference material, allows the direct comparison of results obtained in different laboratories. Differences between laboratories in software, calculation and integration protocols do not appear to introduce excessive variation in the results. Results from several laboratories can therefore be pooled into a single set of data for the purpose of setting the acceptance criteria for the USP 'Heparin sodium' monograph. Figures 2 and 3 summarize the data obtained, and were used in discussion of appropriate acceptance criteria. Figure 2a makes it clear that most of the HP lots have M_w between 16,000 and 18,000, with no batches falling below 15,000 or exceeding 20,000. These values are in agreement with other measurements on recent lots [21]. Although there is not a simple 1:1 correspondence between participants and HP manufacturers, Fig. 2a readily shows that, for example, laboratory 2 reported no values for M_w over 17,000 and laboratory 9 reported no values below 17,000. This indicates that the heparin sodium products analysed by laboratory 2 have consistently lower average MWs than those analysed by laboratory 9, implying that the characteristics of heparin sodium products differ systematically between manufacturers.

Figure 2b summarizes the proportion of material in the lots examined with MW over 24,000 (M_{24000}). There is a strong correlation between this value and M_w (r^2=0.874) and a considerable range, from less than 10 % to over 20 %. There is little correlation between M_{8000} (Fig. 2c) and M_w (r^2=0.139).

Figure 3 illustrates the overall distribution of material in all the lots studied. The four MW ranges shown were chosen to define the MW distribution for HP; values for M_{6000}, $M_{6000-10000}$ and $M_{10000-16000}$ were not used. The largest proportion of HP in all the lots falls between 8,000 and 24,000, distributed unevenly around the midway point of 16,000; all the heparin lots have more material in the $M_{8000-16000}$ range than in the $M_{16000-24000}$ range.

Acceptance criteria for the MW distribution of heparin sodium USP

M_w and distribution slice data were reported for 138 lots of HP (a small number of which may be duplicates), from 13 laboratories. The number of lots analysed by one laboratory

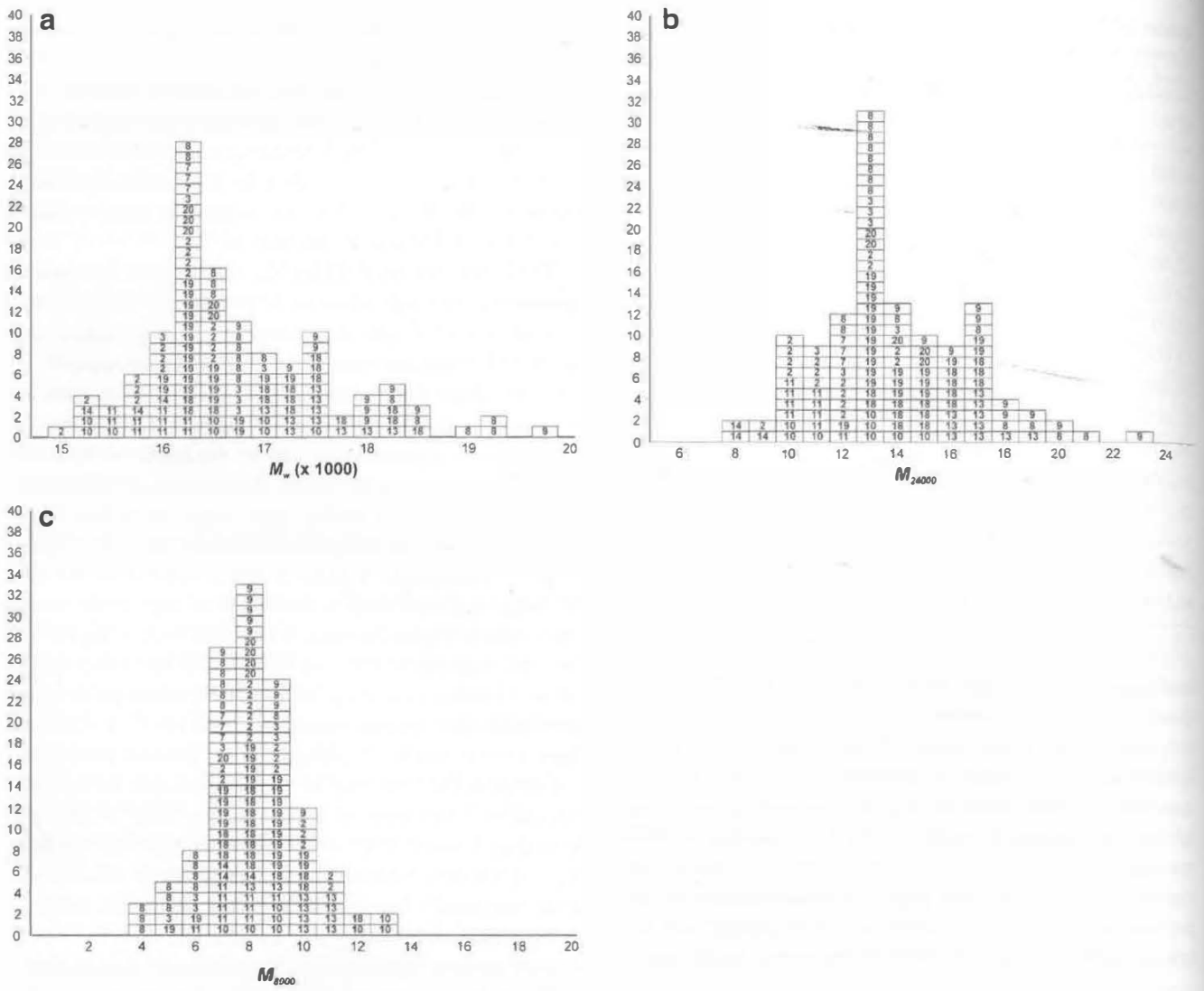

Fig. 2 Values for **a** M_w, **b** M_{24000} and **c** M_{8000} of the heparin sodium lots provided by participants, displayed in histogram format. Each box represents the mean of duplicate determinations in the laboratory specified by its number

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.

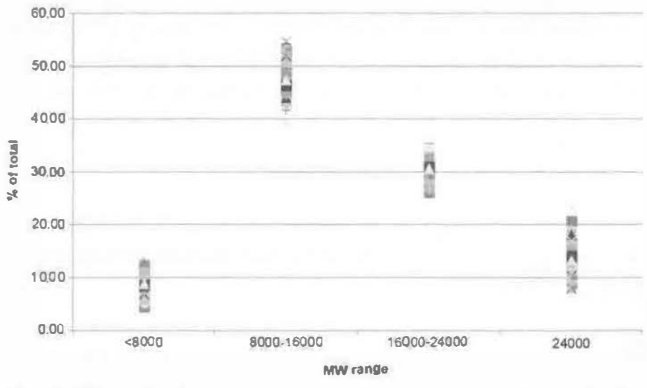

Fig. 3 Values for M_{8000}, $M_{8000-16000}$, $M_{16000-24000}$ and M_{24000} for the active pharmaceutical ingredient lots provided by the participants

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.

The parameters considered were M_w, M_{8000}, $M_{8000-16000}$, $M_{16000-24000}$ and M_{24000}. Polydispersity (M_w/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_{8000} 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 thrombocytopaenia 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 should be interchangeable, although currently this may not be the case [28]. Consistency of physicochemical parameters such as MW helps to ensure this.

Following discussion in the Unfractionated Heparin Expert Panel and a period of public comment, the acceptance criteria to be incorporated in the USP 'Heparin sodium' monograph [29] are as follows:

1. M_{24000} not more than 20 %
2. M_w between 15,000 and 19,000
3. The ratio of $M_{8000-16000}$ to $M_{16000-24000}$ not less than 1.0

The USP Heparin Sodium Molecular Weight Calibrant reference standard and the USP Heparin Sodium Identification reference standard are available from the United States Pharmacopeial Convention (http://www.usp.org/reference-standards).

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 I. Capila and J. Glajch (Momenta Pharmaceuticals, Cambridge, MA, USA), E. K. Chess and W. Wasylenko (Baxter Healthcare, Round Lake, IL, USA), Huihong Fan (NICPBP, Beijing, China), D. Fulchiron (Bay Bioanalytical Laboratories, Hercules, CA, USA), L. Rao and Y. Wang (Scientific Protein Laboratories, Wanaukee, 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, Oss, The Netherlands), P. Torralba (APP, Skokie, IL, USA), S. O. Herr (Leo Pharma, Ballerup, Denmark), H. Chen (Sepax Technologies, Newark, DE, USA), J. Oman (Pfizer, Franklin, OH, USA), R. Van Gorp (Celsus Laboratories, Cincinnati, OH, USA), Z. Xiang (Changzhou Qianhong Biopharm, Cbangzhou City, China), Hao Meixia (Yantai Dongcheng Biochemicals, Yantai, China), H. Stefan-Hinrichs (Sandoz, Kundl, Austria), Li Tan (Shenzhen Hepalink Pharmaceutical, Shenzhen, China), L. Liverani (Opocrin, Corlo di Formigine, Italy), J. Cabañas (Bioiberica, Palafolls, Spain), Yu-juan Song and Huihong Fan (NIFDC, Beijing, China), R. Yin (Chongqing Imperial Biochem, Chongqing, China), G. Gratzl (Boehringer Ingelheim, Ben Vue Laboratories, Bedford, OH, USA), and C. Martinez and P. Anger (Sanofi-Aventis, Aramon, France). Members of the USP Unfractionated Heparin Expert Panel (W. E. Workman, E. Chess, E. Gray, F. Huihong, K. Johansen,P. Torralba and C. Viskov) are thanked for fruitful discussions.

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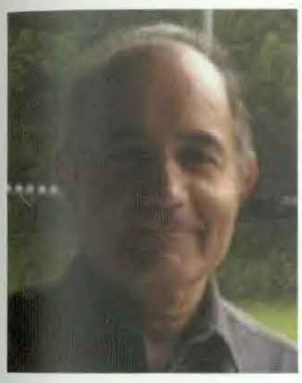

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