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COLLABORATIVE STUDY FOR VALUE ASSIGNMENT OF THE 3rd INTERNATIONAL STANDARD FOR LOW MOLECULAR WEIGHT HEPARIN

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This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Comments MUST be received by 01 October 2012 and should be submitted electronically to the Responsible Officer: Dr Ana Padilla at email: padillaa@who.int, with a copy to Dr David Wood at email: woodd@who.int.

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Summary
Twenty-two laboratories from 13 different countries participated in a collaborative study to value assign the 3rd International Standard for Low Molecular Weight Heparin against the 2nd International Standard for Low Molecular Weight Heparin. Two candidates, sample A (NIBSC code 11/174) and sample B (NIBSC code 11/176) were included in the study

The intra-laboratory variability was low for both candidates. Over 80% of the laboratories obtained potency estimates that had intra-laboratory geometric coefficients of variation (GCV) of less than 5% when the test samples were assayed against the 2nd International Standard for both anti-Xa and anti-IIa activities, indicating that the participants performed these assays reproducibly and with high precision.

For both anti-Xa and anti-IIa activities, inter-laboratory variability was lower for sample B, 11/176 than for sample A, 11/174. The GCVs for anti-Xa and anti-IIa assays for sample B were 3.1% and 3.2% respectively, compared with 6.1% and 8.2% for the anti-Xa and anti-IIa assays for sample A.

Taking into consideration the lower inter-laboratory variation for sample B, we propose to recommend sample B, 11/176, be the 3rd International Standard for Low Molecular Weight Heparin.

It is therefore recommended that the proposed 3rd International Standard for Low Molecular Weight Heparin (11/176) be assigned with potencies for anti-Xa and anti-IIa activity, relative to the 2nd International Standard for Low Molecular Weight Heparin (01/608):

Activity	Proposed 3rd IS IU/ampoule
Anti-Xa	1068
Anti-IIa	342

Introduction
There are at least 8 licensed low molecular weight heparin products and a number of biosimilars available worldwide. The 1st International Standard for Low Molecular Weight heparin established in 1987 successfully harmonised potency labelling of clinical low molecular weight products. The 2nd International Standard (IS) for Low Molecular Weight Heparin (01/608) was established by the Expert Committee on Biological Standardisation (ECBS) of the World Health Organisation (WHO) in October 2003. Because of low stock level, it is now necessary to replace this IS and this study served to evaluate two candidates with a view to value assign a replacement IS against the 2nd IS.

Two prior studies were carried out before the two candidates were chosen for this study. The licensed products are diverse in their biological activity profiles with anti-Xa and anti-IIa ratios ranging from 1.5 to 10. In addition, there are now a new generation of ultra-low low molecular weight heparins with anti-Xa to anti-IIa ratio greater than 80 in phase II and III clinical trials. These pilot studies were required to ensure the best comparators were selected as candidates. The first study was a NIBSC in-house to investigate how well the new generation ultra-low low molecular weight heparin products compare with the current international standard (IS). The results indicated that the current IS can serve as a standard for these products. The second study included 7 licensed low molecular weight heparins and the nine participants of this study were NIBSC, manufacturers of the low molecular weight heparins and pharmacopoeial laboratories. Based on how well they compare with the current IS and other products in anti-Xa and anti-IIa

assays, two preparations were chosen to go forward in the present study as candidates for the replacement of the 2nd IS for Low Molecular Weight Heparin.

Participants

Twenty-three laboratories agreed to take part in the study, with 22 participants from 13 different countries (2 Austria, 1 Australia, 1 China, 1 Denmark, 4 France, 3 Germany, 1 India, 1 Italy, 2 Japan, 1 Sweden, 1 Switzerland, 1 UK, 3 USA) returning data in time for the statistical analysis.

The participants included 8 heparin manufacturers, 3 clinical laboratories, 2 Pharmacopoeias, 4 assay kit manufacturers and 5 regulatory authorities. A list of participants is given in Appendix 1 at the end of this report. Each laboratory is referred to in this report by an arbitrarily assigned number, not necessarily representing the order of listing in the Appendix.

Candidates

Two candidates, NIBSC codes 11/174 and 11/176 were included in the study. The bulk materials were clinical grade APIs. Approximately 10mg/ml was dissolved in sterile water and distributed in glass ampoules, filled and freeze-dried according to guidelines for production of international standards[1]

Samples

The protocol of the study and the following coded samples were sent to each participant:

S - the 2nd International Standard (IS) for Low Molecular Weight Heparin, 01/608
Assigned potency anti-Xa: 1097 IU/ampoule; anti-IIa 326 IU/ampoule.

A - NIBSC code 11/174
Potency range anti-Xa: 925 – 975; anti-IIa 220 – 260 IU/ampoule

B - NIBSC code 11/176
Potency range – anti-Xa: 1000 – 1050; anti-IIa 325 – 375 IU/ampoule

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Assay Methods

Each participant was requested to perform their routine method(s) for anti-Xa and anti-IIa activities. Some laboratories performed more than one method and the data from each method were treated as separate sets of results e.g. Lab 17a and Lab 17b. A list of methods used by the participants is given in Appendix 3.

For both anti-Xa and anti-IIa assays, 5 laboratories carried out the United States Pharmacopoeial (USP) monograph methods and seven laboratories used the European Pharmacopoeial (EP) assays for low molecular mass heparin. One laboratory used the Japanese Pharmacopoeial method. Seven laboratories returned data from their in-house methods. All these methods employed purified antithrombin and proteases (FXa and thrombin). Three laboratories used commercial anti-Xa assay kits that employ plasma as a source of antithrombin, while one laboratory used a commercial kit that involved purified antithrombin. One laboratory used HEPTEST, an anti-Xa clot based assay. Lab 17 also returned data obtained using the Prothrombinase Induced Clotting Time (PiCT) and the Activated Partial Thromboplastin Time (APTT). The laboratory calculated the potencies of the candidates against the anti-Xa value of sample S, the 2nd IS. Since the APTT is based on thrombin inhibition and it is unclear whether

FXa or thrombin inhibition predominates in the PICT assay, the results from these methods were not included in the analysis for the overall potency estimates of samples A and B.

Study Design

Participants were requested to carry out four assays for each activity using fresh ampoules of samples S, A, and B in each assay. Laboratories were requested to assay anti-Xa and anti-IIa activities on the same ampoules. Within each assay, participants were requested to assay three dilutions (preferably four dilutions) of each of the samples S, A, and B in replicate, according to balanced assay designs.

Raw assay data were returned together with calculated estimates for samples A (11/174) and B (11/176) relative to sample S (2nd IS, 01/608) from each individual assay.

Statistical Analysis

An independent statistical analysis of raw data was performed at NIBSC. Potency estimates relative to the 2nd International Standard (01/608), were calculated by parallel-line analysis[2] of assay response (absorbance or clotting time) against log concentration, independently for each test sample included in each assay. For the majority of laboratories untransformed assay responses were used in analysis, with log transformed responses being used in a small number of laboratories in order to obtain a linear dose-response relationship. Assay validity was assessed by analysis of variance and any deviations from linearity and parallelism were considered significant at the 1% level ($p<0.01$). Where significant deviations from the model appeared to result from underestimation of residual error, linearity was assessed by visual inspection of the plotted data and non-parallelism was assessed using deviations from linearity as an alternative residual error. Any assays rejected for deviations from linearity or parallelism are indicated in the tables of results. Results from all valid assays were combined to generate unweighted geometric mean potencies for each laboratory and these laboratory means were used to calculate overall unweighted geometric mean potencies. Variability between assays and laboratories has been expressed using geometric coefficients of variation (GCV = $\{10^s-1\}\times 100\%$ where s is the standard deviation of the $\log_{10}$ transformed potencies). Grubbs' Test[2] was applied to the log transformed laboratory mean potencies in order to detect any significant outliers. Comparisons between methods have been made by unpaired t-tests of log transformed laboratory mean potencies. In general, the laboratories' reported potency estimates were close to those calculated centrally by NIBSC (data not shown).

Results

Assay Data

Twenty two participants returned a total of 188 assays which comprised 100 anti-Xa assays, and 80 anti-IIa assays. Lab 17 returned 8 assays from APTT and PiCT methods and these were not centrally analysed by NIBSC. The individual assay results calculated by NIBSC, together with the geometric mean potencies (GM) and the intra-laboratory variation expressed as GCV are presented in Table 1a, 1b, 2a and 2b.

Assay Validity

All assays were valid as defined by criteria set out in the Statistical Analysis Section of this report.

Sample A, 11/174

Intra- and inter- laboratory variability
Estimates of intra-laboratory variability (between assays) for both anti-Xa and anti-IIa activities in sample A are given as geometric coefficients of variation (GCV) for potency estimates relative to sample S (Tables 1a and 2a). These exceeded 5% in 5 and 4 laboratories for anti-Xa and anti-IIa assays respectively, with 80% of cases giving GCV lower than 5%. Within each assay type, as shown by the range of intra-laboratory GCVs, there were no obvious differences between the reproducibility of the different tests methods used by the participants.

Inter-laboratory variability was low for both anti-Xa and anti-IIa assays, being 6.1 and 8.2%, respectively, showing reasonably good agreement between laboratories.

Potency estimates
The overall potency estimates for sample A (11/174) were calculated relative to the assigned values for the 2nd IS. Laboratory mean potency estimates and overall potency estimates, together with 95% confidence limits, for anti-Xa and anti-IIa activities are shown in Table 1a and Table 2a. The potency estimates relative to the 2nd IS from individual assays are also presented in histogram form in Figures 1a and 2a. The histograms illustrate good agreement between laboratories for sample A relative to the 2nd IS for activities; with no outliers detected. The overall geometric mean anti-Xa and anti-IIa potency estimates were 1030 and 275 IU/ampoule respectively, with an anti-Xa to anti-IIa ratio of 3.75 (Table 3).

Sample B, 11/176

Intra- and inter- laboratory variability
Estimates of intra-laboratory variability for both anti-Xa and anti-IIa activities in sample B are given as geometric coefficients of variation (GCV) for potency estimates relative to sample S (Table 1b and 2b). These exceeded 5% in only 6 and 3 laboratories for anti-Xa and anti-IIa assays respectively, with 80% of cases giving a GCV lower than 5%.

Inter-laboratory variability was exceptionally low at 3.1 and 3.2%, respectively, for anti-Xa and anti-IIa assays, showing good agreement between laboratories.

Potency estimates
The overall potency estimates and 95% confidence limits for test sample B calculated relative to the assigned values for the 2nd IS are shown in Tables 1b and 2b, together with laboratory mean potency estimates. The potency estimates relative to the 2nd IS from individual assays are also presented in histogram form in Figures 2a and 2b. The histograms illustrate good agreement between laboratories for sample B relative to the 2nd IS for both activities; with no detectable outliers. The overall geometric mean anti-Xa and anti-IIa potency estimates were 1068 and 342 IU/ampoule respectively, with the anti-Xa to anti-IIa ratio being 3.12 (Table 3).

Discussion
The primary aim of this study was to value assign a replacement International Standard for Low Molecular Weight Heparin.

For both anti-Xa and anti-IIa activities, over 80% of the participants carried out methods that employed purified antithrombin, factor Xa and thrombin. The majority of the intra-laboratory GCVs were below 5%, indicating that these assays are reasonably robust and reproducible within each laboratory. Approximately 60% of the laboratories carried out pharmacopoeial methods.

There were no significant differences in the anti-Xa and anti-IIa potencies obtained using either the USP or EP monograph methods for both samples(Tables 4a and 4b). The inter-laboratory agreement as expressed using GCVs for both pharmacopoeial methods was also similar. The overall geometric mean potencies by each pharmacopoeial method for sample B, 11/176, were closer to the overall geometric mean potencies than those obtained for sample A (Tables 4a and 4b).

No outliers were detected for either anti-Xa or anti-IIa assays for both samples A and B. The results of PiCT and APTT assays returned by Lab 17 were not included in the calculation of the overall geometric mean anti-Xa potencies of samples A and B as it was unclear whether these data should be included into the anti-Xa group (as performed by the participant). Better agreement between laboratories was obtained for sample B than for sample A. The inter-laboratory variability for sample A, 11/174, expressed as GCVs was 6.1% and 8.2% for anti-Xa and anti-IIa activity respectively. For sample B, 11/176, the inter-laboratory GCVs was 3.1% and 3.2% for anti-Xa and anti-IIa activity (Tables 1a, 1b, 2a and 2b). Table 6 shows the physical characteristics of sample B, in terms of the variability of the fill, residual moisture content and head oxygen space and homogeneity by functional activities were acceptable. In addition, preliminary accelerated degradation study on ampoules of sample B that have been stored at elevated temperature for 5 months showed that there is no apparent loss of anti-Xa and anti-IIa activities when stored at 20°C (Table 7a). Table 7b shows that sample B is relatively stable following reconstitution and no loss of activity was detected 5 hours upon reconstitution when stored at ambient temperature. Since sample B, 11/176, gave lower inter-laboratory variation, closer potency estimates to the overall values by EP and USP methods and reasonable stability, it will serve well as a replacement for the current 2nd IS.

Proposal to Participants

Overall, sample B, 11/176, gave better inter-laboratory agreement for both anti-Xa and anti-IIa activity and the overall geometric mean potencies of sample B were in good agreement with the mean estimates by both the EP and USP monograph methods. It is therefore recommended that sample B, 11/176, be the replacement international standard for low molecular weight heparin, with value assigned using all laboratory mean estimates for both anti-Xa and anti-IIa activities.

Responses from participants and the experts nominated by the SSC/ISTH Control of Anticoagulation Sub-Committee

All participants and SSC nominated experts who have sent in responses agreed with the proposals. There were no comments in relation to the analysis or interpretation of the data in the study.

Proposal and Recommendation to the ECBS

Sample B, 11/176, be the 3rd International Standard for Low Molecular Weight Heparin, 11/176, relative to the 2nd IS

Activity	Proposed 3rd IS IU/ampoule
Anti-Xa	1068
Anti-IIa	342

A draft of "The Instruction for Use" for the proposed Standard, 11/176 is illustrated in Appendix 4.

References

(1) Finney DJ. Statistical Method in Biological Assay. 3rd Edition. London: Charles Griffin 1978.
(2) Grubbs F. Procedures for Detecting Outlying Observations in Samples. Technometrics, 1969; 11: 1-21.

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- Sanofi
- Pfizer
- Leo
- Opocrin
- Rovi
- GSK

Table 1a: Table 1a: Potency estimates, intra- and inter-laboratory GCV for Anti-Xa activity in sample A relative to sample S, the 2nd IS for Low Molecular Weight Heparin

		Estimates (IU/ampoule)					
Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
1	Purified AT	972	963	970	974	970	0.5%
2	Purified AT	987	991	993	986	989	0.3%
4	Purified AT	976	941	973	964	963	1.7%
5	Purified AT	1056	1019	1014	1005	1023	2.2%
7	Purified AT	1040	1006	1041	1032	1030	1.6%
8	Purified AT	973	1021	1008	1094	1023	5.0%
9a	Purified AT	1180	1166	1202	1134	1170	2.5%
9b	Purified AT	1059	1064	1071	1036	1057	1.5%
10	Purified AT	983	985	983	1019	993	1.8%
11	Purified AT	1073	1063	1127	1044	1076	3.3%
13	Purified AT	956	948	962	944	952	0.9%
14	Purified AT	1024	968	970	987	987	2.6%
16	Purified AT	1026	1003	972	975	994	2.6%
17a	Purified AT	1021	1024	960	1092	1023	5.4%
17b	Purified AT	1074	1154	996	1149	1091	7.2%
18	Purified AT	1154	1151	1197	1131	1158	2.4%
19	Purified AT	981	1025	978	1033	1004	2.9%
20	Purified AT	1083	1046	1078	1079	1072	1.6%
21	Purified AT	1009	955	1009	976	987	2.7%
22	Purified AT	909	928	991	926	938	3.8%
23	Purified AT	1042	1049	1219	1088	1097	7.5%
3	plasma AT	1110	1063	1149	1186	1126	4.8%
6	plasma AT	990	956	1034	1121	1023	7.1%
12	plasma AT	1072	1046	1058	1033	1052	1.6%
17c	HEPTEST	859	999	1090	1022	989	10.6%
		Overall GM IU/ampoule		1030			
		95% CL		1005 - 1055			
		Between Lab GCV		6.1%			

GM: geometric mean; GCV: geometric coefficient of variation; CL: confidence limits. Purified AT: assays using purified antithrombin; Plasma AT; assays using plasma as source of antithrombin

Table 1b: Potency estimates, intra- and inter-laboratory GCV for Anti-Xa activity in sample B relative to sample S, the 2nd IS for Low Molecular Weight Heparin

		Estimates (IU/ampoule)					
Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
1	Purified AT	1013	1011	1005	1017	1012	0.5%
2	Purified AT	1076	1085	1124	1061	1086	2.5%
4	Purified AT	1062	1085	1080	1077	1076	0.9%
5	Purified AT	1036	1069	1058	1047	1052	1.3%
7	Purified AT	1059	1073	1060	1073	1066	0.7%
8	Purified AT	1105	1165	1137	1135	1135	2.2%
9a	Purified AT	1007	1059	1076	1087	1057	3.5%
9b	Purified AT	1051	1094	1066	1057	1067	1.8%
10	Purified AT	1019	1073	1085	1098	1068	3.3%
11	Purified AT	1036	1042	1096	1103	1069	3.4%
13	Purified AT	968	990	1059	1077	1022	5.3%
14	Purified AT	1098	1022	1075	1052	1061	3.1%
16	Purified AT	1047	1036	1015	1017	1029	1.5%
17a	Purified AT	1029	1047	1033	1086	1048	2.5%
17b	Purified AT	1131	1092	984	1073	1068	6.1%
18	Purified AT	1035	1035	1098	1085	1063	3.2%
19	Purified AT	1069	1101	1022	997	1047	4.6%
20	Purified AT	1091	1064	1095	1096	1087	1.4%
21	Purified AT	1061	1060	1061	1009	1048	2.5%
22	Purified AT	1012	1164	1017	1084	1068	6.8%
23	Purified AT	1065	1062	1268	1106	1122	8.7%
3	plasma AT	1009	1040	1014	1041	1026	1.7%
6	plasma AT	1065	988	1134	1297	1115	12.2%
12	plasma AT	1095	1072	1069	1032	1067	2.5%
17c	HEPTEST	1048	1208	1165	1157	1143	6.3%
		Overall GM IU/ampoule		1068			
		95% CL		1054 - 1081			
		Between Lab GCV		3.1%			

GM: geometric mean; GCV: geometric coefficient of variation; CL: confidence limits. Purified AT: assays using purified antithrombin; Plasma AT; assays using plasma as source of antithrombin

Table 2a: Potency estimates, intra- and inter-laboratory GCV for Anti-IIa activity in sample A relative to sample S, the 2nd IS for Low Molecular Weight Heparin

		Estimates (IU/ampoule)					
Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
1	Purified AT	249	249	257	236	248	3.5%
2	Purified AT	271	278	280	285	278	2.1%
4	Purified AT	254	244	241	252	248	2.6%
5	Purified AT	279	250	266	275	267	5.1%
7	Purified AT	272	266	268	273	270	1.2%
8	Purified AT	287	271	279	290	281	3.1%
9a	Purified AT	303	361	300	288	312	10.6%
10	Purified AT	280	279	276	266	275	2.5%
11	Purified AT	293	288	297	292	293	1.2%
13	Purified AT	268	268	274	269	270	1.2%
14	Purified AT	251	256	244	250	250	1.9%
16	Purified AT	288	287	283	295	288	1.7%
17	Purified AT	312	278	271	284	286	6.3%
18	Purified AT	313	314	288	320	308	4.8%
19	Purified AT	246	260	285	304	273	9.9%
20	Purified AT	288	288	291	292	290	0.8%
21	Purified AT	263	262	253	274	263	3.3%
22	Purified AT	241	218	230	229	229	4.1%
23	Purified AT	268	271	279	283	275	2.6%
9b	JP Clot	309	308	308	311	309	0.5%
		Overall GM IU/ampoule			275		
		95% CL			265 - 285		
		Between Lab GCV			8.2%		

GM: geometric mean; GCV: geometric coefficient of variation; CL: confidence limits. Purified AT: assays using purified antithrombin; JP Clot: Japanese Pharmacopoeia clot-based assays

Table 2b: Potency estimates, intra- and inter-laboratory GCV for Anti-IIa activity in sample B relative to sample S, the 2nd IS for Low Molecular Weight Heparin

		Estimates (IU/ampoule)					
Lab	Method	Assay 1	Assay 2	Assay 3	Assay 4	GM	GCV
1	Purified AT	347	351	361	368	356	2.7%
2	Purified AT	330	328	340	345	336	2.4%
4	Purified AT	354	349	354	354	353	0.6%
5	Purified AT	333	349	314	342	334	4.7%
7	Purified AT	327	329	326	333	329	0.9%
8	Purified AT	347	354	350	367	354	2.4%
9a	Purified AT	349	348	334	333	341	2.6%
10	Purified AT	344	344	352	345	346	1.2%
11	Purified AT	362	335	334	339	342	3.9%
13	Purified AT	331	328	334	338	333	1.4%
14	Purified AT	341	332	332	335	335	1.3%
16	Purified AT	319	329	321	326	324	1.5%
17	Purified AT	355	319	331	319	331	5.3%
18	Purified AT	340	352	312	340	336	5.3%
19	Purified AT	359	358	346	334	349	3.4%
20	Purified AT	352	346	343	347	347	1.0%
21	Purified AT	348	340	336	356	345	2.5%
22	Purified AT	372	346	355	353	356	3.2%
23	Purified AT	316	313	352	339	330	5.8%
9b	JP Clot	353	359	367	359	359	1.6%
		Overall GM IU/ampoule			342		
		95% CL			337 - 347		
		Between Lab GCV			3.2%		

GM: geometric mean; GCV: geometric coefficient of variation; CL: confidence limits. Purified AT: assays using purified antithrombin; JP Clot: Japanese Pharmacopoeia clot-based assays

Table 3: Summary of Potency estimates and anti-Xa to anti-IIa ratios for samples A and B

	Sample A, 11/174	Sample B, 11/176
Anti-Xa activity IU/ampoule	1030	1068
Anti-IIa activity IU/ampoule	275	342
Anti-Xa:anti-IIa ratio	3.75	3.12

Table 4a: Comparison of potency estimates from USP and EP methods for sample A relative to the 2nd IS

Methods	EP		% from the overall mean potency	USP		% from the overall mean potency	T-Test (p)
	IU/ampoule	GCV		IU/ampoule	GCV		
Anti-Xa	982 (n = 7)	3.0%	4.7	995 (n = 5)	3.1%	3.4	>0.05
Anti-IIa	261 (n = 7)	6.9%	5.1	264 (n = 5)	6.1%	4.0	>0.05

GCV: geometric coefficient of variation;

Table 4b: Comparison of potency estimates from USP and EP methods for sample B relative to the 2nd IS

Methods	EP		% from the overall mean potency	USP		% from the overall mean potency	T-Test (p)
	IU/ampoule	GCV		IU/ampoule	GCV		
Anti-Xa	1055 (n = 7)	1.9%	1.2	1071 (n = 5)	4.2%	0.3	>0.05
Anti-IIa	341 (n = 7)	2.6%	0.3	347 (n = 5)	3.3%	1.6	>0.05

GCV: geometric coefficient of variation;

Table 5a. Sample A, 11/174: Lab 17, Laboratory reported results for PiCT and APTT

	Assay 1	Assay 2	Assay 3	Assay 3	Mean
PiCT	978.2	968.1	930.8	894.4	942.9
APTT	952.8	973.6	1034.3	941.2	975.5

Table 5b. Sample B, 11/176: Lab 17, Laboratory reported results for PiCT and APTT

	Assay 1	Assay 2	Assay 3	Assay 3	Mean
PiCT	1192.4	1201.4	1140.1	999.9	1133.5
APTT	1204.2	1322.3	1347.0	1101.0	1243.6

Table 6: Product characteristics for sample B, 11/176

<table>
<tr><td>NIBSC Code</td><td colspan="3">11/176</td></tr>
<tr><td>Presentation</td><td colspan="3">Sealed, glass 3 ml DIN ampoules</td></tr>
<tr><td>Filling date</td><td colspan="3">22th Sept 2011</td></tr>
<tr><td>Number of Ampoules available</td><td colspan="3">24,000</td></tr>
<tr><td>Liquid filling weight (g) (n=879, measurements taken from all 3 pumps throughout the duration of the fill)</td><td colspan="3">1.0068 (Range 1.0045 - 1.0090)</td></tr>
<tr><td>CV of fill mass (%)</td><td colspan="3">0.097</td></tr>
<tr><td rowspan="3">Homogeneity of the fill by activity: 3 ampoules selected from the beginning of the fill, after every 5000 ampoules and at the end of the fill were assayed against the 2nd IS using EP assays. 2 assays per ampoule were carried out. Effect of fill position assessed by ANOVA of log potencies.</td><td></td><td>GCV</td><td>P</td></tr>
<tr><td>Anti-Xa</td><td>2.62%</td><td>0.814</td></tr>
<tr><td>Anti-IIa</td><td>0.85%</td><td>0.974</td></tr>
<tr><td>Mean dry weight (g) (n=25)</td><td colspan="3">0.00922 (CV 1.39%)</td></tr>
<tr><td>Mean head space oxygen (%) (n=24)</td><td colspan="3">0.47 (CV 33.56%)</td></tr>
<tr><td>Residual moisture (%) (n=3)</td><td colspan="3">3.41 (CV 24.23%)</td></tr>
<tr><td>Storage temperature</td><td colspan="3">-20°C</td></tr>
<tr><td>Address of processing facility</td><td colspan="3">NIBSC, Potters Bar, EN6 3QG, UK</td></tr>
<tr><td>Address of present custodian</td><td colspan="3">NIBSC, Potters Bar, EN6 3QG, UK</td></tr>
</table>

Table 7a: Accelerated Degradation Study on sample B, 11/176

Storage temperature	Anti-Xa activity IU/ampoule (relative to -150°C)	Confidence limits (95 %)	Anti-IIa activity IU/ampoule (relative to -150°C)	Confidence limits (95 %)
-70°C	1.055	1.000-1.114	0.995	0.926-1.070
-20°C	1.093	1.035-1.153	1.019	0.948-1.100
4°C	1.048	0.993-1.106	1.002	0.932-1.077
20°C	1.018	0.965-1.075	0.950	0.884-1.022
37°C	0.946	0.896-0.998	0.928	0.863-0.997
45°C	0.979	0.928-1.033	0.903	0.840-0.971
56°C	0.947	0.897-0.999	0.824	0.766-0.886

An accelerated degradation study of 11/176 was carried out at NIBSC, employing anti-Xa and anti-IIa assays with purified reagents. Table indicates the residual potency for ampoules stored at elevated temperatures for 5 months relative to ampoules stored at -150°C. Data from one time-point (5 months) indicated that the material is stable. No significant loss of activity is observed after storage up to 20°C after 5 months compared to -150°C. Continual real time degradation studies of the –20°C against ampoules stored at –150°C and further accelerated degradation studies at elevated temperatures will be carried out to monitor the stability of the replacement standard.

Table 7b: On-bench stability of sample B, 11/176

Time	Anti-Xa activity (IU/ampoule)	Confidence limits (95 %)	Anti-IIa activity (IU/ampoule)	Confidence limits (95 %)
0 h	1110	1079-1143	341	329-353
5 h	1113	1083-1144	358	339-377

Assessment of on-bench stability was carried out at NIBSC by storage of the reconstituted sample at room temperature (22°C). The anti-Xa and anti-IIa activities at 0 h, and 5 h were estimated relative to freshly reconstituted 2nd IS LMWH 01/608 at each time-point. Two assays were carried out for each method. No significant difference is observed between the potency values for the two time points. This shows that the material is stable after 5 hours storage at room temperature of 22°C.

Figure 1a: Individual assay potency estimates for anti-Xa activity in sample A (11/146), relative to sample S, the 2nd IS. The number in the square denotes the laboratory code.

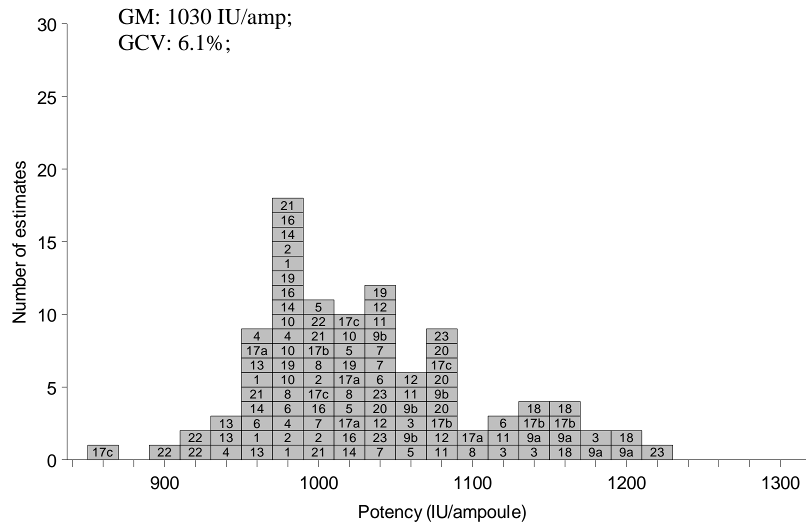


Figure 1b: Individual assay potency estimates for anti-IIa activity in sample A (11/146), relative to sample S, the 2nd IS. The number in the square denotes the laboratory code.

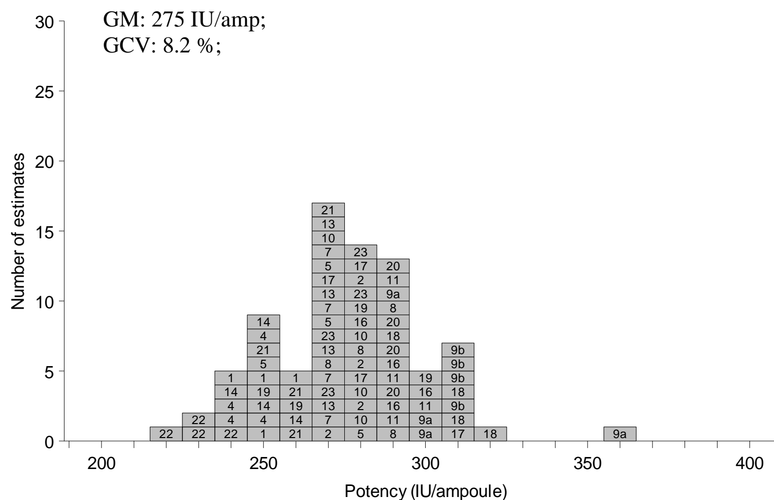


Figure 2a: Individual assay potency estimates for anti-Xa activity in sample B (11/147), relative to sample S, the 2nd IS. The number in the square denotes the laboratory code.

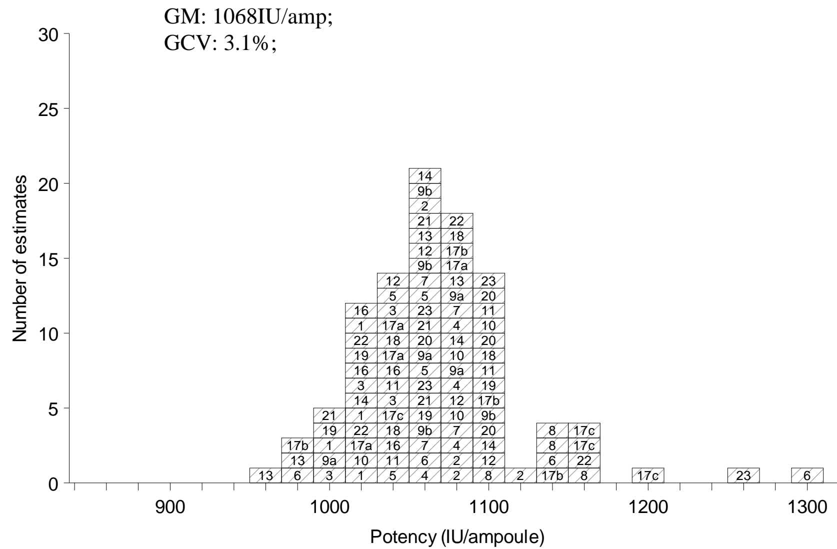
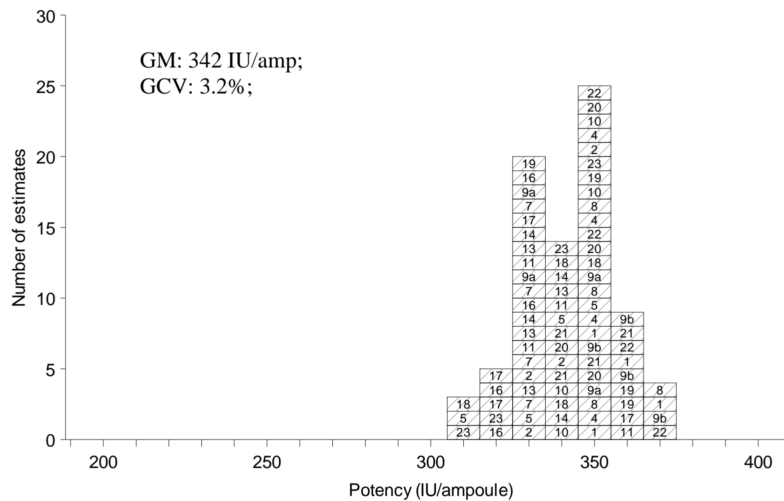


Figure 2b: Individual assay estimates for anti-IIa activity in sample B (11/147), relative to sample S, the 2nd IS. The number in the square denotes the laboratory code.



Appendix 1: List of Participants

Patrick Shaklee, BioCascade Incorporated, USA

Susanne Alban, Christian-Albrechts-University of Kiel, Germany

Stephaine Berrier and Caroline Bisson, Diagnostica Stago, France

Guy Rautmann and Gwenaelle Cozic, European Directorate for the Quality of Medicines, France

C.S. Venkatesan, Gland Pharma Ltd, India

Patrick Rousseau, GlaxoSmithKline, France

Bjørn Fehrmann and Trine Larsen, LEO Pharma A/S, Denmark

Jeanine Walenga and Debra Hoppensteadt, Loyola University Chicago, USA

Fan Huihong and Li Jing, National Institute for Food and Drug Control, P.R. China

Takuo Suzuki, Akiko Ishii and Nana Kawasaki, National Institute of Health Sciences, Japan

John Hogwood, National Institute for Biological Standards and Control, UK

Hinrich Nagel, Nordmark Arzneimittel GmbH & Co KG, Germany

Bruna Parma, Opocrin SpA, Italy

Anders Svensson, Pfizer Health AB, Sweden

Yukari Nakagawa, Pharmaceutical and Medical Device Regulatory Science Society of Japan, Japan

Andreas Weiler and Manuel Seebacher, Sandoz GmbH, Austria

Céline Martinez, Sanofi-Aventis, France

Wolfgang Klein, Siemens Healthcare Diagnostics Products GmbH, Germany

Roger Meier, Swissmedic, Switzerland

Sabine Geiter and Matrina Leiter, Technoclone GmbH, Austria

Allison Jones and Chong Loh, Therapeutics Goods Administration, Australia

Michael Ambrose and Jeanne Fringer, US Pharmacopia, USA

Appendix 2: Protocol of the study

Collaborative study to establish
the 3rd International Standard for Low Molecular Weight Heparin

Aims of Study

The aim of this study is to assay two candidate low molecular weight heparin materials against the 2nd International Standard, 01/608, with the view to establish a new material as the 3rd International Standard for Low Molecular Weight Heparin

Low molecular weight heparin samples provided:

S - the 2nd IS for Low Molecular Weight Heparin, 01/608
Potency anti-Xa: 1097 IU/ampoule; anti-IIa 326 IU/ampoule.

A - Potency range – anti-Xa: 925 – 975; anti-IIa 220 – 260 IU/ampoule

B - Potency range – anti-Xa: 1000 – 1050; anti-IIa 325 – 375 IU/ampoule

Storage and Reconstitution

The samples should be handled as follows:

1. Store all unopened ampoules -20°C or below
2. Ampoules should be allowed to warm to room temperature before reconstitution.
3. Please ensure that all material is at the bottom of the ampoule before opening.
4. DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body. Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
5. Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.
6. Reconstitute the ampoule contents by adding 1 ml of distilled water. Allow the ampoule to stand for 10 minutes at room temperature and aid reconstitution by gentle swirling. Transfer contents to a plastic tube and store at 4°C prior to the assays.

Assay Methods

Please perform in-house routine anti-Xa and anti-IIa methods for low molecular weight heparin, and provide details of the method in the results sheet provide..

Design and number of Assays

Four sets of ampoules are provided. Assays for anti-IIa and anti-Xa should be carried out on each of the 4 sets. Each set should be tested on a different day (see schedule below). A balanced order of testing should be used.

Day 1, ampoule set 1	S1	A1	B1	B2	A2	S2
Day 2, ampoule set 2	A1	S1	B1	B2	S2	A2
Day 3, ampoule set 3	B1	A1	S1	S2	A2	B2
Day 4, ampoule set 4	S1	B1	A1	A2	B2	S2

Each letter refers to a set of three (preferably four) different dilutions (e.g. 1/10, 1/20, 1/40) and **A1, A2** and **S1, S2** etc. refer to separate sets of dilutions (replicates) made independently from the same ampoule. The range of dilutions should be chosen to lie on the linear portion of the dose-response curve.

Report of data

Raw data and estimated potencies should be recorded on in the forwarded excel sheets, sent to participants with this protocol once the "Acknowledgement of samples receipt form" has been returned.

Results should be returned by **Thursday 15th March 2012**, results returned after this date may not be included in the analysis. Please contact john.hogwood@nibsc.hpa.org.uk if this will cause any issues, or you require any further information about the study.

Appendix 3: Methods used by the Participants

Lab	Anti-Xa	Anti-IIa	Other methods
01	USP	USP	
02	EP	EP	
03	Commercial Kit, plasma as source of antithrombin	NT	
04	USP	USP	
05	EP	EP	
06	Commercial Kit, plasma as source of antithrombin	NT	
07	USP	USP	
08	USP	USP	
09	a. In-house b. JP	a. In-house	Clot based, possibly APTT
10	USP	USP	
11	In-house	In-house	
12	Commercial Kit, plasma as source of antithrombin	NT	
13	EP	EP	
14	EP	EP	
15	Results not returned	Results not returned	
16	In-house	In-house	
17	a. Commercial Kit, purified antithrombin b. In-house c. HEPTEST	In-house	APTT and PICT, but used anti-Xa potency of the standard to calculate the estimates of the test samples
18	USP	USP	
19	EP	EP	
20	In-house	In-house	
21	EP	EP	
22	EP	EP	
23	In-house	In-house	

USP: United States Pharmacopoeia; EP: European Pharmacopoeia; JP: Japanese Pharmacopoeia; NT: not tested

Appendix 4: Draft Instruction for Use (IFU) for the Proposed 3rd International Standard for Low Molecular Weight Heparin 11/176

WHO International Standard
The 3rd International Standard for Low Molecular Weight Heparin
NIBSC code: 11/176
Instructions for use
(Version 1.00, Dated)

1. INTENDED USE

The 3rd International Standard for Low Molecular Weight Heparin, consists of ampoules, coded 11/176, containing aliquots of a freeze-dried material prepared from porcine mucosa. This preparation was established as the 3rd International Standard for Low Molecular Weight Heparin by the Expert Committee on Biological Standardisation of the World Health Organisation in 2012. It is intended for anti-Xa and anti-IIa potency estimation of low molecular weight heparin.

2. CAUTION

<u>This preparation is not for administration to humans</u>.

The material is not of human or bovine origin. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The standard was calibrated by 22 laboratories from 13 countries against the 2nd International Standard for Low Molecular Weight Heparin (01/608). Anti-Xa and anti-IIa chromogenic methods were used in the study. Twenty-two laboratories performed anti-Xa assays (100 assays in total) and anti-IIa assays (80 assays in total). The following potencies were assigned based on the geometric mean of all the valid assay results:

1068 International Units of anti-Xa activity per ampoule
342 International Units of anti-IIa activity per ampoule

Uncertainty: the assigned unitage does not carry an uncertainty associated with its calibration. The uncertainty may therefore be considered to be the variance of the ampoule content and was determined to be +/- 0.1%.

4. CONTENTS

Country of origin of biological material: France.
The mean weight of liquid content of 879 check weight ampoules was 1.0068g, with a coefficient of variation of 0.097%. The mean weight of the freeze-dried plug was 9.22mg, with a coefficient of variation of 1.39%. The mean residual moisture was 3.41%.

5. STORAGE

Unopened ampoules should be stored in the dark at or below –20°C.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

<u>No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution</u>
Allow ampoules to warm to room temperature. Open ampoule, taking care to ensure that all material is in the lower part, and reconstitute with 1.0 mL of distilled water. Low molecular weight heparin is reasonably stable and aliquots of the reconstituted solution, at a suitable concentration (eg 100 anti-Xa IU/mL) could be stored frozen at –40°C or below for up to 6 months.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials.

9. REFERENCES

N/A

10. ACKNOWLEDGEMENTS

PAll participants in the international collaborative study. We are also grateful to the following manufacturers for their kind donation of low molecular weight heparin samples, two of which were used as candidates for the collaborative study:

Leo Pharmaceutical Products Ltd
Laboratories Rovi SA
Opocrin SpA
Sanofi Synthelabo
Pfizer
GSK

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.hpa.org.uk
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.ac.uk/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.ac.uk/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.ac.uk/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.hpa.org.uk

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC

code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties			
Physical appearance: White freeze-dried solid		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	Yes	Irritant:	Yes
Flammable:	No	Handling:See caution, Section 2	
Other (specify):	Contains material of porcine origin		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

Information provided by the Institute is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but it is provided without liability to the Recipient in its application and use.
It is the responsibility of the Recipient to determine the appropriateness of the standards or reference materials supplied by the Institute to the Recipient ("the Goods") for the proposed application and ensure that it has the necessary technical skills to determine that they are appropriate. Results obtained from the Goods are likely to be dependant on conditions of use by the Recipient and the variability of materials beyond the control of the Institute.
All warranties are excluded to the fullest extent permitted by law, including without limitation that the Goods are free from infectious agents or that the supply of Goods will not infringe any rights of any third party.
The Institute shall not be liable to the Recipient for any economic loss whether direct or indirect, which arise in connection with this agreement.
The total liability of the Institute in connection with this agreement, whether for negligence or breach of contract or otherwise, shall in no event exceed 120% of any price paid or payable by the Recipient for the supply of the Goods.
If any of the Goods supplied by the Institute should prove not to meet their specification when stored and used correctly (and provided that the Recipient has returned the Goods to the Institute together with written notification of such alleged defect within seven days of the time when the Recipient discovers or ought to have discovered the defect), the Institute shall either replace the Goods or, at its sole option, refund the handling charge provided that performance of either one of the above options shall constitute an entire discharge of the Institute's liability under this Condition.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 9.22 mg
Toxicity Statement: Toxicity not assessed
Veterinary certificate or other statement if applicable. **Attached:** No