

Value Assignment of the WHO 6th International Standard for Unfractionated Heparin and USP Reference Standard, Heparin Sodium for Assay (Lot F0I187)

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Assuring the quality of biological medicines



Heparin Potency Standards



- For the last 30 years or more, there has been a 7 -13% disparity between the USP unit and the International Unit for Heparin
- The implementation of the new USP monograph potency method requires the calibration and establishment of a new USP Reference Standard
- The need to replace the current 5th International Standard for Unfractionated Heparin provided an ideal opportunity to value assign the replacement International Standard and the USP Reference Standard against the 5th IS in the same exercise, thus harmonising the USP unit and the International Unit for Heparin
- The collaborative study also allowed the evaluation of the USP potency method, an anti-factor IIa chromogenic assay based on the 1998 procedure drafted by the WHO drafting group for the measurement of unfractionated heparin

Participants



- Thirty-three laboratories from 18 countries returned results in time for analysis (1 Australia, 4 Austria, 1 Bosnia, 1 Brazil, 1 Canada, 1 China, 2 Denmark, 4 France, 5 Germany, 1 India, 2 Italy, 1 Japan, 1 The Netherlands, 1 Portugal, 3 Spain, 3 Switzerland, 1 UK, 3 USA)
 - **7 Diagnostic companies**
 - **1 Clinical laboratories**
 - **14 Therapeutic manufacturers**
 - **8 Regulatory authorities**
 - **3 Pharmacopoeias**

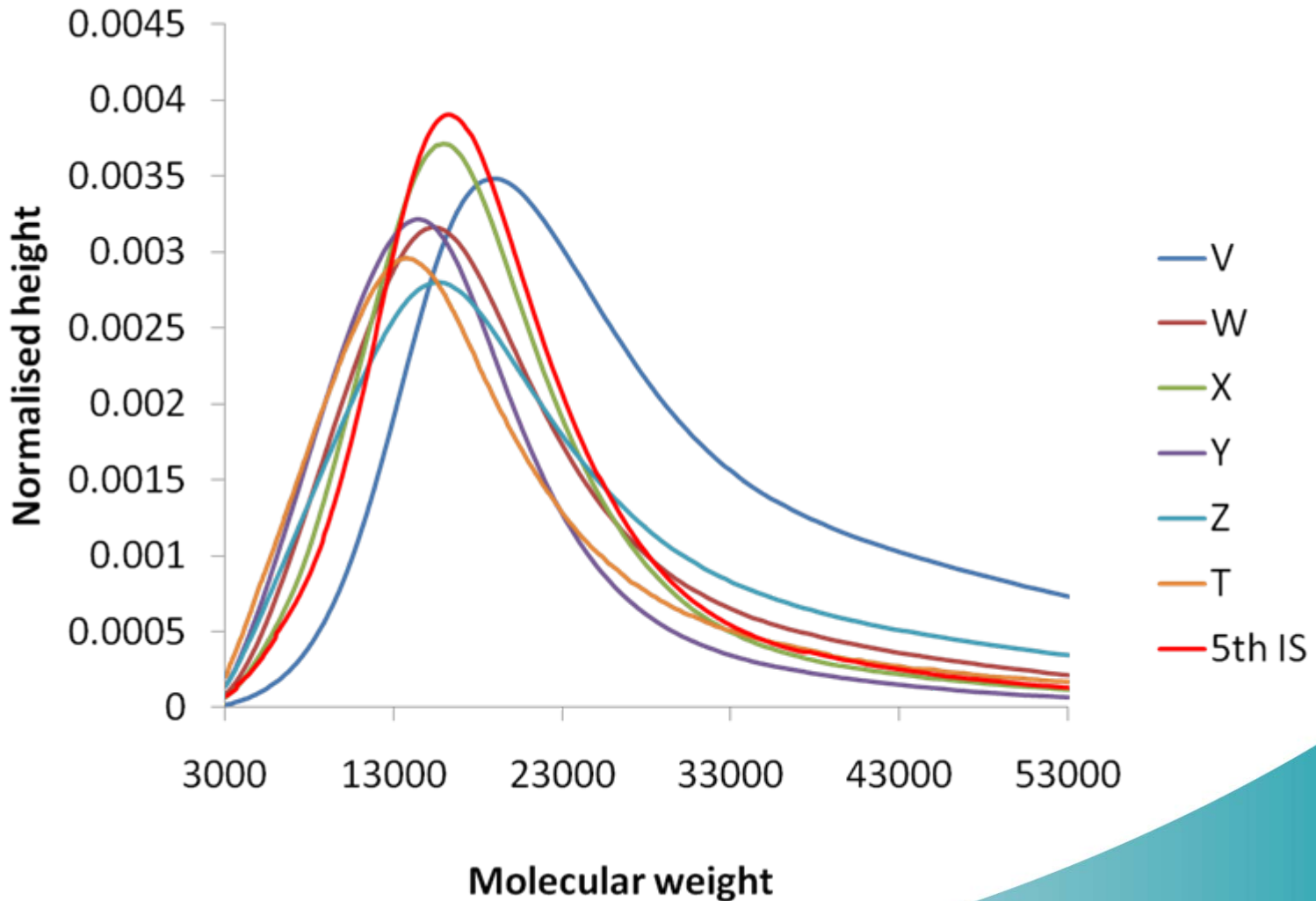
Study Design

- 4 sets of 7 ampoules/vials of samples T, V , W, X, Y, Z and Standard S were provided
- 4 independent assays using fresh ampoules of each sample for each assay type
- Within an assay, at least 3 dilutions for each sample, in replicate
- Variation of assay order design

Sources of Candidates

- Six candidates (T, V, W, X, Y and Z) donated by European and North American Heparin Manufacturers.
- With the exception of candidate V, which has a specific activities close to 300 IU/mg by anti-IIa and anti-Xa assays, all other candidates are clinical APIs from different manufacturers and have specific activities between 170 – 230 IU/mg

Molecular Weight Profile of Candidates



Twelve Different Assay Methods

- Anti-IIa chromogenic assay, purified antithrombin (AT), n = 18
 - USP anti-IIa chromogenic assay, n = 9
- Anti-Xa chromogenic assay, purified AT, n = 23
 - USP anti-Xa chromogenic assay, n = 9
- Anti-IIa chromogenic assay, human plasma, n = 1
- Anti-Xa chromogenic assay, human plasma, n = 3
- Anti-Xa clotting assay, Human plasma, n = 1
- US Pharmacopoeial assay (USP), clot-based sheep plasma assay n = 9
- European Pharmacopoeial assay (EP), clot-based sheep plasma assay n = 12
- Japanese Pharmacopoeial assay (JP), n = 1
- Chinese Pharmacopoeial assay (CP), n = 1
- Human plasma APTT, n = 12
- Thrombin Time (TT), n = 2
- Prothrombinase induced clotting time (PiCT), n = 1

USP Anti-IIa and Anti-Xa Methods for Sub-Group Analysis



- 3 participants stated clearly that they carried out the USP anti-IIa and anti-Xa assays
- 6 participants of the collaborative study requested protocols for anti-IIa and anti-Xa assays
- NIBSC provided protocols (using purified antithrombin) to these participants who subsequently returned data for these assays
- The NIBSC protocol for anti-IIa and anti-Xa methods were based on the USP anti-IIa and anti-Xa monograph methods respectively
- The sub-group USP anti-IIa method includes assays from both USP anti-IIa monograph and NIBSC protocol methods
- The sub-group USP anti-Xa method includes assays from both USP anti-Xa monograph and NIBSC protocol methods

Analysis of Data

- Raw data not available for USP sheep plasma assays. Laboratories' reported individual assay potency estimates were used for combined potencies for each sample
- All other assays analysed centrally at NIBSC as parallel line or slope ratio bioassays, vs the 5th IS. Only valid assays were included in the calculation of potency estimates
- Outliers were determined by Grubb's test and were not included in the subgroup or overall potency estimates
- Intra- and inter-laboratory variations measured by geometric coefficients of variation (%gcv).
- One way analysis of variance and Tukey's test were used to assess any significant differences amongst methods

Intra-Lab Variation by Different Methods

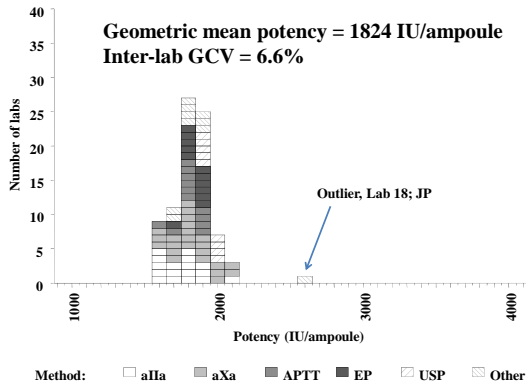
Intra-lab variability (GCV)	No of GCV/total number of GCV									
	Anti-IIa AT	Anti-Xa AT	EP	USP	APTT	Anti-Xa Plasma	TT	Anti-IIa Plasma	CP	PiCT
<5%	63/102	99/139	63/69	50/54	57/71	9/18	6/11	0/6	6/6	5/6
<7%	84/102	117/139	67/69	51/54	63/71	11/18	10/11	0/6	-	6/6
<10%	95/102	128/139	69/69	53/54	67/71	13/18	11/11	2/6	-	-
>10%	7/102	11/139	-	1/54	4/71	5/18	-	4/6	-	-
Range (% GCV)	0.2 – 29.1	0.1 – 26.4	0.1 – 9.7	0.0 – 11.5	0.1 – 30.7	1.2 – 18.3	1.7 – 7.1	8.3 – 19.3	0.9 – 5.0	2.2 – 5.7

Inter-Lab Variation by Different Methods

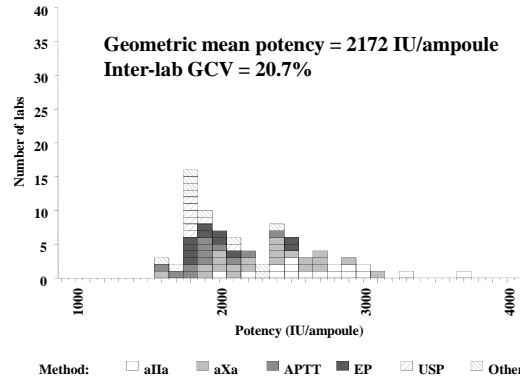
Method	T	V	W	X	Y	Z
	GCV (%)	GCV (%)	GCV (%)	GCV (%)	GCV (%)	GCV (%)
Anti-IIa AT n = 18	8.1	15.6	5.3	5.1	4.9	6.7
Anti-Xa AT n = 23	7.8	18.0	3.5	5.2	7.1	4.7
EP n = 12	3.6	13.5	2.5	2.9	4.4	3.1
USP n = 9	3.3	5.9	3.1	3.0	3.0	1.4
APTT n = 12	2.5	12.1	3.1	5.4	4.6	4.5
Anti-Xa P CH n = 3	8.5	15.9	2.9	11.0	7.3	10.6
Thrombin Time n = 2	2.7	9.9	2.2	-	0.1	2.1
CP n = 1	-	-	-	-	-	-
PiCT n = 1	-	-	-	-	-	-
JP n = 1	-	-	-	-	-	-
A/Xa P CI n = 1	4.6	2.5	6.4	8.2	3.6	3.9

Potency Estimates Vs 5th IS by All Methods

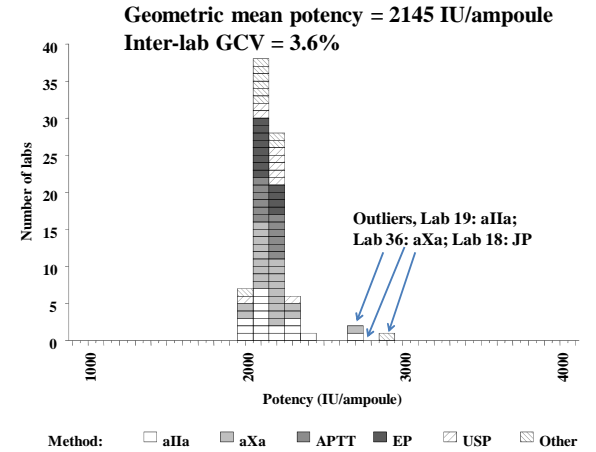
Candidate T



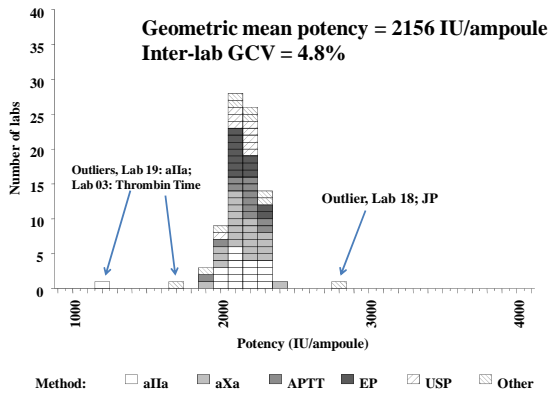
Candidate V



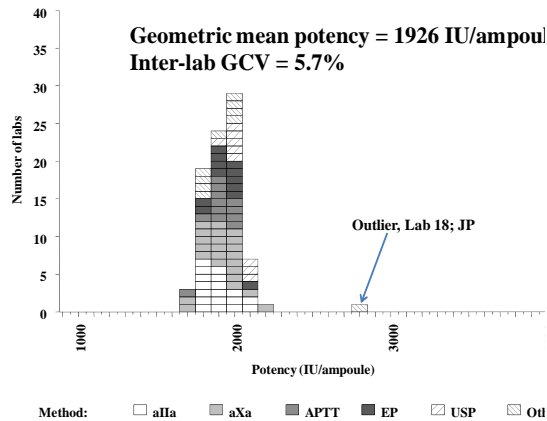
Candidate W



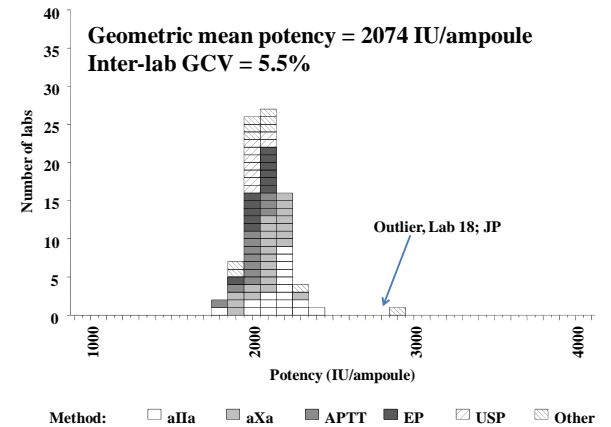
Candidate X



Candidate Y



Candidate Z



Potency Estimates by All Methods: Conventional Way for Potency assignment of potency of WHO Standards



ALL METHODS	T	V	W	X	Y	Z
Overall GM	1824	2172	2145	2156	1926	2074
Lower 95% CL	1799	2084	2128	2134	1903	2050
Upper 95% CL	1850	2265	2161	2178	1949	2099
Between-lab %GCV	6.6	20.7	3.6	4.8	5.7	5.5

Based on the lowest range of intra- and inter-laboratory variation by all methods, Candidate W, 07/328, recommended to be the WHO 6th International Standard for Unfractionated Heparin, with an assigned value of 2145 IU/ampoule

Routes of Calibration for Different Reference Standards



- For WHO International Standards:
 - Value assignment by all methods against previous International Standard
 - Exception when potencies differ because of mechanistic differences in the methods eg functional activity and antigen
- For Pharmacopoeial Reference Standards
 - Calibrate against previous Pharmacopoeial Reference Standard. Pharmacopoeial Reference Standards may not always be traceable to International Standards
 - Using specific Pharmacopoeial monograph method only

EP Sheep Plasma Assays: Intra-Laboratory Variation (% GCV)



Lab	T	V	W	X	Y	Z
01	2.78	2.35	3.94	1.67	1.04	1.67
06b	1.82	1.45	1.62	2.74	3.85	0.65
07a	1.75	1.94	1.76	0.68	0.69	0.73
08d	4.58	3.41	1.07	2.78	5.34	4.93
09	0.18	-	1.04	1.42	0.76	0.92
13d	5.57	1.52	1.94	1.29	3.33	3.05
17a	1.79	1.00	0.72	0.3	1.68	0.33
18e	9.67	3.27	1.29	0.25	1.44	1.44
21	5.62	2.13	4.64	2.78	2.91	7.08
25b	1.78	0.6	1.11	3.71	-	-
27a	5.27	4.32	1.17	0.09	3.92	3.14
31	1.87	2.35	2.96	2.70	3.09	4.05

Range = 0.18 – 9.67%

USP Sheep Plasma Assays: Intra-Laboratory Variation (% GCV)



Lab	T	V	W	X	Y	Z
06	0.6	2.9	0.4	1.7	1.7	0.8
10	1.6	0.0	1.4	1.4	0.9	0.7
12	1.7	1.7	2.6	2.1	1.7	1.5
18	2.9	3.8	1.0	1.8	0.6	3.9
19	8.5	5.9	3.8	7.9	11.5	3.1
24	1.1	1.0	0.5	0.2	1.2	1.2
25	1.9	3.4	2.1	3.5	3.4	3.0
26	0.6	1.2	0.0	2.6	2.5	1.5
28	0.0	1.6	2.0	4.5	1.3	1.5

Range = 0.0 – 11.5%

Anti-Factor IIa Assays by USP

Method: Intra-Laboratory Variation (%GCV)



Lab	T	V	W	X	Y	Z
02	6.2	3.5	2.5	1.4	1.8	3.7
03	7.6	13.6	12.9	16.3	6.1	7.1
06	6.8	3.5	4.6	4.5	2.4	7.5
08	2.6	3.1	2.8	2.6	8.4	3.6
12	1.5	1.8	6.7	6.0	2.0	3.1
13	8.5	10.9	2.6	7.3	6.6	7.1
19	.	29.1	9.2	.	23.4	9.3
25	5.3	1.7	2.0	3.0	5.4	8.5
32	4.6	8.1	1.8	1.9	2.9	2.8

Range = 1.4 – 29.1 %; 27/52 < 5%; 44/52 < 7%; 46/52 < 10%

Intra-Laboratory Variation: Comparison of Anti-Factor IIa Methods



	<5%		<7%		<10%	
	USP method	Other methods	USP method	Other methods	USP method	Other methods
No of estimates	27/52	36/50	44/52	40/50	46/52	49/50
% of estimates	52	72	85	80	88	98

For the USP method: 3 estimates 10 -15%; 3 estimates >15%

Anti-Factor Xa Assays by the USP Method: Intra-Laboratory Variation (%GCV)

Lab	T	V	W	X	Y	Z
02	4.4	1.6	0.3	2.8	2.2	2.9
03	0.4		3.0	14.4		
06	5.1	2.9	3.8	5.9	6.3	2.3
08b	2.4	4.6	2.6	6.2	2.2	1.2
12	1.2	2.7	2.3	0.8	0.6	2.4
13	7.4	7.8	8.3	7.3	6.7	7.3
19	8.0	11.1	5.0	7.8	11.1	5.5
25	2.9	4.3	3.4	5.1	6.8	2.6
27	4.6	4.0	1.5	2.2	3.1	1.2

Range = 0.3 – 14.4 %; 33/51 < 5%; 41/51 < 7%; 48/51 < 10%

Intra-Laboratory Variation: Comparison of Anti-Factor Xa Methods



	<5%		<7%		<10%	
	USP method	Other methods	USP method	Other methods	USP method	Other methods
No of estimates	33/51	66/88	41/51	76/88	48/51	80/88
% of estimates	65	75	80	86	94	91

For the other methods: 5 estimates 10 -15%; 3 estimates >15%

Inter-Laboratory Variation Expressed as % Coefficient of Variation (GCV)

Candidates	Inter-Laboratory Variation (%GCV)						
	USP Sheep Plasma (n=11)	Anti-IIa USP method (n=9)	Anti-IIa Other methods (n=9)	Anti-IIa All methods (n=18)	Anti-Xa USP Method (n=9)	Anti-Xa Other methods (n=15)	Anti-Xa All methods (n=24)
T	3.3	10.0	5.9	8.1	8.5	7.6	7.8
V	5.9	14.9	17.2	15.6	6.1	20.6	18.0
W	3.1	4.9	5.3	5.3	3.6	3.6	3.5
X	3.0	5.3	5.1	5.1	6.4	4.5	5.2
Y	3.0	4.7	4.8	4.9	6.7	7.5	7.1
Z	1.4	7.3	6.6	6.7	4.2	4.8	4.7

Estimated Potencies by Anti-IIa and Anti-Xa Chromogenic Assays using Purified Antithrombin

Candidate	Estimated Potency: Geometric Mean IU/ampoule						
	USP Sheep Plasma (n=11)	Anti-IIa USP method (n=9)	Anti-IIa Other methods (n=9)	Anti-IIa All methods (n=18)	Anti-Xa USP Method (n=9)	Anti-Xa Other methods (n=15)	Anti-Xa All methods (n=24)
T	1935	1736	1784	1758	1804	1832	1823
V	1831	2731	2638	2687	2457	2185	2280
W	2177	2112	2189	2150	2147	2145	2149
X	2154	2144	2171	2158	2158	2170	2163
Y	2026	1866	1932	1899	1889	1925	1912
Z	2036	2158	2150	2154	2112	2102	2105

ANOVA- No significant difference:

anti-IIa USP vs anti-IIa Other method

anti-Xa USP vs anti-Xa Other method

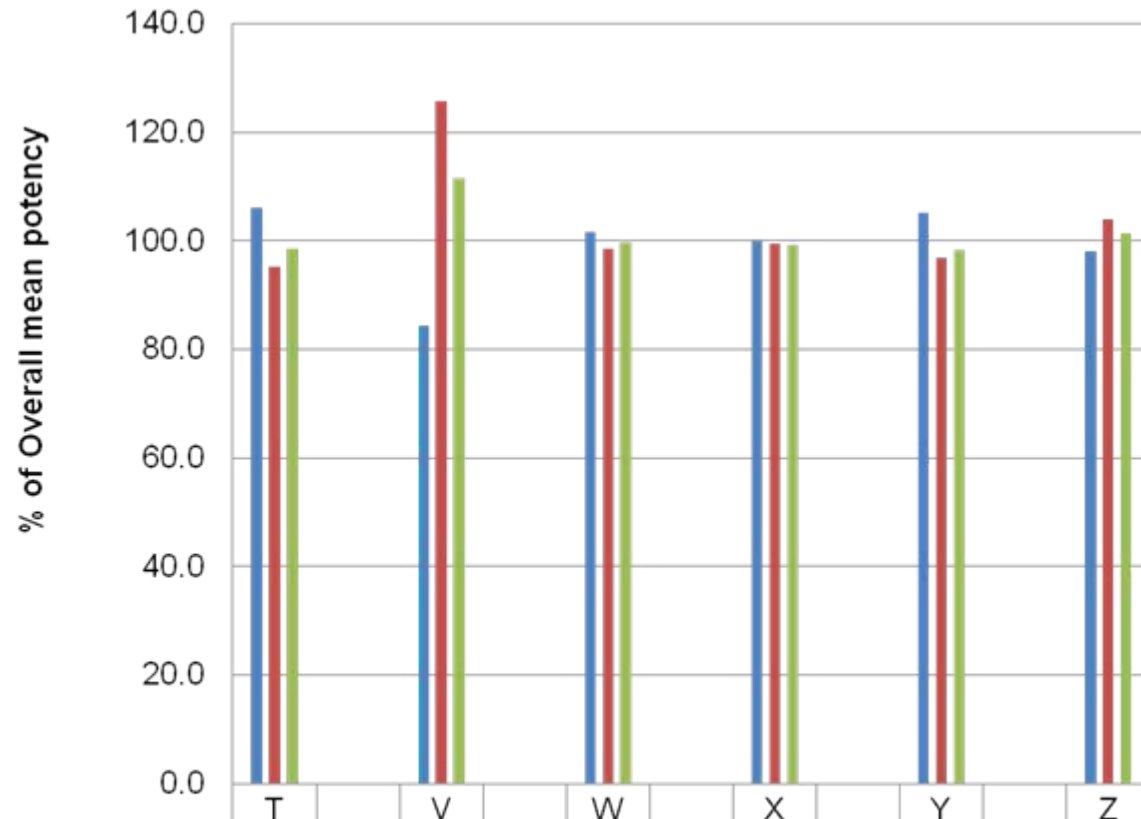
anti-IIa all methods vs anti-Xa all methods

T, V and Z anti-IIa USP significantly different to USP sheep plasma ($p < 0.05$)

Anti-Factor Xa to Anti-Factor IIa Ratios

Candidate	Anti-Xa : Anti-IIa USP Methods	Anti-Xa : Anti-IIa Other Methods	Anti-Xa : Anti-IIa All Methods
T	1.04	1.03	1.04
V	0.89	0.83	0.85
W	1.02	0.98	1.00
X	1.01	1.00	1.00
Y	1.01	1.00	1.01
Z	0.98	0.98	0.98

% Potency Estimates of the Overall Mean Potency by the 3 USP Monograph methods



■ USP sheep Plasma, n=9	106.1	84.3	101.5	99.9	105.2	98.2
■ USP alla:AT, n=9	95.2	125.7	98.5	99.5	96.9	104.0
■ USP aXa:AT, n=9	98.4	111.5	99.7	99.3	98.3	101.4

Value Assignment of USP RS for Potency, Lot F



- Candidates W and X gave the lowest intra-laboratory variability for the USP anti-IIa chromogenic assay
- Candidate X gave marginally lowest inter-laboratory variation for the USP anti-IIa chromogenic assay
- Candidate X gave the closest agreement of potencies relative to the overall mean potency by the 3 USP monograph assays
- Candidate X gave an anti-Xa to anti-IIa ratio closest to 1.0
- It is recommended that candidate X, 07/330, to be the USP RS for potency

Summary

- Candidate V gave the highest variation by all the methods used in the study. This could be explained by the differences in physico-chemical characteristics between V and the other candidates in the study, thus emphasizing the importance of assaying like against like to obtain precise and reproducible results
- The plasma based assays (current EP, USP sheep plasma and APTT assays) , well-established methods, gave the lowest intra- and inter-laboratory variations when the candidates were assayed against the 5th IS
- Although the USP anti-IIa and anti-Xa methods gave more variable results than the plasma based assays, the intra-and inter-laboratory variation as expressed by %GCVs, were generally below 10% , with the majority being around 5%
- The variability of the USP chromogenic methods were similar to other chromogenic methods using purified antithrombin
- Considering these are new methods that the participants have little experience with, the degree of variability is acceptable. It is expected that both the intra-and inter-laboratory variation will be lower once these methods are established
- There was no significant differences between the potency estimates by the USP chromogenic assays and other chromogenic assays
- Anti-Xa to anti-IIa ratio:
 - With the exception of candidate V, all other candidates showed ratios close to 1.0. The atypical behaviour of V could be explained by the presence of higher proportion of high molecular weight material. Other candidates are typical of current clinical unfractionated heparins and exhibited the expected anti-Xa to anti-IIa ratio for unfractionated heparin

Conclusions

- The USP anti-IIa and anti-Xa assays are robust and gave potency estimates similar to other commercial and in-house methods
- For candidates that are typical of current clinical unfractionated heparin preparations, the anti-Xa and anti-IIa ratios are close to 1
- **By calibrating the 6th IS and the USP RS for potency against the 5th IS for Unfractionated Heparin, the USP unit and the International Unit for Unfractionated Heparin are now harmonised.**

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