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WHO International Collaborative Study of the Proposed 2nd International Standard for Sex Hormone Binding Globulin

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Summary

The World Health Organization (WHO) Expert Committee on Biological Standardization (ECBS) has recognized (2008) the need for a replacement international standard for Sex Hormone Binding Globulin (SHBG) for the calibration of assays for the measurement of serum SHBG levels that are important for the diagnosis of conditions associated with abnormal sex steroid function such as infertility and polycystic ovary disease.

We report here the characterization of a candidate standard for SHBG in an international collaborative study carried out by 11 laboratories in 5 countries, and its comparison with the existing 1st International Standard coded 95/560 by immunoassay and binding assay.

The results of this study indicate that the candidate standard, 08/266, shows appropriate immunological and biological activity. Estimates of the SHBG content by immunoassay were also in broad agreement, indicating that it would be suitable to serve as a reference preparation with an assigned content of 180 IU per ampoule, equivalent to 180 pmol per ampoule for immunoassay and binding assay, as assessed by comparison with the 1st International Standard for SHBG, 95/560.

The results of this study also indicate that the candidate standard appears sufficiently stable to serve as an international standard, on the basis of a thermally accelerated degradation study that predicted a yearly loss of 0.159% at a storage temperature of -20°C. .

Introduction

Sex Hormone Binding Globulin (SHBG) is a 93.4 kDa homodimeric glycoprotein, synthesised by hepatocytes, which specifically binds sex steroid hormones such as testosterone and estradiol in the blood with high affinity. The high affinity binding of SHBG to sex steroid hormones limits the amount of free, biologically active hormone available to target cells, and thus SHBG plays an important role in regulating the actions of sex steroid hormones[1].

A number of pathological conditions can affect the circulating levels of SHBG and serum measurements of SHBG are important in the diagnosis of conditions associated with abnormal sex steroid function such as hyperandrogenism, infertility, polycystic ovary disease, impotence and abnormal liver and thyroid function[1,2]. Methods for the measurement of SHBG are primarily based on immunoassay, although assays based on the specific binding of radiolabelled steroid hormones have been used historically. The first International Standard (IS) for Sex Hormone Binding Globulin (SHBG), coded 95/560, was established in 1998 and has been widely used for the calibration of immunoassays and binding assays for the measurement of serum SHBG levels. Stocks of this 1st International Standard are now almost exhausted and there is an urgent requirement to replace the standard.

An international collaborative study was organised with expert laboratories to aid in the value assignment of the proposed 2nd International Standard, and to assess the immunoreactivity and biological (androgen) binding activity of the candidate preparation by both SHBG immunoassay and SHBG binding assay.

The aims of the study were therefore:
1) to calibrate, by immunoassay and binding assay, the candidate standard (08/266) relative to the 1st IS for SHBG (95/560),
2) to demonstrate the suitability of the preparation 08/266 to serve as the 2nd IS for SHBG by examining its behavior in immunoassay and binding assay systems,
3) to assess the relationships among existing local standards and the proposed IS,
4) to determine the stability of the proposed International Standard after accelerated thermal degradation

Participants

11 laboratories in 5 countries took part in the study and are listed alphabetically, by country, in Table 1. Throughout the study each participating laboratory is referred to by a code number. These code numbers were randomly assigned and do not reflect the order of listing.

Table 1: List of participants

Dr Gilles Kertesz, Beckman Coulter, 130 Av. De Lattre de Tassigny, 13009 Marseille, FRANCE

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Materials

Bulk materials and preparation of ampoules of SHBG

The original preparation of 95/560 consisted of freeze-dried human serum from healthy female volunteers. For the candidate replacement material, human serum from healthy female volunteers was obtained from the UK National External Quality Assessment Scheme (UKNEQAS, Birmingham). The source donations were collected by the National Blood Service and then tested by UK NEQAS for SHBG concentration using the DPC Immulite 2000. A total of 18 samples with SHBG concentration above 140 nmol/L were selected by UK NEQAS and sent to NIBSC for use as the candidate 2nd IS material. Mandatory screening tests were performed by the National Blood Service, as required by the Guidelines for the Blood Transfusion Services, and only samples found negative for HBsAg, anti-HIV, anti-HCV, HCV NAT, anti-HTLV and syphilis antibodies were released. The 18 serum samples were pooled, and 2.8 L of the final volume of serum was added to 112 ml of a 1 M HEPES solution to give final approximate concentrations of 170 nmol/L SHBG and 40 mM HEPES which was filled at 1 ml per ampoule, sealed under liquid nitrogen and freeze dried, with each ampoule coded 08/266, following procedures recommended by WHO[3]. A final total of 2608 ampoules were obtained on 02/2009 with a mean fill weight of 1.0080 g (CV 0.20%), a mean dry weight of 0.892 g (CV 0.91%), a residual moisture content, measured using the coulometric Karl Fischer method, of 0.223% (CV 9.62%) and a residual oxygen content, measured by frequency modulated spectroscopy (non invasive) of 0.22% (CV 17.39%).

The preparations for this study, the majority of which were identified only by code letter, are listed in Table 2. Where possible, each participant was allocated the core preparations (the duplicate coded candidate standard ampoules, stored at -20°C and the 1st IS) and a further selection of thermally accelerated degradation samples based on assay capacity and sample availability (some thermally accelerated degradation samples were only available in limited numbers). In addition, participants were asked to include their own in-house standards in the assays.

Table 2: Preparations supplied to participants in collaborative study.

Ampoule code	SHBG preparation	Ampoule unitage and nominal content
Not coded	The 1st IS for SHBG (95/560)	107 pmol (equivalent to 107 IU)
B, E Duplicates	SHBG candidate standard (08/266) stored at -20°C	Nominally 170 pmol
A, D, C and F	Accelerated thermal degradation (ATD) samples of SHBG candidate standard (08/266) stored respectively at +4°C, +20°C, +37°C and +45°C for 6 months	Contents assumed identical to B and E

Design of the study and assay methods contributed

Immunoassay and binding assay of candidate standard, 08/266

Participants were requested to carry out the assay(s) normally in use in their laboratory and, where possible, to perform at least two independent assays, using fresh ampoules, each assay to include all of the preparations allocated at preferably no less than five dose levels in the linear part of the dose-response curve. Handling instructions for the materials were included in the study protocol. In instances where there was not a fresh ampoule for subsequent assays, it was suggested that fresh dilutions be made from frozen stock solutions. Where dilutions of a stored stock solution were used, participants were asked to provide details of its storage and identification of the initial preparation. Participants were asked to ensure that all assays include their local standard where possible and to provide details of the assay method used, including dilution steps, together with all raw assay data in the form of clearly annotated optical densities, counts, etc. for central computation at NIBSC. Participants' own estimates of activity as calculated by the method normally used in their laboratory were also requested.

Assay methods contributed

Summaries of the methods used are given in Table 3.
In the 11 laboratories contributing immunoassay data to the study, there were 12 different assays used from 7 different manufacturers. One laboratory contributed binding assay data to the study using an in-house method[4,5,6].

Table 3: Assay methods used

Lab No.	Assay type	Comments
1	Immunoassay Binding Assay	Siemens Immulite 2000 SPR biosensor, Biacore X[4,5,6]
2	Immunoassay	Beckman Coulter SHBG Access 2 and Dxl platforms
3	Immunoassay	Roche SHBG Elecsys and Cobas
4	Immunoassay	SHBG Abbott Architect
5	Immunoassay	SHBG Abbott Architect
6	Immunoassay	SHBG Abbott Architect
7	Immunoassay	DRG SHBG ELISA
8	Immunoassay	Izotop SHBG IRMA
9	Immunoassay	Siemens Immulite SHBG/ Immulite 2000/Immulite 2500
10	Immunoassay	SHBG Abbott Architect
11	Immunoassay	DSL SHBG ELISA

Statistical analysis

An independent statistical analysis of data was performed at NIBSC. Where raw assay data were available, potency estimates relative to IS 95/560 and to local standards were calculated by fitting a parallel-line model comparing assay response to log concentration[7]. All data were plotted and assay validity was assessed both visually and by analysis of variance with deviations from the model considered significant at the 1% level. Where estimation of the residual error was not possible, i.e. where only one replicate response was provided (laboratory 5, assays 1 and 2), linearity was confirmed visually and non-parallelism was assessed for significance by using the deviations from linearity as an alternative residual error.

Where raw data were returned (laboratories 2a, 2b, 4, 5, 6, 7, 8, 10 and 11), parallel-line analysis was carried out on the linear section of the dose-response curve. In all laboratories, with the exception of laboratory 7, responses were log transformed to achieve linearity. For laboratory 7, a square root transformation was found to be more appropriate. Laboratories 3a, 3b, 9a, 9b, and 9c did not provide any raw data for analysis and the reported results at each dilution were used to calculate mean potency estimates. Laboratory 1a also did not provide raw data but reported duplicate results for each sample which were used to calculate mean potency estimates. Laboratory 1b performed a binding assay and reported a maximum

achievable biosensor binding for all samples, which was used to calculate a direct comparison of 08/266 relative to 95/560.

All mean potencies given in this report are unweighted geometric mean (GM) 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 transformed potency estimates). Duncan's test for multiple comparisons[8] and Grubb's outlier test[9] were applied to the log potencies to detect any outlying laboratories.

Potencies of the accelerated thermal degradation samples relative to 95/560 were used to fit an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay[10] and hence predict the degradation rates for ampoules stored at -20°C.

Results

Laboratory mean potency estimates (IU/ampoule) calculated relative to 95/560 are summarised in Table 4 and represented graphically in Figure 1. Binding assay results (laboratory 1b) are shown in Table 5.

Data returned for analysis

In total 26 assays were analysed. Data were contributed by eleven laboratories. All laboratories carried out immunoassays with some laboratories using more than 1 method. Where this was the case, the laboratory code has been subdivided for method differences, for example 2a and 2b. Laboratory 1b also performed a binding assay. All laboratories performed assays including the candidate 08/266 and the current IS 95/560 and eight laboratories also included the accelerated thermal degradation samples (A, C, D and F).

Assay validity and outliers

For samples calibrated against 95/560, all assays allowed statistically valid estimates to be calculated. For estimates of 08/266 relative to 95/560, laboratory 11 was detected as an outlier and their results were excluded from overall mean calculations. For samples calibrated against local standards, the local standard was found to be non-parallel in laboratories 6, 7 and 11. A potency estimate for these laboratories relative to the local standard has not been calculated.

Potency of 08/266 calculated relative to IS 95/560

The geometric mean potency for 08/266 (Table 4) is 180.10 IU per ampoule (n=14; 95% confidence limits 176.15 – 184.14). Individual potency estimates are provided in Table A1.1 of Appendix 1. Comparison of the mean potencies from all laboratories has been depicted graphically in Figure 1 as log transformed assay estimates. A table of the log transformation of mean potency values is provided in Table A1.2 of Appendix 1. Variability between laboratories is 3.92%. Coded duplicates of 08/266 (B and E) were included in this study to provide an independent measure of the minimum level of variability present in these assays. Variability within laboratories, assessed by GCV's for samples coded B and E in laboratories where at least three potency estimates were obtained, is low for the majority of laboratories ranging from 0.71% to 6.27% with the exception of laboratory 2a which has a GCV of 11.46%.

Individual assay potency estimates relative to local standards (which were reported in nmol/L) are provided in Table A1.3 of Appendix 1, and laboratory means of potency estimates relative to local standards have been provided in Table A1.4. Although there is a larger variability in potency estimates relative to the local standard between laboratories compared to potency estimates calculated relative to 95/560 (CV's 12.1% and 3.9% respectively), the majority of laboratories were in reasonable agreement with the assigned value of 107 pmol per ampoule for the 1st IS, 95/560 (with the exception of laboratories 5 and 10 who measured a higher value of >130 pmol per ampoule for 95/560) and were also in reasonable agreement with a potency of 180 pmol per ampoule for 08/266 (with the exception of laboratories 4, 5 and 10 who reported higher values of >200 pmol per ampoule).

Binding assay results were reported by laboratory 1b as the maximum achievable biosensor binding (RU) for 08/266 ampoules and 95/560 (Table A1.5 of Appendix 1). This was used to calculate a ratio of the maximum binding of 08/266 relative to the maximum binding 95/560, shown in Table 5. All 08/266 ampoules showed an approximately two-fold higher binding ability relative to the 1st IS, 95/560.

Stability of 08/266

Estimates of the potency of ampoules stored at elevated temperatures for a period of six months relative to 95/560 are summarized in Table 4. No significant loss in immunoreactivity was observed at +4°C or +20°C ($p=0.093$ and $p=0.965$ respectively), but greater loss was found at the higher temperatures of +37°C and +45°C ($P<0.0001$). Expressing the potencies relative to ampoules stored continuously at -20°C gives a predicted yearly loss of 0.159% when stored at -20°C.

Table 4: Laboratory mean estimates (IU/ampoule) for 08/266 calculated relative to 95/560

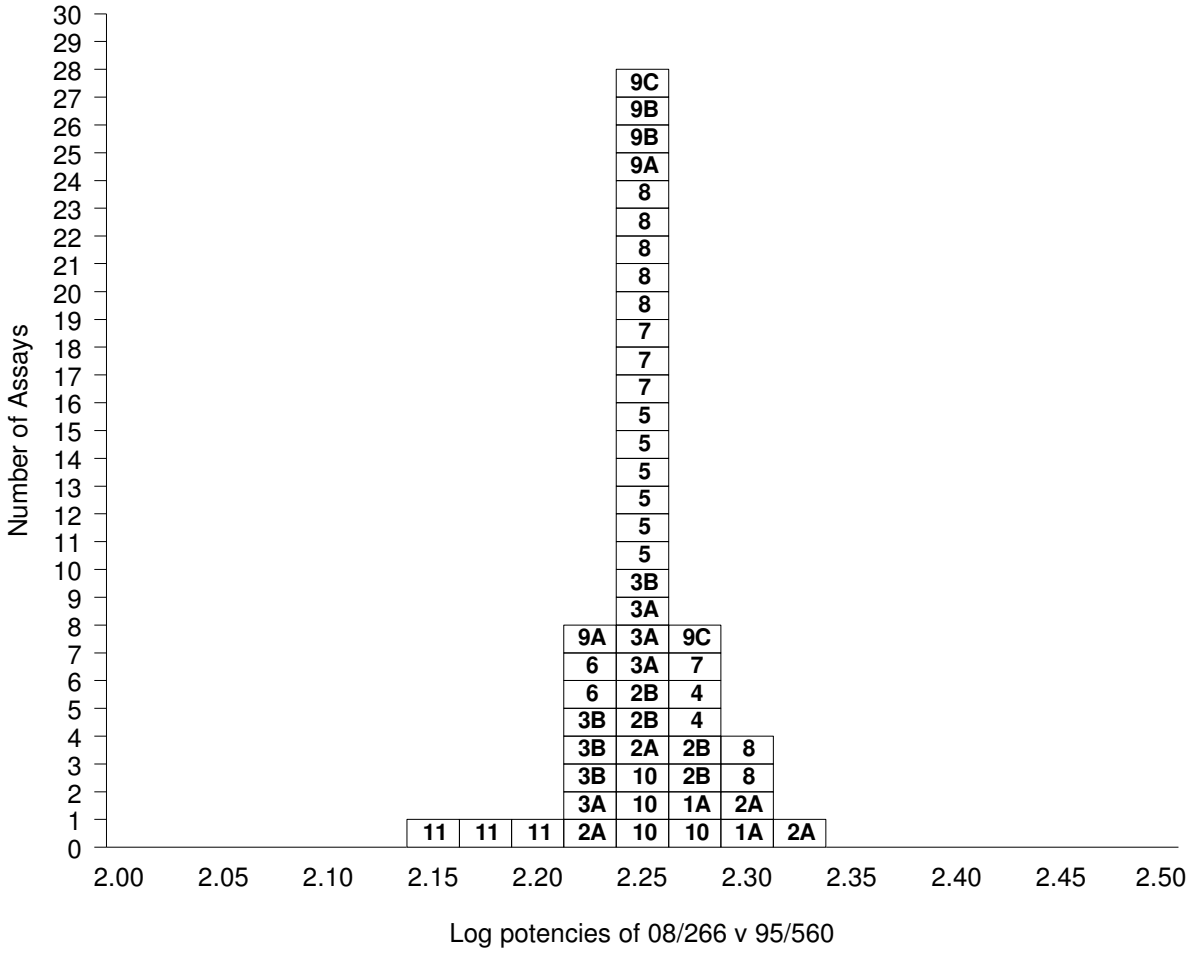
Lab	*GM (B,E) (-20ºC)*	*%GCV (B,E) (-20ºC)*	*GM(A) (+4ºC)*	*GM(D) (+20ºC)*	*GM(C) (+37ºC)*	*GM(F) (+45ºC)*
1a	191.19	--	192.50	183.10	165.23	163.82
2a	186.38	11.46				
2b	183.47	3.97				
3a	174.63	2.17	173.62	171.77	161.83	157.65
3b	171.67	0.71	168.01	167.52	159.62	145.03
4	192.43	--	194.89	187.79	168.17	166.45
5	177.98	1.64	180.26	174.48	162.37	162.09
6	169.08	--	169.13	172.65	154.79	155.14
7	178.40	2.22	172.46	176.81	158.75	167.60
8	183.30	6.27	184.10	181.86	178.15	176.86
9a	174.39	--	166.16	181.17	164.63	152.30
9b	176.16	--	173.75	176.21	161.32	156.17
9c	183.71	--	180.16	181.33	160.21	167.03
10	180.31	1.94				
11	*150.26	--				
Overall GM	***180.10***		***177.50***	***177.61***	***163.09***	***160.70***
95% CI	***176.15–184.14***		***172.10 – 183.06***	***174.22 – 181.07***	***159.67 – 166.58***	***155.70 – 165.86***
%GCV	***3.92***		***5.49***	***3.40***	***3.73***	***5.63***

*Outlier – Excluded from overall calculations
GM = Geometric Mean
%GCV = Geometric Coefficient of Variation
95% CI = 95% Confidence Interval

Table 5: Relative maximum binding of 08/266 ampoules compared to 95/560

	Ampoule					
Lab	***B (-20ºC)***	***E (-20ºC)***	***A (+4ºC)***	***C (+37ºC)***	***D (+20ºC)***	***F (+45ºC)***
1b	2.25	2.45	2.16	2.16	2.22	2.03

Figure 1: Individual assay estimates (IU/ampoule) for 08/266 calculated relative to 95/560



Conclusions and recommendations

The 1st International Standard for SHBG (95/560) has been widely used for the calibration of assays used in the diagnosis of a number of conditions associated with abnormal SHBG levels, for example, SHBG assays are often used in conjunction with total testosterone measurements in order to calculate the free androgen index, or amount of bioavailable testosterone, for the diagnosis of conditions such as hyperandrogenism. The first IS was calibrated based on both immunoassays and binding assays, which utilised a radio-labelled steroid hormone. However, it was noted upon establishment of the 1st IS that rapidly changing

technology may mean that future preparations would be unlikely to be calibrated by both immunoassay and binding assay methods. The majority of SHBG assays now in use in the clinical setting are immunoassays, and although a binding assay based on biosensor measurements has been included in this study, providing valuable information on the binding properties of the standard, it is likely that immunoassays will remain the most widely used assay for clinical purposes in the future.

The 1st IS for SHBG, 95/560, was prepared from pooled human serum. This process has been repeated for the preparation of the candidate replacement IS, coded 08/266, maintaining continuity between the standards. Also, since patient serum is used in SHBG immunoassays for the measurement of SHBG, the adoption of the candidate standard, 08/266, as the 2nd IS for SHBG would adhere to the guidelines of WHO which describe the requirement for International Standards to match, as closely as possible, the analytes to which they are being compared.

By comparison with the 1st IS for SHBG (95/560), the overall mean potency, calculated from immunoassay estimates, for 08/266 was 180 IU per ampoule. The 1st IS was assigned a potency of 107 IU, equivalent to 107 pmol per ampoule, with the assumption that 1 IU was equivalent to 1 nmol SHBG per litre. Therefore, as the candidate preparation 08/266 has been prepared in a similar manner to, and calibrated in terms of, the 1st IS, 95/560, it can also be assigned a potency of 180 pmol per ampoule. This unitage continuity is important as the majority of immunoassay kits are currently calibrated against the 1st IS, 95/560, using nmol/L, which is also the reporting unit for the assays.

The majority of immunoassays used in the study were calibrated against the 1st IS, 95/560, with the exception of the DRG SHBG immunoassay which is calibrated using an SHBG preparation from Scripps laboratories. Potency estimates for the candidate standard 08/266 relative to laboratories' local standards indicate that the majority of laboratories were in good agreement with the overall mean potency (180 pmol per ampoule) calculated relative to the 1st IS, 95/560, and also in reasonable agreement with the assigned potency of 107 pmol per ampoule for the 1st IS. Laboratories 4, 5 and 10, who used a single manufacturer's immunoassay, measured higher values for 08/266. However, the mean recovery of 95/560 in these laboratories was some 23 % higher than the assigned value of 107 pmol per ampoule, demonstrating the requirement for improved harmonisation between immunoassay methods for SHBG.

The candidate standard has been shown to have appropriate immunological activity to serve as the replacement International Standard for SHBG. The candidate standard has also been shown to have biological activity in a steroid binding assay. However, in this assay, 08/266 was shown to bind to dihydrotestosterone derivatives with approximately twice the binding potential compared with the 1st IS, 95/560, indicating that there may be non-continuity of steroid binding activity between the candidate standard, 08/266, and 1st IS, 95/560. Users are therefore recommended to assess the suitability of the candidate replacement standard, 08/266, in their own binding assay systems prior to use.

Data from accelerated thermal degradation studies indicate that the candidate replacement material, 08/266, showed no significant loss of activity at lower temperatures (+4°C, +20°C, P=0.093 and 0.965 respectively) compared to -20°C, the recommended storage temperature, but showed a greater loss of activity at the higher temperatures (+37°C, +45°C, P<0.0001). Measurements by binding assay indicated that binding activity was not affected at elevated

temperatures. Loss of SHBG immunoreactivity after heating is a well known phenomenon, and was also observed at elevated temperatures of +37°C, +45°C and +56°C during preparation of the 1st IS for SHBG, 95/560. At temperatures usually used for storage of biological samples no significant loss of activity was observed, with an estimated yearly loss of activity at -20°C of approximately 0.159%. The candidate replacement preparation 08/266 is therefore deemed sufficiently stable to serve as an international standard.

Proposal

With the agreement of all participants, it is recommended that the preparation in ampoules coded 08/266 be established as the Second International Standard for Sex Hormone Binding Globulin, with an assigned content of 180 IU, where 1 IU is equivalent to 1 nmol SHBG per litre. A total of 2300 ampoules are available to WHO, stored at –20°C at NIBSC, Blanche Lane, South Mimms, EN6 3QG.

Acknowledgements

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Appendix 1: Raw data

Table A1.1: Individual assay estimates (IU/ampoule) for 08/266 calculated relative to 95/560

		Ampoule					
Lab	***Assay***	***A (+4ºC)***	***B (-20ºC)***	***C (+37ºC)***	***D (+20ºC)***	***E (-20ºC)***	***F (+45ºC)***
1a	***1***	192.50	186.01	165.23	183.10	196.53	163.82
2a	***1***	-	203.44	-	-	205.70	-
	2		166.85			172.84	
2b	***1***	-	188.28	-	-	190.64	-
	2		175.35			180.03	
3a	***1***	178.73	179.78	164.81	175.44	174.73	170.87
	2	168.65	173.29	158.90	168.17	170.85	145.46
3b	***1***	168.41	173.05	158.75	167.91	171.11	145.34
	2	167.62	172.25	160.50	167.14	170.30	144.72
4	***1***	194.89	189.35	168.17	187.79	190.01	166.45
5	***1***	182.54	174.56	167.29	184.51	179.49	162.41
	2	179.22	177.43	158.01	167.36	175.41	162.63
	3	179.05	182.50	161.93	172.00	178.61	161.23
6	***1***	169.13	171.05	154.79	172.65	167.14	155.14
7	***1***	172.84	176.19	162.45	179.86	176.38	163.91
	2	172.08	178.40	155.14	173.81	184.65	171.38
8	***1***	198.11	202.33	186.51	195.81	197.39	183.08
	2	175.11	173.68	182.98	189.10	177.15	179.47
	3	181.29	178.71	174.76	170.23	175.24	168.37
	4	182.66	180.65	168.87	173.53	--	--
9a	***1***	166.16	172.63	164.63	181.17	176.16	152.30
9b	***1***	173.75	176.58	161.32	176.21	175.75	156.17
9c	***1***	180.16	179.45	160.21	181.33	188.07	167.03
10	***1***	-	178.69	-	-	176.51	-
	2		181.68			184.45	
11	***1***	-	139.57	-	-		-
			149.24				
			162.86				

Table A1.2: Log transformed laboratory mean estimates for 08/266 ampoules relative to 95/560

	Sample				
Lab	***B,E***	***A***	***D***	***C***	***F***
1A	2.281	2.284	2.263	2.218	2.214
2A	2.270	-	-	-	-
2B	2.264	-	-	-	-
3A	2.242	2.240	2.235	2.209	2.198
3B	2.235	2.225	2.224	2.203	2.161
4	2.284	2.290	2.274	2.226	2.221
5	2.250	2.256	2.242	2.211	2.210
6	2.228	2.228	2.237	2.190	2.191
7	2.251	2.237	2.248	2.201	2.224
8	2.263	2.265	2.260	2.251	2.248
9A	2.242	2.221	2.258	2.217	2.183
9B	2.246	2.240	2.246	2.208	2.194
9C	2.264	2.256	2.258	2.205	2.223
10	2.256	-	-	-	-
11	2.177	-	-	-	-

Table A1.3: Individual potency estimates (pmol/ampoule) for 95/560 and 08/266 relative to local standards

		Ampoule						
Lab	***Assay***	***A***	***B***	***C***	***D***	***E***	***F***	***95/560***
1A	***1***	166.10	155.40	142.58	157.99	169.58	141.35	92.32
2A	***1***	--	195.95	--	--	198.06	--	104.94
	2	--	176.35	--	--	182.70	--	112.95
2B	***1***	--	193.21	--	--	195.57	--	111.17
	2	--	180.19	--	--	184.98	--	109.89
3A	***1***	190.78	191.89	175.92	187.26	186.50	182.39	114.21
	2	179.05	183.98	168.70	178.54	181.39	154.44	113.60
3B	***1***	177.31	182.19	167.13	176.78	180.15	153.02	112.65
	2	177.80	182.71	170.24	177.29	180.64	153.51	113.50
4	***1***	218.09	211.84	188.70	210.07	212.70	185.99	119.05
5	***1***	--	--	--	--	--	--	--
	2	216.06	213.95	191.11	202.12	211.58	196.56	130.74
	3	259.38	264.33	234.81	249.27	258.74	233.81	155.23
6	***1***	Local standard non-parallel						
7	***1***	Local standard non-parallel						
	2							
8	***1***	180.11	183.96	169.50	178.00	179.45	166.37	96.94
	2	182.97	181.46	191.26	197.71	185.11	187.56	111.33
	3	182.72	180.12	176.12	171.08	176.61	169.66	107.63
	4	185.16	182.98	170.21	175.25	--	--	107.73
	5	--	--	--	--	169.20	156.09	--
9A	***1***	166.16	172.63	164.63	181.17	176.16	152.30	115.75
9B	***1***	163.51	166.18	151.82	165.83	165.39	146.97	100.70
9C	***1***	183.60	182.88	163.27	184.80	191.66	170.22	109.04
10	***1***	--	236.07	--	--	233.13	--	139.98
	2	--	227.61	--	--	231.20	--	131.86
11	***1***	Local standard non-parallel						

Table A1.4: Laboratory means of potency estimates (pmol/ampoule) for 95/560 and 08/266 relative to local standards

	Ampoule					
Lab	***95/560***	***GM(B, E)***	***GM (A)***	***GM(C)***	***GM(D)***	***GM(F)***
1A	92.32	162.34	166.10	142.58	157.99	141.35
2A	108.87	188.05	--	--	--	--
2B	110.53	188.39	--	--	--	--
3A	113.90	185.90	184.82	172.27	183.93	167.83
3B	113.07	181.42	177.55	168.68	177.03	153.26
4	119.05	212.27	218.09	188.70	210.07	185.99
5	142.46	235.88	236.73	211.84	224.46	214.38
6	Local standard non-parallel					
7	Local standard non-parallel					
8	105.77	179.80	182.73	176.56	180.23	169.55
9A	115.75	174.39	166.16	164.63	181.17	152.30
9B	100.70	165.78	163.51	151.82	165.83	146.97
9C	109.04	187.22	183.50	162.27	184.80	170.22
10	135.86	231.98	--	--	--	--
11	Local standard non-parallel					

GM = geometric mean

Table A1.5: Maximum binding (RU) of 95/560 and 08/266 ampoules using the Biacore X

	Ampoule						
Lab	***B (-20ºC)***	***E (-20ºC)***	***A (+4ºC)***	***C (+37ºC)***	***D (+20ºC)***	***F (+45ºC)***	***95/560***
1b	155	169	149	149	153	140	69

Appendix 2: Study protocol

STUDY PROTOCOL

REPLACEMENT OF THE 1ST WHO INTERNATIONAL STANDARD FOR SEX HORMONE BINDING GLOBULIN (95/560)

Introduction

Sex Hormone Binding Globulin (SHBG) is a 373 amino acid glycoprotein which specifically binds sex steroid hormones such as testosterone and estradiol. The high affinity binding of SHBG to sex steroid hormones limits the amount of free, biologically active hormone available to target cells, and thus SHBG plays an important role in regulating the actions of sex steroid hormones.

A number of pathological conditions can affect the circulating levels of SHBG, and serum measurements of SHBG are significant for the diagnosis of conditions associated with abnormal sex steroid function such as hyperandrogenism, infertility, polycystic ovary disease, impotence and liver and thyroid function.

The first International Standard (IS) for Sex Hormone Binding Globulin (SHBG) in ampoules coded 95/560 was established in 1998 and has been widely used for the calibration of immunoassays and binding assays for the measurement of serum SHBG levels. Stocks of the 1st IS are almost exhausted and there is an urgent requirement to replace the standard.

The original preparation of 95/560 consisted of freeze-dried human serum from healthy female volunteers. The candidate replacement material consists of serum donations containing 150-200 nmol/L SHBG, obtained from UK NEQAS (Birmingham), which has been pooled and filled into ampoules (NIBSC code 08/266) following procedures recommended by WHO (1). It is intended that an international collaborative study is organised with expert laboratories to aid in the value assignment of the proposed 2nd International Standard, and to assess the immuno-reactivity and biological (androgen) binding activity of the candidate preparation by both SHBG immunoassay and SHBG binding assays.

Aims of the study

Since the collaborative study to establish the first IS demonstrated a content of 107 nmol/L and users of this standard have adopted the molar unitage attributed to the ampoule, we propose to calibrate the 2nd IS in terms of the 1st IS using this unitage. The aims are therefore:

- To calibrate the candidate standard (08/266) relative to the 1st IS for SHBG (95/560).
- To demonstrate the suitability of the preparation 08/266 to serve as the 2nd IS for SHBG by examining its behaviour in immunoassay and binding assay systems.
- To assess the relationships among existing local standards and the proposed IS.
- To determine the stability of the preparation 08/266 by comparison with ampoules stored at elevated temperatures.

Materials

Preparations supplied to participants in collaborative study.

A bulk preparation of human female serum, tested for high levels of SHBG, was obtained from UK NEQAS, Birmingham. Serum samples were also tested and found negative for HBsAg, anti-HIV, anti-HCV and HCV NAT. The serum aliquots were pooled and HEPES (40 mM) added before being dispensed (1 ml aliquots) into glass ampoules, lyophilised and sealed.
The materials for this study, which may be identified only by code letter, are listed in Table 1. Where appropriate, each participant will be allocated a set of core preparations and a further selection of samples based on assay capacity and sample availability (some thermally accelerated degradation samples are only available in limited numbers).

Table 1.

SHBG Preparation	**Ampoule content**
1st SHBG International Standard (95/560)	107 pmol (equivalent to 107 IU)
Candidate standard (08/266) stored at -20ºC	Nominally 170 pmol
Accelerated thermal degradation (ATD) samples of 08/266 stored at +4°C, +20°C, +37°C and +45°C	Content assumed identical to 08/266

Tests requested

Participants are requested to carry out the assay method(s) normally in use in their laboratory, and where possible, to perform at least two independent assays, using fresh ampoules (not a stored aliquot) for each. Each assay should include all of the preparations allocated, **at preferably no less than five dose levels in the linear part of the dose-response curve** in order to provide information on parallelism. In instances where there is not a fresh ampoule for subsequent assays, it is suggested that fresh dilutions are made from frozen stock solutions, and where this is the case, participants are requested to provide details of freeze-thaw steps. **Participants are also asked to ensure that all assays include their local standard where possible**.
The ampoule contents of the test preparations are listed in Table 1. The candidate standard 08/266 and its degradation samples will be coded, in random order, by letter in the final protocol. On receipt, ampoules should be stored at -20°C until use. It is recommended that the contents of each ampoule are reconstituted in 1ml distilled water or appropriate assay diluent according to the protocol used. Appropriate dilutions should be made from this stock using assay diluent according to the assay protocol used or distilled water preferably containing 0.05-0.1% added protein (BSA or HSA) to reduce adsorption.
Participants are asked to provide details of the assay methods used, including detail of the dilution steps made and **all raw assay data** (in electronic excel spreadsheet format if possible) in the form of clearly annotated optical densities, counts etc for central computation at NIBSC. Participants' own estimates of activity as calculated by the method normally used in their laboratory are also requested.

Report

A preliminary report will be prepared and circulated to all participants for comment before submission to the Expert Committee on Biological Standardization of WHO. In the report, participating laboratories will be identified by a laboratory number only and any requests to treat information in confidence will be respected.

References

1. WHO Recommendations for the preparation, characterization and establishment of international and other biological reference standards (revised 2004). In: WHO TRS, No. 932, 2006, Annex 2

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Appendix 3: Draft Instructions for use

Proposed 2nd WHO International Standard for Sex Hormone Binding Globulin
08/266
Instructions for Use (June 2010, first version)

This material is not for *in vitro* diagnostic use

1. Introduction

The candidate 2nd International Standard for Sex Hormone Binding Globulin (SHBG) consists of a batch of ampoules, coded 08/266, containing freeze dried serum obtained from a pool of normal healthy female volunteers for the calibration of immunoassays or binding assays for the measurement of human SHBG.

2. Ampoule contents

Each ampoule contains the freeze-dried residue of 1 ml of human serum with 40 mM HEPES.

3. Unitage

The assigned content is 180 IU SHBG per ampoule, equivalent to 180 pmol per ampoule. SHBG content has been assigned on the basis of an international collaborative study using immunoassay methods. Binding assay users are recommended to assess the suitability of this preparation in their own assays prior to use.

4. Caution

THIS PREPARATION IS NOT FOR ADMINISTRATION TO HUMANS

This preparation contains material of human origin, which has been tested and found negative for HBsAg, anti-HIV antibody, anti-HCV, HCV NAT, anti-HTLV and syphilis antibodies. As with all material 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 will probably include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

A safety data sheet is included in the last page of these instructions.

5. Use of ampoules

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution. For practical purposes each ampoule contains the same quantity of SHBG. The entire content of each ampoule should be completely dissolved in an accurately measured amount of diluent. No attempt should be made to weigh out portions of the freeze-dried powder. Suitable diluents are distilled water or appropriate assay diluent. If the contents are to

be diluted extensively, the addition of 0.05 – 0.1% protein (HSA or BSA) is recommended to minimise adsorption. The material has not been sterilized and the ampoules contain no bacteriostat. A fresh ampoule should be used for each assay as repeated freeze-thawing may lead to loss of immunoreactivity, although if required, users should conduct their own investigations.

Suitable precautions should be taken in the use and disposal of the ampoule and its contents: see **MATERIAL SAFETY SHEET**.

6. Directions for opening ampoule

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. 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. Stability

It is the policy of WHO not to assign an expiry date to their international reference materials. They remain valid with the assigned potency and status until withdrawn or amended. Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Stability studies at elevated temperatures have shown that the material is suitably stable for shipment at ambient temperature without any effect on the assigned value. Reference Materials should be stored on receipt as indicated on the label. Once reconstituted, diluted or aliquoted, users should determine the stability of the material according to their own method of preparation, storage and use. NIBSC follows the policy of WHO with respect to its reference materials. Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

8. Citation

In all publications (or data sheets for kits) in which this preparation is used as an assay calibrant, it is important that the title of the preparation, ampoule code and the name and address of NIBSC are cited and cited correctly.

9. Product liability

9.1 Information emanating from NIBSC is given after the exercise of all reasonable care and skill in its compilation, preparation and issue, but is provided without liability in its application and use.

9.2 This product is intended for use as a standard or reference material in laboratory work in relation to biological research, manufacturing or quality control testing of biological products or in the field of in vitro diagnostics. It is the responsibility of the user to ensure that he/she has the necessary technical skills to determine the appropriateness of this product for

the proposed application. Results obtained from this product are likely to be dependent on the conditions of use and the variability of materials beyond the control of NIBSC.
NIBSC accepts no liability whatsoever for any loss or damage arising from the use of this product, whether loss of profits, or indirect or consequential loss or otherwise, including, but not limited to, personal injury other than as caused by the negligence of NIBSC. In particular, NIBSC accepts no liability whatsoever for :-

i) results obtained from this product; and/or
ii) non-delivery of goods or for damages in transit.

9.3 In the event of any replacement of goods following loss or damage a customer accepts as a condition of receipt of a replacement product, acceptance of the fact that the replacement is not to be construed as an admission of liability on NIBSC's behalf.

10. Material safety sheet

Proposed 2nd WHO International Standard for Sex Hormone Binding Globulin, 08/266

Physical Properties (at room temperature)	
Physical appearance *Freeze-dried powder, yellow colour*	
Fire hazard *None*	
Chemical Properties	
Stable *Yes*	Corrosive *No*
Hygroscopic *Yes*	Oxidising *No*
Flammable *No*	Irritant *No*
Other (specify) *Contains material of human origin*	
Handling *See precautions in section 4*	
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 a virucidal agent. Rinse area with a virucidal agent followed by water. *Absorbent material used to treat spillage should be treated as biologically hazardous waste.*	

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