The third international standard for anti-D immunoglobulin: international collaborative study to evaluate candidate preparations

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Introduction

For 50 years, anti-D immunoglobulin has been used for the prevention of haemolytic disease of the foetus and newborn (HDFN) [1–3] and found to be beneficial in the treatment of primary immune thrombocytopenia (ITP) [4,5]. The mechanism of anti-D action in preventing HDFN and in ITP therapy is not fully understood but is thought to involve antibody-mediated immune suppression (AMIS) [6–9]. Clinical use of anti-D immunoglobulin requires estimation of its potency against a reference preparation [10]. In the 1970s, the World Health Organisation (WHO) and the United States Food and Drug Administration (US-FDA) Center for Biologics Evaluation and Research (CBER) independently established reference standards for anti-D immunoglobulin to ensure that...
appropriate and consistent potency requirements of therapeutic products are met [11,12]. Anti-D immunoglobulin prophylaxis is given to unimmunized D-negative women following the known causes of sensitization; since 125 IU anti-D is recommended per millilitre of RhD-positive foetal red cells, it is critical that therapeutic preparations are rigorously tested to ensure an adequate dose is administered to cover any foetal bleed. In the late 1990s, concern over contamination of the existing standards with hepatitis C virus RNA and declining stocks led to an international collaboration to evaluate anti-D immunoglobulin with the aim of establishing a ‘global’ standard.

In 2003, 01/572 was established by the WHO Expert Committee on Biological Standardization (ECBS) as the 2nd International Standard (IS) for anti-D immunoglobulin; by CBER/FDA as the US Standard for anti-D immunoglobulin, Lot 4; and by the European Pharmacopoeia (Ph Eur) Commission as the 1st Biological Reference Preparation for human anti-D Immunoglobulin potency testing [13]. A reference preparation for quantifying anti-D in immunoglobulin products is unsuitable for quantifying the level of anti-D in plasma and an alternative plasma standard, for use with automated haemagglutination, is available from the National Institute for Biological Standards and Control (currently the CE Marked 3rd British Standard for anti-D (Rho) antibodies; coded: 73/517) [14]. The 2nd WHO IS, distributed by NIBSC, was used by 159 laboratories in 44 countries across the globe and some 15 years later, stocks were almost depleted. This study served to replace the 2nd WHO IS to ensure continuation of harmonized potency units across the globe.

Materials and methods

Anti-D immunoglobulin preparations for the collaborative study

Where possible the clinical grade candidate preparations were reformulated at NIBSC to match the existing IS in terms of excipients and potency. The material used for Sample A and D (coded duplicate) was an anti-D immunoglobulin therapeutic product manufactured from plasma sourced in the United States. Prior to filling approximately 1 ml/ampoule and lyophilization, NIBSC reformulated the material with 0.25 M glycine and 0.4% (w/v) NaCl to give 2% (w/v) IgG at pH 6-7 containing approximately 300 IU/ml. This material was assigned NIBSC code 16/272. The mean weight of the dispensed solution in 699 ampoules was 1.0083 g. The imprecision of the filling (CV) was 0.183%, and the residual moisture was 0.3%.

Sample B consisted of a second anti-D immunoglobulin therapeutic product manufactured from plasma sourced in the United States but with lower specific activity. Prior to filling approximately 1 ml/ampoule and lyophilization, NIBSC reformulated the material with 0.27 M glycine and 0.2% (w/v) NaCl to give 5% (w/v) IgG at pH 6-7 containing approximately 300 IU/ml. This material was assigned NIBSC code 16/278. The mean weight of the dispensed solution in 753 ampoules was 1.0083 g. The imprecision of the filling (CV) was 0.183%, and the residual moisture was 0.3%.

Sample C consisted of the same anti-D immunoglobulin product as Sample A/D and was filled and lyophilized as for all other samples but with a modified formulation for lyophilization; 1-2% (w/v) IgG containing approximately 300 IU/ml with 0.07 M glycine, 0.9% (w/v) NaCl, pH 6-7. This material was assigned NIBSC code 16/272. The mean weight of the dispensed solution in 699 ampoules was 1.0082 g. The imprecision of the filling (CV) was 0.170%, and the residual moisture was 0.6%. Robust ‘cakes’ were not achieved for sample C following lyophilization.

For all samples, the constituent plasma pools were tested and found negative for anti-HIV, anti-HCV, HCV RNA and HBsAg.

Participants were allocated three ampoules of each of the coded materials shown in Table 1. In addition, two ampoules of biotinylated BRAD-5 (02/230) [15] were also provided to those participants performing the competitive enzyme-linked immunoassay (cEIA).

Participants

A total of twenty-one laboratories in fifteen countries across the globe participated in the study (detailed in Appendix A), each of which was assigned a code number. This coding does not reflect the order of listing. The participants included official medicines control laboratories (OMCLs), regulatory authorities and manufacturers of anti-D immunoglobulin. All had experience of performing anti-D quantitation.

Anti-D quantitation methods

A WHO International Standard should be suitable for use in as many different assay methods as possible. Therefore, although most participating laboratories performed one or more of the three Ph Eur reference methods [16,17] (AutoAnalyser [continuous flow analysis], cEIA and flow cytometry), the collaborative study was not limited to such laboratories. Where a laboratory performed more than one different assay method, or variations of the

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same method, each was treated as if performed by different laboratories.

Study design

Microtitre plate layouts for the randomized testing of the samples in both the flow cytometry assay and cEIA were given in the protocol. For each assay method, the layouts resulted in six assay estimates for each of samples A–D relative to sample S, the present IS. For all assay methods, participants were requested to perform two assays on each of three days and regardless of the assay method used, participants were requested to prepare four independent sets of fresh twofold dilutions of each preparation, using a concentration range suitable for their assay, for each assay run or plate. Raw assay data were requested together with a summary of the participants’ own estimates of potency of the four anti-D immunoglobulin samples relative to the WHO 2nd IS for anti-D immunoglobulin, 01/572.

Statistical analysis

The potencies of coded samples A–D were calculated relative to the 2nd International Standard 01/572 by parallel line analysis of the raw assay data using a linear section of the response range with a minimum of three dilutions for all samples. All flow cytometry assays were analysed with a log transformation of the assay response and all competitive EIAs without any transformation. For the AutoAnalyser data, a log transformation, a square transformation and no transformation were used for laboratories 12, 13 and 21, respectively. Calculations were performed using the European Directorate for the Quality of Medicines (EDQM) software, CombiStats Version 5.0 [18]. This software has been developed at the EDQM and performs calculations according to Chapter 5·3 of the Ph Eur (9th Edition). Non-linearity and non-parallelism were considered in the assessment of assay validity. Sample data were visually inspected for non-linearity in all cases. Non-parallelism was assessed by calculation of the ratio of fitted slopes for the test and reference samples under consideration. The samples were concluded to be non-parallel when the slope ratio was outside of the range 0.91–1.10, and no estimates are reported in such cases.

Relative potency estimates from all valid assays were combined to generate an unweighted geometric mean (GM) for each laboratory method, and these laboratory means were used to calculate overall unweighted geometric means for each sample. Variability between assays within laboratories and between laboratories has been expressed using geometric coefficients of variation (GCV = \( \{10^{-s}\} \times 100\% \) where s is the standard deviation of the \( \log_{10} \) transformed estimates). Individual assay estimates of relative potency were log transformed and a mixed effects model used to determine intra-laboratory and inter-laboratory variance components (also expressed as %GCV).

Size exclusion high performance liquid chromatography

Size exclusion high performance liquid chromatography (SE-HPLC) was performed at NIBSC using a TSK G3000SWXL gel filtration column operated by Gilson modular equipment. The equipment was computer controlled by Chromeleon software. Anti-D immunoglobulins were reconstituted, diluted to 2 mg/ml in the mobile phase (0·2 M sodium phosphate, 0·1 M sodium sulphate, pH 6·0), before analysis. An injection volume of 20 µl, using full loop injection, was analysed using a flow rate of 0·5 ml/min. The column eluates were monitored at 280 nm and the software performed peak integration on the 280 nm absorbance channel.

Results

Assay data

The twenty-one participants contributed data from a total of 144 assays which comprised 18 AutoAnalyser assays, 66 competitive EIAs, 54 flow cytometry assays and six in-house assays. Nonconformities from the study protocol and other anomalies were minor and shown in Data S1: Deviations from the study protocol and other anomalies.
Assay validity

The majority of assays gave valid potency estimates when assessed using the validity criteria described above, which were intended for use in the analysis of data from this study only and should not be interpreted as suitable for routine use in the assessment of assay validity within all collaborating laboratories. Around 15% of cases were excluded due to non-linearity or non-parallelism for the competitive EIAs and around 12% due to non-parallelism in the flow cytometry assays. A larger proportion of AutoAnalyser cases were excluded, around 40%, due to non-linearity, non-parallelism or high variability between replicate responses in the assay.

Potencies relative to the WHO 2nd International Standard for anti-D immunoglobulin, 01/572

In general, there was good agreement between laboratory-reported and NIBSC-calculated results. The histograms (Fig. 1a-e) of the NIBSC-calculated geometric mean potencies for each laboratory and sample also show good agreement both between laboratories and assay methods, for all samples.

For each of the assay methods (AutoAnalyser, cEIA and flow cytometry), the NIBSC calculated overall geometric mean potency estimates of each sample relative to the 2nd IS for anti-D immunoglobulin are shown in Table 2. For all samples, the geometric mean potency estimates by the cEIA and flow cytometry assays are similar. However, in most cases, the AutoAnalyser assays gave higher estimates than the other two Ph Eur methods and this is in agreement with the finding of the previous study [13] when using the 2nd IS as the reference. Nevertheless, the data presented in Table 3 show there is little difference between the overall potency estimate for samples A-D when comparing the cEIA plus Flow Cytometry alone to all methods. Therefore, to maintain a consistent approach to assigning potency, the analyses included all methods. Laboratory 12 consistently obtained higher estimates than all the other laboratories. Grubbs' outlier test [19] showed the estimate of potency of sample B by the AutoAnalyser assays of laboratory 12 was significantly higher ($P < 0.05$) than all other estimates from all laboratories and results from laboratory 12 were therefore excluded from analysis of the overall potencies for sample B.

Fig. 1 (a–e) Potency estimates, relative to the WHO 2nd IS for anti-D immunoglobulin. (a) Sample A, (b) Sample B, (c) Sample C, (d) Sample D and (e) Samples A and D* combined. Estimates are expressed on a log scale. The number in the square denotes the laboratory code. Each square represents the geometric mean estimate from that laboratory. The shading represents the different assay methods.
| Assay method      | Laboratory code | A       | %GCV | N | B       | %GCV | N | C       | %GCV | N | D       | %GCV | N | Ratio of A:D |
|-------------------|-----------------|---------|------|---|---------|------|---|---------|------|---|---------|------|---|--------------|
| Competitive EIA   | 01              | 290.6   | 6.0  | 6 | 248.0   | 6.5  | 6 | 289.2   | 8.4  | 6 | 294.6   | 7.9  | 6 | 0.99        |
| Competitive EIA   | 03              | 299.5   | 6.1  | 4 | 274.5   | 2.9  | 4 | 292.2   | 2.9  | 4 | 302.9   | 5.7  | 4 | 0.99        |
| Competitive EIA   | 04              | 309.9   | 11.2 | 3 | 266.0   | 7.9  | 3 | 311.2   | 10.0 | 3 | 303.8   | n/a  | 1 | 1.02        |
| Competitive EIA   | 05              | 310.2   | 0.8  | 6 | 281.2   | 4.5  | 6 | 321.8   | 2.9  | 6 | 313.2   | 2.4  | 5 | 0.99        |
| Competitive EIA   | 07              | 291.1   | 3.2  | 6 | 258.2   | 6.0  | 5 | 289.1   | 3.6  | 5 | 289.7   | 4.0  | 5 | 1.00        |
| Competitive EIA   | 09              | 270.7   | 3.2  | 5 | 252.4   | 6.5  | 5 | 284.4   | 7.7  | 4 | 269.4   | 9.3  | 5 | 1.00        |
| Competitive EIA   | 10              | 295.6   | 1.6  | 6 | 260.7   | 3.5  | 6 | 300.8   | 1.0  | 6 | 293.7   | 2.6  | 6 | 1.01        |
| Competitive EIA   | 11              | 285.8   | 4.2  | 3 | 235.6   | 4.1  | 3 | 278.8   | 5.5  | 4 | 304.9   | 7.3  | 4 | 0.94        |
| Competitive EIA   | 14a             | 301.9   | 3.5  | 6 | 259.6   | 3.7  | 6 | 309.3   | 3.4  | 6 | 298.9   | 2.0  | 5 | 1.01        |
| Competitive EIA   | 14b             | 286.6   | 5.4  | 6 | 246.6   | 6.4  | 5 | 278.2   | 5.0  | 6 | 283.2   | 4.2  | 5 | 1.01        |
| Competitive EIA   | 17              | 293.2   | 5.3  | 6 | 255.0   | 4.8  | 6 | 283.2   | 3.8  | 6 | 296.3   | 3.4  | 6 | 0.99        |
| Flow Cytometry    | 02              | 285.9   | 3.9  | 5 | 264.3   | 1.9  | 4 | 287.8   | 2.5  | 5 | 286.3   | 3.8  | 6 | 1.00        |
| Flow Cytometry    | 03              | 280.0   | 6.0  | 6 | 257.0   | 5.5  | 5 | 282.2   | 4.6  | 6 | 272.1   | 3.8  | 6 | 1.03        |
| Flow Cytometry    | 06              | 293.9   | 1.8  | 5 | 274.8   | 3.0  | 3 | 293.0   | 5.5  | 5 | 294.9   | 1.9  | 3 | 1.00        |
| Flow Cytometry    | 08              | 312.6   | 6.9  | 6 | 291.6   | 1.3  | 6 | 290.1   | 5.4  | 6 | 308.2   | 2.6  | 6 | 1.01        |
| Flow Cytometry    | 14              | 266.0   | 3.7  | 5 | 237.4   | 3.7  | 3 | 273.4   | 3.0  | 6 | 266.4   | 3.1  | 6 | 1.00        |
| Flow Cytometry    | 16              | 300.2   | 2.4  | 5 | 270.2   | 2.6  | 6 | 298.4   | 1.2  | 6 | 294.4   | 1.4  | 6 | 1.02        |
| Flow Cytometry    | 18              | 287.7   | 2.1  | 6 | 270.0   | n/a  | 1 | 289.4   | 2.1  | 6 | 290.8   | 2.4  | 6 | 0.99        |
| Flow Cytometry    | 19              | 305.6   | 6.1  | 6 | 281.6   | 10.8 | 5 | 304.9   | 6.2  | 6 | 296.7   | 7.1  | 5 | 1.03        |
| Flow Cytometry    | 20              | 291.3   | 3.9  | 6 | 262.6   | 4.1  | 5 | 281.9   | 3.2  | 5 | 285.2   | 4.6  | 6 | 1.02        |
| AutoAnalyser      | 12              | 347.6   | 2.3  | 4 | 373.6   | 3.6  | 4 | 354.3   | n/a  | 2 | 338.3   | n/a  | 2 | 1.03        |
| AutoAnalyser      | 13              | 313.7   | 2.1  | 6 | 299.4   | 5.0  | 6 | 312.7   | 2.9  | 6 | 316.1   | 2.0  | 5 | 0.99        |
| AutoAnalyser      | 21              | 334.2   | n/a  | 2 | 296.1   | n/a  | 2 | 344.3   | 2.6  | 3 | 300.2   | n/a  | 2 | 1.11        |

GM, geometric mean; %GCV, geometric coefficient of variation; N, number of valid results
Samples A and D are coded duplicates and their estimates should give a ratio of 1. As shown in Table 2, the ratios of the estimates for A and D from all but laboratory 21 (which showed high inter-assay variability for sample D) were 1.0, indicating good performance by the laboratories. The ratios of the overall potency estimates by different methods for samples A and D were also 1.0 (Table 3). Therefore, the results for samples A and D could be combined with confidence to give an overall potency estimate for the candidate preparation, 16/332 (Table 4a).

Fig. 1e also illustrates the good agreement between laboratories and assay methods for samples A and D. The in-house assay method performed by laboratory 15 produced semi-quantitative results and was unable to be used in the calculation of potency.

Intra-laboratory variability

For four laboratories (04, 12, 18 & 21), it was not possible to calculate the %GCV for one or more samples due to insufficient valid data. The variability within each laboratory, that is, between-assay variability, expressed as % GCV for each sample is given in Table 2. With the exception of two laboratories (laboratory 4, samples A & C; laboratory 19, sample B) that obtained GCVs ranging from 10% to 11%, all GCVs were <10% with the majority <5%. This indicates all participants were able to perform these assay methods with good precision. The combined intra-laboratory % GCVs are similar for all samples (Table 3).

Inter-laboratory variability

There was excellent agreement of potencies within assay methods and between assay methods with the majority of the inter-laboratory GCVs <10% (Table 3). Variability expressed as GCV ranged between 3.2 and 4.2% for the cEIA, 2.8 and 5.4% for the flow cytometry assay and 1.8 and 13.7% for the AutoAnalyser assay. For all assay methods, the variability between laboratories is similar except for the high variability seen with the autoanalyser result for sample B which is more than double the % GCVs obtained from either the cEIA or flow cytometry assay.

Potencies for samples B and C relative to the candidate 3rd International Standard for anti-D immunoglobulin, 16/332

Table 4b shows the estimates for samples B and C for competitive EIA and flow cytometry assays, all methods and all methods excluding the outlier (laboratory 12) for
sample B. Whilst the geometric mean for sample C was identical to sample A/D, sample B was marginally lower.

Size exclusion high performance liquid chromatography

Peak areas for sample S (2nd IS) and samples A-D are shown in Table 5. Aggregate and fragments were present only in negligible amounts for all samples (<5% combined peak areas). Chromatograms are shown in Data S2: SE-HPLC chromatograms.

Stability

Estimates of the potency and SE-HPLC peak areas were determined for ampoules of samples A/D (16/332) and B (16/278) stored at elevated temperatures (+4, +25, +37, +45 and +56°C) for a period of 1.2 and 1.38 years, respectively. The relative potencies of the accelerated thermal degradation samples were used to fit an Arrhenius equation relating degradation rate to absolute temperature assuming first-order decay [20], and hence predict the degradation rates when stored at a range of temperatures. The analysis has shown a predicted loss of potency per year of <0.001% for 16/278 when stored at −20°C. For 16/332, there was insufficient degradation to fit the Arrhenius equation with measurable loss of potency only at +56°C. When viewed alongside SE-HPLC stability data (shown in Data S3: SE-HPLC peak area stability results for Samples A/D (16/332) and B (16/278)) we can be confident that 16/332 is at least as stable as 16/278 and will therefore be stable for decades when stored at −20°C. Stability testing at NIBSC has also shown that reconstituted 16/332 is stable for at least 1 month at 4°C in a tightly lidded tube in the presence of 0.02% (w/v) sodium azide.

Discussion

The origins of the incomplete [21] (IgG) anti-D standard date back to 1956 when Dr AE Mourant (then Director of the WHO International Blood Group Reference Laboratory) highlighted the requirement for an anti-D standard to WHO [22], leading to the establishment of the International Standard for anti-Rho (anti-D) Incomplete Blood-Typing Serum in 1964 [23]. All subsequent anti-D immunoglobulin International Standards and British Standards for serum anti-D are traceable to the Incomplete Blood-Typing Serum and have a potency defined in International Units (IU). In this report, we have presented the results of an international study where the candidate 3rd IS, sample A/D (16/332), was calibrated against the 2nd IS (01/572) and most participating laboratories (20/21) performed AutoAnalyser, flow cytometry and/or competitive EIA methodology all of which are Ph Eur methods described in chapter 2.7.13: Assay of anti-D immunoglobulin [16]. For the autoanlyser, a larger proportion of results were excluded compared to the flow cytometry and competitive EIA. This may be a result of the intricate nature of the autoanlyser for which highly trained dedicated operators and careful equipment maintenance are critical for optimum performance.

There was better overall agreement between all laboratories and between assay methods for the potency of sample A/D (16/332), the candidate 3rd International Standard, than for sample B (16/278). Sample B will be distributed for use as an internal assay control and can

Table 4 Overall potencies

| (a) Sample A/D relative to S (2nd IS) | GM (IU/ml) | Lower 95% CI | Upper 95% CI | N |
| --- | --- | --- | --- | --- |
| Competitive EIA and Flow Cytometry only | 292.5 | 286.8 | 298.3 | 20 |
| All methods | 296.6 | 289.5 | 303.8 | 23 |

(b) Overall estimates for samples B & C relative to sample A/D

| Sample | GM (IU/ml) | Lower 95% CI | Upper 95% CI | N |
| --- | --- | --- | --- | --- |
| Competitive EIA and Flow Cytometry only | B | 266 | 261 | 271 | 20 |
| | C | 297 | 293 | 301 | 20 |
| All methods | B | 269 | 263 | 276 | 23 |
| | C | 298 | 294 | 303 | 23 |
| All methods excluding Laboratory 12 | B | 267 | 262 | 272 | 22 |

GM, geometric mean; N, number of laboratories

Table 5 SE-HPLC peak areas for sample S (2nd IS) and samples A-D

| Sample | % Aggregate | % Dimer + Monomer | % Fragments |
| --- | --- | --- | --- |
| S | 1.2 | 97.8 | 1.0 |
| A/D | 1.4 | 97.8 | 0.8 |
| B | 1.6 | 97.8 | 0.6 |
| C | 2.1 | 96.8 | 1.1 |

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be assayed using the same dilution series as the replacement IS. Sample C was included as a comparability sample and the heterogeneity seen in the lyophilized cake was due to the level of NaCl in the formulation. However, this is only of concern in terms of appearance as molecular integrity was not affected and the overall potencies of samples A/D and C were identical. Unfortunately, there is insufficient stock of sample C for it to be considered as a candidate International Standard.

The overall geometric mean potency for samples A and D is 296.6 IU/ampoule. In addition to showing the least variability between laboratories and between quantitation methods, sample A/D, 16/332, allows for rapid reconstitution due to its low lgG concentration, like the 2nd IS. We conclude that the anti-D immunoglobulin preparation used as sample A/D in the collaborative study is the most suitable preparation for use as the replacement IS. Accelerated degradation studies predict suitable long-term stability when stored at ~20°C.

The participating laboratories have been shown to be highly competent in performing anti-D quantitation which adds confidence to the assigned potency allowing for continuity of the harmonized unitage. Based on the results presented here, 16/332 was established by the WHO ECBS as the 3rd International Standard for anti-D immunoglobulin with an assigned potency of 297 IU/ampoule. The International Standard (16/332) and internal control preparation (16/278) are available from the National Institute for Biological Standards and Control (https://nibsc.org/products/brm_product_catalogue.aspx).

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Author contributions

GS, GR, LS & BF evaluated the candidate samples; EA and PR carried out the statistical analysis; BF lead the study, reviewed the results and drafted the manuscript, which was critically reviewed and approved by all of the authors.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix

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