Chapter

Flow Cytometry Assay for Quantitation of Therapeutical Anti-D IgG during Process Control in the Pharmaceutical Production

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Abstract

Individuals who do not possess the D antigen in their red blood cells generate Anti-D antibodies against an antigenic challenge. Prophylaxis with Anti-D immunoglobulin prevents sensitization. The determination of adequate doses of Anti-D in plasma and pharmaceutical products is carried out by radio immuno assay (RIA) and enzyme immuno assay (EIA) or hemagglutination. An in house technique was developed for the quantitation of Anti-D antibodies, as an alternative test to the reference method. It was specific and with good recovery and did not present false positives or autoagglutination. The dose-response curve (mean fluorescence intensity (MFI) versus logarithm of concentration (log C)) was linear (correlation coefficient of 0.99). The method was validated following the criteria of the NIBSC (National Institute of Biological Standards and Control) and the European Pharmacopoeia. Flow cytometry allowed obtaining accurate, precise, sensitive and specific determinations at different concentrations in different biological matrices. The method can be used in highly diluted samples, has a strong fluorescence signal, is simple, fast, reliable and of relatively low cost. Flow cytometry is more efficient than hemagglutination and easier than RIA. With similar security and efficiency standards, it is cheaper than EIA an RIA. This method as a more suitable choice.

Keywords: cytometry assay, Anti-D IgG (Rho), process control, industrial process

1. Introduction

The Anti-D immunoglobulin (Anti-D IgG) produced from human plasma is used in the immunoprophylaxis of hemolytic disease of the newborn individuals who do not possess the red blood cells D antigen. Health authorities and manufacturers determine the concentration of antibody for each batch of medicine. Initially, the monitoring of Anti-D in the plasma of pregnant women and in finished products (Anti-D IgG) was performed by the anti-globulin test or Coombs test [1, 2] or by the continuous flow analysis of hemagglutination with a Technicon Autoanalyzer [3–8]. The automatic hemagglutination method has been considered as a reference by the European Pharmacopeia (EP); despite this, some groups of experts and regulatory bodies have replaced such methodology by radioimmunoassay (RIA)

[9–11] flow cytometry and a competitive enzyme immunoassay (EIA). These alternatives to the current reference method of European Pharmacopeia have been successfully investigated during international collaborative studies [11–17]. Competitive flow cytometry and EIA assays have been described previously [18–20].

We describe the use of flow cytometry in the control of manufacturing processes of Anti-D globulin, establishing the validation parameters and compliance with the quality standards required by the GMP and established by the producer at the different stages of the industrial process (hyperimmune plasma, Fraction II of the Cohn-Oncley process, and finished product) [13, 14, 21–23].

The rationale of the technique lies in the binding antigen antibodies, which are labeled with fluorescein detected by flow cytometry. The Anti-D immunoglobulin is quantified in comparison with the international reference preparation, calibrated in international units (IU), which allows to give a specification in IU/ml [20, 24]. In relation to flow cytometry, Expert Group No 6B of the NIBSC standardized a procedure to be applied in the evaluation of Anti-D in Anti-D IgG solutions [24]. In our laboratory, a flow cytometry technique was developed for the quantititation of Anti-D antibodies of the Gamma-Rho UNC, designed according to the procedures described in the literature [20, 24] and validated according to the criteria indicated by the NIBSC expert group [16, 24].

2. Materials and methods

2.1 Description of the flow cytometry test

A laser hits the red fluorescein-labeled cells that cross the cytometer in a continuous flow. The light emission produced by the interaction is detected by the forward scatter (FS) and converted into a voltage pulse (FS). A limit of 25,000 events is determined at a flow rate of 600 cells per second with a power of 1300 V. The concentration of Anti-D will be proportional to the size and intensity of fluorescence emitted by the antigen-antibody fluorescein complex of each red blood cell. The mean fluorescence intensity is plotted according to the logarithm of the Anti-D concentration.

2.2 Preparation of cell suspension and Anti-D IgG samples and sensitization and immunoglobulin binding to the cell surface

A Rho red cell suspension (Red Cell phenotypes R1R1/R1R2 and Rh-rr as control), were diluted to 0.2% in phosphate-buffered saline (PBS) pH 72 and plasma samples, Fraction II and finished product were diluted 1/5 to 1/100 with 1% PBS—pH 7.2.

2.3 Sensitization and immunoglobulin binding to the cell surface

0.2% red cell suspension was incubated with different test fractions (Anti-D standard IRP 68/419 OMS-NIBSC, human plasma, Fraction II of the Cohn process and gamma Anti-D globulin) in a 50/50 μl ratio. After stirring 30 min at 4°C, red blood cell suspension is washed with PBS-1% BSA and incubated with a 1/50 dilution of a goat anti-human IgG FITC (**Figure 1**). Red blood cells resuspended in 1 ml of PBS-1% BSA are measured with flow cytometry in Epics XL-MCL Cytometer-Coulter.

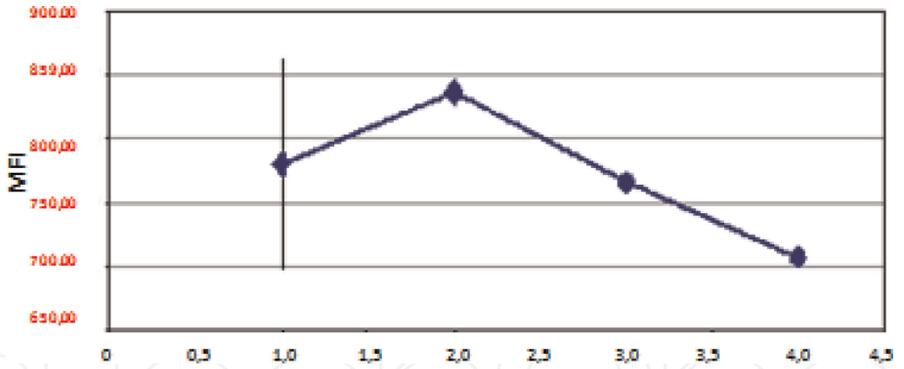

Figure 1.
Calibration curve of the conjugate dilutions.

2.4 Standard curve and statistical treatment

In a concentration range of 1500 ng/ml (7.5 IU/ml)–180 (0.9 IU/ml) ng/ml of international reference for human Anti-D immunoglobulin preparation (IRP 68/419 WHO), we establish the standard curve. No Anti-D sensitized cells were used, to set false positives and the minimum level of detection. Mean fluorescence intensity values were between 600 and 1900 and averaged around 800. The upper and lower limit values were determined using five standard curves. Nonparametric statistics was used and processed with Microsoft Excel 7.0 program and Method Validator 1.15 [25–28].

3. Results

3.1 Calibration of the xytometer

Epics XL-MCL Cytometer (Coulter, Corp., Luston, UK) was checked and calibrated using the standard immunocheck particles (Becton Dickinson, Oxford, UK). The red cell samples automatically passed through the cytometer according to their FS and side scatter (SS). After defining the working conditions and the protocol to be used with the cytometer, it was determined that the most suitable conjugate dilutions were 1/50 and 1/100 (**Figure 1**). It was determined to work with 25,000 events at a flow rate of 600 events/s (**Figure 2**). The intensity of the signal observed in the negative controls is related to non-specific negative unions. There were no other phenomena related to false positives (**Figure 3**).

The most suitable conjugate was determined by processing 26 tubes in duplicate and 1/100 dilutions of the conjugate (Sigma Anti Human IgG -Fc Conjugate-F9512 specific and Kallestad FITC Conjugate #30446). Serial dilutions of commercial gamma globulin (250 μg/ml) were used as control. **Figure 4** shows that the Sigma conjugate presents a greater fluorescence signal. After 10 assays using different fluorescence particles, the fluorescence intensity (MESF) and the fluorescence signal emitted by the cytometer presented good correlation. **Figure 5** shows the linear relationship between the emitted MFI and the MESF. Peak's SD values in a range of 1.4–7.6 represent points with higher and lower intensity.

The histograms of the fluorescence parameters that were plotted according to the anti-Rho IgG concentrations can be seen in **Figure 6**. **Figure 7a** shows the working area determined with the forward and lateral dispersion of a homogenous population of non-sensitized cells. We observed no significant agglutination of auto

aggregated red cells for anti-Rho IgG concentrations less than or equal to 960 ng/ml (4.8 IU/ml). We worked in intervals of ≤960 ng/ml to avoid increments of FS caused by auto aggregation (**Figure 7b**).

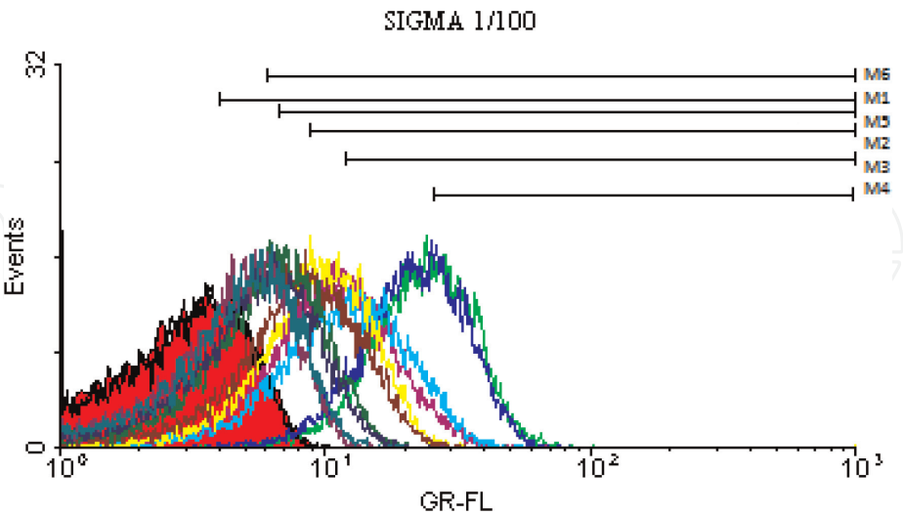

Figure 2.
Work and flow rate events.

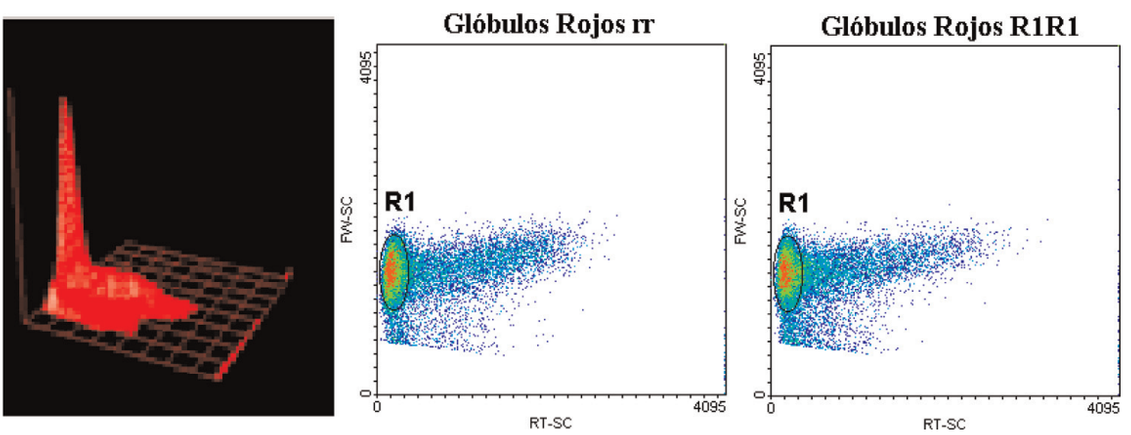

Figure 3.
Forward (FS) and side (SS) scatter graphs of a homogeneous population of non-sensitized cells.

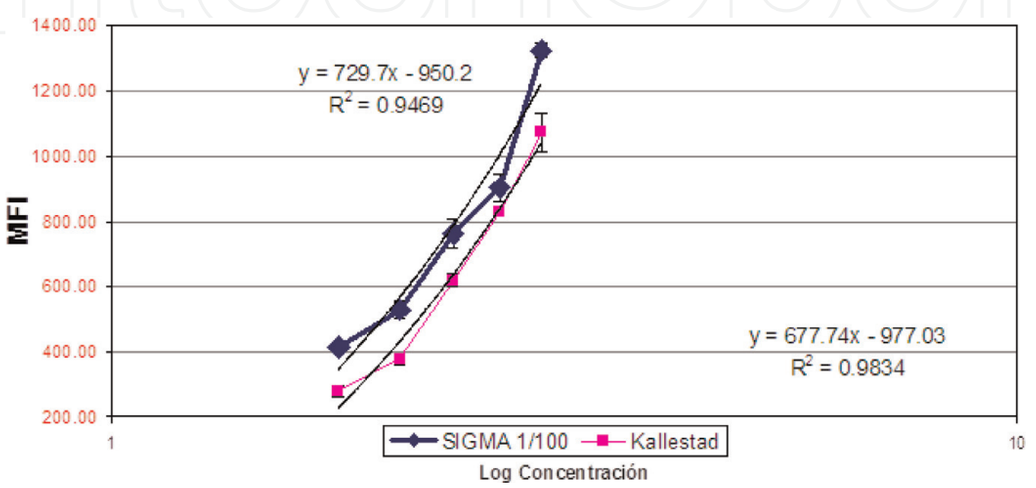

Figure 4.
Comparative curve of the conjugates.

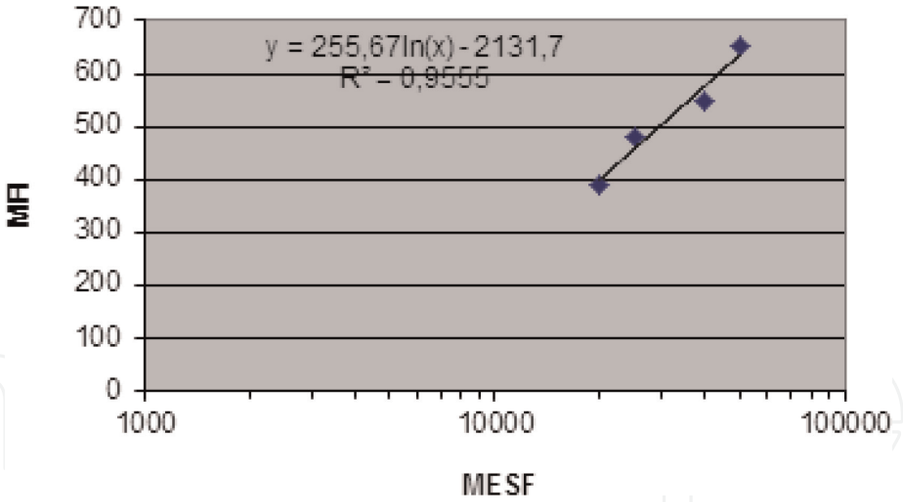

Figure 5.
Calibration curve of the flow cytometer, correlation between MFI and MESF.

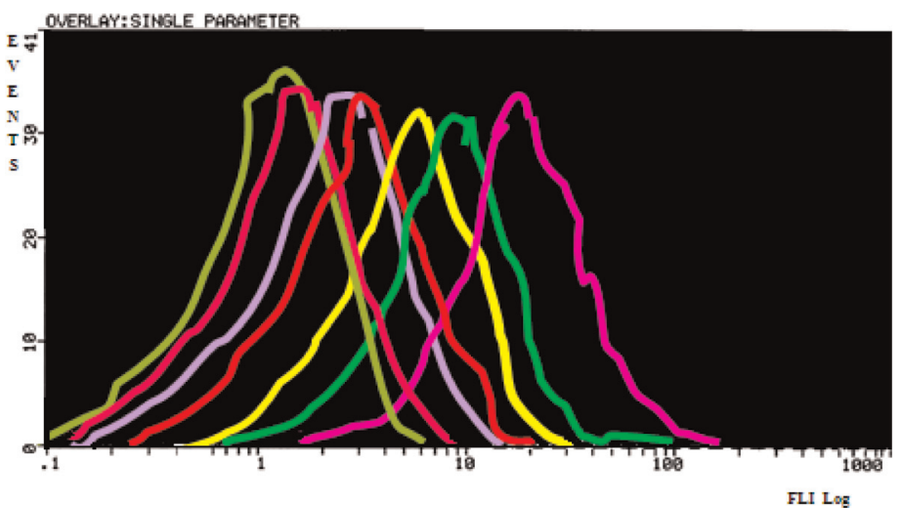

Figure 6.
FL1 histogram of fluorescence stockings obtained from seven dilutions of the WHO 68/419 standard using GR Rho + R1R1, ------- 960 ng/ml AD, ------- 480 ng/ml AD,--- 240 ng/ml AD, ------- 120 ng/ml AD, ------- 60 ng/ml AD, and ------- 15 ng/ml AD.

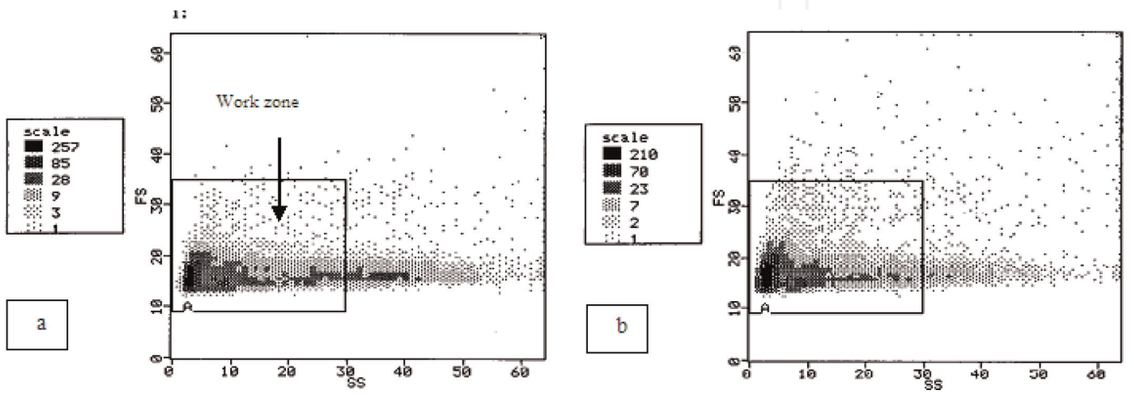

Figure 7.
(a) Working region selected for Rho + R1R1 red blood cells according to the FS and SS of washed cells not sensitized in a concentration of 0.2% in PBS. (b) Increase of FS of GR R1R1 sensitized with anti-Rho IgG standard 68/419 960 ng/ml.

3.2 Effect of the red blood cell phenotype

Phenotypes R1R1, R1R2, R1r´, and R1r were tested to optimize the assay. The values of estimated concentrations (means-standard deviations-CV%) suggest that the tests are reproducible for each sample and each phenotype (**Table 1**). The R1R1 and R1R2 phenotypes presented a greater fluorescence signal, and, therefore, a greater amount of Anti-D was adsorbed than the rest of the phenotypes (**Figure 8**).

3.3 Standard curve and cutoff

To establish the standard curve, dilutions of WHO reference preparation were used in a concentration range of 30–480 ng/ml. The corresponding values of MFI were between 1.4 and 4.5. To establish the minimum level of detection, non-sensitized cells were run, with MFI values averaging 1.2 (**Figure 9**).

A linear and proportional to the log of the Anti-D concentration fluorescence response was obtained in the measured range. The linear regression of 270 points was r = 0.963. The linear measurement range was set between 120 and 240 ng/ml and was the most acceptable for our purpose.

Seven similar curves were run with each other (**Figure 10a**), and the average curve and two SD values were calculated at each point, and the upper and lower

Phenotype		**R1R1**			**R1R2**	
ng STD	480	120	60	480	120	60
ng Rec.	480.45	119.70	60.30	478.75	118.00	60.10
	475.80	118.98	60.00	477.30	119.00	56.90
	481.06	120.00	59.45	476.89	118.5	59.89
	480.03	121.00	60.10	477.00	116.78	60.05
Media	479.33	119.92	59.96	477.48	118.07	59.235
SD	2.39	0.84	0.36	0.86	0.95	1.56
VC%	0.5	0.7	0.6	0.2	0.8	2.6
SD + 2	484.12	121.59	60.69	479.21	119.97	62.35
SD − 2	474.54	118.24	59.23	475.76	116.17	56.12
Phenotype		**R1r´**			**R1r**	
ng STD	480	120	60	480	120	60
ng Rec.	476.35	117.45	60.10	465.98	120.21	60.10
	469.40	117.67	56.90	475.35	114.78	56.90
	474.45	118.25	57.98	474.95	118.25	57.98
	475.60	117.33	58.87	477.60	117.33	56.89
Media	473.95	117.675	58.46	473.47	117.64	57.96
SD	3.13	0.40	1.35	5.12	2254	1.51
VC%	0.7	0.3	2.3	1.08	1.9	2.6
SD + 2	480.21	118.49	61.17	483.72	122.15	60.98
SD − 2	467.68	116.85	55.74	463.21	113.13	54.94

Table 1.
Red blood cell phenotype effect.

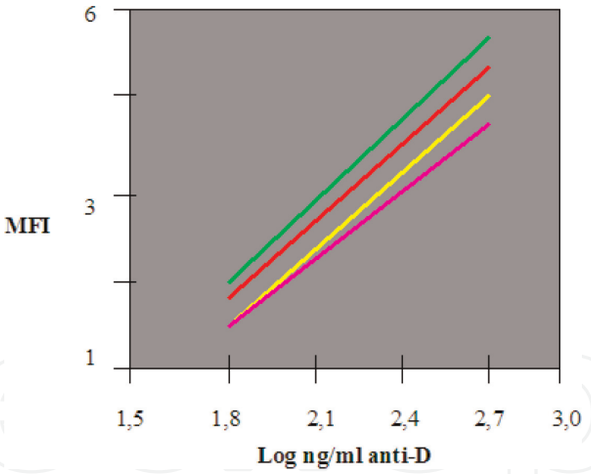

Figure 8.
Standard curves using red blood cells of phenotype: R1R1 (-----), R1R2 (------), R1r´ (------), and R1r (------).

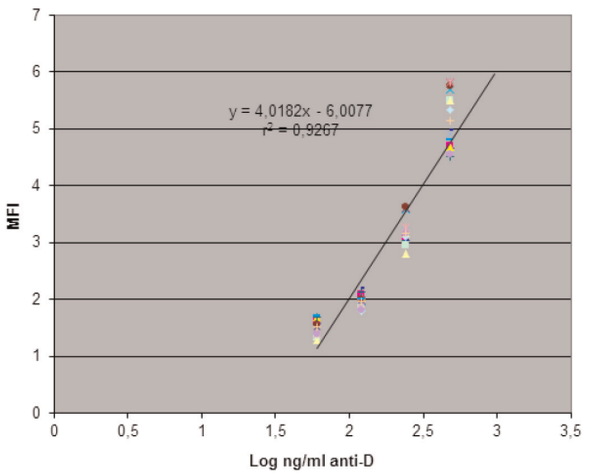

Figure 9.
Construction of the standard curve. A total of 18 curves was run and its linear regression was r = 0.963.

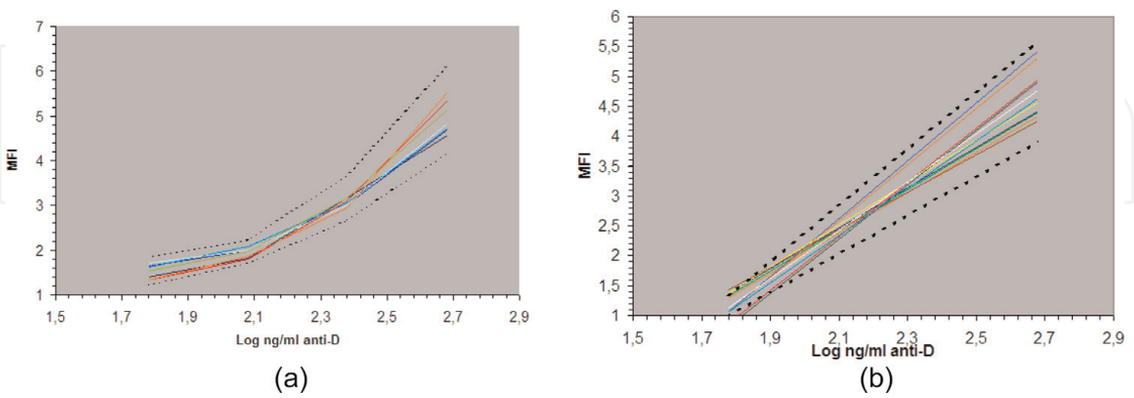

Figure 10.
(a) Determination of the lower and upper limits (------) and the values of ±2DS for the mean standard curve. The limit lines were used for the approval of the standard curves. (b) From 19 standard curves, 15 were found completely within the lower and upper limit lines.

limits of each reading were calculated and plotted. To determine the appropriate limits for our purpose, 19 standard curves were tested. As shown in **Figure 10**, at high concentrations of Anti-D, there are no significant deviations, and in the diluted samples, three showed deviations from the upper limit (0.24 μg/ml) and one below

the lower limit (120 ng/ml). The limits of 120–240 ng/ml were taken as validation criteria of the standard curve.

The cutoff value set at 10 ng/ml (0.05 IU/ml) corresponds to a 1:1024 Anti-D standard (NIBS) dilution. After 135 plasma assays (55 Anti-D positive—80 negative), it was determined.

3.4 Reproducibility and repeatability

Tests in different matrices repeated 10 times simultaneously (intra-assay) during different days (inter-assay) evaluated the reproducibility and repeatability of flow cytometry. The sources of variation found were linked to the preparation of samples and variations in the cytometer (**Table 2**). The test showed good repeatability between tests, indicated by the coefficient of variation in representative samples (CV 4.95%, high concentration; 3.36%, intermediate concentration; and 4.78%, low concentration) (**Table 3**). These results coincide with Thorpe and Schäffner in the collaborative study of the European Union [13].

3.5 Recovery of Anti-D

The estimated concentration of Anti-D for each measured point in three commercial products presented a %CV in the range of 1–7.5% (**Table 4**). Two-thirds of the powers estimated by the manufacturers were within 95% of the acceptance limit of flow cytometry.

Only 1 of the 30 tests performed on 3 known concentration of commercial products showed deviations in the cut of the determined concentrations (87.3–111.9). The assay presented good recoveries of concentration in the samples tested (**Table 4**). The concentrations were estimated using the Wilcoxon test.

Intra-assay	**(a) Reference standard 68/419 WHO in ng/ml**			
Concentration	480	120	30	
n	10	10	10	
Average MFI	4.79	1.98	1.25	
SD	0.24	0.07	0.06	
VC %	4.95	3.36	4.78	
Intra-assay	**(b) Sample control µg/ml**			
Sample	**Pool of plasma***	**Cohn Fraction II ***	**γ1***	**γ2***
Concentration	12.5	30.2	197.2	179.5
n	10	10	10	10
Average MFI	2.08	11.26	3.12	3.49
SD	0.06	0.05	0.16	0.17
VC%	3.06	4.32	5.16	4.93
% Recovery	113.6	91.56	98.33	109.46

**Commercial gamma globulins: concentration 1 = 204 µg/ml, 2 = 164 µg/ml; pool = 11 µg/ml, Cohn's Fraction II = 33 µg/ml.*

Table 2.
Accuracy and reproducibility of the standard (a) and control samples (b).

Inter-assay: (a) reference standard 68/419 WHO in ng/ml											
ng/ml	**MFI**							**SD**	**VC%**	**+2SD**	**−2SD**
	M 1	**M 2**	**M 3**	**M 4**	**M 5**	**M 6**	**_ X**				
480	5.95	5.12	5.06	5.44	4.76	5.22	5.26	0.40	7.69	8.38	4.45
240	3.11	3.17	3.21	2.95	3.01	3.34	3.13	0.14	4.49	5.57	2.85
120	1.8	1.97	1.88	1.84	2.11	2.01	1.93	0.12	6.01	3.97	1.70
60	1.31	1.53	1.39	1.31	1.63	1.59	1.46	0.14	9.71	1.46	1.18
30	1.11	1.23	1.27	1.22	1.3	1.2	1.22	0.06	5.36	1.22	1.09

Inter-assay: (b) Control samples									
Product	**Gamma H***			**Gamma P***			**Gamma B***		
Dilution	1/250	1/500	1/750	1/350	1/700	1/1000	1/250	1/500	1/1000
MFI	1.21	1.16	1.05	1.37	1.24	1.06	1.21	1.15	1.02
	1.18	1.16	1.04	1.39	1.24	1.06	1.23	1.15	1.03
	1.19	1.17	1.04	1.39	1.25	1.06	1.19	1.13	0.98
	1.22	1.19	1.04	1.38	1.23	1.07	1.22	1.14	1.00
	1.20	1.14	1.06	1.3	1.15	1.05	1.21	1.16	1.01
	1.18	1.15	1.05	1.27	1.22	1.04	1.23	1.13	1.02
Average MFI	1.19	1.161	1.05	1.35	1.22	1.06	1.215	1.14	1.01
SD	0.016	0,017	0,008	0.05	0.04	0.01	0.015	0.012	0.018
VC%	1.36	1.48	0.78	3.83	2.99	0.98	1.25	1.06	1.77
µg/ml	149.50	150.25	142.64	236.25	220.86	192.00	151.87	147.41	183.64
% Recovery	99.66	100.16	95.09	107.39	100.39	87.27	92.61	89,89	111.97
+2SD	1.23	1.20	1.06	1.45	1.29	1.08	1.24	1.17	1.04
−2SD	1.16	1.13	1.03	1.25	1.15	1.04	1.18	1.12	0.97

**Declared concentration for H: 150 µg/vial, P: 220 µg/vial, B: 164 µg/vial.*

Table 3.
Accuracy and repeatability of standard (a) control samples (b).

Product	**n**	**Average µg/ml**	**Limit 95%**	**VC%**	**Declared concentration µg/ml**	**% Recovery**
H	6	149.50	145–154	2.6	150	99.7
	6	150.25	146–155	3.3		100.2
	6	142.64	140–144	1.3		95.1
P	6	236.25	218–254	7.2	220	107.4
	6	220.86	208–233	5.7		100.4
	6	192.00	189–196	2.1		87.3
B	6	151.87	147–155	1.9	164	92.6
	6	147.1	145–151	2.7		89.9
	6	183.64	176–189	2.7		111.9

Table 4.
Comparison between anti-D concentration declared by the producer and concentration obtained by flow cytometry.

4. Discussion

The availability of a quantitation method for human plasma, Cohn fractions, and Anti-D gammaglobulin has a relevant importance for the process control of the Anti-D IgG production. Pharmaceutical companies are employing sensitive EIA, RIA, and flow cytometry techniques [10, 11, 15, 20, 24] for the quantitation of Anti-D level in finished products, the use in Anti-D gammaglobulin manufacturing process control have no be reported.

A rapid and low-cost flow cytometry test applied in the control of the industrial process of large-scale production obtaining Anti-D gammaglobulin has been described. This responded to the need of an alternative method to EIA and RIA in the quantitation of Anti-D in different process samples (human plasma, Chon fractions, gammaglobulin) [29].

According to this, the threshold of the assay was adjusted to select the level of antibody content, which will be accepted, to produce an immunoglobulin with a potency of 250 μg/vial (1250 IU/vial), according to the regulatory requirements [13, 14, 28]. To validate the assay, chemical and physical-chemical factors that affect the binding of antigen-antibody influence in the red blood cells were determined. The spatial conditions of cell packing did not affect the assay. The adsorption of antibodies to the cell surface was 1000 times greater when the incubation temperature was 37°C without this affecting the dissociation rate [9]. The different fractions behave similarly in the assay conditions, and there was no interference by autoagglutination at the concentration of red blood cells used (**Figure 7**). The analytical quality assurance of the trial showed that the method presents good reproducibility, repeatability (1–7.5 VC%), and correlation with the standard curve (r: 0.9267). The assay is specific and recovery is 95% of the known Anti-D concentration values. For validation our strategy was based on an international guide accepted by manufacturers and control laboratories [13, 30]. The validation method meets the requirements according to the good manufacturing practices in the pharmaceutical industry. A validation methodology was followed to meet the special requirements of standardization of the good manufacturing practices in the pharmaceutical industry [30, 31].

Biological variability, red blood cells, and matrices were considered in a validation protocol according to international guidelines [14, 30].

5. Conclusions

This flow cytometry method is a sensitive and specific method that allows reproducible results. The concentration values are comparable with those estimated with other methods such as RIA and EIA used by commercial manufacturers of gamma globulins, and the method presents accuracy and precision. The test is completed in 3 h and is easy to perform, allowing quantitative assessment of Anti-D antibodies from plasma, fractions of the Cohn process, and finished products. The flow cytometry method presented shorter processing times (3 h) than the EIA or RIA methods (5 h).

The potential of the flow cytometry method described here represents an alternative to quantify Anti-D in different matrices and meets the criteria of good laboratory practices (simple, fast, and reliable as well as being sensitive and accurate). In the control of industrial processes, this method has shown reproducibility and reliability for this purpose.

The cost of materials is slightly lower for flow cytometry ($ 15/test) than in EIA or RIA ($ 25/test).

Highly significant correlations were observed between the flow cytometry test Anti-D values and EIA values determined in different matrices (Serum, Fraction II, Semi-elaborated, gamma globulin).

For the case of process samples (Fraction II, semi-processed), strong correlations were observed between the flow cytometry test and the EIA values. A potential advantage of the flow cytometry assay could be the higher sensitivity presented than the EIA assay.

It is concluded that the flow cytometry method has advantages over the EIA and RIA method as a substitute for the present standard method.

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