Quality assurance of rituximab (anti-CD 20) antibodies by potency testing: determining the system suitability criteria and sample acceptance criteria

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A validated and robust bioassay is of paramount importance in the various stages of biosimilar development to ensure efficacy, quality and potency. The complement-dependent cytotoxicity assay was validated over six simulated potencies and found specific for rituximab-like antibodies. The bioassay was found robust with linearity parameter $R^2 = 0.99$, %GCV for precision and accuracy was less than 20% for >40 individual performances. Detailed set of system suitability and sample acceptance criteria was determined. The study may play a key part in the development of written and physical potency reference standards for incorporation in different pharmacopeia for effective biosimilar development and regulation.

Keywords: Complement-dependent cytotoxicity, geometric coefficient of variation, quality assurance, rituximab.

BIOSIMILARS are the imitation biological products of their originator molecules with similarity in quality, efficacy and safety[1]. Biosimilars cost less than their originator products owing to their shortened clinical trials[2]. The first biosimilar was approved for use in the European Union (EU) in 2006. A better acceptance of biosimilars will help lessen the burden of healthcare systems through price competition and ease of patient access to vital drugs[3].

Compared to chemical generics, biosimilars are stringently evaluated for quality, safety and efficacy[4]. According to guidance documents on biosimilars issued by European Medicines Agency (EMA) and the US Food and Drug Administration (FDA), pharmacologic activity of protein products showing biosimilarity should be essentially demonstrated by *in vitro* and/or *in vivo* functional assays[5,6]. Guidelines on similar biologics, jointly prepared by the Central Drugs Standard Control Organization (CDSCO) and the Department of Biotechnology (DBT), New Delhi in 2016, state *in vitro* and/or *in vivo* potency assays as one of the critical quality attributes (CQAs) for establishing comparable safety and efficacy of similar biologics and reference biologics[7].

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Rituximab is a chimeric monoclonal antibody wherein mouse variable domains have been grafted over the effector regions derived from humans[8]. The primary target of the antibody is the protein CD20, which is abundantly expressed on the surface of immune system B cells[9]. This mechanism makes rituximab an effective therapy for destruction of B cells and hence is used in the treatment of diseases which are associated with abnormal proliferation, overactive or dysfunctional B cells, such as numerous lymphomas, leukaemia, transplant rejection and autoimmune disorders[10].

A specific, sensitive and robust bio-analytical method for potency or bioactivity evaluation of biosimilars is critical for the development and successful conduct of pre-clinical and clinical pharmacology studies. *In vitro* studies are beneficial in determining the mechanisms of action in a rapid, rigorous and focused way[11]. Previous *in vitro* studies on rituximab suggest three mechanisms for target-cell killing, viz. complement-mediated cytotoxicity of B cells (better known as complement-dependent cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC) and apoptosis of B cells via activation of caspase 3 (ref. 12). Furthermore, it has been proposed and reported in previous studies that CDC is the main pathway involved in *in vivo* effectiveness of rituximab. It has been further suggested that antibody showing poor sensitivity to CDC *in vitro* might show poor clinical response, whereas antibody showing good sensitivity to CDC might show better response to rituximab treatment[13]. Taking clues from these proposals we have developed and validated a robust, efficient and cost-effective CDC-based assay for potency testing of rituximab-like biosimilars. This also encompasses the detailed suitability criteria designed for both the assay and the sample, thus assisting in the batch release and regulation of rituximab-like biosimilars. The study may play a key part in the development of written and physical reference standards for incorporation in different pharmacopeia for effective regulation of the drug.

Chemicals and reagents used in the assay were of cell culture-grade. The WIL2-S cells were procured from American Type Culture Collection (ATCC). RPMI-1640 and FBS were purchased from Sigma-Aldrich, USA. Penicillin and streptomycin solution was purchased from MP Biomedicals, Navi Mumbai, India. Human complement was purchased from Quidel, California, USA. Innovator's rituximab (Ristova) was obtained from the M/s F. Hoffman-La Roche Ltd, Germany for standard solution preparation. All equipment used for CDC assay and validation studies were calibrated and validated. Sterilized plastic ware such as tissue culture flasks, serological pipettes, falcons and 96-well plates was purchased from Nunc, New York, USA.

The WIL2-S cells were grown as suspension in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (heat-inactivated), 5 ml penicillin and streptomycin

Table 1. Dilution Scheme of Drug Antibody

Dilution Step	From step	Concentration (μg/ml)	Fold dilution	Volume (μl)	Volume of assay medium (μl)	Final concentration in plate (μg/ml)
1	Stock	100	NA	NA	NA	NA
2	1	10	10X	30	270	2.5
3	2	5	2X	150	150	1.25
4	3	2.5	2X	150	150	0.625
5	4	1.25	2X	150	150	0.3125
6	5	0.625	2X	150	150	0.1563
7	6	0.3125	2X	150	150	0.0781
8	7	0.1563	2X	150	150	0.0391
9	8	0.0781	2X	150	150	0.0195
10	9	0.0391	2X	150	150	0.0098

Table 2. Assay plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	A.M 150 μl	IRS (2.5)	IRS (1.25)	IRS (0.62)	IRS (0.312)	IRS (0.156)	IRS (0.078)	IRS (0.039)	IRS (0.019)	IRS (0.009)	(+) C	(–) C
B	A.M 150 μl	IRS (2.5)	IRS (1.25)	IRS (0.62)	IRS (0.312)	IRS (0.156)	IRS (0.078)	IRS (0.039)	IRS (0.019)	IRS (0.009)	(+) C	(–) C
C	A.M 150 μl	S1 (2.5)	S1 (1.25)	S1 (0.62)	S1 (0.312)	S1 (0.156)	S1 (0.078)	S1 (0.039)	S1 (0.019)	S1 (0.009)	(+) C	(–) C
D	A.M 150 μl	S1 (2.5)	S1 (1.25)	S1 (0.62)	S1 (0.312)	S1 (0.156)	S1 (0.078)	S1 (0.039)	S1 (0.019)	S1 (0.009)	(+) C	(–) C
E	A.M 150 μl	S2 (2.5)	S2 (1.25)	S2 (0.62)	S2 (0.312)	S2 (0.156)	S2 (0.078)	S2 (0.039)	S2 (0.019)	S2 (0.009)	(+) C	(–) C
F	A.M 150 μl	S2 (2.5)	S2 (1.25)	S2 (0.62)	S2 (0.312)	S2 (0.156)	S2 (0.078)	S2 (0.039)	S2 (0.019)	S2 (0.009)	(+) C	(–) C
G	A.M 150 μl	S3 (2.5)	S3 (1.25)	S3 (0.62)	S3 (0.312)	S3 (0.156)	S3 (0.078)	S3 (0.039)	S3 (0.019)	S3 (0.009)	(+) C	(–) C
H	A.M 150 μl	S3 (2.5)	S3 (1.25)	S3 (0.62)	S3 (0.312)	S3 (0.156)	S3 (0.078)	S3 (0.039)	S3 (0.019)	S3 (0.009)	(+) C	(–) C

IRS, Internal reference standard; Sample 1, S1; Sample 2, S2; Sample 3, S3; (+) C, Control with complement; (–) C, Control without complement.

solution (final concentration to be 100–120 units/ml penicillin and 0.10–0.12 mg/ml streptomycin), 0.25% of glucose and 1 mM sodium pyruvate. Viability and cell count before seeding were carried out using the Neubauer chamber and trypan blue exclusion method. Cells were seeded at the density of $0.1–0.2 \times 10^6$ cells/ml and maintained under a fully humidified atmosphere of 5% CO_2 at 37°C.

CDC assay for potency testing of rituximab was carried out in RPMI-1640 medium supplemented with 1.3% (w/v) bovine serum albumin, 1% penicillin and streptomycin solution (final concentration to be 100–120 units/ml penicillin and 0.10–0.12 mg/ml streptomycin) and 2% 1 M HEPES solution[14]. The assay medium was filtered aseptically through 0.22 μm filter using sterile filtration assembly and stored at 2–8°C.

Dilution scheme used was modified according to the USP Medicines Compendium[14]. Rituximab in case of both reference standard and sample (10 mg/ml) was diluted 100 times through serial dilution to obtain the working stock of 100 μg/ml. Dilutions from the working stock were prepared in assay block using the dilution scheme (Table 1).

As shown in Table 2, assay plates for carrying out CDC assay were setup. Briefly, 50 μl of dilutions prepared were transferred to each assay plate in duplicates. To these dilutions, 50 μl of WIL2-S cell suspension of density $0.8–1.2 \times 10^6$ cells was added. Further, normal human complement was diluted 2.5 times in cold assay medium. Then 50 μl of this diluted complement was added to the reaction mixture. The plates were incubated for 2 h in 5% CO_2 at 37°C. Assay medium control (wherein no reaction component was added), complement control (no antibody was added) and cell culture control (neither antibody nor complement was added) were also setup along with the tests. After 2 h incubation, 50 μl of Alamar Blue was added to each well. The plates were further incubated as in the previous conditions for 16–20 h. After incubation, plates were read for fluorescence using a spectrofluorometer SpectraMax Gemini Spectrofluorometer, Molecular Devices, Shanghai, China at 530/590 nm excitation/emission with cut-off at 590 nm. More than 42 individual performances were conducted to determine the assay suitability and system suitability criteria.

The relative fluorescent units (RFU) signals obtained from SpectraMax plate reader were fit into nonlinear four parameter logistic (4PL) model[15]. The four parameters that need to be estimated in order to 'fit the curve' are A, B, C and D, where A and D are the upper and lower asymptotes respectively, B is the slope and C is the point of infection or half maximal effective concentration (EC_{50}). The equation for the model is

$$y = D + (A - D)/(1 + (x/C)\text{^}B).$$

where x is the independent variable and y is the dependent variable, just as in the linear model[15].

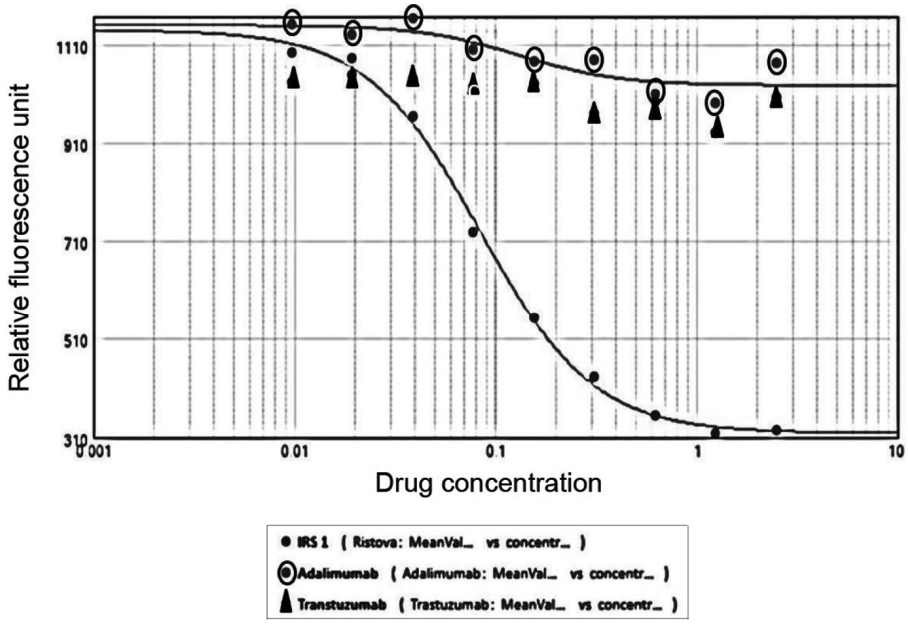

Figure 1. Test for specificity. Dose–response curve of rituximab, adalimumab and trastuzumab using complement-dependent cytotoxicity assay.

The system suitability criteria (SSC) and sample acceptance criteria (SAC) were established based on 95% confidence intervals (mean ± 2SD) or 99% confidence intervals (mean ± 2SD). For determining assay suitability criteria for the bioassay, a detailed comparison of all the 4PL fit terms, viz. R^2 of internal reference standard (IRS) assay, EC_{50}, slope, A/D ratio, mean RFU of cell control, mean RFU of highest concentration of IRS and fold response of $n = 12$ (pre-validation) performances of bioassay was carried out. Similar comparison was done for SSC, viz. % relative potency to IRS and 95% confidence intervals.

The validation parameters, viz. relative accuracy, specificity, intermediate precision, linearity and range of the method were estimated in the method validation study[9,16]. Rituximab test samples were prepared to yield simulated potencies of 50%, 71%, 100%, 122%, 150% and 200% for each parameter.

Specificity parameter was validated to rule out matrix interference and separation selectivity. Anti TNF alpha monoclonal antibody (adalimumab) and anti HER2/neu receptor monoclonal antibody (trastuzumab) samples were put to test along with IRS in a plate to assess the method specificity for rituximab-like antibodies.

For accessing the linearity and range of method, six concentrations of standards with simulated potencies of 50%, 71%, 100%, 122%, 150% and 200% were put to test. A calibration curve for ln (% simulated nominal potencies) versus ln (% observed relative potencies) was plotted and the obtained data were subjected to regression analysis by the least squares method as described by Dafale *et al.*[17].

Repeatability of the method was determined by carrying out CDC assays at various time slots by the same analyst and expressed in the terms of geometric coefficient of variation (%GCV).

$$\%GCV = 100 \times (e^{SD} - 1)\%,$$

where SD is the standard deviation of log-transformed relative potency measurements.

Intermediate precision of the method was assessed by replicating the assay using three different analysts as well as different time periods, and expressed in terms of %GCV (ref. 17).

Accuracy of the bioassay was determined by calculating % relative bias using results of different performances[18]

$$\%\text{Relative bias} = 100 \times ((\text{measured potency}/\text{target potency}) - 1)\%.$$

Biosimilars represent a relatively heterogeneous class of medicinal products that makes their regulation quite challenging. This heterogeneity may be due to higher molecular weight and complexity in structure and function that can be affected by changes in the manufacturing process[19]. According to most guidance documents issued on the regulation of biosimilars, suitable biological assays are required to assess the functional activity and determine the mechanism of action and clinical effect of the product[5–7].

Rituximab has been the choice of treatment in B cell malignancies due to its action against CD20 protein

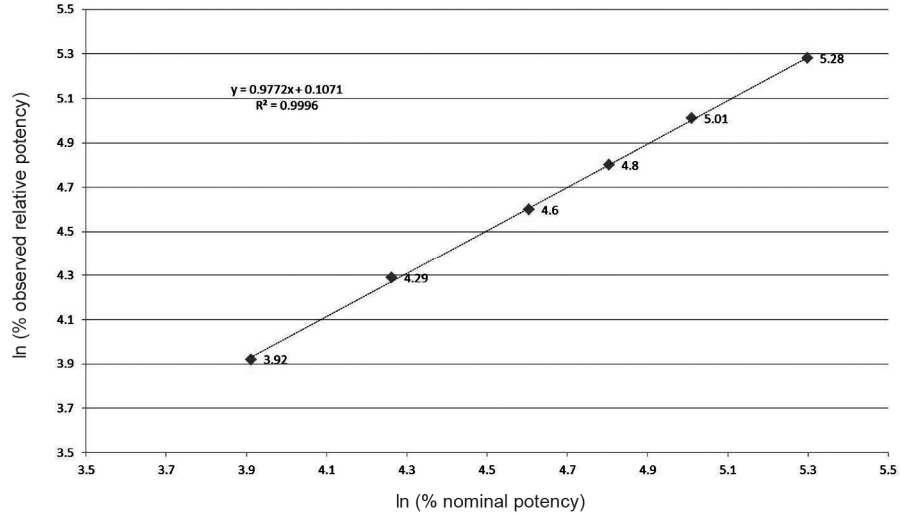

Figure 2. Linear regression model for determining the linearity.

Table 3. Summary data for precision

	Within run (repeatability)		Between run (intermediate precision)	
Nominal potency (%)	CV	%GCV	CV	%GCV
50	0.097	10.24	0.13	13.85
71	0.062	6.36	0.17	18.01
100	0.06	6.14	0.07	7.09
122	0.082	8.59	0.084	8.79
150	0.088	9.29	0.13	14.04
200	0.081	8.54	0.101	10.7

CV, Coefficient of variation; %GCV, Per cent geometric coefficient of variation.

expressed on the surface of proliferating or dysfunctional B cells[8,9]. It is known to act through CDC, ADCC or via activation of caspase 3 under *in vivo* conditions. However, previous studies suggested that *in vitro* CDC response has a direct correlation with effectiveness of rituximab in clinical trials[10]. Many reports have correlated the efficacy of rituximab with CD20 expression[9–12].

A simple and robust CDC bioassay has been developed and validated here for assisting the lot release or regulations of rituximab-like biosimilars. To the best of our knowledge, there are no previous studies wherein a detailed set of the SAC and SSC have been chalked out to establish the biological activity of rituximab like biosimilar. This will also assist in the development of written and physical reference standards for all pharmacopeia.

The developed CDC bioassay was validated for parameters like specificity, linearity and range, repeatability, intermediate precision and accuracy.

Trastuzumab and adalimumab were used as antibody instead of rituximab for testing the specificity of the assay. It can be inferred from Figure 1 that no significant dose response is observed when rituximab was replaced by any other antibody in the assay. Thus, the CDC assay developed complies with criteria of specificity in accordance to the United States Pharmacopeia[16].

Figure 2 shows that linear regression model is obtained for observed and simulated ln(%RP) across the six simulated potency levels. The representative linear equation is $y = 0.9772x + 0.107$. Regression lines for two analysts are nearly coincident and further analysis shows that the method is linear over the range 50–200%, wherein the observed relative potency at each individual simulated level is within 80–120% of the expected potency. Also, the assay complies with the parameter of linearity since relative potency at each individual simulated level is directly proportional to its concentration with the value of $R^2 = 0.99$ (ref. 20).

The reported CDC assay complies with the parameter of precision, which was demonstrated through repeatability and intermediate precision data. For seven assays carried out by a single analyst, %GCV at 100% simulated potency was 6.14% (Table 3). It can also be inferred from Table 3 that the intermediate precision in terms of %GCV for the assay is only 7.09% at 100% simulated potency. The %GCV for precision (repeatability and intermediate precision) is less than 20% over more than 40 individual

Table 4. Summary data for accuracy

Nominal potency (%)	GM*	%GCV**	%Relative bias***	*N*	95% LCL % relative bias	95% UCL % relative bias
50	50.4	13.85	0.76	6	–14.1	17.1
71	72.7	18.01	2.4	6	–15.6	17.6
100	99.76	7.09	–0.24	12	–9.6	10.6
122	125.9	8.79	3.23	7	–8.2	8.8
150	150.03	14.04	2.02	6	–11.3	13.1
200	197.1	10.7	–1.4	6	–11	12.4

*GM = $e^{Average}$, **%GCV = 100 × (e^{SD} – 1)%, ***% Relative bias = 100 × ((measured potency/target potency) – 1)%. LCL, Lower count level; UCL, Upper count level.

Table 5. Assay suitability criteria or system suitability criteria

Slope from 4PL fit	≥1.3
EC_{50} (μg/ml)	0.08–0.32
Fold response	≥5
A/D (max./min.) value	≥3
% CV for 80% of the concentrations duplicate RFUs	≤20

EC_{50}, Half-maximal of the effective concentration; A and D are asymptotes; RFU, Relative fluorescent unit.

Table 6. Sample acceptance criteria

Test for regression, 95% (*F*-test)	Should comply
Test for linearity, 95% (*F*-test)	Should comply
Test for parallelism, 95% (*F*-test)	Should comply
95% confidence interval	69.2–132.6%
Estimated relative potency	80–120%
% CV for 80% of the concentrations duplicate RFUs	≤20%

performances. According to Reed *et al.*[15] and most international guidelines, %CV should not be more than 20% for bioassays to be precise.

The reported assay complied with the parameter of accuracy since the relative bias obtained over numerous performances was only –0.24 at 100% simulated potency (Table 4), which is well in accordance with the criteria of ± 10% for normally distributed data[16]. The %GCV was not more than 20% for any of the simulated potencies tested.

SSC and SAC are necessary to ensure the quality of bioassay results[21]. Based on the 4PL curve fit results obtained from all the performances, a detailed set of SSC or assay acceptance criteria and SAC was determined (Tables 5 and 6). SSC and SAC are primarily based on comparison of dose–response curves of the test sample with a reference sample and will be useful in judging the validity of the assay. SSC and SAC can be reviewed and modified to narrow down the ranges as more data are acquired. These criteria can be critical for assisting the quality assurance of rituximab-like similar biologics. Currently, there are no monographs in any of the pharmacopeia of the world for quality control testing of rituximab-like antibodies. Hence the present study may play a critical role in the development of physical and written reference standards for incorporation in different pharmacopeia.

Extensive performances were carried out to determine SSC and SAC for CDC assay of rituximab-like similar biologics. The reported bioassay is a simple, robust method for assisting the regulation and quality assurance of rituximab-like similar biologics in an efficient way. The study may play a critical role in the development of physical reference standards and monographs for incorporation in different pharmacopeia. Though the bioassay is a critical quality attribute for regulation of similar biologics, however they need to qualify the other physiochemical and bioanalytical parameters supported by clinical trial data before hitting the market and to ensure consistency in production for the lot release.

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Improved square-Z-shaped DNG meta-atom for C- and X-band application

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An improved dual-band square-Z-shaped meta-atom is presented. It shows a bandwidth of 3.61 GHz, where the operating frequency ranges from 2.0 to 14.0 GHz. The meta-atom is split in such a way that it appears as a square-Z-shaped structure and is printed on an epoxy resin fibre substrate material. The dimensions of the single unit cell and array structure are respectively, 10 × 10 mm^2 and 200 × 150 mm^2. Also the unit cell and 1 × 2, 2 × 2 and 4 × 4 arrays are studied for double negative characteristics. CST Microwave Studio 3D-electromagnetic simulator is used to design and perform investigation. The performance of a meta-atom unit cell is measured by wave guide ports. The measured and simulated results matched well and are applicable for C- and X-band applications.

Keywords: Double negative meta-atoms, dual-band, effective medium ratio.

META-ATOMS are artificially engineered resonant materials able to manipulate light at a sub-wave length scale. They can be designed to strongly interact with the electric and/or magnetic fields of incident electromagnetic (EM) waves, thus enabling many unique properties (e.g. perfect absorption, sub-wavelength focusing and negative refractive index). Split-ring resonators are commonly used elements in meta-atoms and can generate a magnetic response that gives a negative permeability. Double negative meta-atoms offer the possibility to obtain certain exotic properties. With considerable efforts, progress has been made towards the realization of high-performance double negative meta-atom with wide operation bandwidth and dynamic EM properties. In 1968, Veselago[1] described the negative permittivity and permeability that showed certain peculiar characteristics of waves, even though no physical material or device was found having negative ε and μ until 1999, when Pendry et *al.*[2] proposed periodically stacked split ring resonators (SRRs) at microwave frequencies which exhibited simultaneous negative ε and μ. Since then, many studies have been reported on related topics of perfect lenses, and potential applications in lenses, absorbers, antennas, optical and microwave components and sensors. In 2000, Smith *et al.*[3] introduced a material that simultaneously showed negative permittivity and negative permeability, with some exceptional characteristics at microwave frequencies. Owing to unusual characteristics of meta-atoms when compared to conventional materials, meta-atoms can be applied in numerous applications such as, EM-band gap structures[4], EM-absorption[5], enhanced antenna performances, polarization resonators, solar energy harvesters and super lenses. Although negative permittivity occurs in some natural conventional materials, negative permeability is hard to observe in natural materials and DNG characteristics are difficult to obtain. To meet the requirements of particular applications, several letter-shaped meta-atoms were discussed earlier. Hasan *et al.*[6] presented a z-shaped DNG, which had wide bandwidth. The dimension of the metamaterial single unit cell was 10 × 10 mm^2 and applicable for dual band applications. A square split z-shape meta-atom was applicable for S-, C-,

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