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Research article

Cost effective and reliable cell based ELISA as an alternative method of flow cytometry for assessment of binding activity of Vedolizumab

Swati Shalini [1], Anu Sharma [1], Nripendra Nath Mishra, Ratnesh Kumar Sharma, Harish Chander, Anupkumar R. Anvikar, Subhash Chand [*]

Therapeutic Antibody Laboratory, National Institute of Biologicals, (Ministry of Health and Family Welfare) Plot No. A-32, Sector-62, Institutional Area, Noida, Uttar Pradesh, 201309, India

A R T I C L E   I N F O

A B S T R A C T

Vedolizumab is a humanized monoclonal antibody used for inflammatory bowel disease treatment. Vedolizumab binds to the α4β7 integrin complex and inhibits its binding to mucosal addressin cell adhesion molecule-1 (MAdCAM-1). To evaluate the binding efficacy and quality control check of Vedolizumab, flow cytometry is performed by using HuT78 cells. As we know, flow cytometer is costly and require high equipment maintenance with a designated technical manpower to handle it. In this regard, the aim of study was to develop and validate an economical, simple and efficient cell based ELISA assay for potency estimation of Vedolizumab which has not been reported in any pharmacopoeia. The proposed bioassay method was optimized by investigating Vedolizumab binding to α4β7 integrin which is expressed by HuT78 cells. The validation of this method was done at different parameters including specificity, linearity, range, repeatability, precision, and accuracy. The Vedolizumab binding by ELISA results were found specific for Vedolizumab with linearity ($R^2 = 0.99$) and precision (%Geometric Coefficient of variance) observed for repeatability and intermediate precision were 3.38% and 2.6% respectively. The relative bias was calculated as 8.68% for repeated performances by different analysts and found in accordance with parameter of accuracy as per various pharmacopoeial guidelines. The developed method is established as robust, effective, and less expensive than high maintenance setup like flow cytometry based assay.

1. Introduction

Vedolizumab is a novel humanized monoclonal antibody, developed for the treatment of inflammatory bowel disease, ulcerative colitis and Crohn's disease. It consists of fully human Fc region and a human variable region with the binding domain of mouse. It is an antagonist to the α4 β7 integrin present on memory T cell and hence prevents its binding to the mucosal addressin cell adhesion molecule-1 (MAdCAM-1) present on the surface of gastrointestinal endothelium [1,2]. The leukocyte internalization into gut mucosa involve multistep procedures such as rolling, adhesion and diapedesis with help of various factors such as PSGL-1, ICAM-1, and LFA-1.

Inhibiting the T cell extravasation to gut mucosa appeared as an effective therapeutic mechanism for treatment of inflammatory bowel disease which otherwise failed to be treated by conventional method [3]. Vedolizumab is considered as clinically safe since it persuades the homing of α4β7 which further reappear in functional manner on withdrawal of the drug from the system [4].

Due to the two point mutations at Fc receptor (FcRγ) binding motif, Vedolizumab fails to elicit antibody-dependent cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) [5]. Therefore, bioactivity assessment of Vedolizumab is exhibited by either apoptosis based method or binding assay by using flow cytometry., ELISA is also a well-known biochemical analytical method for detecting a therapeutic mAb binding activity through a specific interaction between an antibody and its antigen [6–8]. ELISA has advantages of high specificity, simplicity, stability, and rapid analysis. ELISA has become a commonly used tool for analyzing proteins, peptides, and small molecules for research and QC purposes [9–11].

National control laboratories of India are involved in the quality control testing of biological drugs including therapeutic monoclonal antibodies. Bioassay is a critical test parameter to estimate the relative potency of biotherapeutic drugs. Bioassay is required for determination of the biological activity of therapeutic monoclonal antibodies in robust and consistent manner. In order to ensure the standard quality of batches tested, method validation of procedure is necessarily required [12]. Method validation for quality control involves the evaluation of parameters such as accuracy, precision, specificity, repeatability, robustness, range [13].

In order to characterize the binding efficacy of Vedolizumab, generally HuT78 cells are used which express α4β7 integrin. The binding assay of Vedolizumab to α4β7 integrin is conventionally carried out on HuT78 cells by using flow cytometry. An alternate cost effective and reliable assay is reported in this manuscript wherein the potency (biological activity) based quality control evaluation of Vedolizumab has carried out by measuring binding of Vedolizumab to the HuT78 cells by using cell based ELISA method. Method validation of any assay is required for drug development, quality control and characterization, since these criteria ensure about the accuracy, specificity, reproducibility and robustness of the method [14–16]. Therefore, method validation of cell based ELISA procedure was performed for its establishment as an alternative and reliable method to flow cytometry based binding assay. The results obtained from ELISA method have been found statistically reliable and comparable with flow cytometry results. There are certain limitations associated with Flow cytometry based method as it requires an instrumental set up, daily cleaning and trained staff for its operation. The instrumentation is complex and demand regular maintenance by service engineer to ensure the proper functioning of microfludics system as well as calibrations of laser [17]. Hence, the procedure developed in this study will help the startup researchers and quality control analysts for estimating the potency of Vedolizumab in cost effective manner without any complex instrumental set-up as well as designated staff for its execution.

2. Materials and methods

2.1. Chemicals and reagent

Phosphate Buffered Saline 1X (PBS, without Phenol, Calcium and Magnesium, LONZA, Basel, Switzerland), Goat anti-human Fc IgG Horseradish Peroxidase conjugated (Jackson Immuno Research, Pennsylvania, USA) is used as Secondary Antibody. Bovine Serum Albumin (BSA Sigma Aldrich, Darmstadt, Germany) was used in FACS analysis. Vedolizumab sample/reference standard manufactured by Takeda pharmaceuticals. 96-well cell culture plates flat bottom and U bottom (Greiner Bio-One, Frickenhausen, Germany).

2.2. Preparation of dilutions for reference standard, samples and cells

The reference standard/samples were diluted in the 1X PBS to get the 10 μg/ml concentration, furthermore 10 serial dilutions were prepared in duplicate. The HuT78 cells (ATCC-TIB-161), were centrifuged at 1000 rpm for 5 min and resuspended in 1X PBS. To check the viability and number of cells before seeding was carried out by using Neubauer Chamber by using Trypan Blue (Sigma Aldrich, Darmstadt, Germany) dead cell exclusion method [18]. For this experiment the cells were counted and adjusted for seeding 100,000 cells/tube and secondary antibody was used in 1:10,000 dilution.

2.3. Procedure for cell based ELISA assay

Reference standard/samples were two fold serially diluted from 10 ng/ml to 0.015 ng/ml 100 μl of each dilutions of reference standard/sample were incubated with 100 μl (10^6 cells/ml) of cell suspension for 1.5 h at 37 °C (with intermittent shaking after every 30 min). After Incubation cells were washed 3 times with PBS by centrifuging at 2000 rpm for 5 min. After washing, 100 μl of diluted secondary antibody (Peroxidase -conjugated Goat Antihuman IgG) was added to each dilution tube and incubated for 1 h. After incubation the cells were washed with PBS 3 times with by centrifuging at 2000 rpm for 5 min. TMB (Sigma Aldrich, Darmstadt, Germany) were added as substrate, then again the tubes were incubated for 30 min [19]. After appearance of desired color 1 N H_2SO_4 was added to stop the reaction and absorbance was taken by spectrophotometer (Tecan, Spark, Männedorf, Switzerland) at 450 nm.

2.4. Procedure of binding assay by flow cytometry

Flow cytometry (FACS) based binding assay requires 1% w/v BSA/PBS solution as assay buffer. Reference standard/sample was 2 fold serially diluted in assay Buffer to get the concentration of 4 ng/ml and further 9 serial dilutions. The HuT78 cells were centrifuged at 1000 rpm for 5 min and resuspended in FACS Buffer for assay. The cell count was carried by Neubauer Chamber by using Trypan Blue cell exclusion method and adjusted to 1×10^6 cells/ml. 50 μl of each dilution of reference standard and sample were mixed with

100 µl of cell suspension (0.1×10^6 cells per tube) and incubated for 60 min at 37 °C. After completion of incubation period the tubes were washed two times with FACS Buffer while centrifuging at 1100 rpm for 5 min. Secondary Antibody, R-Phycoerythrin antibody produced in goat (Sigma-Aldrich-P9287) was diluted 1:20 times and its 50 µl was added, further the tubes were incubated for 25 min at 37 °C. After incubation two washes were given by centrifuging at 1100 rpm for 5 min with FACS Buffer. Acquisition Buffer was prepared by adding 500 µl of 10% Phosphate Buffered Formalin.For 1000 ml, 100 ml of commercial Formaldehyde (Sigma Aldrich, Darmstadt, Germany) was added to 900 ml of distilled water and 4.0 g of sodium phosphate monobasic (Sigma Aldrich, Darmstadt, Germany) and 6.5g of sodium phosphate dibasic (anhydrous) and pH should be 7.2 ± 0.5 were added to it in a 50 ml conical tube, 5 µl of 1 Mm TO-PRO-3 Iodide (Waltham, MA USA) was added to the 50 ml conical tube and final volume was brought to 50 ml with FACS Buffer. Cells were resuspended in 200 µl of FACS acquisition buffer. 10,000 events were acquired of each dilution of reference standard/sample in Flow Cytometer (BD Lyric). Mean Fluorescence Intensity was measured to estimate the binding of the drug with HUT78 cells. Potency was calculated by plotting the obtained readings in PLA software.

2.5. Statistical analysis

The absorbance signals acquired by spectrophotometer were fit into nonlinear four parameters logistic (4 PL) model. The four parameters required to be estimated in order to "fit the curve" are A, B, C, D. Where A and D are the upper and lower asymptotes respectively, B is the slope, C is the half value of maximal effective concentration (EC_{50}).

The model equation is as follows: y = D+ (A-D)/(1+(x/C)^B)

Here, x is the independent variable and y is the dependent variable respectively.

2.6. Validation of alternate cell based ELISA assay

Method validation analysis was performed as per the guidelines mentioned in ICH Q2 (R1) for determination of parameters such as specificity, linearity and range, repeatability, intermediate precision, accuracy [20].

2.6.1. Specificity
The specificity parameter was analyzed to establish that the test results are true for the potency assay of the Vedolizumab in a sample. Nonspecific antibody Trastuzumab was used for determining the specificity of assay.

2.6.2. Linearity and range
Five concentrations 50%, 75%, 100%, 141% and 200% of sample with respect to standards with simulated potencies of test samples were estimated and statistically tested for calculation of Linearity and Range of the proposed method (USP, 2018). The graph was plotted between ln% simulated potencies and ln% observed relative potency and the obtained data was examined for finding regression parameter.

2.6.3. Repeatability and intermediate precision
Repeatability of the alternate method was determined by performances of same analyst on different days by under similar operational circumstances (USP, 2018). Repeatability was estimated by using the formula % GCV = 100 (eSD-1) %, wherein S.D. is the standard deviation of log values of relative potencies.

Intermediate precision was calculated by obtaining %GCV value from performances of different analyst on separate days.

2.6.4. Accuracy
Accuracy of the bioassay procedure was estimated on the basis of nine distinct performances and providing the result on the basis of % relative biasness.

$$\% \text{ Relative biasness} = 100 \left(\frac{Measured\ Potency}{Target\ potency} - 1 \right) \%$$

wherein the measured potency was obtained from % of mean potencies found during intermediate precision performances.

Table 1
Binding assay of Vedolizumab by using Cell based ELISA and Flow cytometry.

Validation parameter	Mean	Standard Deviation	% GCV	Geometric mean	Relative biasness	%RSD
Cell based ELISA	101.8	0.34	0.349	101.16	8.68	0.074%
Flow Cytometry	108	1.63	1.63	108.68	7.90	0.346%

3. Results

3.1. Flow cytometry assay

The binding assay of Vedolizumab by flow cytometry was performed and validated as per the process available in the literature [21, 22]. The results were obtained from three independent performances of flow cytometry and after statistical analysis the relative potencies of Vedolizumab were depicted within the range of 70–130%. To establish the robustness and precision of the proposed method, we performed cell based ELISA on different validation parameters. The comparative study of method validation parameters for both methods has been shown in Table 1. The drug response curve of cell based ELISA and flow cytometry procedure is shown in Fig. 1 (a) & (b) respectively. In Fig. 2 (a – c), represents the cell control, primary and secondary antibody controls respectively, while Fig. 2 (d - h) represents flow cytometry graphs showing the cell surface epitopes binding to Vedolizumab as per the dose response. Three independent experiments were performed. Results obtained from both procedures were found in accordance with the method validations criteria as per US pharmacopoeia and ICH guideline.

3.2. Method validation

Method validation of bioassay procedures have been performed for determining their fitness for quality control testing. The proposed Cell based ELISA procedure was validated as per the parameters of specificity, linearity, repeatability, intermediate precision and accuracy. The variance were calculated and found within the recommended limit (Table 1).

3.2.1. Specificity

The curve fit response of this experiment was only obtained with Vedolizumab sample and no matrix interference was observed with media components (Fig. 3). Trastuzumab was used as nonspecific antibody and no dose response was observed when Vedolizumab was replaced by the trastuzumab as nonspecific antibody in the assay (Fig. 3). Thus it was inferred that the alternate cell based ELISA assay was in compliance with the specificity criteria as per USP.
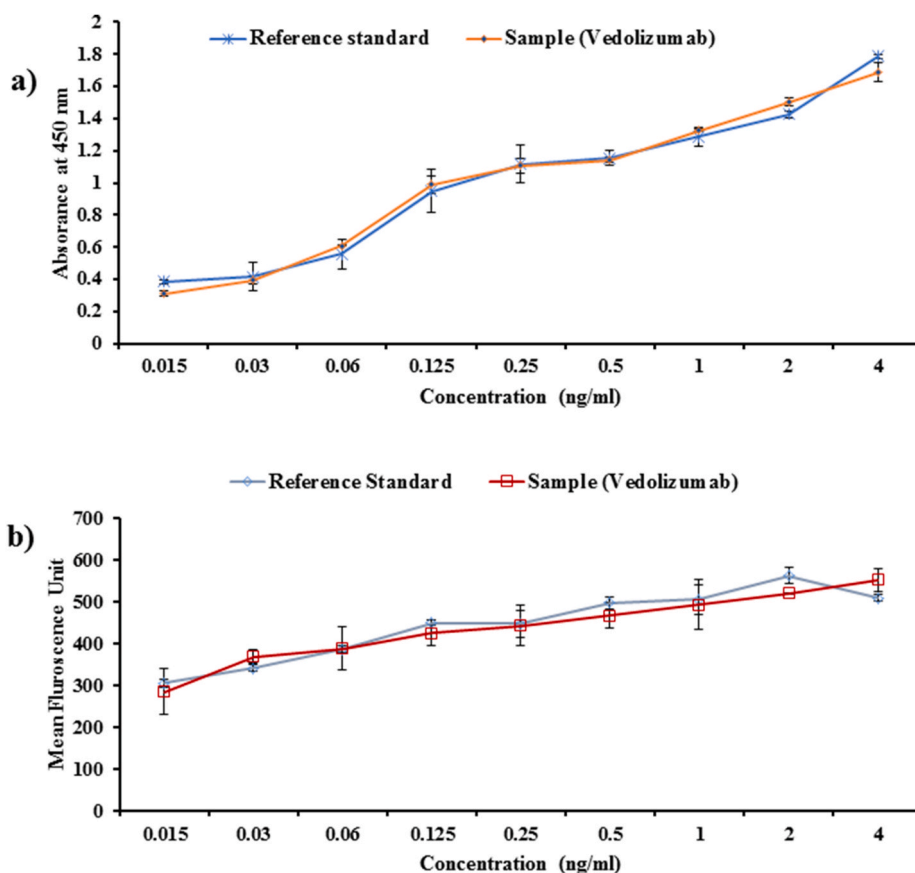

Fig. 1. Dose dependent response of Vedolizumab binding to the HuT78 cells has shown in this figure. Fig. 1 a) The graph was plotted between the concentration of Vedolizumab used in the assay and the absorbance (O.D. at 450 nm) obtained by cell (HuT78) based ELISA. Fig. 1 b) The graph depicts here dose response binding activity of Vedolizumab on HuT78 cells by using Flow cytometry technique.
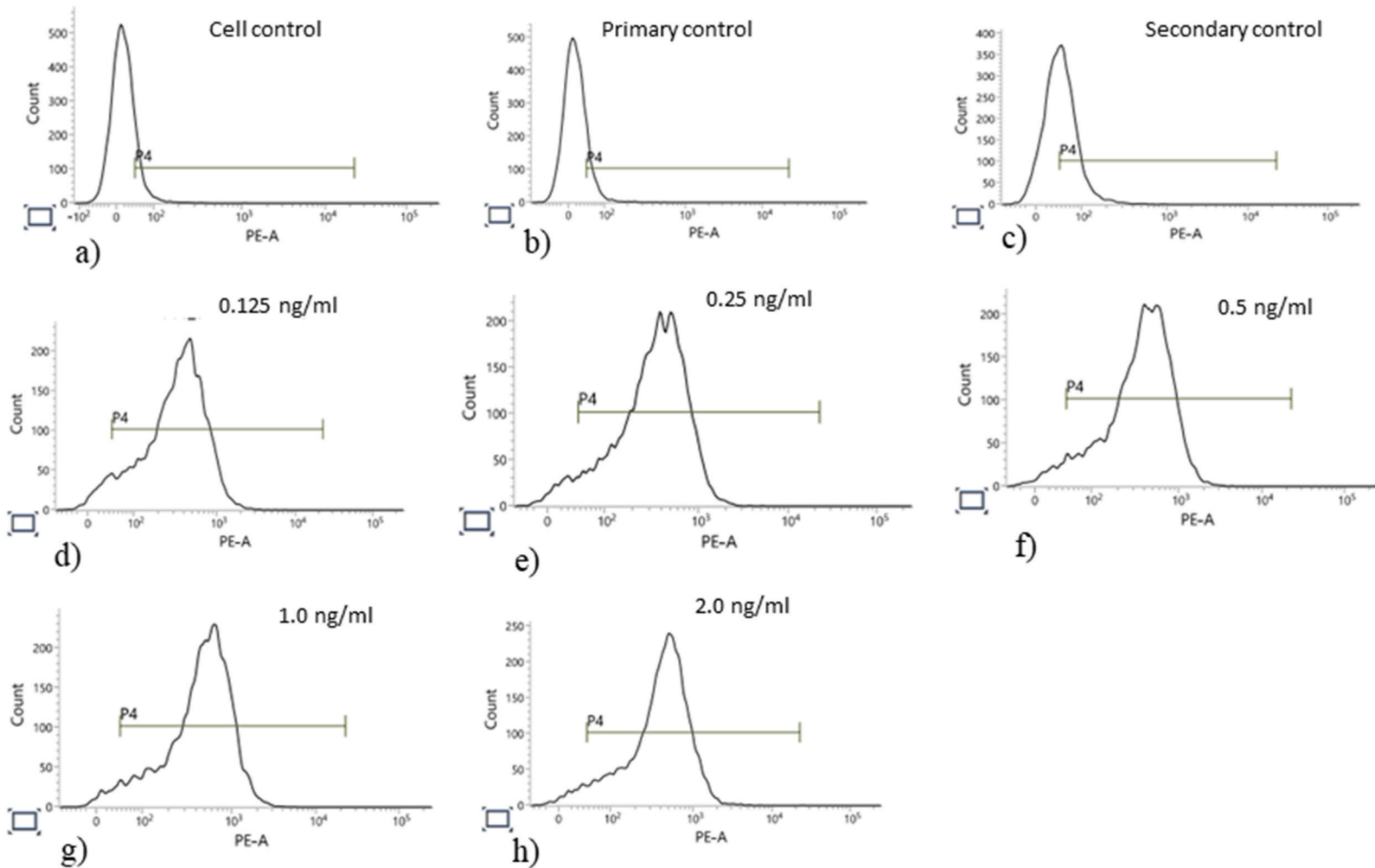
Fig. 2. Detection of binding activity of Vedolizumab by using flow cytometry. HuT78 cells were harvested and incubated Vedolizumab at different dilution (4 ng/ml to 0.015 ng/ml) and stained with FITC labeled human anti-IgG. a). Unstained cells were used as Primary control b) only Vedolizumab incubated with cells and no secondary antibody was used. c) Only FITC labeled human anti-IgG were used the same concentration as secondary control. d-h) Histogram of Flow cytometry result at different concentration of Velolizumab from 4 ng/ml to 0.015 ng/ml. The figures represent percent of positive cells.
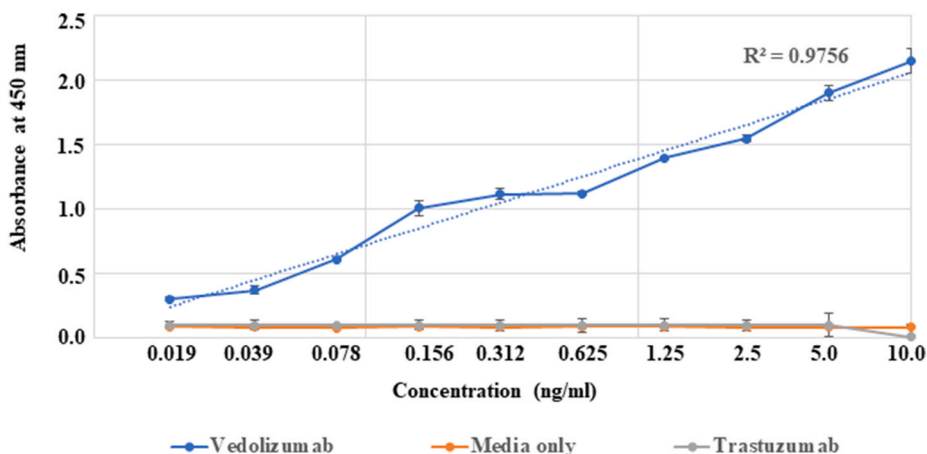
Fig. 3. Cell based ELISA procedure to establish the binding activity of Vedolizumab has depicted here, in the graph Trastuzumab used as non-specific antibody and did not show any dose response against HuT78 cells. Media control and cell control also did not show any response. Therefore, no interference was found by matrix.

3.2.2. Linearity and range

The linear regression model is used to explain the relation between expected and observed relative potencies. To estimate the linearity, five different concentrations (50%, 75%, 100% 141% and 200%) were used. The graph was plotted between natural log (ln) of % relative potencies observed along with expected (Fig. 4) and R^2 value was 0.99. It could be comprehended from the graph that the proposed method was linear over the entire range of 50%–200%. The observed relative potency found at each individual level was within the range of acceptance criteria 70–130%. It was inferred from the graph plotted between ln observed % relative potency and ln expected % relative potency that proposed assay complies with the criteria of parameter linearity as all stimulated levels were directly proportional to the dosages of drug (Fig. 4).

3.2.3. Repeatability and intermediate precision

The repeatability is also an important parameter to analyze the variance in results of experiment on different days by same analyst. To establish the repeatability criteria, the n = 6 experimental performances were performed at 100% concentration level against reference standard of Vedolizumab and estimated % Geometric Coefficient of Variance (GCV) at 100% expected potency was 3.38% (Table 2). The method validation parameters were calculated for two analysts separately and the intermediate precision values were found within the range of ICH guidelines (Table 3).

The precision criterion of method validation is analyzed to know the robustness of proposed method. For estimation of intermediate precision, the n = 9 performances were by two different analysts at different dates and the % GCV for the assay was observed as 2.66% for simulated potency (Table 3). It were analyzed that the % Coefficient of Variance (CV) for precision was less than 20% for all 9 distinct performances which is within the range of acceptance criteria so the proposed assay complies for the parameters of intermediate precision and repeatability and most international guidelines % CV should not be more than 20% for bioassays to be precise (Fig. 5).
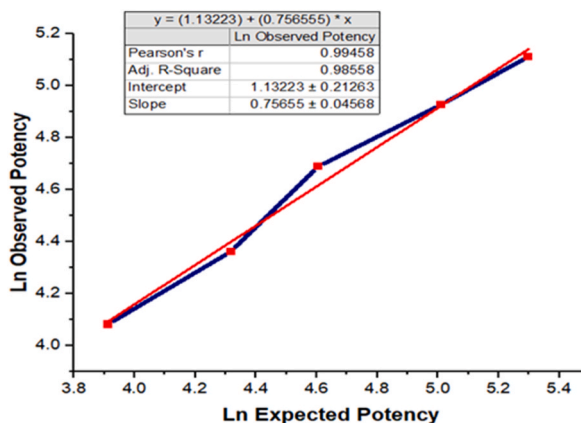

Fig. 4. Linearity of the assay procedure determined by Linear regression mode showed here. The graph depicts the relation between expected potency and observed potency.

Table 2
System suitability criteria and acceptance criteria for proposed cell based ELISA assay.

Parameter	Assessment	Output	%RSD
Accuracy	% Relative Bias (Should be ≤ **15**%)	% Relative Bias = **8.68%**	0.558%
Intermediate Precision	Variance Component Analysis ≤ **20**%	%GCV = **2.66%**	0.558%
Repeatability	Variance Component Analysis ≤ **20**%	%GCV = **3.386**	0.705%
Specificity	No Matrix interference and no dose response curve observe from media control, cell control and nonspecific monoclonal antibodies	Complies	NA

Table 3
Summary data for interpretation of repeatability, Intermediate precision and accuracy calculation for cell based ELISA procedure.

Analyst	Mean	Standard Deviation	% GCV	Geometric mean	Relative bias	%RSD
Analyst 1	112.13	3.73	3.386	112.02	12.14	0.705%
Analyst 2	112.2	0.173	0.154	112.14	12.02	0.032%

3.2.4. Accuracy and robustness

In method validation, accuracy is analyzed to nullify the biasness of the procedure. In this manner accuracy of the proposed cell based ELISA method was evaluated at 100% in the specified range of 10–0.019 ng/ml. The mean accuracy for potency was 112.15% with RSD 0.558% (Table 2, Fig. 5). In this manner relative biasness was also estimated for the proposed method. The relative bias was calculated on the basis of relative potency obtained during the 9 performances by two different analysts at different dates. The calculated result has shown that the proposed assay complies with the parameter of accuracy as the relative biasness with 8.68% (Table 2, Fig. 5). The relative biasness was also calculated for each analyst and result showed 12.14% and 12.04% for analyst 1 & 2 respectively (Table 3, Fig. 5). The % CV observed during every analysis was found less than 20% for all of the parameter tested. This data confirms the ability of the method to determine with accuracy.

4. Discussion

Bioassay offers a range of services based on *in-vivo* and *in-vitro* systems including potency assays and cytotoxicity studies. In this study, National Control Laboratory (NCL) has developed a product specific, precise, accurate and robust assay as per the various pharmacopoeial requirements for bioassay and guidelines issued by International Council for Harmonisation (ICH).

In the presented manuscript, investigations were done for developement and validation of the quantitative bioassay of Vedolizumab by cell based ELISA method as compared to flow cytometry. Vedolizumab binding activity to α4β7 integrin has been assessed by using flow cytometry, which is an expensive and skilled analyst requiring assay. We aimed to compare binding activity results between flow cytometry and newly developed an alternate cell based ELISA assay to determine the relative potency (biological activity) of
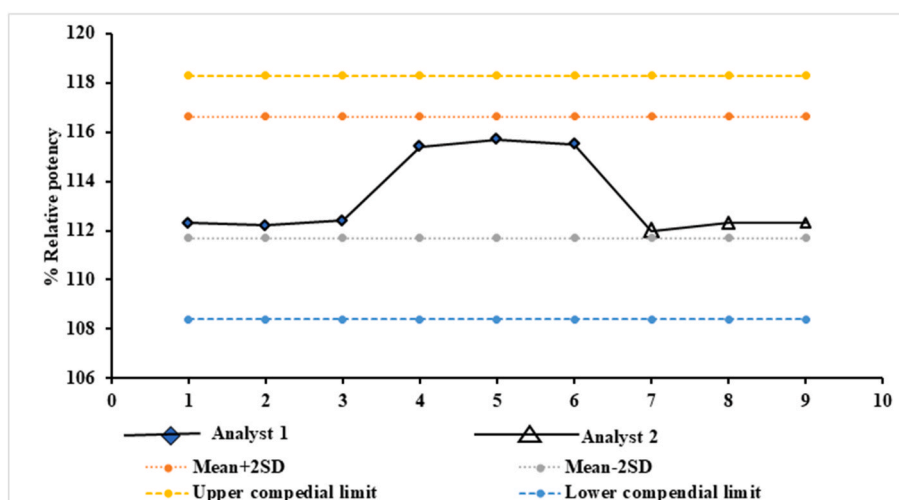

Fig. 5. Summary data of nine performances of two different analysts for Repeatability (Intra-analyst assay) and Intermediate precision (Inter-analyst assay) is lying between compendia limit. In the figure, diamond shaped points showed the performance of analyst 1 and hollow triangles showed performance of analyst 2.

Vedolizumab using HUT78 cells. The alternate assay developed in the current study is simple, precise, reliable and economical as compared to flow cytometry. The experimental conditions were optimized on the basis of different parameter such as cell density, drug concentration, incubation period and secondary antibody. Desired dose-dependent curves were observed at a seed density of 1×10^6 cells/ml. All the experiments were performed on HUT78 cells between passage number 3 to 30 from the initial master culture form ATCC, considering the receptor expression varies with cell passage number and may affect drug binding activity. As earlier described in this manuscript that, flow cytometry is an expensive procedure as well as require a technically skilled person to perform. In this regard we developed a cell based ELISA method (binding assay) for quality control testing of Vedolizumab.

This newly developed method has been validated according to current global guidelines for bioanalytical methods of validation. This cell based ELISA bioassay was validated on the basis as per the various pharmacopoeial requirements for bioassay and guidelines issued by International Council for Harmonisation (ICH) including US pharmacopoeia, US FDA "guidance for industry, bioanalytical method validation" and the European "Guideline on bioanalytical method validation" [23]. This newly developed cell based ELISA is a quantitative assay and it is currently not available in any of the pharmacopoeia. The developed cell based ELISA method has been validated for various parameters for bioanalytical method validation including specificity, linearity, range, repeatability, accuracy and precision.

The assay described here is more readily comparable with the sandwich-type ELISA described in detail by Maple *et al.* (2004) [24]. The method is comparable in terms of performance to that assay, with similar precision, dynamic range and accuracy, but is simpler to execute and is not limited by access to reagents of restricted availability. The dynamic range of the assay of 10 ng/ml to 0.015 ng/ml. The result of this method was also comparable to the flow cytometry method.

Specificity of a method is necessary to establish that there is no interference of any matrix in obtaining the test results. For specificity of the proposed assay, media control, cell control and nonspecific antibodies were analyzed along with Vedolizumab dilutions. Specificity of a method is performed to nullify the interference of any matrix, which is used in the method to obtaining the test results. In this proposed assay, media control, cell control and nonspecific antibodies such as trastuzumab were analyzed along with Vedolizumab dilutions and no matrix interference was observed (Fig. 3). Thus it was confirmed that the alternate cell based ELISA assay was in compliance with the specificity criteria as per USP, European and other pharmacopoeial guidelines.

Linearity assesses the ability of the method to obtain test results that are directly proportional to the concentration of the mAb in the sample. The linear range of the method must be determined regardless of the phase of drug development. ICH guidelines recommend evaluating a minimum of five concentrations to assess linearity. The five concentration levels should bracket the upper and lower concentration levels evaluated during the accuracy study [25]. In this manuscript five concentration ranges (50%, 75%, 100% 141% and 200%) were evaluated for linearity analysis during the study. The linear regression model was used to explain the relation between expected and observed relative potencies. The graph was plotted between natural log (ln) of % relative potencies observed along with expected (Fig. 4) and R^2 value was 0.99.

Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision. Repeatability is sometimes also termed within-run or within-day precision. In this method development process the repeatability criteria were also established by the single analyst six times performance of experiment and estimated % Geometric Coefficient of Variance (GCV) was 3.38% that is within the range of guidelines (Table 2). Intermediate precision expresses within-laboratories variations by different days, different analysts, different equipment, etc. [26]. Intermediate precision is sometimes also called between-run, between-day, or inter-assay precision. The precision criterion of method validation is analyzed to know the robustness of proposed method. In this regard the assay was performed 9 times by two analysts and the % GCV for the assay was observed within the range of accepted criteria (Table 3).

According to different guidelines, relative bias (accuracy) is the difference between the expectation of test results and an observed reference value. It may consist of more than one systematic error component. Bias can be measured as a percent deviation from the accepted reference value. The term trueness expresses the deviation of the mean value of a large series of measurements from the accepted reference value. It can be expressed in terms of bias. Due to the high workload of analyzing such large series, trueness is usually not determined during method validation, but rather from the results of a great number of quality control samples (QC samples) during routine application [27]. In method validation, accuracy is analyzed to nullify the biasness of the procedure and was also estimated for the proposed method. The relative bias was calculated for all performances and the result has shown that the proposed assay complies with the parameter of accuracy as the relative biasness is 8.68% (Table 2). The relative bias was also calculated for performances by different analysts and the result has shown that the proposed assay complies with the parameter of accuracy as the average relative biasness is 12.06% (Table 3) with %CV observed less than 20% for all of the parameters tested [28].

To the best of our knowledge, this is the first report demonstrating the cell based ELISA of Vedolizumab binding as compared to the flow cytometry assay to determine the efficacy of Vedolizumab. Thus, based on results of method validation parameters, this newly developed method qualified all the criteria for pharmacopoeia bioassay method validation and established itself as an economic, robust and reliable method.

5. Conclusion

Bioassay is a critical quality attribute for regulatory testing of biological drugs since it is performed for the potency estimation. It helps to ensure the uniform quality and consistency in the quality of drug released in the market. In this study, an economical, reliable, robust and efficient cell based ELISA assay for potency estimation of Vedolizumab was developed and compared to the Flow cytometer based binding assay. The proposed assay was analyzed for the validation of parameters specificity, linearity, range, accuracy, repeatability, and intermediate precision. It was inferred from the results obtained that the developed assay is an efficient and reliable

alternate method to flow cytometry based assay for determining the relative potency of Vedolizumab using HUT78 cells.

Declarations

Data availability statement

Data included in article/supp. Material/referenced in article.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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