

<https://doi.org/10.48047/AFJBS.6.14.2024.12978-12997>



African Journal of Biological Sciences

Journal homepage: <http://www.afjbs.com>



Research Paper

Open Access

Development and Validation of an ELISA to Evaluate Pharmacokinetics of Rituximab

Manjunath S Devaramani^{1,2#}, Dinesh Kumar Saini^{2,3}, Kiran Kumar Mudnakudu Nagaraju^{1*}, Siddesha Jalahalli Mariswamy³, Ashna P Thampi², Kavya J²

¹Department of Biotechnology & Bioinformatics, School of Life Sciences, JSS Academy of Higher Education & Research, Mysuru, Karnataka 570015, India

²Denovo Biolabs Pvt. Ltd., No. A-112, Electronic City Phase 1, Electronic City, Bengaluru, Karnataka 560100

³Division of Biochemistry, School of Life Sciences, JSS Academy of Higher Education & Research, Mysuru, Karnataka 570015, India

***Corresponding authors:** kirankumarmn@jssuni.edu.in * Dr. Kiran Kumar MN
Department of Biotechnology & Bioinformatics School of Life Sciences JSS Academy of Higher Education & Research SS Nagar, Mysuru- 570015, India
Email: kirankumarmn@jssuni.edu.in

Volume 6, Issue 14, Sep 2024

Received: 15 July 2024

Accepted: 25 Aug 2024

Published: 05 Sep 2024

doi: [10.48047/AFJBS.6.14.2024.12978-12997](https://doi.org/10.48047/AFJBS.6.14.2024.12978-12997)

Abstract

Rituximab, a chimeric monoclonal antibody targeting CD20, is widely used in the treatment of various malignancies and autoimmune disorders. In this study, an ELISA was developed to quantitate the rituximab levels, using mouse anti-rituximab (CDR specific) antibody as capture antibody. Accurate pharmacokinetic (PK) monitoring is essential for optimizing dosing regimens and improving therapeutic outcomes. Initially, surface plasma resonance showed acceptable binding kinetics (Kd) of 0.5×10^{-9} for rituximab and non-cross reactive to human IgG. A standard curve of rituximab (1.56ng/ml to 800ng/ml), with five quality control points (6.25, 18.75, 25, 150, and 200ng/ml) was evaluated for inter and intra-assay accuracy and precision, using serum matrix, by four independent validations. The linear range of the validated assay was 1.56ng/ml to 800ng/ml, and ULOQ and LLOQ were 200ng/ml and 6.25ng/ml, respectively. The assay specificity was validated by testing cross-reactivity of rituximab with adalimumab, which found to be non-reactive. Further, the hook effect was over-ruled by diluting the highest concentration of rituximab tested to assay linear range, and dilution integrity was observed for the entire concentrations within linear range (%RE $\leq 20\%$), as recommended by European Medicines Agency. Collectively, this binding-based ELISA method is highly sensitive, reproducible, and useful for monitoring rituximab.

Keywords: Rituximab, CD-20, Sandwich ELISA, Pharmacokinetics, Validation, SPR, Bioanalysis, EMA.

1. Introduction

A chimeric anti-CD20 monoclonal antibody, rituximab (Mabthera, Roche Pharmaceuticals, Basel, Switzerland) targets the CD20 antigen on the surface of B cells (Reff et al. 1994). IDEC Pharmaceuticals initially created rituximab using a murine monoclonal antibody (mAb) with high affinity (Reff et al. 1994). While the human Fc component enables the efficient application of complement and cell-mediated lysis processes, the variable murine sections are unique to the CD20 antigen (Tedder and Engel 1994). Mature B-cells produce CD20, which is found on 95% of B-cell lymphomas and is believed to function as a calcium channel that controls the cell cycle's advancement (Tedder and Engel 1994). Anti-CD20 mAb cross-links CD20, which causes apoptosis via a caspase-dependent mechanism. It can also result in antibody-directed cell cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) (Tedder and Engel 1994). Significant immunological impairment and increased susceptibility to infection have

not been observed, despite the expected reduction of B-lymphocytes after rituximab treatment (Maloney et al. 1994; Berinstein et al. 1998). Early in 1993, the first patient enrolled in phase I dosage-escalation clinical trials of rituximab, demonstrating the safety of a weekly dose of 375 mg/m² administered for four weeks. In the highest dose group, no dose-limiting toxicities were found, and no maximum tolerated dose (MTD) was attained (Maloney et al., 1997a). Later studies (Maloney et al. 1994; Berinstein et al. 1998) and the pivotal multicenter phase II trial (Berinstein et al. 1998) also used this dose level, and in November 1997 the FDA approved it for the treatment of follicular B-cell non-Hodgkin's lymphoma and low grade relapsed or refractory lymphoma (Piro et al. 1999; Berinstein et al. 1998). Rituximab, which can be administered alone or in conjunction with chemotherapy, has been the standard treatment for many B-cell lymphomas since receiving FDA approval (Berinstein et al. 1998; Piro et al. 1999). Rituximab has been tested in recent years for the treatment of many B-cell dependent autoimmune diseases as well as non-malignant conditions such rheumatoid arthritis (Gürçan et al. 2009). Wide inter-individual heterogeneity has been observed in the pharmacokinetics (PK) of rituximab, with high serum drug concentrations seeming to correspond with clinical response (Berinstein et al. 1998). Higher rituximab concentrations are thought to enable more successful tumour regression, even though serum rituximab concentrations may serve as a proxy indicator of disease burden (Hampson et al. 2010b; Ward et al. 2009; Davis et al. 1999; Davies et al. 2014; Berinstein et al. 1998). The ELISA for rituximab has been validated to improve understanding of the drug's PK, its relevance to patient response and outcome, and the development of more stratified patient dosage schedules. The use of polyclonal (Iacona et al. 2000; Beum, Kennedy, and Taylor 2004; Berinstein et al. 1998) or monoclonal (Cragg et al. 2004; Hong et al. 2004; Blasco et al. 2007; Hampson et al. 2010a; Ward et al. 2009; Hampson et al. 2010b) antibodies in ELISA for measuring serum rituximab levels has been described in previous studies. In this case, the assay has undergone sufficient validation to guarantee an exact assessment of the rituximab levels in patient samples. As per the European Clinical Trials Regulations (Cummings et al. 2010; 2008), this is to fully validate a serum rituximab assay for use in clinical applications. We believe the level of validation performed on the assay, meets the expected criteria (da Silva et al. 2014; Findlay et al. 2000; Smolec et al. 2005a; 2005b).

2. Materials and methods

2.1. Materials

The anti-idiotypic mouse anti-rituximab monoclonal antibody 8A12 was developed in-house, HRP-conjugated goat anti-human IgG Fc specific for the Fc fragment was purchased from Denovo Biolabs, India. Mabthera (rituximab) 100mg supplied as 2×10 mg/ml vials, as a solution for infusion, was obtained from Roche Pharmaceuticals (Basel, Switzerland). All reagents used were of the highest grade appropriate and are supplied by Sigma-Aldrich, Qualigens (India), Himedia (India), Denovo Biolabs (India) of extra pure grade were procured, unless otherwise stated.

2.2. Specificity by SPR

The affinity of the purified mouse anti-rituximab antibody and cross-reactivity to human IgG was determined by using P4SPR (Affinite Instruments, Canada). The 16-MHA coated Au-sensor chip (Affinite Instruments, Canada) was used to covalently couple the rituximab and human IgG onto the sensor surface using EDC/NHS-based coupling chemistry. Briefly, the Au-sensor chip was equilibrated with the running buffer (1XPBS pH 7.4, 0.05% Tween-20) and 200µl of fresh EDC/NHS was injected for 4 minutes. The Sensor chip was washed with 250µl of running buffer, followed by 250µl of immobilization buffer (0.01M Sodium Acetate, pH 5.0) for 10 seconds. Rituximab at 0.02mg/ml was injected and incubated for 10 minutes. The channel was washed with 250µl of immobilization buffer for 60 seconds and blocked with 250µl of blocking buffer (1M ethanolamine pH 8.5), which was incubated for 4 minutes and washed the channels. The mouse anti-rituximab f(ab) antibodies at different concentrations (6.67, 13.33, 26.67, 53.33, 106.67nM) were injected and analyzed in 400 seconds. All the above-mentioned steps were repeated for the immobilization of human IgG onto the sensor surface and mouse anti-rituximab f(ab) antibodies at the concentrations mentioned above were passed onto the human IgG immobilized channel to determine the cross-reactivity of anti-rituximab antibody to human IgG.

2.3. Rituximab PK ELISA method

The mouse anti-rituximab antibody 8A12 was diluted to a concentration of 6µg/ml in a coating buffer consisting of 1X PBS, pH 7.4. Corning™ Costar Plates (96-well, flat-bottom plates) were coated with 100µl of the antibody solution and incubated overnight at 4°C. After coating, the plates were blocked with 300µl/well of 3% (w/v) BSA, 0.05% tween-20, 1X PBS, pH 7.4 for 1 hour at room temperature (RT). The plates were washed three times with wash buffer containing 1X PBS, pH 7.4, 0.05% tween-20 using ELISA microplate washer, with incubation of 1 minute between each wash.

The rituximab stock was prepared in a human serum matrix as per the dilutions and stored at -80°C for the preparation of standards and quality control (QC), to mimic the clinical subject samples received. The minimum required dilution of human serum matrix used for this assay was 1:200, where the signal-to-noise (S/N) ratio was found to be optimum after evaluating S/N of 1:10, 1:20, 1:100, and 1:200 dilutions. The human serum matrix at the described minimum required dilution (MRD) was used as an assay matrix.

Rituximab calibration standards were prepared in the following concentrations: 800, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.56 and 0ng/ml, and 100µl of standard was added to the plate in two replicates. Quality control samples (QC samples) 200, 150, 25, 18.75 and 6.25ng/ml (ULOQ, HQC, MQC, LQC, LLOQ respectively) were prepared in the serum matrix diluted in blocking (to mimic the clinical subject samples received) and to prevent nonspecific matrix interference, all samples utilized in the validation procedure were diluted to a minimum of 1:200 and added to the ELISA plate. The plate was incubated for 60 minutes at RT. The plates were washed three times with ELISA wash buffer.

A horseradish peroxidase (HRP)-conjugated goat-anti-human detection antibody, diluted 1:3000 in blocking buffer, was added at 100 µl per well and incubated for 1 hour at RT. Following the incubation, the plates were washed three times with ELISA wash buffer.

For color development, 100 µl of 1X TMB/H₂O₂ was added and the plate was incubated in the dark at RT for up to 15 minutes, and the reaction was stopped by adding 50µl/well of 2N sulfuric acid. Absorbance was measured at 450 nm with reference measurement at 620nm in an ELISA plate reader (F50, Tecan, Switzerland) and analyzed with Graph-pad prism-10 and Microsoft excel software.

2.4. Assay validation

The drug calibration curve was developed using a total of ten calibrators ranging from 1.56 ng/ml to 800ng/ml. The calibrators were prepared by diluting the analyte rituximab in a diluent buffer spiked with human serum matrix in 1:250 dilution. The back-calculated concentrations of the calibrators were within 20% of the nominal value as per EMA guidelines, for at least 75% of calibration standards. The anchor calibrators do not require acceptance criteria, since they are beyond the curve quantifiable range. The data analysis was performed using software generating four parameter logistics (4PL) fit.

For the further validation parameters mentioned below, the calibration curve was run in the assay followed by other sample in the parameters to be validated (Findlay et al. 2000; Smolec et al. 2005a; da Silva et al. 2014; Cummings et al. 2010; 2008; Hampson et al. 2010b).

2.5. Repeatability - Inter-assay precision

The QC samples were prepared and examined in twelve replicates on a single plate. Five different concentrations of QC samples such as ULOQ, high-level quantification (HQC), mid-level quantification (MQC), low-level (LQC), and LLOQ within the calibration range were calculated based on the following criteria. Briefly, HQC is one-third of the ULOQ of the calibration curve, MQC is near the midpoint on the calibration curve, and LQC is three times the LLOQ. The repeatability was acceptable if the relative error (%RE) of the back-calculated value for each level was $<20\%$ ($\leq 25\%$ at ULOQ and LLOQ), coefficient of variation (%CV) $\leq 20\%$ ($\leq 25\%$ at ULOQ and LLOQ), and total error (sum of the %RE and %CV) $\leq 30\%$ ($\leq 40\%$ at ULOQ and LLOQ).

2.6. Reproducibility - Intra-assay precision

The same set of QC samples as in repeatability section, was evaluated in duplicates by two independent analysts to determine the reproducibility through intra-assay precision. The reproducibility was acceptable if the relative error (%RE) of the back-calculated value for each level $\leq 20\%$ ($\leq 25\%$ at ULOQ and LLOQ), coefficient of variation (%CV) $\leq 20\%$ ($\leq 25\%$ at ULOQ and LLOQ), and total error (sum of the %RE and %CV) $\leq 30\%$ ($\leq 40\%$ at ULOQ and LLOQ).

2.7. Sensitivity

The sensitivity of the assay is to detect the minimum analyte concentration (rituximab) reliably. The QC at LLOQ (Lower limit of detection) was determined by testing the twelve replicates at LLOQ concentration on four independent occasions. The coefficient of variation (%CV) and relative error (%RE) $\leq 25\%$ and total error (sum of the %RE and %CV) $\leq 40\%$ was acceptable at LLOQ concentration.

2.8. Selectivity

The selectivity of the rituximab binding-based ELISA method was determined by testing 11 matrices by spiking rituximab at LLOQ. A minimum of 80% of the near-LLOQ QC samples

should have assay values that are within 20% of the nominal concentration (or within 25% at the LLOQ).

2.9. Dilution linearity

The rituximab was prepared at 1000X of 200ng/ml and diluted 10 times to the concentration within the assay range, using an assay diluent. Different concentrations of rituximab were prepared and the hook effect in the assay was determined.

2.10. Specificity

The specific binding of rituximab to anti-rituximab antibody was evaluated in the ELISA during the method validation. A non-specific drug, adalimumab was tested against anti-rituximab coated ELISA plate to ensure the specific binding of rituximab to anti-rituximab antibody in presence of interfering drug adalimumab.

3. Results

3.1. Affinity of mouse anti-rituximab to rituximab

SPR-binding assay for the anti-rituximab Antibody showed specific binding to rituximab and no cross-reactivity to human IgG. The K_d value of the anti-rituximab antibody was determined to be 0.5×10^{-9} , which shows a high affinity against rituximab (*Figure. 1*). Thus, confirming that the antibody was an anti-idiotypic antibody to rituximab.

3.2. Calibration curve of rituximab

The calibration curve generated using 10 calibrator standards was fitted using the 4PL model (*Figure 2*). The regression model was accepted as the %RE of the back-calculated value for at least 75% calibrators, within 20% of nominal concentration. The standard curve was within the acceptance criteria as %RE and %CV of $\leq 20\%$ and total error within 30% for all calibrators (*Table 1*).

3.3. Precision and accuracy of ELISA

The precision and accuracy of ELISA were determined based on the data of inter-assay and pooled intra-assay precision (%CV) accuracy (%RE) of five different concentrations of QC

samples. For the inter batch assay the %CV was 9.3 to 13.78 and for pooled intra batch assay the %CV was 4.78 to 15. The %RE were found to be <6.98 and total error was <20.76. All the parameters analyzed were within $\leq 20\%$ ($\leq 30\%$ for total error), which is accepted for the ligand-binding assay. (Figure 3a, 3b).

3.4. Selectivity of ELISA

The selectivity of the ELISA was determined by testing 11 different matrices by spiking rituximab at ULOQ and LLOQ points. A minimum of 80% of the ULOQ and LLOQ samples showed assay %RE values $\leq 20\%$ of the nominal concentration, which showed the recovery of spiked rituximab (Table 2).

3.5. Sensitivity of the ELISA

The sensitivity of ELISA was evaluated by inter and intra-batch accuracy and precision. Notably, both inter and intra-batch accuracy and precision at LLOQ of 6.25ng/ml, with %CV of 11.35 and 6.26 respectively. The %RE of 6% and total error of 17.35% of the LLOQ sample was found to be $\leq 20\%$ range (Figure 4a, 4b). This confirms the assay sensitivity of rituximab at 6.25ng/ml.

3.6. Dilution linearity of the ELISA

The dilution linearity was expressed as %RE for QC samples tested. The Hook's effect of the developed ELISA was determined by measuring rituximab concentrations 1000x of 200ng/ml to 1:10 till 0.25ng/ml concentrations. Further, it was found that the rituximab above the assay range (>200 ng/ml) displayed the Hook effect (Figure 5). The QC samples were within the linear range exhibiting the acceptance criteria ($\leq 20\%$) as per EMA guidelines (Table 3).

3.7. Specificity of ELISA

The specificity of rituximab was confirmed by measuring the levels of rituximab and in presence of a high concentration of adalimumab. Notably, rituximab alone bound to the capture antibody in a concentration-dependent manner pre-coated microtiter plate and not the adalimumab, thus confirming the specificity and no cross-reactivity with a similar antibody. The ELISA data analysis of the QC points prepared in presence of adalimumab showed values, which was within the acceptance criteria as %RE and %CV of $\leq 20\%$ and total error within 30% for all calibrators (Table 4).

4. Discussion

The development and validation of ELISA to quantify rituximab in human serum could help validate immunoassays to quantify biopharmaceuticals and their pharmacokinetics. Therefore, the present study aimed to develop a reliable, robust, and sensitive ELISA method to assess the pharmacokinetics of rituximab in the serum matrices. An anti-rituximab antibody developed in mice had a high affinity of 0.5×10^{-9} , the specificity of which was confirmed by SPR technique. This antibody also showed no cross-reactivity towards the human backbone of rituximab, and was only specific to the CDR region. Moreover, this antibody binds to rituximab with a higher affinity (K_d) which was comparable to the monoclonal antibody panel developed in rats against rituximab (Tada, Suzuki, and Ishii-Watabe 2018). The sandwich ELISA developed and validated in this study could detect rituximab rapidly, sensitively, and precisely in serum matrix when compared to the previously reported methods (Desvignes et al. 2015).

The rituximab PK ELISA was validated for parameters including linearity, precision, accuracy, specificity, selectivity, sensitivity and dilution linearity and the results obtained were within the recommended guidelines of EMA (DeSilva et al. 2003; Smolec et al. 2005a; 2005b; Findlay et al. 2000). The linearity results depicted that the standard calibrator curve range was determined to be from 800ng/ml to 1.56ng/ml.

The precision and accuracy for the standard calibrator points in the inter and intra assay batches the assay batches for the %CV were <13.8% and <12.1% respectively and the, %RE were <6.5%, %Total error was <15.4%. For the quality control points in the inter and intra assay batches the assay batch, the %CV were <13.78% and <15% respectively and the, %RE were <6.98%, %Total error was <20.76%. The obtained values were within the limits of recommended errors of $\leq 20\%$ for %CV, %RE and $\leq 40\%$ (LLOQ, ULOQ), $\leq 40\%$ (for other QC points), which were within the accepted limits.

Moreover, the sensitivity of the assay was tested and found to be 6.25 ng/ml, which qualifies the acceptance criteria. The method developed was demonstrated to be robust, accurate, sensitive, and reproducible for rituximab concentrations between 800ng/ml to 1.56ng/ml.

Of note, the Hook effect frequently observed in ligand-binding assays, is primarily due to high antibody concentration leading to saturation and precipitation of antigen, and false negative results. Hence, the Hook effect of the newly developed ELISA was determined by measuring rituximab concentrations. Further, it was found that the rituximab above the assay range (>800

ng/ml) displayed the Hook effect. Remarkably, reports emphasize the impact of assay matrix and dilution linearity on the quality of the antibody-based assays (Cummings et al. 2008; Keystone and Haraoui 2004; Li et al. 2022; Walker 2004; Tracey et al. 2008; Luchavez et al. 2011). In this line, the current ELISA was also validated using various levels of rituximab, and found that the dilution linearity was maintained between 2.5ng/ml to 200ng/ml. However, due to high variability in quantification below LLOQ, this assay might not be appropriate for measuring rituximab concentrations below 2.5ng/ml. Furthermore, the serum matrix did not affect the measurement of rituximab and thus ruled out matrix interference in the assay.

5. Conclusions

In this study, an ELISA method using a anti-rituximab antibody was used as capture antibody in developed ELISA and validated to quantify high and low rituximab concentrations. Further, the method was validated for its analytical performance, to ensure its robustness, sensitivity, and reliability for therapeutic drug monitoring in the biological matrices. The overall outcome of this study highlights the potential use of the validated ELISA as a standard procedure for batch release in QC laboratories during the drug manufacturing, test for rituximab's characterization and bio-comparability during the biosimilar development and routine tests during drug therapy.

Funding

This work was not funded by any government or non-government agencies.

Ethics approval

The study did not require ethical approval.

Credit authorship contribution statement

Manjunath S Devaramani, developed the research idea; Manjunath S. Devaramani, Dinesh Kumar Saini, Ashna P Thampi and Kavya J performed and analyzed experiments; Kiran Kumar Mudnakudu Nagaraju, Siddesha Jalahalli Mariswamy, Dinesh Kumar Saini, and Manjunath S. Devaramani, contributed to the design of the study, evaluation of the results, and writing of the manuscript. All authors have read and approved the manuscript.

Acknowledgments

The authors thank JSS Academy of Higher Education & Research (Deemed to be University), Mysuru, and Denovo Biolabs Pvt Ltd, Bengaluru for providing laboratory facilities and resources.

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.

References

1. Berinstein, N.L., A.J. Grillo-López, C.A. White, I. Bence-Bruckler, D. Maloney, M. Czuczman, D. Green, J. Rosenberg, P. McLaughlin, and D. Shen. 1998. "Association of Serum Rituximab (IDEC-C2B8) Concentration and Anti-Tumor Response in the Treatment of Recurrent Low-Grade or Follicular Non-Hodgkin's Lymphoma." *Annals of Oncology* 9 (9): 995–1001. <https://doi.org/10.1023/A:1008416911099>.
2. Beum, Paul V, Adam D Kennedy, and Ronald P Taylor. 2004. "Three New Assays for Rituximab Based on Its Immunological Activity or Antigenic Properties: Analyses of Sera and Plasmas of RTX-Treated Patients with Chronic Lymphocytic

- Leukemia and Other B Cell Lymphomas.” *Journal of Immunological Methods* 289 (1–2): 97–109. <https://doi.org/10.1016/j.jim.2004.03.012>.
3. Blasco, H., G. Lalmanach, E. Godat, M.C. Maurel, S. Canepa, M. Belghazi, G. Paintaud, et al. 2007. “Evaluation of a Peptide ELISA for the Detection of Rituximab in Serum.” *Journal of Immunological Methods* 325 (1–2): 127–39. <https://doi.org/10.1016/j.jim.2007.06.011>.
 4. Cragg, Mark S., Mike B. Bayne, Alison L. Tutt, Ruth R. French, Stephen Beers, Martin J. Glennie, and Timothy M. Illidge. 2004. “A New Anti-Idiotypic Antibody Capable of Binding Rituximab on the Surface of Lymphoma Cells.” *Blood* 104 (8): 2540–42. <https://doi.org/10.1182/blood-2004-05-1733>.
 5. Cummings, J, F Raynaud, L Jones, R Sugar, and C Dive. 2010. “Fit-for-Purpose Biomarker Method Validation for Application in Clinical Trials of Anticancer Drugs.” *British Journal of Cancer* 103 (9): 1313–17. <https://doi.org/10.1038/sj.bjc.6605910>.
 6. Cummings, J, T H Ward, A Greystoke, M Ranson, and C Dive. 2008. “Biomarker Method Validation in Anticancer Drug Development.” *British Journal of Pharmacology* 153 (4): 646–56. <https://doi.org/10.1038/sj.bjp.0707441>.
 7. Davies, Andrew, Francesco Merli, Biljana Mihaljevic, Noppadol Siritanaratkul, Phillippe Solal-Céligny, Martin Barrett, Claude Berge, et al. 2014. “Pharmacokinetics and Safety of Subcutaneous Rituximab in Follicular Lymphoma (SABRINA): Stage 1 Analysis of a Randomised Phase 3 Study.” *The Lancet Oncology* 15 (3): 343–52. [https://doi.org/10.1016/S1470-2045\(14\)70005-1](https://doi.org/10.1016/S1470-2045(14)70005-1).
 8. Davis, T. A., C. A. White, A. J. Grillo-López, W. S. Velásquez, B. Link, D. G. Maloney, R. O. Dillman, et al. 1999. “Single-Agent Monoclonal Antibody Efficacy in Bulky Non-Hodgkin’s Lymphoma: Results of a Phase II Trial of Rituximab.” *Journal of Clinical Oncology* 17 (6): 1851–1851. <https://doi.org/10.1200/JCO.1999.17.6.1851>.
 9. DeSilva, Binodh, Wendell Smith, Russell Weiner, Marian Kelley, JoMarie Smolec, Ben Lee, Masood Khan, Richard Tacey, Howard Hill, and Abbie Celniker. 2003. “Recommendations for the Bioanalytical Method Validation of Ligand-Binding Assays to Support Pharmacokinetic Assessments of Macromolecules.”

- Pharmaceutical Research* 20 (11): 1885–1900.
<https://doi.org/10.1023/B:PHAM.0000003390.51761.3d>.
10. Desvignes, Céline, Soujanya R Edupuganti, François Darrouzain, Anne-Claire Duveau, Amy Loercher, Gilles Paintaud, and Denis Mulleman. 2015. “Development and Validation of an Enzyme-Linked Immunosorbent Assay to Measure Adalimumab Concentration.” *Bioanalysis* 7 (10): 1253–60.
<https://doi.org/10.4155/bio.15.30>.
 11. Findlay, J.W.A., W.C. Smith, J.W. Lee, G.D. Nordblom, I. Das, B.S. DeSilva, M.N. Khan, and R.R. Bowsher. 2000. “Validation of Immunoassays for Bioanalysis: A Pharmaceutical Industry Perspective.” *Journal of Pharmaceutical and Biomedical Analysis* 21 (6): 1249–73. [https://doi.org/10.1016/S0731-7085\(99\)00244-7](https://doi.org/10.1016/S0731-7085(99)00244-7).
 12. Gürcan, Hakan M., Derin B. Keskin, Joel N.H. Stern, Matthew A. Nitzberg, Haris Shekhani, and A. Razzaque Ahmed. 2009. “A Review of the Current Use of Rituximab in Autoimmune Diseases.” *International Immunopharmacology* 9 (1): 10–25. <https://doi.org/10.1016/j.intimp.2008.10.004>.
 13. Hampson, G, T H Ward, J Cummings, M Bayne, A L Tutt, M S Cragg, C Dive, and T M Illidge. 2010a. “Validation of an ELISA for the Determination of Rituximab Pharmacokinetics in Clinical Trials Subjects.” *Journal of Immunological Methods* 360 (1): 30–38. <https://doi.org/https://doi.org/10.1016/j.jim.2010.05.009>.
 14. Hampson, G., T.H. Ward, J. Cummings, M. Bayne, A.L. Tutt, M.S. Cragg, C. Dive, and T.M. Illidge. 2010b. “Validation of an ELISA for the Determination of Rituximab Pharmacokinetics in Clinical Trials Subjects.” *Journal of Immunological Methods* 360 (1–2): 30–38.
<https://doi.org/10.1016/j.jim.2010.05.009>.
 15. Hong, Kyu, Leonard G. Presta, Yanmei Lu, Ashley Penn, Camellia Adams, Anan Chuntharapai, Jihong Yang, Wai Lee Wong, and Y. Gloria Meng. 2004. “Simple Quantitative Live Cell and Anti-Idiotypic Antibody Based ELISA for Humanized Antibody Directed to Cell Surface Protein CD20.” *Journal of Immunological Methods* 294 (1–2): 189–97. <https://doi.org/10.1016/j.jim.2004.09.003>.
 16. Iacona, Isabella, Mario Lazzarino, Maria Antonietta Avanzini, Maurizio Rupolo, Luca Arcaini, Cesare Astori, Francesca Lunghi, et al. 2000. “Rituximab (IDEC-C2B8): Validation of a Sensitive Enzyme-Linked Immunoassay Applied to a

- Clinical Pharmacokinetic Study.” *Therapeutic Drug Monitoring* 22 (3): 295–301. <https://doi.org/10.1097/00007691-200006000-00010>.
17. Keystone, Edward, and Boulos Haraoui. 2004. “Adalimumab Therapy in Rheumatoid Arthritis.” *Rheumatic Diseases Clinics of North America* 30 (2): 349–64, vii. <https://doi.org/10.1016/j.rdc.2004.02.004>.
 18. Li, Ruo-Jing, Lian Ma, Hyewon Kim, Insook Kim, Lesley Hanes, Tara Altepeter, Jessica Lee, Jiang Liu, Hao Zhu, and Yaning Wang. 2022. “Model-Informed Approach Supporting Approval of Adalimumab (HUMIRA) in Pediatric Patients with Ulcerative Colitis from a Regulatory Perspective.” *The AAPS Journal* 24 (4): 79. <https://doi.org/10.1208/s12248-022-00730-0>.
 19. Luchavez, Jennifer, Joanne Baker, Sheila Alcantara, Vicente Belizario, Qin Cheng, James S McCarthy, and David Bell. 2011. “Laboratory Demonstration of a Prozone-like Effect in HRP2-Detecting Malaria Rapid Diagnostic Tests: Implications for Clinical Management.” *Malaria Journal* 10 (September):286. <https://doi.org/10.1186/1475-2875-10-286>.
 20. Maloney, D G, T M Liles, D K Czerwinski, C Waldichuk, J Rosenberg, A Grillo-Lopez, and R Levy. 1994. “Phase I Clinical Trial Using Escalating Single-Dose Infusion of Chimeric Anti-CD20 Monoclonal Antibody (IDEC-C2B8) in Patients with Recurrent B-Cell Lymphoma.” *Blood* 84 (8): 2457–66.
 21. Piro, L.D., C.A. White, A.J. Grillo-López, N. Janakiraman, A. Saven, T.M. Beck, C. Varns, et al. 1999. “Extended Rituximab (Anti-CD20 Monoclonal Antibody) Therapy for Relapsed or Refractory Low-Grade or Follicular Non-Hodgkin’s Lymphoma.” *Annals of Oncology* 10 (6): 655–61. <https://doi.org/10.1023/A:1008389119525>.
 22. Reff, M E, K Carner, K S Chambers, P C Chinn, J E Leonard, R Raab, R A Newman, N Hanna, and D R Anderson. 1994. “Depletion of B Cells in Vivo by a Chimeric Mouse Human Monoclonal Antibody to CD20.” *Blood* 83 (2): 435–45.
 23. Silva, Antonio da, Ulrich Kronthaler, Vera Koppenburg, Martin Fink, Ines Meyer, Anastassia Papandrikopoulou, Matthias Hofmann, Thomas Stangler, and Jan Visser. 2014. “Target-Directed Development and Preclinical Characterization of the Proposed Biosimilar Rituximab GP2013.” *Leukemia & Lymphoma* 55 (7): 1609–17. <https://doi.org/10.3109/10428194.2013.843090>.

24. Smolec, JoMarie, Binodh DeSilva, Wendell Smith, Russell Weiner, Marian Kelly, Ben Lee, Masood Khan, et al. 2005a. "Bioanalytical Method Validation for Macromolecules in Support of Pharmacokinetic Studies." *Pharmaceutical Research* 22 (9): 1425–31. <https://doi.org/10.1007/s11095-005-5917-9>.
25. EMA. 2005b. "Bioanalytical Method Validation for Macromolecules in Support of Pharmacokinetic Studies." *Pharmaceutical Research* 22 (9): 1425–31. <https://doi.org/10.1007/s11095-005-5917-9>.
26. Tada, Minoru, Takuo Suzuki, and Akiko Ishii-Watabe. 2018. "Development and Characterization of an Anti-Rituximab Monoclonal Antibody Panel." *MAbs* 10 (3): 370–79. <https://doi.org/10.1080/19420862.2018.1424610>.
27. Tedder, Thomas F., and Pablo Engel. 1994. "CD20: A Regulator of Cell-Cycle Progression of B Lymphocytes." *Immunology Today* 15 (9): 450–54. [https://doi.org/10.1016/0167-5699\(94\)90276-3](https://doi.org/10.1016/0167-5699(94)90276-3).
28. Tracey, Daniel, Lars Klareskog, Eric H Sasso, Jochen G Salfeld, and Paul P Tak. 2008. "Tumor Necrosis Factor Antagonist Mechanisms of Action: A Comprehensive Review." *Pharmacology & Therapeutics* 117 (2): 244–79. <https://doi.org/10.1016/j.pharmthera.2007.10.001>.
29. Walker, D K. 2004. "The Use of Pharmacokinetic and Pharmacodynamic Data in the Assessment of Drug Safety in Early Drug Development." *British Journal of Clinical Pharmacology* 58 (6): 601–8. <https://doi.org/10.1111/j.1365-2125.2004.02194.x>.
30. Ward, Tim H., Grace Hampson, Jeff Cummings, Mike Bayne, Alison Tutt, Mark Cragg, Caroline Dive, and Tim Illidge. 2009. "Abstract C184: Validation of an ELISA for the Determination of Rituximab Pharmacokinetics in Clinical Trials Subjects." *Molecular Cancer Therapeutics* 8 (12_Supplement): C184–C184. <https://doi.org/10.1158/1535-7163.TARG-09-C184>.

Figures

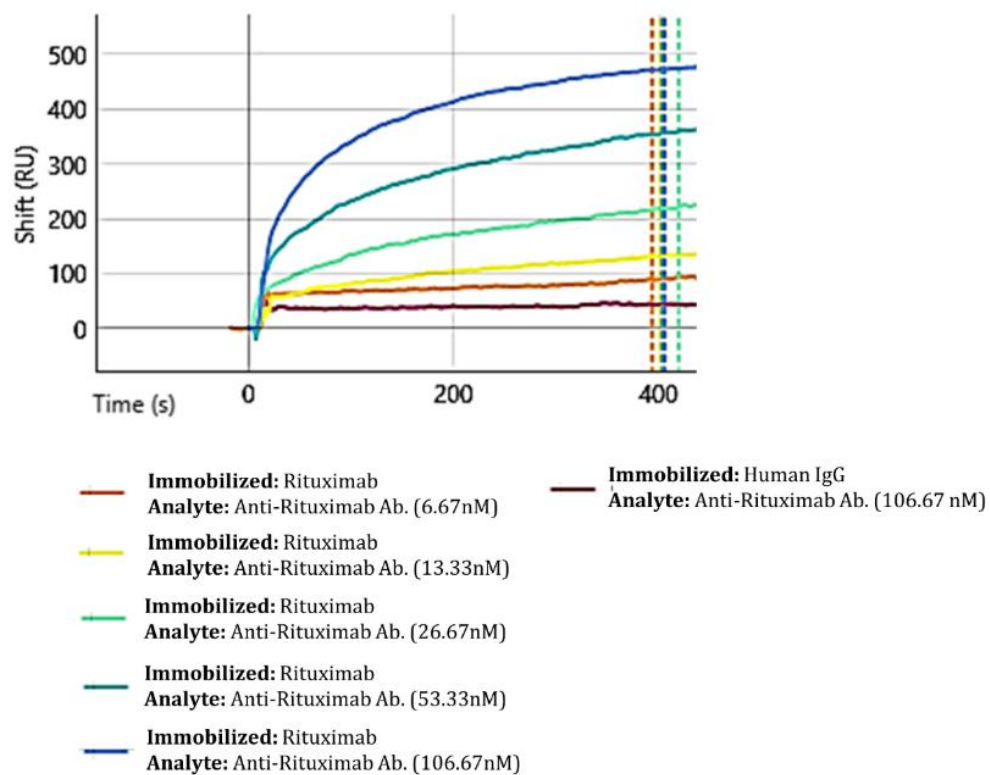


Figure 1. Specificity of mouse anti-rituximab antibody to rituximab and human IgG

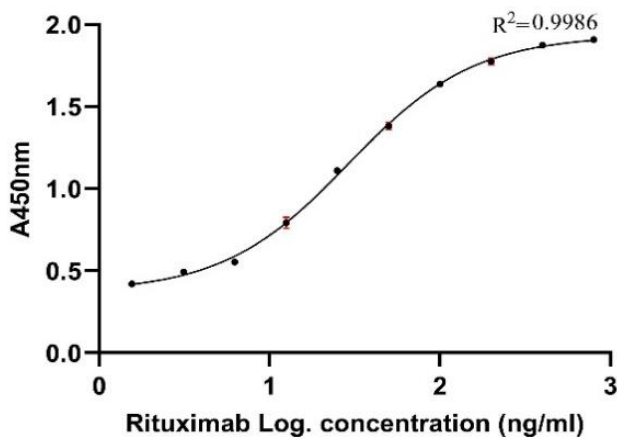


Figure 2. Calibration curve determined for PK ELISA method

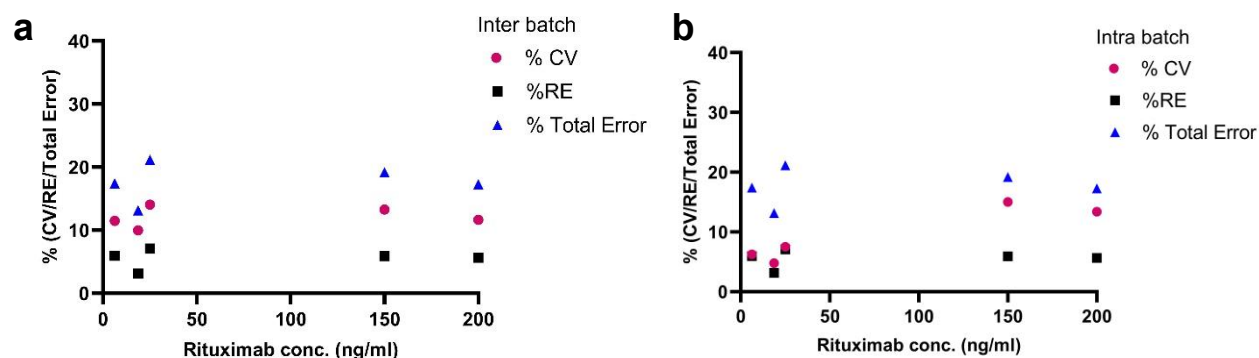


Figure 3. Precision and accuracy determination showing the %CV, %RE and total error values obtained during analysis of QC points in, (a) inter batch assay, (b) intra batch assay

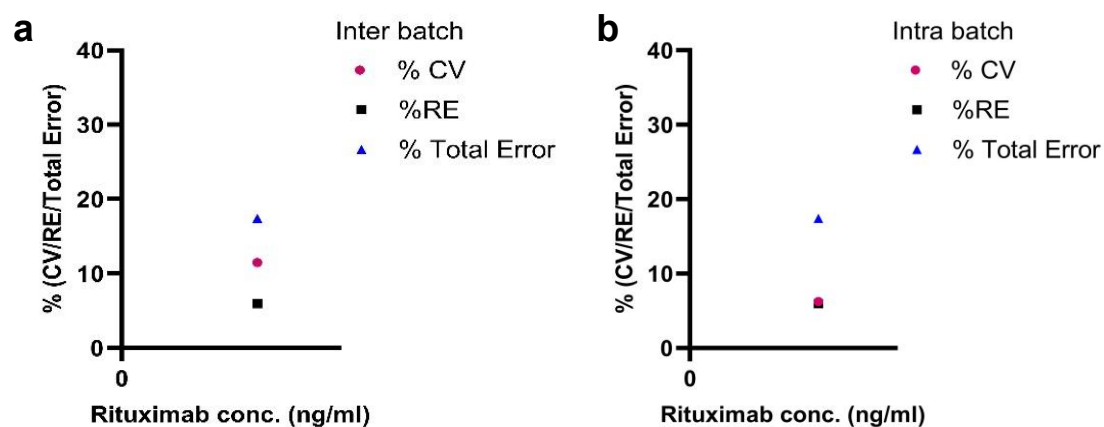


Figure 4. Precision and accuracy determination showing the %CV, %RE and total error values obtained during analysis of LLOQ point in, (a) inter batch assay, (b) intra batch assay

Tables**Table 1.** Calibration curve; ELISA data analysis

<i>Std</i>	<i>RTX (ng/ml)</i>	<i>Sd</i>	<i>%CV</i>	<i>%RE</i>	<i>%Total error</i>
S1	800.00	0.005	0.26	14.06	14.33
S2	400.00	0.018	0.96	13.66	14.62
S3	200.00	0.021	1.17	7.33	8.50
S4	100.00	0.010	0.60	0.47	1.07
S5	50.00	0.023	1.64	2.86	4.50
S6	25.00	0.009	0.83	5.78	6.62
S7	12.50	0.035	4.38	0.11	4.49
S8	6.25	0.018	3.22	19.01	22.22
S9	3.13	0.013	2.57	14.54	17.11
S10	1.56	0.001	0.29	5.82	6.11

Table 2. Selectivity; ELISA data analysis and depiction of %CV, %RE and total error values

Serum Matrix ID	ULOQ tested conc. (ng/ml)	Measured Mean Conc (ng/ml)	%CV	%RE	%Total error	LLOQ tested conc. (ng/ml)	Measured Mean Conc (ng/ml)	%CV	%RE	%Total error
1	200	237.30	1.290	18.65	19.94	6.25	5.66	10.31	9.36	19.67
2		198.65	1.793	0.68	2.47		5.49	0.28	12.21	12.48
3		201.63	4.397	0.81	5.21		5.84	2.04	6.63	8.67
4		177.34	0.719	11.33	12.05		5.24	2.24	16.19	18.43
5		182.44	0.000	8.78	8.78		5.34	0.56	14.51	15.07
6		173.08	2.167	13.46	15.63		6.03	9.29	3.57	12.86
7		207.50	2.582	3.75	6.33		6.06	3.89	2.99	6.88
8		161.57	2.874	19.21	22.09		6.01	16.41	3.92	20.33
9		190.17	0.379	4.91	5.29		6.44	6.60	2.98	9.58
10		177.49	1.534	11.26	12.79		6.14	23.89	1.79	25.68
11		162.75	4.046	18.63	22.67		7.18	13.81	14.93	28.74

Table 3. Dilution linearity; ELISA data analysis and depiction of %CV, %RE and total error values

QC Sample	Predicted Concentration (ng/ml)	Measured Concentration (ng/ml)	Avg. %AR	%CV	%RE	%Total error	Result
1000x DL-1	200000	AQL	1.2	1.99	98.83	100.8	AQL
100x DL-2	20000	AQL	1.8	7.82	98.17	106.0	AQL
10x DL-3	2000	AQL	36.0	39.20	64.05	103.2	AQL
ULOQ 1X DL-4	200	191.11	95.6	3.13	4.45	7.6	Passed
HQC-DL-5	150	148.59	99.1	1.15	0.94	2.1	Passed
HQC-DL-6	100	100.97	101.0	1.54	0.97	2.5	Passed
MQC-DL-7	50	45.51	91.0	3.40	8.98	12.4	Passed
MQC-DL-8	25	25.01	100.0	7.74	0.04	7.8	Passed
LQC-DL-9	15	13.10	87.3	5.04	12.67	17.7	Passed
LLOQ DL-10	5	5.27	105.4	4.53	5.40	9.9	Passed
BLLOQ DL-11	2.5	2.26	90.5	0.65	9.51	10.2	Passed
BLLOQ DL-12	0.5	BQL	178.9	3.29	78.94	82.2	BQL
BLLOQ DL-13	0.25	BQL	346.4	1.47	246.42	247.9	BQL

AQL: Above Quantitative Limits

BQL: Below Quantitative Limits

Table 4. Specificity; ELISA data analysis and depiction of %CV, %RE and total error values

QC Sample	Rituximab in (ng/ml)	Recovered Average Conc. (ng/ml)	%AR	%CV	%RE	%Total error
ULOQ	200	197.91	98.96	0.56	1.06	1.61
HQC	150	173.22	115.48	4.22	13.41	17.63
MQC	25	21.73	86.93	0.22	15.04	15.26
LQC	18.75	17.75	94.66	4.82	5.65	10.47

LLOQ	6.25	7.01	112.22	2.16	10.89	13.04
-------------	------	------	--------	------	-------	-------