Development and validation of a novel ELISA for accurate and precise pharmacokinetic analysis of bispecific antibody drug Faricimab.

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ABSTRACT

Faricimab, a bispecific monoclonal antibody drug, targets both vascular endothelial growth factor (VEGF) and angiopoietin 2 (Ang-2). It is indicated for the treatment of age-related macular degeneration and diabetic macular edema via stabilization of blood vessels in the retina. By blocking the action of these two growth factors, Faricimab decreases migration and replication of endothelial cells allowing for stabilization of vascular structures, thereby decreasing vascular leakage.

It holds immense potential for therapeutic applications and additional treatments, however, precise pharmacokinetic analysis is crucial for understanding its behaviour in vivo. Characterizing the ability of bispecific antibody drugs to bind to both targets simultaneously is critical for development of biotherapeutics. Compared to SPR, which is complex to set up and is not widely used, and cell-based flow cytometry, that lacks precision in complex matrices, ELISA is readily available method, being widely and extensively applied in almost every laboratory, and can be easily optimized to offer accuracy and precision in complex matrices.

In this study, we developed an immunoassay for accurate and sensitive quantification of Faricimab concentrations in biological samples. The immunoassay employs a combination of monoclonal antibodies, specifically designed for selective recognition of Faricimab's antigen-binding sites. We validated the assay's specificity, precision, accuracy, and linearity with extensive analytical characterization and comparison to standard methods. Our results demonstrate excellent performance, with an assay range of 0-10 ng/ml and low limits of detection (0.2 ng/ml) and quantification (0.3 ng/ml). The unique immunoassay presented in this study offers a valuable tool for PK analysis of Faricimab, facilitating clinical development and optimization, and enabling a deeper understanding of bispecific antibody drugs' pharmacokinetic properties and additional therapeutic possibilities.

INTRODUCTION

Retinal vascular disease (RVD) understanding and treatment has increased tremendously over the last decade, with an increased focus on improving the current anti-angiogenesis mechanisms and agents. Different disease mechanisms exist perpetuating a variety of different diseases; commonly, these include diabetic macular edema (DME), “wet” age-related macular degeneration (nAMD), and central or branch retinal vein occlusion. These disease processes are very prevalent, with diabetes mellitus (Type I or Type II) estimated to increase by 56% in the U.S. by 2030 (1) and DME causing visual imparity in up to 25% of patients (2). For both nAMD and DME, current widely used treatments include intravitreal agents target VEGF-A to suppress the abnormal growth of blood vessels and show a reduction in disease progression, including monoclonal antibody drugs like Ranibizumab. VEGF, present in four isoforms (VEGF-A, -B, -C, and -D), signal through three transmembrane tyrosine kinase receptors and are key regulators of disease.

Despite the success of these drugs reducing vessel leakage and preventing further vessel growth, patients require frequent administration of anti-VEGF medications to maintain their visual acuity; further, some may experience vision loss due to recurrent leakage from persistent CNV despite intensive treatment. In addition, real-world data suggest that in many cases, patients do not receive optimal dosing frequency or achieve optimal visual outcomes, and therefore the lack of total therapeutic penetrance and success in both nAMD and DME indicate that factors other than VEGF contribute to pathology. This has led to development of drugs targeting other anti-angiogenic targets like Angieopoitin-2 (Ang-2).

Faricimab (trade name faricimab-svoa; Vabysmo™) is a first-of-its-kind bispecific antibody (bsAb) that binds to and inhibits both VEGF-A and Ang-2. By blocking the action of these two growth factors, faricimab decreases migration and replication of endothelial cells allowing for stabilization of vascular structures, thereby decreasing vascular leakage. Approved in 2022, it is indicated for the treatment of both nAMD and DME and has shown considerable therapeutic success both alone and in combinations with other anti-VEGF treatments.

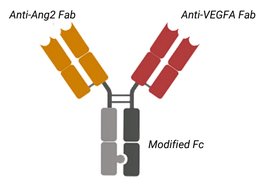


Figure 1: Structure of the Faricimab antibody.

Faricimab is also now under investigation for improving efficacy, additional combination therapies, switching therapies for existing patients as well as in patients who have shown drug resistance to previous anti-VEGF therapies and others. For these studies, scientists currently develop and manufacture their own Faricimab in-house assays to quantify serum and plasma drug levels, for pharmacokinetic research or in dosage response studies. These assays are often based on traditional protein quantification techniques and are developed and validated by the individual laboratory or company. While these assays can provide accurate results after validation, they are also associated with several limitations.

They are often labour-intensive and time-consuming - this is especially true for bispecific antibody assays which require an additional validation to ensure specificity and the right assignment of proteins as capture and detection. This can be a significant burden for drug development and manufacturing teams, particularly when multiple assays are needed to quantify different aspects of the drug product. Additionally, in-house assays can be prone to variability between laboratories, making it difficult to compare results between different research groups or to reproduce results over time. This can lead to inconsistencies in data and reduced confidence in the results.

The aim of this study was to develop and validate a commercially available, sensitive immunoassay for the detection and quantification of Faricimab and other VEGF/Ang2 antibodies. The principal of the assay was based on the use of the target proteins in a sandwich assay format.

METHODS

A step by step optimization and validation protocol was followed for the development of this assay. Using a checkerboard testing format, recombinant VEGF165 and Ang2 proteins were analysed for their binding affinity to the standard. Accordingly, VEGFA165 (the most abundant and biologically active form) was coated overnight onto Corning CoStarTM microwell plates using a proprietary coating solution and blockers for long term immobilization and stability of the protein. The standard used was a research grade Faricimab antibody (expressed in CHO cells). It was run at six dilutions to form the standard curve of the kit. Ang-2 protein was conjugated to HRP using an in-house conjugation protocol and was used as the detection in a sandwich assay format. The assay scheme is depicted in Fig. 2.

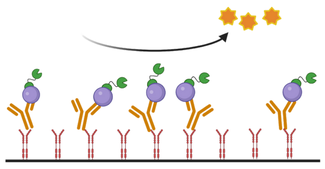


Figure 2: Faricimab assay principle: a direct sandwich assay

For determining optimized concentrations of the coating and detection antigen, various concentrations for each were tested until an optimal differentiated signal was obtained. To determine antibody titers, the assay was optimized using checkerboard titration experiments. Various incubation and wash steps were used to optimize removal of unbound proteins at various steps. The substrate solution of 3,3’,5,5’-Tetramethylbenzidine (TMB) was added and incubated. The enzyme reaction was terminated by stop solution dispensed into the wells turning the solution from blue to yellow. The optical density (OD) of the solution read at 450 was directly proportional to the specifically bound amount of Faricimab present in the sample. Absorbances were read on a Tecan Safire2.

Each assay step was optimized for optimal noise-to-signal ratio and working range using a checkboard experiment format. This included running the standards in duplicates in various diluents, at various coating and detection conjugate levels, and other variables. The optimized kit was then validated using the guidelines set by the ICH M10 (USA FDA / EMA). This included determining precision, sensitivity, stability and robustness. Repeatability was determined using ten replicates of the same extract in one assay. Intra-assay reproducibility was evaluated by analyzing ten extracts of the same sample in one assay. Inter-assay reproducibility was determined analyzing three extracts of the same sample in three independent assays. Additional optimization and spiking experiments were performed for minimal %CV and relative error. Assay precision was determined by both intra (n=10 assays) and inter assay (n=10 assays) reproducibility on two pools with low, medium and high concentrations, run in duplicates. Robustness was estimated by introducing deliberate changes in the established procedure in the same experiment. The Limit of Detection (LOD) was estimated as the average concentration of ten replicates of the zero standard plus three. Finally, an accelerated stability test was conducted by keeping various temperature sensitive parameters at 37°C and calculating deterioration via %CV. Other in-house and regulatory validation processes were also compeleted. Calculations and statistical analysis were performed using the GraphPad Prism Software v5.

RESULTS - METHOD OPTIMIZATION

The sandwich ELISA was optimized for Faricimab concentration and buffer composition of coating and detection antibodies, washing buffer composition, as well as incubation temperature and time of the different steps of the assay to give a commercially acceptable assay that surpasses current industry standards. The standard range of drug assays is set via analysis of the serum trough levels expected to be found in patients. The serum trough levels for Faricimab are between 2 ng/ml - 3 ng/ml, and therefore, the assay range was set as 0 - 10 ng/ml, allowing for more concentrated samples to be tested as well.

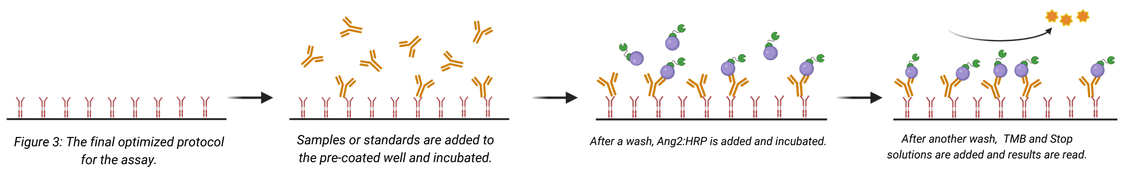


Figure 3: The final optimized protocol for the assay.

Samples or standards are added to the pre-coated well and incubated.

After a wash, Ang2:HRP is added and incubated.

After another wash, TMB and Stop solutions are added and results are read.

Working Conditions Optimization: Optimal concentrations of coating and detection conjugate, appropriate diluents and incubation times were determined through a series of checkerboard titrations till optimal noise:signal ratio was observed.

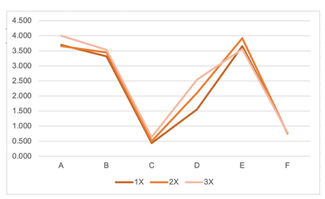


Figure 4: Optimization of development and working conditions. (A) - (F) represent various proprietary diluents, and 1X - 3X are the coating antibody concentrations included in the checkerboard experiments. These diluents and coating antibodies were run in checkerboard experiments to determine the optimal concentrations and noise:signal ratios. Absorbance values are depicted on the Y axis.

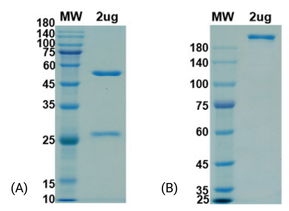


Figure 5: SDS-PAGE analysis of the recombinant Human ANGPT2/VEGFA protein expressed in CHO cells using a recombinant Faricimab antibody. (A) The lane was run under reducing conditions. (B) The lane was run under non-reducing conditions.

RESULTS - ASSAY VALIDATION

Standard Lyophilization: To ensure stability of the standard over the 12 month expiry, it was lyophilized using in-house proprietary solutions and methods. Validation was performed for the quality of lyophilization over various lots to ensure that it provides robust and reliable results for each run. In each complete run, acceptable recovery results were considered when between 8-12%CV only. The final concentration of the lyophilized standard was set at 1000 ng/ml, which was diluted by the user to the required standard range.

Limit of Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ was found to be 0.3 ng/ml.

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of ‘0’ standard plus 2* SD. 10 replicates of ‘0’ standards were evaluated and the LOD was 0.2 ng/ml.

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.108	0.118	0.113	0.0	--
0.5	0.199	0.200	0.199	0.5	104.9
1	0.282	0.297	0.289	0.9	94.7
2	0.518	0.527	0.522	1.9	97.1
4	1.051	1.077	1.064	4.1	101.9
6	1.611	1.568	1.590	6.1	101.3
8	2.051	2.054	2.053	7.8	98.0
10	2.590	2.670	2.630	10.1	100.7

Table 1: Results from the Faricimab ELISA - showing a full standard range, interpolated concentrations and percentage recovery again actual concentration.

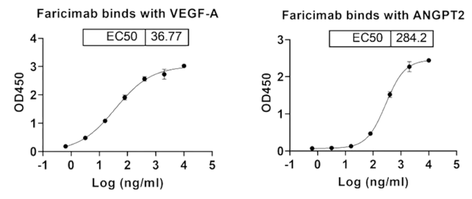


Figure 6: Results of bioactivity analysis of the recombinant Faricimab standard used. EC50 which refers to the concentration of a drug that induces a response halfway between the baseline and maximum, is inversely related to the potency of a compound.

Specificity: The recombinant proteins used in the kit are specific for Faricimab bispecific antibody using a sandwich ELISA technique with VEGF-165 as the capture protein and ANG-2 protein as the detection protein. The standard used in the kit was also analysed for specificity against the two proteins via bioactivity assays, as shown in Figure 4.

High Dose Hook Effect: It is a reduction in measured signal that occurs in the presence of very high concentrations. Over several duplicate runs, the ELISA kit did not experience a high dose hook effect when it was tested up to a Faricimab concentration of 10 ng/ml.

Matrix Effect Recovery: It is used to determine whether analyte detection can be affected by the difference between diluent used for preparation and the experimental sample matrix. It is an important technique for analyzing the accuracy of the Faricimab ELISA. Known amounts of Faricimab was “spiked” into sample diluent (1X), one set diluted with normal human serum (1:10, 1:100, 1:1000) and the other diluted with normal human plasma (1:10, 1:100, 1:1000) and run.

The resulting concentration, or “recovery” of the spiked material demonstrated if the expected value can be measured accurately. It was observed that serum and plasma diluted at 1:1000 obtained the best recoveries (+/-10%). The recovery of this assay was assessed by comparing observed vs. expected values based on non-spiked and/or neat (undiluted) samples across several lots of samples.

	Standard (ng/ml)	Absorbance	% Recovery against Actual
1:10 Human Serum	0	0.100	
	5	2.448	104.68
1:100 Human Serum	0	0.094	
	5	2.862	89.52
1:1000 Human Serum	0	0.101	
	5	2.648	96.75
1:10 Human Plasma	0	0.101	
	5	2.674	95.82
1:100 Human Plasma	0	0.087	
	5	2.558	100.17
1:1000 Human Plasma	0	0.095	
	5	2.670	95.97

Table 2: Serum and Plasma spiking data for Faricimab.

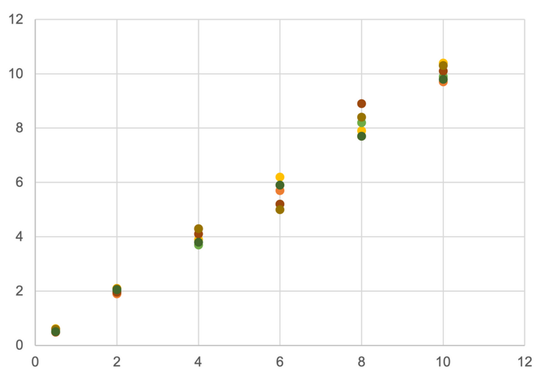


Figure 7: Recoveries for each standard observed over ten experiments in duplicates. All recoveries (in percentage) were within +/- 10% of actual concentration, showing robustness and reproducibility in data.

Precision: It is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=10 assays) and inter assay (n=10 assays) reproducibility on two pools with low (0.5 ng/ml), medium (4 ng/ml) and high (10 ng/ml) concentrations. Additionally, when running the complete standard range as well in duplicates, deviation within and between plates was under 10% CV, ensuring robust precision and reproducibility.

	Lot 1	Lot 2	Lot 3	Lot 4	Lot 5
Standards (ng/ml)	Means Abs	Means Abs	Means Abs	Means Abs	Means Abs
0	0.113	0.207	0.175	0.137	0.208
1	0.289	0.318	0.368	0.356	0.390
2	0.522	0.510	0.547	0.581	0.543
4	1.064	0.933	1.022	1.028	1.003
6	1.590	1.337	1.430	1.483	1.499
8	2.053	1.778	1.995	1.956	1.878
10	2.630	2.456	2.454	2.562	2.513

Table 2: Over 10 lots of complete standards run on different days to observe the deviation in absorbance. They were run in duplicates following the protocol, and mean absorbance was noted. In all runs, satisfactory recoveries were observed, and statistical results showed low standard deviation between wells, and minimal co-effecient of variation.

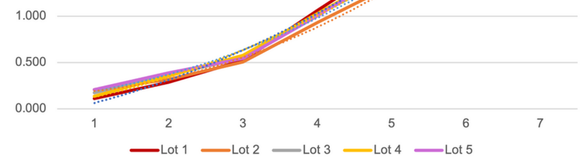


Figure 8: Graphical representation of Table 1. Five lots were mapped on graphpad prism with a 2nd order polynomial best-fit curve trendline. For each lot, the the R2 of 0.99 or higher, well accepted for bioassays as per EMA / FDA guidelines.

Accelerated Stability: Accelerated stability testing increases the rate of degradation and physical change of components by using exaggerated storage conditions as part of the formal stability testing program. Three Faricimab kits from the same lot were subjected to a fourteen day accelerated stability study, with one critical component from each kit at stored at 37 degrees Celsius. The entire standard range was run on days 1, 2, 4, 6, 11 and 14 as per the protocol, meant to represent the stability of the kit over a period of 12 months. Inter- and intra- assay precision and recovery was analysed for each lot at each run. Satisfactory results were obtained from the accelerated stability studies under the acceptable 20% CV over all standards across all types of runs (detection conjugate, standard and plate).

CONCLUSION

Upon completion of the validation process of the assay as per both internal and regulatory standards, we report that the KRIBIOLISA Faricimab ELISA kit successfully and accurately detected Faricimab with high accuracy in both human sample and plasma samples. The ELISA was designed for 0 - 10 ng/ml as assay range and achieved a sensitivity of 0.3 ng/ml.

In conclusion, this Faricimab ELISA for the quantification of bispecific antibody drug Faricimab offers numerous advantages over in-house developed assays. They are optimized for sensitivity, specificity, and reproducibility, and are rigorously validated against a stringent SOP for use in drug development and manufacturing, following guidelines set by US FDA / EMA as the ICH for the validation of bioassays. This can provide greater confidence in the results and reduces the risk of variability between laboratories.

Additionally, the development of this standardized, well validated ELISA for Faricimab can aid in the development of new therapies and treatments by providing researchers with a reliable and accurate tool for studying the drug's efficacy.

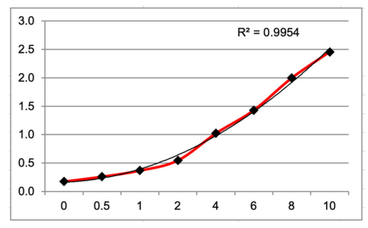
Pool	Intra Assay %CV	Inter Assay %CV
Low	<10%	<10%
Medium	<8%	<8%
High	<5%	<5%

Table 4: Inter and Intra Assay Precision for the ELISA

Standard Concentration (ng/ml)	Absorbance A	Absorbance B	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.116	0.137	0.127	0.0	--
0.5	0.210	0.216	0.213	0.5	104.8
1	0.277	0.306	0.292	0.9	93.5
2	0.494	0.524	0.509	2.0	99.2
4	0.966	0.986	0.976	4.1	101.7
6	1.446	1.466	1.456	6.1	101.6
8	1.817	1.923	1.870	7.8	97.3
10	2.437	2.465	2.451	10.1	100.9

Table 4: Typical results from the KRIBIOLISA Faricimab ELISA run.

- Developed as a direct sandwich assay format, the first of its kind, validated for both human serum and plasma.
- It is well validated as per ICH M10 guidelines and performs within required precision parameters, as demonstrated over many lots of kits and testing.
- Final assay range was set at 0 - 10 ng/ml, with a sensitivity of 0.3 ng/ml.
- Offers robust inter- and intra- assay precision of under <10% CV each.
- Ships at room temperature or 2-8 degrees Celsius, owing to lyophilized standards.
- Provides 95% - 105% recovery and provides accurate, reproducible data.
- Can be used for Faricimab or other biosimilars that target VEGFA/Ang2.



A typical graph that is included with each kit as part of the certificate of analysis. It represents the lot characteristics and expected graph.

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ClinicalTrials.gov. A study to evaluate the efficacy and safety of faricimab (RO6867461) in participants with diabetic macular edema.
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