T2047

An ELISA for the Quantitative Determination of Free and Partially Bound Bevacizumab in Human Serum

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Purpose

Bevacizumab (Avastin®) is a recombinant humanized monoclonal antibody that inhibits angiogenesis by binding to the vascular endothelial growth factor A (VEGF-A). Bevacizumab is currently licensed to treat various cancers, including colorectal, lung, breast, glioblastoma, kidney and ovarian.

The purpose of this study was to develop and validate a sensitive, quick, and cost-effective ELISA for the quantitative determination of free and partially bound bevacizumab innovator in human serum to support clinical studies. The assay format is designed to detect both bevacizumab innovator and biosimilar drug materials. Therefore, biosimilar assay comparability can be quickly validated and a single pharmacokinetic assay may be used to support biosimilar and follow-on clinical trials.

Methods

The analytical method is a classic bridging ELISA using an anti-idiotypic monoclonal capture antibody that specifically recognizes the bevacizumab CDR VEGF binding region (Figure 1). After washing and blocking, bevacizumab in the serum sample is captured. Detection is performed by HRP labeled second anti-bevacizumab monoclonal antibody (not directed against the CDR VEGF binding region), and visualized using TMB substrate.

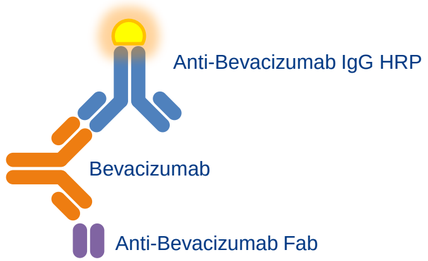

Figure 1. Assay format: A bridging ELISA using anti-idiotypic capture and detection reagents.

Results

Standard Curve. The method demonstrates detection range from 50 to 12,800 ng/ml. Calibration standards were prepared in pooled human sera by spiking bevacizumab at 50 (anchor point), 100 (LLOQ), 200, 400, 800, 1,600, 3,200, 6,400 (ULOQ), and 12,800 (anchor point) ng/mL (Figure 2). Bevacizumab concentrations are interpolated from the 5-parameter regressed standard curve ranging from a Lower Limit of Quantitation (LLOQ) of 100 ng/mL to the Upper Limit (ULOQ) of 6,400 ng/mL. The bevacizumab quality control (QC) samples were prepared in pooled human sera at 100, 400, 1,500, 5,000 and 6,400 ng/mL and analyzed in 4 different assays on two different days by 2 analyst (n = 3 at each level).

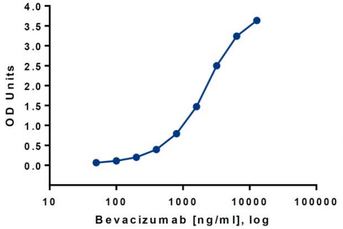

Figure 2. Typical Standard Curve. Standards ranging from 50 to 12,800 ng/mL are prepared in pooled normal human serum, then subject to the 1:50 minimum required dilution prior to adding to the plate.

Dilution Linearity. QC's were spiked with bevacizumab at 500,000 ng/mL and diluted using negative pooled sera. The diluted QC samples had an overall % CV of 4.0% and bias ranging from 4.1 to 7.4%. Maximum dilution after 1:50 MRD is 1:1600 with no hook effect observed at 50,000 and 500,000 ng/mL (Table 1).

Results (ng/mL)			Dilution Factor	Mean Corrected (ng/mL)	Accuracy (%RE)
1st	2nd	3rd			
>ULOQ	>ULOQ	>ULOQ	1		
>ULOQ	>ULOQ	>ULOQ	10		
5,338	5,307	5,081	100	52,4200	4.8
2,668	2,606	2,532	200	52,0400	4.1
1,419	1,302	1,235	400	52,7467	5.5
346	321	340	1,600	53,7067	7.4

Table 1. Dilutional Linearity. A serum sample spiked to 500 µg/mL was run at several different dilutions. No hook effect was seen. Dilutions up to 1:1600 had acceptable recovery.

Accuracy and Precision. The QC samples had intra and inter-assay precision range of 5.6% to 18.4% with bias ranging from -8.7% to 2.6%, using a 5PL regression.

Validation Parameter	Expected Concentration (Units)	Inter-Assay Precision (%CV)	Inter-Assay Accuracy [a](%RE)	Total Error [b](%TE)
ULOQ	6,400	10.2	-10.0	20.2
HQC	5,000	15.2	-7.7	22.9
MQC	1,500	7.7	-6.6	14.3
LQC1	400	10.9	-3.2	14.1
LQC2	200	14.9	4.4	19.3
LLOQ1	100	17.6	-0.7	18.3
LLOQ2	50	32.6	-25.8	58.4

Table 2. Accuracy and Precision: Seven levels of validation samples were run in four assays over 2 days, n = 3 per assay A &P was acceptable within the range of 100 to 6400 ng/mL. (a) %RE: Bias compared to nominal concentration. (b) Total Error = |%RE| + %CV.

Selectivity. Samples (ten independent human sera lots) were spiked with bevacizumab at 100 and 3,200 ng/mL. Nine out of ten lots at 100 ng/mL and all ten lots at 3,200 ng/mL were within ±20% recovery compared to either nominal buffer and pooled serum spiked concentrations (Figure 3).

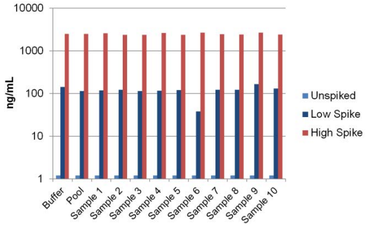

Figure 3. Ten serum samples, buffer and a pool of the samples were spiked with 0, 100 and 3,200 ng/mL of bevacizumab and analyzed in the assay. The resulting signals from the serum samples were compared to the signal in buffer. 9 of 10 samples were within 20% of the pool response at all levels.

Impact of Free Target. The assay format detects free bevacizumab and partially bound VEGF; however, does not detect complexes of bevacizumab completely bound with VEGF. When both VEGF binding sites are occupied on the bevacizumab antibody, the assay format will not allow detection and lower concentration of bevacizumab will be recovered in the sample. Expression levels of VEGF are low and free VEGF concentrations are in low pmol/L range; however, upon Anti-VEGF administration, 100- to a 1,000-fold difference between free VEGF concentration and the concentration of VEGF bound to the anti-VEGF (12.5 to 125 ng/mL) have been reported.

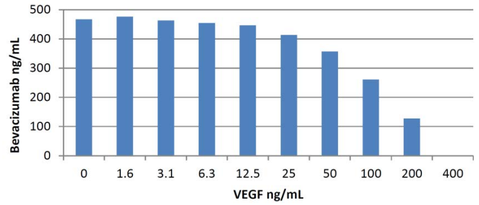

Figure 4. VEGF interference: A sample containing 400 ng/mL of drug was spiked with varying amounts of human VEGF. And pre-incubated for 30 minutes before assay. VEGF at concentrations of 50 ng/mL or more were observed to interfere with the detection of bevacizumab in this assay.

Conclusion

An ELISA method for quantitation of bevacizumab in human serum was developed and qualified. The assay is sensitive, with an LLOQ of 100 ng/mL. The ELISA method exhibits a broad dynamic range, with a ULOQ of 6,400 ng/mL, extendable by dilution up to 10 mg/mL. Concentrations of VEGF above 25 ng/mL may interfere with detection of drug. Thus this should be considered a method for quantitation of free, rather than total, bevacizumab. A sensitive, robust, selective and precise method was qualified to support the development of biosimilar bevacizumab.

Criteria	Results
Validated Range	100 ng/mL to 6,400 ng/mL
Accuracy	-8.7% to 2.6%
Precision Intra-assay Inter-assay	 Range 5.6% to 18.4% Range 5.6% to 18.4%
Specificity / Selectivity	9 out of 10 lots within ±20% RE of buffer nominal with 100 ng/mL spike. 10 out of 10 lots within ±20% RE of buffer nominal with 3,200 ng/mL spike.
Dilutional Linearity	%RE Range: 4.1% to 7.4% Maximum dilution 1:1,600 (in addition to MRD) No hook effect was observed