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Determination of Rituximab in Human Serum by a Gyrolab Assay

Xiaodong F. Liu, Roni J. Weaver, Laurelle Calliste, Christina Xia, Yuyan Joy He, Xun Wang, and LingSing Chen

QPS, One Innovation Way, Suite 200, Newark, DE 19711

INTRODUCTION

Rituximab was the first monoclonal antibody approved as a drug for clinical use. The drug targets CD20 on B-cell surface and has been successfully used to treat diseases and disorders that are characterized by having too many B cells or dysfunctional B cells, such as a solid tumor of lymphoid cells and rheumatoid arthritis. ELISA is the most common immunoassay platform used for pharmacokinetic analysis of such macromolecules. However, ELISA assays are considerably more labor intensive, consume significant amounts of critical reagents, and usually have a narrow dynamic range. New assay platforms with miniaturization and automation such as Gyrolab® are always desirable for quick turn around and 'fit-for-purpose' assay development in the CRO environment. Here we report the development and validation of a Gyrolab assay to determine rituximab levels in human serum. The rituximab assay used a sandwich immunoassay format on a Bioaffiy CD200, in which the analyte is immobilized by a biotinylated monoclonal antibody and is detected by an Alexa-labeled anti-human IgG antibody. The dynamic range of the assay was established to be 90 – 60,000 ng/mL in human serum. Assay selectivity was evaluated and found to be acceptable for spiked serum samples of both healthy individuals and solid tumor patients. The method was fully validated according to the current industry standards for immunoassays. This is part of our continued effort to implement automation in ligand-binding assays for large molecule bioanalysis at QPS, LLC.

Table 1. Summary of the Gyrolab Assay of Rituximab in Human serum

Analytical Method Type	Gyrolab assay		
Minimum Required Dilution	1:30 in PBS-T (0.01%Tween 20), 10% rat serum and Rexxip H-max		
Assay Range	90 – 60000 ng/mL		
Standard Concentrations	90, 255, 750, 2250, 6600, 20000, 60000 ng/mL with anchor points 30 and 180000 ng/mL		
QC Concentrations	90 (LLOQ), 250, 3500, 45000, 60000 (ULOQ) ng/mL in human serum		
	Experimental Design	Acceptance Criteria	Observation
QC Intra-batch Precision (%CV)	1 run, n = 6, 5 levels	≤ 20% (≤ 25% at LLOQ & ULOQ)	2.1 to 8.5%
QC Intra-batch Accuracy (%Diff)	1 run, n =6, 5 levels	≤ 20% (≤ 25% at LLOQ & ULOQ)	7.4 to 18.3 %
QC Inter-batch Precision (%CV)	6 runs, n = 3, 5 levels	≤ 20% (≤ 25% at LLOQ & ULOQ)	4.9 to 10.6%
QC Inter-batch Accuracy (%Diff)	6 runs, n = 3, 5 levels	≤ 20% (≤ 25% at LLOQ & ULOQ)	2.0 to 12.0%
Selectivity (10 healthy individual lots, spiked 250 and 45000 ng/mL)	n = 1, 3 levels	≥ 80% blank lots BQL ≥ 70% spiked lots with 100±25% recovery	90% blank lots BQL 80% spiked lots with 100±25% recovery
Selectivity (10 solid tumor lots, spiked 250 and 45000 ng/mL)	n = 1, 3 levels	≥ 80% blank lots BQL ≥ 70% spiked lots with 100±25% recovery	100% blank lots BQL 90% spiked lots with 100±25% recovery
Dilutional Linearity and Hook effect	500 µg/mL sample was undiluted and diluted 10, 50, 250, 1000 –fold before assay. n = 3, 5 levels	The back-calculated concentrations after applying the dilution factors are within 100 ± 20% of the nominal value and %CV ≤ 20%. Samples above the quantifiable limit shown AQL to be considered no Hook effect.	All dilutions to the assay range were acceptable. No hook effect was detected.
Benchtop Stability in Human Serum	n = 3, 2 levels	The back-calculated conc. within 100 ± 20% of the nominal value to be considered stable.	Stable at benchtop for at least 7 hours.
Freeze/thaw Stability in Human Serum	n = 3, 2 levels	The back-calculated conc. within 100 ± 20% of the nominal value to be considered stable.	Stable for at least 3 cycles at -70°C.

Table 2. Back-calculated conc. of calibration standards

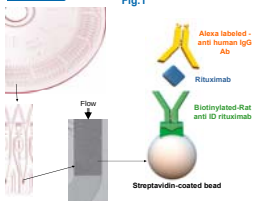
Test#	Concentration (ng/mL)						
	90	255	750	2250	6600	20000	60000
1	87.3	258	744	2250	7060	18300	59900
	85.2	260	765	2210	6420	20900	64100
2	92.4	254	760	2240	6430	20000	62700
	87.7	255	742	2260	6740	20200	57200
3	72.8	244	739	2210	6590	19900	59900
	95.0	276	810	2210	6710	19600	61800
4	86.3	260	727	2210	6700	20000	58400
	107	256	748	2240	6570	20700	59500
5	72.7	275	770	2140	6830	19800	57200
	71.7	266	778	2200	6560	20200	62700
6	97.5	255	719	2120	6830	22600	50800
	108	258	706	2250	7320	18100	71200
Mean	88.7	260	751	2210	6730	20000	60500
S.D.	12.4	8.96	28.3	43.2	289	1160	4870
%CV	14.0	3.4	3.8	2.0	3.8	5.8	8.0
%Diff	-1.4	2.0	0.1	-1.8	2.0	0.0	0.8
n	12	12	12	12	12	12	12

Table 3. Back-calculated conc. of validation samples

Test#	Concentration (ng/mL)				
	LLOQ	Low	Mid	High	ULOQ
	90	250	3500	45000	60000
1	106	283	3850	50700	73400
	89.2	272	3730	56500	73300
	102	304	3740	52700	67000
	108	264	3790	50400	67600
	116	280	3800	53200	68000
	102	264	3620	48900	76900
2	101	266	3500	48800	62700
	91.2	266	3290	46500	59300
	93.7	264	3410	46400	63100
3	113	260	3440	49000	61900
	113	289	3410	47700	61900
	103	248	3360	48100	62700
4	92.9	258	3560	46100	58400
	95.0	261	3660	49800	69200
	95.6	245	3840	48100	64200
5	121	261	3490	48300	60500
	97.9	272	3440	48400	69000
	102	260	3570	48100	71900
6	84.9	243	3460	61900	82200
	79.6	236	3340	50400	79100
	103	278	3670	57400	55700
Mean	100	265	3570	50400	67000
S.D.	10.3	16.2	175	4000	7090
%CV	10.3	6.1	4.9	7.9	10.6
%Diff	11.1	6.0	2.0	12.0	11.7
n	21	21	21	21	21

EXPERIMENTAL

Fig.1



Gyrolab Bioaffy CD contains streptavidin-bead packed microstructures. Rituximab is captured on the CD200 by a biotinylated rat anti-idiotypic monoclonal antibody against rituximab and detected by an Alexa-labeled anti-human IgG antibody (Fig. 1). The rituximab-capture antibody was biotinylated using Sulfo-NHS-LC-Biotin kit and the detection antibody was labeled using Alexa Fluor 647 Mono-clonal Antibody Labeling Kit. The concentrations of the capture reagent and the detection reagent and the buffer system were optimized to provide a high signal to background ratio for the entire method validation study.

Standards and validation samples were prepared in pooled human serum. All samples were diluted 15 fold in dilution buffer before mixed with Rexxip H-max at 1:1 ratio. The overall minimum required dilution was MRD30.

Data acquisition at 1% PMT level was used throughout the validation. Regression was performed by Gyrolab Evaluator (v 3.1.5.137, Gyros AB, Sweden) with 5-parameter logistic fit without the blank and with weighting for response. The validation acceptance criteria and the statistical parameters were determined according to QPS' SOPs for Validation of Ligand Binding Assays and Sample Analysis in Binding Assays.

In comparison, ELISA assay was conducted using the rat anti-idiotypic monoclonal antibody against rituximab as the coating reagent. The assay plate was incubated with HRP-labeled goat anti-human IgG antibody before adding HRP substrate TMB.

Fig. 2. A standard curve for rituximab

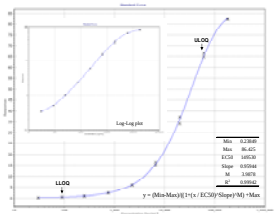
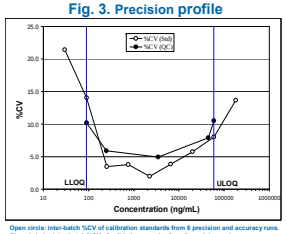
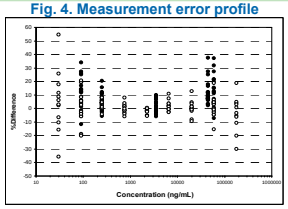


Fig. 3. Precision profile



Open circle: inter-batch %CV of calibration standards from 6 precision and accuracy runs.
Closed circle: inter-batch %CV of validation samples from 6 precision and accuracy runs.

Fig. 4. Measurement error profile



Open circle: %difference of each replicate of calibration standards from 6 precision and accuracy runs. Closed circle: %difference of each replicate of validation samples from 6 precision and accuracy runs.

Fig. 5. Dilutional linearity

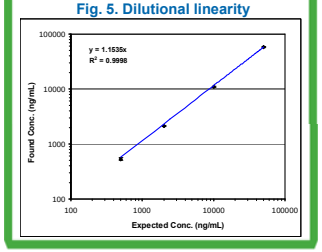
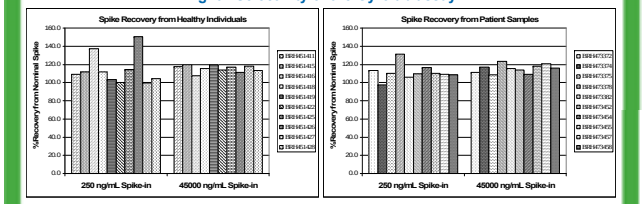


Fig. 6. Selectivity of the Gyrolab assay



RESULTS

The validation of the Gyrolab rituximab assay is summarized in Table 1. Fig. 2 shows typical calibration standards of rituximab in human serum and a 5-parameter logistic fit from 30 to 180000 ng/mL. Table 2 lists the back-calculated standard concentrations and inter-batch statistical analysis.

Precision and accuracy of the method were evaluated by analyzing validation samples at 5 different concentrations. The intra-batch precision and accuracy were determined by analyzing 6 replicates of each validation sample in the same run and the results were acceptable (Table 1). The inter-batch precision and accuracy of the assay were determined by analyzing validation samples from 3 consecutive validation runs and 3 additional runs and the results were acceptable (Table 3). Setting LLOQ of the assay at 90 ng/mL and ULOQ at 60,000 ng/mL was appropriate since inter-batch %CV values at 90 and 60,000 ng/mL were around 10% (Fig. 3). A tighter limit of acceptable precision allows the overall assay imprecision and inaccuracy observed during pre-study or in-study method validation. Measurement error profile (Fig. 4) shows inaccuracy increased at 90 ng/mL and 60,000 ng/mL. In addition, S/B ratio at 90 ng/mL was 1.3 – 2.5 indicating LLOQ should not be lower than 90 ng/mL.

Stability of rituximab in human serum was evaluated at low and high QC concentrations. The results indicate that rituximab in human serum is stable at room temperature for at least 7 hrs and stable for 3 freeze/thaw cycles between -70°C and room temperature.

Dilutional linearity test showed that there was no Hook effect. The found concentration had a linear relationship with the expected concentration after dilution on a log-log scale with R^2 of 0.9998 (Fig. 5).

Human serum lots of 10 healthy individuals and 10 solid tumor patients were analyzed before and after spiked with rituximab at low and high QC levels. Eighty percent of the normal human lots and 90% of the solid tumor patient lots had a recovery between 75-125%, which is acceptable (Fig. 6). All 20 blank serum lots were BQL except one, which was not reportable due to imprecision between the 2 measurements.

Preliminary results of an ELISA sandwich assay for rituximab in human serum using the same antibodies and similar experimental conditions gave a dynamic range of 100 – 5000 ng/mL suggesting that the Gyrolab platform has a greater assay range but may not provide improved sensitivity over traditional immunoassays.

CONCLUSIONS

A Gyrolab method has been validated for the quantitation of rituximab in human serum from 90 to 60,000 ng/mL. With a validated dilution factor of 1000, this assay is suitable for measuring rituximab in human serum from 90 ng/mL to 60 mg/mL. The Gyrolab assay was proved to be accurate and selective, with a comparable sensitivity as the ELISA method, but provides a significantly wider assay dynamic range for determination of rituximab in human serum.