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Celine Desvignes, David Ternant, Thierry Lecomte, Astrid Lièvre, Marc Ohresser, Romain Chautard, William Raoul, Gilles Paintaud

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A robust enzyme-linked immunosorbent assay to measure serum ramucirumab concentrations

Abstract:

Introduction: Ramucirumab, an anti-VEGFR2 monoclonal antibody, has been approved for the treatment of metastatic gastric and colorectal cancer. An assay measuring ramucirumab serum concentrations was needed to investigate its pharmacokinetics and concentration-response relationship.

Results: An ELISA was developed and validated according to the international guidelines for ligand binding assays. Ramucirumab calibration standards ranged from 0.125 to 40 mg/L. Low, Middle and High Quality Controls were spiked at 0.2, 4 and 8 mg/L, respectively. The limits of quantification were established to be 0.125 and 10 mg/L for LLOQ and ULOQ, respectively. No cross-reactivity with anti-VEGF or anti-EGFR was detected.

Conclusion: This in-house-developed ELISA is sensitive, accurate, reproducible, and suitable for pharmacokinetic studies of ramucirumab.

Keywords: Monoclonal antibody, ramucirumab, enzyme-linked immunosorbent assay, Pharmacokinetics, Therapeutic drug monitoring.

Introduction

During the last two decades, the therapeutic arsenal against advanced malignancies was strengthened by the use of anti-angiogenic monoclonal antibodies (mAbs) or fusion proteins in addition to standard chemotherapy, as bevacizumab, aflibercept and later ramucirumab [1-4]. Ramucirumab was approved in gastric cancer [5, 6], aggressive non-small cell lung cancer [7], metastatic colorectal cancer [8] and hepatocellular carcinoma [9].

Ramucirumab, developed as IMC-1121B (ImClone Systems) and marketed as Cyramza® (LY3009806, Lilly), is a 147 kDa fully human immunoglobulin G1 (IgG1κ) mAb that targets extracellular domain of vascular endothelial growth factor receptor 2 (VEGFR-2). VEGFR-2 ligands include VEGF-A, formerly known as vascular permeability factor (VPF) and one of the most potent anti-angiogenic factors discovered and characterized in the 80s [10, 11]. The intended target of ramucirumab is VEGFR-2 expressed on endothelial cells surface and its expected effect is an inhibition of the VEGF-A/VEGFR-2 axis involved in pathological angiogenesis. However, it is worth noting that the concept of direct effects of anti-angiogenic therapies on tumor cells themselves was only recently highlighted and reviewed by Simon *et al*. [12].

The administration of approved doses of ramucirumab leads to a large pharmacokinetic variability, which is influenced by several individual sources of variability as body weight [13]. In addition, other factors, as serum albumin levels or tumor burden may influence ramucirumab pharmacokinetics [14]. This variability is relevant, since ramucirumab concentrations lead to better clinical response. Indeed, higher ramucirumab exposure was

associated with longer overall and progression-free survivals [15]. Thus, the interpretation of clinical efficacy of ramucirumab might benefit from accurately defined pharmacokinetic parameters [16, 17].

The present work focused on the development of an Enzyme Linked Immunosorbent Assay (ELISA) that measures accurately and reproductively ramucirumab concentrations for a large range of concentrations. A high level of sensitivity (*i.e.* very low LLOQ - Low Limit Of Quantification) is also required for future pharmacokinetic studies. This ELISA technique was developed under both European Medicines Agency (EMA) and US Food and Drug Administration (FDA) guidelines.

Material and Methods

Reagents

96-well plates were purchased from ThermoFisher Scientific (Nunc plates, Elancourt, France). Phosphate-buffered saline (PBS) was provided by Immuno Concepts (Sacramento, CA). Tween 20 was purchased from Biosolve (Dieuze, France), Bovine serum albumin (BSA), a polyvalent peroxidase-conjugated goat anti-IgG specific for Fc fragment (HRP-anti-human IgG; lot number 067M4885V and 127M4800V), and FAST o-phenylenediamine dihydrochloride tablets were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). A human recombinant protein of 82.5 kDa, soluble form of VEGFR-2 from Clinisciences (Nanterre, France), was used as the capture antigen. A stock solution of 10 mg/ml of ramucirumab (Cyramza®) was purchased from Eli-Lilly (Indianapolis, IN, USA) through [redacted].

Reagent preparation

The calibration standards and quality controls (QCs) prepared from a 10 mg/ml ramucirumab stock solution were aliquoted and stored at +4°C until use or at -20°C for long term. Concentrations of standard calibrators were the following 0.125, 0.25, 0.625, 2.5, 12.5, 25 and 40 mg/l and the concentrations of QCs were 0.2, 4 and 8 mg/l. A 1:100 dilution in 1% (w/v) PBS-BSA was prepared for all calibration standards, QCs and samples.

Assay procedure

Assay validation followed the procedure previously described [18-20]. Notably, coating was performed overnight at +4°C, by incubating 100 µL/well of 2 mg/l sVEGFR-2 in carbonate-bicarbonate buffer. Calibration standards, QCs and samples were incubated at 37°C during 1 h under agitation. One hundred microliters per well of HRP conjugated-anti IgG antibody (1:8000 dilution in 1% PBS-BSA) was used. The final absorbance was read at 490 and 630 nm (for correction) on an ELx808 ELISA plate reader (BioTek, VT, USA). Non-specific background was determined with carbonate-bicarbonate buffer only. Serum ramucirumab concentrations were calculated with a 4-parameter logistic calibration curve (Response = (Min-Max)/(1 + (Concentration/Inflection point)^Slope + Max) implemented in Gen5 software (BioTek, VT, USA).

Precision, accuracy, limit of quantification

The precision and accuracy were assessed as previously described [18-20]. Briefly, intra- and inter-day precisions and accuracies were defined as coefficient of variation (% CV = Standard deviation (SD)/mean) and absolute bias (% RE, where relative error (RE) = [measured value – actual value]/actual value), respectively. The limit of detection (LOD) was determined as 3 times the mean SD of the concentration of 11 blank samples. The LLOQ corresponded to the

lowest calibration standard. The upper limit of quantification (ULOQ) was defined as the highest amount of ramucirumab that could be quantified with SD and RE < 20%. The low QC value was within three times the LLOQ, the mid QC value was the geometric mean of the curve and the high QC was spiked at 80% of ULOQ.

Matrix effect and other parameters

To assess the matrix effect, we compared the precision and accuracy when QCs were prepared in PBS-BSA or normal human serum (n = 4) against calibrators prepared in PBS-BSA. The recovery percentage was calculated as (recovered concentration / spiked concentration) x 100. These results were compared using a non-parametric Mann-Whitney test. Dilution linearity, prozone, parallelism and stability were determined in agreement with our published methods without any particular modification [18-22].

Selectivity & specificity

Selectivity was assessed using serum from healthy controls (n=18) and serum from healthy controls spiked with rheumatoid factor (55 UI/ml or 30 UI/ml or 15 UI/ml) (n=6 for each). Analytical interference with other mAbs used in colorectal cancer patients was evaluated using serum samples from patients treated with anti-VEGF-A bevacizumab (n=9), anti-EGFR (Epidermal Growth Factor Receptor) cetuximab (n=9) or VEGF-targeting fusion protein aflibercept (n=9). Identical samples (except for aflibercept) were spiked with ramucirumab at a final concentration equal to 4 mg/L. Normal human serum was spiked with aflibercept and ramucirumab at a ratio equal to 1:1 or 1:5. The impact of hemolyzed and lipemic samples spiked or not with ramucirumab (= 4 mg/L) was also tested. In all cases, to consider there is no cross-reactivity, the concentrations of these samples had to be below the LLOQ of

ramucirumab, ie 0.125 mg/l.

Detection of unbound ramucirumab

Ramucirumab is a bivalent molecule and is able to bind two molecules of VEGFR2. When ramucirumab is binding to VEGFR2, it blocks activity and prevents fixation of VEGFA, VEGF-C and VEGF-D. During ramucirumab treatment, three different species may be present in the serum: free ramucirumab and ramucirumab bound to either 1 or 2 VEGFR2 molecules. Among these three species, biologically active species are free ramucirumab and ramucirumab monovalently bound to a molecule of VEGR2. Therefore, we tested our in-house ELISA by mixing serum samples containing ramucirumab (4 mg/L) with increasing ramucirumab:sVEGFR2 ratios of: 0:25, 1:0, 1:1, 1:5 and 1:25. We also tested ramucirumab (4 mg/L) mixed with increasing ramucirumab:VEGF-A ratios of: 0:100, 1:0, 1:1, 1:10 and 1:100. All mixes were incubated for 30 minutes at 37°C. Each ratio was assayed on four occasions on the same day.

Data interpretation: acceptance and rejection criterion

Measurements were accepted if differences between duplicates were lower than 20% for more than 75% of the calibrators. Calibrators and QCs performances were validated when the intra- and inter-day precision and accuracy were between ±20% (± 25% at LLOQ and ULOQ) and the total error [|CV| + |RE|] was between ±30% (±40% at the LLOQ and ULOQ). In order to be validated, the precision of each back-calculated concentration, had to be within ± 20% (dilution linearity) and ± 30% (parallelism) of the target concentration [23-29].

Patients and blood samples

Concentrations were measured in serum samples collected from two patients treated for advanced colorectal cancer by 8 mg/kg ramucirumab infusions every 2 weeks. These samples are part of a declared biological collection (N° [redacted]) and were in conformity with the National Data Information and Freedom Commission (CNIL). Trough (n=9) and peak (n=9) samples were collected just before infusion and 2 hours after the end of the infusion, respectively. This study was an ancillary study performed after the constitution of the declared biological collection.

Results

Enzyme-Linked Immunosorbent assay

The chosen coating concentration led to reproducible standard calibration. A mean curve from six individual calibration curves on 6 different days is given on Figure 1. This latter was generated using ramucirumab concentrations from 0 to 40 mg/l. The limits of quantification (LOD, LLOQ and ULOQ) (table 1) of the assay are within the range of our previously published ELISA methods [18-20].

Precision & accuracy of the assay

Intra-day and inter-day reproducibility, precision and accuracy of calibration standards and QCs were within -/+ 15% of the nominal value (Table 2). The total error was below 30% for all calibration standards.

Matrix effect and other parameters

Mean concentrations (CV, bias) of low (0.2 mg/l), medium (4 mg/l), and high (8 mg/l) QCs prepared in PBS-BSA were 0.19 mg/l (3.63%, -3.45%), 3.92 mg/l (5.93%, -2.09%), and 8.12 mg/l

(4.77%, 1.52%), respectively. In human serum, mean concentration of QCs were 0.13 mg/l (7.93%, -36.07%), 3.23 mg/l (3.92%, -19.30%), and 6.36 mg/l (3.63%, -20.56%), respectively. Concentrations of low QC prepared in human serum were significantly lower than those measured in PBS-BSA (Mann–Whitney test, $P < 0.05$). Therefore, the calibration standards and QCs were prepared in the same matrix as the samples as previously recommended by our team [19].

Dilution linearity & prozone

A serum sample was spiked with ramucirumab at a concentration of 200 mg/l and was diluted 1:10, 1:20, 1:50, 1:100, 1:500, 1:1000, 1:2000 and 1:5000 in normal human serum ± 20%, which is in accordance to EMA guideline (Figure 2) [26]. Therefore, samples can be diluted up to 1,000-fold. No prozone effect was observed until a ramucirumab concentration of 1,000 mg/l (Figure 3). The dynamic range of the assay is therefore between 0.125 – 10 mg/l., i.e. between the LLOQ and ULOQ of the assay.

Parallelism

Parallelism was assessed using different dilutions (1:25 to 1:1000) with a panel of three patient serum samples (n = 1 for each dilution). Patient's serum concentrations were respectively 192.56, 153.25 and 145.10 mg/l. All measured concentrations were within the target concentration of ±30% as recommended by guidelines on bioanalytical method validation [24, 26-28, 30], indicating that samples can be diluted up to 1,000-fold (Figure 4).

Selectivity

Selectivity of the assay was determined by using sera from healthy donors, from normal

human serum spiked with rheumatoid factor and from patients treated with bevacizumab, cetuximab or aflibercept (Figure 5). All measured concentrations were below LLOQ. The same samples (except for aflibercept) were spiked with ramucirumab and all concentrations were equal to 4 mg/L (±20%). For normal human serum spiked with aflibercept and ramucirumab at a ratio equal to 1:1 or 1:5, measured concentrations were also equal to 4 mg/L (±20%). The same results were obtained with hemolyzed and lipemic samples spiked or not with ramucirumab. These results revealed no analytical interference.

Ramucirumab concentrations after addition of sVEGFR2 or VEGF-A

Mean ramucirumab concentrations (SD) were 0.1 (0.01), 3.6 (0.28), 1.7 (0.19), 0.3 (0.01) and 0.2 (0.00) mg/L for 0:25 (negative control), 1:0 (positive control), 1:1, 1:5 and 1:25 ramucirumab to sVEGFR2 respectively.

Mean ramucirumab concentrations (SD) were <LLOQ (not calculated), 4.1 (0.27), 4.3 (0.24), 3.4 (0.33) and 2.0 (0.14) mg/L for 0:100 (negative control), 1:0 (positive control), 1:1, 1:10 and 1:100 ramucirumab to VEGF-A respectively.

Stability

The three concentration levels maintained at room temperature, +4°C and -20°C were all stable up to 14 days, 28 days and 180 days, respectively (Supplemental figure 1). Samples were stable up to 7 freeze-thaw cycles (Supplemental figure 2). In all cases, the relative error was below 20%.

Patients samples concentrations

Ramucirumab serum concentrations were measured in 2 patients treated for metastatic

colorectal cancer and were both above LLOQ except for baseline concentration (0 mg/l). Median (range) trough and peak concentrations during treatment were respectively 26.6 (9.3-56.9) mg/l and 156.0 (93.3-243.0) mg/l.

Discussion

To our knowledge, this is not the first ELISA technique that was published to measure ramucirumab concentrations. Three other techniques have been published so far, but present considerable differences with our technique, including limits of quantification. The advantage of our in-house ELISA is characterized by a lower LLOQ (0.125 mg/l *vs* 0.5 mg/l [31] and 2.5 mg/l [32]) and a higher ULOQ (10 mg/l *vs* 0.2 mg/l) [33]. Furthermore, we report here more detailed information about intra- and inter-run variabilities, selectivity, interferences, and dilution-induced inaccuracy or imprecision on our development and validation method.

This high sensitivity of our assay allowed measuring very low concentrations. To our knowledge, the pharmacokinetics of ramucirumab was investigated in only one study using population PK modelling. We therefore believe that this pharmacokinetic, notably the elimination decay still has to be thoroughly investigated. This can only be done using an assay able to measure low concentrations that are observed after single doses or when treatment is discontinued. These measurements are needed for an accurate estimation of pharmacokinetic parameters. A sensible assay is even more mandatory if a nonlinear target-mediated elimination has to be studied. This level of sensitivity may not be useful for clinical trials, but is mandatory for pharmacokinetic studies a feature needed to estimate pharmacokinetic parameters in future studies.

An exposure-response relationship has been observed from randomized trials of ramucirumab

in lung cancer, gastric cancer and colorectal cancer [15, 34, 35]. Therefore, a fully validated assay is required to thoroughly investigate ramucirumab pharmacokinetics (dose-concentration relationship) and concentration-effect relationship, either in clinical trials or in routine clinical use of this drug. Moreover, the understanding of the interindividual variability of ramucirumab clinical response has to be made with respect to a sound assessment of its concentration-effect relationship, in order to determine the optimal dosing regimen which maximizes patients survival, while minimizing the risk of adverse events [36, 37].

In the context of daily clinical practice with samples from our registered biological collection, concentrations ranged from 26.6 mg/l to 156 mg/l), corresponding to through and peak concentrations, respectively. This range of quantification is in accordance with the values reported in clinical studies, i.e. 49.5 [6.3-228 mg/l] to 74.4 mg/l [13.8- 234 mg/l]) [38] or 49.7 to 81.1 mg/L for Cohn *et al*. [34] which confirms the relevance of measurements provided by our ELISA technique.

To eliminate matrix effect for low QC concentration in human serum, the calibration standards and QCs were prepared in human pooled serum. In the present study, we observed no analytical interference with bevacizumab, cetuximab or aflibercept. This result reveals that our ELISA may be also useful to measure serum ramucirumab concentration in patients concomitantly or previously treated with an anti-VEGF or an anti-EGFR biopharmaceutical. Moreover, since our ELISA is based on the capture of ramucirumab by sVEGFR2, i.e. ramucirumab unbound or bound to one sVEGFR2. In order to confirm this hypothesis, we tested ramucirumab mixed with increasing concentrations of sVEGFR2. Measured ramucirumab concentration decreased when the amount of sVEGFR2 increased. When

ramucirumab and sVEGFR2 were equimolar, measured ramucirumab concentration decreased to nearly 50% of that obtained in the absence of sVEGFR2. Our test may reflect unbound ramucirumab, since almost total signal inhibition occurs for molecular ratio above 1:5. The influence of VEGFA was also tested by measuring concentrations in normal human serum spiked with ramucirumab with or without VEGFA. The ramucirumab concentration is not affected by for equimolar ratio and decreased by half for ratio 1:100. The difference between the two targets is explained by a higher affinity of ramucirumab for VEGR2. These two experiments revealed that our concentration measurement reflects the unbound fraction of ramucirumab.

Conclusion

Overall, we developed and validated an immunoassay that is suitable for measuring serum concentrations of ramucirumab in treated patients according to guideline criteria. Our assay is sensitive, reproducible and accurate and thus can be used in both pharmacokinetic studies and future therapeutic drug monitoring of ramucirumab.

Future perspective

In the [redacted]-BLINDED-[redacted] clinical trial [redacted]-BLINDED- [redacted] the measure of ramucirumab concentrations, will allow better understanding of the relationship between ramucirumab concentrations and clinical outcomes in advanced gastric cancer in elderly patients, which has never been evaluated before.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Background

- Pharmacokinetic and concentration-response studies of ramucirumab, a full human anti-VEGFR-2 monoclonal antibody, require a validated and robust analytical method.

Experimental section

- An ELISA using sVEGFR-2 coated microtiter plates was developed.
- The assay was validated according to International guidelines (EMA & FDA).

Results

- The assay enables the reproducible measurement of ramucirumab concentrations in human serum.
- The range of quantification is 0.125 to 10 mg/l.

Conclusion

- The assay is sensitive and accurate, and suitable for both pharmacokinetic studies and, potentially, therapeutic drug monitoring of ramucirumab.

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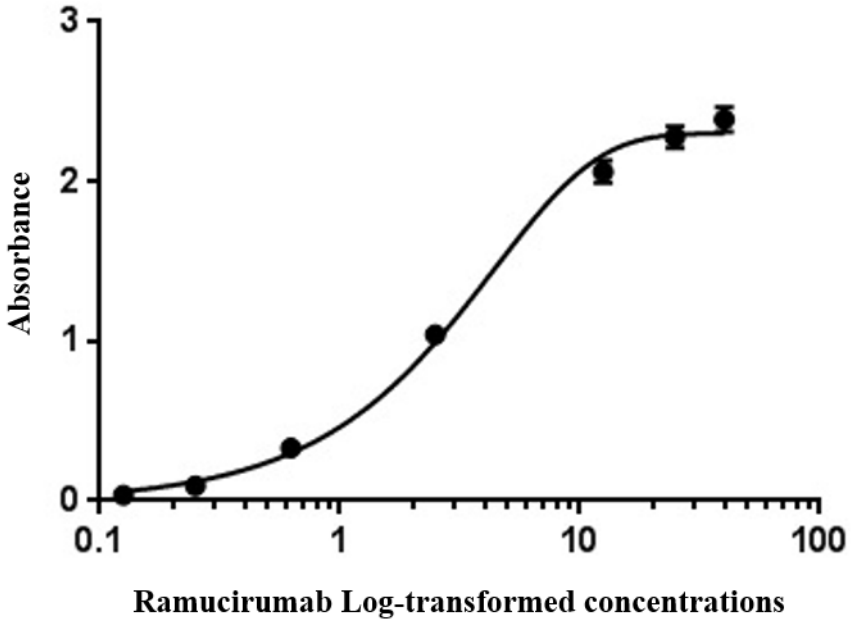
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Table 1. Limits of detection and quantification of the assay.

Parameter	Value (mg/l)
LOD	0.02
LLOQ	0.125
ULOQ	10.00

Table 2. Intra and interday precision, accuracy and total error of calibration standards and quality controls.

Spiked concentration	Measured concentration (mg/l)		Precision (CV%)		Accuracy (%RE)		Total error (%CV + %RE)	
	Intraday *(n=9)*	Interday *(n=12)*	Intraday *(n=9)*	Interday *(n=12)*	Intraday *(n=9)*	Interday *(n=12)*	Intraday *(n=9)*	Interday *(n=12)*
Calibration standards (mg/l)								
40	40.50	42.59	6.97	13.60	1.25	6.49	8.22	20.08
25	24.04	26.65	7.18	7.88	3.83	2.60	11.01	10.48
12.5	12.96	12.42	2.67	3.25	3.67	0.61	6.35	3.87
2.5	2.45	2.51	1.13	2.26	1.86	0.45	3.00	2.71
0.625	0.67	0.65	2.21	5.04	7.71	4.03	9.93	9.07
0.25	0.22	0.22	3.35	5.67	10.46	13.06	13.81	18.73
0.125	0.11	0.12	6.34	10.44	10.37	5.90	16.72	16.34
QCs (mg/l)								
8 *(n=12)*	7.58	7.80	7.92	8.96	5.29	2.49	13.21	11.45
4 *(n=12)*	3.53	3.75	6.02	8.31	11.79	6.35	17.81	14.66
0.2 *(n=12)*	0.20	0.22	3.48	5.57	1.40	10.63	4.87	18.20



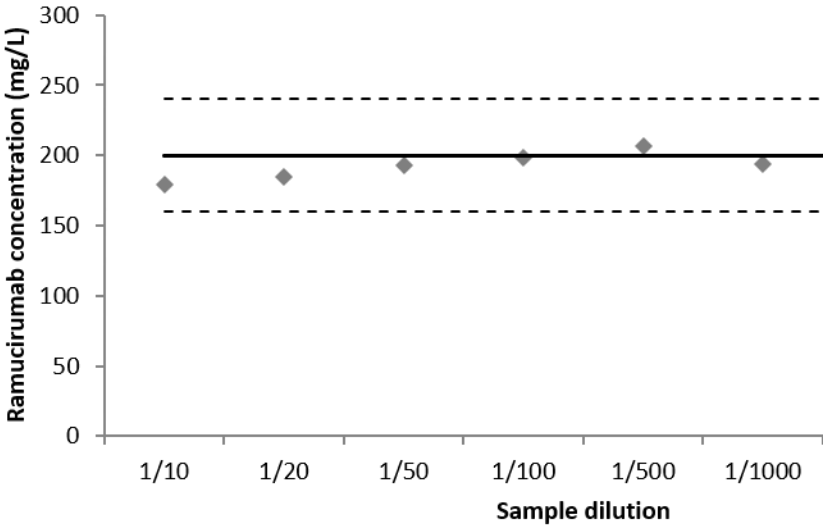
425

426

427 **Figure 1. Mean standard curve of ramucirumab ELISA.**

428 Six curves were obtained on 6 different days. Eight calibration standards were prepared in
429 normal human serum in a wide range of concentrations (0 to 40 mg/l). Results were used to
430 assess the four-parametric logistic standard curve. The error bars represent standard
431 deviations.

432



433

434 **Figure 2. Dilution linearity of ramucirumab ELISA.**

Samples containing 200 mg/l of ramucirumab were diluted up to 1,000 folds and analyzed in duplicate. Target concentration is represented as solid line and ± 20% of target concentrations are displayed as dashed lines.

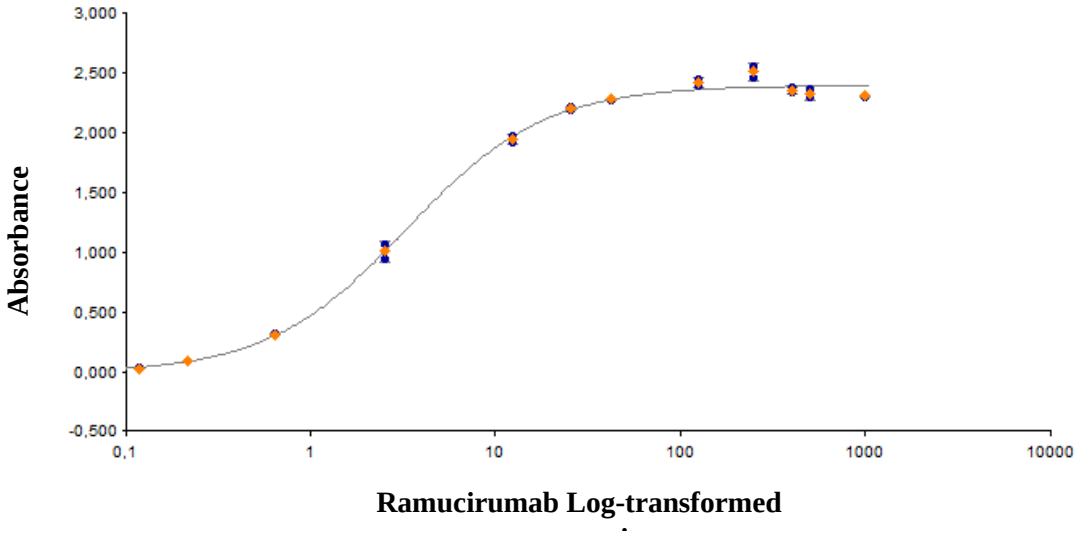


Figure 3. Research of a prozone with ramucirumab ELISA. A four parametric curve was produced using ramucirumab concentrations up to 1,000 mg/l.

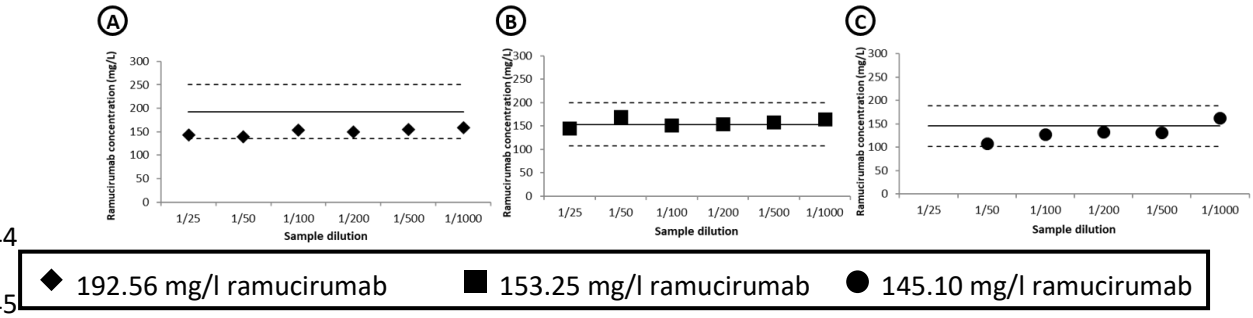


Figure 4. Parallelism of ramucirumab ELISA. Three different patient sera samples containing 192.56 mg/l **(A)**, 153.25 mg/l **(B)** or 145.10 mg/l **(C)** were diluted up to 1,000 fold. Target concentration is represented as solid line and ± 30% are figured with dashed lines.

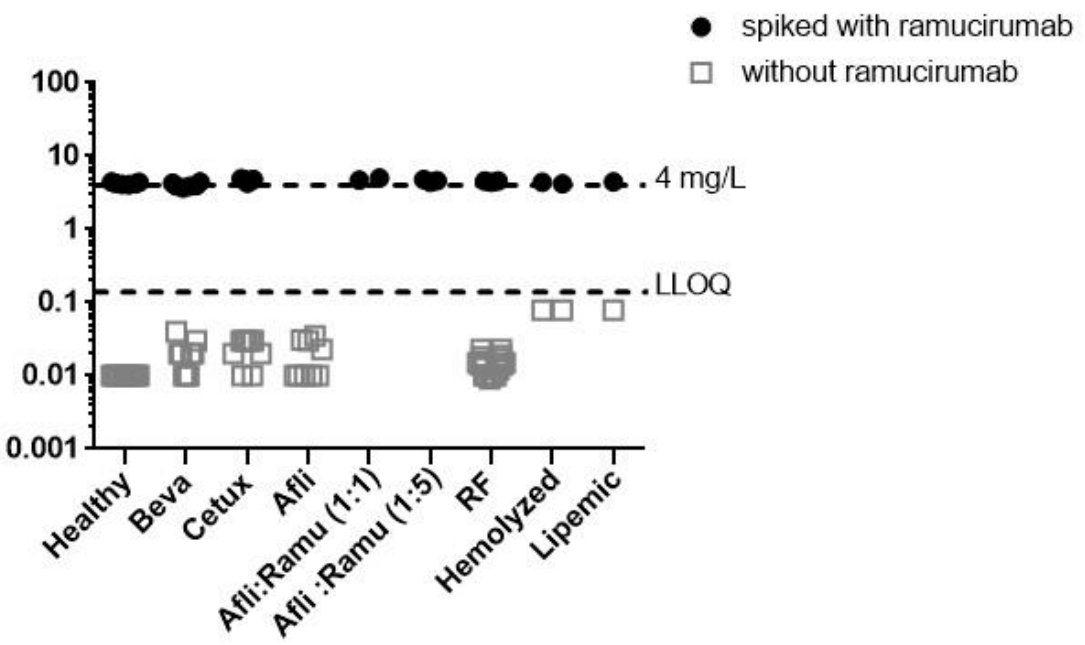
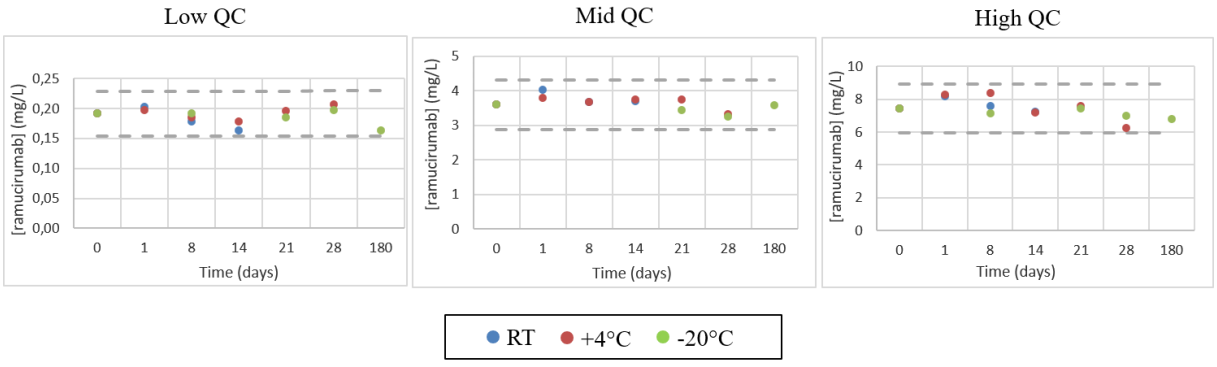
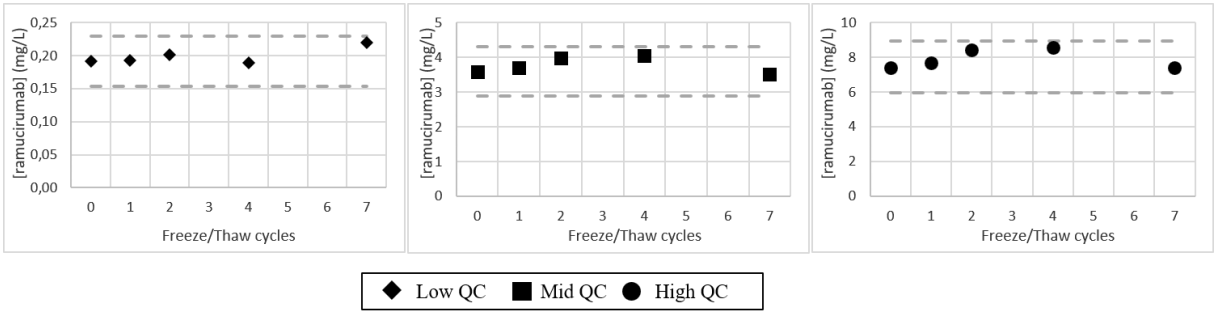


Figure 5. Assay selectivity. Selectivity was tested with sera from healthy donors, sera from patients treated with bevacizumab, cetuximab, normal human serum spiked with aflibercept or rheumatoid factor (RF). Ramucirumab concentration was measured in all samples spiked or not with ramucirumab. The same tests were done in hemolyzed and lipemic samples. (LLOQ: Lower limit of quantification).



Supplemental figure 1. Stability of the samples according to storage temperature. Quality controls were stored at different conditions for various durations. Ramucirumab

concentration was then measured. Dashed lines represent ±20% of the concentration of the corresponding QC.



Supplemental figure 2. Stability of the samples according to the number of freeze/thaw cycles. Samples were frozen at -20°C and thawed at room temperature. The freeze/thaw cycle was repeated up to 7 times and ramucirumab concentrations was then measured. Dashed lines represent ±20% of the concentration of the corresponding QC.