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Simultaneous quantification of rituximab and eculizumab in human plasma by liquid chromatography-tandem mass spectrometry and comparison with rituximab ELISA kits

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Abbreviations

ACN, acetonitrile

ADA, anti-drug antibodies

COFRAC, Comité Français d'Accréditation

C5, C5 fraction of complement

ECU, eculizumab

ELISA, enzyme-linked immunosorbent assay

ESI, electrospray ionization

FDA, Food and Drug Administration

IgG, immunoglobulin G

IQC, internal quality controls

LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry

LLOQ, lower limit of quantification

mAbs, therapeutic monoclonal antibodies

MeOH, methanol

MRM, multiple-reaction monitoring

nSMOL, nano-surface and molecular orientation limited proteolysis

PBS, phosphate-buffered saline,

Q-TOF, quadrupole time-of-flight

RTX, rituximab

SIL-ADM, stable isotope-labeled adalimumab

TDM, therapeutic drug monitoring

ULOQ, upper limit of quantification

Abstract

Objectives

Specific and sensitive analytical techniques to quantify therapeutic monoclonal antibodies (mAbs) are required for therapeutic drug monitoring. The quantification of mAbs has been historically performed using enzyme-linked immunosorbent assays (ELISAs), for which the limitations in terms of specificity have led to the development of alternative analytical strategies.

Methods

Here, we describe the validation of a liquid chromatography tandem mass-spectrometry (LC-MS/MS) method for the simultaneous quantification of rituximab (RTX – anti-CD20) and eculizumab (ECU – anti-C5). Sample preparation was based on our previously published method, using protein G purification and trypsin digestion. A new specific peptide for RTX, containing an N-terminal pyroglutamine and a trypsin miss-cleavage, enables better sensitivity, while peptide of ECU was chosen thanks to an *in silico* trypsin digestion and the Skyline[®] software. Full-length stable-isotope-labeled adalimumab was added to plasma samples as an internal standard. RTX in 50 human serum samples was quantified by LC-MS/MS and the concentrations obtained compared to those obtained with two commercial ELISA kits (Lisa Tracker[®] and Promonitor[®]).

Results

Calibration curves were linear from 1 to 200 $\mu\text{g}\cdot\text{mL}^{-1}$ for RTX and 5 to 200 $\mu\text{g}\cdot\text{mL}^{-1}$ for ECU, and within-day and between-day accuracy and precision fulfilling Food and Drug Administration validation criteria. Comparison of the LC-MS/MS method with ELISA showed a negligible bias with the Lisa Tracker[®] kit (4%), but

significant bias with the Promonitor[®] assay (mean underestimation of 69% for the Promonitor[®] assay).

Conclusions

This new LC-MS/MS method allows the simultaneous quantification of RTX and ECU in human samples and could be used for TDM.

Highlights:

- An accurate and precise LC-MS/MS method to simultaneously quantify rituximab and eculizumab in human samples was validated.
- Bias between LC-SM/SM and ELISAs for rituximab quantification was negligible with the Lisa Tracker[®] kit, but 69% with the Promonitor[®] kit.
- This multiplex LC-SM/SM method opens the field of mAbs therapeutic drug monitoring.

Keywords:

Rituximab, eculizumab, liquid-chromatography tandem mass spectrometry, therapeutic drug monitoring, ELISA.

1. Introduction

Over the last 20 years, therapeutic monoclonal antibodies (mAbs) have emerged as one of the major new classes of biologics. The use of mAbs has revolutionized therapeutic strategies for numerous diseases, such as solid cancers and inflammatory immune diseases, and considerably improved the prognosis of patients (1,2). However, the clinical response to mAbs and their pharmacokinetics are highly variable (3), suggesting that therapeutic drug monitoring (TDM) could be a useful tool to personalize the dose for each patient and thus improve the benefit/risk ratio.

In nephrology, several mAbs, such as rituximab (RTX) and eculizumab (ECU), are candidates for TDM. RTX, a chimeric anti-CD20 IgG₁ monoclonal antibody, is indicated notably for primary membranous nephropathy (4,5), whereas ECU, a humanized anti-C5 IgG_{2/4} monoclonal antibody that inhibits the complement system, is approved for the treatment of paroxysmal nocturnal hemoglobinuria and atypical hemolytic uremic syndrome (6,7). These two biologics exhibit high pharmacokinetic variability, which can potentially affect treatment outcomes (8–10). The link concentration-efficacy was especially well demonstrated for RTX in primary membranous nephropathy (8,11). In addition, the financial stakes are high, especially for ECU, which is one of the world's most expensive drugs. TDM of mAbs could thus be a useful tool to reduce costs without modifying treatment efficacy (12,13).

TDM requires an accurate and precise method to quantify mAbs in human samples (8,14–16). Numerous enzyme-linked immunosorbent assay (ELISA)-based methods are available for the quantification of mAbs, notably for RTX and ECU (8,14,17). However, these approaches are time-consuming and may be limited by lack of specificity or systematic bias (18). Moreover, different ELISA methods are not interchangeable (15,16). Thus, we and others have developed liquid

chromatography-tandem mass spectrometry (LC-MS/MS)-based methods to quantify mAbs in human samples (17,19–22). This highly specific analytical approach allows multiplexing for the quantification of several mAbs in a single analysis (19,20,22), which saves instrument time and reagents and may be very useful given the increasing number of available mAbs with different specific targets.

Our previously published method (20) did not integrate ECU quantification and showed a poor limit of quantification for RTX of $5 \mu\text{g}\cdot\text{mL}^{-1}$, which may be insufficient for some patients with low serum RTX concentrations (8). Here, we describe an improved LC-MS/MS method, adapted from our previously published one (20), that allows simultaneous quantification of RTX and ECU. In addition, we compared RTX concentrations determined with this new method with those obtained with two different commercial ELISA kits (Lisa Tracker[®] and Promonitor[®]).

2. Materials and methods

2.1. Multiplex LC-MS/MS method

2.1.1. Chemicals and reagents

RTX (Mabthera[®], Roche, Basel, Switzerland) and ECU (Soliris[®], Alexion, Boston, MA, United States) were obtained from the institutional pharmacy and reconstituted in water to produce stock solutions at concentrations of 10 mg.mL⁻¹. Full-length stable isotope-labeled adalimumab (Arginine ¹³C₆-¹⁵N₄ and Lysine ¹³C₆-¹⁵N₂) (SIL-ADM) was purchased from Promise Advanced Proteomics (Grenoble, France). This internal standard has an estimated purity > 95% and isotope incorporation of arginine ¹³C₆, ¹⁵N₄ and lysine ¹³C₆,¹⁵N₂ > 99%. Sequencing-grade modified trypsin was purchased from Promega (WI, USA). BioXtra urea, ammonium bicarbonate, 30% hydrogen peroxide solution (H₂O₂), and tris (hydroxymethyl) aminomethane (TRIS) Trizma[®] base were purchased from Sigma Aldrich[®] (MI, USA). LC-MS-grade methanol (MeOH), acetonitrile (ACN), and formic acid were purchased from Honeywell, Sigma-Aldrich[®] Co (St Louis, MO, USA), and Carlo Erba[®] Reagents (Val de Reuil, France), respectively. Ammonia solution (25%) was purchased from Merck (Darmstadt, Germany). Ultrapure water (H₂O) with a resistivity ≥ 18.0 MΩ.cm was produced with a Milli-Q Plus[®] system (Millipore, Molsheim, France). Dulbecco's phosphate-buffered saline (PBS) solution and 96-well Pierce™ Protein-G Spin Plates for IgG Screening were purchased from Thermo Scientific[®] (Waltham, MA, United States). Drug-free human plasma from volunteers was provided by the French National Blood Service (Grenoble, France).

2.1.2. Preparation of working solutions, calibration standards, and quality-control samples

Calibration curves and internal quality controls (IQC) were designed according to the therapeutic range of each mAb. Two batches of RTX and ECU plasma solutions were prepared from the stock solutions, the first for the calibration curve ($200 \mu\text{g}\cdot\text{mL}^{-1}$ of each mAb) and the second for the IQC ($150 \mu\text{g}\cdot\text{mL}^{-1}$ of each mAb). Calibrator points containing 0, 5, 10, 40, 80, and $200 \mu\text{g}\cdot\text{mL}^{-1}$ of ECU and 0, 1, 2.5, 5, 10, 40, 80, and $200 \mu\text{g}\cdot\text{mL}^{-1}$ of RTX (i.e. 6 and 8 calibrator points for ECU and RTX, respectively) and 3 IQCs containing 5.6, 45, and $150 \mu\text{g}\cdot\text{mL}^{-1}$ of both mAbs were generated by successive dilution in drug-free plasma.

2.1.3. Sample preparation: protein-G purification and trypsin digestion

The sample preparation was performed as previously described (20), with minor changes. Each point contained 20 μL working solution, 3 μL SIL-ADM, and 80 μL PBS. Following incubation for 1 h at ambient temperature with smooth agitation, protein-G wells containing the samples were washed three times with 500 μL PBS buffer. Then, elution was performed by two applications of 175 μL 50/50 $\text{H}_2\text{O}/\text{ACN}$ + 0.1% formic acid before evaporation. The following steps, consisting of reconstitution, trypsin digestion, and oxidation, were the same as previously described (20).

2.1.4. LC-MS/MS analysis

2.1.4.1. Configuration of two-dimensional liquid chromatography (LC)

The Shimadzu[®] (Kyoto, Japan) LC system used consisted of an LC-20AD quaternary pump (pump A) and two LC-20AD XR quaternary pumps (pumps B+C), equipped with a SIL-20AC XR autosampler and a CTO-20AC column compartment. Online sample (15 μL injected) clean-up was performed with mobile phase A (98/2

H₂O/ACN + 0.1% formic acid) and pump A on a μ -precolumn (C18 PepMap100, 5 μ m, 100 Å, Thermo Scientific®, MA, USA). Chromatographic separation was performed in backflush mode, on an XB C₁₈ analytical column (Phenomenex® Kinetex, 2.6 μ m, 2.1 mm \times 50 mm, Aschaffenburg, Germany) (Supplemental Figure 1). The column temperature was maintained at 60°C. Elution was performed at a flow rate of 0.4 mL min⁻¹ under the following conditions: mobile phase B from pump B (same composition as mobile phase A) and mobile phase C from pump C (H₂O/ACN 20/80% + 0.1% formic acid): 0 to 4 min: 8% C; 4 to 6.3 min: 8 to 60% C; 6.3 to 6.4 min: 60 to 90% C; 6.4 to 6.9 min: 90% C; 6.9 to 7 min: 90 to 8% C, and 7 to 8.6 min: 8% C (Figure 1).

2.1.4.2. Mass-spectrometry conditions

Measurements were performed on an API 5500 QTRAP Tandem Mass Spectrometer (Sciex®, Toronto, Canada) equipped with a Turbo V® ion source, operating in the positive electrospray ionization mode (ESI). Quantification was performed using the MRM mode. The source-dependent parameters were set as follows: ESI voltage, 5500 V; ion source temperature, 500°C; turbo heater gas, 45 psi; nebulizer, 60 psi; curtain gas, 30 psi; and collision gas: medium. Proteotypic peptides were selected after *in silico* trypsin digestion using the online software Peptide Cutter from ExPASy® (Swiss Institute of Bioinformatics, Lausanne, Switzerland) and their MRM setting was optimized using version 4.1 Skyline® software (MacCoss Lab Software, Washington, USA). Analyte peak areas were integrated using Analyst software (version 1.6.3) (Table 1).

2.1.5. Method validation

The method was validated according to the Food and Drug Administration (FDA) Guidelines for Industrial Bioanalytical Method Validation and the analytical technique guide from the French Committee of Accreditation (COFRAC) (23,24). Acceptance criteria to validate our improved LC-MS/MS method according to the FDA guidelines are summarized in Supplemental Table 1.

Briefly, within- and between-day accuracy (bias) and precision were examined by performing replicate analyses ($n = 6$) for all IQC standards. Between-day accuracy and precision were also assessed for each calibrator points. Sample test dilution, carry-over interference, stability and selectivity were performed as described in Supplemental Table 1. The upper limit of quantitation (ULOQ) was fixed at the highest concentration of the calibration curve and validated with the same levels of precision and accuracy criteria as those required for the medium and high levels of the IQC. In addition, residual plasma samples of 2 patients suffering from paroxysmal nocturnal hemoglobinuria and treated with ECU were analyzed using our LC-MS/MS method.

2.2. Comparison of the LC-MS/MS method with the two ELISA kits for the quantification of rituximab in human serum

To compare the LC-MS/MS method with the two ELISA kits for RTX concentration, serum RTX concentrations of 50 samples from primary membranous nephropathy patients (from the study NCT02199145 entitled 'Role of Anti-mouse PLA2R1 ELISA in Membranous Nephropathy') were determined during routine TDM with Lisa Tracker[®] Duo Rituximab (Theradiag, Croissy Beaufourg, France). The

serum samples were then analyzed using our new LC-MS/MS method and the Promonitor[®] RTX 1-DV kit (Grifols Progenika Biopharma, Vizcaya, Spain).

Both ELISA kits were used according to the manufacturer's instructions. The logistical adjustment logarithmic calibration curve was performed using myassays.com for the Promonitor[®] RTX 1-DV kit. The performance characteristics of both ELISA kits are summarized in Supplemental Table 2.

The detection of anti-drug antibodies (ADA) was performed during routine TDM using Lisa Tracker[®] Duo Rituximab (Theradiag, Croissy Beaufourg, France) and was confirmed by the Promonitor[®] anti-RTX kit. Both ADA kits are drug-sensitive bridging tests and would not well detect ADAs in presence of the drug.

2.3. Statistical analysis

Continuous data are expressed as medians (10th-90th percentiles) and categorical variables as numbers (percentages). The concentrations of RTX obtained by LC-MS/MS were compared to those obtained by the ELISA kits using non-parametric Passing and Bablok analysis and Bland and Altman plots (25). Statistical tests were performed using Medcalc 12.5 (MedCalc Software, Ostende, Belgium).

3. Results

3.1. Validation of the LC-MS/MS method

3.1.1. Chromatograms

Representative chromatograms are shown in Figure 2 of a double blank (2a), a blank (2b), a calibration curve point set at 10 $\mu\text{g}\cdot\text{mL}^{-1}$ for ECU and RTX (2c), and samples from patients treated with RTX (2d) or ECU (2e).

The signal measured in the double blank (2a) showed the absence of any interference due to signals from endogenous compounds overlapping with the selected ion transition for the peptide selected for both mAbs and SIL-ADM. The blank (2b) showed the MRM signals measured for the transitions corresponding to unlabeled peptides to be at the level of the background noise in drug-free serum spiked with SIL-ADM. These levels of interference were not significant relative to the level of signal obtained at the LLOQ ($1 \mu\text{g}\cdot\text{mL}^{-1}$ for RTX and $5 \mu\text{g}\cdot\text{mL}^{-1}$ for ECU). Presence of RTX in human samples of patients treated did not alter ECU quantification and conversely, as illustrated in figures 2d and 2e.

3.1.2. Accuracy and precision

Within-day and between-day imprecision and accuracy are presented for the three IQCs in Table 2, while between-day precision and accuracy of each calibration point are indicated in the Supplemental table 3. All calculated concentrations were within acceptance limits ($< 15\%$ for precision and within $\pm 15\%$ of the nominal value for accuracy, except for the LLOQ for which $< 20\%$ is accepted), except for the between-day bias of IQC for RTX which is at 15.2% .

3.1.3. Linearity and limits of quantification

Ratios of the peptide peak areas and SIL-ADM peptide peak areas (y-axis) were plotted against the ratios of mAb and SIL-ADM concentrations (x-axis) and the calibration curve calculated for a $1/x$ -weighted least squares linear regression, with a coefficient of determination (r^2) > 0.99 for both RTX and ECU. The LLOQ for RTX was $1.0 \mu\text{g}\cdot\text{mL}^{-1}$ (bias = 1.3% , CV = 17.0% , $n = 6$) and $5.0 \mu\text{g}\cdot\text{mL}^{-1}$ for ECU (bias = -0.5% , CV = 16.1% , $n = 6$). The ULOQ was $200 \mu\text{g}\cdot\text{mL}^{-1}$ for both analytes (RTX: bias

= -0.9%, CV = 9.9% – ECU: bias = 0.2%, CV = 3.8%, n = 6). These limits comply with FDA and COFRAC guidelines (24).

3.1.4. Selectivity and carry-over interference

Analysis of six blank plasma samples with or without SIL-ADM showed no interference of endogenous compounds from the matrix with RTX and ECU quantification. Carryover interference assays revealed concentrations below the LLOQ for both analytes, with mean carryover effects of 0 and 0.72% for RTX and ECU, respectively. These low contamination rates exclude any intersample contamination.

3.1.5. Stability of prepared samples

The relative bias (relative bias = $\frac{(\text{concentration } J0 - \text{concentration } J24h)}{\text{concentration } J24h}$) was < 15% (n = 6) for both RTX and ECU after storage of the IQC samples in injection vials at 4°C for 24 or 96 hours (Supplemental Table 4).

3.1.6. Sample test dilution

The ¼ dilution of a plasma sample spiked at 500.0 µg.mL⁻¹ of RTX or ECU were was associated with bias of -13.4 and 1.9 %, and CVs of 5.5 and 7.8 % for RTX and ECU (n=6), respectively.

3.1.7. Eculizumab quantification in human plasma

Eculizumab plasma concentrations were determined for two patients at 258.0 and 667.0 µg.mL⁻¹ in samples handled just before infusion (Figure 2e).

3.2. Comparison of the LC-MS/MS method with the ELISA methods for the quantification of RTX in human serum

RTX concentrations determined with the LC-MS/MS method were compared with those obtained with the two commercial ELISA kits. Passing and Bablok regression analysis and Bland-Altman plots for each LC-MS/MS-ELISA pair are shown in Figure 3. Results of the samples with RTX concentrations outside the analytical range for at least one method are presented in Table 3.

3.2.1. LC-MS/MS versus Lisa Tracker[®]

No significant deviation from linearity was shown by the Passing and Bablok regression analysis (Figure 3a) (Custum test, $p = 1.00$, slope = 1.04, $n = 28$). Only one patient (P17) had a notable discrepancy (circled with a dotted line in Figure 3a): $17.2 \mu\text{g.mL}^{-1}$ with our LC-MS/MS method vs $38.5 \mu\text{g.mL}^{-1}$ with the Lisa-Tracker[®] assay. The Bland and Altman plot showed the mean bias of these two methods to be close to zero (4%) (Figure 3c), with a large span (140%)

Agreement between the two methods was also good for samples with RTX concentrations outside the analytical range (Table 3). RTX was undetectable in 17 samples by both the Lisa Tracker[®] and LC-MS/MS methods. RTX was undetectable in three samples (P01, P34, P45) with Lisa Tracker[®], but low concentrations were measured by LC-MS/MS. Conversely RTX was undetectable in one sample (P16) by LC-MS/MS but a low concentration was measured by Lisa Tracker[®]. One sample (P02) had a RTX concentration above the ULOQ for the Lisa Tracker[®] assay, with a concentration of $74.7 \mu\text{g. mL}^{-1}$ by LC-MS/MS.

3.2.2. LC-MS/MS versus Promonitor®

Passing and Bablok regression analysis revealed no significant deviation from linearity (Custum test, $p = 0.53$, $n = 26$) (Figure 3b). The slope from the equation for the linear regression (1.69) indicates that LC-MS/MS overestimated the concentrations by 69% relative to those estimated using the Promonitor® kit. The Bland and Altman chart (Figure 3d) plot showed the mean difference to be 71.1%, regardless of the concentration, with a large span of 142 %.

The two methods were in agreement for RTX concentrations outside the analytical range (Table 3). RTX was undetectable by both Promonitor® and LC-MS/MS for 14 samples. It was undetectable by Promonitor® but low concentrations were measured by LC-MS/MS for five samples (P01, P12, P32, P34, P43) and it was undetectable by LC-MS/MS but low concentration were measured by Promonitor® for four (P04, P18, P25, P38). The RTX concentration of sample P02 was above the ULOQ of the Promonitor® assay, as it was for the Lisa-Tracker® kit.

3.2.3. Impact of anti-drug antibodies on RTX quantification

ADA was detected in 11 samples (italicized rows in Table 3). Among them, RTX was undetectable in nine, regardless of the method used. Low concentrations of RTX were measured in two samples by the LC-MS/MS method, whereas it was undetectable for both ELISA kits (P34) or only the Lisa-Tracker® kit (P45).

4. Discussion

Our LC-MS/MS method allows the simultaneous quantification of RTX and ECU, two mAbs widely used. The new specific peptide for RTX quantification enables better sensitivity. In addition, the large range of concentrations measured with this

LC-MS/MS method are consistent with its use for TDM of both RTX and ECU (8,14,16).

RTX serum concentrations are highly variable (26) and can be particularly low, even undetectable in some patients with primary membranous nephropathy treated twice a year (8,11,27). As a threshold of 1 $\mu\text{g}\cdot\text{mL}^{-1}$ for the trough RTX concentration has been associated with a long-term response in primary membranous nephropathy (11), our previous LC-MS/MS method of quantification of RTX (20), like others (17,28), with a LLOQ of 5 $\mu\text{g}\cdot\text{mL}^{-1}$, was not suitable for RTX TDM. To improve the sensitivity of our previous LC-MS/MS method (20), two new proteotypic peptides with several unique features were monitored for RTX quantification (see Table 1). First, both peptides have an N-terminal glutamine cyclized in pyroglutamate, corresponding to the loss of an $-\text{NH}_3$ group, i.e. -17 Da. Although the spontaneous *in vivo* formation of pyroglutamate has been well described (29), its origin is hypothetical and may occur at various moments: during mAb production in the bioreactor, sample preparation, or the analytical process (liquid chromatography and/or electrospray ionization). Moreover, the percentage of N-terminal pyroglutamine that occurs during the preparation process appears to depend on the temperature and buffer composition (30,31). Second, the sequence of the RTX quantification peptide includes an arginine (K) followed by a proline (P), which is a site of miss-cleavage by trypsin (32) (Table 1). Finally, MS sensitivity turned out to be better when monitoring this miss-cleaved peptide than that previously monitored (20), allowing us to obtain a LLOQ of 1 $\mu\text{g}\cdot\text{mL}^{-1}$, making our LC-MS/MS method compatible for RTX TDM, including for patients with increased RTX clearance (8,11).

ECU, another mAb used by nephrologists, also shows highly variable pharmacokinetics, with an established exposure-effect relationship (13). Simulation data have shown that ECU TDM can decrease the variability of ECU concentrations and the inhibition of C5 activity, while reducing costs (13). As there are currently few analytical methods for ECU TDM (10,22,33,34), we integrated ECU into our LC-MS/MS method.

Our LC-MS/MS method had within-day and between-day accuracy and precision fulfilling FDA validation criteria (24). The range of concentrations measured with our LC-MS/MS method was broad for both mAbs (from 1 to 200 $\mu\text{g}\cdot\text{mL}^{-1}$ for RTX and 5 to 200 $\mu\text{g}\cdot\text{mL}^{-1}$ for ECU), as for other previously described LC-MS/MS methods (17,22,33) and could be expanded by additional dilution. Such a wide range allows direct quantification of RTX in human samples, whereas ELISA methods require systematic dilution (see supplemental Table 2 and (10,13,34)), as illustrated here by the results with sample P02 (see Table 3). Our analytical approach is thus suitable for performing TDM of RTX and ECU, while saving instrument time and reagents.

The second aim of this study was to compare RTX concentrations measured with our LC-MS/MS method with those measured with two commercial ELISA kits: Lisa Tracker[®] and Promonitor[®]. The RTX concentrations measured by LC-MS/MS correlated strongly with those determined by both ELISA assays. Agreement between the results of the LC-MS/MS method and the Lisa-Tracker[®] kit was excellent (Figure 3a and 3b), as previously found for ADM (21). Such a finding suggests that the therapeutic range of RTX proposed in clinical studies based on results of the Lisa-Tracker[®] kit (8) could be transposable to RTX TDM with our LC-MS/MS method.

Conversely, comparison of RTX concentrations measured using our LC-MS/MS method and the Promonitor[®] kit revealed a high systematic bias, with RTX

concentrations approximately 69% higher for LC-MS/MS (Figure 3b and 3d). Such discrepancies between LC-MS/MS and ELISA have been widely reported for the quantification of mAbs and could be multifactorial. The lack of specificity of ELISA, with non-specific binding of interfering proteins or the lack of reference materials may be responsible. Moreover, the presence of ADA could differentially affect methods for the quantification of RTX. Indeed, commercially available ELISA only quantifies the unbound excess of the drug and/or ADA and not when they are in a complex with each other (15). Drug/ADA complexes may also interfere with the protein-G purification step in our LC-MS/MS method (35).

In our study, RTX was undetectable in 9/11 samples with ADA, regardless of the method used (Table 3), which means that the systematic bias observed between the LC-MS/MS method and the Promonitor[®] kit did not seem to be related to ADA. For the other two samples with ADA, RTX could be quantified at low concentrations by LC-MS/MS, whereas it was undetectable using one or both ELISA kits. Although this finding needs to be confirmed in a larger cohort (using especially drug tolerant ADA assays, which is the case of both ADA kits used here), this result suggests that our LC-MS/MS approach may be better for patients who have become immunized against their biological.

Otherwise, Bland-Altman graphs revealed large spans (almost 140%) for comparisons of the LC-SM/SM method with both ELISA kits. Such spans were higher than those reported for similar comparisons performed for infliximab or adalimumab (21,36) and underlines the importance to use the same quantification method for one patient during longitudinal TDM.

Our study had several limitations. First, the quantification of a RTX biosimilar using our LC-MS/MS method is uncertain and needs to be verified, as a single

change in the amino-acid sequence would change the mass/charge ratio of the peptides. Moreover, our study did not include any clinical data, which limited investigations to explain discrepancies between the analytical methods, especially for sample P17 (Figure 3). Finally, quantification of ECU in human samples with our method was not compared with previously described methods, including LC-MS/MS methods using specific purification method for IgG4 (37) or home-made ELISA (34).

5. Conclusion

We describe a precise and accurate LC-MS/MS method for the simultaneous quantification of RTX and ECU in human samples. This method could be used in further clinical studies to determine the therapeutic concentrations of RTX and ECU associated with clinical efficacy.

Figure Legends

Figure 1. Gradient of the mobile phase throughout the run of 8.6 min.

Step 1. Loading of the sample onto the purification column

Step 2. Elution of the analytes and transfer to the analytical column, followed by analysis in the mass spectrometer

Figure 2. Chromatograms illustrating the selected proteotypic peptides of rituximab (RTX), eculizumab (ECU), and the internal standard (SIL-ADM).

- a. Double blank showing no interference by RTX, ECU, or SIL-ADM*
- b. Drug-free plasma with SIL-ADM (blank)*
- c. Calibration curve point at $10 \mu\text{g.mL}^{-1}$ of RTX (1) and ECU (2)*
- d. Sample from a patient treated with RTX and measured at $19.4 \mu\text{g.mL}^{-1}$*
- e. Sample from a patient treated with ECU and measured at $258.0 \mu\text{g.mL}^{-1}$*

Figure 3. Interassay comparison of the methods of RTX quantification: LC-MS/MS vs Lisa Tracker® (1) or Promonitor® (2).

a-b: Passing & Bablok representations

c-d: Bland-Altman plots. The difference between two measurements ($\mu\text{g.mL}^{-1}$) is plotted on the y-axis and the average of the two measurements ($\mu\text{g.mL}^{-1}$) on the x-axis. Dashed lines represent the 95% limits of agreement of the bias.

Table 1. Mass spectrometry settings and proteotypic peptides: Position of the peptide in the antibody (H: heavy chain, L: light chain), MRM transitions, voltage setting declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) for each analyte. Dwell times were set automatically by the scheduled MRM mode of the software. Scheduled MRM detection was set at 30 s and the target scan time was fixed at 0.5 s. Underlined ions were used as quantifiers and the second ions were used for confirmation. The trypsin miss-cleavage is represented in bold.

Analytes	Signature peptide	RT (min)	Position in the antibody	Q1 (m/z)	Q3 (m/z)	DP(V)	EP (V)	CE (eV)	CXP (V)
Rituximab	Q[PGQ]VQLQQPGAELV K PGASVK +2y6	5.62	H-FR1 aa. 1-19	<u>980.5</u>	<u>558.3</u>	75	10	55	15
	Q[PGQ]IVLSQSPAILSASPGEK +2y11	5.97	L-FR1 aa. 1-18	904.5	1069.6	75	10	37	12
Eculizumab	LLIYGATNLADGVPSR +2y5	5.76	L-CDR2 aa. 46-61	<u>830.45</u>	<u>515.1</u>	55	10	30	15
	LLIYGATNLADGVPSR +2y3	5.76		830.45	359.2	55	10	30	15
SIL-Adalimumab	[¹³ C ₆ - ¹⁵ N ₂] APYTFGQG T K +2y7	5.10	L-CDR3 aa. 94-103	539.3	746.4	100	10	28.3	15

Q[PGQ]: N-terminal glutamine cyclized in pyroglutamate = N-terminal pyroglutamine; RT, retention time; SIL, stable isotope-labeled.

Table 2. Within- and between-day precision (expressed as coefficients of variation) and accuracy (expressed as bias) of the LC-MS/MS method for the quantification of rituximab (RTX) and eculizumab (ECU).

Analyte	IQC concentration ($\mu\text{g.mL}^{-1}$)	Within-day precision and accuracy (n = 6)			Between-day precision and accuracy (n = 6)		
		Mean concentration ($\mu\text{g.mL}^{-1}$)	CV (%)	Bias (%)	Mean concentration ($\mu\text{g.mL}^{-1}$)	CV (%)	Bias (%)
RTX	5.6	4.8	6.7	-13.8	4.7	7.8	-15.2
	45	47.0	5.8	-4.5	45.3	7.6	0.78
	150	131.8	14.1	-12.1	133.8	9.0	-10.8
ECU	5.6	6.6	12.3	17.9	6.3	5.4	11.7
	45	39.6	7.2	-12.0	45.1	14.4	0.3
	150	131.0	11.5	-12.7	151.7	10.8	1.1

RTX: rituximab, ECU: eculizumab, IQC: Internal quality control, CV: coefficient of variation.

Table 3. Agreement between rituximab (RTX) concentrations that were out of the analytical range for at least one of the techniques (< 1 µg.mL⁻¹ for LC-MS/MS, < 2 µg.mL⁻¹ or > 50 µg.mL⁻¹ for Lisa Tracker[®], and < 0.75 µg.mL⁻¹ or > 51 µg.mL⁻¹ for Promonitor[®]). Characters in italics indicate samples with antidrug antibodies (ADA) and undetectable RTX concentrations, regardless of the method used (n = 9). Bold characters indicate samples with ADA and RTX detected by the LC-MS/MS method but not ELISA (n = 2).

Sample (n = 25)	RTX LC-MS/MS ($\mu\text{g.mL}^{-1}$)	RTX Lisa Tracker [®] ($\mu\text{g.mL}^{-1}$)	RTX Promonitor [®] ($\mu\text{g.mL}^{-1}$)	Presence of ADA
P01	1.2	< 2	< 0.75	Negative
P02	74.7	> 50	> 51.0	Negative
<i>P04</i>	< 1	< 2	3.96	<i>Positive</i>
P05	< 1	< 2	< 0.75	Negative
P08	< 1	< 2	< 0.75	Negative
P10	< 1	< 2	< 0.75	Negative
P12	1.8	4.31	< 0.75	Negative
P13	< 1	< 2	< 0.75	Negative
P14	< 1	< 2	< 0.75	Negative
P16	< 1	4.98	< 0.75	Negative
P18	< 1	< 2	5.29	Negative
<i>P19</i>	< 1	< 2	< 0.75	<i>Positive</i>
<i>P20</i>	< 1	< 2	< 0.75	<i>Positive</i>
P25	< 1	< 2	3.25	Negative
<i>P27</i>	< 1	< 2	< 0.75	<i>Positive</i>
<i>P29</i>	< 1	< 2	< 0.75	<i>Positive</i>
P30	< 1	< 2	< 0.75	Negative
P32	3.0	2.58	< 0.75	Negative
P34	3.7	< 2	< 0.75	Positive
P36	< 1	< 2	< 0.75	<i>Positive</i>
P38	< 1	< 2	3.37	<i>Positive</i>
P43	3.3	2.31	< 0.75	Negative
P45	5.3	< 2	1.4	Positive
<i>P47</i>	< 1	< 2	< 0.75	<i>Positive</i>
<i>P48</i>	< 1	< 2	< 0.75	<i>Positive</i>

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Conflict of interest

The authors report no conflicts of interest related to this work.

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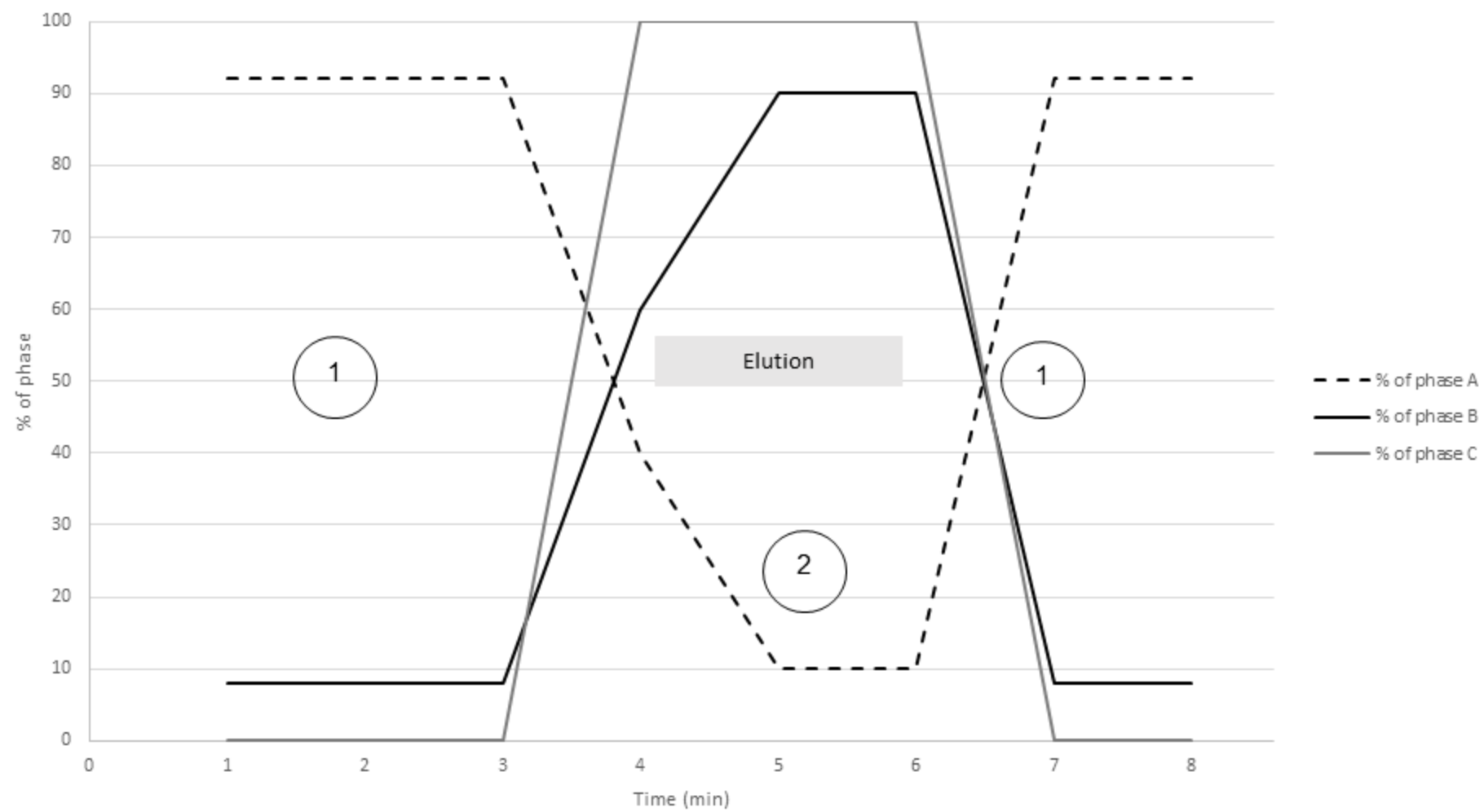
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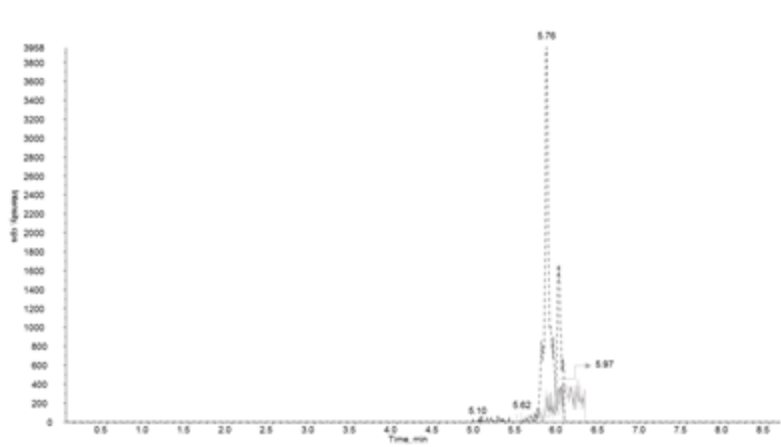
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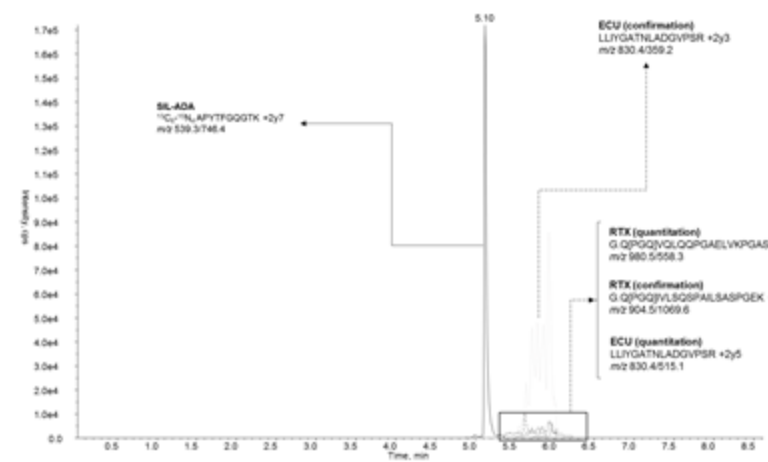
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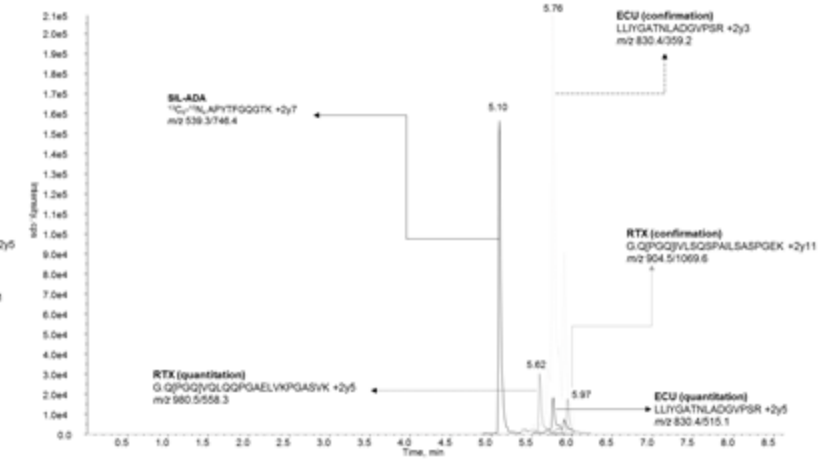




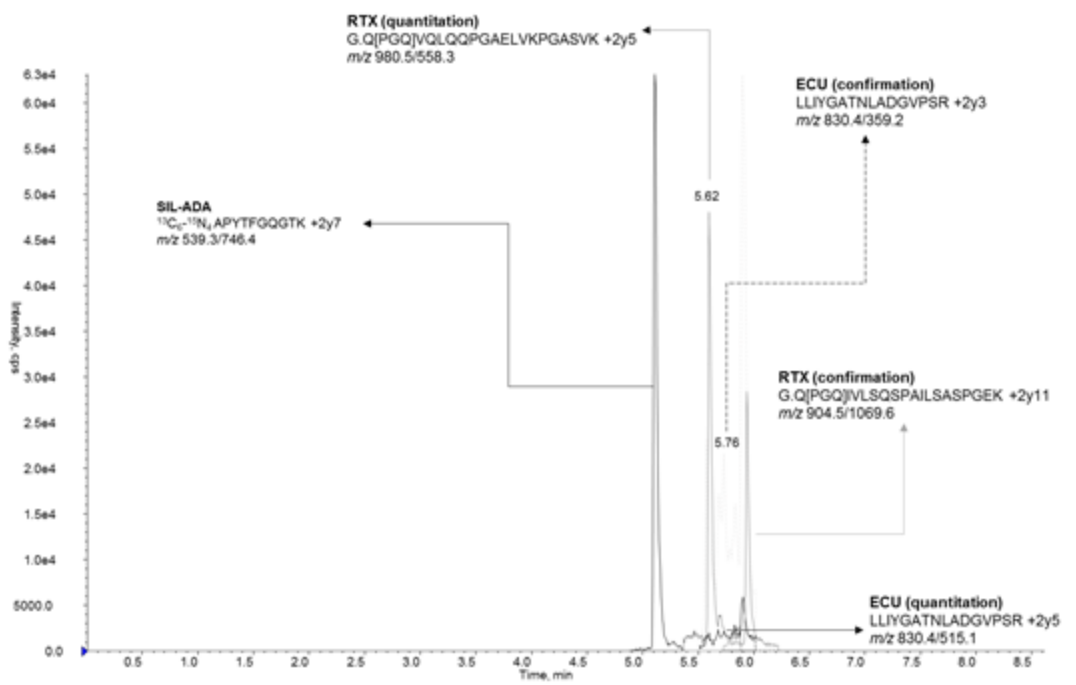
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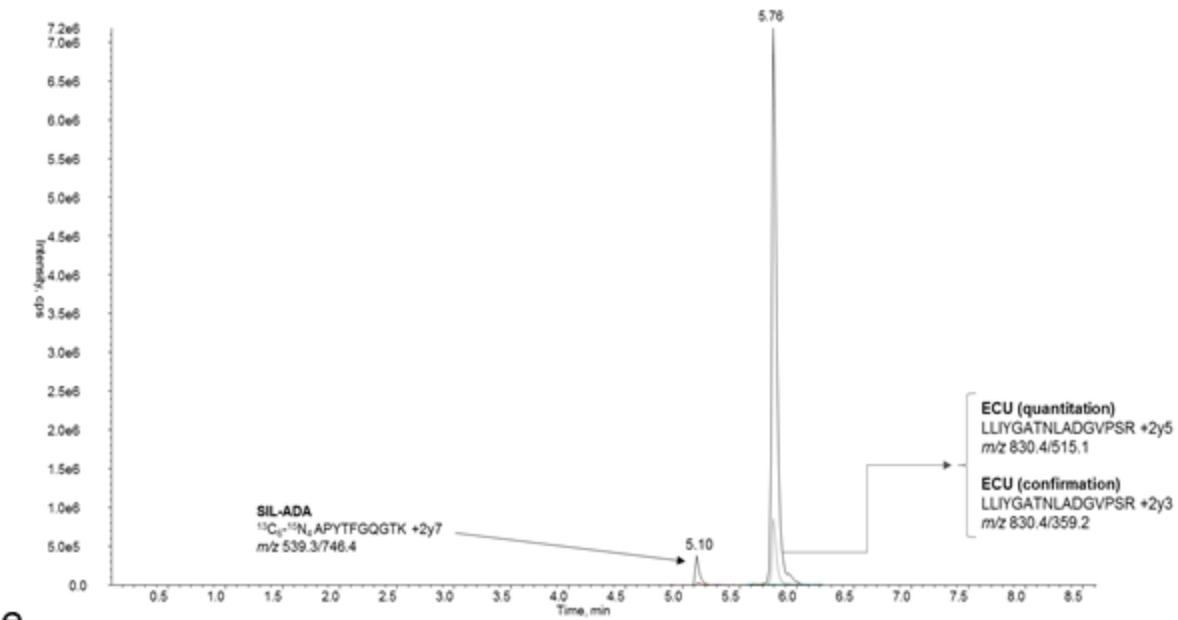
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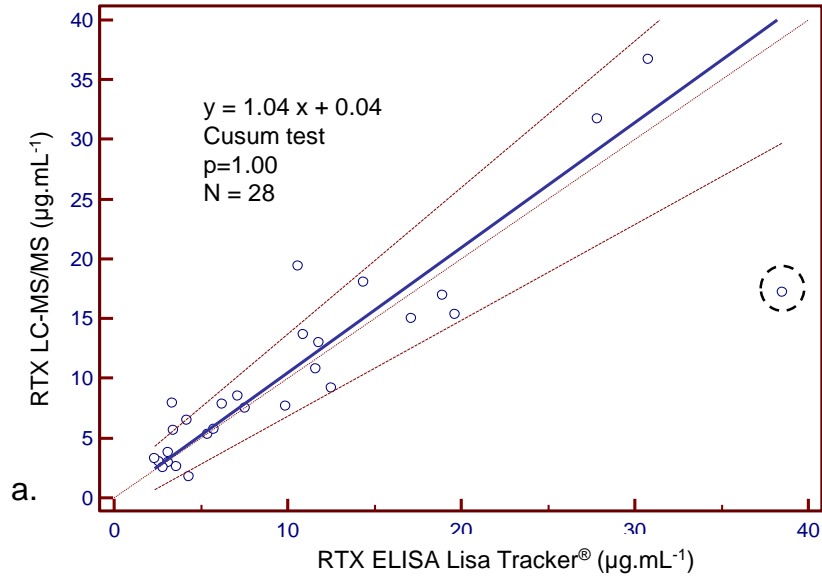


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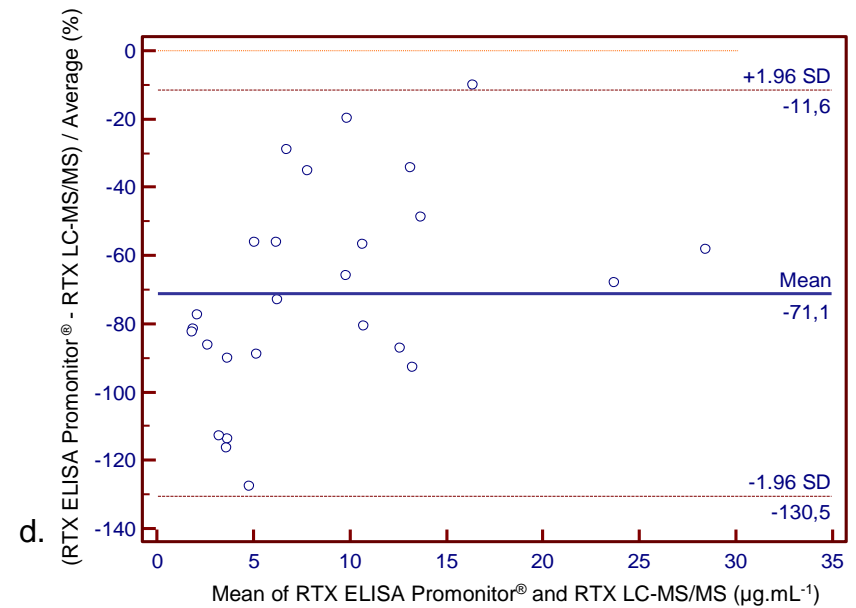
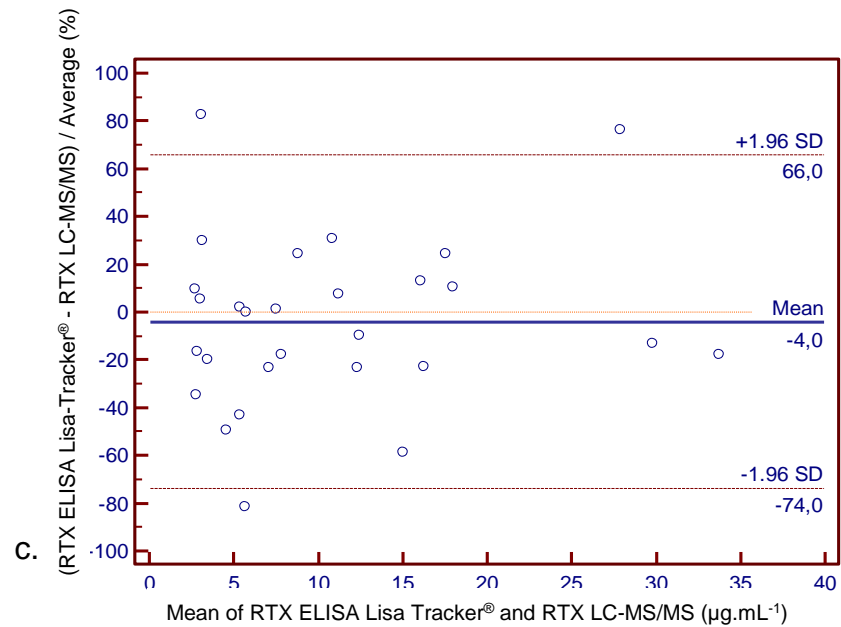
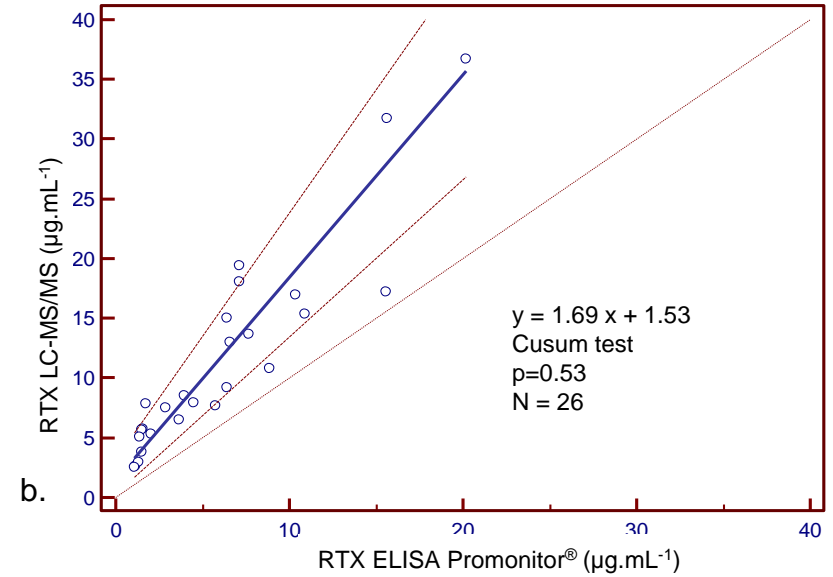


e.

LC-MS/MS vs Lisa Tracker®

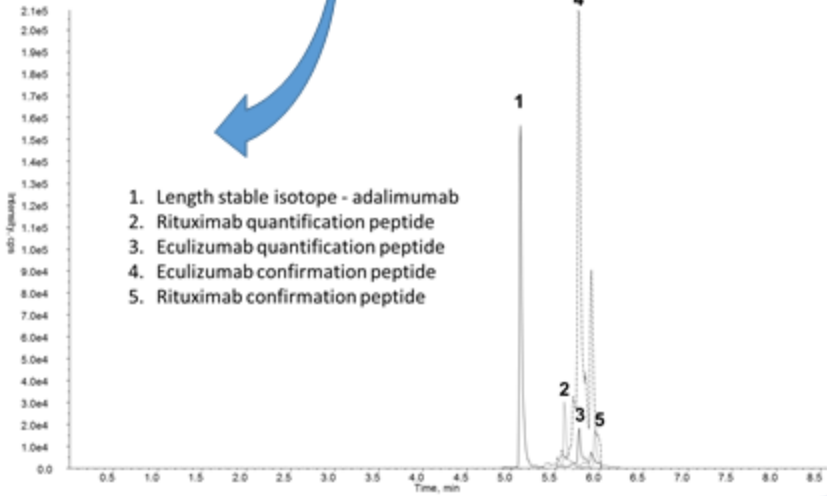


LC-MS/MS vs Promonitor®

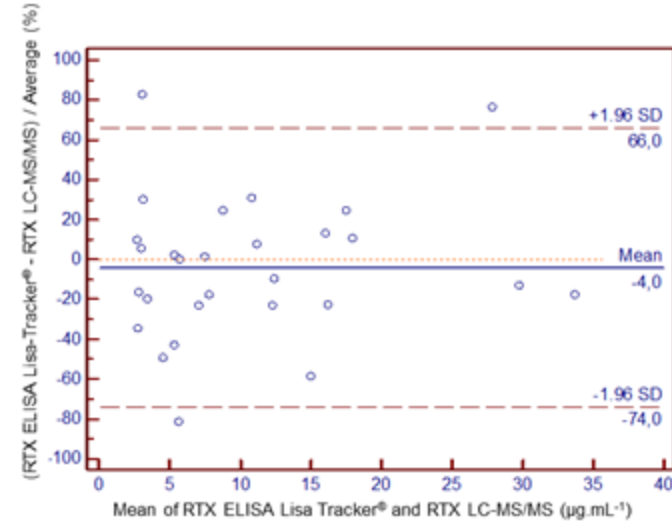


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Purification by
protein-G immunocapture
and trypsin digestion



- ✓ Accuracy
- ✓ Precision
- ✓ Linearity
- ✓ Stability



Development of a **LC-MS/MS**
method for quantification rituximab
(RTX) and eculizumab (ECU)

Validation of the
method

Comparison of RTX
concentrations obtained by
LC-MS/MS with existing
ELISA kits (Lisa Tracker® and
Promonitor®)



Towards a clinical
application
&
**Therapeutic Drug
Monitoring**