Journal of Mass Spectrometry and Advances in the Clinical Lab 21 (2021) 10–18

Contents lists available at ScienceDirect

Journal of Mass Spectrometry and Advances in the Clinical Lab

journal homepage: www.sciencedirect.com/journal/journal-of-mass-spectrometry-and-advances-in-the-clinical-lab

Research Article

Ravulizumab: Characterization and quantitation of a new C5 inhibitor using isotype specific affinity purification and high-resolution mass spectrometry

Paula M. Ladwig [1,*], Maria A.V. Willrich [1]

Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA

ARTICLE INFO

Keywords:
Ravulizumab
Eculizumab
Therapeutic monoclonal antibody
Mass spectrometry
Intact light chain
Orbitrap
Time of flight

ABSTRACT

Introduction: Ravulizumab (RAVUL) is a new complement inhibitor, with a difference of 4 amino acids in the heavy chain from a predecessor compound, eculizumab (ECUL).
Objectives: First, to utilize mass spectrometry (MS) to characterize RAVUL and verify differences from its predecessor and, second, to validate and implement a lab developed test (LDT) for RAVUL that will allow for quantitative therapeutic monitoring.
Methods: A time-of-flight mass spectrometer (TOF-MS) was used to characterize and differentiate the molecular weight differences between RAVUL and ECUL by both digest and reduction experiments. In parallel, an LDT for RAVUL was validated and implemented utilizing IgG4 enrichment with light chain detection and quantitation on a high throughput orbitrap MS platform.
Results: The TOF-MS platform allowed for the mass difference between RAVUL and ECUL to be verified along with providing a proof of concept for a new intact protein quantitation software. An LDT on an orbitrap MS was validated and implemented using intact light chain quantitation, with the limitation that it cannot differentiate between ECUL and RAVUL. The LDT has an analytical measuring range from 5 to 600 mcg/mL, inter-assay imprecision of ≤13% CV (n = 13) and accuracy with <4% error from expected values (n = 20).
Conclusion: The TOF-MS is a versatile development platform that can be used to characterize and verify the molecular weight differences between the ECUL and RAVUL heavy chains. Routine laboratory testing for RAVUL was viable using an orbitrap-MS to quantitate using the mass of the intact light chain. These two platforms, combined, provide incomparable value in development of LDTs for the clinical laboratory.

Introduction

Ravulizumab (RAVUL), or Ultomiris®, is a humanized monoclonal IgG2/4 kappa therapeutic monoclonal antibody (t-mAb) engineered by Alexion Pharmaceuticals for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) and atypical hemolytic uremic syndrome (aHUS) [1]. RAVUL has been engineered from a predecessor therapeutic, eculizumab (ECUL), as a longer-acting t-mAb to reduce the frequency of infusions. RAVUL was FDA-approved in 2019. Both t-mAbs are directed against complement component 5 (C5) [2]. By association with C5, ECUL and RAVUL inhibit the terminal complement pathway through simultaneous blockade of the generation of the potent pro-thrombotic and pro-inflammatory molecule, C5a, and the formation of the membrane attack complex initiator, C5b [3]. Since all three arms of the complement cascade converge at the point of C5 activation, targeted by both ECUL and RAVUL, these therapeutics have broad potential application in disorders with complement over-activation. In PNH, ECUL has become the standard of care, proving to be a safe and effective therapy

Abbreviations: aHUS, atypical hemolytic uremic syndrome; AMR, analytical measuring range; C5, complement component 5; Da, daltons; DTT, dithiothreitol; ECUL, eculizumab; Fc, crystallizable fragment; HPLC, high performance liquid chromatography; IRB, Institutional Review Board; IS, internal standard; LC, liquid chromatography; LDT, lab-developed test; LLOD, lower limit of detection; LLOQ, lower limit of quantitation; LOB, limit of blankMS, mass spectrometry; MW, molecular weight; NHS, normal human serum; NIVOL, nivolumab; PBS, phosphate buffered saline; PNH, paroxysmal nocturnal hemoglobinuria; Q-TOF, quadrupole time-of-flight; RAVUL, ravulizumab; t-mAb, therapeutic monoclonal antibody; XIC, extracted ion chromatogram.

* Corresponding author.
E-mail addresses: Ladwig.paula@mayo.edu (P.M. Ladwig), willrich.mariaalice@mayo.edu (M.A.V. Willrich).
[1] Address reprint requests to: Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA.

https://doi.org/10.1016/j.jmsacl.2021.08.002
Received 12 March 2021; Received in revised form 2 August 2021; Accepted 5 August 2021
Available online 12 August 2021

with long-lasting effects, potentially enabling patients to become transfusion-independent and extending their survival.

RAVUL differs from ECUL by 4 amino acid substitutions in the heavy chain region, 2 substitutions in the complementarity-determining variable region and 2 in the crystallizable fragment (Fc) region (CH3); Fig. 1. These modifications result in a terminal half-life for RAVUL that is approximately 4-fold longer than that of ECUL [2], and a change in the t-mAb's binding affinity to C5. In comparison to ECUL, RAVUL produces immediate, complete, and sustained inhibition of C5 that results in an extended 8-week dosing interval in comparison to ECUL's shorter 2-week dosing intervals [2].

Therapeutic monitoring of ECUL has allowed cost-savings along with improved quality of life for patients by extending infusion intervals when drug concentrations are above therapeutic thresholds, with patients maintaining complete complement activity blockage [4–7]. Although not yet common practice, we expect that therapeutic monitoring of RAVUL may similarly assist in personalizing therapy regimens and contribute to improved outcomes.

Our goal was to utilize our available mass spectrometer (MS) platforms to characterize the differences between RAVUL and ECUL and then establish and implement a clinical lab-developed test (LDT) for RAVUL quantitation. We currently offer a LDT for ECUL quantitation by IgG4 enrichment followed by detection of the ECUL light chain on a high resolution orbitrap MS platform [8,9]. While this method can be utilized for the enrichment and detection of RAVUL, the MS detection and quantitation method would not be able to distinguish between the therapeutics, as the method targets the intact light chain which is identical in both RAVUL and ECUL. Here, we describe the characterization experiments performed for RAVUL, the MS platform and software options explored for an LDT, and finally provide the validation summary data for the implemented RAVUL LDT.

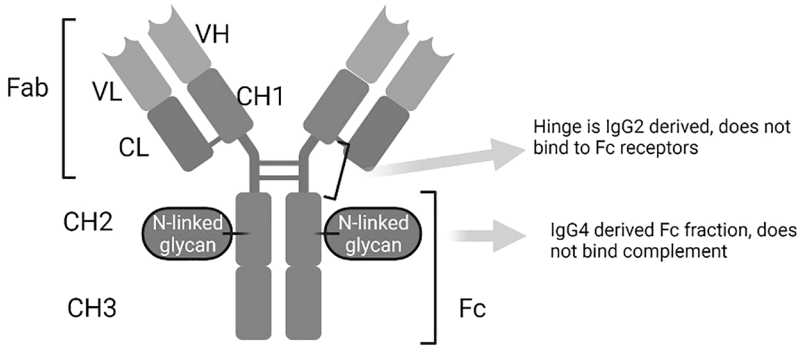
Materials and methods

Chemicals and reagents

Acetic acid, acetone, ammonium bicarbonate, dithiothreitol (DTT), formic acid, and isopropyl alcohol were purchased from MilliporeSigma (St. Louis, MO). IdeS protease was purchased from Promega (Madison, WI). Water and acetonitrile were purchased from Honeywell Burdick and Jackson (Muskegon, MI). CaptureSelect™ IgG4 (Hu) Affinity Matrix was purchased from Life Technologies (Carlsbad, CA). Phosphate buffered saline (PBS) was purchased from Fisher Healthcare (Hampton, NH). Normal human serum (NHS) was purchased from EMD Millipore (Billerica, MA).

Therapeutic monoclonal antibodies

ECUL, RAVUL (Alexion Pharmaceuticals), and nivolumab (NIVOL) (Bristol-Myers Squibb) were obtained from the institution's pharmacy. Standards (5, 10, 25, 75, 150, 300, 500 and 600 mcg/mL) and controls (15, 100, 400 mcg/mL), as well as artificial samples were prepared by spiking ECUL or RAVUL into purchased NHS. NIVOL was utilized as a surrogate protein level internal standard (IS) due to the general lack of isotopically labelled t-mAbs and the expense of having them made. NIVOL was chosen as a favorable surrogate due to its similar light chain chromatographic retention time to ECUL, being another IgG4 t-mAb and, importantly, an unlikely co-prescribed medication. Institutional



Eculizumab

VH:
QVQLVQSGAEVKKPGASVKVSCKASG**Y**IFSNYWI
QWVRQAPGQGLEWMGEILPGSG**S**TEYTENFKDR
VTMTRDTSTSTVYMELSSLRSEDTAVYYCARYFFG
SSPNWYFDVWGQGTLVTVSS
CH1:
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEP
VTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVP
SSNFGTQTYTCNVDHKPSNTKVDKTVER
Hinge: KCCVECPPCP
CH2:
APPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVS
QEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVV
SVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
CH3:
GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRL
TVDKSRWQEGNVFSCSV**M**HE ALH**N**HYTQK
SLSLSLGK(448aa)

Ravulizumab

VH:
QVQLVQSGAEVKKPGASVKVSCKASG**H**IFSNYWI
QWVRQAPGQGLEWMGEILPGSG**H**TEYTENFKDR
VTM TRDTSTSTVYMELSSLRSEDTAVYYCARYFFG
SSPNWYFDVWGQGTLVTVSS
CH1:
ASTKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPV
TVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
NFGTQTYTCNVDHKPSNTKVDKTVER
Hinge: KCCVECPPCP
CH2:
APPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ
EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVV
SVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAK
CH3:
GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPS
DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRL
TVDKSRWQEGNVFSCSV**L**HE ALH**S**HYTQK
SLSLSLGK (448aa)

Fig. 1. ECUL and RAVUL IgG structure and heavy chain amino acid sequences. The 4 differing amino acids are highlighted in bold and underlined, with a theoretical molecular weight difference of 21.1 Da between the 2 heavy chains. The variable and constant regions are noted. There is a conserved site for N-linked glycans in the Fc fraction of all immunoglobulins. In IgGs, the N-glycans are bound to the asparagine 297 in the CH2 domain.

Review Board (IRB) approval was not required since no patient samples were used.

Characterization experiments

A volume of 5 mcL ECUL or RAVUL 10 mg/mL pharmaceutical stock was added to 100 mcL water and 100 mcL of 100 mM DTT in 50 mM ammonium bicarbonate. The 200 mcg/mL preparations were reduced at 55 °C for 30 min to disrupt the immunoglobulin disulfide bonds, giving separate, but intact, heavy and light chains. This would allow for 1 mcg to be injected on column (5 mcL injection volume).

A volume of 5 mcL ECUL or RAVUL 10 mg/mL pharmaceutical stock was added to 100 mcL water with 2 mcL of IdeS protease. The 467 mcg/mL preparations were digested at 37 °C for 1 h. This would allow for 2 mcg to be injected on column (5 mcL injection volume). A 50 mcL aliquot of the IdeS protease digest was then reduced at 55 °C for 30 min with 50 mcL of 100 mM DTT in 50 mM ammonium bicarbonate. This would allow for 1 mcg to be injected on column (5 mcL injection volume).

IgG4 enrichment

Both ECUL and RAVUL LDTs utilize an IgG4 enrichment step for all standards, controls, and unknowns to enrich for the IgG4 t-mAbs while lowering the protein content before LC-MS. A volume of 100 mcL CaptureSelect™ IgG4 (Hu) affinity matrix was added to each well of a 0.2-μm PVDF Corning 96 well filter plate (Sigma-Aldrich; St. Louis, MO). The matrix was washed 2 times with 200 mcL 1XPBS. A positive pressure manifold 96 processor (Agilent; Santa Clara, CA) was used to push the washes through the filter plate into a waste plate by manually increasing the pressure to approximately 30 psi using the flow control knobs. Volumes of 30 mcL standard, control, or unknown, 30 mcL surrogate protein level IS (50 mcg/mL NIVOL in 1XPBS) and 100 mcL 1XPBS were added to the pre-washed matrix. The plate was covered and mixed at room temperature for 1 h on an Eppendorf ThermoMixer (Fisher Healthcare; Hampton, NH) at 800 rpm. The filter plate was then washed 4 times with 150 mcL water using positive pressure. A volume of 200 mcL 5% acetic acid was added and the plate incubated for 15 min at room temperature on the ThermoMixer at 800 rpm. Positive pressure was used to collect the eluate in a 96 well, deep well collection plate. A volume of 100 mcL of 100 mM DTT in 1 M ammonium bicarbonate was added, and the samples were reduced at 55 °C for 30 min.

Research microLC-Q-TOF MS platform

The prepared samples (digested and/or reduced preparations from section 2.3 or IgG4 enriched standards, controls, and unknowns from section 2.4) were injected onto an Eksigent Ekspert 200 microflow liquid chromatography platform (LC) (Dublin, CA); mobile phase A was water + 1% formic acid, and mobile B was 90% acetonitrile + 10% 2-propanol + 0.1% formic acid. Five mcL injections were made onto a 1.0 × 75 mm Poroshell 300 SB-C3 column (Agilent Technologies; Santa Clara, CA) with 5 μm particle size flowing at 45 mcL/min, while the column was heated at 60 °C. A 13 min gradient was started at 95%A/5%B held for 1.25 min, ramped to 75%A/25%B over 0.25 min, ramped to 63%A/37% B over 5.5 min, ramped to 2%A/98%B over 1 min and held for 2 min, ramped to 95%A/5%B over 0.5 min and re-equilibrated for 2.5 min.

Spectra were collected on a Sciex 5600 quadrupole time-of-flight (Q-TOF) MS (Sciex; Framingham, MA) in ESI positive mode with a Turbo V dual ion source with an automated calibrant delivery system. Source conditions were IS: 5500 V, Temp: 500 °C, CUR: 45 psi, GSI: 35 psi, GS2: 30 psi, CE: 50 V. TOF MS scans were acquired from *m/z* 600–2500 with an acquisition time of 100 ms.

Analyst TFv1.7 was used for instrument control while Sciex's newest software, Sciex OS, was used for qualitative viewing along with being evaluated for intact protein quantitation to correctly identify and quantitate in batch mode. The Mass Reconstruction Processing feature is a part of the MQ4 integration algorithm. The user inputs a *m/z* range in Daltons (Da) to produce an extracted ion chromatogram (XIC). We used a 0.2 Da window around the theoretical *m/z* for the +34-charge state for the respective RAVUL and ECUL heavy chains. Forty-nine spectra centered from the highest peak of the XIC for a specified retention time window (±0.1 min) were averaged to give a summed spectrum used for reconstruction. Reconstruction was performed at 1500 resolution from 1000 to 2200 Da for start and stop masses of 50,700 to 50,900 Da. A theoretical molecular weight (MW) (±5 Da) of 50,810 Da was used to verify and integrate the reconstructed peak for the heavy chains of ECUL and 50,790 Da for RAVUL. Theoretical MWs were obtained from inputting the amino acid sequences into ProteinProspector v6.2.2 (UCSF, CA). The theoretical masses were confirmed by the characterization experiments that employed the pharmaceutical preparations; see section 3.1.1.

HPLC orbitrap MS platform

Standards, controls and unknowns, enriched per section 2.4, were injected onto a Cohesive TLX4 Transcend multi-plex high performance liquid chromatography (HPLC) system (ThermoFisher Scientific; Waltham, MA) used for separation; mobile phase A was water + 1% formic acid, and mobile phase B was 89% acetonitrile + 10% isopropyl alcohol + 1% formic acid. A 10 mcL injection was made onto an Agilent 2.1 × 75 mm 5-μm Poroshell 300SB-C3 column flowing at 300 mcL/min set in a column oven sleeve at a temperature of 60 °C. An 18.5 min run was started at 90% A/10% B, held for 1.5 min, ramped to 73% A/27% B over 1 min, ramped to 64% A/36% B over 3 min, ramped to 50% A/50% B over 1 min, and finally ramped to 2% A/98% B over 1 min. The method then performed 3 ramps between 85% A/15% B to 10% A/90% B to reduce carryover and finally re-equilibrated at 90% A/10% B before the next injection.

Spectra were collected on a Q Exactive Plus high-resolution accurate mass MS (ThermoFisher Scientific; Bremen, Germany). The HESI source utilized a spray voltage of 3.5 kV, capillary temperature of 300 °C, sheath gas flow of 50 psi, auxiliary gas flow of 12 psi, and s-lens RF level of 5. A full scan and t-SIM methods were run in parallel. The full scan method utilized a scan window from 1900 to 2400 *m/z* at 140 kDa resolution, maximum IT of 125 ms and AGC target of 2e5. T-SIMS were collected for 4 mass windows, 2314.05, 2103.77, 1928.54 and 2142.06, at 140 kDa resolution, max IT of 500 ms and AGC target of 1e6.

TraceFinder (ThermoFisher Scientific; Bremen, Germany) software was used for data acquisition, processing, and reporting. The t-SIM method was used for quantitation. The XIC consists of a combination of the 6 most abundant isotopes from 3 charge states (+10, +11, +12). This quantitation method has been described elsewhere, as it was originally developed for the ECUL LDT, which detects and quantitates using the light chain mass [9].

Results

TOF-MS research platform

Characterization of RAVUL

RAVUL and ECUL differ by 4 amino acids in their respective heavy chain amino acid sequences: Fig. 1. Our first step after obtaining the RAVUL stock was to characterize the drug to confirm the difference in mass between RAVUL and ECUL using the IdeS digest and DTT reduction experiments described in section 2.3. Fig. 2A shows the mass spectrum from the DTT reduction experiment and verifies that the same light chain mass (23,131.4 Da theoretical) is seen for both ECUL, 23,131.4 Da, and RAVUL, 23,131.6 Da. Fig. 2A also confirms a difference in the heavy chain masses; 50,812.8 Da and 50,792.3 Da for ECUL and RAVUL, respectively. The measured mass difference between the main peak of the glycosylated heavy chains (20.5 Da) is consistent with the

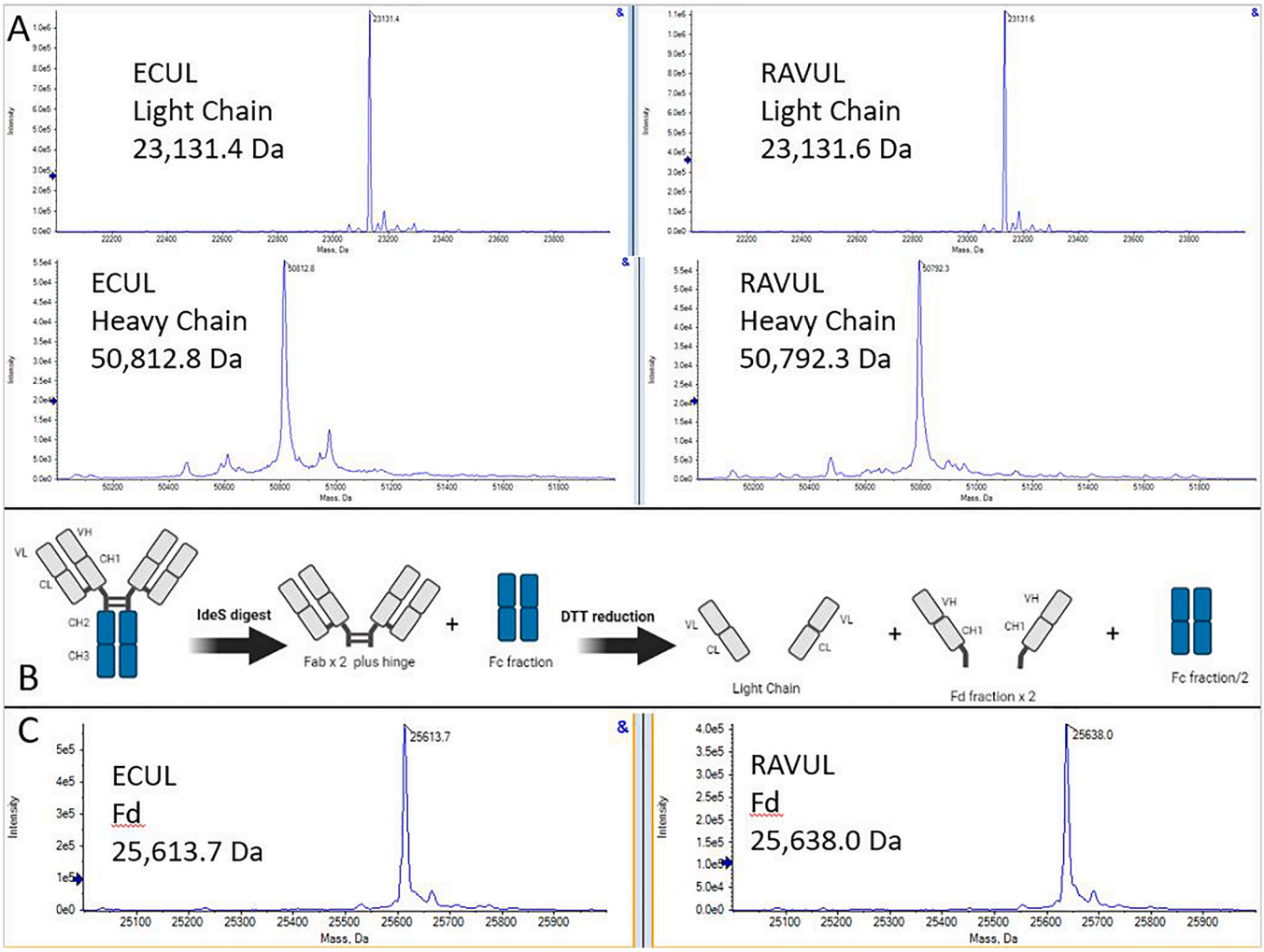


Fig. 2. ECUL and RAVUL TOF characterization. (A) DTT reduction of ECUL and RAVUL to give intact light and heavy chains verifies that the t-mAbs have the same light chain mass (theoretical 23,131.4 Da; <1 Da ppm error), but differ in the mass of the heavy chain; 50,812.8 Da for ECUL (theoretical 49,519 Da + glycosylation mass ~ 1,293 Da) and 50,792.3 Da for RAVUL (theoretical 49,498 Da + glycosylation mass ~ 1,294 Da). The measured mass difference for the heavy chain (20.5 Da) is consistent with the theoretical difference (21.1 Da). (B) IdeS protease digestion and DTT reduction yields several different immunoglobulin fragments. The Fd fragment of the heavy chain is composed of VH + CH1 and is the heavy chain fragment without glycosylation. The Fc fraction includes CH2, which contains a conserved site for N-linked glycans. The light chains are freed from the heavy chains after DTT reduction of the disulfide bonds. (C) The Fd unglycosylated subunit masses differ by 24.3 Da, the mass difference expected by the 2 amino acid substitution (theoretical 23.9 Da) between ECUL and RAVUL for the Fd fragment.

theoretical difference due to the 4 known amino acid substitutions in the heavy chain (21.1 Da theoretical). In Fig. 2B, IdeS + DTT breaks down the immunoglobulins into specific fragments, as shown. The combined fragmentation gives the Fd subunit of the heavy chain (VH + CH1) that would not contain any glycosylation. The measured difference of 24.3 Da aligns with the 2 known substitutions in the complementarity-determining region; two histidines in the RAVUL sequence for the tyrosine and serine in ECUL (23.9 Da theoretical), Fig. 2C.

Intact heavy chain quantitation

As the difference in the 2 t-mAbs is in the heavy chain, in theory an analytically specific quantitation method should target the heavy chain. Currently, MS quantitation of t-mAbs target either a tryptic peptide from the heavy or light chain, or the intact light chain [9]. We performed a proof of concept for intact heavy chain detection and quantitation using our research and development Q-TOF MS platform with a new version of software; Sciex OS with intact protein quantitation. We prepared standards and controls using the IgG4 enrichment method outlined in section 2.4 with LC and MS method details, along with a description of the intact protein deconvolution method used, described in section 2.5.

The quantitation function of Sciex OS allows for intact protein reconstruction in batch mode. As the Q-TOF MS collects full scan data, we can revisit and re-process the raw files to identify the protein utilizing the heavy chain with the confirmed mass difference. Fig. 3 contains 3 examples demonstrating how the software can correctly identify ECUL and RAVUL when they are spiked alone or in combination. Example 3 in Fig. 3 shows that the software correctly identifies both ECUL and RAVUL when spiked together in serum.

Finally, we performed a single proof of concept experiment to evaluate whether we could use this same software to both correctly identify and simultaneously quantitate the appropriate t-mAb using the differences in the heavy chain. In a single experiment, we prepared 2 unknowns by spiking NHS with differing concentrations of ECUL and RAVUL from pharmacy stock. These 2 unknowns were extracted along with both ECUL and RAVUL standards and controls per section 2.4. The tray was analyzed using the research Q-TOF-MS platform and Sciex OS software for quantitation. Sciex OS was used to quantitate the sample set first using the ECUL standards and then a second time using the RAVUL standards. When the unknowns were quantitated by the light chain by either set of standards, the measured concentration for ECUL and RAVUL showed a sum or combination of the therapeutics, as expected; see Table 1. This summed/combination of the therapeutics is a

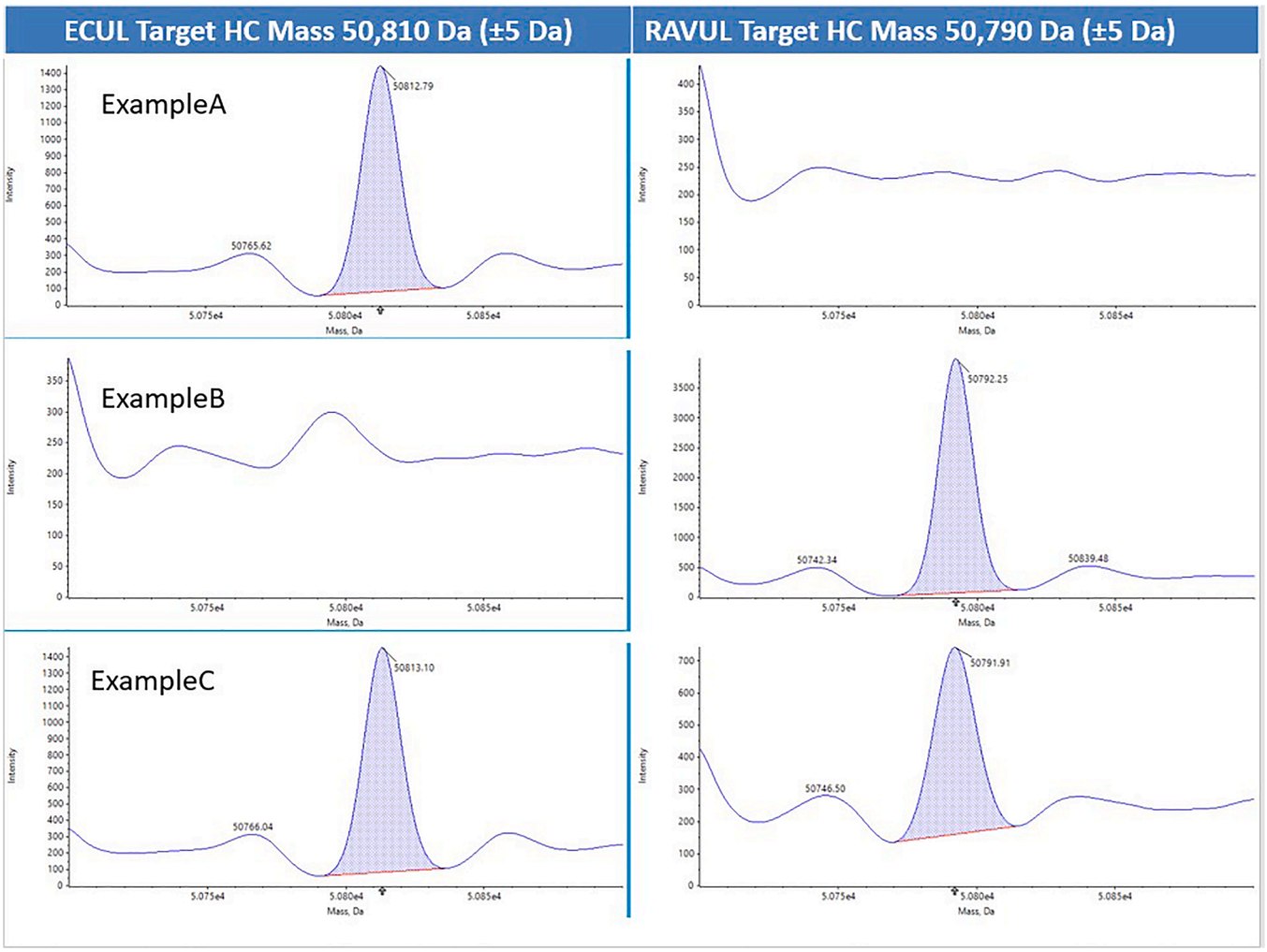


Fig. 3. ECUL and RAVUL identification using their respective heavy chain. Sciex OS Intact Protein Quantitation allows for the correct identification of ECUL and/or RAVUL. Each row shows the intact mass reconstruction window for ECUL on the left and RAVUL on the right. Example A was spiked with ECUL only, B for RAVUL only and C was spiked with both ECUL and RAVUL.

Table 1
Light chain versus heavy chain intact protein quantitation.

	SPIKED ECUL Expected concentration (mcg/mL)	SPIKED RAVUL Expected concentration (mcg/mL)	ECUL measured (mcg/mL)	RAVUL measured (mcg/mL)
			Quantitation by Light Chain	
Sample A	300	75	426 (142%)	314 (419%)
Sample B	75	300	514 (685%)	380 (127%)
			Quantitation by Heavy Chain	
Sample A	300	75	366 (122%)	78 (104%)
Sample B	75	300	92 (123%)	354 (118%)

Note: Percent recovery in parenthesis, single experiment.

limitation when both therapeutics are present. The heavy chain quantitation presented results closer to the spiked concentrations, with recovery ranging from 104 to 123%, which allows for the discrimination between the two therapeutics.

HPLC orbitrap platform

Initial validation experiments

For pursuing a clinical LDT for RAVUL, the platform that is available in our clinical laboratory is an HPLC orbitrap platform currently utilized for 2t-mAb LDTs, ECUL and vedolizumab, targeting the respective intact light chain for detection and quantitation [10,11] with robust performance over the years. Therefore, this is the platform we chose to utilize for our RAVUL LDT method.

We first tried to quantitate spiked RAVUL samples using the current ECUL LDT without any change to the method. The ECUL method quantitates ECUL utilizing the intact light chain mass of the t-mAb, which is identical to RAVUL's light chain. Therefore, the ECUL LDT cannot distinguish between ECUL and RAVUL, but could potentially provide accurate quantitation when not co-prescribed. The ECUL LDT employs an ECUL set of standards (5 mcg/mL to 600 mcg/mL) prepared from spiking NHS from an ECUL pharmacy stock.

RAVUL quantitation precision was tested by analyzing a 50 mcg/mL pool of RAVUL spiked into NHS; 3 replicates over 5 days. The RAVUL spiked pool was analyzed on the ECUL LDT method and quantitated using ECUL standards. The within run precision was 3.4% while the between run precision was 6.2%. Of note, 15 measurements of the 50 mcg/mL pool did quantitate at a mean of 62 mcg/mL; approximately 20% higher in concentration than expected.

Linearity was performed over 6 runs for RAVUL spiked

concentrations of 5, 75, 150, 300, and 550 mcg/mL analyzed using the ECUL LDT method: quantitation using ECUL standards. While we were able to show that the method was linear, we noticed a bias in the measured concentrations trending above that expected; Fig. 4. Without a comparative test for RAVUL, we performed recovery experiments to further address this inaccuracy. The recovery experiment was performed using RAVUL stock spiked into NHS over the range of 5 to 600 mcg/mL: with 3 spikes for each concentration. Again, we noticed a bias evidenced by a mean recovery of >20% suggesting over-quantitation of RAVUL, Table 2. The recovery did not meet the acceptance criteria of <±20% mean recovery with no visible bias in direction.

In reviewing the initial validation with ECUL standards, we hypothesized that the 4 amino acid difference and potential structural differences between ECUL and RAVUL could affect the binding affinity of the two t-mAbs for the camelid IgG4 beads used in the enrichment for the assay and play a role in the over-recovery observed for RAVUL. The glycosylation pattern in the CH2 region of the molecules was assessed with the initial characterization experiments using IdeS and DTT on the Q-TOF MS platform. As seen in Fig. 5, ECUL and RAVUL do have different glycosylation profiles, although the specific configuration and composition of the N-glycan moiety cannot be determined without additional experiments.

RAVUL LDT and validation

We proceeded then to develop an LDT using standards and controls specific for RAVUL on the orbitrap platform to avoid any possible bias that could result from using the ECUL LDT method and ECUL set of standards.

Standards were prepared by spiking RAVUL from stock into NHS for 8 standards: 5, 10, 25, 75, 150, 300, 500 or 600 mcg/mL. Quality controls were prepared by spiking RAVUL into NHS for 3 controls: 15, 100, and 400 mcg/mL. The IgG4 enrichment was carried out in the exact same manner as for the ECUL LDT; see section 2.4. The LC, MS, and quantitation parameters were also copied over and renamed for RAVUL but remained the same as for the ECUL LDT. The RAVUL LDT utilizes the mass of the RAVUL light chain for detection and quantitation and, as such, even though the test would be offered specifically for RAVUL, just like ECUL LDT, it cannot distinguish between RAVUL and ECUL.

RAVUL validation was then performed. A summary of validation studies addressing linearity, analytical specificity, precision, and accuracy follow.

Linearity

Linearity was assessed over 6 different runs using a mixture of standards and controls set as unknowns, which were picked to allow for full coverage of the analytical measuring range (AMR); 5, 15, 100, 225 and 500 mcg/mL run. We did not notice a trend in accuracy with this linearity as we had with the initial experiment using the ECUL standards. The average percentage error of the data set was −0.4%, with all ±12% of the expected value. Fig. 6 shows the linear regression.

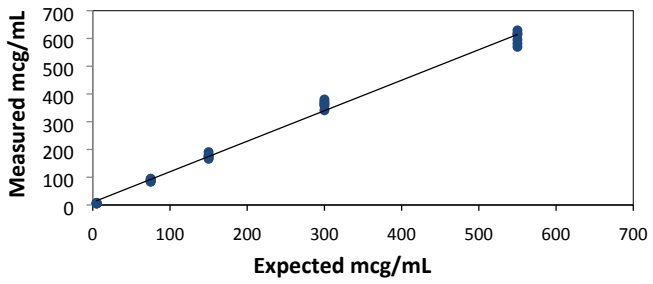


Fig. 4. Initial RAVUL validation linearity against ECUL standards Linearity was performed over 6 runs for concentrations 5, 75, 150, 300 and 550 mcg/mL.

Table 2
Initial RAVUL validation recovery against ECUL standards.

Spike mcg/mL	Mean measured, mcg/mL (n = 3)	% Recovery
5	6.2	124%
10	11.4	114%
25	31.0	124%
50	65.5	131%
100	129	129%
200	266	133%
300	386	129%
400	503	126%
500	625	125%
600	712	119%

Note: Recovery was performed using RAVUL pharmacy stock spiked into NHS over the range 5 to 600 mcg/mL.

Analytical sensitivity

Limit of blank (LOB), lower limit of detection (LLOD) and lower limit of quantitation (LLOQ) were determined by analyzing 0, 2.5, and 5 mcg/mL over 17 runs. The results are shown in Table 3. The LLOD was determined to be approximately 1.3 mcg/mL. The LLOQ was then set at 5 mcg/mL (8.8% inter-assay CV) to be greater than the LLOD along with an acceptable CV of <20%. The RAVUL assay LLOQ matched the previously published LLOQ for the ECUL assay. With the establishment of the LLOQ, the full AMR of the RAVUL assay was set as 5 to 600 mcg/mL. Results below the LLOQ were reported as <5 mcg/mL and results greater than upper limit of quantitation were reported as >600 mcg/mL. No dilutions were performed on the specimens.

Analytical specificity

RAVUL should not quantitate higher than the LLOQ in patients who are not taking RAVUL or ECUL. We analyzed 73 de-identified residual waste serum samples with IgG4 concentrations above the reference interval established by the laboratory (>121 mg/dL) and total IgG concentrations greater than 1700 mg/dL (hypergammaglobulinemia). Seventy-two samples measured less than the LLOQ of the assay, <5 mcg/mL. One unknown would have had a measurable RAVUL concentration: 6 mcg/mL. A comment was added to the validation summary and to the lab test catalog to acknowledge that it is rare, but possible, for patients with high levels of immunoglobulins, especially high levels of IgG4, to have an endogenous clone that can mimic RAVUL. The likelihood that the residual sample contained ravulizumab is very small, but cannot be fully excluded, since chart-review in the de-identified residual samples was not possible and bottom-up MS/MS was not performed on the sample.

Precision

Both intra- and inter-assay precision studies were performed utilizing pools of RAVUL spiked into NHS at 15, 50, 100, 225, and 400 mcg/mL with an additional level at 35 mcg/mL for inter-assay studies; Table 4. For intra-assay analyses, 20 replicates were set per run for each level. For inter-assay, one replicate was set on each of 13 runs for each level. Intra-assay precision CVs were below 10% (range: −8.0% to 9.7%) for all levels. For inter-assay, all CVs were below 10%, except for at 15 mcg/mL (13.3%) and 35 mcg/mL (11.2%).

Accuracy

As RAVUL has no comparator assay available in the United States, recovery experiments were performed to determine accuracy. Recovery experiments consisted of 5 different experiments with 5 levels of RAVUL with concentrations across the AMR; 10, 25, 150, 225, and 400 mcg/mL. We did not see a bias in accuracy across the AMR; mean % recovery was 104%, well within our acceptance limits of 80–120%; Table 5.

We also performed spiked recoveries from 4 different vials of the pharmaceutical preparation stock. NHS was spiked at 5 different levels; 25, 75, 150, 300 and 400 mcg/mL from 4 different pharmacy bottles.

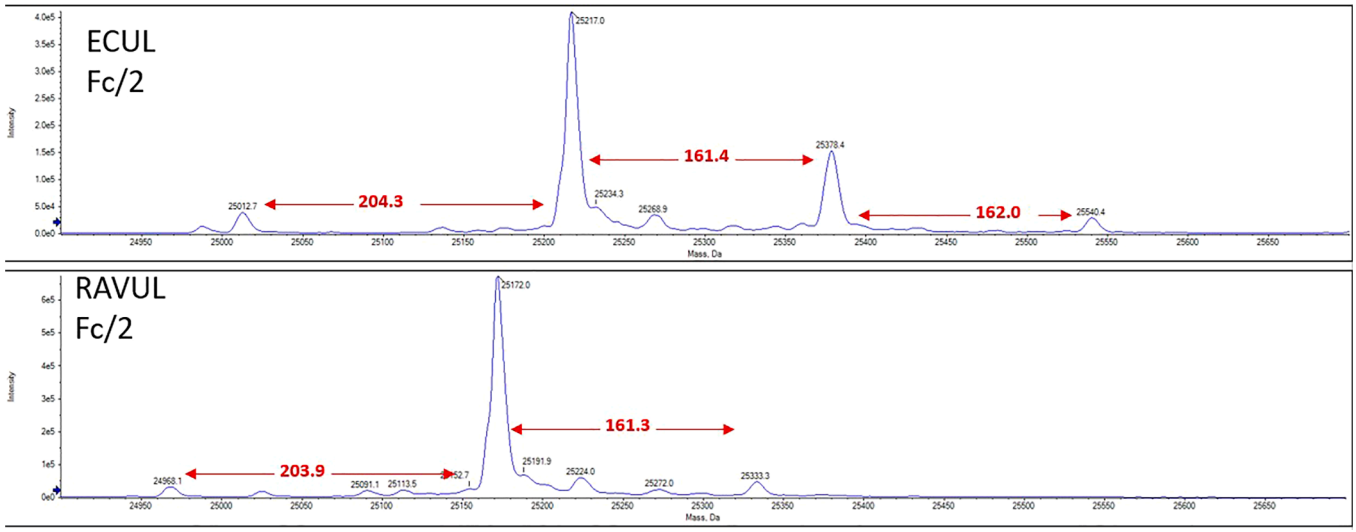


Fig. 5. ECUL and RAVUL glycosylation patterns Ides digest + DTT reduction gives the Fc/2 fragment composed of CH2 + CH3 for ECUL and RAVUL. This fragment carries the glycosylation site in Asn297 of the CH2. This representation suggests different glycosylation patterns between ECUL and RAVUL.

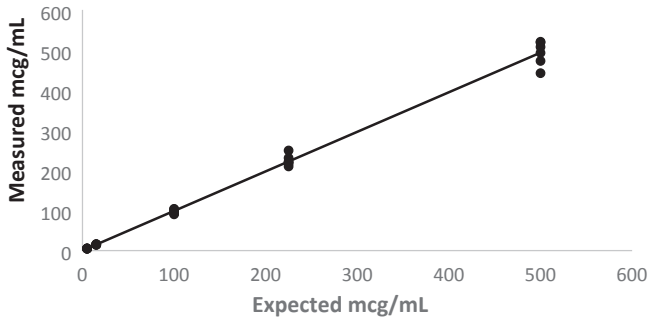


Fig. 6. RAVUL LDT linearity against RAVUL standards. Linearity was performed over 6 runs for concentrations 5, 15, 100, 225 and 500 mcg/mL.

Table 3
Analytical sensitivity of the RAVUL LDT.

ANALYTICAL SENSITIVITY					
	Area counts			Concentration	
	LOB 0 mcg/mL	2.5 mcg/mL	LLOQ 5 mcg/mL		LLOQ 5 mcg/mL
N of runs	17	16*	17	**N**	17
Mean Area Counts	697,708	2,246,348	5,578,238	**Mean**	5.0
SD of Area Counts	316,042	724,819	2,736,152	**SD**	0.44
				CV%	8.8%
Calculated LLOD = Mean(0mcg/mL) + 3*SD(0mcg/mL)	1,645,834 counts or approximately 1.3 mcg/mL				

*Note: the 2.5 mcg/mL was only run on 16 of the 17 runs as it was missed on the first run. *Note: Calculated LLOD concentration of 1.3 mcg/mL was calculated from regression line of 0, 2.5, and 5 mcg/mL and their mean area counts; y = 976106x + 400500.

Mean % recovery from the original stock (used to make standards and controls for the LDT) was 99% while the other 3 vials had mean % recoveries of 100%, 91% and 92%, respectively (Table 6).

Implementation of the RAVUL LDT

In summary, the evidence shown here demonstrates that RAVUL quantitation is possible using the same enrichment and LC-MS method used for ECUL, although using specific RAVUL sets of standards. Samples submitted for analysis of ECUL or RAVUL can undergo IgG4 enrichment and reduction steps simultaneously, optimizing workflows in the laboratory for low volume tests such as these. The use of specific RAVUL standards and controls is necessary for accurate quantitation.

Table 4
Precision of the RAVUL LDT.

PRECISION					
INTRA-ASSAY					
	15 mcg/mL	50 mcg/mL	100 mcg/mL	225 mcg/mL	400 mcg/mL
N of replicates	20	20	20	20	20
Measured Range	13 to 50	46 to 59	93 to 131	208 to 294	367 to 494
Measured Mean	14	52	104	247	413
SD	0.63	3.10	7.60	21.37	35.48
%CV	4.6%	5.9%	7.3%	8.7%	8.6%
%Error	−8.0%	4.8%	3.5%	9.7%	3.3%
INTER-ASSAY					
	15 mcg/mL	**35 mcg/mL**	**50 mcg/mL**	**100 mcg/mL**	**225 mcg/mL**	**400 mcg/mL**
N of runs	13	13	13	13	13	13
Measured Range	13 to 21	31 to 43	42 to 58	82 to 113	203 to 245	355 to 451
Measured Mean	15	37	48	96	225	404
SD	2.01	4.13	4.08	9.14	12.90	25.24
%CV	13.3%	11.2%	8.5%	9.6%	5.7%	6.3%
%Error	0.6%	5.7%	−4.0%	−4.3%	0.1%	0.9%

Discussion

RAVUL and ECUL have an identical light chain mass, with a 4 amino acid difference in their respective heavy chains. The Q-TOF MS platform characterization experiments made it possible to document the identical light chain mass between ECUL and RAVUL in addition to confirming the differences in the heavy chain sequence of the two complement inhibitors. The Sciex OS software package is innovative in its protein quantitation function, suggesting it could soon be possible to accurately

Table 5
RAVUL LDT recovery using RAVUL standards.

Spike (mcg/mL)	Measured (mcg/mL)		Mean (mcg/mL)	Recovery
10	10.5	10.4	10.6	106%
	10.0	11.2		
	10.9			
25	27.2	26.3	28.0	112%
	26.0	29.8		
	30.9			
150	152	139	152	101%
	152	152		
	163			
220	235	221	228	101%
	227	237		
	218			
400	369	407	400	100%
	424	406		
	394			

Note: Recovery was performed using RAVUL pharmacy stock spiked into NHS at 5 different levels to span the AMR. Mean is an average of the 5 spikes at each concentration.

Table 6
RAVUL LDT recovery of different vials of pharmaceutical preparations.

	Spike (mcg/mL)	Measured (mcg/mL)	Recovery	Mean Recovery
Bottle 1	25	27.5	110%	99%
	75	76.6	102%	
	150	140	93%	
	300	323	108%	
	400	337	84%	
Bottle 2	25	28.4	114%	100%
	75	88.8	118%	
	150	149	100%	
	300	271	90%	
	400	306	76%	
Bottle 3	25	22.1	88%	91%
	75	77.7	104%	
	150	143	95%	
	300	274	91%	
	400	309	77%	
Bottle 4	25	25.7	103%	92%
	75	73.6	98%	
	150	136	90%	
	300	252	84%	
	400	333	83%	

Note: Recovery was performed using RAVUL pharmacy stock spiked into NHS at 5 different levels to span the AMR from 4 different bottles of pharmacy stock.

quantitate using the heavy chain mass of t-mAbs. The quantitation of immunoglobulin heavy chains is unusual in the clinical laboratory, as they are very heavy (~50 KDa) with possible glycosylation making their mass spectrums more complex, with implications on the limit of detection. The proof of concept experiment in this study for both ECUL and RAVUL did not attempt to define a LLOQ for the heavy chain quantitation, and only tested the recovery of therapeutic concentrations of the t-mAbs, which are expected to be greater than 50 mcg/mL. We anticipate the need for a test that accurately distinguishes ECUL and RAVUL would only exist when patients are transitioning from ECUL to RAVUL, and, hence, would present with very high concentrations of the t-mAbs in circulation (>100 mcg/mL) during that initial induction stage where infusions happen more often, according to pharmacokinetics data from the clinical trials [12]. The heavy chain quantitation is a potential future application if further development is undertaken in this area, but it is not yet ready for implementation in a clinical mass spectrometry laboratory due to requirements for complex spectra review and data manipulation.

The QExactive orbitrap platform, which already houses other t-mAb LDTS and conveniently, the ECUL LDT, became an easy choice of platform for implementation of an LDT for RAVUL. Initial experiments attempted to quantitate RAVUL using the current ECUL LDT and ECUL standards. There was a trend in over-recovery of RAVUL in the initial validation experiments, and we went back to the TOF-MS full scan data to review the heavy chain differences between the t-mAbs. Since the 4 amino acid difference was sufficient to change RAVUL affinity for C5, and also increase its half-life by 4-fold when compared to ECUL based on the clinical trials data, we thought the structural difference could also affect the binding of RAVUL to the IgG4 camelid beads used in the immunoenrichment step. In addition to the different amino acid compositions, the TOF-MS data hinted that the glycosylation pattern between the molecules was different. N-glycans have an invariable core structure composed of N-acetylglucosamine and three mannose moieties, with additional variable monosaccharides, such as fucose, galactose and others [13], which can result in more than 400 different combinations. With the experiment performed, we cannot fully identify the composition of the N-glycans present in the two molecules. N-glycans have been a target for optimization of therapeutic monoclonal antibody efficacy, a term coined glycoengineering. Their diversity and heterogeneity contributes to antibody effector function and impacts molecule stability, conformation and aggregation, so it seems likely that the combination of the 4 amino acid change, plus the glycosylation pattern differences, did impact the enrichment step with a preference for RAVUL over ECUL. The use of the RAVUL specific standards eliminated the bias initially observed and allowed accurate quantitation in subsequent validation experiments.

There is no known process or application to test for RAVUL concentration at the beginning of therapeutic regimens, but the TOF-MS Sciex OS software could be useful for rare situations when testing is ordered while individuals are switching from one medication to another. Considering that RAVUL is a newer medication with a longer half-life, the most common transition direction should be to discontinue ECUL and start therapy with RAVUL. To move forward with test implementation in an expedited manner, which wouldn't require new software or new instrumentation, we validated a standalone LDT for RAVUL. Both ECUL and RAVUL LDTs are performed clinically in our Clinical Mass Spectrometry Laboratory on a high throughput QExactive orbitrap MS platform. When patients are transitioning from ECUL to RAVUL, the results reported will be the sum of ECUL + RAVUL, if ECUL is still present in the sample; the specific standards were shown to be inequivalent. For those cases, it is most appropriate to wait until the first maintenance dose trough measurement before ordering testing for RAVUL quantitation. If testing is warranted during the transition period, when measured concentrations of the t-mAb using the orbitrap light chain method are in the therapeutic range, running the samples on the research Q-TOF MS can help determine if the sample contains both RAVUL and ECUL, or only one or the other, by using the heavy chain mass for detection and quantitation.

Importantly, this study reports an analytical characterization of the 2t-mAbs and the analytical validation of an LDT for RAVUL using spiked NHS. Real patient samples were not part of this validation, as the diseases for which RAVUL is prescribed are very rare and in our institution only a few patients have made the transition from ECUL to RAVUL or are at the stage of remission where a potential dose de-escalation is being considered. Hence, samples were not available at the time of validation, which is a limitation of this study. It is unknown from this validation study if the patient serum samples tested for RAVUL will behave the same as the spiked pharmaceutical preparations, but considering that the t-mAb should circulate free or in an immune complex with C5, it is likely that patterns of binding affinity from serum samples of patients undergoing therapy will mimic the ones from spiked pharmaceutical preparations, based on our experience with other t-mAbs. The half-life of RAVUL in circulation is 4-fold longer than ECUL, and the stability of RAVUL in serum from patients taking RAVUL is estimated to be at least the same as ECUL (28 days after blood draw, data not shown).

Dose de-escalation or therapy discontinuation have become common practice in academic centers for ECUL [14–17], but data does not yet exist for RAVUL. Having a validated laboratory assay for RAVUL will

enable clinical practice to establish correlations between RAVUL therapeutic monitoring and improved clinical outcomes.

Conclusion

Personalizing therapy for patients taking ECUL or RAVUL is possible. aHUS patients with low risk of relapse have benefited from improved quality of life with fewer ECUL infusions [16]. Furthermore, the cost to healthcare can be decreased when full complement blockage may be achieved by giving patients a smaller drug dose and still maintaining the full complement blockage for therapeutic effect. It is likely that RAVUL use will improve quality of life for patients even more, given its favorable dosing regimen every 8 weeks when contrasted to ECUL every 2 weeks. For aHUS patients achieving remission on RAVUL, TDM and discontinuation of therapy may become hot topics, and strategies to measure both complement blockage using assays such as AH50 [18], and drug quantitation that can accurately measure the t-mAb will prove as helpful tools to clinicians enrolling patients in such studies in the near future.

Conflict of interests

Paula Ladwig has no competing interests to declare. Maria Alice V. Willrich has intellectual property on measurement of therapeutic monoclonal antibodies by LC-MS/MS (US patent 42580) and this research was conducted in compliance with Mayo Clinic Conflict of Interest policies.

Acknowledgements

The authors would like to thank Dr. David Murray for reviewing the glycosylation data and helpful discussions for this manuscript.

References

[1] K. McKeage, Ravulizumab: first global approval, Drugs 79 (3) (2019) 347–352.
[2] A.G. Kulasekararaj, et al., Ravulizumab (ALXN1210) vs eculizumab in C5-inhibitor-experienced adult patients with PNH: the 302 study, Blood 133 (6) (2019) 540–549.
[3] R.P. Rother, et al., Discovery and development of the complement inhibitor eculizumab for the treatment of paroxysmal nocturnal hemoglobinuria, Nat. Biotechnol. 25 (11) (2007) 1256–1264.
[4] C. Passot, et al., Feasibility and safety of tailored dosing schedule for eculizumab based on therapeutic drug monitoring: lessons from a prospective multicentric study, Br. J. Clin. Pharmacol. (2020).
[5] M. Cugno, et al., Complement functional tests for monitoring eculizumab treatment in patients with atypical hemolytic uremic syndrome, J. Thromb. Haemost. 12 (9) (2014) 1440–1448.
[6] G. Ardissino, et al., Discontinuation of eculizumab maintenance treatment for atypical hemolytic uremic syndrome: a report of 10 cases, Am. J. Kidney Dis. 64 (4) (2014) 633–637.
[7] G. Ardissino, et al., Discontinuation of eculizumab treatment in atypical hemolytic uremic syndrome: an update, Am. J. Kidney Dis. 66 (1) (2015) 172–173.
[8] M.A.V. Willrich, et al., The impact of eculizumab on routine complement assays, J. Immunol. Methods 460 (2018) 63–71.
[9] P.M. Ladwig, D.R. Barnidge, M.A.V. Willrich, C.J. Papasian, Mass spectrometry approaches for identification and quantitation of therapeutic monoclonal antibodies in the clinical laboratory, Clin. Vaccine Immunol. 24 (5) (2017).
[10] P.M. Ladwig, D.R. Barnidge, M.A.V. Willrich, Quantification of the IgG2/4 kappa monoclonal therapeutic eculizumab from serum using isotype specific affinity purification and microflow LC-ESI-Q-TOF mass spectrometry, J. Am. Soc. Mass Spectrom. 28 (5) (2017) 811–817.
[11] K.W. Cradic, et al., Vedolizumab quantitation using high-resolution accurate mass-mass spectrometry middle-up protein subunit: method validation, Clin Chem Lab Med 58 (6) (2020) 864–872.
[12] L. Sahelijo, et al., First in human single-ascending dose study: safety, biomarker, pharmacokinetics and exposure-response relationships of ALXN1210, a humanized monoclonal antibody to C5, with marked half-life extension and potential for significantly longer dosing intervals, Blood 126 (23) (2015) 4777.
[13] M. Kiyoshi, et al., Glycosylation of IgG-Fc: a molecular perspective, Int Immunol 29 (7) (2017) 311–317.
[14] G. Ardissino, et al., Discontinuation of eculizumab treatment in atypical hemolytic uremic syndrome: an update, Am. J. Kidney Dis. 66 (1) (2015) 172–173.
[15] G. Ardissino, et al., Discontinuation of eculizumab maintenance treatment for atypical hemolytic uremic syndrome: a report of 10 cases, Am. J. Kidney Dis. 64 (4) (2014) 633–637.
[16] F. Fakhouri, et al., Pathogenic variants in complement genes and risk of atypical hemolytic uremic syndrome relapse after eculizumab discontinuation, Clin J Am Soc Nephrol 12 (1) (2017) 50–59.
[17] J.F.M. Wetzels, N.C.A.J. van de Kar, Discontinuation of eculizumab maintenance treatment for atypical hemolytic uremic syndrome, Am. J. Kidney Dis. 65 (2) (2015) 342.
[18] M.A.V. Willrich, et al., Monitoring Ravulizumab effect on complement assays, J. Immunol. Methods 490 (2021) 112944.