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Ocrelizumab quantitation by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Introduction: Ocrelizumab is a monoclonal anti-CD20 antibody approved for the treatment of multiple sclerosis (MS). The clinical value of therapeutic drug monitoring (TDM) for this antibody in treatment of MS is unknown, and an adequately specific and precise quantitation method for ocrelizumab in patient serum could facilitate investigation. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based quantitation methods have been shown to have higher analytic specificity and precision than enzyme-linked immunosorbent assays.
Objectives: To establish and validate an LC-MS/MS-based quantitation method for ocrelizumab.
Methods: We present an LC-MS/MS-based quantitation method using immunocapture purification followed by trypsinization and analysis by a triple quadrupole mass analyzer obtaining results within the same day.
Results: We found that the ocrelizumab peptide GLEWVGAIYPGNGDTSYNQK (Q1/Q3 Quantifier ion: 723.68^{3+}/590.77 $y11^{2+}$ Qualifier ion: 723.68^{3+}/672.30 $y12^{2+}$) can be used for quantitation and thereby developed a method for quantifying ocrelizumab in human serum with a quantitation range of 1.56 to 200 μg/mL. The method was validated in accordance with EMA requirements in terms of selectivity, carry-over, lower limit of quantitation, calibration curve, accuracy, precision and matrix effect. Ocrelizumab serum concentrations were measured in three MS patients treated with ocrelizumab, immediately before and after ocrelizumab infusion, with additional sampling after 2, 4, 8 and 12 weeks. Measured serum concentrations of ocrelizumab showed expected values for both Cmax and drug half-life over the sampled time period.
Conclusion: We have established a reliable quantitation method for serum ocrelizumab that can be applied in clinical studies, facilitating the evaluation of ocrelizumab TDM in MS.

Introduction

Multiple sclerosis (MS) is a chronic immune-mediated disease causing lesions with demyelination and axonal damage in the central nervous system [1]. The exact pathogenesis is not known, but evidence strongly suggests the involvement of both *T*-cells and B-cells. Along with the development of B-cell depletion therapies for MS, the importance of B-cells in the pathogenesis of the disease has increasingly been studied [2–4]. This includes possible disease mechanisms through antigen presentation, autoantibody production, or cytokine secretion [5–7]. As a central role of B-cells in the pathogenesis of MS was revealed, therapeutic monoclonal antibodies (*t*-mAbs) depleting B-cells through binding to CD20 proved efficient [8,9,10,11]. CD20 is a cell surface antigen located on pre-B-cells, mature B-cells and memory B-cells.

Abbreviations: EMA, European Medicines Agency; IDA, Information dependent acqusition; IgG1, immunoglobulin G1; LC-MS/MS, Liquid chromatography-tandem mass spectrometry; MS, Multiple sclerosis; MWCO, molecular weight cut-off; OVERLORD-MS, Ocrelizumab vErsus Rituximab Off-Label at the Onset of Relapsing MS; QTOF, quadrupole time-of-flight; QTRAP, quadrupole ion trap; ROS-MS, Rituximab and Ocrelizumab in Serum with Multiple Sclerosis study; TDM, therapeutic drug monitoring; *t*-mAb, therapeutic monoclonal antibody; UHPLC, ultra high performance liquid chromatography.

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Ocrelizumab (Ocrevus) is a humanized CD20 binding *t*-mAb engineered by Genentechfor Roche Pharma [12]. According to the assessment report, the drug molecule is based on the human immunoglobulin G1 (IgG1) framework, containing heavy chain VHIII and light chain VκI subgroup sequences [13]. The recombinant mAb is produced in Chinese hamster ovary cells and is reported to consist of two identical 213 residue light chains and two identical 451 or 452 residue heavy chains.

A standard dose of 600 mg ocrelizumab every-six months is currently used for the treatment of MS [14]. However, available data suggests that the clinical effect may exceed this treatment interval and, accordingly, that the interval could be increased [14]. Although generally well tolerated, side effects such as infections, as well as reduced vaccine response, are associated with ocrelizumab [15,16]. Secondary therapy failure, with *t*-mAbs apparently losing effect over time, is also a clinical challenge [17]. Further, the population included in drug development studies may differ from the actual patient population [18]. Taken together, individualization of the treatment with ocrelizumab, both with regard to dosage and dosing interval, could be of potential benefit to patients [19].

Serum concentrations of a drug can be a useful tool in personalization of drug treatment if incorporated in a therapeutic drug monitoring (TDM) approach. With regard to ocrelizumab, no treatment approach using TDM has been published. In studies describing the pharmacokinetics of ocrelizumab, enzyme-linked immunosorbent assay (ELISA) methods were used for determination of serum concentrations [20]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) methods have been shown to offer several advantages compared to ELISA, such as a higher analytic specificity and precision, no reliance on mAb-specific reagents, such as recombinant antigens, a shorter development time and possibility of multiplexing [21,22].

Here, we present a novel LC-MS/MS method for quantitation of ocrelizumab in serum. After establishing the method, it was evaluated in accordance with the EMA guideline for bioanalytical method validation and applied to measure ocrelizumab serum concentrations in three patients with MS followed for 12 weeks after ocrelizumab administration. This highly specific method, with a clinically relevant quantitation range of 1.56–200 μg/mL, can be utilized to examine various pharmacological aspects of ocrelizumab treatment in a reliable manner.

Material and methods

Chemicals and reagents

Sodium chloride (Cat. 0241-500G), sodium phosphate (Cat. 0404-500G), methanol (Cat. 85800.320) and acetonitrile (Cat. 83642.320) were purchased from VWR Life Science (Radnor, PA, USA). Ammonium bicarbonate (Cat. 11213–1 KG) was purchased from Honeywell (Charlotte, NA, USA). Acetic acid (Cat. 1.000.63.100), formic acid (Cat. 1.00264.1000), calcium chloride (Cat. 2382) and hydrochloric acid (Cat. 1.00317.1000) were purchased from Merck (Darmstadt, Germany). Dried synthetic peptides were purchased from Vivitide (Gardner, MA, USA) with greater than 95 % purity, isotope-labelled lysine (13C6,15 N2) and arginine (13C6,15 N4) residues. Peptide stock solutions were made by resuspension in ultrapure water to a concentration of 10 μg/mL and stored at −20 °C in glass vials. Pierce mass spectrometry-grade trypsin protease (Cat. 90058) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Trypsin stock solutions were made by resuspension in acetic acid (50 mM) to a concentration of 0.1 g/L and stored at 4 °C. Purified horse IgG (AS10867) was purchased from Agrisera (Vännäs, Sweden). For spiking of serum samples we used minute surplus ocrelizumab (Ocrevus, Roche, Basel, Switzerland) volumes available after infusion of patients included in the OVERLORD-MS study (see below). Ultrapure water was obtained from a Milli-Q Advantage A10 (Merck Millipore, Burlington, MA, USA).

Patient samples

The patient samples included in this study were obtained through the *Rituximab and Ocrelizumab in Serum from patients with Multiple Sclerosis* (ROS-MS) study. This study was approved by the Western Norway Regional committee for medical and health research ethics (REK approval no. 228768) and all patients provided written informed consent. ROS-MS is a sub-study of *Ocrelizumab* vErsus *Rituximab Off-Label at the Onset of Relapsing MS Disease* (OVERLORD-MS) (REK approval no. 66391). In OVERLORD-MS, treatment-naïve male and female patients aged 18–60 years, with newly diagnosed MS, are randomized 2:1 to treatment with rituximab or ocrelizumab. Ocrelizumab is administered as a 600 mg intravenous infusion every 6 months. The duration of infusion is 4 h. Blood samples in ROS-MS were collected in tubes without additives. Serum was obtained and stored at −80 °C until analysis. One sample was drawn before infusion, and one within 30 min after the infusion. Further, patients included in ROS-MS visited the hospital to provide a blood sample 2, 4, 8, 12 and 24 weeks after the infusion. In the present study, 0–24-week samples from 3 patients were analyzed.

Signature peptide identification

The protein sequence of ocrelizumab was obtained from Inxight Drugs [23]. PeptideMass [24] was used for generating lists of theoretical tryptic peptides. BLAST [25] was used to search for endogenous peptide sequences in the human proteome. Protein sequence alignments were generated using Clustal Omega [26]. The sample analyzed with QTOF was digested ocrelizumab (1 μL), prepared by mixing ocrelizumab (3 g/L), ammonium bicarbonate (50 mM, pH 7.8), urea (2 M), and $CaCl_2$ (10 mM) with trypsin (5 mg/L) and incubated at 60 °C for 3 h, followed by incubation at 37 °C for 16 h. The digestion was stopped with formic acid (1 %, v/v) and ultrafiltration, using an AcroPrep Advance 96 well 350 μL 3 kDa MWCO plate (8033, Pall Corporation, Port Washington, NY, USA). Initial time course experiments showed that an incubation time of 1 h gave the highest amount of signal (data not shown). For experimental peptide identification analyses, we used an ExionLC AD UHPLC with a Kinetex 2.6 μm XB-C18, 100 × 2.1 mm column (Phenomenex, Torrance, CA, USA) connected to a QTOF X500R (SCIEX, Concord, ON, Canada). We used 0.1 % formic acid in ultrapure water for mobile phase A and 0.1 % formic acid in acetonitrile for mobile phase B, with a flow rate of 0.2 mL/min. Peptide separation was achieved using a gradient from 5 % B at 1 min to 10 % B at 2 min, 30 % B at 12 min, 90 % B at 14.5 min and re-equilibration with 5 % B for 5.5 min. The total run time was 20 min. The mass analyzer was set to positive mode and data collected from 4 to 13 min of the LC run. Ion source gas 1 and 2 were 40 psi; curtain gas 30 psi; CAD gas 7; ion source temperature 500 °C; and ion spray voltage 5500 V. The single and tandem mass spectrometry (MS/MS) data was collected through an information dependent acquisition (IDA) experiment, selecting peaks for MS/MS between 350 and 2000 Da, with accumulation time for 0.125 s. The declustering potential was 100 V (spread 0 V), and collision energy 10 V (spread 0 V). IDA criteria for peak selection were set to peptide mode with maximum 15 candidate ions; 100 cps intensity threshold; dynamic background subtraction and dynamic collision energy for MS/MS were enabled: picking charge states between 1 and 7 and excluding former candidate ions for 3 s after 2 occurrences. For MS/MS, data was collected from 50 to 2000 Da, the declustering potential was 100 V (spread: 0 V), accumulation time of 0.05 s and collision energy 35 V (spread at 15 V).

Sample preparation

The serum samples for ocrelizumab quantitation underwent partial purification by isolation of immunoglobulins, using Protein G Spin Plates for IgG Screening (45204, Thermo Scientific, Rockford, IL, USA). The plate containing Protein G resin was centrifuged to remove the storage solution. All centrifugations of this plate were at 1000 g for 1

min using a swing out centrifuge at 25 °C. A plate incubator with shaking was used for all plate incubations. The resin was washed using a binding buffer, consisting of a saline phosphate buffer (phosphate, 100 mM, pH 7.3 adjusted using hydrochloric acid; sodium chloride, 100 mM). The serum sample (20 µL) was premixed with the binding buffer (80 µL), containing 150 µg/mL of purified horse IgG. The sample mixture was added to the Protein G resin plate and incubated with mild agitation for 30 min at 22 °C. After a centrifugation step to let the sample mixture pass through the Protein G resin, the resin was washed three times by additional centrifugation; first with 500 µL of binding buffer, then with 500 µL phosphate buffer (100 mM, pH 7.3), and finally with 500 µL of ultrapure water. The bound immunoglobulins were eluted by addition of 150 µL of elution buffer (acetonitrile, 50 % v/v; formic acid, 0.1 % v/v), followed by incubation with mild agitation for 10 min at 22 °C and elution by centrifugation to a 1 mL Nunc 96 DeepWell PP plate (260252, Thermo Fisher Scientific, Waltham, MA, USA). An additional volume of 150 µL of elution buffer was added to the Protein G resin with incubation and elution as mentioned above. The pooled 300 µL of immuno-purified sample was dried by a nitrogen gas flow for 3 h at 40 °C using a TurboVap 96 (Caliper LifeSciences, Hopkinton, MA, USA) solvent evaporator. The dried sample was resuspended in 100 µL of a digestion buffer (ammonium bicarbonate, 50 mM, pH 7.8; methanol, 5 %; MS-grade trypsin, 5 µg/mL; isotope labeled ocrelizumab peptide, 100 ng/mL; isotope labeled horse IgG peptide, 25 ng/mL), incubated for 10 min with mild agitation at 22 °C, and finally incubated for 50 min at 53 °C to digest the proteins. After the proteolytic digestion, the sample was cooled on ice for 10 min before an ultrafiltration step using an AcroPrep Advance 96 well 350 µL 10 kDa MWCO plate (8034, Pall Corporation, Port Washington, NY, USA). The filter plate containing the digested sample was placed on top of a 96 well PP microplate (651201, Greiner Bio-One, Frickenhausen, Germany), used as a collection plate, and centrifuged at 1500 g for 20 min at 25 °C. The filtered sample was injected into an LC-MS/MS system for quantitation.

Quantitative LC-MS/MS analysis

The LC-MS/MS system used for quantitation of ocrelizumab was an ExionLC AD UHPLC connected to a QTRAP 6500 + mass analyzer (SCIEX, Concord, ON, Canada). 0.1 % formic acid in ultrapure water was used as mobile phase A and 0.1 % formic acid in acetonitrile as mobile phase B. The prepared samples (5 µL) were loaded onto a Kinetex 2.6 µm XB-C18, 100 × 2.1 mm column (Phenomenex, Torrance, CA, USA) with a mobile phase mixture of 5 % B and flow rate of 0.5 mL/min. Peptide separation was achieved using a gradient from 5 % B at 0.5 min to 10 % B at 1.5 min, 30 % B at 6.5 min, 100 % B at 7 min and maintained for 1 min, then re-equilibration with 5 % B for the final 2 min. The total run time of one sample was 10 min. The mass analyzer collected data in positive mode from 3.0 to 6.2 min during the run with a declustering potential of 60 V; entrance potential: 12 V; collision cell exit potential: 19 V; ion source curtain gas: 35 psi; collision gas: high; ion spray voltage: 5500 V; ion source temperature: 500 °C and ion source gas 1/2 at 40/45 psi. The mass transitions were analyzed using a dwell time of 50 ms and are listed in Table 1.

Data processing

The LC-MS/MS data obtained for quantitation was processed using Analyst 1.7 (SCIEX, Concord, ON, Canada). Integrated analyte peak areas were normalized to the corresponding peak area of the labeled peptide internal standard. The normalized ocrelizumab peptide peak area was then normalized again to the normalized horse IgG peptide peak area of the same sample. This signal response was used to create a standard curve against 8 levels of spiked concentrations of ocrelizumab in blank serum. A quadratic fit with 1/x2 weighting was used for calibration. LC-MS/MS data obtained with the QTOF X500R was processed in Sciex OS 2.0.1.48692 (Concord, ON, Canada).

Method validation

The quantitation method was validated in accordance with the EMA guideline for bioanalytical method validation [27] in for selectivity, carry-over, lower limit of quantitation, calibration curve, accuracy, precision and matrix effect.

Results

A signature peptide suitable for quantitation

Theoretical trypsinization of the ocrelizumab protein sequence resulted in one cysteine-free peptide with appropriate mass for the mass analyzer, as well as a unique sequence in the human proteome according to BLAST database searches. Peptide identification of trypsinized ocrelizumab using a QTOF mass analyzer could detect this peptide (Fig. 1), while it was not detected in trypsinized blank serum. The sequence of the peptide, located in the heavy chain of ocrelizumab, is GLEWVGAIYPGNGDTSYNQK. The peptide could also be detected using a QTRAP mass analyzer, yielding a measurable signal with satisfactory sensitivity and without interference, allowing quantitation.

Quantitation method overview

The sample preparation method presented is based on isolation of immunoglobulin using immobilized protein G, followed by rapid trypsinization and filtration prior to LC-MS/MS analysis. Similar methods have been described to quantify other monoclonal antibodies [28,29], but were adapted by us for quantitation of ocrelizumab. For calibration, we spiked blank serum with known amounts of ocrelizumab and analyzed these samples in parallel with samples of unknown concentration. A calibration range of 1.56–200 ug/mL was found to be appropriate considering the serum levels found in MS patients within 3 months of ocrelizumab treatment (Fig. 2). To monitor the preparation of each sample during isolation of immunoglobulins, a small amount of horse serum IgG was added as an internal standard. Errors due to liquid handling or sample matrix effects interfering with immunoglobulins binding to protein G can be accounted for due to the known amount of spiked foreign IgG. This method has previously been used for quantitation of monoclonal antibodies [30]. Synthetic stable isotope-labeled (SIL) peptides were added along with the trypsinization buffer and

Table 1
Analyte peptide sequences, fragment ions and fragmentation collision energy used for quantitation of ocrelizumab.

Protein	Analyte peptide sequence	Ion type	Precursor ion	Fragment ion	CE (V)
Ocrelizumab	GLEWVGAIYPGNGDTSYNQK	Quantifier	723.68 (3 +)	590.77 ($y11^{2+}$)	25.0
		Qualifier	723.68 (3 +)	672.30 ($y12^{2+}$)	20.0
	GLEWVGAIYPGNGDTSYNQK*	Quantifier	726.34 (3 +)	594.77 ($y11^{2+}$)	25.0
	K* = K(13C,15 N2)	Qualifier	726.34 (3 +)	676.30 ($y12^{2+}$)	20.0
Horse IgG	VNNQALPQPIER	Quantifier	689.90 (2 +)	739.40 ($y6^{1+}$)	35.1
		Qualifier	689.90 (2 +)	514.30 ($y4^{+1}$)	35.1
	VNNQALPQPIER*	Quantifier	694.90 (2 +)	749.40 ($y6^{1+}$)	35.1
	R* = R(13C,15 N4)	Qualifier	694.90 (2 +)	524.30 ($y4^{+1}$)	35.1

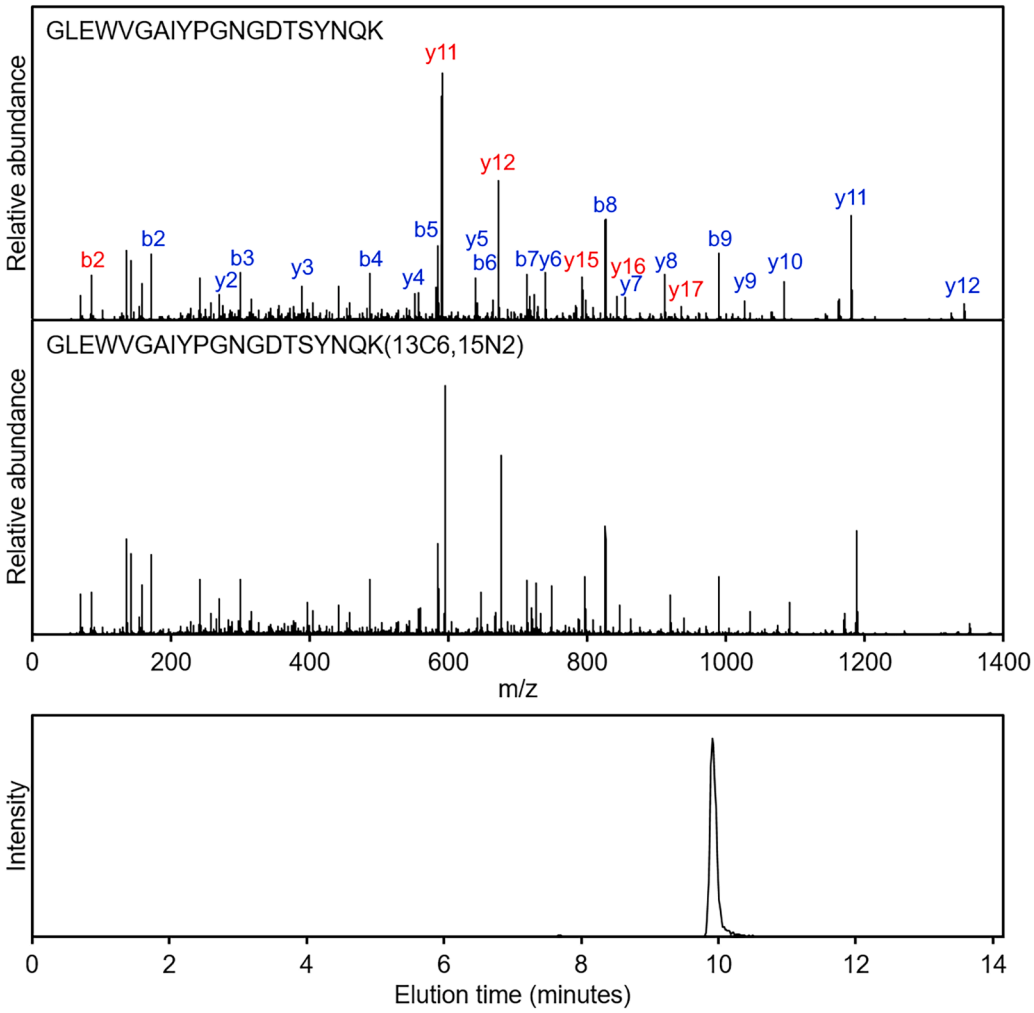

Fig. 1. Mass fragmentation spectra of the signature peptide (723.68 *m/z*) and SIL-peptide (726.39 *m/z*) collected using a QTOF mass analyzer. Ions with 1 + charge in blue, 2 + charge in red. Below, an extracted-ion chromatogram for the mass 723.68 ± 0.05 Da, representing the signature peptide mass. Note that a longer gradient was used compared to QTRAP analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

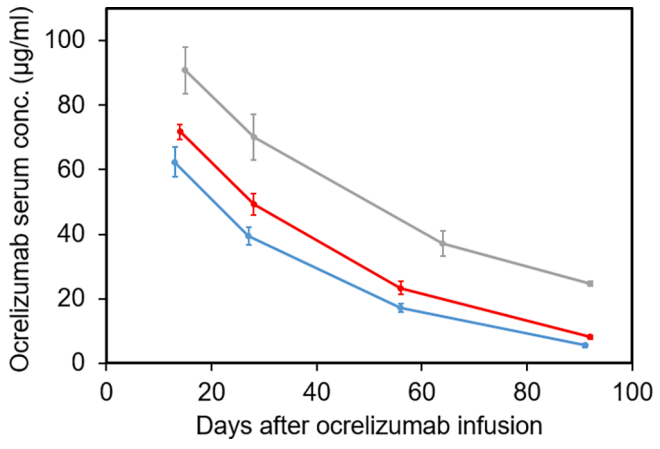

Fig. 2. Ocrelizumab serum concentrations in three patients with MS, undergoing treatment with ocrelizumab. Error bars display ± one standard deviation. Cmax concentrations were 243 (gray), 220 (red) and N/A (blue) µg/mL. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

used as internal standards for each peptide to be quantified in the mass analyzer. Extracted-ion chromatograms representing quantifier and qualifier ions of peptides from ocrelizumab, horse IgG and their isotope-labeled variants are shown in Supplementary Fig. S1. With the full quantitation process including all required sample preparations performed within a working day and LC-MS/MS analyses running at night, serum levels of ocrelizumab can be obtained for 96 samples within 24 h.

Method validation

The selectivity of the method was verified by analyzing blank serum from 15 individuals. The average response of all these blank samples was 6 % (6.4 % for the quantifier ion and 6.2 % for the qualifier ion) of the lower limit of quantitation, fulfilling the requirement of < 20 %. The lower limit of quantitation was 1.56 µg/mL, the lowest concentration used in the calibration curve, which fulfilled the requirement of having at least a fivefold response compared to blank samples. The upper limit of quantitation was set to 200 µg/mL, the highest concentration of the calibration standard. Samples above this limit were diluted with the blank serum used for the calibration curve. Ocrelizumab quantifier and qualifier responses versus a blank serum sample are shown in Fig. 3. The selectivity of the internal standards also fulfilled the criteria by giving a response from prepared blank serum lower than 5 % of the response in

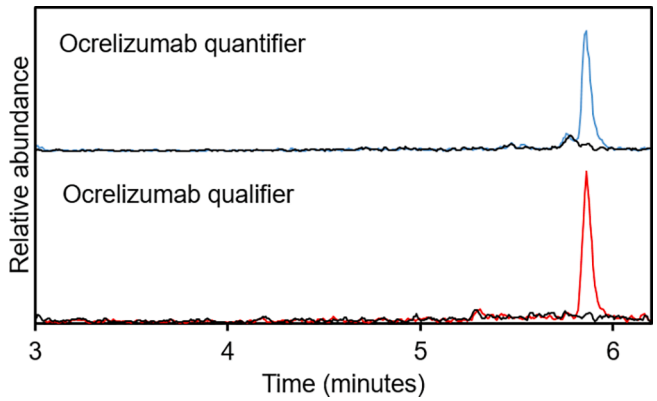

Fig. 3. Extracted-ion chromatogram of ocrelizumab quantifier (blue) and qualifier (red) ions from analyzing the lowest standard concentration versus a blank serum sample (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

samples spiked with internal standards (n = 8, data not shown). By analyzing spiked blank samples with ocrelizumab and/or internal standards in different combinations at the highest concentration used, we could confirm that the response of one analyte did not affect the response from any of the other analytes. The carry-over effect was tested by injecting samples corresponding to the highest calibrator concentration, followed by injection of 2 samples prepared from blank serum (n = 3). There was no detectable carry-over above what was detected in the blank serum. The linearity of the quantitation model was tested by preparing and analyzing 3 replicates of the standard curve (Fig. 4, Table 2). A quadratic fit with weighting $1/x^2$ was determined to be the best fit giving the highest r^2 and best accuracy over all calibration levels. The total variation (Table 3), and variation within a sample series (Table 4), show that the method yields values well within < 15 % CV and ± 15 % accuracy across the entire quantitation range, as required by the EMA guidelines. Matrix effects were assessed by performing sample preparation and analysis of 12 different blank serum samples from 12 different individuals, spiked with either a low (2.0 μg/mL, n = 6) or high (200 μg/mL, n = 6) concentration of ocrelizumab (Table S1). The CV for the calculated concentration of ocrelizumab was 9.4 % for samples spiked with 2.0 μg/mL, and 3.1 % for samples spiked with 200 μg/mL ocrelizumab, well within the EMA guideline requirement of < 15 % CV. The accuracy of the calculated concentration was 98.2 % for samples spiked with 2.0 μg/mL and 101 % for samples spiked with 200 μg/mL ocrelizumab. To test the stability of the prepared samples during a run with the mass analyzer, a pool of prepared samples, extracted from blank serum spiked with 50 μg/mL ocrelizumab, was reinjected 50 times after injection of the calibration standards. All samples were stored inside the instrument autosampler at 10 °C. The CV of the calculated

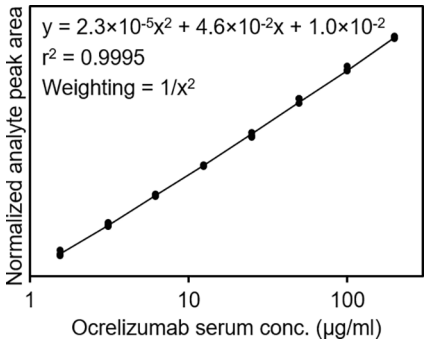

Fig. 4. Calibration curve of measured LC-MS/MS response to spiked ocrelizumab concentration in blank serum (n = 3). Precision and accuracy are presented in table 2.

Table 2
Statistics related to the calibration curve (n = 3) plotted in Fig. 4.

Spiked conc. (μg/mL)	Measured conc. (μg/mL)	Precision (%)	Accuracy (%)
1.56	1.56 ± 0.12	7.5	100
3.13	3.15 ± 0.14	4.4	101
6.25	6.26 ± 0.19	3.1	100
12.5	12.2 ± 0.14	1.2	97
25.0	24.3 ± 1.18	4.9	97
50.0	52.0 ± 3.19	6.1	104
100	102 ± 5.79	5.7	102
200	197 ± 4.89	2.5	99

Table 3
Total variation for quantitation of ocrelizumab (n = 12, days = 6).

Spiked conc. (μg/mL)	Measured conc. (μg/mL)	Precision (%)	Accuracy (%)
1.56	1.67 ± 0.11	7.5	107
6.25	6.41 ± 0.39	6.0	103
25.0	25.5 ± 1.26	5.0	102
200	207 ± 10.5	5.1	104

Table 4
Within-run variation for quantitation of ocrelizumab (n = 6).

Spiked conc. (μg/mL)	Measured conc. (μg/mL)	Precision (%)	Accuracy (%)
1.56	1.76 ± 0.19	10.8	113
6.25	6.56 ± 0.44	6.7	105
25.0	25.7 ± 1.57	6.1	103
200	223 ± 12.5	5.6	111

concentration of the 50 reinjections was 2.3 % and did not show any indication of drifting during the run. The prepared samples, therefore, appeared stable within the time course of analyzing 50 samples. We did not test analyses of more than 50 samples within the same run. The stability of ocrelizumab in serum was tested by spiking blank serum from 4 individuals to a concentration of 50 μg/mL, followed by storage at 4 °C. There was no significant change in ocrelizumab concentration within 4 weeks of storage. Moreover, we could not detect any significant change in ocrelizumab serum concentration in spiked blank serum after one freeze/thaw cycle at −80 °C (n = 3).

Serum concentrations in patients with MS

The serum concentration of ocrelizumab in patients undergoing treatment with ocrelizumab was successfully quantified using our developed method. None of the three patients had a detectable amount of ocrelizumab before treatment as determined by a sample taken immediately prior to initiation of ocrelizumab infusion. This test demonstrates that the patients did not have interfering compounds appearing as the analyte. A serum sample was taken directly after the infusion with ocrelizumab for two of the patients, giving a value for Cmax. These two concentrations were 243 and 220 μg/mL and are close to the Cmax reported by the manufacturer, 212 μg/mL [12], after an identical dosing and serum sampling scheme. The samples taken at subsequent time points showed a decrease in serum concentration of ocrelizumab (Fig. 2), as expected. Using the measured serum concentrations after 2, 4, 8 and 12 weeks after infusion, the half-life of ocrelizumab was calculated as 22, 26 and 39 days for the 3 patients. The first two values come close to 26 days, the reported terminal elimination half-life by the manufacturer [12], while the third patient had a constant higher serum concentration and longer half-life of ocrelizumab.

Discussion

To our knowledge, the method presented in this study is the first published LC-MS/MS-based quantitation method for ocrelizumab. The

quantitation range of the method is 1.56–200 μg/mL and appears to be well suited for serum levels registered in MS patients undergoing treatment with ocrelizumab [20]. The quantitation method was validated in terms of selectivity, carry-over, lower limit of quantitation, calibration curve, accuracy, precision and matrix effect in line with EMA requirements for bioanalytical method validation [27].

Finding a suitable signature peptide for LC-MS/MS-based quantification becomes more is challenging for humanized and fully human *t*-mAbs, where almost the entire protein sequence can be found in native immunoglobulins. However, the uniqueness of *t*-mAbs is located at the antigen-binding site, which contains peptide sequences not present in other immunoglobulins. Indeed, we identified the peptide used for quantitation at the antigen-binding site of ocrelizumab. All other potential peptides for quantitation were excluded based on occurrences in other native immunoglobulins. Using sequence alignments of ocrelizumab and rituximab, a chimeric anti-CD20 *t*-mAb used off-label for treatment of MS, and the solved 3D structure (PDB entry 6VJA) of rituximab in complex with CD20 [31], revealed the location of the signature peptide used for quantitation of ocrelizumab. The peptide is a two-stranded beta-sheet located at the tip of the antigen-specific site, embracing a helix-loop structure of CD20 (Fig. 5a). The equivalent signature peptide in rituximab has an identical sequence with the exception of the fifth residue, which is isoleucine in rituximab and valanine in ocrelizumab. The location of the signature peptide in the antigen-binding site underlines the functional importance of the peptide. Metabolomic modification of this region is expected to affect the interaction with CD20. Functionally active ocrelizumab is, therefore, more likely to have the signature peptide intact compared to regions outside the antigen-binding site.

We here assume that the binding interaction between ocrelizumab to CD20 and rituximab to CD20 are the same. The sequence similarity is high and epitope mapping shows that the binding regions to CD20 are the same for rituximab and ocrelizumab [32,33]. Of note, one should not exclude the possibility that ocrelizumab has some variations in terms of binding to CD20. Ocrelizumab has a few sequence variations compared to rituximab, located within 6 Å of CD20 (Fig. 5b). First, the *N*-terminal residue of the light chain has a modification from glutamine in rituximab to aspartate in ocrelizumab and the residues at position 92 and 93 are serine and phenylalanine in ocrelizumab, but threonine and serine in rituximab. The most significant sequence variations are located in the heavy chain at one beta-loop structure where a S95V mutation is located, followed by G98S, G99N and an additional serine residue, extending the loop in ocrelizumab compared to the rituximab sequence. However, all of the listed sequence variations above, except for the light chain *N*-terminal residue, are present in the crystal structure of a 2H7 Fab domain [34]. This structure overlaps well with the structure of rituximab Fab and, in combination with epitope mapping, strongly suggests that the binding regions are the same for ocrelizumab and rituximab.

The method presented in this study is the final product of screening involving various techniques and parameters. Some aspects concerning method alternatives deserve mentioning. The signature peptide chosen for quantitation was observed to have a low yield after trypzination of ocrelizumab at 37 °C overnight. Trypsination under denaturing conditions with 2 M of urea did not result in a significant improvement, but the issue was resolved through addition of methanol during trypsinization. An increase in the temperature during trypsinization improved the yield further, but could also result in a shoulder peak of the analyte, present in both the quantifier and qualifier chromatogram for both analyte peptide and isotope labeled peptide. This shoulder peak could be reduced by shorter incubation time during trypsinization, further increasing the amount of measured peptide. In terms of the amount of signature peptide, the trypsinization reaction yield appeared complete after approximately 1 h, which also prevented the formation of the shoulder peak. After the high temperature exposure during the trypsinization, there was no sign of instability or signal loss of the signature peptide in the sample. Using the QTOF mass analyzer, we observed that the shoulder peak is caused by a + 1 Da (0.98 Da) mass shift of the signature peptide (data not shown). Fragmentation of this peptide showed that the Asn residue at position 12 carried this + 1 Da mass shift, in line with a deamidation. The following residue after the Asn is a Gly, which increases the mobility of the peptide backbone and the risk of aspartimide formation by a nucleophilic attack by the backbone nitrogen [35]. Apart from the mass spectrometry transitions used as quantifier and qualifier, 2 more ions were in use during method development, but later excluded: the b8-ion with 1 + charge (Q1/Q3 723.68/826.45), which cannot be used together with a C-terminal labeled peptide internal standard, and the y11-ion with 1 + charge (Q1/Q3 723.68/1180.52), where interference problems arose, requiring a longer gradient for proper peak separation. The ideal internal standard for

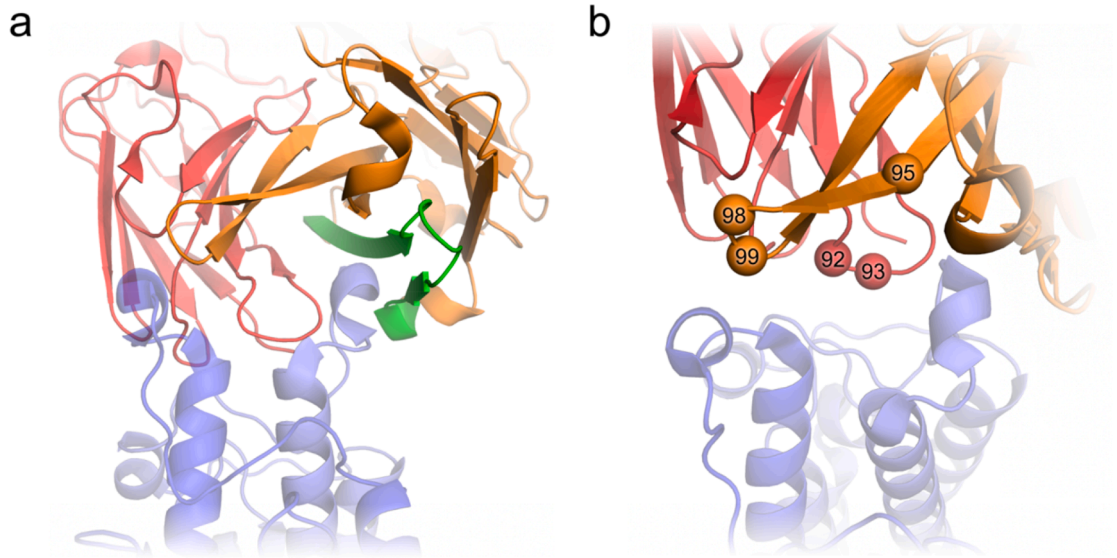

Fig. 5. Structure of rituximab in complex with CD20 (PDB entry 6VJA). Heavy chain in orange, light chain in red, CD20 in blue. (a) Alignment-based, structural location of the signature peptide (green) used for quantitation of ocrelizumab. (b) Sequence variations between rituximab and ocrelizumab within 6 Å to CD20, marked with spheres. Mutations from rituximab to ocrelizumab lcT92S, lcS93F, hcS95V, hcG98S, hcG99N and an extra S residue after position hc99 in ocrelizumab. Figures are generated using PyMol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sample preparation would be isotope labeled ocrelizumab, produced in an identical way as the ocrelizumab given to the patient. This internal standard is not commercially available at present and would lead to a drastic increase in cost of the analysis. However, as also shown in other studies, alternative mAb or IgG molecules can be used as an internal standard for sample preparation in combination with SIL-peptides as internal standards for the LC-MS/MS analysis [30,21,36].

Serum quantitation of ocrelizumab in patients with MS was successful, giving no false positive signal for samples collected before ocrelizumab treatment and expected values after infusion of ocrelizumab. A treatment dose of 600 mg ocrelizumab to an average person, with a blood volume of approximately 5 L of blood and a plasma volume of ~ 55 %, should result in an ocrelizumab concentration of around 218 μg/mL. This is in line both with ELISA-based results from Gibiansky et al. (2021) and with results produced by our LC-MS/MS-based method. The serum levels obtained by LC-MS/MS in this study 2, 4, 8 and 12 weeks after ocrelizumab infusion showed an expected half-life of ocrelizumab in 2 of 3 patients. One of the patients had elevated levels of ocrelizumab and an increased ocrelizumab half-life. The collected dataset is not large enough to draw specific patient-related conclusions, but the method shows potential for use on a larger scale. The previous data from phase 2 and phase 3 trials reported by Gibiansky et al. (2021) were collected using an ELISA-based method. ELISA-based quantitation is commonly used in hospital settings, but frequently suffers from reduced analyte specificity compared to LC-MS/MS-based methods [21,22]. A reliable and specific quantitation method for ocrelizumab allows a more solid ground for investigating future TDM and dosing strategies.

In conclusion, we have developed an LC-MS/MS-based method for quantitation of ocrelizumab in serum, validated the method in accordance with EMA requirements and successfully utilized it for quantitation of serum concentrations in multiple sclerosis patients undergoing treatment with ocrelizumab. The method could be used as a tool to examine the pharmacokinetic and pharmacodynamic properties of ocrelizumab, and potentially in evaluating whether TDM models may be useful in personalized dosing of ocrelizumab in patients.

Ethics statement

The clinical studies described in this publication were approved by the regional committees for medical and health research ethics (REK approval nos. 66391, 228768) and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent to participation in the OVERLORD and ROS-MS studies.

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CRediT authorship contribution statement

Erik I. Hallin: Conceptualization, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Trond Trætteberg Serkland:** Conceptualization, Resources, Project administration, Funding acquisition, Writing – original draft, Writing – review & editing. **Kjell-Morten Myhr:** Conceptualization, Resources, Project administration, Writing – review & editing. **Øivind Grytten Torkildsen:** Conceptualization, Resources, Project administration, Writing – review & editing. **Silje Skrede:** Conceptualization, Resources, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2022.07.004.

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