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METHODOLOGICAL ARTICLE

Quantification of emicizumab by mass spectrometry in plasma of people with hemophilia A: A method validation study

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Abstract

Background: Emicizumab is a new treatment option for people with hemophilia A. Emicizumab was approved with a body-weight-based dosage regimen, without laboratory monitoring requirements. Guidelines, however, recommend measuring emicizumab concentrations when the presence of antidrug antibodies is suspected. Furthermore, drug monitoring can be useful in clinical decision making, in adherence checking, and for research purposes. Therefore, we developed a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for quantifying emicizumab. We performed a validation study on this LC-MS/MS method quantifying emicizumab in the plasma of people with hemophilia A.

Methods: Sample preparation for LC-MS/MS analysis included ammonium sulfate protein precipitation and trypsin digestion. A signature peptide of emicizumab and a matching stable isotope-labeled internal standard were used to quantify emicizumab by LC-MS/MS analysis. Validation was performed in accordance with the "Guideline on Bioanalytical Method Validation" of the European Medicines Agency (EMA). The LC-MS/MS method was cross validated against a modified and calibrated (r^2 Diagnostics) one-stage clotting assay (OSA).

Conclusions: The LC-MS/MS method demonstrated linearity over a wide range of emicizumab concentrations, far exceeding the concentrations observed in people with hemophilia A. Precision and accuracy were excellent, and all other validation parameters were also within the acceptance EMA criteria. Cross validation showed that the LC-MS/MS method and the OSA-based method can be used interchangeably for drug monitoring of emicizumab without the application of a correction factor.

KEYWORDS
drug monitoring, emicizumab, hemophilia A, mass spectrometry, validation study

Essentials

- A method with mass spectrometry (MS) had been developed to quantify emicizumab in human plasma.
- This MS method was validated analytically and cross validated against a current standard method.
- All MS method validation results were well within the acceptance criteria of the European Medicines Agency guideline.
- Excellent agreement between both methods allows interchangeable use in the future.

1 | INTRODUCTION

Hemophilia A is a congenital bleeding disorder resulting from a deficiency or malfunction of coagulation factor VIII (FVIII).[1] This cofactor is required to bridge activated factor IX (FIXa) and factor X (FX) for adequate hemostasis. The recommended treatment to prevent bleeding for patients with FVIII levels of ≤1 IU/dL is FVIII replacement therapy on a regular basis.[2,3] Despite its efficacy, prophylaxis is burdensome due to frequent intravenous injections.[4,5] In addition, a major complication is the formation of anti-FVIII antibodies (called inhibitors), which renders treatment with FVIII products less effective.[6]

Emicizumab (ACE910, Hemlibra; by Roche [Basel, Switzerland] and Chugai [Tokyo, Japan]) is the first licensed non–factor replacement product. The European Medicines Agency (EMA) and the US Food and Drug Administration approved emicizumab for the prophylaxis of people with hemophilia A in 2018.[7,8] This recombinant, humanized, and bispecific IgG4 antibody binds both FIXa and FX and mimics the function of activated FVIII in coagulation reactions.[9] The advantages of emicizumab compared with FVIII products are subcutaneous instead of intravenous administration, longer dosing intervals, and lack of interference by anti-FVIII antibodies.[10] Emicizumab has been approved with a body-weight-adjusted regimen without the requirement of drug monitoring.[11,12] However, guidelines recommend measuring the emicizumab plasma concentration when suspecting the presence of antidrug antibodies (ADAs) against emicizumab.[13-17] In addition, drug monitoring of emicizumab can be useful in clinical decision making, in detecting lack of adherence, and for research purposes.[18,19]

Consequently, efforts have been made to determine emicizumab concentrations in human plasma. An ELISA was used for this purpose in the HAVEN (Study to Evaluate the Efficacy, Safety, and Pharmacokinetics of Prophylactic Emicizumab Versus No Prophylaxis in Hemophilia A Participants With Inhibitors) premarket approval studies but is not commercially available.[20-23] Instead, the manufacturer supplies emicizumab-specific calibrators and controls to use in combination with a modified activated partial thromboplastin time (aPTT)-based one-stage clotting assay (OSA), commonly used in a clinical setting.[18,24] The modified, calibrated OSA (mcOSA) has shown agreement with the noncommercial ELISA.[25] However, disadvantages of the mcOSA are interference by FVIII or by ADAs and its availability at specialized hematologic laboratories.[19,26,27]

A novel liquid chromatography–tandem mass spectrometry (LC-MS/MS) method for quantification of emicizumab was developed by our research group.[28] The objective was to perform a validation study on this LC-MS/MS method quantifying emicizumab in the plasma of people with hemophilia A.

2 | MATERIAL AND METHODS

2.1 | LC-MS/MS method

The development of the LC-MS/MS method was reported previously.[28] Here, the protocol and validation procedures are described in detail.

2.1.1 | Chemicals and reagents

The vials containing emicizumab (batch no. B2002) at a concentration of 150 μg/μL were obtained from F. Hoffmann-La Roche Ltd. (Basel, Switzerland). A stable isotope-labeled (SIL) internal standard (IS) was used to correct for variations during sample preparation and to eliminate the matrix effect. The amino acid sequence of this SIL-IS, matching the signature peptide, was SGGSIYNEEFQD(R*), where (R*) = Arg ($^{13}C_6$,$^{15}N_4$). The SIL-IS was synthesized by and obtained from Pepscan (Lelystad, The Netherlands). The tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin was supplied by Thermo Scientific (Breda, The Netherlands) as a lyophilized powder and was dissolved in acetic acid (50 mM) to a concentration of 10 mg/mL; aliquots were stored in LoBind microcentrifuge tubes (Eppendorf, Hamburg, Germany) at −80°C. The methanol mobile phase solvent (LC-MS grade) and all remaining reagents were obtained from Sigma-Aldrich (Saint Louis, MO, USA).

2.1.2 | Standard working solution, calibration standard, internal standard, and quality controls

The working emicizumab standard solution was prepared by pipetting a 10-μL stock solution of Hemlibra® (150 mg/mL) and 140 μL pooled human plasma in a LoBind tube (10 mg/mL). Calibration standard solutions with concentrations of 512, 256, 128, 64, 32, 16, 8, and 4 μg/mL were prepared freshly from the working standard solution by serial dilution in pooled human plasma, and aliquots were stored at −80°C. The working IS solution (50 μg/mL) was prepared in tris(hydroxymethyl)aminomethane (Tris) buffer pH 8.5, 100 mM, containing 0.5% octyl glucoside (OG). The following quality control (QC) samples were prepared in pooled human plasma: lower limit of quantification (LLOQ; 4 μg/mL), low (10 μg/mL), medium (200 μg/mL), and high concentration (400 μg/mL). Aliquots of QC samples were stored at −80°C.

TABLE 1 Optimized SRM transition information for signature tryptic peptides and SIL-IS of emicizumab

Signature peptide sequence	Analyte	Function	Precursor (*m/z*)	Product (*m/z*)	Product ion	Charge	CE (V)
SGGSIYNEEFQDR	EMI	Quantifier	751.331	1100.46	y_8	1+	23.8
QAPGQGLEWMGDINTR	EMI	Qualifier	886.923	787.375	y_{14}	2+	26.4
ASGYTFTDNNMDWVR	EMI	Qualifier	888.886	1150.50	y_9	1+	28.5
SGGSIYNEEFQDR*[$^{13}C_6$,$^{15}N_4$]	IS	SIL-IS	756.335	1110.47	y_8	1+	23.8

Abbreviations: CE, optimized collision energy; EMI, emicizumab; SIL-IS, stable isotope-labeled internal standard; SRM, selected reaction monitoring.

2.1.3 | Sample preparation for LC-MS/MS

An ammonium sulfate (AS) protein precipitation method was chosen for simplicity and fast workflow.[29] From the plasma sample, 10 µl was taken and diluted with a 5-µl IS solution and 85 µL of Tris buffer (50 mM, pH 8, 0.5% OG) in a 1-ml LoBind 96 deep-well plate and mixed for 1 minute at 1350 rpm. Then, 100 µL of saturated AS solution was added to each sample and mixed for 1 minute at room temperature at 1350 rpm to precipitate both therapeutic and endogenous immunoglobulins from the plasma samples. The 96-well plate was centrifuged at 4000 *g* for 5 minutes to collect the IgG pellet at the bottom. The supernatant containing albumin was decanted, and the pellet was redissolved in 50 µL of Tris buffer (100 mM, pH 8.5, 6 M guanidine chloride, 20 mM 1,4-dithiothreitol [DTT]). Then, the 96-well plate was placed in a ThermoMixer (Eppendorf) at 60°C, at 1000 rpm for 30 minutes to denature the proteins and enable the DTT to reduce the disulfide bonds. The thiol groups were alkylated by adding 20 µL of iodoacetamide (IAA) solution (100 mM) and placed on the ThermoMixer at 37°C for 30 minutes of mixing in the dark. Subsequently, 150 µL of ultrapure water was added and mixed for 1 minute to dilute guanidine and IAA. After mixing, 400 µL of methanol was added to precipitate the IgG fragments, and the 96-well plate was centrifuged at 4000 *g* for 5 minutes. The supernatant, containing guanidine and IAA, was decanted. Subsequently, 90 µL of Tris buffer (pH 8.5, 50 mM) with 0.5% OG was added to the pellet, followed by addition of 10 µl of TPCK-trypsin solution (2 mg/mL). Samples were placed on the ThermoMixer for overnight digestion at 37°C at 1000 rpm. Trypsin activity was stopped by adding 20 µL of 10% formic acid in acetonitrile (pH 3) and centrifugation at 4000 *g* for 5 minutes. Finally, a 5-µL sample was injected into the LC-MS/MS system.

2.1.4 | Instrumentation and chromatographic conditions

Instrumentation and chromatographic conditions were as described previously.[28]

2.1.5 | Signature peptide selection

The amino acid sequence of emicizumab was obtained from the International Immunogenetics Information System (http://imgt.org). From in silico (tryptic) digestion of emicizumab, potential signature peptides within the variable chains with amino acids $6<n<20$, were identified with Skyline software (University of Washington, Seattle, WA, USA). These peptides were screened for absence from the human genome using the basic local alignment search tool (Blast) (https://blast.ncbi.nlm.nih.gov/Blast). Finally, the retention time and the signal intensity of peptides were assessed with Skyline. Three stable, unique signature peptides on the heavy chain were identified: The SGG (serine-glycine-glycine) peptide had the smallest isobaric interferences, a high signal-to-noise ratio and was selected as the quantifier; the remaining peptides QAP (glutamine-alanine-proline) and ASG (alanine-serine-glycine) were adequate to function as qualifiers (Table 1).

2.2 | Analytical validation study

The analytical validation was performed in accordance with the EMA guideline on bioanalytical method validation.[30] The selectivity and matrix effect were investigated with 12 blank human plasma samples from 12 different individuals. The linearity of the standard curve was assessed with $1/x$ weighting. The within-run and between-run accuracy values and precision were evaluated for the QC samples of LLOQ, QC low, QC medium, and QC high, corresponding to concentrations of 4, 10, 200 and 400 µg/mL, respectively. Stability was tested using QC low and high samples in the autosampler (after sample preparation, at 10°C) and for three freeze (−80°C)-and-thaw cycles. Samples were analyzed in quintuplicate on three different days.

2.3 | Cross-validation study

2.3.1 | Patient sampling

The cross-validation study had a cross-sectional design and was performed on patients from the Van Creveldkliniek (University Medical Center Utrecht, The Netherlands) in accordance with our local institutional Medical Ethics Review Board–approved, opt-out procedure. People with hemophilia A received emicizumab loading doses of 3 mg/kg/wk for 4 weeks, followed by maintenance doses of 6 mg/kg/4 wks with varying dosing intervals (from 7 to 28 days) using entire vials according to local clinical protocol.[31] Samples were taken in loading and maintenance phases during clinical visits (usually at

weeks 1, 2, and 4 and month 3, then annually) in the period between June 2018 and February 2021. All peripheral blood samples from patients receiving emicizumab were collected through venipuncture in 4.5 mL tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA), containing 1/10 volume of 105 mM trisodium citrate. Plasma samples were prepared from blood samples by two subsequent centrifugation steps at 2000 *g* for 5 minutes at room temperature. Samples were aliquoted, stored at −80°C, and analyzed with mcOSA and LC-MS/MS.

2.3.2 | Modified and calibrated one-stage clotting assay

The emicizumab concentration was measured with the mcOSA on a Sysmex CS2500, a coagulation analyzer (TOA Medical Electronics Co., Ltd., Hamburg, Germany) with Actin FS aPTT reagent (Siemens, Marburg, Germany). Standard dilutions for CS2500 were applied and were followed by an extra dilution 1:8 with Owren's Veronal Buffer (calcium system buffer) to minimize FVIII interference, then FVIII-deficient plasma, Actin FS, and $CaCl_2$ were added (Siemens, Marburg, Germany). Emicizumab concentrations were deduced from an emicizumab calibration curve, based on the plasma calibrator (r^2 Diagnostics, South Bend, IN, USA; catalog #152-401-RUO, 102 µg/mL, lot no. EC0140). The plasma controls (r^2 Diagnostics; catalog no. 152-401-CE) of level 1 (26.6 µg/mL; lot no. E10310) and level 2 (73.4 µg/mL; lot no. E20410) were used as internal quality controls. The calibration curve was linear over a concentration range of 10 to 200 µg/mL with an R^2 of 1.00. The within-run and between-run precision (relative standard deviation [RSD], %) of the control samples ranged between 3.5% and 5.7%. The RSDs of the two control samples were similar after four freeze-and-thaw cycles. The LLOQ was 2 µg/mL.

2.3.3 | Cross-validation parameters

Plasma samples from people with hemophilia A were measured with mcOSA and LC-MS/MS. The following EMA criterion for cross-validation was applied: "the difference between the two values obtained should be within 20% of the mean for at least 67% of the repeats."[30] Samples with >20% difference were reanalyzed with LC-MS/MS method.

Cross-validation results were analyzed with weighted Deming regression and Bland-Altman analysis. The regression was performed with jackknife-based calculation of 95% confidence intervals (CIs) according to Linnet's method and a Pearson's correlation coefficient, with a validated web-based tool.[32]

The Bland-Altman analysis of absolute and relative differences included mean bias (in µg/mL or %, respectively) with standard deviation (SD) and 95% limits of agreement (LoAs). Relative differences (%) were calculated as:

$$\frac{(\text{Reference method} - \text{New method})}{(\text{Reference method} + \text{New method})/2} \times 100\,\%.$$

The influence of covariates on absolute differences was assessed with an unpaired Student's *t* test (dichotomous). Anti-FVIII antibodies and FVIII in samples were scored based on laboratory results and reviewing the electronic patient records. Titers of anti-FVIII antibodies were determined when indicated by the local protocol with the Bethesda assay (Nijmegen modified chromogenic assay with bovine reagents) for which the clinical cutoff ≥0.6 Bethesda units per milliliter was used.[16] No FVIII activity was measured during emicizumab therapy in our clinic. Statistics were performed in Prism version 8.3.0 (GraphPad Software LLC, San Diego, CA, USA).

2.4 | Ethical approval

The study was conducted in accordance with the local opt-out policy of the hospital, and the Medical Ethics Review Board provided a waiver for use of samples and to review electronic patient records (study approval no. 21-77/C).

The source of biological material was people with congenital hemophilia A receiving emicizumab at the Van Creveldkliniek (University Medical Center Utrecht, The Netherlands).

A statement on animal welfare was not applicable.

3 | DISCUSSION

3.1 | Analytical validation study

Linearity of the LC-MS/MS method was established from 4 to 512 µg/mL with an R^2 of 1.00. The RSD of the within-run precision ranged from 2.1% to 4.9% and the RSD of the between-run precision ranged from 2.8% to 7.4%. The accuracy (%bias) ranged from −4.1% to 6.1%. All other validation parameters were also well within the acceptance criteria of the EMA guideline (Table 2). The validated LLOQ was 4 µg/mL and had a signal-to-noise ratio of 88, which indicated that an even lower LLOQ can be achieved. Emicizumab in QC samples was stable during three freeze-and-thaw cycles. The QC low and high samples remained stable after sample preparation for 1 week at 10°C.

In addition to the analytical validation results, two plasma control samples from r^2 Diagnostics were measured with the LC-MS/MS method. The assigned values of these controls were 26.6 and 78.3 µg/mL, and LC-MS/MS results were 25.9 and 79.2 µg/mL. Also, a sample with an unknown amount of emicizumab from a pilot external quality assessment study of the WFH (UK-NEQAS, Sheffield, UK. sample: WFH EMI 21:01, July 2021) was tested. The LC-MS/MS result was 59.0 µg/mL for a median of 57.5 µg/mL derived from 11 laboratories. Both of these findings corroborate the results of the analytical performance of the LC-MS/MS method.

TABLE 2 Summary of LC-MS/MS method validation performance[a]

Validation parameter	Sample	Expressed as	Result	Acceptance[b]
Within-run precision	QC LLOQ	RSD (%)	4.9	<20
	QC low		4.2	<15
	QC medium		2.4	<15
	QC high		2.1	<15
Between-run precision	QC LLOQ	RSD (%)	7.4	<20
	QC low		4.5	<15
	QC medium		2.8	<15
	QC high		3.4	<15
Accuracy	QC LLOQ	Bias (%)	6.1	<20
	QC low		−4.1	<15
	QC medium		−3.8	<15
	QC high		1	<15
Selectivity in plasma	Human samples #1–12	Max relative to LLOQ (%)	0.2	<20
LLOQ	LLOQ of 4 μg/ml	Signal/noise	88	>5×
Linearity	Standards 4, 8, 16, 32, 64, 128, 256, 512 μg/ml	R^2	0.999	>0.99
Freeze-and-thaw stability	QC low	Bias (%)	−4.5	<15
	QC high		4.3	<15
Spiked recovery in plasma	Human samples #1–12 low	Min/max Bias (%)	−1.1/11.8	<15
	Human samples #1–12 high	Min/max Bias (%)	−6.3/1.2	<15
Autosampler stability	Day 1 reinjected after 7 days	Min/max Bias (%)	−9.6/8.8	<15
Carry over	Blank after highest standard	Relative to LLOQ (%)	0.3	<20
Zero sample	Pool human plasma with IS	Relative to LLOQ (%)	0.2	<20

Abbreviations: IS, internal standard; LLOQ, lower limit of quantification; max, maximum; min, minimum; QC, quality control; RSD, relative standard deviation.

[a]SGG as signature peptide for SRM transition of 751.33 --> 1100.46.

[b]In accordance with 'Guideline on bioanalytical method validation' of the European Medicines Agency.[29]

3.2 | Cross-validation study

A total of 77 samples obtained from 41 patients were used for cross validation (Table 3). Most patients were male with a diagnosis of severe congenital hemophilia A. The mean age at sampling was 28 years (range, 0-78 years), and the mean treatment week at sampling was 20 weeks (range, 1-133 weeks). The mean plasma concentration of emicizumab measured with LC-MS/MS was 49 μg/mL (range, 11-106 μg/mL), and also 49 μg/mL (range, 8-104 μg/mL) when measured with mcOSA.

The correlation between observations of the emicizumab concentrations measured with mcOSA and the LC-MS/MS method, using weighted Deming regression, is depicted in Figure 1. The slope of the regression line was 1.02 (95% CI, 0.891-1.144) with an intercept of −1.61 (95% CI, −7.18 to 3.95) (Pearson's r = .99). The line of identity, with a regression slope of 1, lies within the 95% CI of the weighted Deming regression line (Figure 1).

A Bland–Altman analysis was performed on absolute and relative differences. The absolute differences had a mean bias of 0.03 μg/mL (SD, 4) with 95% LoAs ranging from −9 to 9 μg/mL (Figure 2A). No trends or outliers were observed. The relative differences (Figure 2B) had a mean bias of 2% (SD, 11), with 95% LoAs from −20 to 25%. The mean difference between methods was <20% in 71 of 77 samples (92%), which is well within the acceptance criterion of >67% of samples. The six samples with >20% difference had a mean emicizumab concentration ranging between 4 and 35 μg/mL; the mean absolute difference of these six samples was 3.9 μg/mL. Four of six samples retained a difference of >20% after reanalysis with LC-MS/MS.

The influence of anti-FVIII antibodies and FVIII on the emicizumab concentration differences obtained by both methods was assessed. The absolute differences were similar (P = .30) for samples in presence (n = 19) and absence (n = 58) of anti-FVIII antibodies. The absolute differences were also similar (P = .17) for samples in presence (n = 19) and absence (n = 58) of FVIII. The presence of both covariates resulted in minor increases of mcOSA results (positive absolute differences), but these were neither statistically significant nor clinically relevant. This is in line with reported spike experiments; despite a 1:80 dilution, mcOSA remains sensitive to the presence of replacement FVIII or endogenous FVIII and cannot be made completely specific to emicizumab by using higher dilutions.[33] Especially in a clinical setting, patients receive large amounts of FVIII products during bleeding episodes or perioperative periods

TABLE 3 Patient characteristics from samples in cross validation

Total number of patients = 41					**Number of patients**
Severe congenital HA					38[a]
Male					40
Total number of samples = 77	**Number of samples**	**Mean**	**Min**	**Max**	**SD**
Emicizumab concentration (μg/ml)[b]	77	49	11	106	23
Age at sampling (year)	77	28	0	79	26
Treatment week of sampling	77	20	1	133	29
Albumin concentration					
Measured (g/L)	39	42.0	32.1	47.8	4.3
Not measured	38				
aFVIII titer					
>0.5 BU/ml	19	549	0.6	2790	951
≤0.5 BU/ml	58				
FVIII in sample					
Present[c]	19				
Absent	58				

Abbreviations: aFVIII, anti-FVIII antibodies (inhibitors); BU, Bethesda units; FVIII, coagulation factor VIII; FVIII:C, factor VIII activity; HA, hemophilia A; SD, standard deviation.

[a]Remaining patients: one woman with acquired HA (three samples); two men with moderate HA (two samples).

[b]Measured with LC-MS/MS.

[c]FVIII:C was not quantified in presence of emicizumab.

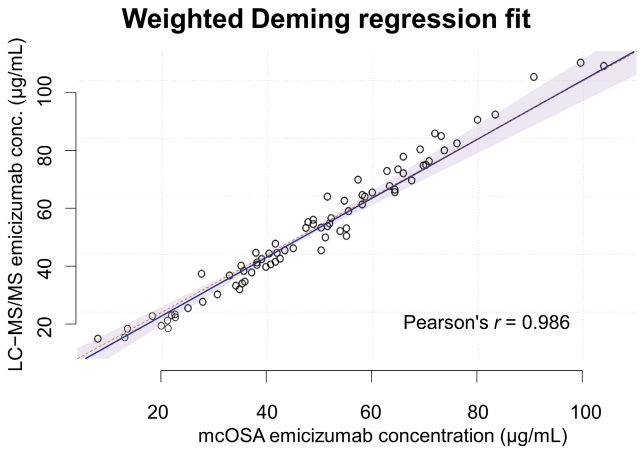

FIGURE 1 Weighted Deming regression for cross validation. Emicizumab concentrations using the modified, calibrated one-stage clotting assay (mcOSA) are plotted against emicizumab concentration using liquid chromatography-tandem mass spectrometry (LC-MS/MS) method in patient samples (n = 77). Purple line is the regression fit (−1.61 + 1.02*X; Pearson's r = .99); purple area represents the 95% confidence interval (jackknife method) of the fit; dashed red line is line of identity

during emicizumab therapy.[34] Fortunately, the LC-MS/MS method is unaffected by FVIII interference owing to its principle, which is one of the strengths of this method.

The ideal comparator for the LC-MS/MS method would be a total ELISA; that is, an ELISA with a preceding dissociation step to release the drug from any other potential binding target. This classical cross-validation approach cannot be applied in our study because the sole existing ELISA, which was used in the HAVEN studies,[20-23] detects only the free, dual-binding competent drug and cannot detect emicizumab in complex with either FIXa or FX. Nevertheless, a cross-validation can still be of value to determine whether the data obtained are reliable and can be compared between laboratories. As the ELISA from the HAVEN studies was not commercially available, the LC-MS/MS method was compared with the standard mcOSA. The principle of this type of OSA-based assay relies upon measuring emicizumab activity as a factor VIII mimetic and is based on clotting (enzymatic) reactions in FVIII-deficient plasma.[24] In contrast, the principle of the LC-MS/MS method relies upon measuring the exact amount of a signature peptide of emicizumab per sample using SIL-IS for quantification. These different principles explain the slight negative trend in absolute differences above 50 μg/mL (Figure 2A). Despite the fundamental differences, a very strong correlation between the methods was found. The 95% CI of the intercept contained "zero" in the weighted Deming regression fit, and the 95% CI of the slope contained "one" (Figure 1). The relative differences were well within the EMA's acceptance criteria for cross-validation (Figure 2B). Therefore, the application of a correction factor for interchangeable method use is not required.

The six samples with relative difference of >20% had emicizumab concentrations <34 μg/mL. These six samples were obtained during the loading phase because concentrations in maintenance phase range between 38 and 67 μg/mL.[11,35] While

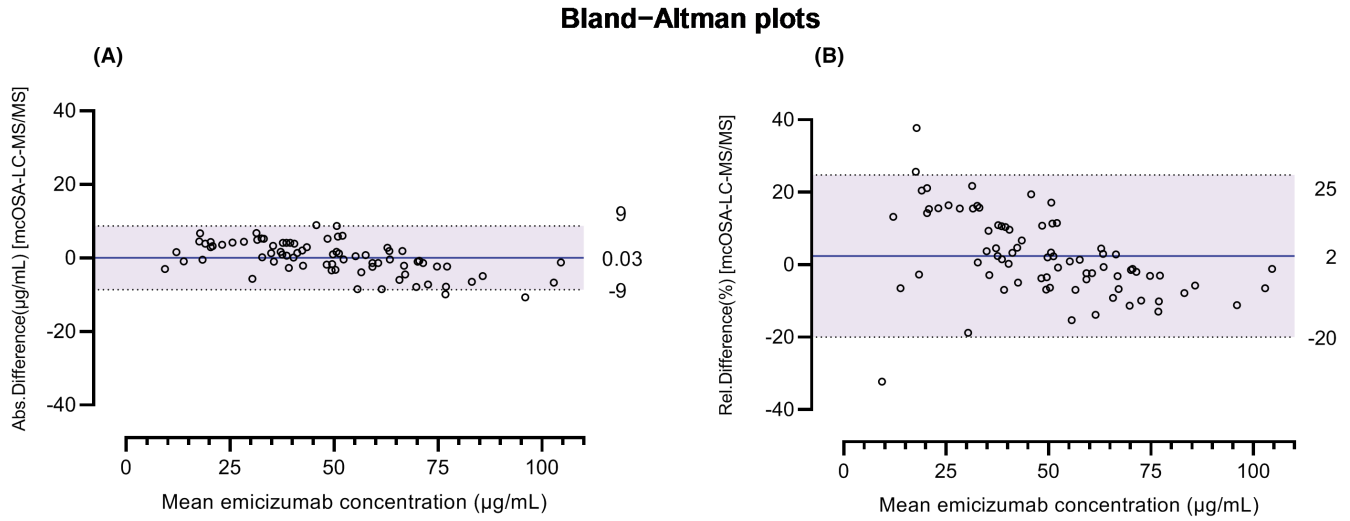

FIGURE 2 Bland-Altman difference plots for cross validation. Absolute (A) and relative (B) differences in emicizumab concentrations obtained by modified, calibrated one-stage clotting assay (mcOSA) and liquid chromatography–tandem mass spectrometry (LC-MS/MS) method in patient samples (n = 77) were plotted against the corresponding mean emicizumab concentration (µg/mL). Straight line is mean bias; purple area (between the dotted lines) represents 95% limits of agreement

relative differences are of analytical importance, they have low clinical value. The absolute differences were small, especially from a clinicians' perspective, and relevant outliers or trends were lacking. The EMA criterion was evidently met, making this finding not clinically relevant.

3.3 | Strengths and limitations

This is the first report on clinical use of an LC-MS/MS method quantifying emicizumab in plasma, further building on our previous work measuring FVIII in plasma with LC-MS/MS.[36,37] The strengths of this LC-MS/MS method over the mcOSA method are the lack of interference, a high-throughput and easy-to-implement design, and the opportunity to multiplex with other therapeutic monoclonal antibodies. In addition, the sampling volume for this LC-MS/MS method is only 0.25 mL (minimal required volume of tube), which is particularly beneficial to the pediatric population. Furthermore, the LC-MS/MS–based methods have become the standard for measuring drug concentrations in clinical laboratories worldwide[38]; making this method for emicizumab quantification accessible to routine practice.

Another form of assay interference might result from the formation of ADAs against emicizumab. This immune response generally enhances drug clearance and removal from the circulation but might also form neutralized emicizumab-ADA complexes that remain in the circulation.[39,40] These neutralized complexes could potentially lead to falsely high emicizumab concentrations using the LC-MS/MS method. The occurrence of such complexes remaining in the circulation has rarely been reported for therapeutic monoclonal antibodies and has not been reported for emicizumab as well. Unfortunately, no robust assays for ADA detection or neutralized complexes are commercially available. The presence of ADAs in our study samples is highly unlikely, however, as it is extremely rare (reported incidence of <0.8%[41,42]) and the clinical response of our patients was excellent.[31] This validation study was not powered for the development of emicizumab-ADA. Therefore, future studies should further investigate the impact of potential interference by this phenomenon, especially for the mcOSA and the ELISA, and to demonstrate the complementary role of LC-MS/MS.

A limitation of the LC-MS/MS method is the sample preparation time of 24 hours, due to the overnight trypsin digestion step, and an analysis run time of 13 minutes per sample. Fast drug monitoring of emicizumab is not required according to clinical guidelines but might be supportive in an acute bleeding setting.[15,34] Consequently, the work-flow may need to be optimized.

In conclusion, the LC-MS/MS method for the quantification of emicizumab in the plasma of people with hemophilia A was performed successfully in this validation study. The strong correlation between the current reference method and the LC-MS/MS method allows interchangeable use. This LC-MS/MS method can be implemented for drug monitoring of emicizumab.

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RELATIONSHIP DISCLOSURE

None.

AUTHOR CONTRIBUTIONS

AAMTD designed the study, retrieved data, performed statistical analysis, and drafted the manuscript. MEA, LG, and MPM

performed analytical analysis. All authors assisted in data analysis and interpretation, and critically reviewed and approved the final manuscript.

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