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A Rapid LC–MS/MS Method for Quantifying Serum Tocilizumab Using Immobilized Trypsin Without IgG Purification

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ABSTRACT

The clinical implementation of liquid chromatography–tandem mass spectrometry (LC–MS/MS) for quantifying serum tocilizumab has been limited due to labor-intensive and expensive sample preparation procedures. This study sought to establish and validate an LC–MS/MS assay for serum tocilizumab measurement employing immobilized trypsin digestion without the need for immunoglobulin G (IgG) purification, and to assess its applicability in rheumatoid arthritis (RA) patients receiving either intravenous or subcutaneous tocilizumab therapy. A tocilizumab-specific signature peptide was identified using a nano-LC system coupled with a hybrid quadrupole-Orbitrap mass spectrometer. Serum samples underwent rapid enzymatic digestion with immobilized trypsin for 30 minutes. The signature peptide and its corresponding internal standard were chromatographically resolved from serum digests within a total analysis time of 15 minutes. The assay demonstrated linearity over a concentration range of 2–200 µg/mL. Intra- and inter-day accuracy ranged from 90.7% to 109.4%, with relative standard deviations below 10%. Measured serum tocilizumab concentrations ranged from 5.8 to 28.9 µg/mL in patients receiving intravenous administration and from 2.4 to 63.5 µg/mL in those receiving subcutaneous administration. Concentrations obtained using this LC–MS/MS method showed a positive correlation with values determined by enzyme-linked immunosorbent assay, although a consistent systematic bias between the two methods was observed. Overall, this study presents a validated LC–MS/MS approach with simplified sample preparation suitable for monitoring serum tocilizumab levels in patients with rheumatoid arthritis.

Keywords: LC-MS/MS, Tocilizumab, Immobilized trypsin, Rheumatoid arthritis, IgG purification, Proteomics

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Introduction

By blocking the interaction between interleukin-6 (IL-6) and its receptors, tocilizumab—a humanized monoclonal antibody of the immunoglobulin G (IgG) 1κ subclass—reduces systemic inflammatory responses [1, 2]. This agent is widely prescribed for patients with moderate to severe rheumatoid arthritis (RA), particularly for inducing and maintaining remission [3]. Several studies have demonstrated that circulating levels of tocilizumab are linked to prognostic indicators in RA [4, 5], indicating that monitoring serum concentrations may support therapeutic optimization. Nevertheless, analytical approaches for routine clinical measurement of serum tocilizumab remain limited.

Quantification of therapeutic monoclonal antibodies in human serum has been achieved using liquid chromatography–tandem mass spectrometry (LC–MS/MS) techniques [6-9]. However, conventional LC–MS/MS workflows involve complex pretreatment procedures, notably extensive enzymatic digestion and purification of IgG. When soluble trypsin is employed, digestion typically requires prolonged incubation periods of 10–14 hours [6, 7], and enzyme concentrations must be carefully controlled to prevent autolytic degradation in serum matrices [10, 11]. Additionally, exhaustive digestion produces a large number of peptides that can suppress ionization and adversely affect mass spectrometric performance. Rapid digestion, in contrast, can be achieved using trypsin

immobilized on monolithic silica supports, enabling efficient centrifugal processing [11, 12]. Although IgG enrichment using protein A or G is commonly applied to reduce matrix effects and enhance analytical sensitivity [7, 9, 13, 14], this step is costly and may introduce quantitative variability. The combined burden of time, expense, and variability has restricted the clinical applicability of existing LC–MS/MS assays. Accordingly, development of an LC–MS/MS method that allows rapid digestion without the need for IgG purification is warranted.

Depending on clinical requirements and patient adherence, tocilizumab may be delivered either intravenously or subcutaneously. These administration routes differ in dosing regimens and injection intervals [15], which subsequently influence drug absorption and elimination kinetics. Compared with intravenous administration, subcutaneous delivery has been associated with greater interindividual variability in serum tocilizumab concentrations among RA patients [5, 16]. Therefore, an LC–MS/MS assay capable of covering a wide concentration range is particularly valuable for therapeutic drug monitoring, especially in patients who transition between administration routes or exhibit secondary loss of response.

While LC–MS/MS-based measurements of serum tocilizumab following both intravenous and subcutaneous administration have been reported, further simplification of analytical procedures remains necessary. The aim of the present study was to validate an LC–MS/MS method for serum tocilizumab quantification based on rapid tryptic digestion without IgG purification and to apply this approach to RA patients treated via intravenous or subcutaneous injection.

Materials and Methods

Signature peptide determination

Digestion of tocilizumab (Actemra; Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was carried out using immobilized trypsin (MonoSpin Trypsin; GL Science, Tokyo, Japan) following previously described protocols [17]. The resulting peptide mixtures containing tocilizumab-derived fragments were analyzed using a nano-liquid chromatography system interfaced with a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific Inc., Waltham, MA, USA).

Identification of candidate signature peptides was performed using Proteome Discoverer software version 2.1 (Thermo Fisher Scientific Inc.) in combination with FASTA databases from the International ImMunoGeneTics Information System® (IMGT/3Dstructure-DB and IMGT/2Dstructure-DB, version 4.12.2), which include the primary amino acid sequence of tocilizumab [18]. Candidate peptides were selected based on close agreement between experimentally observed m/z values and those theoretically predicted from the FASTA entries. Final selection of a signature peptide was guided by the following criteria: inclusion of the complementarity-determining region (CDR) of tocilizumab; chromatographic separation from serum-derived contaminant peptides; complete enzymatic cleavage with no remaining lysine or arginine residues; and absence of carbamidomethylation or oxidation within the peptide sequence.

Sample preparation for LC-MS/MS quantitation

Preparation procedure

With 440 µL of 50 mM ammonium bicarbonate buffer (pH 8.1; Fujifilm Wako Pure Chemical Industries, Osaka, Japan) and 50 µL of internal standard solution (1 µg/mL), 10 µL of serum was thoroughly mixed. The mixture then received 50 µL of RapiGest SF surfactant (10 mg/mL; Waters, Milford, MA, USA), followed by heating for 10 minutes at 80 °C to unfold proteins. Disulfide bonds were reduced upon addition of 25 µL of 500 mM dithiothreitol (Thermo Fisher Scientific Inc.), with incubation for one hour at 60 °C. Alkylation of cysteines occurred after adding 50 µL of 500 mM iodoacetamide (Fujifilm Wako Pure Chemical Industries), allowing the reaction to continue for 30 minutes in darkness at room temperature. Onto an immobilized trypsin column, the prepared sample was loaded, circulated three times, and centrifuged gently for 10 minutes at 37 °C and 100 g. Using 300 µL of ammonium bicarbonate buffer, the column was rinsed. Residual RapiGest SF was removed by acidifying the digest using 25 µL of 2 M hydrochloric acid (pH 2.2; Nacalai Tesque, Kyoto, Japan), then centrifuging for 30 minutes at 37 °C and 15, 000 g to precipitate the surfactant. On an Oasis HLB cartridge (3 cm^3, 60 mg sorbent, 30-µm particles; Waters), the supernatant was desalted. With 1 mL of 70% acetonitrile containing 0.5% acetic acid (Fujifilm Wako Pure Chemical Industries), bound peptides were eluted, and the eluate was dried completely by evaporation at ambient temperature. In 80 µL of 14% acetonitrile with 0.1% acetic acid,

the dry residue was reconstituted. The solution was clarified by cold centrifugation for 30 minutes at 4 °C and 10, 000 g, filtered, and finally, 10 µL was injected into the HPLC system.

Optimization of sample preparation

To optimize conditions, the number of digestion cycles in centrifugal mode and the starting dilution factor for serum were explored. By examining chromatographic peak areas of surrogate peptides, dilution factors from three- to eight-fold were compared to identify the best one. Peak areas were likewise used to assess the outcomes from one to four cycles of centrifugal digestion. The ratio of peak areas was calculated for surrogate peptides generated through tocilizumab proteolysis versus those spiked externally into serum digests, thereby measuring digestion effectiveness.

Characterization of trypsin digestion

Digestions using solubilized trypsin and immobilized trypsin

Against digests produced by free trypsin in solution (Thermo Fisher Scientific Inc.), those from immobilized trypsin for tocilizumab were compared. Using the solution-based method, 200 µg of tocilizumab (already reduced and alkylated) was incubated with 40 µL of trypsin solution (100 µg/mL) for 12–14 hours at 37 °C, and the process was halted by adding 25 µL of 2 M hydrochloric acid. For the column-based approach, 200 µg of pretreated tocilizumab was applied to the immobilized trypsin and passed either once or thrice. On an HPLC instrument with photodiode array (PDA) detection, all resulting digests were examined.

Evaluation of tryptic digestion products

Peptide mixtures generated from tocilizumab digestion were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) to compare digestion profiles. Analyses were conducted using a UFLCXR system (Shimadzu Corporation, Kyoto, Japan) equipped with an SPD-M30A photodiode array (PDA) detector and operated via LabSolutions software (version 5.73; Shimadzu Corporation). Separation was achieved using an Aeris WIDEPORE XB-C18 column (150 mm × 2.1 mm i.d., 3.6 µm; Phenomenex Inc., Torrance, CA, USA), which was thermostatically controlled at 85 °C.

Chromatographic separation employed a binary mobile phase system consisting of solvent A (0.3% trifluoroacetic acid in water) and solvent B (0.3% trifluoroacetic acid in isopropanol; Fujifilm Wako Pure Chemical Industries). A linear gradient was applied in which the proportion of solvent B was increased from 5% to 35% over 60 minutes, followed by an isocratic hold at 5% B between 60.1 and 70 minutes. The total analysis time was 70 minutes with a constant flow rate of 0.5 mL/min. Ultraviolet detection was carried out at 210 nm, and each sample was injected at a volume of 10 µL. Chromatographic peak patterns obtained from immobilized-trypsin digests were compared directly with those produced by solubilized-trypsin digestion.

LC–MS/MS assay

LC–MS/MS chromatographic setup

For LC–MS/MS analysis, peptide separation was performed using a Nexera X2 HPLC system (Shimadzu Corporation) controlled by LabSolutions software version 5.91. An Aeris Peptide C18 column (150 mm × 3.0 mm i.d., 2.6 µm; Phenomenex Inc.) fitted with a SecurityGuard Ultra guard cartridge (3.0 mm i.d.; Phenomenex Inc.) was used, and the column temperature was maintained at 85 °C.

Elution was conducted using a stepwise gradient composed of mobile phase A (0.1% acetic acid in water) and mobile phase B (0.1% acetic acid in acetonitrile). The elution program consisted of an initial hold at 14% B for 8 minutes, followed by an increase to 70% B from 8.1 to 11.0 minutes, and re-equilibration to 14% B between 11.1 and 15.0 minutes. The flow rate was set at 0.3 mL/min during the initial phase (0–8 minutes) and the final phase (12.6–15.0 minutes), while an increased flow rate of 0.6 mL/min was applied from 8.1 to 12.5 minutes.

During analysis, the switching valve directing the eluate to the tandem mass spectrometer was activated between 6.0 and 8.0 minutes. To minimize carryover, the external surface of the autosampler injection needle was washed with 30% methanol in water (Fujifilm Wako Pure Chemical Industries).

Mass spectrometric conditions of LC-MS/MS

Custom synthesis of the signature peptide LLIYYTSR and its stable isotope-labeled counterpart [R ($^{13}C_6$ $^{15}N_4$)] as the internal standard (IS) was performed by Scrum Inc. (Tokyo, Japan). Analysis of the chromatographic eluate occurred on a Shimadzu LCMS-8050 triple quadrupole mass spectrometer equipped with electrospray ionization, operating in positive-ion multiple reaction monitoring (MRM) mode. Control of the system was handled via LabSolutions software (version 5.91). Each MRM transition employed a 100 ms dwell time: m/z 514.95 → 689.45 (+) for the analyte peptide LLIYYTSR, and m/z 519.90 → 699.30 (+) for the labeled IS. Turbo ion spray maintained the interface at 350 °C, with +4000 V applied. Argon collision gas was supplied at 270 kPa. Flow settings included 10 L/min for drying gas, 3 L/min for nebulizing gas, and 10 L/min for heating gas. Collision energies were optimized to −19 V for the analyte and −20 V for the internal standard.

Method validation

Compliance with U.S. FDA guidance was ensured during validation of this assay [19]. To confirm selectivity, blank serum samples processed from six healthy individuals and six RA patients were checked for any interfering peaks at the expected retention times of the target peptides. Standard curves were generated from peak area ratios (analyte/IS) across tocilizumab levels in serum ranging from 2 to 200 μg/mL (specifically 2, 5, 10, 20, 50, 100, 150, and 200 μg/mL). The LLOQ was set at the concentration where RSD remained ≤20%. Three quality control levels were established per FDA recommendations: low (LQC) at 4 μg/mL, medium (MQC) at 40 μg/mL, and high (HQC) at 160 μg/mL. Precision (RSD) and accuracy, both within-day and between-day, were determined using these QCs in human serum, with accuracy calculated as percent bias from nominal values.

Using synthetic peptide at QC-equivalent concentrations, extraction recovery and matrix effects were investigated. Matrix factor derived from dividing post-extraction spiked peak areas in blank serum (from six healthy sources) by those in pure water. Recovery was computed from peak areas of peptide subjected to full solid-phase extraction in digested serum versus direct post-extraction addition. Tocilizumab stability in serum (LQC and HQC) was tested under multiple conditions: 24 hours at ambient or refrigerated (4 °C) temperatures, one month frozen at −80 °C, and following three freeze-thaw cycles. Processed sample stability for the peptide in mobile phase was verified after 24 hours at 4 °C in the autosampler. All stability data were reported as percent accuracy against curves from newly prepared standards. Carry-over assessment involved injecting blank serum immediately following a high (200 μg/mL) tocilizumab sample and comparing analyte signals to those at the low (2 μg/mL) level.

Ethics and clinical application

Approval for this research was granted by the Ethics Committee at Hamamatsu University School of Medicine (code: 19–130). Twenty-two Japanese RA patients undergoing tocilizumab treatment (Actemra) at Hamamatsu University Hospital (Hamamatsu, Japan) were included. Dosing followed either intravenous delivery of 8 mg/kg every 4–5 weeks or subcutaneous administration of 162 mg every two weeks. Through blood samples were obtained right before the next infusion after a minimum of six IV doses or within one week prior to injection after at least twelve SC doses. All serum samples were kept frozen at −80 °C prior to testing.

Comparison of LC-MS/MS and enzyme-linked immunosorbent assay (ELISA) methods

Tocilizumab concentrations in serum from patients on either intravenous or subcutaneous therapy were determined using both the current LC-MS/MS approach and an ELISA kit (ImmunoGuide, TANI Medikal Ltd., Ankara, Türkiye). Strength of association between methods was evaluated via Pearson correlation coefficient. Potential systematic differences were explored through Bland-Altman analysis [20]. Statistical processing was carried out with IBM SPSS version 23 (IBM Japan Ltd., Tokyo, Japan), considering results significant at $P < 0.05$.

Results and Discussion

Signature peptide determination

Analysis using a hybrid quadrupole–Orbitrap mass spectrometer yielded a total of 37 peptide species originating from tocilizumab. Among these, twelve peptides were assigned to the variable regions of the antibody **(Table 1)**. Five of the variable-region peptides—TL 46–53, TL 25–42, TL 25–45, TH 83–98, and TH 103–123—contained complementarity-determining regions (CDRs). Two candidates (TL 25–42 and TL 25–45) were removed from further consideration because they exhibited incomplete tryptic cleavage. An additional two peptides (TH 83–98

and TH 103–123) were excluded due to the presence of chemical modifications. Following this stepwise evaluation, the TL 46–53 fragment, corresponding to the amino acid sequence LLIYYTSR, was selected as the most suitable signature peptide for quantitative measurement of serum tocilizumab.

Table 1. Characteristics of candidate peptides derived from variable regions of tocilizumab identified by hybrid quadrupole-Orbitrap mass spectrometry.

Peptide ID	Amino acid sequence	Structural location	Chemical alteration	Missed tryptic site	Protonated mass [M+H]+ (m/z)
TL 1–18	DIQMTQSPSSLSASVGDR	Light chain	None	None	1878.9
TL 19–24	VTITcR	Light chain	C5: carbamidomethylation	None	749.4
TL 1–24	DIQMTQSPSSLSASVGDRVTITcR	Light chain	C23: carbamidomethylation	R18	2609.3
TL 25–42	ASQDISSYLNWYQQKPGK	Light chain, CDR1	None	K15	2113.0
TL 25–45	ASQDISSYLNWYQQKPGKAPK	Light chain, CDR1	None	K15, K18	2409.2
TL 46–53	LLIYYTSR	Light chain, CDR2	None	None	1028.6
TL 54–61	LHSGVPSR	Light chain	None	None	852.5
TH 66–72	SRVTMLR	Heavy chain	None	R2	862.5
TH 73–82	DTSKNQFSLR	Heavy chain	None	K4	1195.6
TH 77–82	NQFSLR	Heavy chain	None	None	764.4
TH 83–98	LSSVTAADTAVYYcAR	Heavy chain, CDR3	C14: carbamidomethylation	None	1747.8
TH 103–123	TTAmDYWGQGSLVTVSSASTK	Heavy chain, CDR3	M4: oxidation	None	2190.0

Peptide nomenclature was assigned based on the location within the tocilizumab molecule: peptides derived from the heavy and light chains were designated as TH and TL, respectively, while the numerical values indicate the N-terminal and C-terminal amino acid positions relative to the N-terminus of tocilizumab. In the amino acid sequences, lowercase letters denote post-translational or chemical modifications, with *c* representing carbamidomethylated cysteine residues and *m* indicating oxidized methionine residues. Missed cleavage refers to trypsin-cleavable amino acid residues that remained uncleaved. CDR denotes the complementarity-determining region.

Optimization of sample preparation

When serum samples were diluted three-, six-, seven-, and eight-fold, the peak area ratios of the signature peptide relative to a five-fold dilution were 14.4%, 77.7%, 83.6%, and 79.7%, respectively. In experiments evaluating the number of centrifugal digestion cycles, peak areas obtained with one, two, and four passes were reduced to 47.9%, 70.4%, and 89.6%, respectively, when compared with three passes. Under the condition of three centrifugal digestions, the digestion efficiency reached 50.1% (n = 3), with a relative standard deviation of 5.2%.

Comparison between solubilized and immobilized trypsin digestion

Chromatographic analysis showed that intact tocilizumab eluted at 45.7 min, whereas the signature peptide appeared at 6.2 min. No detectable peak corresponding to intact tocilizumab remained following digestion with either solubilized or immobilized trypsin. Compared with solubilized trypsin, digestion using immobilized trypsin produced a greater number of peaks in the 30.0–35.0 min elution window. In contrast, peak patterns observed between 0 and 25.0 min were comparable between the two digestion approaches. Increasing the number of centrifugal digestions enhanced signal intensity, with three digestion cycles yielding stronger peaks in the 0–15.0 min range than a single cycle.

Chromatographic resolution and assay selectivity

Representative LC-MS/MS chromatograms of the signature peptide and the internal standard (IS) obtained from human serum are presented in **Figure 1**. Both analytes were clearly separated from endogenous serum components, eluting at an identical retention time of 7.6 min. No interfering signals were detected at the retention times of the signature peptide or IS in serum samples collected from six healthy volunteers and six RA patients who had not received tocilizumab treatment (data not shown).

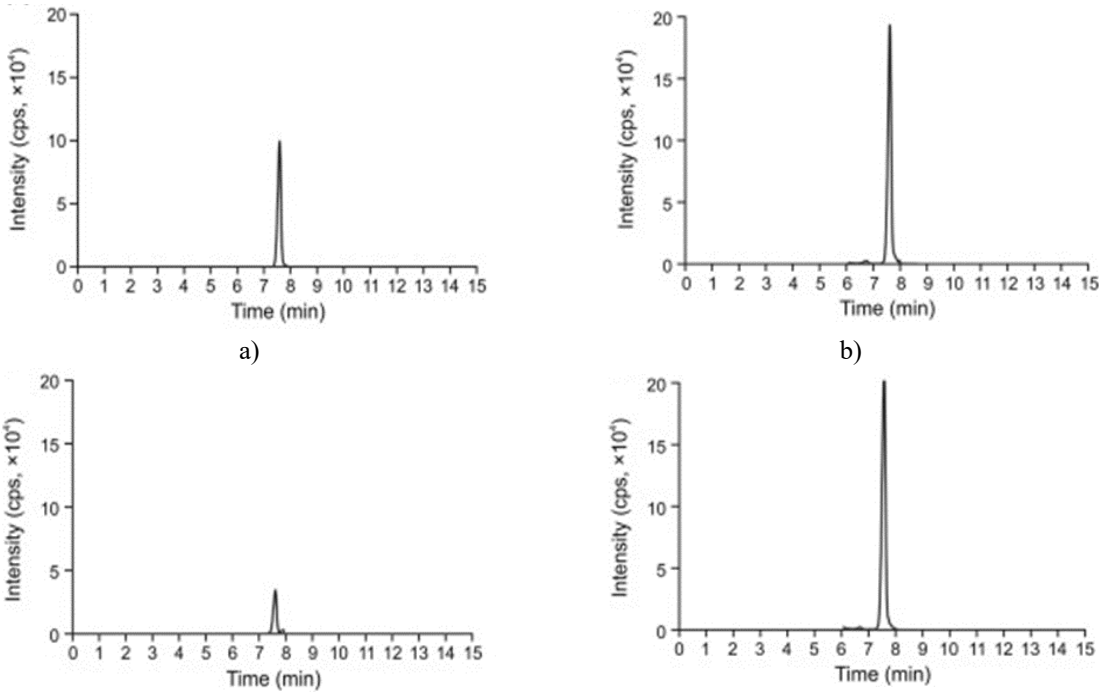


Figure 1. Representative selective reaction monitoring chromatograms for the tocilizumab-derived signature peptide. Chromatograms obtained from drug-free human serum spiked with 160 µg/mL tocilizumab show (a) the signature peptide and (b) the internal standard (IS, 1 µg/mL). Chromatograms from serum samples of rheumatoid arthritis patients receiving subcutaneous tocilizumab treatment display (c) the signature peptide and (d) the IS at 1 µg/mL. The concentration of the IS corresponds approximately to the amount of signature peptide generated from about 360 µg/mL of tocilizumab.

Analytical performance

Linearity and sensitivity

A linear response was obtained for serum tocilizumab concentrations spanning 2 to 200 µg/mL. Using a 1/x weighting factor, the calibration model yielded a correlation coefficient of 0.999. The lower limit of quantification (LLOQ) was established at 2 µg/mL. At the LLOQ, evaluation across three analytical runs with five replicates per run demonstrated accuracy values ranging from 89.6% to 100.5%, while the corresponding RSD values varied between 8.5% and 18.0%.

Precision and accuracy of the assay

The intra- and inter-day performance of the assay, assessed using three quality control (QC) levels (n = 5), is summarized in **Table 2**. Intra-day accuracy ranged from 90.7% to 109.4%, whereas inter-day accuracy fell between 97.5% and 101.3%. Precision, expressed as RSD, was 2.3%–8.5% for intra-day measurements and 2.9%–6.1% for inter-day evaluations.

Table 2. Intra-day and inter-day accuracy and precision of tocilizumab quantification in human serum.

Target concentration (µg/mL)	**Between-day performance (n = 5)**			**Within-day performance (n = 5)**		
	Measured value ± SD (µg/mL)	Variability (RSD, %)	Trueness (%)	Measured value ± SD (µg/mL)	Variability (RSD, %)	Trueness (%)

4	3.9 ± 0.1	2.9	97.5	4.4 ± 0.1	2.3	109.4
40	40.5 ± 2.5	6.1	101.3	40.9 ± 3.5	8.5	102.2
160	161.7 ± 6.2	3.8	101.1	145.2 ± 4.4	3.0	90.7

RSD: relative standard deviation; SD: standard deviation.

Matrix effects and recovery from solid-phase extraction

Matrix effects and extraction recovery were assessed using low (LQC), medium (MQC), and high (HQC) quality control samples prepared in serum from six healthy volunteers. The uncorrected matrix factors for the signature peptide were 50.4% (CV 7.3%) at LQC, 51.8% (CV 2.7%) at MQC, and 50.8% (CV 4.5%) at HQC. After internal standard normalization, these values improved to 99.6% (CV 3.7%), 97.4% (CV 2.0%), and 101.0% (CV 2.4%), respectively. Extraction recoveries for the signature peptide across the three QC levels were consistently high: 96.5% (CV 4.5%) at LQC, 93.8% (CV 1.6%) at MQC, and 94.5% (CV 3.2%) at HQC.

Stability and carry-over assessment

Table 3 summarizes the stability results for tocilizumab in human serum under various storage and handling conditions. No degradation was observed after 24 hours at room temperature or at 4 °C. Stability was maintained through up to three freeze-thaw cycles. Samples stored at −80 °C remained stable for at least one month. Processed samples kept in the autosampler at 4 °C for 24 hours showed negligible changes in the peptide-to-internal standard peak area ratio (n = 3; 98.8% of initial for LQC and 100.7% for HQC). Blank serum samples injected immediately after high-concentration (200 µg/mL) tocilizumab samples exhibited no detectable signature peptide peaks, confirming the absence of carry-over.

Table 3. Stability of tocilizumab in human serum under various conditions (n = 3).

Tocilizumab Nominal Concentration (µg/mL)	**Room Temperature (24 h) Mean ± SD (%)**	**RSD (%)**	**−80 °C (1 month) Mean ± SD (%)**	**RSD (%)**	**4 °C (24 h) Mean ± SD (%)**	**RSD (%)**	**Three Freeze-Thaw Cycles Mean ± SD (%)**	**RSD (%)**	**Two Freeze-Thaw Cycles Mean ± SD (%)**	**RSD (%)**	**One Freeze-Thaw Cycle Mean ± SD (%)**	**RSD (%)**
4	105.2 ± 3.3	3.1	97.7 ± 9.4	9.6	93.0 ± 7.6	8.2	91.7 ± 6.3	6.8	98.4 ± 10.9	11.1	105.4 ± 8.4	8.0
160	104.4 ± 12.5	12.0	103.0 ± 4.8	4.6	99.0 ± 4.0	4.0	97.2 ± 5.9	6.1	95.4 ± 1.7	1.7	111.2 ± 5.8	5.2

RSD: relative standard deviation; SD: standard deviation.

Measurement of serum tocilizumab levels in patients with rheumatoid arthritis

A total of eight and fourteen patients with rheumatoid arthritis received tocilizumab via intravenous and subcutaneous administration, respectively. Using the validated calibration model, serum drug concentrations were successfully quantified for all individuals. In the intravenously treated group, tocilizumab levels ranged from 5.8 to 28.9 µg/mL, with an average concentration of 15.9 ± 8.5 µg/mL. In contrast, patients treated by subcutaneous injection exhibited a broader concentration distribution, spanning 2.4–63.5 µg/mL, and a higher mean value of 25.6 ± 19.6 µg/mL **(Figure 2)**.

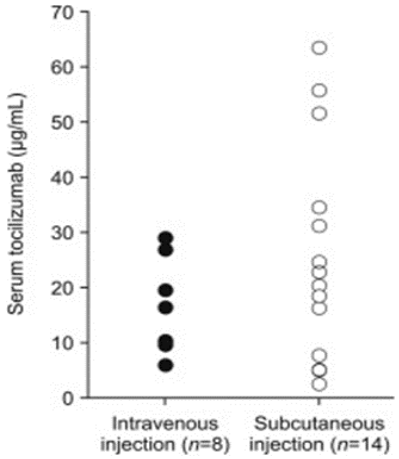
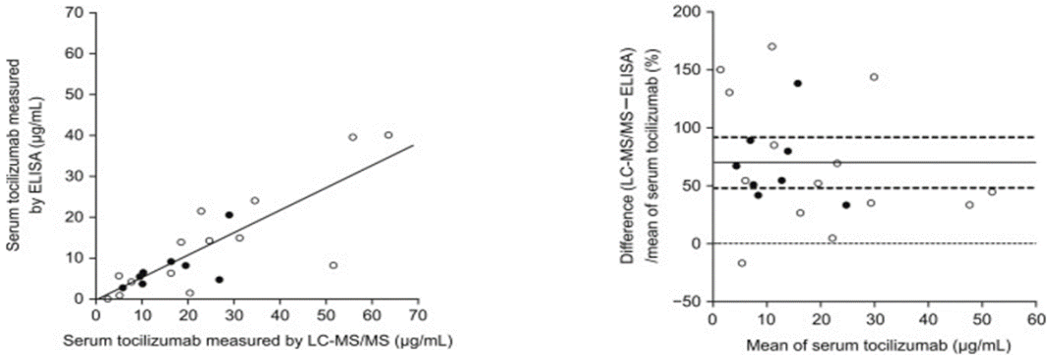


Figure 2. Serum concentrations of tocilizumab observed in a cohort of twenty-two individuals diagnosed with rheumatoid arthritis. Patients received either intravenous dosing of 8 mg/kg at intervals of 4–5 weeks (solid markers) or subcutaneous dosing of 162 mg per individual every two weeks (open markers).

Cross-method comparison of tocilizumab measurements

To evaluate agreement between analytical platforms, serum samples from twenty-two rheumatoid arthritis patients were analyzed using both LC-MS/MS and enzyme-linked immunosorbent assay techniques. Concentrations obtained by LC-MS/MS increased proportionally with those measured by ELISA, demonstrating a strong positive relationship characterized by the linear equation $y = 0.55x - 0.33$ (correlation coefficient $r = 0.82$, $P < 0.01$) **(Figure 3)**.

Despite this correlation, systematic divergence between the two methods was evident. Bland–Altman analysis showed that LC-MS/MS measurements differed from ELISA results by an average of 69.8%, with limits of agreement defined by a 95% confidence interval ranging from 47.9% to 91.7%. These findings indicate a statistically significant method-dependent bias between LC-MS/MS and ELISA.



a) b)

Figure 3. Comparative analysis of serum tocilizumab concentrations obtained using liquid chromatography–tandem mass spectrometry (LC-MS/MS) and enzyme-linked immunosorbent assay (ELISA) in a cohort of twenty-two patients with rheumatoid arthritis. (a) Correlation plot illustrating the relationship between concentrations measured by LC-MS/MS and those determined by ELISA. (b) Bland–Altman analysis depicting the agreement between the two analytical methods. Patients receiving intravenous tocilizumab therapy (n = 8) are represented by filled symbols, whereas those treated via subcutaneous administration (n = 14) are shown as open symbols. In the Bland–Altman plot, the central solid line indicates the mean bias, the dotted line corresponds to zero difference, and the heavy dotted lines represent the 95% confidence limits.

Reliable and rapid determination of serum tocilizumab concentrations is increasingly important for therapeutic drug monitoring in rheumatoid arthritis (RA), particularly to sustain disease remission in routine clinical practice. In this study, a rapid LC-MS/MS workflow was established by replacing conventional solubilized trypsin with immobilized trypsin, enabling completion of enzymatic digestion within 30 min. The resulting calibration model covered a broad concentration range (2–200 µg/mL), allowing sensitive and reproducible measurement of serum tocilizumab, including trough levels. To our knowledge, this is the first report describing an LC-MS/MS-based

quantification strategy for serum tocilizumab that combines immobilized trypsin digestion with omission of IgG purification in RA patients receiving either intravenous or subcutaneous administration.

Selection of an appropriate signature peptide is a critical determinant of quantitative accuracy in LC-MS/MS assays for monoclonal antibodies. Using a nano-LC system coupled to a hybrid quadrupole–Orbitrap mass spectrometer, five peptides derived from complementarity-determining regions (CDRs) were initially identified as candidates. Four of these were excluded due to analytical limitations, including the presence of missed cleavage sites or chemical modifications introduced during sample preparation. Peptides with incomplete tryptic cleavage may lack digestion specificity, while carbamidomethylation of cysteine or oxidation of methionine alters peptide mass and compromises quantitative reliability. Such structural heterogeneity can introduce variability in measured concentrations of therapeutic antibodies. Consequently, peptides containing internal lysine or arginine residues or modified amino acids were excluded, and LLIYYTSR was selected as the optimal signature peptide due to the absence of missed cleavages and chemical modifications.

Unlike many previously reported LC-MS/MS methods, the present approach does not incorporate an IgG purification step prior to digestion. Affinity-based purification using protein A or G is often employed to reduce ion suppression effects [7, 9, 13, 14, 17]; however, these procedures are labor-intensive and may introduce artificial variability in measured antibody concentrations [21]. Such complexity represents a major obstacle to routine clinical implementation. In the current method, although a moderate matrix effect was observed for the signature peptide, this effect was effectively compensated for by the stable isotope-labeled internal standard. In addition, solid-phase extraction minimized the introduction of non-target peptides into the MS/MS system. As a result, sufficient sensitivity was achieved to quantify trough serum tocilizumab concentrations in RA patients without IgG purification.

Optimization of sample preparation focused on maximizing the signal intensity of the signature peptide while minimizing analytical interference. High serum viscosity necessitated dilution to ensure efficient passage through the immobilized trypsin column. At the same time, excessive digestion was avoided to limit the generation of abundant non-specific peptides. A five-fold serum dilution combined with three centrifugal digestion cycles provided the optimal balance between signal intensity and reproducibility.

Immobilized trypsin enabled rapid proteolysis while reducing autolysis, owing to its dense binding to monolithic silica within the centrifugal column [10, 11]. Although cleavage of lysine- or arginine-rich regions buried within the protein structure may be less efficient with immobilized trypsin, chromatographic analysis using a PDA detector demonstrated that low-molecular-weight peptide profiles were comparable between immobilized and solubilized trypsin digestion. In contrast, digestion with immobilized trypsin yielded a greater number of higher-molecular-weight peptide peaks. A single digestion cycle was insufficient to generate adequate amounts of the signature peptide, whereas three centrifugal digestions produced optimal results.

Chromatographic conditions were carefully refined to ensure selective detection of the signature peptide while minimizing analysis time. A core-shell column was employed to achieve high-resolution separation [22], and elevated column temperature (85 °C) was used to reduce mobile phase viscosity and enhance peptide diffusion. A stepwise gradient incorporating a high acetonitrile phase facilitated removal of residual protein digests from the analytical column, enabling continuous sample analysis. Importantly, no interfering peaks corresponding to the signature peptide or internal standard were detected in serum digests from healthy individuals or RA patients not receiving tocilizumab.

The calibration range of 2–200 μg/mL was selected to accommodate the wide variability in serum tocilizumab concentrations associated with different administration routes. Method accuracy and precision met established international bioanalytical validation criteria. Although the MQC concentration was slightly below 30% of the upper calibration limit, assay reproducibility was confirmed through evaluation of calibration standards and three QC levels. Tocilizumab and its signature peptide remained stable throughout sample handling and LC-MS/MS analysis. Long-term serum storage at −80 °C and up to three freeze–thaw cycles were permissible without compromising analytical integrity. Carry-over was effectively prevented by routine needle rinsing procedures within the HPLC system.

Application of the validated method to RA patients revealed substantial interindividual variability in serum tocilizumab concentrations, particularly among those receiving subcutaneous injections. Blood samples represented both trough and non-trough conditions. Observed concentrations were higher than those reported in a previous phase III study involving intravenous (12.4 ± 7.9 μg/mL) and subcutaneous (10.6 ± 7.8 μg/mL)

administration [23], yet all values remained within the established calibration range. These findings demonstrate that the method is suitable for clinical use regardless of dosing route or sampling timing.

Comparison with ELISA revealed a positive correlation between the two analytical approaches; however, LC-MS/MS consistently yielded higher concentration values. This discrepancy likely reflects fundamental differences in assay targets. The ELISA employed here detects only unbound tocilizumab, whereas LC-MS/MS quantifies both free and receptor-bound forms through measurement of a proteolytic signature peptide [24]. Consequently, results obtained by the two methods should not be interpreted interchangeably without careful consideration of assay principles.

Several limitations should be acknowledged. First, digestion efficiency was lower than that typically achieved with solubilized trypsin, potentially reducing sensitivity and introducing variability at low concentrations. Second, the method was not evaluated in patients with hyperproteinemia, where elevated immunoglobulin levels may further impair digestion efficiency and increase ion suppression [25, 26]. Third, patients receiving weekly subcutaneous dosing were not included, although the established calibration range should accommodate such regimens. Fourth, applicability in juvenile RA and patients with abnormal physiological conditions remains unconfirmed. Finally, the study cohort was relatively small, though patient characteristics reflected typical Japanese RA populations, and no significant demographic or clinical differences were observed between administration groups. Larger-scale validation across diverse patient populations will be necessary to confirm broad clinical applicability.

Conclusion

A rapid and validated LC-MS/MS method was successfully developed for quantifying serum tocilizumab using immobilized trypsin digestion without IgG purification. The method demonstrated acceptable accuracy, precision, and stability across a wide calibration range, enabling reliable measurement of both trough and non-trough concentrations. Considerable variability in serum tocilizumab levels was observed among RA patients, highlighting the value of therapeutic drug monitoring. This approach is applicable to both intravenous and subcutaneous dosing regimens and offers a practical alternative for pharmacokinetic evaluation and clinical monitoring of tocilizumab in routine healthcare settings.

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