# A rapid analytical method using an LC–MS/MS for quantitation of serum tocilizumab and its clinical application

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## Background and purpose

Tocilizumab, a humanized anti-interleukin-6 receptor monoclonal antibody, is used for treatment of rheumatoid arthritis (RA). Serum tocilizumab concentration was reported to be inversely correlated with serum inflammatory markers level and disease activity score in RA patients. Enzyme-linked immunosorbent assay (ELISA) has been generally used for quantitation of serum tocilizumab. However, ELISA has several problems such as cross-reactivity with endogenous components. Although a proteomics-based quantitative method using a liquid chromatography-tandem mass spectrometry (LC–MS/MS) has been developed, this method is not practical for clinical settings because of time-consuming pretreatments including tryptic digestion and IgG purification. The aims of this study were to develop a rapid LC–MS/MS method for quantitation of serum tocilizumab with immobilized trypsin and without IgG purification and to apply it to patient samples.

## Methods

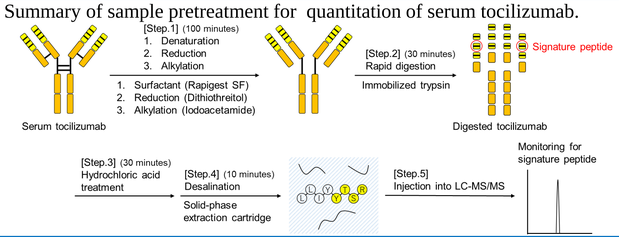
### Signature peptide determination

Tryptic digests including tocilizumab-derived peptides were separated and measured by nano LC system and hybrid quadrupole-Orbitrap mass spectrometer. Signature peptide candidates were searched by Proteome Discoverer software Version 2.1 with FASTA file including the amino acid sequences of tocilizumab.

### Sample pretreatment

Reduced-alkylated serum tocilizumab was digested for 30 minutes using immobilized trypsin. In the digested products, residual RapiGest SF surfactant was precipitated by adding hydrochloric acid. The supernatant was desalinated using a solid-phase extraction cartridge. The sample solution was filtrated and 10 µl of the solution was injected into the HPLC system (Figure.1).

Figure 1 Summary of sample pretreatment for quantitation of serum tocilizumab.




### Chromatographic conditions

The signature peptide and internal standard (IS) in human serum were separated using an LC system. The analytical column of Aeris Peptide C18 with a SecurityGuard Ultra cartridge was used for separation. The column was kept at 85°C. Stepwise elution program was combined with the following mobile phase: A solution, 0.1% acetic acid in water; B solution, 0.1% acetic acid in acetonitrile. The mobile phase consisted of 14% B solution/86% A solution for the first 8 minutes, followed by 70% B solution/30% A solution from 8.1 to 11.0 minutes and 14% B solution/86% A solution from 11.1 to 15.0 minutes. The flow rate was 0.3 mL/min for the first 8 minutes and from 12.6 to 15.0 minutes, and 0.6 mL/min from 8.1 to 12.5 minutes. The position of the switching valve was set to connect the MS/MS system from 6 to 8 minutes.

### Mass spectrometer conditions

The column effluent was monitored using a triple quadrupole tandem mass spectrometer equipped with an electrospray probe in positive ion multiple reaction monitoring mode. The ion transitions were monitored for each compound: LLIYYTSR as signature peptide, m/z 514.95/689.45(+); LLIYYTSR[R($^{13}C_6$$^{15}N_4$)] as IS, m/z 519.90/699.30(+).

### Clinical application

This study enrolled 22 Japanese patients receiving tocilizumab treatment for RA at Hamamatsu University Hospital. Tocilizumab was administrated by intravenous injection at 8 mg/kg every 4–5 weeks or subcutaneous injection at 162 mg/body every 2 weeks. Serum samples were collected just before intravenous injection or within a week before subcutaneous injection.

## Results

- The LLIYYTSR peptide including complementary determining region of tocilizumab was determined as signature peptide for quantitation of serum tocilizumab (Table 1).
- The retention time of signature peptide and IS was 7.6 minutes and the total run time was 15 minutes. No peaks interfering with signature peptide and IS were observed from serum digests of six healthy subjects and RA patients without tocilizumab administration (Figure 2).
- The LLOQ for serum tocilizumab was 2 µg/mL. The accuracies and imprecisions of LLOQ in five replicates at three runs were 89.6–100.5% and 8.5–18.0%, respectively. The intra- and inter-day accuracies and imprecisions were shown in Table 2 and allowed for three QC samples.
- Tocilizumab was stable in human serum under different storage conditions (Table 3).
- The calibration curve of serum tocilizumab concentration was liner within a range of 2–200 µg/mL and the coefficient of determination was 0.998 (Figure 3).
- The matrix factor were calculated for three QCs in six healthy subjects. The matrix factors of signature peptide in LQC, MQC, and HQC were 50.4% (RSD, 7.3%), 51.8% (2.7%), and 50.8% (4.5%), respectively. The IS-corrected matrix factors in LQC, MQC, and HQC were 99.6% (RSD, 3.7%), 97.4% (2.0%), and 101.0% (2.4%), respectively.
- Serum tocilizumab concentrations in RA patients receiving intravenous and subcutaneous injection were 5.8–28.9 µg/mL (interquartile range, 9.8–23.1 µg/mL) and 2.4–63.5 µg/mL (7.6–34.5 µg/mL), respectively (Figure 4).

Table 1 Characteristics of candidate peptides derived from variable regions of tocilizumab.

| Peptide name | Sequence | Region | Missed cleavage | Modification | Mass of $[M + H]^+$ |
|---|---|---|---|---|---|
| TL 46–53 | LLIYYTSR | Light, CDR2 | No | No | 1028.6 |
| TL 25–42 | ASQDISSYLNWYQQKPGK | Light, CDR1 | K15 | No | 2113.0 |
| TL 25–45 | ASQDISSYLNWYQQKPGKAPK | Light, CDR1 | K15, K18 | No | 2409.2 |
| TH 83–98 | LSSVTAADTAVYYcAR | Heavy, CDR3 | No | C14: Carbamidomethylation | 1747.8 |
| TH 103–123 | TTAmDYWGQGSLVTVSSASTK | Heavy, CDR3 | No | M4: Oxidation | 2190.0 |
| TL 1–18 | DIQMTQSPSSLSASVGDR | Light | No | No | 1878.9 |
| TL 1–24 | DIQMTQSPSSLSASVGDRVTITcR | Light | R18 | C23: Carbamidomethylation | 2609.3 |
| TL 19–24 | VTITcR | Light | No | C5: Carbamidomethylation | 749.4 |
| TL 54–61 | LHSGVPSR | Light | No | No | 852.5 |
| TH 66–72 | SRVTMLR | Heavy | R2 | No | 862.5 |
| TH 73–82 | DTSKNQFSLR | Heavy | K4 | No | 1195.6 |
| TH 77–82 | NQFSLR | Heavy | No | No | 764.4 |

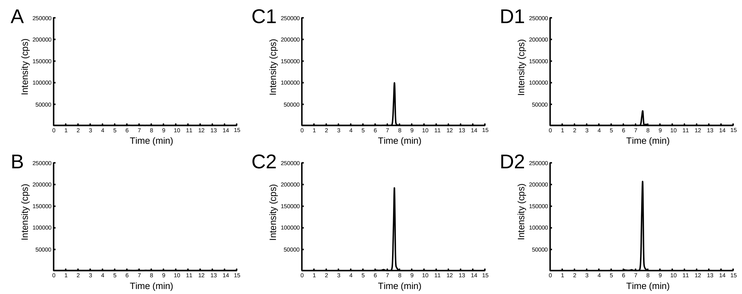
Peptides were named after its region in the tocilizumab (TH, heavy chain; TL, light chain; the numbers, the position of N- and C-terminal amino acid from N-terminus of tocilizumab).

A small letter c and m in the sequences represents a carbamidomethylated cysteine residue and an oxidized methionine residue, respectively.

Missed cleavage indicates the residue of sequences that can be digested by trypsin.

CDR: Complementarity determining region.

Figure 2 Selective reaction monitoring chromatograms of the signature peptide derived from tocilizumab.




(A) Drug-free human serum. (B) Serum of a RA patient not treated with tocilizumab. (C) Drug-free human serum spiked with 160 µg/mL tocilizumab: signature peptide (C1) and 1 µg/mL internal standard (IS) (C2). (D) Serum of a RA patient treated with tocilizumab: signature peptide (D1) and 1 µg/mL IS (D2).

Table 2 Intra- and inter-day accuracies and imprecisions of tocilizumab in human serum.

| Nominal value (µg/mL) | Intra-day (n = 5) | | | Inter-day (n = 5) | | |
|---|---|---|---|---|---|---|
| | Mean ± SD (µg/mL) | Accuracy (%) | RSD (%) | Mean ± SD (µg/mL) | Accuracy (%) | RSD (%) |
| 4 | 4.38 ± 0.10 | 109.4 | 2.3 | 3.90 ± 0.11 | 97.5 | 2.9 |
| 40 | 40.9 ± 3.5 | 102.2 | 8.5 | 40.5 ± 2.5 | 101.3 | 6.1 |
| 160 | 145 ± 4 | 90.7 | 3.0 | 162 ± 6 | 101.1 | 3.8 |

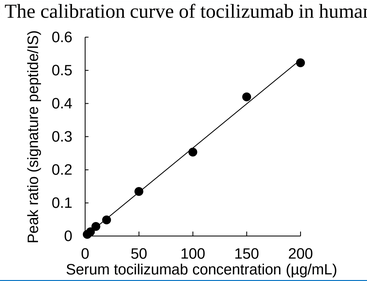
SD standard deviation and RSD relative standard deviation.

Table 3 Stability of tocilizumab in human serum.

| Tocilizumab concentration | 4 (n = 3) | | 160 (n = 3) | |
|---|---|---|---|---|
| (µg/mL) | Stability ± SD (%) | RSD (%) | Stability ± SD (%) | RSD (%) |
| Room temperature, 24 hours | 105.2 ± 3.3 | 3.1 | 104.4 ± 12.5 | 12.0 |
| 4°C, 24 hours | 93.0 ± 7.6 | 8.2 | 99.0 ± 4.0 | 4.0 |
| -80°C, 1 month | 97.7 ± 9.4 | 9.6 | 103.0 ± 4.8 | 4.63 |
| Freeze-thaw cycle (-80°C), once | 105.4 ± 8.4 | 8.0 | 111.2 ± 5.8 | 5.2 |
| Freeze-thaw cycle (-80°C), twice | 98.4 ± 10.9 | 11.1 | 95.4 ± 1.65 | 1.7 |
| Freeze-thaw cycle (-80°C), three times | 91.7 ± 6.3 | 6.8 | 97.2 ± 5.9 | 6.1 |

SD standard deviation and RSD relative standard deviation.

Figure 3 The calibration curve of tocilizumab in human serum.



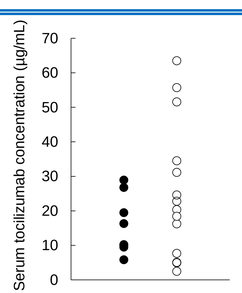




Figure 4 Serum tocilizumab concentrations in RA patients receiving intravenous (closed circle) and subcutaneous (open circle) injection. Tocilizumab was administrated by intravenous injection at 8 mg/kg every 4–5 weeks or subcutaneous injection at 162 mg/body every 2 weeks.

## Conclusions

This study developed a validated LC-MS/MS method for rapid quantitation of serum tocilizumab with immobilized trypsin and without IgG purification. The present method can be applied to evaluating the pharmacokinetics of intravenous and subcutaneous tocilizumab in RA patients.

## Disclosures

Nothing to disclose.