Simultaneous quantification of trastuzumab and pertuzumab in human serum using accurate mass spectrometry

Sensitive multiplexed quantification of 2 commonly co-administered mAbs using the ZenoTOF 7600 system with SCIEX OS software

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This technical note demonstrates a highly sensitive quantification workflow for trastuzumab and pertuzumab in human serum on a high-end accurate mass spectrometer. Lower limits of quantification (LLOQs) of 0.15 µg/mL were achieved for trastuzumab and pertuzumab. Strong linearity with a wide linear dynamic range (LDR) of 3.3 orders of magnitude with exceptional accuracy and precision was achieved. The assay included a simple immunoprecipitation and on-bead digestion method paired with an 8-minute LC-MS/MS analysis.

Monoclonal antibodies (mAbs) are increasingly being used in combination with other mAbs for cancer and COVID-19 treatements.[1] In breast cancer treatment, trastuzumab and pertuzumab are commonly co-administered to target unique human epidermal growth factor receptor 2 (HER2) epitopes.[2] With the increasing application of mAb co-administration, simple and robust quantification methods are necessary to meet the needs of pharmacokinetic (PK) studies.

Conventional analytical methods involving ligand-binding assays, such as enzyme-linked immunosorbent assays (ELISAs), are employed during PK evaluations due to high sensitivity and sample throughput. However, multiplexing can be a significant challenge for such assays with limited selectivity. In addition to selectivity, sensitivity is also a critical factor because PK studies are often challenged by low sample volumes. Therefore, LC-MS/MS-based techniques are being increasingly adopted for PK measurements of combination mAb therapeutics given their enhanced specificity, selectivity and sensitivity.[3]

Here, an immunoaffinity purification workflow with on-bead digestion was employed to simultaneously quantify trastuzumab and pertuzumab in human serum on the ZenoTOF 7600 system (Figure 1).

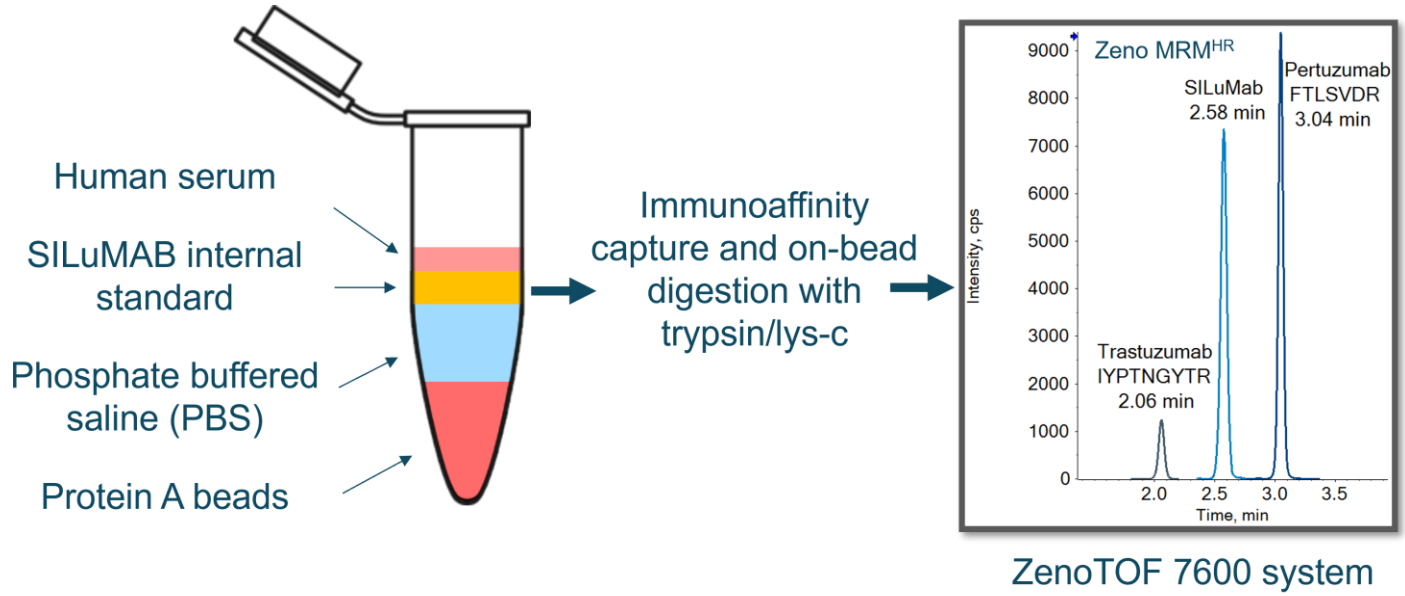

Figure 1. Summarized workflow for the simultaneous quantification of pertuzumab and trastuzumab in human serum. Pertuzumab and trastuzumab were extracted from human serum using immunoprecipitation. On-bead digestion was performed to capture digested peptides, which were analyzed on the ZenoTOF 7600 system.

Key features of the trastuzumab and pertuzumab quantification workflow on the ZenoTOF 7600 system

- **Sensitive quantification of co-administered mAbs**: Trastuzumab and pertuzumab were quantified in human serum with excellent linearity, accuracy and precision.
- **Reduced sample volume**: Highly sensitive results were acquired with a low sample volume of 10 µL by leveraging the Zeno trap, which enables greater MS/MS sampling efficiency.
- **Improved selectivity**: High-resolution analysis using the ZenoTOF 7600 system reduced background and provided high selectivity and sensitivity for quantification.
- **User-friendly interface**: Productivity was increased with a user-friendly interface and integrated platform for data acquisition, processing and management in SCIEX OS software.

Methods

Samples and reagents: Trastuzumab and pertuzumab were purchased from MedChemExpress. Normal human serum and SILuMAB were purchased from Sigma Aldrich. Trypsin/lys-C was purchased from Promega.

Sample preparation: A 3 mg/mL stock solution of trastuzumab was prepared by dissolving 1 mg of lyophilized trastuzumab in 333 µL of phosphate buffered saline (PBS) with a pH of 7.4. A 3 mg/mL stock solution of pertuzumab was prepared by adding 300 µL of PBS to a 30 mg/mL stock solution. Subsequent dilutions were performed in normal human serum to prepare spiked serum samples ranging from 0.03 µg/mL to 300 µg/mL. A 500 µg/mL stock solution of SILuMAB was prepared by dissolving 0.1 mg of lyophilized SILuMAB in 0.2 mL of PBS. The stock was diluted 250:1 (v/v) in PBS to prepare a final solution at a concentration of 2 µg/mL. Protein A beads were diluted 8:1 with PBS and washed three times with PBS before use.

Each sample contained a 10 µL sample of normal human serum, 20 µL of 2 µg/mL SILuMAB, 200 µL of diluted protein A beads and 200 µL of PBS. After samples were shaken for 30 minutes at room temperature, the beads were washed twice with PBS. The beads were resuspended in 150 µL of digestion buffer containing 150 mM ammonium carbonate and 1 mM calcium chloride, and they were denatured at 95°C for 5 minutes. After allowing it to cool to room temperature, 2 µg of trypsin/lys-c was added to each sample and on-bead digestion was performed for 2 hours at 50°C. Digestion was stopped by adding 3 µL of formic acid. Samples were separated from the beads and placed in vials or plates for LC-MS/MS analysis.

Chromatography: Chromatography was performed on a Shimadzu LC-40 X3 system using a Phenomenex Biozen XB-C18 (2.1 × 100 mm, 2.6 µm, 100 Å) column. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. The operating flow rate was 0.5 mL/minute. Gradient conditions are summarized in Table 1. Column temperature was set at 40°C. A 20 µL sample was injected for analysis.

Table 1. Chromatography gradient.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	*95*	*5*
5	*70*	*30*
5.1	*5*	*95*
5.6	*5*	*95*
5.7	*95*	*5*
7.5	*95*	*5*

Mass spectrometry: The ZenoTOF 7600 system with the Turbo V ion source operated in positive Zeno MRM^{HR} mode was used for analysis. The source and gas conditions and Zeno MRM^{HR} transitions are summarized in Tables 2 and 3, respectively.

Table 2. Source and gas conditions.

Parameter	Settings
Polarity	Positive
Curtain gas	35 psi
Ion source gas 1	80 psi
Ion source gas 2	80 psi
CAD gas	11
Ion spray voltage	4000 V
Source temperature	700°C

Data analysis: Quantitative data analysis was performed using SCIEX OS software 3.0. Exact product ion masses were calculated using SCIEX OS software, and a peak width of 0.02 Da was used to generate extracted ion chromatograms (XICs). Linear calibration curves (1/x^2 weighted) for each compound normalized to the SILuMAB internal standard.

Table 3. MRM^{HR} transitions.

Name	Peptide	Precursor ion (*m/z*)	Product ion (*m/z*)	DP (V)	CE (V)
*Trastuzumab_1**	*IYPTNGYTR*	*542.8*	*404.7010*	*60*	*28*
Trastuzumab_2	*IYPTNGYTR*	*542.8*	*808.3948*	*60*	*28*
*Pertuzumab_1**	*FTLSVDR*	*419.2*	*589.3304*	*20*	*24*
Pertuzumab_2	*FTLSVDR*	*419.2*	*476.2463*	*20*	*24*
*SILuMAB**	*DTLMISR*	*423.2*	*516.2838*	*20*	*24*

**MRM^{HR} transition was used for quantification.*

Trastuzumab and pertuzumab quantification results

Trastuzumab and pertuzumab were selected as model mAb therapeutics to evaluate the quantification of combination mAbs in human serum on the ZenoTOF 7600 system. The calibration curve included concentrations ranging from 0.15 µg/mL to 300 µg/mL for trastuzumab and pertuzumab. The concentration of the internal standard (IS), SILuMAB, was 0.25 µg/mL. Each calibration point was measured in three replicate injections.

Defined quantitative criteria for the coefficient of variation (%CV) and accuracy were followed for assay performance assessment. At the level of the LLOQ, the %CV was required to be <20% with accuracy within ±20% of the nominal concentration. For the remaining concentrations, the %CV was required to be <15%, and the accuracy was required to be within ±15% of the nominal concentration.

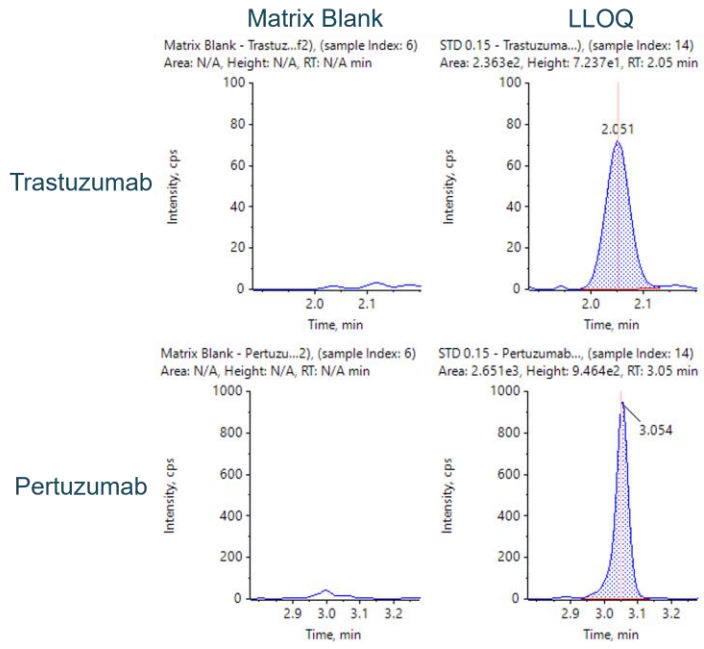

Figure 2. Extracted ion chromatograms (XICs) of trastuzumab (top) and pertuzumab (bottom) in matrix blanks (left) and human serum at the LLOQ concentrations (right). An LLOQ of 0.15 µg/mL was achieved for both mAbs, trastuzumab and pertuzumab.

An LLOQ of 0.15 µg/mL was achieved for trastuzumab and pertuzumab (Figure 2). Matrix interferences in the blank were more than 5x lower than the peak areas in the LLOQ. The overall %CV was <10.1% for trastuzumab and <8.12% for pertuzumab (Table 4). The overall accuracy was within ±8% of the nominal concentration for trastuzumab. While for pertuzumab, the overall accuracy was within ±12% of the nominal concentration.

Strong linearity was achieved with correlation coefficients (r) of >0.996 for both trastuzumab and pertuzumab. A wide LDR of up to 3.3 orders of magnitude was reached (Figure 3).

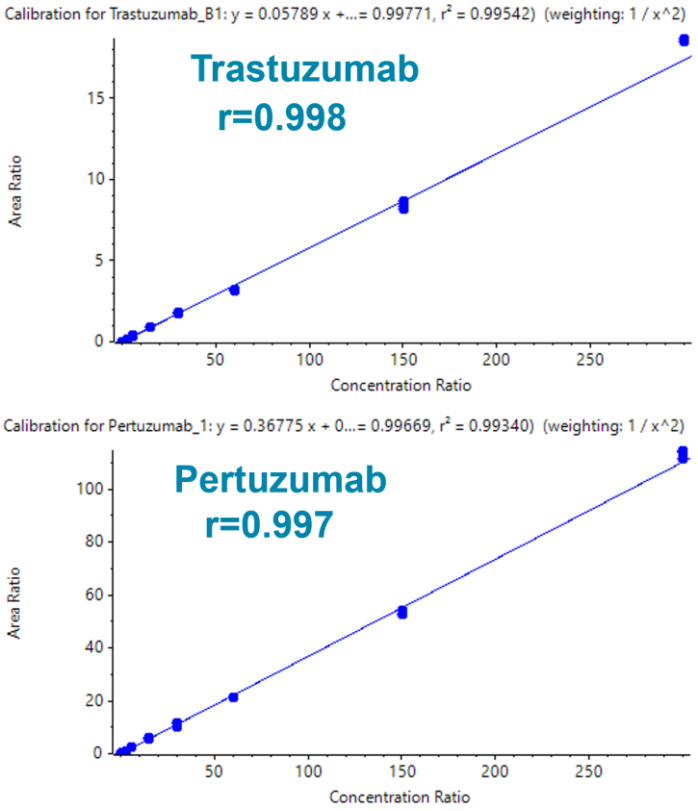

Figure 3. Calibration curve for trastuzumab (top) and pertuzumab (bottom). For trastuzumab, the linear range was 0.15–300 µg/mL. For pertuzumab, the linear range was 0.15–300 µg/mL. An LDR of up to 3.3 orders of magnitude was reached.

Carryover was also evaluated by analyzing a blank immediately following the upper limit of quantification (ULOQ) standard at 300 µg/mL. The carryover was observed to be minimal with peak area of <0.003% relative to the ULOQ and <5% relative to the LLOQ for both mAbs. Assay conditions demonstrated optimal conditions and a setup suitable for accurate determination of combination mAbs at low levels with high confidence.

Table 4. Accuracy and precision results for trastuzumab and pertuzumab for three replicate injections at each calibration level.

Trastuzumab			Pertuzumab		
Concentration (µg/mL)	Accuracy (%)	CV (%)	Concentration (µg/mL)	Accuracy (%)	CV (%)
0.15	*94.0*	*5.52*	*0.15*	*104*	*7.62*
0.3	*93.0*	*10.1*	*0.3*	*88.0*	*5.40*
0.6	*99.0*	*3.49*	*0.6*	*107*	*1.26*
3	*96.0*	*4.42*	*3*	*92.0*	*3.15*
6	*107*	*1.87*	*6*	*106*	*2.08*
15	*104*	*2.48*	*15*	*107*	*1.93*
30	*104*	*1.66*	*30*	*100*	*8.12*
60	*92.0*	*1.64*	*60*	*97.0*	*0.46*
150	*98.0*	*2.83*	*150*	*98.0*	*1.54*
300	*108*	*0.47*	*300*	*103*	*1.36*

The Zeno MRM^{HR} method achieved excellent linearity, accuracy and precision from 0.15 µg/mL to 300 µg/mL for trastuzumab and pertuzumab. The on-bead digestion immunoprecipitation workflow resulted in clean samples that enabled a minimal serum volume of 10 µL of serum for sensitive and simultaneous quantification of trastuzumab and pertuzumab.

The on-bead digestion protocol provided clean and low-background samples resulting in a simple workflow. Combining the consistent integration and automated quality control flagging in SCIEX OS software enabled a rapid turnaround of quantitative results for trastuzumab and pertuzumab in a single assay.

Conclusions

- LLOQs of 0.15 µg/mL were reached for the quantification of trastuzumab and pertuzumab in human serum.
- Sensitive analysis of low serum volume was achievable with the improved MS/MS sampling efficiency offered by the Zeno trap.
- Excellent linearity, accuracy and reproducibility were achieved in the quantification of combination mAb therapeutics using the ZenoTOF 7600 system in MRM^{HR} mode.
- A stand-alone streamlined software platform was demonstrated on SCIEX OS software for data acquisition, analysis, quality control flagging and reporting.

References

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