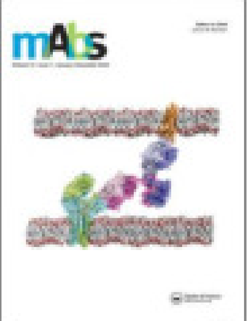

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REPORT

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Development and validation of an LC-MS/MS method for simultaneous quantification of co-administered trastuzumab and pertuzumab

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ABSTRACT

Given the increasing use of combination therapy with multiple monoclonal antibodies (mAbs), there is a clinical need for multiplexing assays. For the frequently co-administered anti-human epidermal growth factor receptor 2 (HER2) mAbs trastuzumab and pertuzumab, we developed a high-throughput and robust hybrid ligand-binding liquid chromatography-mass spectrometry (LC–MS)/MS quantitative assay. Nanomolar concentrations of trastuzumab and pertuzumab were determined in 10 µL serum samples after extraction by affinity purification through protein A beads, followed by on-bead reduction, alkylation, and trypsin digestion. After electrospray ionization, quantification was obtained by multiple reaction monitoring LC-MS/MS using SILuMab as an internal standard. The method was validated according to the current guidelines from the US Food and Drug Administration and the European Medicines Agency. Assay linearity was established in the ranges 0.250–250 µg/mL for trastuzumab and 0.500–500 µg/mL for pertuzumab. The method was accurate and selective for the simultaneous determination of trastuzumab and pertuzumab in clinical samples, thereby overcoming the limitation of ligand binding assays that cannot quantify mAbs targeting the same receptor. Furthermore, this method requires a small blood volume, which reduces blood collection time and stress for patients. The assay robustness was verified in a clinical trial where trastuzumab and pertuzumab concentrations were determined in 670 serum samples. As we used commercially available reagents and standards, the described generic bioanalytical strategy can easily be adapted to multiplex quantifications of other mAb combinations in non-clinical and clinical samples.

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Trastuzumab; pertuzumab; mAbs; monoclonal antibodies; lc-MS; pharmacokinetics; multiplex; validation

Introduction

With a rapidly growing number of registered monoclonal antibodies (mAbs) in different fields of medicine, mAbs are also increasingly used in combination. Co-administration of immunomodulating mAbs such as nivolumab and ipilimumab is common practice, as well as dual HER2-targeting mAbs, and a sharp rise in co-administration of other mAbs can be expected.[1–3]

Approximately 10–15% of breast cancer and esophagogastric cancer patients are human epidermal growth factor receptor 2 (HER2) positive, and the oncogenic transmembrane HER2 can be used for targeted therapy in these patients.[4,5] Dual HER2 targeting through trastuzumab and pertuzumab, which target different HER2 epitopes, is a standard of care in breast cancer treatment and being investigated in, for example, esophageal cancer and gastric cancer.[5–7] For both mAbs, a clear exposure–response relationship is lacking.[8,9] However, since these two mAbs are increasingly used together, simple and robust quantification methods are needed for pharmacokinetic (PK) evaluation in clinical studies. In addition, considering the invasive nature of blood withdrawal, there is an unmet need to reduce blood sampling volume and thereby minimize patient harm in clinical trials, as well as clinical practice. The use of multiplex bioanalytical assays for co-administered mAbs is an effective way to achieve these goals.

Ligand-binding assays (LBAs), in particular enzyme-linked immunosorbent assays (ELISAs), have long been the standard method for PK measurements of therapeutic antibodies, because of their high sensitivity and throughput. However, depending on the available reagents, LBAs often lack the selectivity to distinguish between significant molecular differences (such as point mutations in the target protein or post-translational modifications) and display limited multiplexing capabilities.[10–12] For this reason, simultaneous quantification of multiple antibodies remains challenging with LBA and is impossible for quantification of mAbs like trastuzumab and pertuzumab, which recognize the same receptor, albeit targeting two distinct epitopes.[12] Specific enzyme-linked immunosorbent assay methods using anti-idiotype mAbs against either trastuzumab or pertuzumab have been used previously, but this approach still necessitates two assays.[13] Although superior in specificity, selectivity, multiplexing ability, and requiring a small sample volume, liquid

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chromatography-mass spectrometry (LC-MS) techniques are not yet able to deliver the sensitivity, throughput and miniaturization provided by modern ligand binding approaches. However, when used together in hybrid methods, the two techniques allow the association of functional knowledge with high molecular detail, providing more accurate insights into PK and pharmacodynamic behavior of biotherapeutics. Due to the practical limits in mass range and resolution, surrogate signature peptides that are released from protein drugs via enzymatic digestions are monitored to obtain absolute quantitative data. Essential for minimizing technical variations during sample preparation is the addition of an internal standard (IS), displaying similar or identical physical and chemical features, which allows monitoring of the full sample preparation workflow.[10,12,14] LC-MS/MS methods using multiple reaction monitoring (MRM) for quantification of only trastuzumab in human serum have been described previously, including a method employing two surrogate peptides reaching a sensitivity of 5 µg/mL, and another reaching a sensitivity of 20 ng/mL with the latter using high resolution MS detection.[15–18]

To facilitate quantification of co-administered mAbs, multiplex LC-MS/MS methods have been developed. However, until recently these assays always required specific reagents for affinity purification or specific stable isotope labeled (SIL) ISs.[19,20] Recently, SIL universal mAbs have become commercially available, and an LC-MS/MS assay capable of quantifying multiple co-administered mAbs using nonspecific affinity purification through protein G, and a single commercially available SIL IS, has been described.[21] However, this method has a rather low sensitivity, does not reach the throughput times of LBA, and has not been used for multiplexing the quantification of mAbs that are co-administered in clinical practice.

To date, no validated multiplex assays for the quantification of co-administered trastuzumab and pertuzumab in human samples have been published. In fact, the recently published PK profiling of co-administered trastuzumab and pertuzumab in a clinical trial was obtained with two separate methods, since trastuzumab was quantified by LC-MS/MS and pertuzumab through ELISA.[8] Here, we present a fully validated and high-throughput 2-in-1 LC-MS/MS method for the simultaneous quantification of trastuzumab and pertuzumab in human serum using commercially available reagents and standards. The method, which uses only 10 µL of patient serum, was employed for PK assessment of the co-administered antibody drugs in support of a Phase 2 clinical trial in patients with resectable HER2-positive esophageal adenocarcinoma.[6]

Results

Method development

Selection of surrogate peptides

The availability of unique surrogate peptides is essential for quantification of proteins by LC-MS/MS methods. Because of the high similarity in amino acid sequence between trastuzumab and pertuzumab, unique suitable peptides can only be found in the highly variable complementarity-determining region (CDR). *In silico* trypsin digestion was used to predict surrogate tryptic peptides, based on the known amino acid sequences and functional structures of the mAb drugs.[22] Selectivity of each tryptic peptide in human serum was initially screened by querying the human protein databases with BLAST and later experimentally validated.[23] Tryptic peptides IYPTNGYTR, FTLSVDR and DTLMISR*($^{13}C_6{}^{15}N_4$-labeled arginine) were selected as quantifier peptides for trastuzumab, pertuzumab and SILuMab, respectively (see Table 1 for surrogate peptides and their MRM transitions). During method development, the trastuzumab surrogate peptide FTISADTSK was used as a monitoring peptide for information purposes, but this peptide was not included for quantification during validation. The tryptic peptide DTLMISR* ($^{13}C_6{}^{15}N_4$) is one of the labeled surrogate peptides in the SILuMab sequence. It was chosen because during chromatography the peptide elutes between the chosen surrogate peptides for trastuzumab and pertuzumab and resulted in a stable response throughout validation and bioanalysis of study samples. Because of the labeling, the mass can be resolved from its native counterpart, thereby overcoming selectivity issues.

Sample clean up for sensitivity

Nonselective affinity purification with protein A was used. The sample volume, dilution, amount of beads added for affinity purification, and incubation time were all optimized to ensure maximum binding and to avoid saturation throughout the full calibration range, as well as to reduce background interference.

Trypsin digestion optimization

The amount of trypsin (20 µg per sample), incubation time (60 min), and temperature (37°C) were optimized for sufficient recovery of peptides and a reproducible and time-effective sample processing. During method development it became clear that, although none of the peptides contain cysteine residues and the CDR region is exposed on the surface of the antibody and therefore readily accessible to proteolysis, the addition of a reduction and alkylation step contributed to an increased and consistent recovery of tryptic peptides. A graphic depiction of the method workflow is shown in Figure 1.

Table 1. Surrogate peptides and MRM transitions.

Analyte	Surrogate peptide	Q1 mass (amu)	Q3 mass (amu)	Time (min)
Trastuzumab	IYPTNGYTR	542.8	404.7	1.3
Pertuzumab	FTLSVDR	419.5	589.2	2.7
SILuMab	DTLMISR*($^{13}C_6{}^{15}N_4$)	423.2	516.3	2.2

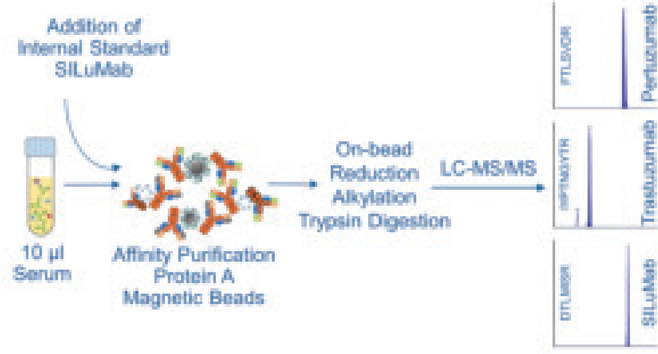


Figure 1. Graphic description of method workflow.

Throughput

This method allows a throughput similar to LBA of at least 120 samples per day on one LC-MS/MS platform, due to a relatively short injection to injection time of 10 minutes in the chromatography step.

Method validation

Selectivity

In the blank human serum samples, no interfering peaks were detected at the retention times of the trastuzumab and SILuMab surrogate peptides. Interfering peaks were observed for the pertuzumab surrogate peptide, but these were within the acceptance criteria for the selectivity experiment. Furthermore, spike-in selectivity was within criteria for both trastuzumab and pertuzumab.

Sensitivity and linearity

The lower limit of quantification (LLOQ) was determined at 0.250 µg/mL for trastuzumab and at 0.500 µg/mL for pertuzumab. The calibration range for trastuzumab was established at 0.250 to 250 µg/mL and for pertuzumab at 0.500 to 500 µg/mL. The calibration curves were fitted using linear regression with 1/concentration^2 ($1/x^2$) weighting. Correlation coefficients of ≥0.9924 and ≥0.9914 were recorded during the validation study for trastuzumab and pertuzumab, respectively.

Accuracy and precision

The results of the determination of accuracy and precision are presented in Table 2. Both intra-run and inter-run accuracy and precision were acceptable as all results met the general acceptance criteria of ±20% of the nominal value for hybrid LC-MS/MS methods.

Table 2. Intra-run and inter-run precision and accuracy of trastuzumab and pertuzumab.

Analyte	Concentration (µg/ml)	Intra-Run Precision & Accuracy		Inter-Run Precision & Accuracy	
		CV (%)	RE (%)	CV (%)	RE (%)
Trastuzumab	0.250	7.0	−0.7	7.9	−0.6
	LLOQ	7.6	−6.6		
		4.5	5.6		
	0.750	13.2	−10.4	12.7	−7.9
	LQC	10.3	−16.5		
		3.3	3.2		
	20.0	4.8	−13.7	10.8	−4.6
	MQC	6.7	−7.4		
		4.9	7.4		
	200	6.4	−15.3	15.1	−5.9
	HQC	7.0	−13.2		
		10.0	10.7		
Pertuzumab	0.500	8.5	7.2	13.2	8.0
	LLOQ	18.9	13.6		
		8.6	3.2		
	1.50	8.2	8.0	5.9	7.8
	LQC	5.8	8.8		
		4.0	6.6		
	40.0	4.0	2.1	10.1	3.0
	MQC	14.8	8.5		
		5.7	−1.5		
	400	4.2	−4.4	6.5	−4.3
	HQC	5.2	−5.3		
		9.8	-3.1		

Intra-run precision & accuracy results of three independent runs with six QC samples at each concentration. Inter-run precision & accuracy results based on these three runs. LLOQ: Lower Limit Of Quantification, LQC: Low Quality Control, MQC: Medium Quality Control, HQC: High Quality Control

Cross-analyte interference

No interference between the IS and trastuzumab, and pertuzumab individually was observed. In addition, the presence of trastuzumab did not interfere with measurement of pertuzumab concentrations. For trastuzumab, the presence of pertuzumab did not interfere with trastuzumab determination at mid-quality control (MQC) and high quality control (HQC) concentrations, whereas high pertuzumab concentrations could have an effect on determination of lower trastuzumab concentrations. In fact, the presence of pertuzumab at the upper limit of quantification (ULOQ; 500 µg/mL) showed a bias of 40.8% at determination of trastuzumab at LLOQ (0.250 µg/mL).

Recovery

The apparent recovery associated with the Protein A affinity capture was within criteria, with a recovery variation of 11.7% over the quality control (QC) range for trastuzumab and 12.7% for pertuzumab. Recovery of the internal standard is not a required item for validation of chromatographic methods. However, during method development, recovery of SILuMab was maximized by optimization of the amount of protein A beads added for affinity purification, with the structural positioning of the DTLMISR*($^{13}C_6{}^{15}N_4$) peptide not affecting the recovery of SILuMab. Validation of matrix effect excluded any matrix interference on the internal standard response.

Further validation results, including matrix effect, stability, reinjection reproducibility, dilution linearity, and carryover are detailed in the Supporting Information. In line with earlier recommendations, the acceptance criteria for hybrid LC-MS/MS were set in the pre-specified validation plan.[24] However, at completion of the validation study we observed that, with the exception of a few results, most precision and accuracy parameters were within ±15% as required for the validation of small molecule LC-MS/MS. The apparent lower accuracy of the trastuzumab determination could be a consequence of a more pronounced retention time gap between the trastuzumab and SILuMab surrogate peptides compared to the more similar retention times of the pertuzumab and SILuMab surrogate peptides.

Application to clinical studies: the TRAP trial

After validation, the assay was successfully employed for PK assessment of co-administered trastuzumab and pertuzumab in support of a Phase 2 clinical trial. Between April 2014 and September 2016, 40 esophageal cancer patients were enrolled in seven centers in the Netherlands. A total of 670 serum samples were analyzed for pertuzumab and trastuzumab. The concentrations of trastuzumab ranged from <0.250 µg/mL (<LLOQ) to around 250 µg/mL, and for pertuzumab from <0.500 µg/mL (<LLOQ) to around 525 µg/mL. The linearity of the calibration curves of both trastuzumab and pertuzumab was good, with a mean correlation coefficient of 0.9928 across 10 accepted

analysis runs for trastuzumab and 0.9871 for pertuzumab. Overall accuracy of the calibration runs was between −2.0% and 4.6% for trastuzumab, and between −3.7% and 7.9% for pertuzumab. The overall QC accuracy and precision were between −4.4% and 1.2% for trastuzumab and between −3.0% and 1.3% for pertuzumab. Evaluable PK data were available from 37 patients. The mean C_{min} and C_{max} for trastuzumab were 38.4 µg/mL (±10.8) and 128.9 µg/mL (±23.2), respectively; for pertuzumab these were 89.5 µg/mL (±24.8) and 292.3 µg/mL (±52.5), respectively (see Figure 2).

Discussion

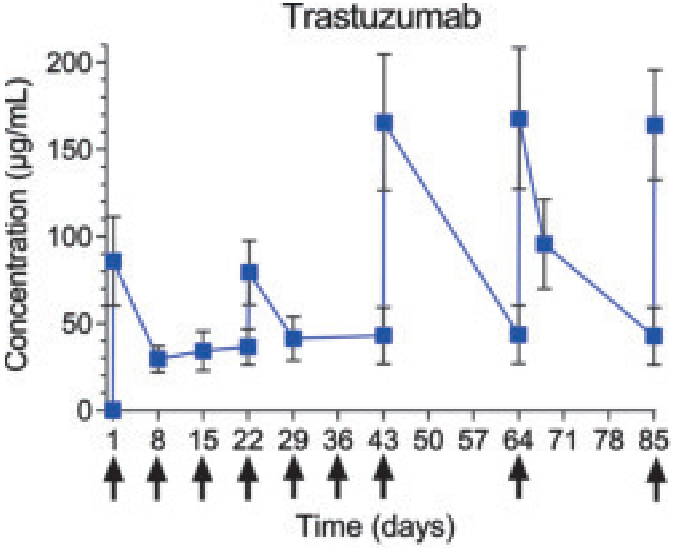
This is the first report of a validated LC-MS/MS quantification method able to quantify the frequently co-administered mAbs trastuzumab and pertuzumab simultaneously using 10 µL of serum.

Compared with LBA, the use of an internal standard to correct for quantitative bias and variation in LC-MS/MS makes it easier to maintain inter-batch and inter-laboratory consistency because of correction for matrix effects. Furthermore, through superior transferability to other matrices and species, and the limited requirement for critical reagents, an LC-MS/MS-based method can more efficiently be transferred throughout the entire process from preclinical drug development to clinical trials and clinical practice.[12,25,26] As argued for the cetuximab LC-MS/MS assay described by Becher *et al*., the use of triple quadrupole instruments and commercially available reagents and standards facilitates inter-laboratory exchanges and maintains cost-effectiveness.[25] Compared with the most recent multiplex LC-MS/MS method using nonselective affinity purification and a commercially available IS, our method has multiple advantages.[21] Using a sampling amount compatible with microsampling, we validated a method achieving a higher sensitivity and broader dynamic range (LLOQ of 0.250 µg/mL vs 1 µg/mL and range of 0.250–250 µg/mL vs 1–100 µg/mL for trastuzumab).[27] The use of a small sample volume generally results in reduced background interference, contributing to a higher signal/noise (S/N) ratio even at the lowest analyte concentrations. Theoretically, the sensitivity of the assay could have been enhanced further with prolonged incubation with protein A and with the introduction of a solid phase extraction at peptide level prior to electrospray ionization. However, the current assay range was chosen to meet the clinical study requirements.

We found no cross-analyte interference except when determining low concentrations of trastuzumab in the presence of high concentrations of pertuzumab, probably pertaining to a suppression effect where adequate resolution performance cannot be guaranteed. This finding did not influence the results of the PK results in the clinical trial, as all timepoints where pertuzumab was present at concentrations around ULOQ were post-dose for both pertuzumab and trastuzumab, with determined trastuzumab concentrations above 40 µg/mL The same holds true in general clinical trial practice, where trough and peak samples for both mAbs are taken at different time points.

The method has a throughput similar to LBA, and is therefore sufficient to provide a time- and cost-effective solution for therapeutic drug monitoring and PK profiling in large clinical studies, but, since this method relies on manual sample preparation, throughput can be accelerated further with the introduction of automatization.[6] Apart from the small sample consumption, LC-MS/MS offers additional advantages over LBA in the clinical trial setting. After validation, LC-MS methods can be used for an extended period of time since they are independent of the variability of critical reagents. Where LBA methods sometimes require bridging, LC-MS methods can readily be used to monitor long-lasting clinical trials and at the same time ensure homogeneity of the data.

This method was used for PK analyses in support of a Phase 2 clinical trial with generation of trastuzumab and pertuzumab concentrations that are in line with PK analyses of previous trials with trastuzumab or co-administered trastuzumab and



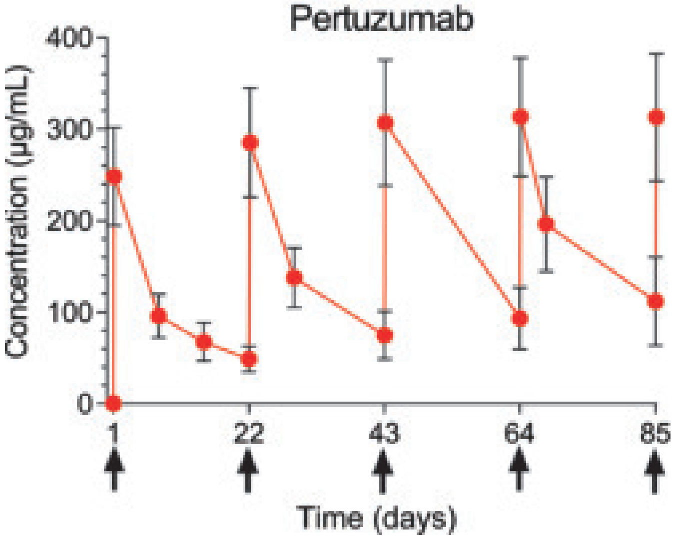


Figure 2. Pharmacokinetic analyses of trastuzumab and pertuzumab in the Phase II TRAP trial. Mean minimum concentrations (Cmin) and maximum concentrations (Cmax) of trastuzumab (A) and pertuzumab (B) in serum, measured throughout treatment (n = 37). Arrows indicate mAb administration days and horizontal bars indicate standard deviations. Reprinted with permission from Stroes C, Schokker S *et al*: J Clin Oncol 38(5), 2020: 462–471. © 2020 American Society of Clinical Oncology. All rights reserved.

pertuzumab.[28] For pertuzumab, concentrations were also consistent with differences found between gastrointestinal and breast cancer indications.[28,29] Furthermore, the limits of quantification of the described method are sufficient for clinical trial purposes, as almost all concentrations found in other large trials are below the ULOQ.[8] To cover the occurrence of concentration above the ULOQ, dilution accuracy evaluation was included in method validation, and therefore concentrations up to 10-fold above ULOQ can be adequately quantified.

Regarding method development, there are a number of limitations. The CDR peptide IYPTNGYTR is a frequently used surrogate peptide for trastuzumab quantification, but it contains an asparagine residue followed by a glycine (NG motif), indicating a susceptibility for deamidation to IYPTDGYTR.[30,31] Deamidation of asparagine is a common post-translational modification that can occur both *in vitro* and *in vivo*. The *in vitro* deamidation will be minimal in the current method, as temperature and pH stress are reduced during sample preparation, which is obtained in less than 4 h with incubation at 37°C limited to 60 minutes and incubation at 60°C and pH 7.4 limited to 15 minutes.[31] Deamidation of IYPTNGYTR can also occur *in vivo* and has been demonstrated to hamper the functionality of trastuzumab, most likely due to a conformational change that affects antigen binding.[18,31] Consistent evidence is lacking on whether surrogate peptides that reflect the total trastuzumab concentration (e.g., FTISADTSK, DTYIHWVR) or surrogate peptides more reflective of *in vivo* modifications (e.g., IYPTNGYTR, IYPTDGYTR) should be used.[15,16,18,31] Considering the small differences in mass (1 Da) and polarity between the IYPTNGYTR peptide and its deamidated form, the current method cannot resolve the two forms, and is therefore more reflective of the total trastuzumab concentration.

While a whole-sequence SIL IS would most likely yield the highest accuracy, the use of a labeled reference peptide for normalization of multiple unlabeled signature peptides has been demonstrated to be a robust and cost-effective alternative.[32] Although we chose SILuMab because of its commercial availability, we did not utilize the labeled version of any of the target surrogate peptides because those cannot be found in the SILuMab primary sequence. The labeled version of the tryptic peptide DTLMISR*($^{13}C_6{}^{15}N_4$) was used instead as analogue internal standard for both trastuzumab and pertuzumab. Nevertheless, the validation results demonstrate high accuracy for both trastuzumab and pertuzumab, probably pertaining to the structural similarity and identical behavior during affinity purification, making SILuMab a good representative internal standard.

In conclusion, we developed and fully validated a high-throughput and robust LC-MS/MS quantification method able to simultaneously quantify the frequently co-administered mAbs trastuzumab and pertuzumab using 10 µL of serum. Hereby we also addressed the unmet need for a reduction of blood sampling volume and collection time in clinical trials, consequently reducing the burden and discomfort of sampling for patients. Furthermore, due to the use of readily available reagents and standards, this bioanalytical strategy can easily be adapted to multiplex quantifications of other mAb combinations.

Materials and methods

Chemicals and reagents

Affinity purification: PureProteome™ Protein A Magnetic Bead System (Merck Millipore), internal standard: SILuMab (Sigma-Aldrich, catalog number MSQC3), sequencing grade modified trypsin (Promega, catalog number V5113), blank human serum (BioIVT), trastuzumab, and pertuzumab solutions (Hoffmann-La Roche).

Selection of surrogate peptides process

Accuracy and linearity of the tryptic digestion and LC-MS/MS method were demonstrated with pure trastuzumab and pertuzumab without addition of an IS at the start of method development. For experimental selection and optimization of the MRM ion transitions based on the m/z values of the theoretical precursor and product ions, neat solutions of trastuzumab, pertuzumab and SILuMab digested with trypsin were analyzed with LC-MS/MS using a 20-min high performance LC gradient program. Tryptic peptides IYPTNGYTR and FTLSVDR were used as surrogate for quantification of trastuzumab and pertuzumab, respectively. The tryptic peptide DTLMISR*($^{13}C_6{}^{15}N_4$) from SILuMab was monitored as internal standard. See Table 1 for surrogate peptides and their MRM transitions.

Preparation of calibration standards and quality control samples

Calibration samples were prepared fresh for each analysis batch by spiking 0.250, 0.500, 1.00, 2.50, 10.0, 25.0, 100 and 250 µg/mL of trastuzumab and 0.500, 1.00, 2.00, 5.00, 20.0, 50.0, 200, and 500 µg/mL of pertuzumab in human serum from diluted stock solutions. QC samples of trastuzumab were prepared in human serum at the concentrations of 0.250 (LLOQ), 0.750 (Low Quality Control; LQC), 20.0 (MQC), and 200 µg/mL (HQC), and of pertuzumab at the concentrations of 0.500 (LLOQ), 1.50 (LQC), 40.0 (MQC), and 400 (HQC).

LC-MS/MS sample pretreatment

Aliquots of 10 µL of serum samples (blanks, standards, QCs, or study samples) were dispensed into a 96-well polypropylene deep-well plate; 20 µl of a 5 µg/mL SILuMab solution was added to each sample prior to dilution to 200 µl with Buffer A (50 mM TRIS-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% tween 20, 0.1% BSA, pH = 7.4). Subsequently, 200 µl of an eightfold diluted Protein A beads suspension were added to each sample and incubated at room temperature on a plate shaker under constant shaking for 30 minutes, ensuring sufficient speed to keep the beads well suspended during incubation. Before use, protein A beads were washed once with Buffer A, maintaining the same dilution. Thereafter, the beads were briefly washed twice with 200 µl of buffer B (50 mM TRIS HCl, 150 mM NaCl, 1 mM EDTA, pH = 7.4). On-bead reduction (in 10 mM dithiothreitol for 15 minutes at 60°C) followed by alkylation (in 55 mM iodoacetamide for 30 minutes at room temperature) were performed prior to trypsin digestion, which was obtained by adding 20 µg

of trypsin to each sample. After 1 h of incubation at 37°C under constant shaking, proteolysis was stopped by addition of formic acid in water solution, to a final concentration of 2%. Samples were filtered on a Multiscreen HTS filter plate and filtrates were centrifuged for 1 minute at 3000 *g* prior to LC-MS /MS analysis.

LC and MS conditions and settings

A volume of 5 µl of each protein digest was separated onto an Acquity Ultra Performance LC ethylene-bridged hybrid (BEH) C18 column (2.1 × 100 mm, 1.7 µm (Waters)) mounted on a 1290 Infinity Ultra High-Performance Liquid Chromatography system. Peptides were eluted by applying a 3-min gradient program of 10% to 22% acetonitrile in 0.1% formic acid at a flow-rate of 0.5 mL/minute prior analysis on a triple-quadrupole mass spectrometer (SCIEX 6500) operated in positive electrospray MRM mode. The MRM parameters (such as gas flow rates, ionization spray voltage, temperature, ion transitions, declustering potential, collision energy, and collision cell exit potential) were individually optimized using step values for each parameter (curtain gas, 35 units; CAD gas, 10 units; gas 1 and 2, 60 units; ion spray voltage, +5500 V; temperature, 600°C). The acquired chromatographic peaks were integrated by the Analyst software (version 1.6.2, AB SCIEX), for calibration curve regression and back calculation of the concentrations of QCs and study samples.

Method validation

The method was validated according to the current guidelines from the US Food and Drug Administration and the European Medicines Agency for bioanalytical chromatographic methods (detailed experimental description in Supporting Information).[33–35] Acceptance criteria of ±20% for calibration standards and QCs (±25% at the LLOQ) were applied, in line with the recommendations for hybrid LC-MS /MS methods.[24]

Application to clinical studies: the TRAP trial

The TRAP trial was a Dutch, investigator-initiated, multicenter, Phase 2 feasibility study where trastuzumab and pertuzumab were added to neoadjuvant chemoradiation with carboplatin and paclitaxel (NCT02120911).[6] The trial was approved by the institutional review board of the Amsterdam University Medical Centers and conducted in accordance with Good Clinical Practice guidelines and the Declaration of Helsinki. All patients provided written, voluntary informed consent prior to inclusion. In short, HER2-positive patients with resectable esophageal adenocarcinoma received paclitaxel (50 mg/m^2), carboplatin (area under the curve = 2) and radiotherapy (23 × 1.8 Gy) over the course of 5 weeks. Intravenous trastuzumab was administered in a loading dose of 4 mg/kg with subsequent weekly doses of 2 mg/kg in weeks 2–6, and ultimately 6 mg/kg every 3 weeks in weeks 7–13. Intravenous pertuzumab was dosed at 840 mg every 3 weeks until week 13. Doses could be delayed, but not reduced. Surgery was performed around 14 weeks after start of neoadjuvant treatment. PK sampling was done pre-dose on days 1, 8, 15, 22, 29, 43, 64, and 85, and post-dose on days 1, 22, 43, 64, and 85. Further methods of this study have been published previously.[6]

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