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LC-MS/MS-Based Monitoring of In Vivo Protein Biotransformation: Quantitative Determination of Trastuzumab and Its Deamidation Products in Human Plasma

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Supporting Information

ABSTRACT: An LC-MS/MS-based method is described for quantitatively monitoring the in vivo deamidation of the biopharmaceutical monoclonal antibody trastuzumab at a crucial position in its complementarity determining region (CDR). The multiplexed LC-MS/MS assay using selected reaction monitoring (SRM) allows simultaneous quantitation of five molecular species derived from trastuzumab after tryptic digestion: a stable signature peptide (FTISADTSK), a deamidation-sensitive signature peptide (IYPTNGYTR), its deamidated products (IYPTDGYTR and IYPTisoDGYTR), and a succinimide intermediate (IYPTsucGYTR). Digestion of a 50 μL plasma sample is performed at pH 7 for 3 h at 37 °C, which combines a reasonable (>80%) digestion efficiency with a minimal (<1%) formation of deamidation products during digestion. Rapid in vitro deamidation was observed at higher pH, leading to a (large) overestimation of the concentrations of deamidation products in the original plasma sample. The LC-MS/MS method was validated in accordance with international bioanalytical guidelines over the clinically relevant range of 0.5 to 500 μg/mL with bias and CV values well below 15%. Deamidation of trastuzumab was observed in plasma both in a 56 day in vitro forced degradation study (up to 37% of the total drug concentration) and in samples obtained from breast cancer patients after treatment with the drug for several months (up to 25%). Comparison with a validated ELISA method for trastuzumab showed that deamidation of the drug at the CDR leads to a loss of recognition by the antibodies used in the ELISA assay.

In recent years, there has been a considerable increase in the development and marketing of protein-based pharmaceuticals (or biopharmaceuticals) and, consequently, in the interest for reliable analytical techniques for their quantification in samples of biological origin, to support e.g. pharmacokinetic studies or therapeutic drug monitoring. Traditionally, protein quantification is performed by ligand-binding assays (LBAs), but over the past decade liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been gaining popularity as an alternative analytical platform. LC-MS/MS is extensively used to study structural modifications of proteins in pharmaceutical formulations, e.g. for quality control purposes. Important spontaneously occurring reactions are the deamidation of asparagine (Asn) and glutamine (Gln) as well as oxidation reactions, particularly of methionine (Met) or tryptophan (Trp) residues. These may cause biologically relevant changes in the protein structure if the modified amino acids are present in the active part of the protein molecule, such as in one of the several complementarity determining regions (CDRs) of an antibody. Deamidation and oxidation can potentially also occur in vivo and their form of a protein responds in an assay. No separate concentration results are therefore obtained for the different protein forms, and probably for that reason, protein biotransformation has been traditionally somewhat neglected.

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monitoring could give important information about the inactivation of the protein drug during its residence in the body. This is especially relevant for monoclonal antibodies (mAbs), a major class of biopharmaceuticals, because of their relatively long half-life and consequently their long exposure to conditions that might induce deamidation and/or oxidation. Monitoring these reactions in vivo, however, is less straightforward than in pharmaceutical preparations in vitro, because of the lower analyte concentrations and the much more complicated sample matrix, which contains a significant number and amount of endogenous proteins that may interfere in the LC-MS/MS assay.

One of the most widely prescribed therapeutic monoclonal antibodies is trastuzumab (Herceptin). It consists of two heavy and two light chains, is composed of a total of 1330 amino acids, and has an average molecular weight of approximately 145.5 kDa (Supplemental Figure S-1). Trastuzumab is a humanized monoclonal immuno-globulin gamma 1 (IgG1) antibody directed against the human epidermal growth factor receptor-2 (HER2/neu). This receptor is overexpressed in approximately 25% of all breast cancer patients, and trastuzumab inhibits proliferation and induces cell death via extracellular and intracellular mechanisms.9–11

Trastuzumab can be deamidated in several places along its peptide chain. Most in vitro studies have focused on the deamidation of Asn30 in the light chain, which is known to take place under the slightly acidic conditions of a typical pharmaceutical formulation. The conversion of this amino acid to the corresponding aspartate (Asp) does, however, not lead to any significant decrease in pharmacological activity.6,12 Asn55, located in the CDR2 region of the heavy chain, is another deamidation site. This amino acid occurs in several mAbs of the IgG1 subclass and is therefore considered to play a key role in the biological functioning of these proteins.13 Since Asn55 deamidation does not appear to occur to a large extent in pharmaceutical preparations, it has not been studied as closely as other reactions. The conversion to aspartic acid (Asp55) and the concomitant isomerization to iso-aspartic acid (isoAsp55) for another, not specified deamidation site, is potentially relevant for trastuzumab as well.

Asn55 or its modified forms. In addition, a second peptide from a metabolically stable part of the molecule is quantified for comparative reasons. The digestion conditions were carefully optimized to avoid additional in vitro deamidation and to ensure sufficient accuracy. The results are compared to those obtained with a standard enzyme-linked immunosorbent assay (ELISA) to investigate the suitability of this technique to quantify trastuzumab in the presence of its deamidation products.

### MATERIALS AND METHODS

#### Chemicals and Biological Materials. Trastuzumab, supplied in a vial containing 150 mg as a lyophilized sterile powder, was obtained from Roche (Basel, Switzerland). For the LC-MS/MS method, custom synthesized peptides (IYPTNDG, IYPDLDG, IYPDEG, and their C-terminal arginine 15N2-labeled internal standards, FTISADTSDK and its C-terminal lysine 15N2-labeled internal standard were obtained from JPT Peptide Technologies (Berlin, Germany). Acetonitrile, methanol, formic acid, acetic acid, ammonium hydroxide solution (25%), ammonium acetate, and hydrochloric acid (37%) were obtained from Merck (Darmstadt, Germany). Tween-20, Trizma base, and Trypsin from porcine pancreas (Type IX-S, lyophilized powder, 13 000–20 000 BAEE units/mg protein) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

For the ELISA method, human anti-trastuzumab HCA169 and human anti-trastuzumab HCA177 were obtained from AbD Serotec (Puchheim, Germany). A biotin Protein labeling kit was obtained from Roche Diagnostics (Almere, The Netherlands). Phosphate-buffered saline 10× (PBS), 3,3′,5,5′-tetramethylbenzidine (TMB) Liquid Substrate System for ELISA and stop reagent for TMB substrate were obtained from Sigma-Aldrich. Superblock T20 (PBS) blocking buffer and Streptavidin-HRP were obtained from Thermo Fisher Scientific (Etten-Leur, The Netherlands). PBS-Tween tablets (Calbiochem) were obtained from Merck. HPLC-grade water was prepared using a water purification system from Merck. Human K,EDTA plasma from healthy volunteers (hereafter referred to as blank human plasma) was obtained from Seralabs (Haywards Heath, U.K.).

#### Preparation of Calibration, Validation, and Quality Control Samples. A trastuzumab stock solution at 20.8 mg/mL was prepared by dissolving the contents of a vial of lyophilized protein (label claim: 150 mg) in 7.20 mL of water according to the manufacturer’s instructions for use. The stock solution was divided into 0.5 mL aliquots in Eppendorf Protein LoBind tubes (obtained from VWR International, Amsterdam, The Netherlands) and stored at −80 °C. Aqueous standard solutions at 500 and 50.0 μg/mL were prepared freshly from this stock solution, and from these solutions, calibration samples were prepared in blank human K,EDTA plasma at 0.500, 1.00, 2.50, 10.0, 25.0, 100, 250, 400, and 500 μg/mL. Similarly, validation and quality control (QC) samples were prepared at 0.500, 1.50, 25.0, and 400 μg/mL. For the ELISA method, a plasma stock was prepared at 100 μg/mL, from which calibration samples were prepared by serial dilution with blank human K,EDTA plasma to 60.1, 102, 172, 290, 490, 829, 1400, 2370, and 4000 ng/mL. Similarly, validation and quality control (QC) samples were prepared at 102, 250, 1000, 3000, and 4000 ng/mL. All calibration, validation, and QC samples were stored in Eppendorf Protein LoBind tubes at −80 °C (or −20 °C for stability assessment).

#### LC-MS/MS Sample Pretreatment. For the LC-MS/MS determination of trastuzumab in human K,EDTA plasma, 50.0
μL aliquots of sample were pipetted into the 1.2 mL wells of an Eppendorf Protein LoBind 96-well plate (obtained from VWR International, Amsterdam, The Netherlands), and 200 μL of methanol was added. After vortex-mixing for 2 min, the proteins were pelleted by centrifugation for 10 min at 1500g. The supernatant was discarded by inverting the plate above a waste receptacle and placing it upside down on a tissue for 15 min. The protein pellet was reconstituted by vortex-mixing in 400 μL of a solution consisting of 50 mM Trizma buffer at a pH of 7.0, 0.02% Tween-20, 3% (v/v) acetonitrile and 200 μg/mL trypsin. Next, 50.0 μL of a solution was added containing 2.00 μg/mL of the SIL-peptide internal standards. The sample was digested at 37 °C and 1250 rpm for 3 h using an Eppendorf Thermomixer comfort, after which the digestion was stopped by the addition of 50.0 μL of 10% formic acid in water. Finally, the plate was sealed, vortex-mixed and placed in an autosampler at 10 °C for analysis.

LC and MS Conditions and Settings. Experiments were conducted using an Acquity UPLC system (Waters, Milford, MA, U.S.A.) coupled to an AB Sciex API 6500 triple quadrupole mass spectrometer equipped with a Turboionspray source (Concord, Canada). Chromatographic separation was performed at 40 °C on a 2.1 × 50 mm, 1.8 μm particle-size, Acquity UPLC HSS T3 column (Waters). Mobile phase A consisted of 10 mM ammonium acetate in water (pH = 5) and mobile phase B was acetonitrile. Samples were separated by gradient elution using the following settings: 0.0−5.0 min: 5−10% B; 5.0−5.1 min: 10−50% B; 5.1−5.6 min: 50% B; 5.6−5.7 min: 50−70% B; 5.7−6.2 min: 70% B; 6.2−6.3 min: 70−90% B; 6.3−6.8 min: 90% B; 6.8−6.9 min: 90−5% B; 6.9−8.0 min: 5% B. The flow rate was 0.600 mL/min, and the sample injection volume was 7 μL. Mass spectrometric settings were optimized by infusion of the peptides into the mass spectrometer. Optimal conditions for all compounds were as follows: ionspray voltage (IS) + 5500 V, temperature 750 °C, nebulizer gas (GS1) 70, TurboIonSpray gas (GS2) 45, collision-activated dissociation gas (CAD) 10, curtain gas (Cur) 40, dwell time 40 ms, declustering potential (DP) 35 V, collision exit potential (CXP) 14 V and entrance potential (EP) 10 V. Analyte-specific mass spectrometer parameters are represented in Table S-1 (Supporting Information), as well as the SRM transitions used to monitor the peptides and the corresponding SIL internal standards.

ELISA Method. An ELISA Maxisorp plate (Thermo Fisher Scientific) was coated overnight with 100 μL of a solution containing 1.00 μg/mL of human anti-trastuzumab HCA169, after which the plate was washed three times with 300 μL wash buffer (one PBS-Tween tablet per liter of water). After it was thawed, 15.0 μL of plasma was prediluted with 285 μL of Superblock T20, and subsequently, 100 μL of diluted sample was transferred in duplicate to the plate and incubated for 1 h at 22 °C and 500 rpm. Next, the plate was washed three times with 300 μL of wash buffer; 100 μL of detection solution (Biotin-labeled human anti-trastuzumab HCA177 at 0.500 μg/mL) was added, and the plate was incubated for 1 h at 22 °C and 500 rpm. Then the plate was washed three times with 300 μL of wash buffer, and 100 μL of conjugate solution (10 000-fold diluted Streptavidin HRP) was added followed by incubation for 0.5 h at 22 °C and 300 rpm. Subsequently, the plate was washed three times with 300 μL of wash buffer, and 100 μL of TMB was added and the plate was incubated for 9 min at 22 °C and 300 rpm. Next, 100 μL of stop solution was added, and the plate was analyzed within 10 min of stopping the reaction by measuring the absorbance at a wavelength of 450 nm on a SpectraMax MSe plate reader (Molecular Devices, California, U.S.A.).

LC-MS/MS and ELISA Method Validation. Trastuzumab concentrations in the validation and control samples were determined by applying the methods for trastuzumab (LC-MS/MS for peptides IYPTNGYTR and FTISADTSK, and ELISA) and comparing the results to a calibration curve spiked with trastuzumab in blank plasma, which was analyzed with the same method in the same run. All methods for trastuzumab determination were validated according to international guidelines for bioanalytical method validation6,17 by assessing precision, accuracy, selectivity, and stability using calibrators and validation samples in plasma (detailed experimental description in Supporting Information).

In Vitro Deamidation of Trastuzumab. Plasma was set at pH 8 and spiked with trastuzumab at 400 μg/mL, after which the sample was aliquoted into 1 mL portions in Eppendorf protein LoBind tubes. The samples were prepared and stored under sterile conditions, to avoid external influences during the experiment. They were stored at 37 °C for 56 days, and at several time points, a 1 mL portion was taken out and stored at −70 °C until analysis. All samples were subsequently analyzed with the described ELISA and LC-MS/MS methods.

Patient Samples. Blood samples were collected from patients with HER2-positive breast cancer, who were receiving trastuzumab intravenously every 3 weeks (8 mg/kg as initial dose, followed by 6 mg/kg, every 3 weeks) for a period of 1 year, as part of their adjuvant treatment for early breast cancer. Samples were withdrawn prior to the first dose and after a minimum of three administrations immediately before the administration of a next dose of trastuzumab. Patient samples were collected under appropriate ethical approval and after written informed patient consent. Plasma was prepared immediately after blood collection and stored at −20 °C. All samples were subsequently analyzed with the described ELISA and LC-MS/MS methods.

RESULTS AND DISCUSSION

Selection of Signature Peptides. To follow in vivo deamidation of Asn55, two signature peptides were selected for trastuzumab: one containing the Asn55 residue and another one from a chemically stable part of the protein. An in silico tryptic digestion of the trastuzumab sequence was used to generate a list of candidate peptides using mMass18 resulting in 62 theoretical peptides that are expected after digestion with trypsin. To avoid interferences from the human plasma proteome, both signature peptides had to be unique for trastuzumab: one containing the Asn55 residue and another one from a chemically stable part of the protein. An in silico tryptic digestion of the trastuzumab sequence was used to generate a list of candidate peptides using mMass18 resulting in 62 theoretical peptides that are expected after digestion with trypsin.

In addition, to facilitate LC-MS/MS quantification, peptides containing unstable amino acids (other than Asn55), such as methionine and tryptophan, and glycosylated peptides and peptides forming disulfide bonds as well as peptides outside the 500−2000 mass range were excluded.

The Asn55-containing peptide IYPTNGYTR (heavy chain amino acids S1−S9, see Figure S-1 Supporting Information) fulfilled all criteria, from which digestion with trypsin was concluded to be a suitable approach. A potentially useful second tryptic peptide was FTISADTSK (heavy chain amino acids 68−76), which falls outside the CDRs of trastuzumab. To confirm the suitability for LC-MS/MS analysis, peptide
mapping experiments were conducted using trypsin digestion of trastuzumab after protein precipitation with methanol. Both peptides showed appropriate LC-MS/MS characteristics such as good chromatographic retention, efficient ionization, and fragmentation and were thus used for further work. Reference standards were obtained for the signature peptides as well as for the deamidated products IYPT\textsubscript{DGYTR} and IYPT\textsubscript{isoDGYTR} and their corresponding stable-isotope labeled (SIL) forms. For all compounds, the parent ion was the doubly charged protonated molecule and the product ion the singly charged y7 fragment. Since the unstable succinimide intermediate could not be obtained in pure form, the same general MS settings were used for this compound, while the SRM transitions were based on a theoretical prediction of the \( m/z \) values of its molecular and fragment ions. The suitability of these settings was confirmed by the analysis of a solution of the non-deamidated peptide, which had been stored under conditions that induced succinimide formation and in which the succinimide form was present at relatively high levels.

**Separation and Identification of the Peptides.** The Asp55- and isoAsp55-containing peptides have the same mass, which differs only 1 Da from that of the Asn55-containing peptide. This means that the difference in their \( m/z \) values is just 0.5 for the doubly charged molecules (Table S-1 in the Supporting Information). With the triple quadrupole MS having unit mass resolution, the Asp55- and isoAsp55-

**Figure 1.** LC-MS/MS chromatograms for the two signature peptides and three degradation products of trastuzumab, recorded for a processed patient plasma sample. (A): FTISADTSK (\( m/z \) 485.3 → 721.4); (B): IYPTNGYTR (\( m/z \) 542.8 → 808.4); (C): IYPTDGYTR and IYPT\textsubscript{isoDGYTR} (\( m/z \) 543.3 → 809.4); (D): IYPT\textsubscript{succGYTR} (\( m/z \) 534.3 → 791.4).

**Figure 2.** Release of the FTISADTSK signature peptide, as well as release and degradation of the IYPTNGYTR signature peptide during trypsin digestion of trastuzumab at four different pH values.
containing forms cannot be distinguished mass spectrometrically, while the Asn-containing peptide will give a response in the mass transition of the deamidated forms. Hence, all three peptides need to be chromatographically separated.

The best separation (nearly baseline), while keeping sensitivity, was achieved by applying a relatively shallow gradient from 5 to 10% acetonitrile in 5 min. All relevant peptides could thus be individually determined with a still reasonable sample throughput at 10 min from injection to injection (see Figure 1A–D for representative chromatograms).

**Sample Digestion and Deamidation.** It has been reported that the rate of asparagine deamidation increases at alkaline pH because of the deprotonation of the peptide bond nitrogen atom, which is the first step in the formation of a succinimide intermediate. Because tryptic digestion is typically performed at slightly alkaline pH, it is essential to investigate the pH dependence of the kinetics of Asn deamidation for trastuzumab under the digestion conditions. This is especially important because the deamidation rate for small peptides can be considerably higher than for intact proteins, and once formed after digestion of trastuzumab, the Asn55-containing signature peptide will be subjected to the deamidation-inducing digestion conditions for a relatively long time.

Tryptic digestion was performed under four different conditions in which only the pH of the digestion buffer was varied between pH 7.0 and 8.5. The other conditions were the same as described under the materials and methods section. Aliquots of a plasma sample spiked with trastuzumab at 400 μg/mL were digested for up to 20 h and digests were analyzed at several time points. Figure 2 shows a clear pH effect on the stability of IYPTNGYTR, whereas FTISADTSK is stable at all values between 7.0 and 8.5. IYPTNGYTR is deamidated at pH 7.5 and above, with a higher deamidation rate when the pH increases. This is most apparent after around 3 h when trastuzumab has been completely digested, but the effect will also play a role during the first 3 h, when the peptide is being liberated from the protein. It was found that the deamidated forms of IYPTNGYTR and the succinimide intermediate were all formed under the various digestion conditions with an increase in concentration at higher pH values (Figure S-2 in the Supporting Information).

The isoAsp-containing peptide is formed to an approximately 4-fold larger extent than the Asp-containing form and has an abundance of about 8% of that of the nondeamidated peptide after 3 h of digestion at pH 8.5, which increases to 150% after 20 h.

To avoid deamidation during digestion and to enable reliable monitoring of the actual deamidation products in vivo, it is clear that a pH of 7 is suitable. It combines a reasonable digestion efficiency (>80%) with minimal formation (<1%) of deamidation products during the first 3 h of digestion.

**LC-MS/MS Method Validation.** The described LC-MS/MS method for trastuzumab was validated by quantitation of both signature peptides. All obtained results are included in the Supporting Information Tables S-2 to S-10. For the peptide IYPTNGYTR, all results meet the criteria set for small-molecule bioanalytical method validations, which shows that trastuzumab can be determined with high accuracy and precision and that it can be kept sufficiently stable for a reliable determination in patient samples, despite the presence of a deamidation site in its sequence. For the peptide FTISADTSK, there is interference in the SRM transition, corresponding to approximately 100% to 200% of the response at the lower limit of quantitation (LLOQ), which affects the quantitation of this peptide at low levels of trastuzumab. This interference may be caused by the release of a peptide with a very similar amino acid sequence from an endogenous plasma protein or matrix compound. All other validation results are well within internationally accepted criteria. Monitoring of this signature peptide is therefore reliable at concentrations of trastuzumab above approximately 10 μg/mL.

**ELISA Method Validation.** All obtained results from the validation of the trastuzumab ELISA method are included in the Supporting Information Tables S-11 to S-15. For trastuzumab, all results meet the criteria set for large molecule bioanalytical method validations and thus show that trastuzumab can be determined with high accuracy and precision and that it is sufficiently stable during storage and analysis for a reliable determination in patient samples.

**Forced In Vitro Degradation.** With the deamidation and isomerization of the Asn55-containing peptide under control during LC-MS/MS sample preparation, and the LC-MS/MS method efficiently stable during storage and analysis for a reliable determination in patient samples.
and ELISA methods validated, the methods were applied to investigate the deamidation and isomerization process in an in vitro forced degradation experiment. Trastuzumab was stored in plasma at 37 °C for 56 days, which mimics its residence in the body after intravenous administration (the half-life of trastuzumab in humans ranges up to 32 days). The LC-MS/MS results for all forms of the protein are shown in Figure S-3 (Supporting Information). There is a 37% decrease in concentration of the nondeamidated form over a storage time of 56 days, which can be attributed to the formation of IYPTisoDGYTR (∼31% increase), IYPTDGYTR (∼4% increase), and IYPTSuccGYTR (∼2% increase). The response ratio between the isoAsp55 and Asp55 peptides remained constant between 7 and 8 during the entire experiment. Thus, the formation of isoAsp is favored over the formation of Asp. Comparison of Figures S-2 and S-3 shows that the rate of trastuzumab deamidation is much lower in plasma than in digestion buffer at the same pH (8) and temperature (37 °C). In digestion buffer, 37% degradation was observed after 4 to 6 h, while this took 56 days in plasma. This confirms earlier findings that deamidation of peptides occurs much more rapidly than of intact proteins, probably because of conformational constraints that prevent the formation of the succinimide intermediate.

Application of the LC-MS/MS method to quantify trastuzumab using the stable and unstable signature peptides and of the ELISA method gave three different concentration profiles (Figure 3). The FTISADTSK concentrations remain stable at the initial level of 400 μg/mL, while the IYPTNGYTR concentrations decrease to 250 μg/mL after 56 days (37.5% decrease compared to t = 0). In the same period, the ELISA concentrations decrease to 100 μg/mL (75% decrease). Although all results are expressed as “the” concentration of trastuzumab, it should be realized that each analytical technique measures a different feature of trastuzumab. The signature peptide FTISADTSK represents the total amount of trastuzumab and does not discriminate between nondeamidated and deamidated forms at Asn55, or any other modification outside this peptide, for that matter. The signature peptide IYPTNGYTR specifically represents the concentration of trastuzumab which has not been deamidated at Asn55. Finally, the ELISA result, which decreases exactly 2-fold faster than the LC-MS/MS results for IYPTNGYTR, corresponds to the immunoreactive concentration of trastuzumab. Trastuzumab contains two identical light and heavy chains and therefore two IYPTNGYTR sequences. Since the sandwich ELISA format uses two anti-idiotypic antibodies, it is very likely that both IYPTNGYTR sequences need to remain intact to form a detectable immune complex with trastuzumab. Therefore, when one of the two IYPTNGYTR sequences in a trastuzumab molecule is deamidated, LC-MS/MS still gives 50% of the original response, whereas with ELISA the complete signal is lost, which explains the 2-fold faster decrease of the measured concentration.

Analysis of Patient Samples. The validated LC-MS/MS and ELISA methods were used to quantify concentrations in plasma samples collected from breast cancer patients, who were treated with trastuzumab. Figure 4 presents LC-MS/MS results for a representative patient showing that there is deamidation and isomerization in all postdose samples with an increase in abundance of the deamidation products over time. Because of the absence of pure reference standards of the intact protein containing Asp55, isoAsp55, and Asu55, the concentrations of the deamidated forms of trastuzumab were calculated by reference to the LC-MS/MS response factors for the peptides. For all deamidated peptides, this was found to be equal to that of the nondeamidated peptide IYPTNGYTR, and therefore, the peak area ratios found for the deamidated forms of trastuzumab could be directly substituted into the calibration curve equation for trastuzumab, quantified through its signature peptide IYPTNGYTR. After 365 days of treatment of this particular patient, a total trastuzumab plasma concentration (as represented by peptide FTISADTSK) of 117 μg/mL was found, while Asn55 nondeamidated trastuzumab (peptide IYPTNGYTR) circulated at 89.2 μg/mL. A total of 24% of the drug was thus deamidated and this was mainly to isoAsp55-trastuzumab and much less to Asp55-trastuzumab. The ELISA concentration data are lower than the IYPTNGYTR results measured by LC-MS/MS, but in fact the data are considerably lower than the factor of 2, which was expected on the basis of the in vitro experiments. This might be explained by other (e.g., enzymatic) protein degradation processes which further decrease the ELISA response by affecting the binding epitope(s). Alternatively, there could be antidrug antibodies and ELISA results in plasma obtained from a breast cancer patient on long-term treatment with trastuzumab. LC-MS/MS concentrations obtained for peptides FTISADTSK, IYPTNGYTR, IYPTDGYTR, and IYPTisoDGYTR.
in the circulation, formed as a response to long-term exposure to trastuzumab, competing with the ELISA antibodies for binding and reducing the signal.\textsuperscript{23,24} Although this was not further investigated in the present study, the results do show that an ELISA is potentially hampered by multiple effects and that it is difficult to ascertain which fraction of the drug in plasma is actually measured. Results from the other patients are summarized in the Supporting Information Table S-16. They show that the degree of deamidation varies considerably between patients, which may have an effect on the efficacy of the therapy.

\section*{CONCLUSION}

We have developed and validated an LC-MS/MS-based method for quantitatively monitoring the \textit{in vitro} and \textit{in vivo} deamidation of trastuzumab at a potentially crucial position in one of its complementarity determining regions.\textsuperscript{12,25} The Asn55 residue is located at the top of the CDR2 loop, which is involved in the binding of trastuzumab to the HER2/neu receptor. It is, therefore, not unlikely that a modification of Asn55 may result in a conformational change in the CDR2, thus leading to changes in binding affinity of trastuzumab to its receptor and, potentially, to an altered pharmacological effect.

Application of the method to samples of \textit{in vitro} and \textit{in vivo} studies showed deamidation and isomerization with an increase in abundance of the deamidation products over time (weeks to months). Comparison of the LC-MS/MS results for the two signature peptides with ELISA data from the same \textit{in vitro} samples indicated that three different concentrations were found, each of which represented a different entity: total trastuzumab (FTISADTSK), trastuzumab which has not been deamidated and isomerized at Asn55, each of which represented a different concentration of a protein, since this value depends on the actual part(s) of the protein molecule which are the basis for analysis as well as on the underlying principle of the analytical technique, be it LC-MS/MS or ELISA.\textsuperscript{4} LC-MS/MS results from patient plasma samples revealed deamidation and isomerization of Asn55, but these translated into much lower values for the corresponding ELISA results than the factor that was expected on the basis of \textit{in vitro} experiments. This may indicate the effect of trastuzumab binding to antidrug antibodies or other specific proteins in the sample in addition to deamidation and further emphasizes the complex nature of protein quantification.

\section*{ASSOCIATED CONTENT}

\section*{Supporting Information}

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b04276.

Additional material as described in the text (PDF)

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\section*{Notes}

The authors declare no competing financial interest.

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