Technical note

Development and validation of cell-based ELISA for the quantification of trastuzumab in human plasma

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Abstract

Trastuzumab is a therapeutic monoclonal antibody against the Her2 oncoprotein, which is over-expressed in approximately 30% of breast cancers, and is now used routinely in the management of early and metastatic Her2+ disease. However, not all Her2+ breast cancer patients respond to trastuzumab and the pharmacodynamic and pharmacokinetic parameters behind this variation in response are unknown. Pharmacological investigations into variable response to trastuzumab have been hampered by the lack of a published feasible method to determine trastuzumab concentration in plasma. Here we describe the development and validation of a cell-based ELISA to measure trastuzumab in human plasma. The assay specifically measures the interaction between trastuzumab and Her2 and has a dynamic range of between 10 and 120 µg/ml. The mean intra-assay and inter-assay variability of the ELISA was 9%. Trastuzumab in plasma was stable for at least 10 weeks at −20 °C and 72 h at 4 °C, and was unaffected by 5 freeze/thaw cycles. Having validated the assay, the trough plasma trastuzumab concentrations of 30 patients being treated for metastatic or early disease were measured. The median trough concentration was 62 (range 21 to 441) µg/ml.

This cell-based ELISA method has undergone appropriate validation and is suitable for quantification of trastuzumab in the plasma of patients treated with Herceptin.

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1. Introduction

Breast cancer is the most commonly diagnosed cancer and, despite improvements in treatment, remains the leading cause of cancer-related mortality in women in Europe (Ferlay et al., 2007). Incidence of breast cancer has been rising over the last 30 years, however improvements in early detection and therapeutic management of the disease have led to a concomitant decrease in mortality over the last 20 years in many countries (Botha et al., 2003). The improvements in therapy have been largely due to an increased understanding of the molecular aetiology of the disease. Breast cancer is a heterogeneous disease, and bio-molecular elements responsible for this heterogeneity have been exploited in attempts to specifically target cancer cells. For example ~70% of breast cancers are estrogen and/or progesterone receptor positive. Many of these tumours are initially dependent on estradiol for proliferation and estrogen antagonism is central to the management of hormone receptor positive tumours (EBCTCG, 1998). More recently the over-expression, due to gene amplification, of the receptor tyrosine kinase human epidermal growth factor receptor 2 (cErb2, Her2) has been exploited in cancer chemotherapy (Cobleigh et al., 1999).

Her2 gene amplification occurs in up to 30% of breast cancers (Slamon et al., 1987) and is an oncogenic event, driving ligand-independent proliferation (Worthylake et al., 1999) and survival via aberrant PI3K/AKT signalling (Yakes et al., 2002). The oncogenic overexpression of Her2 has provided a tumour specific target for the therapeutic management of breast cancer.

Trastuzumab (Herceptin) is a humanised monoclonal antibody targeting an epitope resulting from the tertiary structure of Her2 (Cho et al., 2003). It is currently used...
Clinically as an adjuvant therapy in early breast cancer and in the treatment of metastatic disease (Baselga et al., 2006). The exact mechanism of action of trastuzumab is uncertain, but appears to be multifactorial and includes the direct inhibition of proliferative signalling and induction of antibody-dependent cell-mediated cytotoxicity (ADCC) (Cooley et al., 1999). Four major trials have shown that trastuzumab reduces the risk of recurrence of early Her2 positive breast cancer by approximately 50% (Piccart-Gebhart et al., 2005; Romond et al., 2005; Slamon et al., 2005). When coadministered with docetaxel in the treatment of metastatic disease, trastuzumab improves overall survival from 23 to 31 months (Marty et al., 2005). However only 38% of patients with Her2-positive metastases respond to single agent trastuzumab therapy (Vogel et al., 2002), and cancer recurs in the majority of patients with metastatic disease (Slamon et al., 2001).

There are few published clinical investigations of biomarkers that may be predictive of response or susceptibility to resistance to trastuzumab. Several studies have reported pharmacokinetic data on trastuzumab and interindividual variability in trough concentrations of 10-fold and 1000-fold have been observed, with low concentrations being associated with high circulating Her2 extracellular domain (ECD) in plasma (Baselga et al., 2005). It has also been observed that response is associated with a 1.6-fold higher mean trough trastuzumab concentration than that seen in non-responders (Cobleigh et al., 1999).

A barrier to investigating potential predictive pharmacokinetic parameters and pharmacodynamic biomarkers is the lack of a published assay to measure trastuzumab concentration in plasma. Pharmacokinetic studies published so far have failed to provide sufficient detail to allow the assay to be carried out independently (Baselga et al., 1996), (Cobleigh et al., 1999), (Tokuda et al., 1999), or have used purified Her2 protein that is not commercially available (Pegram et al., 1998). Although a very detailed method for quantification of trastuzumab has been published (Maple et al., 2004) the method described requires a capture antigen that is supplied only as a standard in a commercially-available Her2 ELISA kit. The quantities required mean that only a limited number of samples could be analysed for each kit purchased, in addition to the cost of capture antibodies and routine ELISA reagents.

We are currently undertaking clinical trials in breast cancer where measurement of trastuzumab pharmacokinetics would be advantageous. Given the lack of commercially-available purified trastuzumab antigens, we have developed and validated a cell-based ELISA to quantify trastuzumab in diluted human plasma. The assay uses a high density of formaldehyde-fixed SKBR3 Her2-positive breast cancer cells as the capture antigen in what is otherwise a conventional direct ELISA.

2. Materials and methods

Trastuzumab (Herceptin) was purchased from Roche (Weybridge, UK). Fetal calf serum (FCS), HRP conjugated goat anti-human antibody and Alexa Fluor488 conjugated goat anti human antibody were purchased from Invitrogen (Paisley, UK). Goat serum was purchased from Millipore (Herts, UK). Human plasma from nine individuals was purchased from the Blood Transfusion Service (Newcastle upon Tyne, UK). Phosphate buffered saline, Tris base, sodium chloride, concentrated hydrochloric acid, Tween 20, sodium azide, poly-o-lysine, Hoescht 33342, RPMI 1640, Formalin and Costar 96 well tissue culture plates were all purchased from Sigma (Poole, Dorset, UK). Sterile μ-clear black-walled optical 96 well plates were purchased from Greiner (Gloustershire, UK). SKBR3 breast adenocarcinoma cells and MDA MB 231 breast adenocarcinoma cells were gifts from Dr. Felicity May at the NICR, UK.

2.1. Patient samples

Plasma samples from 30 patients with Her2-positive breast cancer were collected during 2008. All the patients received trastuzumab every three weeks (8 mg/kg loading dose followed by 6 mg/kg) as part of their treatment of metastatic or non metastatic disease at the Newcastle General Hospital, Newcastle, UK. Samples were withdrawn immediately before the administration of the next dose of trastuzumab, after a minimum of 6 weeks of treatment. Patient samples were collected under appropriate ethical approval and with full informed patient consent.

2.2. Immunocytochemistry

SKBR3 and MDA MB 231 cells were plated at 5000 cells per well in optical 96 well plates and allowed to adhere for 24 h at 37 °C and 5% CO₂. After washing with PBS cells were fixed with 10% formalin for 20 min. The cells were blocked for 1 h with 20% goat serum in Tris buffered saline (TBS) with 0.1% Tween 20 for 1 h at room temperature and subsequently incubated with human plasma spiked with trastuzumab and diluted 1/4000 in wash buffer (TBS with 0.1% Tween 20 and 1% goat serum). Each well was washed 3 times with wash buffer, then incubated for 1 h at room temperature with an Alexafluor 488 tagged goat anti-human antibody diluted 1/500 in wash buffer with 10 μg/ml Hoescht 33342. Wells were washed 3 times with PBS and imaged with a ×20 objective on a Pathway HT Inverted fluorescent microscope (BD, Oxford, UK).

2.3. Trastuzumab ELISA

SKBR3 cells were propagated in RPMI 1640 with 10% FCS at 37 °C and 5% CO₂. To prepare single-use aliquots, SKBR3 cells were maintained and trypsinised at approximately 90% confluence, and split 1 in 2 until thirty two 125 cm² tissue culture flasks with cells at approximately 90% confluence were attained. The cells were trypsinised, pooled and counted on a Coulter Z1 cell counter (BD, Oxford, UK). The resuspended cells were centrifuged at 1000 rpm in a Centaur 2 bench top centrifuge (MSE, London, UK) for 5 min, the medium was aspirated and the pellet resuspended in RPMI 1640 with 10% FCS and 10% DMSO at a concentration of 2.6 × 10⁷ ml⁻¹. This suspension was stored at −80 °C in 0.5 ml aliquots until needed for the assay.

On day one of the assay the inner 60 wells of two 96 well tissue culture plates were incubated with 100 μl per well of 100 μg/ml 70,000–150,000 kDa poly-d-lysine for 5 min at room temperature under sterile conditions. The poly-d-lysine was aspirated and each well was washed with sterile deionised water.
water and the plates were allowed to dry under sterile conditions. Once dry, a single-use aliquot of SKBR3 cells was thawed and re-suspended to a total volume of 13 ml in RPMI 1640 with 20% FCS and a concentration of $1 \times 10^6$ ml$^{-1}$. This cell suspension was distributed into the poly-d-lysine coated wells of the previously-coated plates at a density of $1 \times 10^5$ cells per well in 100 µl volumes. The plates were incubated for exactly 24 h at 37 °C and 5% CO2 to allow the cells to recover and adhere to the poly-d-lysine coated substrate. On day two of the assay the medium was gently aspirated by vacuum and the cells were washed with cold PBS and fixed with 100 µl per well 10% formalin for 20 min. The formalin was aspirated and the cells washed 3 times with 100 µl PBS per well. Following washing, the fixed cells were incubated overnight at 4 °C in 100 µl blocking buffer (Tris buffered saline with 0.1% Tween 20, 20% goat serum and 0.2% sodium azide).

On day 3 of the assay all samples, standards and QCs were diluted 1/4000 in wash buffer (TBS with 0.1% Tween 20 and 1% goat serum). The block buffer was aspirated from the plate, replaced with 100 µl per well of the diluted samples, standards and QCs in triplicate (or replicates of 15 to determine intra assay variability) and incubated for 1 h at room temperature on a Gyro rocker STR9 (Bibby Scientific, Stone, UK) at 20 rpm. Following the primary incubation the samples were removed by aspiration and each well was washed 3 times with 100 µl PBS wash buffer. The final wash was replaced with 100 µl per well HRP goat anti-human antibody diluted 1/1000 in wash buffer and the plate was incubated for 1 h at room temperature on a Gyro rocker at 20 rpm. Each well was then washed 5 times with 300 µl wash buffer. HRP activity remaining was detected with TMB Substrate Kit as per manufacturer’s instruction. Briefly 100 µl of the combined TMB substrate and H2O2 solution was added to each well and was incubated in darkness at room temperature for 20 min on a Titertek plate shaker on setting 6. After 20 min the reaction was stopped with the addition of 100 µl per well 180 mM sulphuric acid and agitation for 10 s on a Titertek plate shaker on setting 6. The absorbance at 450 nm of each well was read on a Spectramax plate reader. Sample and QC values were interpolated from the standard curve using a Power equation in Excel ($y = cx^b$).

### 2.4. Validation

The robustness of the assay was characterised by a set of validation experiments designed to determine dynamic range, inter and intra assay variability, lower limit of detection, dilutional linearity, parallelism, stability at different storage temperatures and stability over five freeze–thaw cycles. A 150 mg vial of trastuzumab was dissolved in 2 ml sterile deionised water and aliquots stored at −80 °C. The 75 mg/ml solution was diluted to 1 mg/ml solutions in human plasma from one individual. For the initial determination of a dynamic range, trastuzumab at 1 mg/ml was diluted in human plasma by serial 1 in 2 dilutions to give a range of concentrations from 1 mg/ml to 60 ng/ml. These solutions were further diluted 1/4000 in wash buffer. Subsequent validation experiments and patient determinations used a standard curve with concentrations of 120, 100, 80, 60, 40, 20 and 10 µg/ml and concentrations of 100 and 25 µg/ml for quality control samples. Standard curves were made up fresh for each experiment. Quality control samples were made as

**Fig. 1.** Immunocytochemistry detection of trastuzumab in human plasma. Trastuzumab in human plasma was used as the primary antibody at 120 µg/ml (a and d), 10 µg/ml (b) or 0 (unspiked human plasma, c) with formalin fixed Her2+ SKBR3 cells (a, b, c) or Her2− MDA MB 231 cells (d) as the capture antigen. All primary antibodies were detected with an Alexafluor 488 tagged goat anti-human antibody. Signal above background was detected with 120 and 10 µg/ml trastuzumab on the SKBR3 cells but not by 120 µg/ml trastuzumab on MDA MB 231 cells.
single use aliquots and were stored at −80 °C. These samples were used to validate the assay as described below.

3. Results

3.1. Specificity of interaction between trastuzumab and Her2

We first wanted to know if the selective binding of trastuzumab at pharmacological concentrations in a plasma matrix could be observed by immunocytochemistry. Using as the antigen SKBR3 breast carcinoma cells, which over-express Her2, a strong fluorescent signal was observed when the cells were incubated with 120 µg/ml trastuzumab in human plasma diluted 1/4000 in wash buffer (Fig. 1a), and a signal above background detected when 10 µg/ml trastuzumab in human plasma diluted 1/4000 in wash buffer was used as the primary antibody (Fig. 1b). No signal was detected when SKBR3 cells were incubated with unspiked human plasma diluted 1/4000 (Fig. 1c). When MDA MB 231 cells, which do not over-express Her2, were incubated with 120 µg/ml trastuzumab no fluorescence above background was observed (Fig. 1d).

3.2. Validation of ELISA for quantification of trastuzumab

Having determined the specificity of the interaction between trastuzumab and Her2 in the SKBR3 cells we went on to develop a cell-based ELISA that would allow the quantification of trastuzumab in plasma from breast cancer patients. Initial experiments identified a dynamic range for the assay of between 8 and 125 µg/ml when samples had been diluted 1/4000 in wash buffer. Seven trastuzumab concentrations between these values were used to generate standards and these concentrations were used in all subsequent validation experiments and analysis of patient samples. The plot of signal versus concentration was best fitted by a power curve ($y = cx^b$) with an $R^2$ value of 0.996 and a $p$ value of 0.714 for deviation from the model (Fig. 2). Intra assay variability was determined by running 15 replicates of the low and high QC, and this was repeated twice, for a total of 3 independent experiments, and the mean values were used to contribute towards the acquisition of data to determine inter assay variability. The mean intra assay variability was 9% and on one occasion only was above 15% (Table 1). Inter assay variability was assessed over 11 independent experiments performed as part of the validation. Mean inter-assay variability was 12% for the low QC and 11% for the high QC. The variability of the back-calculated concentrations of trastuzumab from the standard curve were between 5 and 12% and all calculated values of the standards were within ±8% of the intended value (Table 1). The mean percentage recovery for the low and high QC samples was 98% and 95% respectively.

3.3. Stability of Herceptin QC samples in different storage conditions

The stability of trastuzumab under numerous storage conditions was determined. Both the high and low QCs were stable over 72 h at 4 °C (CV = 4 and 5% respectively) and

| Table 1 |

| Intra- and inter-assay variability ($n =$ intra-assay replicates in individual experiments for the intra-assay variability determination and number of independent experiments for the inter-assay experiments). |

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<th>Low QC 2</th>
<th>Low QC 3</th>
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<th>High QC 2</th>
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10 weeks at −20 °C (CV = 4 and 9% respectively) with no downward trend. QC samples were also stable for at least 6 months at −80 °C. There was a slight downward trend in the absorbance values of the QC samples over the period of the validation but this had no effect on the calculated QC concentrations and was attributed to a decrease in the HRP activity of the secondary antibody. Absorbance values returned to those seen at the beginning of the method development when new secondary antibody was purchased. There was no loss of signal over 5 freeze/thaw cycles from −80 °C to RT.

3.4. Limit of quantification

The lower limit of quantification was determined by analysing the plasma of eight individuals who had not received trastuzumab. The apparent plasma concentration was 5.6 ± 1.1 µg/ml (Mean and SD). Therefore a concentration of 9 µg/ml trastuzumab (Mean ± 3SD) was established as the lower limit of detection and at this value it is predicted that less than 1 in 200 positive values will be false.

3.5. Dilutional linearity and parallelism

Dilutional linearity was demonstrated by spiking matrix with 1 mg/ml trastuzumab and serially diluting the spiked sample 1 in 2 followed by a 1/4000 dilution in wash buffer. No hook effect was observed up to 1 mg/ml. Dilutions between 1/16 and 1/64 had predicted and measured results within the dynamic range of the assay and the trastuzumab concentrations corrected for dilution of these samples were within 20% of the predicted concentration. Three patient samples with measured concentrations above the dynamic range of the assay were used to determine parallelism. The samples were serially diluted in matrix 1 in 2 followed by a 1/4000 dilution in wash buffer. For each sample the lowest dilution that resulted in a measured concentration within the dynamic range of the assay and a calculated concentration less than 20% different from the preceding concentration was reported as the determined concentration. In 2 of the patients the minimum required dilution was 1/4 and in 1 patient a 1/16 dilution was required.

3.6. Clinical characteristics of patients and determination of patient samples

The trough trastuzumab concentrations of plasma samples from 30 patients being treated for metastatic breast cancer were measured using the cell-based ELISA method described. The median age of the patients was 55. The majority (77%) of patients were being treated for non metastatic disease and 61% of these patients had node-negative disease. Of the 30 patients, 1 was receiving concomitant lapatinib, 2 paclitaxel, 2 docetaxel, 2 vinorelbine and 1 anastrozole. The remaining 22 patients were receiving trastuzumab as a single agent. The median trastuzumab trough concentration was 62 µg/ml (range 21 to 441 µg/ml) (Fig. 3). Trastuzumab concentration was not normally distributed due to the presence of three samples from three individuals with high concentrations. No demographic or disease parameters explained the high trastuzumab concentration in these three patients. Excluding the three high outliers from analysis revealed a mean trastuzumab concentration of 63 ± 23 µg/ml (mean and SD).

4. Discussion

Herceptin is now routinely used clinically as adjuvant therapy in the treatment of Her2-positive breast cancer. However both de novo and acquired resistance remains a substantial barrier to prolonged efficacy. The pharmacodynamic and pharmacokinetic parameters that contribute to the variability in response to trastuzumab remain to be fully elucidated. The lack of a freely-available assay to quantify trastuzumab has precluded pharmacokinetic investigations independent of the manufacturers of Herceptin. This paper describes a robust and reliable method to quantify trastuzumab concentrations in human plasma. It is not possible to directly compare the performance of this assay with other trastuzumab assays used in clinical investigations, due to the lack of detail in the literature. However, the Genentech assay reports a sensitivity of 156 ng/ml (Baselga et al., 1996; Pegram et al., 1998). This is approximately two orders of magnitude greater than sensitivity of the assay described here (9 µg/ml). This is expected and explicable by the difference in purity of the capture antigen. The Genentech assay uses a purified human recombinant Her2 protein that is not available commercially, compared with the multiple antigens exposed on the non-permeabilized formalin-fixed SKBR3 cells. The sensitivity of the assay described here is sufficient to discriminate plasma concentrations less than the pharmacological target of a trough trastuzumab concentration of 20 µg/ml (Leyland-Jones et al., 2003). The assay is specific for the interaction between trastuzumab and Her2 as illustrated by the immunofluorescence experiments. No fluorescence signal was observed when a 1 in 4000 dilution of human plasma without trastuzumab was used as the primary antibody on the Her2 positive SKBR3 cells, nor when a 1 in 4000 dilution of human plasma spiked with
120 μg/ml trastuzumab was used on the Her2 negative cell line MDA MB 231.

The assay described here is more readily comparable with the sandwich-type ELISA described in detail by Maple et al. (2004). The method is comparable in terms of performance to that assay, with similar precision, dynamic range and accuracy, but is simpler to execute and is not limited by access to reagents of restricted availability.

The dynamic range of the assay of 10 to 120 μg/ml encompasses the expected mean trough trastuzumab concentration, as determined by previously-published trastuzumab pharmacokinetic studies. The mean and CV (63 μg/ml, 37% CV) of the patient samples measured was also comparable to previously published pharmacokinetic papers (Cobleigh et al., 1999; Baselga et al., 2005).

The use of formalin-fixed cells as capture antigens for the detection and quantification of therapeutic antibodies in plasma is not limited to trastuzumab. A similar assay to that described here that can detect and quantify the antibody in a plasma matrix (Yang et al., 2006).

Other therapeutic antibodies that may be quantifiable by this approach include rituximab and cetuximab. The described assay for the measurement of trastuzumab in human plasma is robust, reliable and will allow future studies to be undertaken into the effect of trastuzumab pharmacokinetics on variability of efficacy.

Acknowledgements

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References


