Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 861–866

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

The bioanalysis of the monoclonal antibody trastuzumab by high-performance liquid chromatography with fluorescence detection after immuno-affinity purification from human serum

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ARTICLE INFO

Article history:
Received 5 March 2009
Received in revised form 26 April 2009
Accepted 29 April 2009
Available online 7 May 2009

Keywords:
Monoclonal antibody
Trastuzumab
Herceptin
Immuno-affinity purification
HPLC-fluorescence

ABSTRACT

For the quantification of therapeutic monoclonal antibodies in biological specimens, enzyme-linked immunosorbent assay (ELISA) is the most widely used technique. ELISA's have some limitations and therefore alternative analytical techniques are being explored. In this study we describe the development of a bioanalytical assay using high-performance liquid chromatography (HPLC) coupled with fluorescence detection for the bioanalysis of the monoclonal antibody trastuzumab. Different extraction procedures were explored, like isolation using protein A and protein G. Finally a method using immuno-affinity purification has been developed. Trastuzumab is isolated from human serum using sepharose coupled with anti-trastuzumab idiotype antibodies. After extraction samples are injected onto a Zorbax 300SB C_8 column at 75 °C using the organic solvents isopropanol and acetonitrile with high eluotropic strengths. The assay quantifies trastuzumab from 5 to 40 μg/mL in human serum with accuracies <20%. Samples with concentrations above the upper limit of quantification (>ULOQ; >40 μg/mL) can be diluted 5 times with control human serum prior to sample pre-treatment. The assay can now be used to analyse serum samples of patients treated with trastuzumab. The obtained results are comparable to those obtained using ELISA. This is the first report describing a bioanalytical assay using HPLC and fluorescence detection for the quantification of a monoclonal antibody at the intact protein level in human serum. This unique approach has the advantage compared to ELISA that a HPLC separation step is introduced to improve the selectivity. This method is a potential alternative to ELISA to support pharmacokinetic evaluations. However, for purification of trastuzumab from serum anti-idiotype antibodies are necessary. These anti-idiotype antibodies are also used in ELISA and as ELISA is more sensitive and less labor-intensive, ELISA probably remains the analytical technique of first choise.

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1. Introduction

In recent years, monoclonal antibodies (mAbs) have been used increasingly for targeted therapy. Around 20 mAbs are currently on the market and are used in various therapeutic areas including oncology, infectious diseases and immune diseases [1]. The number of registered mAbs will likely increase in the coming years as more than 100 mAbs are currently in clinical development [2].

The preclinical and clinical development of these mAbs requires sensitive and selective bioanalytical methods to support pharmacokinetic studies. Immunoassay techniques are the most frequently used bioanalytical methods for the quantification of mAbs. Although immunoassays are efficient for high throughput analysis and are very sensitive, there are also some disadvantages. For example proteins in the analyzed biological fluid can interfere with the assay and give high background signals. Recently an enzyme-linked immunosorbent assay (ELISA) for the quantification of rituximab in human serum has been described [3]. Two different approaches were followed. The first approach used the antigen as catcher on the ELISA plates, the second approach used anti-rituximab idiotype antibodies as catcher on the ELISA plates. Although both assays were validated and fulfilled the required criteria a discrepancy was observed when clinical samples were analysed using both assays. This may be due to the fact that ligand binding assays for macromolecules are typically run without separation of the molecule of interest from any of its biotransformation products or endogenous macromolecules, which are also present in serum or plasma [4]. Another explanation can be that one catcher can only bind the free rituximab and that the other catcher can bind total rituximab leading to different results.

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doi:10.1016/j.jpba.2009.04.031

In the search for an alternative for the quantification of mAbs in biological specimen, high-performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MS/MS) has been described [5–8]. These methods typically create a trypsine digest of the serum sample and quantitate the mAb of interest by analysis of a peptide specific for that mAb. As internal standard the same peptide, but then isotopically labelled [6] or another protein can be used [5,8]. The most variable step, however, is the execution of the trypsin digestion. To correct for this variable step, Heudi et al. [7] labelled the entire mAb isotopically and added this as internal standard to the sample before trypsin digestion. This approach appeared to be very successful. Unfortunately, the labelling of mAbs is expensive and limited to settings where the mAb is produced itself.

Trastuzumab (Herceptin®) is a humanized immunoglobulin gamma 1 (IgG1) mAb, which is directed against the human epidermal growth factor receptor-2 (HER2/*neu*). This receptor is over-expressed in about 25% of breast cancer patients [9,10]. In the search for an alternative for immunoassays and to avoid the error prone trypsin digestion step reported LC–MS methods we aimed to develop a bioanalytical method for the intact trastuzumab protein. In this paper we describe the development of the assay using HPLC coupled with fluorescence detection.

2. Materials and methods

2.1. Chemicals and materials

Trastuzumab (Herceptin®) was supplied as a lyophilized powder and originated from Roche (Basel, Switzerland). This pharmaceutical formulation contains additionally L-histidine hydrochloride, L-histidine, α-α-trehalose dihydrate and polysorbate 20. Glycine was purchased from Sigma–Aldrich (St. Louis, MO, USA). Sterile phosphate buffered saline (PBS) and sterile water were purchased from Fresenius-Kabi (Bad Homburg, Germany). Acetonitrile (HPLC grade) was from BioSolve Ltd. (Amsterdam, the Netherlands). Cyanogen bromide (CNBr-) activated sepharose 4B originated from Pharmacia/GE Healthcare (Uppsala, Sweden). Pepsin was from Sigma–Aldrich (St. Louis, MO, USA). Freeze buffer was obtained from Sanquin Diagnostics (Amsterdam, the Netherlands). Anti-trastuzumab idiotype antibodies were produced in-house as described elsewhere [11] and were present as 0.5 mg/mL solution in PBS. All other used reagents were of analytical reagent grade and purchased from Merck (Darmstadt, Germany). Serum was obtained from healthy volunteers from the Slotervaart Hospital (Amsterdam, the Netherlands).

2.2. Instrumentation

The HPLC procedure was carried out using an 1100 series binary solvent delivery system with a column heater, an online mobile-phase degasser and an autosampler (Agilent Technologies, Santa Clara, CA, USA). The system was used without solvent mixer [12]. Mobile phase A consisted of 0.1% (v/v) TFA in water and mobile phase B consisted of 0.1% (v/v) TFA in isopropanol–acetonitrile–water (70:20:10, v/v/v). Gradient elution was applied at a flow rate of 0.5 mL/min through a Zorbax 300SB C_8 column (150 × 2.1 mm ID, particle size 5 μm, pore size 300 Å (Agilent Technologies)) protected with a 0.5 μm filter (Upchurch Scientific, Oak Harbor, WA, USA) and thermostatted at 75 °C. After an isocratic hold of 2 min with 10% B, a linear gradient was started from 10% to 25% B in 2 min followed by a gradient from 25% to 40% B over 21 min. Then the percentage B was increased immediately to 100% to wash the column for 5 min before the eluent composition returned to the starting conditions. The column was equilibrated for 5.5 min before the next injection, leading to a total runtime of 35.5 min. The HPLC column was coupled to a FP2020 fluorescence detector (Jasco, Easton, MD, USA) set at an excitation wavelength of 278 nm and an emission wavelength of 343 nm. The band width was set to 40 nm and the gain to 100. Chromatograms were acquired and processed using Chromeleon Software version 6.5 (Dionex Corporation, Sunnyvale, CA, USA).

2.3. Trastuzumab isolation using protein G

Prepacked protein G columns (Nab Protein G Spin columns 0.2 mL (Pierce Biotechnology/Thermo Fisher Scientific, Rockford, IL, USA)) were used for sample purification according to the instructions from the manufacturer. In brief, columns were equilibrated with PBS. A volume of 200 μL of serum was applied and incubation was performed with end-over-end mixing during 10 min. The columns were washed 3 times using PBS. Finally, elution was performed using 400 μL 0.1 M glycine pH 2.5. Elution was performed 3 times and fractions were collected separately and injected on the LC-fluorescence system. Besides blank serum and serum spiked with 500 μg/mL trastuzumab, also PBS with 500 μg/mL trastuzumab was tested. From this aqueous solution all incubation and washing steps were injected on the HPLC to get information about the various processes.

2.4. Trastuzumab F(ab')2 isolation using protein A

Trastuzumab was cleaved in F(ab')2 fragments using pepsin. Serum was adjusted to pH 4.3 and pepsin was added in amounts of 130, 220, 500 and 1250 μg/mL serum. Digestion was allowed for 18 h at 37 °C. Subsequently, the pepsin was inactivated by the addition of 1 M Tris pH 7.5. After digestion extraction was performed using prepacked protein A columns (Nab Protein A Spin columns 0.2 mL (Pierce Biotechnology/Thermo Fisher Scientific, Rockford, IL, USA)) according to the instructions from the manufacturer as described in paragraph 2.3 for protein G spin columns. Tests were performed using blank serum, serum spiked with 210 μg/mL trastuzumab and an aqueous trastuzumab solution of 210 μg/mL.

2.5. Coupling of anti-idiotype antibodies to sepharose

Anti-trastuzumab idiotype antibodies were coupled to CNBr-activated sepharose according to the instructions from the manufacturer. In brief, an amount of sepharose, corresponding 100 times the mass of anti-idiotypes, was added to PBS containing 0.01 M EDTA and 0.3% (w/v) bovine serum albumin (BSA). A volume of 100 mL 1 mM HCl was added and the CNBr-activated sepharose was allowed to swell for 15 min by end-over-end mixing. The suspension was centrifuged and the supernatant was discarded. The sepharose was washed 3 times with coupling buffer (0.5 M NaCl, 0.1 M $NaHCO_3$, 4 N NaOH, pH 8.5) prior to antibody coupling. An amount of 4.1 mg anti-trastuzumab idiotype antibodies was diluted to 10 mL using coupling buffer and this mixture was added to the sepharose. The coupling was allowed by end-over-end mixing for 18 h at 4 °C. After centrifugation, the anti-trastuzumab concentration in the supernatant was determined by measuring the absorbance at 280 nm.

After coupling, the sepharose was washed alternated with 1 M NaCl, 0.1 M sodium acetate, 0.046 M citric acid, pH 4 and 1 M NaCl, 0.2 M boric acid, 0.05 M NaOH, pH 8 to remove non-covalently bound proteins. The sepharose was inactivated with 0.5 M glycine buffer pH 8.5 during end-over-end mixing for 2 h at 4 °C. Groups that did not react were blocked by this procedure. The mixture was centrifuged and the supernatant was discarded. Finally the

sepharose anti-trastuzumab suspension was diluted and stored as a 2 mg/mL suspension in PBS containing 0.01 M EDTA and 0.3% (w/v) BSA.

2.6. Trastuzumab isolation using anti-trastuzumab idiotype antibodies

The affinity purification was executed using 1 mL of the 2 mg/mL sepharose-anti-trastuzumab suspension per sample. For optimal mixing during incubation, the volume of the sepharose suspension was reduced. The sepharose-anti-trastuzumab suspension was centrifuged for 1 min and 450 μL of the supernatant was rejected. Subsequently, 150 μL serum sample and 100 μL Freeze buffer were added. The mixture was incubated for 18 h using end-over-end mixing at ambient temperatures.

After incubation, the samples were centrifuged during 1 min. Subsequently, the samples were washed by rejecting 600 μL of the supernatant and adding 600 μL washing buffer (0.15 M NaCl in 8.6 mM Na_2HPO_4–1.3 mM NaH_2PO_4 buffer, pH 7.5). This procedure was repeated 5 times. After the last washing step, a volume of 75 μL washing buffer remained behind.

Trastuzumab bound to sepharose was eluted by adding 100 μL 0.1 M glycine, pH 2.5 and using mechanical shaking (200 rpm) for 1 h at ambient temperatures. Thereafter the tubes were centrifuged and 100 μL of the supernatant was transferred to an autosampler vial with insert. Volumes of 13 μL aliquots were injected onto the LC-fluorescence system for quantification.

2.7. Preparation of stock solutions, calibration standards and quality control samples

Trastuzumab was dissolved in water for injections according to the instruction from the manufacturer resulting in a stock solution of 21 mg/mL. This stock solution was stored at nominally 4 °C and was found to be stable for at least 6 months [12].

The stock solution was serially diluted with drug-free human serum to obtain calibration standards at concentrations of 5, 10, 15, 20, 25, 30, 35 and 40 μg/mL trastuzumab.

For the preparation of quality control (QC) samples a similar procedure was followed. Another stock solution was serially diluted in a different batch of drug-free human serum as was used to prepare the calibration samples to obtain QC samples containing trastuzumab at concentrations of 15, 40 and 85 μg/mL. The validation sample at 85 μg/mL was prepared to assess the accuracy and precision after dilution in drug free human serum.

2.8. Plasma pharmacokinetics

Samples of patients treated with trastuzumab were analysed with the developed assay. The Committee on the Medical Ethics of The Netherlands Cancer Institute authorized this study and written informed consent was obtained from all patients. The calibration standards were prepared freshly and were processed and analysed in triplicate. Calibration curves (peak area versus nominal concentration) were fitted by Logit regression according to the formula Ln((response-Blank)/(Bmax-response)) = slope × Ln conc + intercept, where the highest (40 μg/mL) en lowest (5 μg/mL) points were used as anchoring points.

QC samples were processed and analysed in triplicate, besides the QC mid level (15 μg/mL) that was processed and analysed in quintuplicate. The QC sample of 85 μg/mL was diluted 5 times with control human serum before processing. Deviations from the nominal concentrations should be within 80–120%. At the lower limit of quantification level (LLOQ level, 5 μg/mL) and upper limit of quantification (ULOQ, 40 μg/mL) level a deviation of 25% was permitted [13–15].

3. Results and discussion

3.1. HPLC-fluorescence

For structural characterization and investigation to the heterogeneity of mAbs, reversed-phase chromatography was investigated extensively by Dillon et al. [16]. Using a Zorbax-300SB C_8 column at high temperatures (>70 °C) with a mobile phase containing solvents of high eluotropic strength like isopropanol and acetonitrile, they developed a chromatographic system that is capable of separating different isoforms of a mAb. Due to the high resolution of this system, we used it as a starting point for our bioanalytical method, making separation of trastuzumab from endogenous IgG molecules possible. We made a small adjustment to the system. Dillon et al. [16] used a linear gradient from 25% to 32% B in 21 min. However, by applying this gradient no trastuzumab peak was observed in the chromatograms. As trastuzumab strongly interacts with the stationary phase it was found in the final washing step of the gradient (100% B). Therefore the gradient was changed to run from 25% to 40% B in 21 min, leading to a trastuzumab retention time of 16.8 min. Additionally we investigated whether enough selectivity could be obtained. Therefore we injected other therapeutic mAbs like adalimumab, bevacizumab and rituximab together with trastuzumab. Other therapeutic mAbs were almost baseline separated from trastuzumab and therefore was likely that trastuzumab could additionally be separated from endogenous IgG.

At first we tried to couple the system to an API365 triple quadrupole mass spectrometer equipped with a turbo ion spray source (Applied Biosystems, Foster City, CA, USA). Although Dillon et al. [16] managed to combine the system with a mass spectrometer (MS), our results were not satisfactory. The sensitivity was very poor and this was probably due to two effects. First of all, the viscosity of the mobile phase was high due to the presence of isopropanol, resulting in a poor spray performance. Secondly, trifluoroacetic acid (TFA) was present as ion pairing agent in the mobile phase, leading to severe ion suppression [17]. Dillon et al. [16], however, used a Z-spray source and these authors claim that this configuration might circumvent these problems. As we have demonstrated before [12] the dynamic range of a mass spectrometer for intact mAbs is very small due to spray saturation. Therefore, we have decided to discontinue the use of MS as detection method.

The dynamic range of LC coupled to UV detection at 278 nm by a previously described method is 19–200 μg/mL [12]. mAbs contain three aromatic amino acids: phenylalanine, tryptophan and tyrosine explaining the intrinsic fluorescence. We therefore investigated if LC-fluorescence could improve the selectivity and sensitivity of our assay. With an excitation wavelength set to 278 nm, emission was found at 343 nm. Trastuzumab was diluted to different concentrations in 0.1 M glycine pH 2.5 in water to determine the LLOQ with the LC-UV and LC-fluorescence system. The LLOQ was defined as a signal to noise ratio (S/N) of at least 5. Volumes of 20 μL were injected onto the HPLC column. The LLOQ for the LC-UV method was 12 μg/mL (240 ng on column) and for LC-fluorescence method 2 μg/mL (40 ng on column). A calibration curve in 0.1 M glycine pH 2.5 was constructed and linearity using the fluorescence detector was observed until 30 μg/mL. Concentrations of 35 μg/mL and higher were out of range of the fluorescence detector.

In summary, our final chromatographic system consisted of a reversed phase column using a mobile phase with isopropanol for high eluotropic strength and TFA as ion paring

agent with fluorescence detection ($\lambda_{excitation}$: 278 nm; $\lambda_{emission}$: 343 nm).

3.2. Extraction procedure

The major challenge in developing a chromatographic method for an intact mAb in biological specimens is the extraction of the analyte from the matrix. Human serum from healthy adults consists of approximately 11 mg/mL IgG, 1.5 mg/mL IgA and 1.1 mg/mL IgM [18,19]. The group of IgG's can be subdivided into subgroups with concentrations of approximately 7 mg/mL IgG1, 3 mg/mL IgG2, 0.7 mg/mL IgG3 and 0.5 mg/mL IgG4 [20]. Trastuzumab is a humanized IgG1 molecule that shows high resemblance to endogenous immunoglobulins. However, serum concentrations of trastuzumab are rather low in comparison with endogenous immunoglobulin levels. Serum trough concentrations of approximately 27–52 μg/mL are reported during a 3 weekly trastuzumab monotherapy schedule and the steady state concentration was calculated to be 65 μg/mL [21]. To extract trastuzumab from human serum we investigated different approaches.

3.2.1. Evaluation of trastuzumab isolation using protein G

The first approach was to extract trastuzumab from human serum using protein G columns. Protein G is a bacterial cell wall protein isolated from group G streptococci and is known for its ability to bind selectively immunoglobulins of the IgG isotype. Protein G does not bind to human IgM, IgA or IgE. The results from the aqueous solution revealed that all trastuzumab was bound to the protein G columns, additionally no losses were observed during the washing steps. A fraction of 56% of all trastuzumab was found in the first elution fraction and the total recovery was 59%. However, the results obtained with blank and spiked serum were not satisfactory. Separation of trastuzumab from endogenous compounds was not accomplished, even the HPLC gradient was prolonged.

To improve the selectivity of the sample pre-treatment using Protein G, the influence the pH of the elution step was then investigated. For protein A it is known that different IgG subclasses could be separated by changing the pH of the elution solvent [22–26]. In an aqueous solution no trastuzumab was found in the elution fraction when using 0.1 M glycine pH 4 or higher. At pH 3.5 a small amount of trastuzumab eluted from the protein G column. Therefore the effect of an extra washing step with respectively 0.1 M glycine pH 4 or 0.1 M glycine pH 3.5 was investigated using serum samples. Results revealed that endogenous immunoglobulins are still present in the final extracts, interfering with the detection of trastuzumab.

Our last effort to optimize the protein G extraction was to test an additional washing step consisting of 3 M NaCl, 1 M glycine, pH 8.9 as from literature it is known that non-specific bound serum compounds can be washed from protein G using glycine with a high salt content [27–29]. However, addition of this extra washing step did not improve the selectivity of the sample pre-treatment.

In conclusion, Protein G columns are not capable to extract trastuzumab at the intact protein level selectively from serum. The separation between trastuzumab and these endogenous compounds using the described HPLC method was insufficient.

3.2.2. Evaluation of trastuzumab F(ab')2 isolation using protein A

The interaction between protein A and human IgG is established through the Fc regions of the immunoglobulin. The protein A binding site is located on the CH2 and CH3 domains of the Fc region of human IgG [30,31]. For trastuzumab however, we found that the F(ab')2 fragments also bind to protein A [11] and this is an exceptional but known property of some IgG and IgA antibodies [32]. To obtain more selectivity for trastuzumab during the extraction procedure we tried to use this rather unique property of trastuzumab.

F(ab')2 fragments in an aqueous solution were created by adding pepsin and then the sample was injected on the HPLC system. A single peak with a retention time of 18.0 min was observed. As the chromatogram showed a single peak with a different retention time compared to intact trastuzumab (Rt = 16.8 min) we concluded that the cleavage was complete. The created F(ab')2 fragments were subjected to extraction using protein A columns. As no F(ab')2 fragments were present after the incubation nor in the washing steps, it could be concluded that all trastuzumab F(ab')2 fragments were bound to the column. Elution with 0.1 M glycine pH 2.5 showed 100% recovery in the first elution step.

However, digestion of serum using pepsin and subsequent extraction using protein A, revealed that the selectivity was poor. First we tested pepsin amounts of 130 and 220 μg/mL serum, based on IgG and total immunoglobulin concentrations in serum. The chromatogram revealed a peak with a retention time of 16.7 min between the endogenous compounds. The hypothesis was that the digestion of intact trastuzumab to F(ab')2 fragments was not complete. As the total protein concentration in serum is approximately 80 mg/mL, much higher pepsin concentrations of 500 and 1250 μg pepsin per millilitre serum were tested. The peak at 16.7 min disappeared, however too many interfering compound were observed in the chromatogram, no distinctive trastuzumab F(ab')2 peak was visible.

In conclusion, not enough selectivity could be reached using production of F(ab')2 fragments and subsequential extraction using protein A. Therefore, this approach was abandoned and considered as not useful for the bioanalysis of trastuzumab in human serum.

3.2.3. Evaluation of trastuzumab isolation using anti-trastuzumab idiotype antibodies

As the previously described extraction procedures did not obtain sufficient selectivity, an extraction procedure more specific for trastuzumab itself was explored. Therefore, anti-trastuzumab idiotype antibodies, which were produced in-house [11], were covalently coupled to a solid support. As solid support cyanogen bromide (CNBr-)activated sepharose 4B was selected. In alkaline environment the CNBr reacts with the hydroxyl groups on sepharose. The result is a cyanate ester or a cyclic imidocarbonate. These groups react with primary amines in the antibody which results in covalent coupling of the antibody to the sepharose.

After execution of the coupling the protein concentration in the supernatant was analysed using UV absorbance at 280 nm. No protein could be detected in the supernatant leading to the conclusion that all added anti-trastuzumab idiotype antibodies were bound to the sepharose carrier.

The anti-trastuzumab idiotype sepharose was used to extract trastuzumab from serum. The sepharose was not packed in columns but used batchwise as in a radio immunoassay (RIA) format, which means that the serum sample and the sepharose were brought together with Freeze buffer in a tube and binding was allowed by end-over-end mixing.

First, the binding capacity of the sepharose was determined. Therefore, 2 mg of sepharose was incubated with 150 μL serum spiked with trastuzumab in different concentrations from 0.5 to 33 μg/mL. Binding was allowed during 18 h using end-over-end mixing. Thereafter the tubes were centrifuged and the amount of trastuzumab in the supernatant was determined. In the sample of 33 μg/mL 2% of the trastuzumab was not bound. At lower concentrations the unbound amounts were between 0% and 1%, leading to the conclusion that the binding capacity of the sepharose is excellent in the range of the LC fluorescence method (2–30 μg/mL).

In the next step blank serum and serum samples spiked with trastuzumab were analysed. Fig. 1A shows the chromatogram of blank human serum extracted with the anti-trastuzumab idiotype sepharose. No interfering peaks were observed at the retention

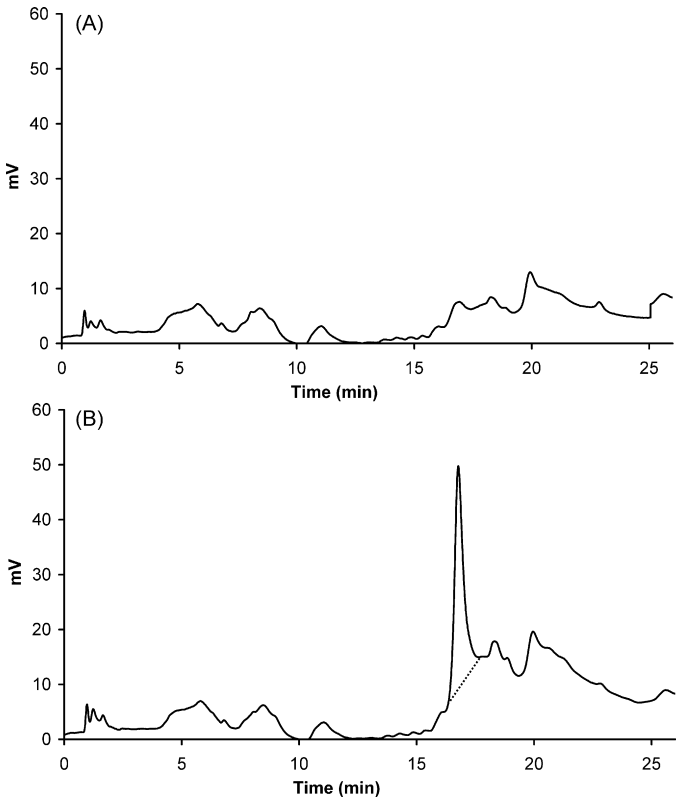

Fig. 1. Chromatograms of blank serum (A) and spiked at LLOQ level (5 μg/mL, B) after immuno-affinity purification using anti-trastuzumab idiotype antibodies. The retention time of trastuzumab is around 16.8 min.

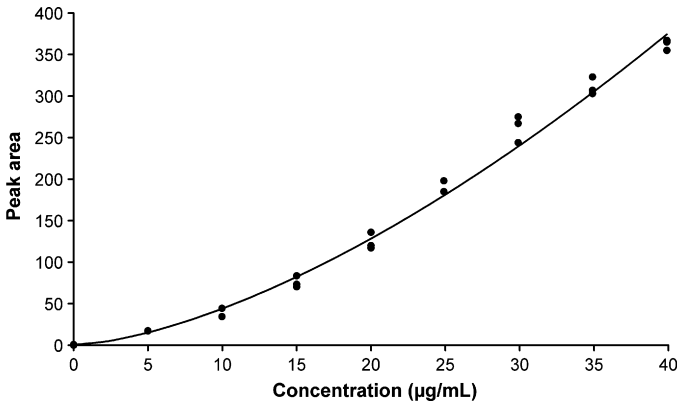

Fig. 2. Representative calibration curve of trastuzumab ($n = 3$).

time of trastuzumab. Serum samples spiked with 35 and 40 μg/mL trastuzumab were processed and fluorescence responses above the range of the detector were obtained. Therefore it was decided to lower the injection volume from 20 to 13 μL. The LLOQ was evaluated again but now in extracted serum samples instead of aqueous solutions. With 5 μg/mL a S/N of 6.6 was obtained and therefore this concentration was chosen as LLOQ. The extraction recovery was determined in triplicate at all concentrations of the calibration curve by comparing the peak area of an extracted serum sample with the peak area obtained with trastuzumab in 0.1 M glycine pH 2.5. The average recover was 90.1% (range 74.8–108%). With an injection volume of 13 μL a dynamic range from 5 to 40 μg/mL trastuzumab in human serum was established. Fig. 1B shows a chromatogram of a spiked serum sample at the LLOQ of 5 μg/mL. S/N was 6.6 and the peak shape was acceptable for a mAb with an asymmetry factor (As) of 2.3 and the retention factor was 16.8.

Fig. 2 shows a representative calibration curve ($n = 3$. This curve was fit with a Logit regression according to the formula: $\mathrm{Ln}((\text{response}-0.4957)/(7.31 \times 10^{11} - \text{response})) = 1.5533 \times \mathrm{Ln}\,\text{conc} - 27.1$ with a regression coefficient of 0.9949. The curve is S-shaped, which is common for antibody binding experiments [4]. QC samples were analysed together with clinical samples. Accuracies and precisions of the QC samples are presented in Table 1 and fulfilled the required criteria [13–15]. Additionally it can be concluded that acceptable accuracy and precision values were obtained when a serum sample was diluted 5 times with control human serum before processing and this is necessary as it was known on forehand that at the end of trastuzumab infusion these high concentrations could be expected. From the results in Table 1 the use of an internal standard appeared to be unnecessary. However, the extraction procedure is labour intensive and error prone. Therefore, the use of an internal standard would be advantageous, however due to the extraction with anti-idiotype antibodies for trastuzumab it is not possible to add an internal standard that corrects for the extraction procedure.

In conclusion, an extraction procedure with high specificity has been developed. With this extraction procedure in combination with HPLC with fluorescence detection trastuzumab concentrations in human serum can be quantified in the therapeutic relevant range of 5–40 μg/mL [21]. Additionally, samples above the upper limit of quantification (>ULOQ) can be diluted 5 times prior to the extraction procedure. However, anti-idiotype antibodies are necessary to extract trastuzumab from serum. These anti-idiotype antibodies are used in the ELISA as well, so basically the method is based on the same principle but with a chromatographic separation step. As the ELISA is more sensitive and less labour-intensive than this HPLC-fluorescence method, the ELISA will stay the method of first choice for bioanalysis of trastuzumab. Therefore, not a full validation procedure was executed but QC samples were analysed together with clinical samples to demonstrate the possibilities with the LC-fluorescence method.

4. Application of the method

The developed assay for trastuzumab in serum was used to analyse serum samples of patients treated with trastuzumab. Fig. 3A shows an example of the serum concentration–time curve one of our patients who started with trastuzumab therapy. Initially, a loading dose of 310 mg was given (equivalent to 4 mg/kg) as

Table 1
Assay performance data of quality control serum samples containing trastuzumab.

Nominal concentration (μg/mL)	Mean measured concentration (μg/mL)	Intra-assay bias (%)	Intra-assay precision (%)	No. of replicates
15.0	15.8	106	17.0	5
39.9	42.8	107	5.12	3
84.8**	93.1	110	3.95	2*

Conc.: concentration, No.: number.
* One result was rejected due to a failure during sample processing.
** Sample was diluted 5 times in control human serum before processing.

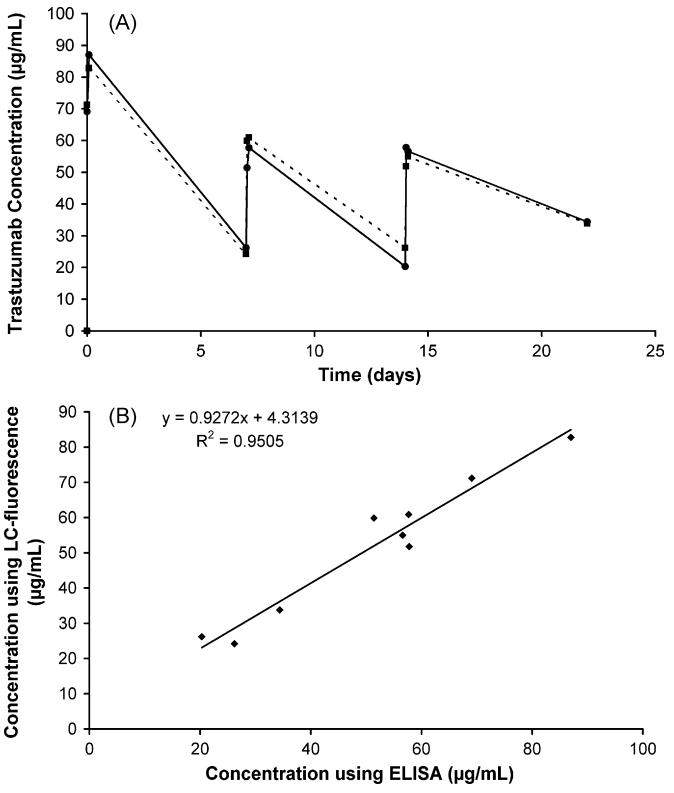

Fig. 3. (A) Concentration versus time profile of a 77 kg female treated with a starting dose of 310 mg trastuzumab followed by 155 mg trastuzumab weekly. Samples were taken before the start of infusion, at the end of infusion and 2 h after the end of infusion. (●) Samples analysed using the described immuno-affinity purification and LC-fluorescence assay. (■) Samples analysed using ELISA. (B) Comparison of LC-fluorescence and ELISA analysis.

an intravenous infusion during 1.5 h. Thereafter a dose of 155 mg (equivalent to 2 mg/kg) was given weekly. Serum samples were taken pre-dose, immediately at the end of infusion and 2 h after infusion. Additionally, Fig. 3A also shows the results of the same samples analysed using ELISA [11]. In Fig. 3B the obtained concentrations using ELISA and LC-fluorescence method are compared. The quantified trastuzumab levels are similar: only at one time point the deviation between the methods is 29.1%. At all other time point deviations between −10.4% and 16.5% were found.

5. Conclusion

The goal of this study was to develop a bioanalytical method for the quantification of trastuzumab at the intact protein level in human serum using a different approach than an ELISA. Different strategies were followed to extract trastuzumab from serum. Unfortunately, no general applicable sample pre-treatment method appeared efficient. Specific antibodies against trastuzumab were necessary to extract the drug from serum. The production of anti-idiotype antibodies using rabbits is a simple procedure and therefore the described method may also be applicable for other therapeutic monoclonal antibodies than trastuzumab. However, these anti-idiotype antibodies can be used in an ELISA format as well and as an ELISA is more sensitive and less labour-intensive, ELISA is at this moment still the analytical method of first choice.

The dynamic range of the assay is within the therapeutic range of trastuzumab. Additionally, the LLOQ of 5 µg/mL makes quantification of trastuzumab in serum possible for several weeks after the last dose, as the terminal half-life of trastuzumab is approximately 3 weeks. The results obtained with the described assay are in consensus with the results obtained using when ELISA.

A bioanalytical assay for a monoclonal antibody at the intact protein level using chromatography with fluorescence detection has not been described before, as far as we know. We have now demonstrated that there are other possibilities than ELISA for the bioanalysis of trastuzumab although a very specific extraction procedure from human serum is needed.

Acknowledgement

We gratefully acknowledge Lucien A. Aarden and Henk de Vrieze from the Department of Immunopathology, Sanquin Research, Amsterdam, for their help with the anti-idiotype sepharose coupling and the immunoaffinity purification procedure.

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