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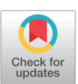
Original Article

Development of a competitive enzyme-linked immunosorbent assay for therapeutic drug monitoring of afatinib



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ABSTRACT

Afatinib is an oral tyrosine kinase inhibitor (TKI) approved for treating advanced non-small cell lung cancer. It is necessary to develop a simple quantification method for TKIs in order to facilitate therapeutic drug monitoring (TDM) in clinical settings. This study sought to develop a simple and sensitive competitive enzyme-linked immunosorbent assay (ELISA) to quantify afatinib in plasma for routine pharmacokinetic applications. An anti-afatinib antibody was obtained using (S)-*N*-4-(3-chloro-4-fluorophenyl)-7-(tetrahydrofuran-3-yloxy)-quinazoline-4,6-diamine (CTQD), which has the same substructure as afatinib, as a hapten. Enzyme labeling of afatinib with horseradish peroxidase was similarly performed using CTQD. A simple competitive ELISA for afatinib was developed based on the principle of direct competition between afatinib and the enzyme marker for the anti-afatinib antibody, which had been immobilized on the plastic surface of a microtiter plate. Plasma afatinib concentrations below the limit of quantification of 30 pg/mL were reproducibly measurable. Also, the values of plasma afatinib levels measured from 20 patients were comparable with those measured by high-performance liquid chromatography, and there was a strong correlation between the values determined by both methods ($Y = 0.976X - 0.207$, $r = 0.975$). As indicated by its specificity and sensitivity, this newly developed ELISA for afatinib is an important tool for TDM and studies of the pharmacokinetics of afatinib.

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1. Introduction

Afatinib is a second-generation epidermal growth factor-tyrosine kinase inhibitor (TKI) and an irreversible ErbB family blocker that has been approved for the treatment of epidermal growth factor receptor-mutated non-small cell lung cancer [1,2]. Treatment with this drug has considerable clinical advantages, but it is also associated with a high incidence of adverse events such as diarrhea (95.2% of cases), rash/acne (89.1%), stomatitis/mucositis (72.1%), and paronychia (56.8%), and thus management of these adverse events is vital [3,4]. Grade ≥ 3 diarrhea and rash are significantly more common with afatinib versus gefitinib or erlotinib [5], and serious adverse events interrupt treatment in many patients. Accordingly, an important issue is maximizing the efficacy of afatinib by minimizing its adverse events.

In recent years, increasing evidence has been gathered regarding the likely advantages of therapeutic drug monitoring (TDM) in cancer TKI therapy [6,7]. TDM can improve the efficacy of TKIs and decrease the risk of toxicity. For afatinib, dose adjustment is reported to reduce the incidence and severity of treatment-related adverse events without affecting efficacy [8]. In addition, afatinib trough plasma concentrations are increased with the severity of diarrhea and rash/acne [9]. Therefore, if its dose can be adjusted early by TDM, this may lead to a further reduction in the incidence and severity of adverse events.

Typically, the range for afatinib plasma concentration at clinical doses is from 5 to 70 ng/mL [9]. A method of quantification with ultra-high sensitivity is therefore needed for TDM and pharmacokinetic studies of afatinib. Existing modalities of analysis for afatinib include high-performance liquid chromatography (HPLC) [10] and liquid chromatography with tandem mass spectrometry [11,12]. However, these methods require expensive equipment and a high degree of technical expertise that might not be available in busy clinical chemistry departments. Thus, the development of a simpler method for the quantification of afatinib is needed. The enzyme-linked immunosorbent assay (ELISA) should be suitable

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for quantitative analyses of this drug given its high sensitivity and specificity with the added benefits of being simple, safe, and cost-effective, as well as allowing the processing of numerous samples. To date, however, no ELISA system for afatinib has been developed.

Previously, we established ELISAs for other TKIs, each exhibiting simplicity, sensitivity, and utility for TDM and pharmacokinetic studies of the drugs in question [13–17]. Therefore, in this study, we sought to develop an ELISA for use in TDM and pharmacokinetic studies of afatinib. We report here the successful development of a specific and sensitive competitive ELISA for afatinib, using a polyclonal antibody against part of the structure of afatinib. We measured plasma afatinib levels of 20 patients by using this ELISA and found the measured values equivalent to those obtained using HPLC measurement.

18.7 Å
Afatinib
(MW:485.95)
16.0 Å
CTQD
(MW:374.81)

Fig. 1. Chemical structures and molecular sizes of afatinib and CTQD.

2. Materials and methods

2.1. Chemicals and reagents

Afatinib was obtained from ChemScene LLC (Monmouth Junction, NJ, USA). (S)-*N*-4-(3-Chloro-4-fluorophenyl)-7-(tetrahydrofuran-3-yloxy)-quinazoline-4,6-diamine (CTQD) was obtained from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). *N*-4-(3-Chloro-4-fluorophenyl)-7-methoxyquinazoline-4,6-diamine (CMQD) was obtained from Ark Pharm, Inc. (Libertyville, IL, USA). Vandetanib was obtained from AdooQ BioScience LLC (Irvine, CA, USA). 2,4,6-Trinitrobenzene sulfonic acid was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Keyhole limpet hemocyanin (KLH) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Horseradish peroxidase (HRP) and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Boehringer Ingelheim Pharma GmbH (Ingelheim, Germany). The other reagents and solvents were all of the highest commercially available grade.

2.2. N-Succinylation of CTQD and CMQD

A solution of CTQD (0.1 mg, 26.6 μmol) or CMQD (0.1 mg, 31.3 μmol) and succinic anhydride (3.2 mg, 32 μmol) in pyridine (0.1 mL) was stirred overnight at 60 °C [14,15]. The resultant *N*-succinyl derivatives were used for cross-reactivity experiments with no additional purification. The yields of the individual *N*-succinyl derivatives were estimated at 100% based on HPLC measurements of the quantity of non-reacted drug.

2.3. Preparation of the immunogen for afatinib

The afatinib immunogen was prepared using part of the structure of afatinib (CTQD) as shown in Fig. 1. As in a previous study [14], a solution of CTQD (4 mg, 10.6 μmol) and succinic anhydride (1 mg, 10.1 μmol) in pyridine (0.5 mL) was stirred overnight at 60 °C. The solvent was removed by evaporation, and the residue, carboxylic modified CTQD, was dissolved in 95% dioxane (0.5 mL). The dioxane solution was added to 1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride (EDC; 20.3 mg, 106 μmol) and *N*-hydroxysuccinimide (2.4 mg, 21 μmol), and the resulting solution was incubated for 2 h at room temperature. The reaction mixture containing succinimidyl CTQD was mixed immediately with KLH (5 mg) in 1 mL of 0.1 M phosphate buffer (pH 7.0) containing 3 M urea and again incubated at room temperature for 2 h. The mixture was subjected to chromatography on a 2.0 cm × 30 cm Sephadex G-50 column with 20 mM phosphate buffer (pH 7.0) containing 3 M urea. The purified conjugate was used as the ELISA immunogen. The primary amine was determined using the trinitrobenzene sulfonic acid method [18], and the extent of conjugation was determined using the formula $(A_{CTQD\text{-}KLH}/A_{KLH}) \times 100$, where $A_{CTQD\text{-}KLH}$ and A_{KLH} are the absorbances obtained from the reaction of 2,4,6-trinitrobenzene sulfonic acid with CTQD-KLH and KLH, respectively. The extent of conjugation was found to be 71.1% of the total amino group residues on KLH.

2.4. Preparation of the afatinib antibody

We used five 5-week-old female BALB/c mice (Kyudo Exp. Animals, Kumamoto, Japan), injected intraperitoneally with 0.1 mg CTQD-KLH conjugate emulsified in complete Freund's adjuvant [14,15]. In total, the mice received 3 injections of the conjugate (0.05 mg) alone at 2-week intervals. Seven days after the final injection, the mice were killed and serum was collected. The serum samples (5 mL) were centrifuged at 1048 *g* at 4 °C for 10 min and then heated at 55 °C for 30 min [14,15]. IgG fractions were purified using a HiTrap Protein G column (GE Healthcare, Stockholm, Sweden) with 20 mM sodium phosphate (pH 7.0) as the binding buffer and 0.1 M glycine HCl (pH 2.7) as the elution buffer, according to the manufacturer's protocol [13]. The fraction that was eluted through the column was lyophilized and used as the anti-afatinib antibody for ELISA [13].

The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation at Sojo University, Japan.

2.5. Preparation of the afatinib-HRP conjugate

Afatinib was bound to HRP for labeling, basically using the same method used for preparing the afatinib immunogen. As in a previous study [14], solution of CTQD (4 mg, 10.6 μmol) and succinic anhydride (1 mg, 10.1 μmol) in pyridine (0.5 mL) was stirred overnight at 60 °C. The solvent was removed by evaporation, and the residue, carboxylic modified CTQD, was dissolved in 95% dioxane (0.5 mL). The dioxane solution was added to EDC (20.3 mg, 106 μmol) and *N*-hydroxysuccinimide (2.4 mg, 21 μmol), and the resulting solution was allowed to stand at room temperature for 2 h. An aliquot (50 μL) of the reaction mixture containing succinimidyl CTQD was added directly to HRP (0.5 mg, 12.5 nmol) in 1 mL of 0.1 M phosphate buffer (pH 7.0) containing 3 M urea, followed by incubation for 1 h at room temperature. The mixture was subjected to chromatography on a 2.0 cm × 30 cm Sephadex G-50 column using phosphate-buffered saline (PBS) containing 0.1% BSA to remove any remaining small molecules. Fractions (4 mL) were collected, and fractions 8 and 9, consistent with the main peaks indicative of enzyme activity, were used as a label for ELISA.

2.6. ELISA procedure

The underlying principle of ELISA is enzyme-labeled and unlabeled drugs competing for a purified immobilized antibody, followed by measuring marker enzyme activity of the resulting immunocomplex bound to the solid phase. Briefly, following previously described methods [13], microtiter plate wells (Nunc F Immuno plates I; Nunc, Roskilde, Denmark) were coated by loading 100 μL anti-afatinib antibody (4 μg/mL) in 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM NaN_3, and allowed to stand for 1 h at 37 °C. The plates were washed twice with PBS containing 0.1% BSA, and then incubated with 100 μL of 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM NaN_3 with 1% skim milk for 30 min at 37 °C to prevent nonspecific adsorption. The anti-afatinib antibody-coated wells were filled with 50 μL of either afatinib-treated samples or PBS containing 0.1% BSA as a control, and followed immediately by the addition of 50 μL of the pooled afatinib-HRP conjugate (diluted 1:50 (v/v) in PBS containing 0.1% BSA for afatinib). The wells were then incubated for 3 h at 37 °C and again washed carefully with PBS containing 0.1% BSA.

The activity of the enzyme conjugate that was bound to each well was measured by adding 100 μL of 0.42 mM TMB in 0.05 M acetate-citric acid buffer (pH 5.5) containing 3% dimethyl sulfoxide and 0.01% hydrogen peroxide, and then incubating the wells at 37 °C for an appropriate duration [13]. Next, 100 μL of 2.0 M H_2SO_4 was added to each well to stop the reaction, and the ensuing color intensity was measured using a spectrophotometer at 450 nm with an ELISA analyzer (ImmunoMini NJ-2300; Nalge Nunc Int. Co., Ltd., Tokyo, Japan). Concentrations were then determined from the standard curve using semi-logarithmic graph paper.

2.7. Plasma samples

Plasma samples were collected in sterilized vacuum tubes from 20 patients for plasma separation. Samples were subjected to centrifugation (2610 × g at 4 °C for 20 min), and plasma was collected and stored at −80 °C until use. The plasma was diluted with control plasma to obtain afatinib concentrations appropriate for their measurement by ELISA as described below. The prepared samples were immediately measured by the ELISA. This study was approved by Saga University of Medical Science Hospital Ethics Committee (2017-06-04) and the patients provided informed consent.

2.8. HPLC method

HPLC for afatinib in human plasma was performed consistent with the HPLC procedure for gefitinib [19].

Fig. 2. Scheme showing the preparation of the immunogen and enzyme conjugate.

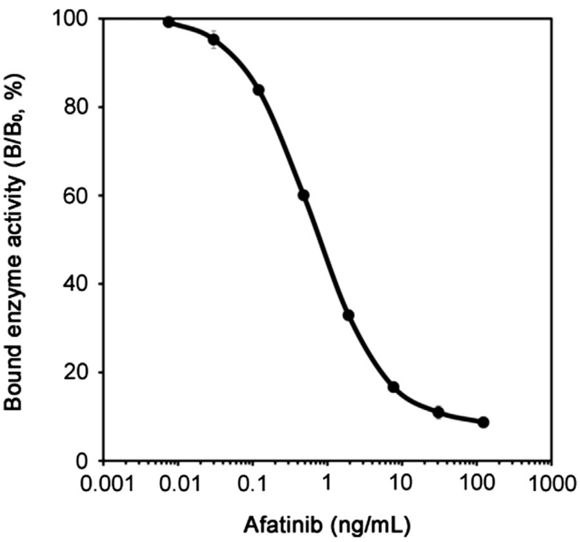

Fig. 3. Calibration curve of the developed ELISA for afatinib in human plasma. The curve shows the amount (%) of bound enzyme activity for various doses of afatinib (B) as a ratio of that bound using afatinib-HRP alone (B0). Each point represents the mean ± standard deviation ($n = 6$).

3. Results and discussion

3.1. Preparation of the immunogen and enzyme conjugate for afatinib

High-affinity antibodies become more difficult to obtain as the molecular weight and size of the hapten decrease [20,21]. Accordingly, hapten molecules of a certain size are needed to generate an antibody for high-sensitivity analysis in ELISA. We have previously produced specific antibodies for a large number of haptens [13–17]. On the basis of this experience, we consider that the hapten requires a molecular weight of approximately ≥ 250 and a molecular length of approximately ≥ 8.5 Å. Afatinib has a molecular weight of 485.95 and a molecular length of 18.7 Å, and it is thus considered to be a hapten of sufficient size. However, because afatinib does not have an appropriate reactive structure for producing such immunogens as the afatinib-KLH conjugate (Fig. 1), CTQD (molecular weight: 374.81, molecular length: 16.0 Å), which has the same substructure as afatinib, was used as the hapten. The CTQD carboxylate was coupled to KLH using the hydroxysuccinimide ester technique [22], and the resulting afatinib-KLH conjugate (afatinib immunogen) induced the formation of specific antibodies in each of the five mice immunized. An afatinib-HRP conjugate (as a tracer) was also prepared by the same procedure (Fig. 2). The conjugate remained stable for over 6 months in the elution buffer (pH 7.0) at 4 °C with no loss of enzyme or immunoreactive enzyme activity [13].

Table 1
Recoveries of afatinib from human plasma and precision of the developed ELISA for afatinib.

Assay	Added (ng/mL)	Estimated (ng/mL)	Recovery (%)	CV (%)
Intra-assay	0.03	0.0295 ± 0.0020	98.3	6.8
	0.16	0.1633 ± 0.0144	102.1	8.8
	0.8	0.7750 ± 0.0582	96.9	7.5
	4	3.9417 ± 0.2668	98.5	6.8
Inter-assay	0.03	0.0315 ± 0.0018	105.0	5.7
	0.16	0.1642 ± 0.0086	102.6	5.2
	0.8	0.7940 ± 0.0281	99.3	3.5
	4	4.0310 ± 0.1588	100.8	3.9

Values represent the mean ± SD ($n = 6$).

Table 2
Percent cross-reactivity of analogs measured by the developed ELISA.

Compound	Cross-reactivity (%)
Afatinib	100
CTQD	19.6
N-Succinyl CTQD	100
CMQD	4.7
N-Succinyl CMQD	22.8
Vandetanib	0.006

3.2. Validation of method

Calibration curve model, limit of detection, limit of quantification, precision (intra-assay and inter-assay), spike-in recovery and linearity under dilution of ELISA for afatinib were determined for human plasma. The parameter requirements applied in the following sections comply with the current bioanalytical guidelines of FDA [23].

Fig. 3 shows the calibration curve of afatinib as obtained in the human plasma system. The calibrator range was 7.5 pg/mL to 122.88 ng/mL afatinib. The best curve fitting was obtained by applying the 4-parameter logistics regression algorithm. The mean correlation coefficient from these 7 calibration curves was 0.99936 ± 0.00051. The lower limit of detection was determined to be 24.6 pg/mL by interpolation at 3 SD above the mean background signal. The lower limit of quantification was determined to be 30 pg/mL by interpolation at 10 SD above the mean background signal. Intra-assay precision in the low, mid, and high assay ranges (four levels, $n = 6$ each) resulted in CVs between 6.8% and 8.8% (Table 1). Inter-assay precision in the low, mid, and high assay ranges (four levels, over five independent runs) resulted in CVs between 3.5% and 5.7% (Table 1). Spike-in recovery in the low, mid, and high assay ranges (four levels) was between 96.9% and 105.0% (Table 1). Dilutional linearity of a spiked sample showed a recovery between 96.4% and 113.7% over the working range when diluted 5–100 fold, respectively. The developed ELISA fulfilled acceptance criteria for all addressed validation parameters [23].

The limit of quantification of afatinib was 0.03 ng/mL. Given that the plasma concentration range for the usual clinical dose of afatinib is 5–70 ng/mL [9], this ELISA seems to have adequate sensitivity to quantify afatinib in TDM and pharmacokinetic studies.

3.3. Specificity

Antibody cross-reactivity was determined by displacement of bound afatinib-HRP by comparable compounds [13]. The values for cross-reactivity were set as the ratio of each compound to afatinib at the concentration needed for a 50% inhibition of afatinib-HRP binding to the antibody. The anti-afatinib antibody showed 100% cross-reactivity with afatinib, 19.6% with CTQD as the hapten antigen, 100% with *N*-succinyl CTQD, 4.7% with CMQD, 22.8% with *N*-succinyl CMQD, and 0.006% with vandetanib (Table 2). The cross-reactivity of CTQD when used as the hapten antigen decreased to approximately one-fifth. When CTQD is conjugated with succinic acid, it has the same cross-reactivity as afatinib. CMQD, which does not possess the CTQD furan group, shows decreased cross-reactivity to approximately one-fourth of that of CTQD. It also has virtually no cross-reactivity with vandetanib, which has large structural differences from the chloro-4-fluorophenyl moiety of afatinib. Based on these results, we suggest that anti-afatinib antibody recognition extends at least as far as the succinic acid-conjugated amide bond and covers almost the entire structure of CTQD. In addition, based on the predicted target area of the anti-afatinib antibody, we estimate the length of the epitope to be approximately 13 Å (Fig. 4).

Afatinib undergoes almost no enzyme-mediated oxidative metabolism in vivo, and its main metabolite in plasma is the covalent protein adduct. The cross-reactivity of the afatinib-protein conjugate has not yet been confirmed. It is considered that the afatinib-protein conjugate exhibits a similar cross-reactivity to afatinib based on the specificity of the anti-afatinib antibody. However, their maximum concentrations in human plasma are relatively low [24]. Accordingly, these metabolites are thought to have a negligible influence on plasma afatinib quantification. These findings suggest that this novel ELISA could have adequate

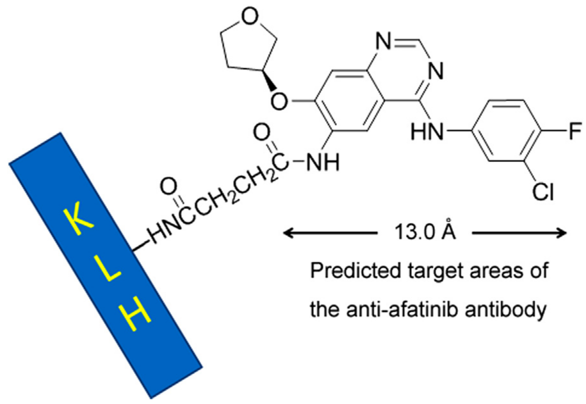

Fig. 4. The predicted target areas of the anti-afatinib antibody. The length of the epitope was approximately 13 Å.

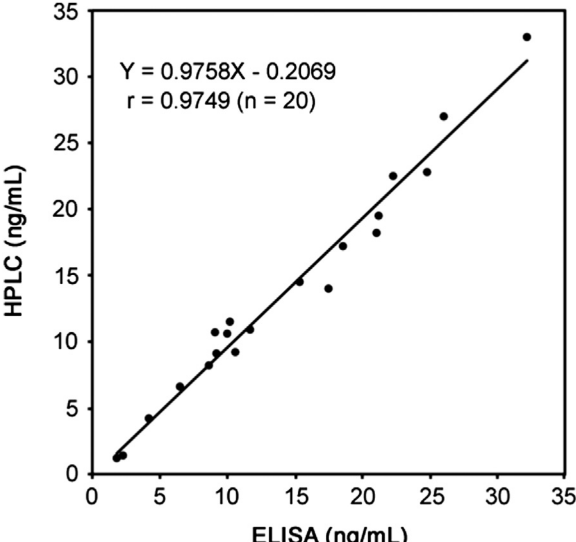

Fig. 5. Correlation between plasma afatinib concentrations of patients determined by the developed ELISA and HPLC.

specificity for quantifying afatinib to carry out TDM and pharmacokinetic studies in humans.

3.4. Correlation between patients' plasma afatinib concentrations determined by the developed ELISA and HPLC

The new ELISA quantification method was compared with an existing HPLC method for afatinib quantification. Plasma samples from 20 afatinib-treated patients were used to verify the suitability of the ELISA for TDM and pharmacokinetics studies of afatinib (Fig. 5). A good correlation was found between the values of both methods. The equation $Y = 0.976X - 0.207$ was derived, with Y as the concentration determined by HPLC analysis and X as the concentration determined by ELISA. A correlation coefficient of 0.975 was obtained ($n = 20$). These results strongly suggest that this ELISA has adequate sensitivity and specificity to quantify afatinib for TDM and pharmacokinetic studies in humans.

4. Conclusion

By using a hapten antigen that has the substructure of afatinib, we succeeded in producing the first specific antibody with strong affinity for afatinib. The generated antibody was used to develop a competitive ELISA, with a detection limit of 24.6 pg/mL, which demonstrated simplicity, sensitivity, specificity, and adaptability for high-throughput analyses. This ELISA promises to be an invaluable tool for routine pharmacokinetic applications in TDM and pharmacokinetic studies of afatinib. In addition, our approach should be helpful in the development of new immunoassays for low-molecular-weight drugs that do not have an appropriate reactive structure for generating immunogens.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

[1] J.C. Yang, J.Y. Shih, W.C. Su, et al., Afatinib for patients with lung adenocarcinoma and epidermal growth factor receptor mutations (LUX-Lung 2): a phase 2 trial, Lancet Oncol. 13 (2012) 539–548.
[2] J.C. Soria, E. Felip, M. Cobo, et al., Afatinib versus erlotinib as second-line treatment of patients with advanced squamous cell carcinoma of the lung (LUX-Lung 8): an open-label randomised controlled phase 3 trial, Lancet Oncol. 16 (2015) 897–907.
[3] L.V. Sequist, J.C. Yang, N. Yamamoto, et al., Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations, J. Clin. Oncol. 31 (2013) 3327–3334.
[4] R.T. Dungo, G.M. Keating, Afatinib: first global approval, Drugs 73 (2013) 1503–1515.
[5] M. Takeda, I. Okamoto, K. Nakagawa, Pooled safety analysis of EGFR-TKI treatment for EGFR mutation-positive non-small cell lung cancer, Lung Cancer 88 (2015) 74–79.
[6] P. Herviou, E. Thivat, D. Richard, et al., Therapeutic drug monitoring and tyrosine kinase inhibitors, Oncol. Lett. 12 (2016) 1223–1232.
[7] H. Yu, N. Steeghs, C.M. Nijenhuis, et al., Practical guidelines for therapeutic drug monitoring of anticancer tyrosine kinase inhibitors: focus on the pharmacokinetic targets, Clin. Pharmacokinet. 53 (2014) 305–325.
[8] J.C. Yang, L.V. Sequist, C. Zhou, et al., Effect of dose adjustment on the safety and efficacy of afatinib for EGFR mutation-positive lung adenocarcinoma: post hoc analyses of the randomized LUX-Lung 3 and 6 trials, Ann. Oncol. 27 (2016) 2103–2110.
[9] S. Wind, D. Schnell, T. Ebner, et al., Clinical pharmacokinetics and pharmacodynamics of afatinib, Clin. Pharmacokinet. 56 (2017) 235–250.
[10] S.X. Xiang, H.L. Wu, C. Kang, et al., Fast quantitative analysis of four tyrosine kinase inhibitors in different human plasma samples using three-way calibration-assisted liquid chromatography with diode array detection, J. Sep. Sci. 38 (2015) 2781–2788.
[11] R.W. Sparidans, H. Rosing, J.J.M. Rood, et al., Liquid chromatography-tandem mass spectrometric assay for therapeutic drug monitoring of the B-Raf inhibitor encorafenib, the EGFR inhibitors afatinib, erlotinib and gefitinib and the O-desmethyl metabolites of erlotinib and gefitinib in human plasma, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1033–1034 (2016) 390–398.
[12] A.A. Kadi, A.S. Abdelhameed, H.W. Darwish, et al., A highly efficient and sensitive LC-MS/MS method for the determination of afatinib in human plasma: application to a metabolic stability study, Biomed. Chromatogr. 30 (2016) 1248–1255.
[13] T. Saita, M. Shin, H. Fujito, Development of a specific and sensitive enzyme-linked immunosorbent assay for the quantification of imatinib, Biol. Pharm. Bull. 36 (2013) 1964–1968.
[14] T. Saita, Y. Yamamoto, M. Shin, et al., Preparation of antibodies and development of an enzyme-linked immunosorbent assay for the tyrosine kinase inhibitors lapatinib and nilotinib, Biol. Pharm. Bull. 38 (2015) 1652–1657.
[15] T. Saita, Y. Yamamoto, S. Noda, et al., Quantification of sorafenib in human serum by competitive enzyme-linked immunosorbent assay, Biol. Pharm. Bull. 38 (2015) 1788–1793.
[16] T. Saita, Y. Yamamoto, K. Hosoya, et al., An ultra-specific and sensitive sandwich ELISA for imatinib using two anti-imatinib antibodies, Anal. Chim. Acta 969 (2017) 72–78.
[17] Y. Yamamoto, T. Saita, Y. Yamamoto, et al., Quantitative determination of erlotinib in human serum using competitive enzyme-linked immunosorbent assay, J. Pharm. Anal. 8 (2018) 119–123.

[18] A.F. Habeeb, Determination of free amino groups in proteins by trinitrobenzenesulfonic acid, Anal. Biochem. 14 (1966) 328–336.
[19] M. Miura, K. Sato, H. Miura, et al., A limited sampling strategy for estimation of the area under the plasma concentration-time curve of gefitinib, Ther. Drug Monit. 36 (2014) 24–29.
[20] O.N. Chappey, P. Sandouk, J.M. Scherrmann, Monoclonal antibodies in hapten immunoassays, Pharm. Res. 9 (1992) 1375–1379.
[21] A. Morel, M. Darmon, M. Delaage, Recognition of imidazole and histamine derivatives by monoclonal antibodies, Mol. Immunol. 27 (1990) 995–1000.
[22] H. Hosoda, Y. Sakai, T. Nambara, A direct enzyme immunoassay of 6 beta-hydroxycortisol in human urine, Chem. Pharm. Bull. 29 (1981) 170–175.
[23] Guidance for industry: bioanalytical method validation, US Department of Health and Human Services, Food and Drug Administration, U.S. DHHS, F.D.A., CDER, Center for Drug Evaluation and Research and Center for Veterinary Medicine, 2018. Available at: 〈http://www/fda.gov/cder/guidance/index.htm〉.
[24] P. Stopfer, K. Marzin, H. Narjes, et al., Afatinib pharmacokinetics and metabolism after oral administration to healthy male volunteers, Cancer Chemother. Pharmacol. 69 (2012) 1051–1061.