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Multimodality approach towards individualized non-small cell lung cancer treatment

Schaake, E.E.

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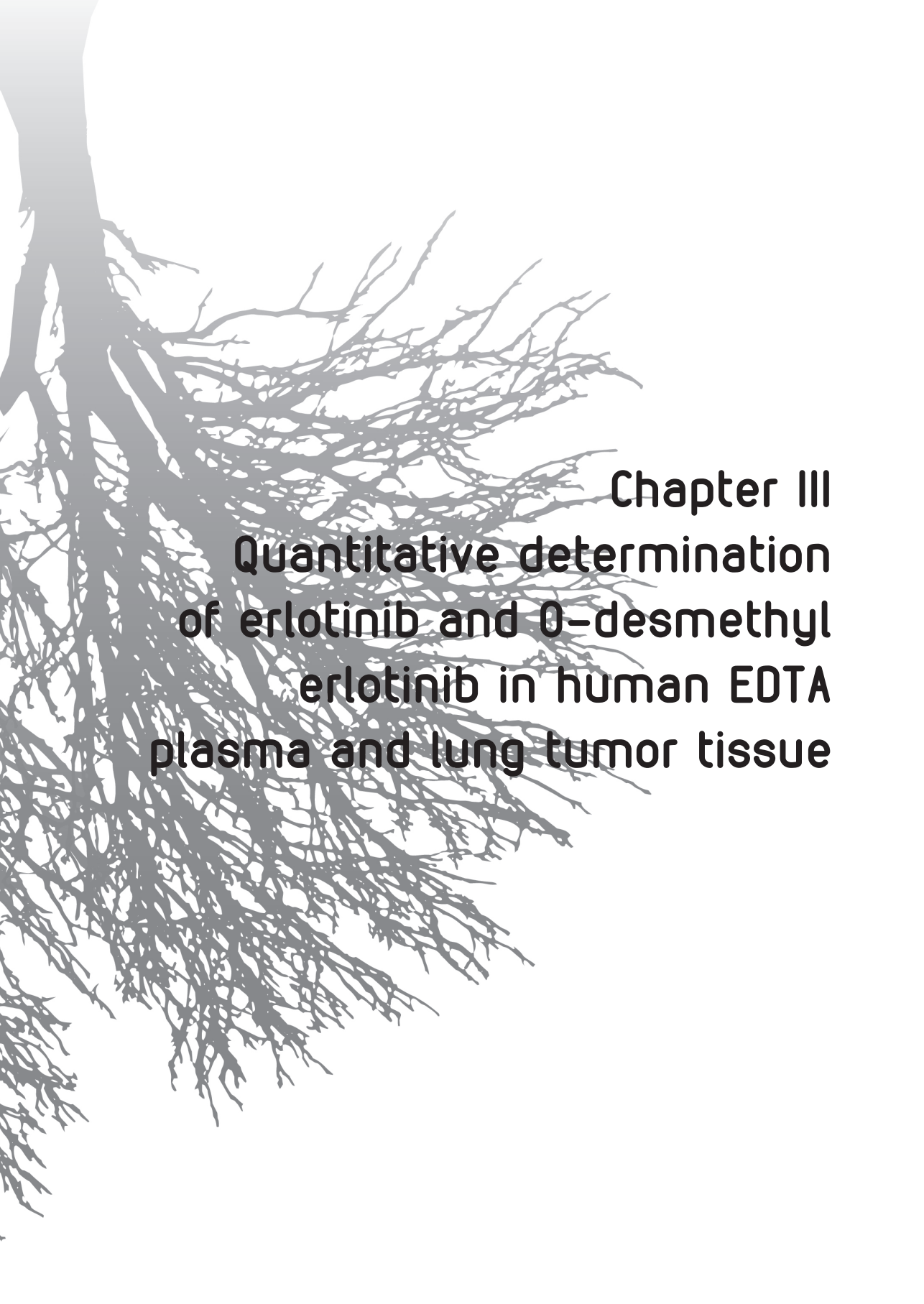
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N.A.G. Lankeet
E.E. Schaake
H. Rosing
J.A. Burgers
J.H.M. Schellens
J.H. Beijnen
A.D.R. Huitema

Bioanalysis, November 2012



Chapter III
Quantitative determination
of erlotinib and O-desmethyl
erlotinib in human EDTA
plasma and lung tumor tissue

ABSTRACT

Background | To increase knowledge about lung tumor tissue levels of erlotinib and its primary active metabolite and about erlotinib plasma levels in intercalated dosing schedules, a sensitive and accurate method for determination of erlotinib and O-desmethyl erlotinib (OSI-420) in human plasma and lung tumor tissue has been developed.

Results | A method with high-performance liquid chromatography and detection with tandem mass spectrometry (HPLC-MS/MS) was validated over a linear range from 5 to 2,500 ng/mL in plasma and from 5.0 to 500 ng/mL for lung tumor tissue homogenate (50- 5000 ng/g for lung tumor). Calibration curves in plasma were used to quantify analytes in lung tumor tissue homogenate. Lung tumor tissue of 15 patients has been collected and analyzed with the presented method.

Conclusion | This method has been successfully validated and applied to determine plasma and lung tumor tissue concentrations of erlotinib and O-desmethyl erlotinib in patients with non-small cell lung cancer.

INTRODUCTION

Erlotinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI). Erlotinib is approved for first-line treatment of non-small cell lung cancer (NSCLC) with mutated EGFR, second-line treatment of NSCLC and first-line treatment of advanced pancreatic adenocarcinoma.

Erlotinib drug exposure may be altered by pharmacokinetic drug-drug interactions leading to high inter-patient variability in plasma concentrations (1). It has been established that the magnitude of the pharmacological effect (tyrosine kinase inhibition) *in vitro* is concentration dependent. Moreover, in clinical studies trough plasma concentrations of erlotinib and its metabolite O-desmethyl erlotinib (OSI-420) seemed to correlate with treatment outcome (2,3). At the advised daily dose of erlotinib (150 mg/d) trough plasma concentrations are 1200 (SD 600) ng/mL (2). To provide an adequate level of tyrosine kinase inhibition minimal trough plasma concentrations of approximately 500 ng/mL are required, based on animal pharmacodynamic studies (2). Furthermore, an association has been observed between erlotinib plasma exposure and severity of skin toxicity (1). However, no clear cut-off values for efficacy and toxicity have been established in human, hitherto (1,2). Therefore, further pharmacokinetic investigations are needed to design individual treatment strategies.

Additionally, pharmacodynamic drug-drug interactions between chemotherapeutics and erlotinib can negatively influence treatment outcome (4). Since erlotinib interferes with the mechanism of action of chemotherapeutics, intermittent dosing schedules with a wash-out period of five days for erlotinib have been introduced for combination therapy regimens (4,5). In these intermittent schedules it is important that concentrations of erlotinib in plasma are subtherapeutic before the chemotherapeutic drugs are administered and, therefore, this has to be established by determination of erlotinib plasma concentrations. Moreover, since the pharmacodynamic target of erlotinib is located in tumor tissue, knowledge about concentrations of erlotinib in tumor tissue is even more informative (5).

To support the pharmacokinetic analysis in clinical trials, compound specific analytical methods are essential. Five bioanalytical assays for the determination of erlotinib in plasma have been reported so far (2,6,7,8,9). The determination of the active metabolite of erlotinib was only incorporated in three assays (7,8,9). None of the assays was validated to analyse erlotinib and O-desmethyl erlotinib in tissue samples. Additionally, these methods require a minimal sample volume of 100-250 μ L during sample pretreatment and have a lower limit of quantification (LLOQ) of 10-12.5 ng/mL (7,8,9). For these reasons, the reported methods are not suitable for analysis of our study samples, which include samples with restricted volumes (e.g. tumor tissue homogenates) and with low expected drug concentrations (e.g. tissue homogenates or plasma samples drawn after an erlotinib wash-out period). Therefore, we developed and validated a sensitive and specific HPLC-MS/MS method for quantification of erlotinib and O-desmethyl erlotinib in human EDTA plasma and in human lung tumor

tissue homogenates with an LLOQ of 5.0 ng/mL in plasma and 50 ng/g in tumor tissue, respectively, using 50 μ L of sample.

EXPERIMENTAL

Chemicals

Reference standards and internal standard (ISTD) were provided by the following manufacturers: erlotinib. HCl ($C_{22}H_{23}N_3O_4 \cdot HCl$) by Sequoia Research Products (Oxford, United Kingdom), O-desmethyl erlotinib ($C_{21}H_{21}N_3O_4$) by Toronto Research Chemicals (North York, Canada), stable isotopically labelled erlotinib- $^{13}C_6$ ($C_{16}^{13}C_6H_{23}N_3O_4 \cdot HCl$) by Alsa Chim (Illkirch, France). The chemical structures of erlotinib, O-desmethyl erlotinib and erlotinib- $^{13}C_6$ are depicted in Figure 1. HPLC-grade acetonitrile and methanol were purchased from Biosolve (Valkenswaard, The Netherlands). HPLC grade Lichrosolve water and formic acid 98-100% were purchased from Merck (Darmstadt, Germany).

Drug-free matrices

Drug-free human plasma with EDTA as anticoagulant was obtained from the Slotervaart Hospital (Amsterdam, The Netherlands) and stored at $-20^\circ C$ until use. Drug-free lung tumor tissue was obtained from patients that functioned as a untreated control group to a phase II trial in the Antoni van Leeuwenhoek Hospital (Amsterdam, The Netherlands) (10). Fresh lung tumor material of resected lung tumors of ten control patients was frozen at $-70^\circ C$ directly after surgery. Subsequently, of each lung tumor specimen 50 to 150 mg was weighted accurately. On first sight, the used parts of the tumors were not contaminated with blood. Therefore, no sample clean-up was performed before sample homogenization.

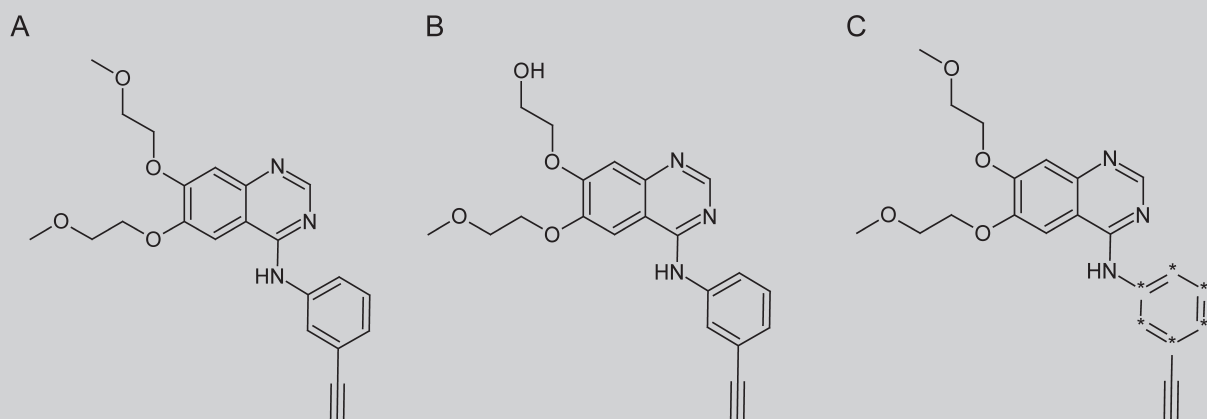


Figure 1. structural formulas of erlotinib (A), O-desmethyl erlotinib (B) and erlotinib- $^{13}C_6$ (C). (* represents ^{13}C)

An accurate volume of 500 to 1500 μL of drug-free human EDTA plasma was added to obtain samples containing 100 mg of lung tumor tissue per 1.0 mL of plasma. Lung tumor tissue homogenate was prepared by using a rotor/stator-type mechanical homogenizer until no tissue fibers were visible. Lung tumor tissue homogenate samples were stored at nominally -20°C until use.

Chromatographic and mass spectrometric conditions

An HPLC system (LC-20AD Prominence binary solvent delivery system) with a column oven, DGU-20A3 online degasser and a SIL-HTc controller (all: Shimadzu, Kyoto, Japan) and a cooled autosampler (4°C) were used. Chromatographic separation was carried out at 40°C on a reversed phase system with a Synergi Fusion-RP 80 column (150×2.0 mm ID, $4.0 \mu\text{m}$ particle size, Phenomenex, Torrance, CA, USA) protected with a Securityguard Synergi Fusion precolumn (4×2.0 mm ID, $4.0 \mu\text{m}$ particle size, Phenomenex). The injection volume was $10 \mu\text{L}$. A stepwise gradient was applied at a flow rate of $250 \mu\text{L}/\text{min}$. The mobile phase consisted of a mixture of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Before each new injection, the column was reconditioned for 4.7 minutes with 25% B (volume/volume) resulting in a total run time of 10 min. The chromatographic separation conditions are given in Table 1. The divert valve was directed to waste during the first 1.0 min and last 2.5 min to prevent the introduction of endogenous compounds into the mass spectrometer. A TSQ Quantum Ultra triple quadrupole mass spectrometer equipped with an electrospray ionisation source (ESI) operating in the positive ion mode (Thermo Scientific, Waltham, MA, USA) was used. For quantification, multiple reaction monitoring (MRM) chromatograms were acquired with LCquanTM software version 2.5 (Thermo Scientific). Positive ions were created at atmospheric pressure and the quadrupoles were operating in unit resolution (0.7 Da). Mass transitions from m/z 394 to 278 for erlotinib, m/z 380 to 278 for O-desmethyl erlotinib and m/z 400 to 284 for ISTD erlotinib $^{-13}\text{C}_6$ were optimised (see Figure 2). The ESI-MS/MS operating parameters used in this assay are listed in Table 2.

Preparation of calibration standards and validation samples

A set of stock solutions of erlotinib and O-desmethyl erlotinib were prepared from two independent weighings; one for the calibration standards and one for the validation samples. Approximately 2.2 mg of erlotinib.HCl was accurately weighted and dissolved in 1 mL of DMSO in a volumetric flask to give a $1.0 \text{ mg}/\text{mL}$ stock solution of the free base. Approximately 0.7 mg of O-desmethyl erlotinib.HCl was accurately weighted and dissolved in 1 mL of DMSO in a volumetric flask to give a $0.7 \text{ mg}/\text{mL}$ stock solution. Stock solutions of the ISTD erlotinib $^{-13}\text{C}_6$ were prepared in methanol at a concentration of approximately $500 \mu\text{g}/\text{mL}$. A $1,000 \text{ ng}/\text{mL}$ working solution of the ISTD was prepared by dilution of the stock solution in methanol.

For the preparation of the calibration standards, working solutions containing erlotinib and O-desmethyl erlotinib in the range from 100 to $50,000 \text{ ng}/\text{mL}$ were used. These working solutions were prepared by dilution of erlotinib and O-desmethyl

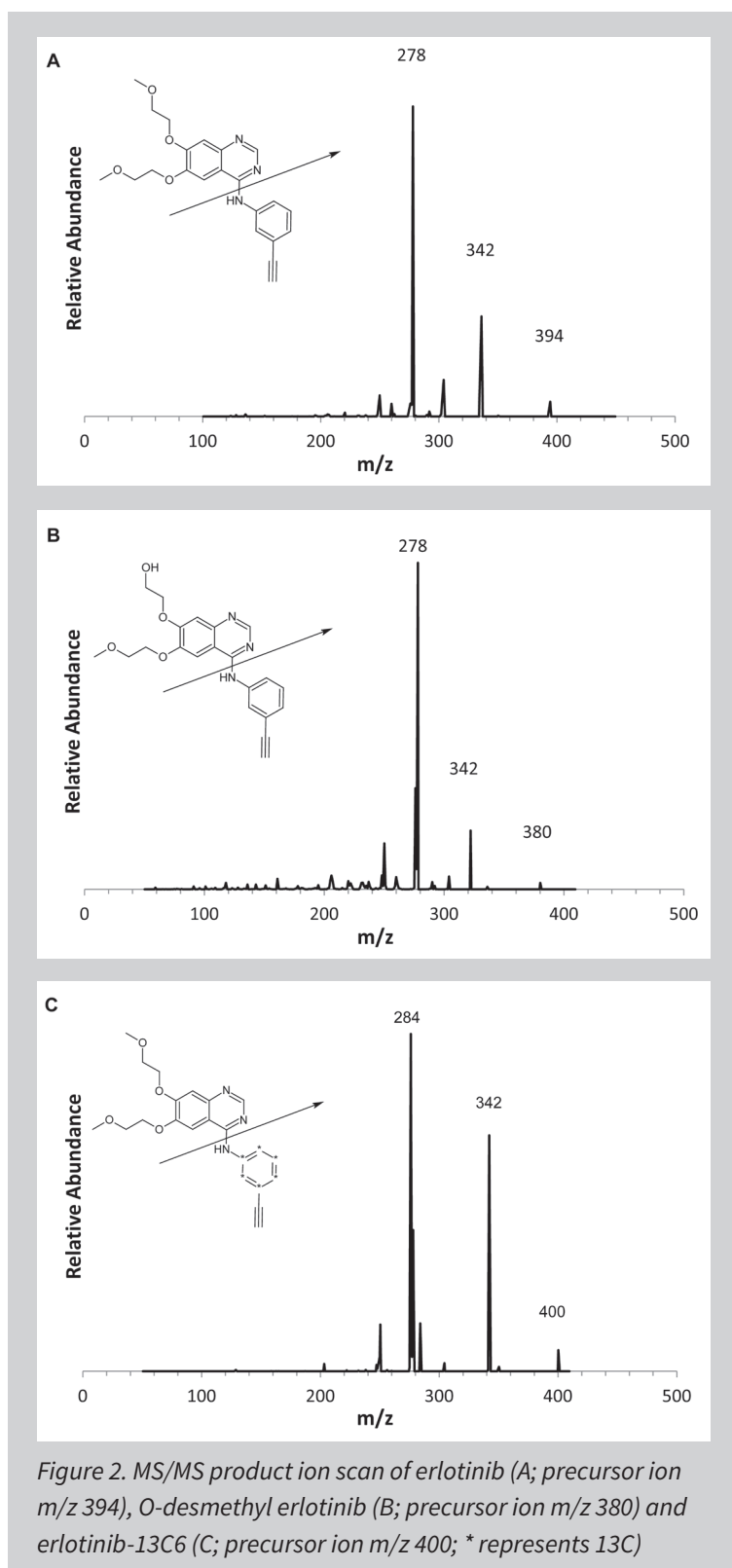


Figure 2. MS/MS product ion scan of erlotinib (A; precursor ion m/z 394), O-desmethyl erlotinib (B; precursor ion m/z 380) and erlotinib- $^{13}C_6$ (C; precursor ion m/z 400; * represents ^{13}C)

erlotinib stock solutions in methanol. A volume of 50 μL of each working solution was added to 950 μL of drug-free human EDTA plasma to obtain calibration standards in the range from 5 to 2,500 ng/mL.

Five working solutions in the range from 100 to 40,000 ng/ml were prepared by dilution of independently prepared erlotinib and O-desmethyl erlotinib stock solutions in methanol. To obtain validation samples of 5, 10, 400 and 2,000 ng/mL in plasma, 50 μL of each working solution was added to 950 μL of drug-free human EDTA plasma. To obtain validation samples of 5, 10, 80, 400 ng/mL lung tumor tissue homogenate in plasma, 50 μL of each working solution was added to 950 μL of drug-free human tumor tissue homogenate. The stock and working solutions in methanol were stored at nominally -20 oC until use.

During the first two method validation runs validation samples and calibration samples were prepared freshly since the stability of the analytes in the different matrices was not established at that moment. However, the validation samples were prepared independently of the calibration samples by using different stock solutions and working solutions. When short term analyte stability was established, freshly prepared calibration samples and aliquoted validation samples were used for the final validation run.

To establish the accuracy and precision of the method after dilution of samples containing analyte concentrations above the upper limit of quantification (ULOQ), a plasma sample at 5,000 ng/mL erlotinib and O-desmethyl erlotinib was spiked. Before processing, this sample was diluted ten times in drug-free human EDTA plasma.

Sample pre-treatment

Protein precipitation (PP) was used as sample pre-treatment for plasma and lung tumor tissue homogenate samples. 20 μL of ISTD working solution (1,000 ng/mL) was added to 50 μL of plasma and tissue homogenate sample. Subsequently 150 μL of acetonitrile (-20 °C) was added. After vortex mixing for 15 s, samples were centrifuged at 15,000 x g for 15 min. A volume of 50 μL of the clear supernatant was diluted with 50 μL of eluent A (0.1% formic acid in water) before injection of 10 μL onto the column.

Validation procedures

A full validation of the assay was performed according to the FDA guidelines for validation of bioanalytical assays including linearity, inaccuracy, precision, specificity, selectivity, cross-analyte/ISTD interference, recovery, ion suppression, carry-over and stability (11,12).

Application of assay in patient blood and tissue samples

The validated erlotinib and O-desmethyl erlotinib assay was used to measure trough plasma levels in patients on a sequential dosing regimen in a Phase II trial conducted in multiple centers in the Netherlands. Patients were treated with erlotinib for 14 days followed by a wash-out period of 5 days. During the last day of the wash-out period

EDTA plasma samples were collected and then directly sent to the laboratory. Within 48 h after blood draw, plasma was stored at -20°C until analysis.

Additionally, the validated assay was used to measure erlotinib and O-desmethyl erlotinib levels in human lung tumor tissue homogenates of patients on a neoadjuvant continuous dosing regimen of erlotinib until three days before surgery in a phase II trial in the Antoni van Leeuwenhoek Hospital (Amsterdam, The Netherlands). Lung tumor tissue homogenates of patients were obtained using the same procedure as was used for preparation of drug-free lung tumor tissue homogenates (see Section 1.2). Both trials were approved by the local institutional review boards and informed consent was given according to the Declaration of Helsinki.

RESULTS AND DISCUSSION

Method development

The stable isotopically labeled analogue of erlotinib, erlotinib -13C6, was used as ISTD to normalize for variations in the response of erlotinib and O-desmethyl erlotinib. The physical and chemical similarities between erlotinib -13C6, erlotinib and O-desmethyl erlotinib, makes this ISTD very suitable to compensate for variations in the response of the analytes introduced by sample preparation, injection, and matrix effects. However, during method development erlotinib and the co-eluting stable isotopically labeled ISTD appeared to suppress each other's responses due to competition in the electrospray ionisation (ESI) process of the MS source (13,14). This resulted in non-linear calibration curves and high inaccuracies of the validation samples when using an ISTD solution of 2,000 ng/mL, mainly due to a fluctuation of the ISTD response. Since the extent of suppression was supposed to be concentration dependent, the concentrations of ISTD solutions were varied (20, 200, 500, 1,000, 2,000, 20,000 ng/mL). It was expected that an exceptionally high ISTD concentration would lead to saturation of the electrospray even in presence of low concentrations of the analyte and would diminish the effect of high analyte concentrations on the ISTD response. However, fluctuation of the ISTD responses was observed at all ISTD concentrations (see Figure 3). In the first part of the calibration range (5,0 – 250 ng/mL) the response of the ISTD signal seemed to be enhanced with increasing analyte concentrations. In contrast, in the second part of the calibration range (250- 2,500 ng/mL) the ISTD signal seemed to be suppressed with increasing analyte concentrations. It was considered that this double-directed effect could be due to cross-interference of analyte into ISTD trace, followed by suppression of ISTD at higher levels. However, this mechanism was not plausible, since cross-analyte interference and selectivity were assessed and the interference of an ULOQ concentration of erlotinib into the ISTD trace was only 0.07% of the LLOQ level of the ISTD. Remarkably, no correlation between ISTD concentration and degree of response fluctuation was observed. Sequentially, suitability of a HPLC-APCI-MS/MS system in the quantification of erlotinib was investigated, since the ionisation process in atmospheric pressure chemical ionisation (APCI) is known to be less susceptible for ion saturation in

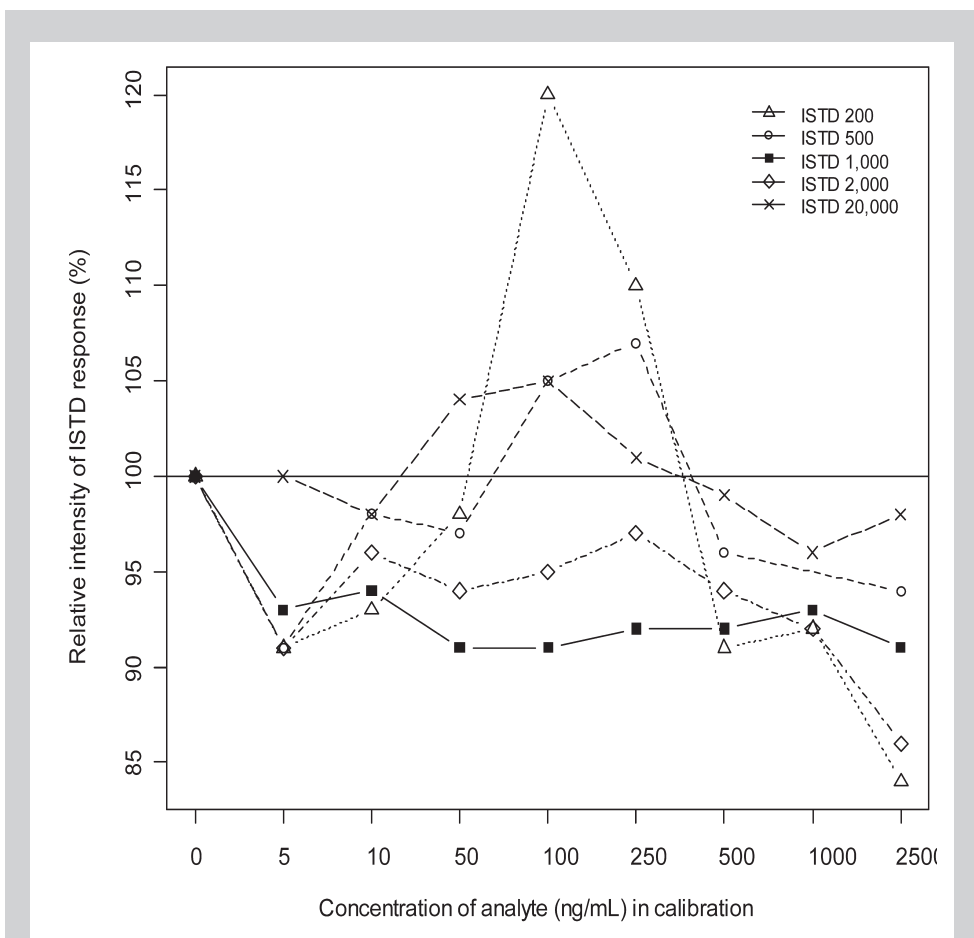


Figure 3. Representative, typical patterns of fluctuating ISTD response using different concentrations of ISTD erlotinib- $^{13}\text{C}_6$ (200, 500, 1,000, 2,000, 20,000 ng/mL) in calibration samples with increasing analyte concentrations (range 5.00 – 2,500 ng/mL) determined using LC-ESI-MS/MS. ISTD response in blank plasma sample processed with ISTD is defined as 100%. This figure shows results that are representative for two experiments performed on different days.

the source (13). The ISTD responses of a set of calibration samples using APCI were not influenced by concentration of analytes. However, the assay sensitivity was decreased leading to an inevitable and undesirable increase of the lower limit of quantification (LLOQ) to at least 10.0 ng/mL for both analytes. For this reason, it was decided to use the HPLC-ESI-MS/MS system and consequently to tighten the range to 5.0 – 2,500 ng/mL in order to avoid the tremendous effect of the highest calibration level (5,000 ng/mL) on the ISTD response. The ISTD concentration of 20,000 ng/mL showed the smallest deviations from the ISTD response of a plasma sample without analyte, as shown in Figure 3. However, this high ISTD concentration gave signal suppression of erlotinib in

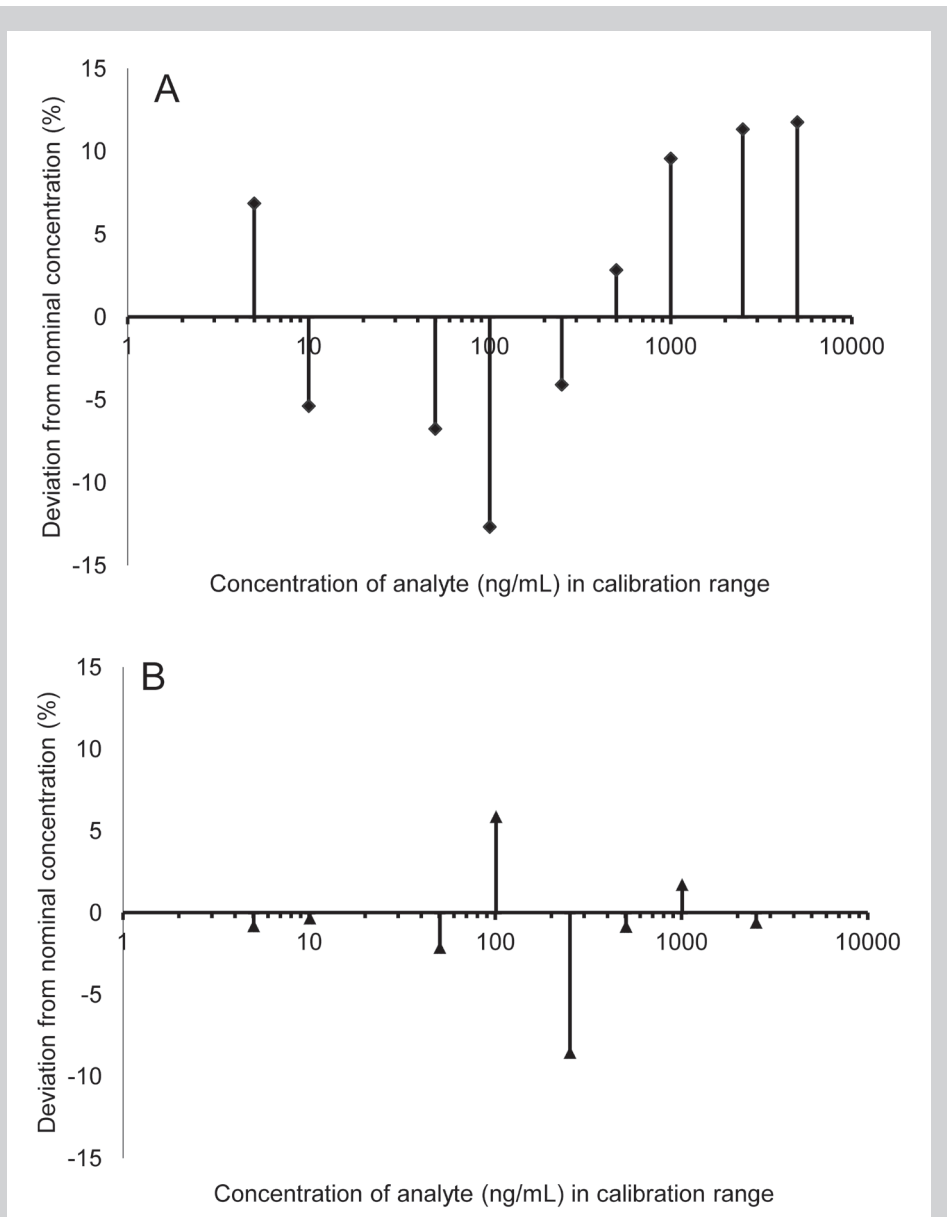


Figure 4. Deviations from nominal erlotinib concentrations based on area ratio responses in samples of a calibration range using different concentrations of ISTD erlotinib- $^{13}\text{C}_6$ ((A) 2,000 ng/mL ISTD; (B) 1,000 ng/mL ISTD). This figure shows results that are representative for two experiments performed on different days. Figure 4A, using 2,000 ng/mL ISTD, shows negative deviations from nominal concentrations in the first part of the calibration curve and positive deviations in the second part of the calibration curve, leading to non-linear calibration curves. Figure 4B, using 1,000 ng/mL ISTD, shows small deviations from nominal concentrations, which are evenly distributed across the calibration range with as a result good linearity of the calibration curve.

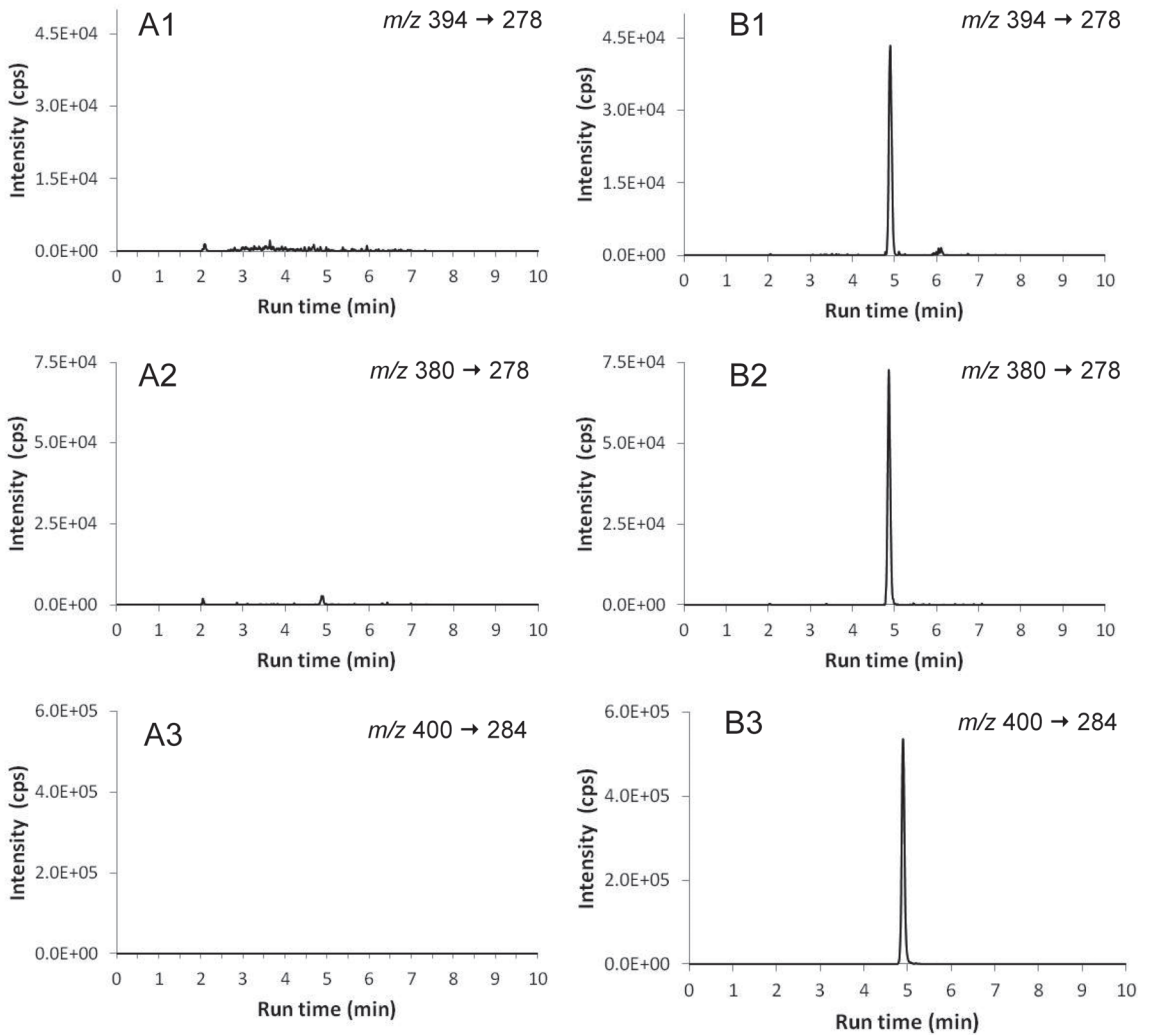


Figure 5. Representative LC-MS/MS chromatograms of a blank human plasma sample (A1, erlotinib; A2, O-desmethyl erlotinib; A3, ISTD erlotinib- $^{13}\text{C}_6$) and of a spiked human plasma sample at the LLOQ level of 5.0 ng/mL (B1, erlotinib; B2, O-desmethyl erlotinib; B3, ISTD erlotinib- $^{13}\text{C}_6$).

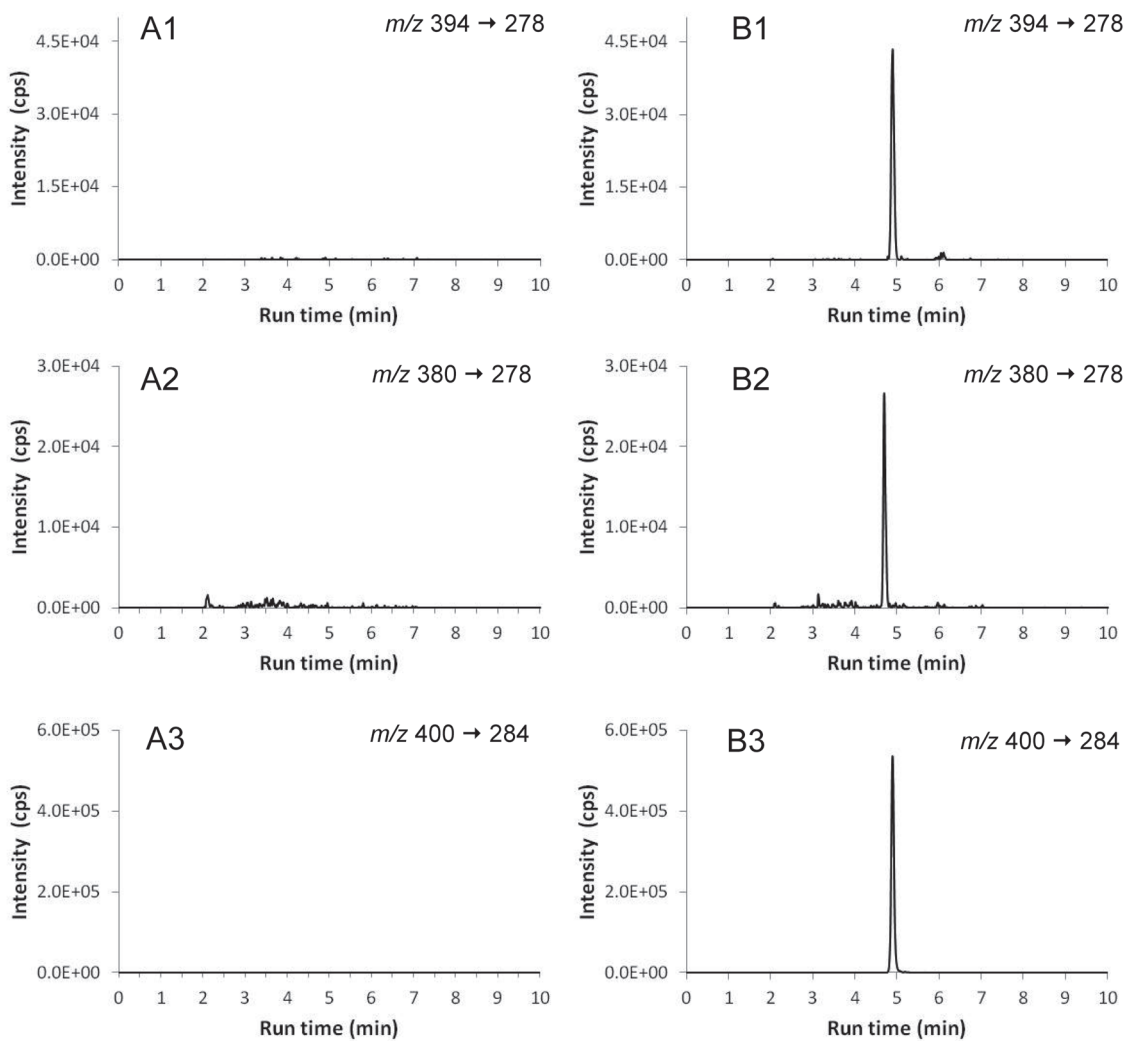


Figure 6. Representative LC-MS/MS chromatograms of a blank human lung tumor tissue homogenate sample (A1, erlotinib; A2, O-desmethyl erlotinib; A3, ISTD erlotinib- $^{13}\text{C}_6$) and of a spiked human lung tumor tissue homogenate sample at the LLOQ level of 5.0 ng/mL (B1, erlotinib; B2, O-desmethyl erlotinib; B3, ISTD erlotinib- $^{13}\text{C}_6$).

the samples, leading to decreased assay sensitivity. The ISTD concentration of 1,000 ng/mL showed the smallest fluctuation in ISTD response over the entire calibration range and was, therefore, used in the validation experiments. This ISTD concentration showed good linearity, accuracy and precision within the validated range, as shown in Figure 4.

Using a stable isotopically labeled ISTD for either a parent drug and for its metabolite risks the problem that the quantification of the metabolite is affected by ISTD ionization suppression in presence of high concentrations of the parent drug. However, it was assumed that this was not an issue in our method, since the effect of erlotinib on the ISTD (in a concentration of 1,000 ng/mL) was not concentration dependent, as shown in Figure 3. Moreover, calibrations curves of the metabolite in presence and absence of erlotinib could both be fitted linear (correlation coefficients 0.9917 and 0.9918, respectively) with inaccuracies <15% (data not shown).

Chromatographic and mass spectrometric conditions

Peaks with satisfying peak shapes were obtained when the stepwise gradient starting on 25% eluent B was followed by a block gradient with 55% eluent B at a flow rate of 0.25 mL per min (see Table 2). Typical chromatograms of LLOQ samples are depicted in Figure 5 and 6. At LLOQ level (5.0 ng/mL) a signal to noise ratio (S/N-ratio) of >10 was obtained. In neither the validation samples nor the patient plasma and tissue samples more than one peak was observed in the mass transition of O-desmethyl erlotinib. Thus, the isomeric forms of O-desmethyl erlotinib (OSI-420 and OSI-413), were not chromatographically separated.

During optimization of the mass spectrometric parameters, the Q1 spectrum of erlotinib and O-desmethyl erlotinib showed the singly charged molecular ion as most intense ion at m/z 394 and 380, respectively. For erlotinib-¹³C₆ the most intense peak in the Q1 spectrum also corresponded to the singly charged molecular ion at m/z 400. MS/MS experiments were carried out to determine the most abundant product ions for multiple reaction monitoring (MRM). MS/MS product ion scans and the proposed fragmentation pathways for the chosen transitions of erlotinib, O-desmethyl erlotinib and erlotinib-¹³C₆ are shown in Figure 2. The analytes and the ISTD could be detected with the electrospray source operating in the positive mode.

Validation experiments

Linearity. Eight non-zero plasma calibration samples were prepared and analysed in duplicate in three separate analytical runs. Calibration curves in plasma were also used to quantify tissue homogenate samples. The linear regression of the ratio of the areas of the analyte and the ISTD peaks versus the concentration were weighted with weighing factor $1/x^2$ (where x=concentration). The linearity was evaluated by means of back-calculated concentrations of the calibration standards. The assay was linear over the validated concentration range from 5.0 to 2,500 ng/mL of erlotinib and O-desmethyl erlotinib in human plasma and from 5.0 to 500 ng/mL for these analytes in lung tumor

tissue homogenates. Correlation coefficients (r^2) were at least 0.995. The deviation from the nominal concentrations should be within $\pm 20\%$ for the LLOQ and within $\pm 15\%$ for the other concentrations with coefficient of variation (CV) values less than 20% and 15% for both the LLOQ and the other concentrations respectively. At all concentration levels the inaccuracies were within -6.7 and 7.7% with CV values less than 10.1% for erlotinib and its metabolite in plasma. For tissue samples the levels the inaccuracies were within -6.0 and 7.4% with CV values less than 7.7%.

Inaccuracy and precision. The intra- and inter-assay performance data are presented in Table 3 and 4. Inaccuracy and precision of the assay were established by analysing validation samples with analyte concentrations at the LLOQ and in the low, mid and high concentration ranges of the calibration curves. Five determinations of each validation sample were measured in three separate analytical runs. The coefficient of variation (CV%) was used to report the intra- and inter-assay precision. The intra- and inter-assay inaccuracies should be within $\pm 20\%$ for the LLOQ and $\pm 15\%$ for all other concentrations. The precisions CV% should be less than 20% for the LLOQ and less than 15% for all other concentrations (12). The intra-assay inaccuracies (% bias) for erlotinib and O-desmethyl erlotinib in human EDTA plasma were within respectively $\pm 14.0\%$ for all concentration levels. The intra-assay precisions (CV%) for the analytes were less than 10.0% for all concentration levels. In conclusion, the validated range for erlotinib and O-desmethyl erlotinib based on 50 μL human EDTA plasma is from 5.0-2,500 ng/mL. Additionally, the validated range for erlotinib and O-desmethyl erlotinib based on 50 μL human tissue homogenate is from 5.0-500 ng/mL. The intra-assay inaccuracies (% bias) for erlotinib and O-desmethyl erlotinib in human tissue homogenates were within $\pm 12.5\%$ for all concentration levels. The intra-assay precisions (CV%) for the analytes in this matrix were less than 15.9% for the LLOQ level and less than 13.2% for the other concentration levels. Samples with analyte concentrations above the ULOQ (2,500 ng/mL) were diluted 1:10 (v/v) in drug-free human EDTA. These samples were processed in 5-fold and measured in one analytical run to assess the accuracy and precision. The intra-assay inaccuracy for diluted samples was -5.0 and -7.4% and the intra-assay precision was 2.1 and 2.5% for erlotinib and O-desmethyl erlotinib, respectively. When concentrations above 2,500 ng/mL are expected, samples can be diluted 10 times with drug-free human EDTA plasma. Inaccuracies and precisions fulfilled the requirements (11).

Specificity and selectivity. To investigate whether endogenous compounds from plasma could interfere with the detection of the analyte or the ISTD, six different batches of drug-free human EDTA plasma and five different batches of human tissue homogenate were processed as double blanks (containing neither analyte nor ISTD) and LLOQ samples. Samples were analysed according to the described procedures. Areas of peaks co-eluting with the analytes should not exceed 20% of the area at the LLOQ level. In MRM chromatograms of six batches of drug-free EDTA plasma no interference of endogenous compounds from plasma could be detected with the analyte or the ISTD. No co-eluting peaks $>20\%$ of the erlotinib and O-desmethyl erlotinib peak area at the LLOQ level were found and also no co-eluting peaks $>5\%$

of the ISTD were detected. In MRM chromatograms of five batches of drug-free tissue homogenates no interference of endogenous compounds from plasma could be detected with the analyte or the ISTD. No co-eluting peaks >20% of the erlotinib and O-desmethyl erlotinib peak area at the LLOQ level were found and also no co-eluting peaks >5% of ISTD were detected. The deviation of the nominal concentration for the LLOQ samples should be within $\pm 20\%$ and were between -2.1 and 7.6% for erlotinib and -13.0 and 2.6% for O-desmethyl erlotinib in plasma samples. In lung tumor tissue homogenates the deviations of the nominal concentration at the LLOQ level were between -17.0 and 14.2% for erlotinib and -27.2 and 17.4% for O-desmethyl erlotinib. For the metabolite only one out of five LLOQ samples in lung tumor tissue homogenate showed deviation >20%. Therefore, it can be concluded that the method is selective and specific and that endogenous compounds do not interfere with the assay.

Cross analyte interference. To investigate possible cross interference between erlotinib, O-desmethyl erlotinib and ISTD, a cross interference check was performed. Drug-free human EDTA plasma was spiked at ULOQ level and was processed without ISTD. Also drug-free plasma with only ISTD erlotinib- $^{13}\text{C}_6$ was processed. The response of any interfering peak with the same retention time as erlotinib or O-desmethyl erlotinib should be less than 20% of the response of a LLOQ sample. The response of any interfering peak with the same retention time as the ISTD should be less than 5% of the response of the ISTD. No cross-analyte/ISTD standard interference was detected and, therefore, cross-analyte/ISTD interferences of the assay fulfilled the requirements.

Recovery and matrix effect. Recovery and matrix effect were tested in EDTA plasma samples and not in lung tumor tissue homogenates, since these tumor tissue homogenates were scarce and, therefore, difficult to obtain in large quantities. Moreover, since selectivity assessments in different batches of lung tumor tissue homogenate established that endogenous compounds did not interfere with the assay's accuracy, it was assumed that the stable isotopically labeled internal standard corrected for potential matrix effects.

The protein precipitation (PP) recovery of erlotinib and its metabolite was determined at two concentrations (10.0 and 2,000 ng/ml) by comparing the analytical response of processed samples with those of processed blanks spiked with analyte (representing 100% recovery). The mean PP recovery was 71.0% (CV 4.3%) and 73.0% (CV 4.8%) for erlotinib and O-desmethyl erlotinib, respectively.

Ion suppression (matrix factor) was examined by comparing the analytical response of processed blanks spiked with analyte with those unprocessed samples in precipitation reagent. These experiments were performed in triplicate. The mean matrix factor detected for erlotinib and O-desmethyl erlotinib in plasma was 1.14 (CV% 9.5) and 1.14 (CV% 8.0), respectively.

Carry-over. Carry-over was tested by injecting two processed blank matrix samples sequentially after injecting an ULOQ sample. The response in the first blank matrix at the retention times of erlotinib, O-desmethyl erlotinib and erlotinib- $^{13}\text{C}_6$ should be less than 20% of the response of a LLOQ sample. Apparent carry-over was

observed after injection of spiked plasma samples (28.6% and 26.1% of the LLOQ for erlotinib and O-desmethyl erlotinib, respectively). To solve this carry-over problem a systematic approach, as described before, was used (15,16). The carry-over seemed to arise from contamination of the autosampler needle and the divert valve due to adsorption of the analyte after multiple injections. Contamination in the divert valve was diminished by performing multiple valve switches (>15) during the equilibration of the column before each analytical run. Contamination of the autosampler needle was diminished by using an acidic flush solvent (1% formic acid in ACN) instead of 100% methanol and increasing the rinse dip time from 5s to 30s. Carry-over was reduced to 16.8% and 14.5% of the LLOQ for erlotinib and O-desmethyl erlotinib, respectively, observed in a processed blank sample after injection of an ULOQ sample.

Stability. Stability data are summarized in Table 5. The stability of erlotinib and O-desmethyl erlotinib in spiked human EDTA plasma after three freeze/thaw cycles from nominally -20 °C to ambient temperatures and after 48 hours at ambient temperature was investigated in triplicate at two concentrations. Additionally, the stability of erlotinib and O-desmethyl erlotinib in spiked human EDTA plasma kept at -20°C for 1.5 months was investigated in triplicate. The analytes were considered to be stable in the matrix or final extract if 85–115% of the initial concentrations was recovered. Erlotinib and O-desmethyl erlotinib are stable in human plasma for at least three freeze (-20 °C) / thaw cycles. Short term stability of the analyte in plasma at ambient temperatures is established up to at least 48 h and at -20 °C up to at least 1.5 months.

The processed sample stability of erlotinib and O-desmethyl erlotinib was investigated at three concentrations (10.0, 200 and 2,000 ng/mL) after 7 days (2-8 °C). Both analytes were stable in the final extract at least 7 days at nominally 2-8 °C. Re-injection reproducibility was established and an analytical run can be re-injected after at least 7 days of storage in the autosampler at 4 °C.

Stability of stock solutions of erlotinib, O-desmethyl erlotinib and ISTD stored at ambient temperature for 6 h was established in triplicate. The analyte was considered to be stable in stock solutions if 90-110% of the initial concentration was recovered. Investigation of the long term stability of the analytes in stock solutions and plasma at -20°C is still ongoing.

Application of assay in patient blood and tissue samples

The validated assay was used to support translational research within two Phase II trials of erlotinib in patients with non-small cell lung cancer. Plasma samples and lung tumor tissue samples were collected and thereafter processed and analyzed by the methods described in this report. Subsequently, lung tumor tissue homogenates were quantified on plasma calibration curves.

The bioanalytical assay did not distinguish between the two isomer forms of the primary metabolite of erlotinib, OSI-420 and OSI-413. However, both isomers possess similar pharmacological activity compared to the parent compound (17). In

addition, it was assumed that both isomers possess similar ionization efficiencies. Therefore, quantification of the sum of both isomers is thus justified.

In the first trial, the assay was used to measure trough plasma levels after an erlotinib wash-out period of 5 days to ensure that erlotinib levels had reached subtherapeutic levels at that time point. The minimal effective therapeutic level of erlotinib, as deduced from IC₅₀ values after correction for plasma protein binding, is approximately 235 ng/mL (18,19). Therefore, the assay sensitivity and range (5.0 – 2,500 ng/mL) are sufficient to discriminate between samples underneath and above the therapeutic level of erlotinib.

In the second trial, as a proof of concept, lung tumor tissue homogenates of patients treated with erlotinib until three days before surgery were analysed. Erlotinib levels of approximately 5.0-30 ng/mL (50 - 300 ng/g tissue) and O-desmethyl erlotinib levels of approximately 7.0 ng/mL (70 ng/g tissue) were measured (10). Therefore, a range of 5.0 – 500 ng/mL in tissue homogenate appeared to be sufficient for analyses of tumor tissue samples of patients treated with erlotinib.

CONCLUSION

We have developed and validated a fast LC-MS/MS method for the quantitative analysis of erlotinib and O-desmethyl erlotinib in human plasma and lung tumor tissue samples. To our knowledge, this is the first LC-MS/MS method for analysis of erlotinib and O-desmethyl erlotinib in human lung tumor tissue. Human EDTA plasma and human lung tumor tissue homogenate samples with erlotinib and O-desmethyl erlotinib are pre-treated by protein precipitation with acetonitrile after addition of ISTD erlotinib-¹³C₆. Chromatography is performed under acidic conditions. A linear dynamic range from 5.0 to 2,500 ng/ml has been validated for plasma samples and a range from 5.0 to 500 ng/mL for lung tumor tissue homogenates (50 - 5000 ng/g for lung tumor). Calibration curves in plasma are used to quantify lung tumor tissue homogenate samples. Validation results show that the method is accurate and precise. Proof of concept experiments demonstrated the applicability of the method for quantification of the analytes in clinical samples.

Future perspective

Quantitative analysis of drugs in tissues is important to gain knowledge about drug uptake at the site of action, particularly, for chemotherapeutic drugs that have to be selectively destructive to malignant cells and tissues. In near future, tissue analysis may be increasingly used to support the determination of target levels for optimal therapeutic effects of targeted chemotherapeutic drugs. However, inconsistency between extraction recovery of the drug in calibration samples and patient samples remains one of the major challenges of tissue analysis. To minimize this potential bias, areas for future research should concentrate on new tissue preparation techniques with improved extraction efficiency and reproducibility.

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