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Quantification of afatinib, alectinib, crizotinib and osimertinib in human plasma by liquid chromatography/triple-quadrupole mass spectrometry; focusing on the stability of osimertinib

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ABSTRACT

The development and full validation of a sensitive and selective ultra-performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) method are described for the simultaneous analysis of afatinib, alectinib, crizotinib and osimertinib in human lithium heparinized plasma. Afatinib-d6, crizotinib-d5 and erlotinib-d6 were used as internal standards. Given osimertinib's instability in plasma and whole blood at ambient temperature, samples should be solely processed on ice (T = 0 °C). Chromatographic separation was obtained on an Acquity UPLC ® BEH C18; 2.1 × 50 mm, 1.7 µm column, which was eluted with 0.400 mL/minute flow on a linear gradient, consisting of 10 mM ammonium formate (pH 4.5) and acetonitrile. Calibration curves for all compounds were linear for concentration ranges of 1.00 to 100 ng/mL for afatinib and 10.0 to 1000 ng/mL for alectinib, crizotinib and osimertinib, herewith validating the lower limits of quantification at 1.00 ng/mL for afatinib and 10.0 ng/mL for alectinib, crizotinib and osimertinib. Within-run and between-run precision measurements fell within 10.2%, with accuracy ranging from 89.2 to 110%.

1. Introduction

Molecular pathology has become a diagnostic keystone of the treatment for non-small cell lung cancer (NSCLC). As a result of precise determination of driver mutations and better insight in tumor behavior, a more tailored therapy can be given to patients. In the last decade, this resulted in the development and registration of targeted therapies with multi-kinase inhibitors (MKIs). With the introduction of MKIs, promising outcome data have been achieved; both survival and quality of life improved significantly [1,2].

Epidermal growth factor receptor (EGFR) is with approximately 17% the most frequently determined NSCLC mutation, followed by anaplastic lymphoma kinase (ALK) with *circa* 7% [3]. Afatinib is an orally bioavailable MKI registered as first line treatment for locally advanced and metastatic NSCLC with EGFR mutations [4]. Disease progression under first line EGFR MKI therapy is in 49 to 66% caused by the EGFR exon 20 T790M point mutation [5–8]. For this escape mechanism, osimertinib was developed as second line MKI [9]. However, recently, promising data prompted registration of osimertinib to become first line treatment for EGFR mutated NSCLC [10]. Crizotinib is registered as first line treatment of ALK and ROS1 mutated locally advanced and metastatic NSCLC [11]. Alectinib is also an ALK inhibitor and is approved as first line treatment for ALK mutated NSCLC or as second line MKI after crizotinib [12].

Ultra-performance liquid chromatography tandem mass spectrometric (UPLC-MS/MS) assays to quantify these lung cancer MKIs (afatinib, alectinib, crizotinib and osimertinib) have already been developed separately using different methods [13–16]. However, a multi-assay for these agents has not been developed before. Strikingly, Rood et al. and Dickenson et al. reported contrasting data on osimertinib's stability in plasma [15,17]. Dickenson et al. reported data suggesting osimertinib to be highly unstable, whereas Rood et al. suggested that osimertinib remains stable for a few hours at room temperature. This discrepancy needs further clarification, since the outcome will have vast implications for handling of patient samples.

Pharmacokinetics of MKIs are increasingly being studied to investigate for example exposure-response or exposure-toxicity relationships, the impact of drug-drug interactions [18], or the contribution of MKI pharmacokinetics in the development of drug resistance. Subsequent implementation of Therapeutic Drug Monitoring could

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optimize individual treatment further by increasing efficacy and lower toxicity [19]. Therefore, it is very important to quantify the selected MKIs in plasma. Aim of this study was hence to develop a quantitative UPLC-MS/MS method for the simultaneous determination of afatinib, alectinib, crizotinib and osimertinib in human plasma.

2. Experimental

2.1. Chemicals

Afatinib ($C_{24}H_{25}ClFN_5O_3$), alectinib ($C_{30}H_{34}N_4O_2$) as hydrochloride (HCl) salt and osimertinib ($C_{28}H_{33}N_7O_2$) were purchased from LC Laboratories (Woburn, USA). Crizotinib ($C_{21}H_{22}Cl_2FN_5O$) was purchased from Sigma-Aldrich (Saint Louis, USA). All MKIs had purities of > 99%. Labeled internal standards afatinib-d6, crizotinib-d5 and erlotinib-d6 were acquired from Toronto Research Chemicals (Toronto, Canada). Different batches of human drug-free lithium heparinized plasma originated from Biological Specialty Corp. (Colmar, USA). Nitrogen (Nitrogen 3.0, purity 99.9%) was supplied by LindeGas (Schiedam, The Netherlands). Acetonitrile, pure water and methanol originated from Biosolve BV (Valkenswaard, The Netherlands). Dimethylsulfoxide (DMSO) was purchased from Sigma-Aldrich. Formic acid was provided by JT Baker BV (Deventer, The Netherlands). All chemicals were of analytical grade or higher.

2.2. Calibration

Three separate stock solutions, all within 5% of each other, of afatinib, alectinib, crizotinib and osimertinib were formulated with DMSO at concentrations of 1.00 mg/mL. The stock solutions were preserved at T < −70 °C. Independent stock solutions were used for the formation of calibration standards and preparation of quality control (QC) samples. An additional solution containing 10.0 µg/mL afatinib and 100 µg/mL alectinib, crizotinib and osimertinib was prepared to serve in the titration of calibration curve standards.

Internal standards afatinib-d6, crizotinib-d5 and erlotinib-d6 were dissolved in DMSO to obtain concentrations of 1 mg/mL and preserved at T < −20 °C. Internal standard solutions were subsequently 10,000-fold diluted with acetonitrile, obtaining a 100 ng/mL internal standard working solution which was stored at T < 8 °C and no longer used than three months.

For each run fresh calibration standards were prepared *in duplo* by addition of 10-µL aliquots of appropriate dilutions to 190-µL aliquots of human lithium heparinized plasma to obtain concentrations of 1.00, 2.00, 5.00, 10.0, 20.0, 50.0, 90.0 and 100 ng/mL for quantitation of afatinib and 10.0, 20.0, 50.0, 100, 200, 500, 900 and 1000 ng/mL for quantitation of alectinib, crizotinib and osimertinib.

Pools of QC samples for the calibration standard curve of 1.00–100 ng/mL were formulated in human plasma to obtain concentrations of 1.00, 3.00, 40.0, 80.0 and 800 ng/mL for the lower limit of quantification (LLQ) and QC's low, middle, high and diluted respectively. The QC sample concentrations for the 10.0–1000 ng/mL calibration standard curve were 10.0, 30.0, 400, 800 and 8000 ng/mL for LLQ and QC's low, middle, high and diluted respectively. Prior to processing, QC diluted was processed after a 20-fold dilution in blank plasma. Pools of QC samples were aliquoted and stored at T < −70 °C.

2.3. Sample pretreatment

Aliquots of 100 µL internal standard solution were added to 25 µL of plasma sample in polypropylene vials of 1.5 mL. Hereafter, all samples underwent vigorous vortex mixing for 5 s and were centrifuged (18,000 × *g*) for 10 min at room temperature. Subsequently, supernatants were pipetted into a 350 µL 96-well plate, that was placed within a self-cooled (T = 10 °C) autosampler, which injected quantities of 5 µL into the UPLC-MS/MS column.

2.4. Chromatography

The UPLC-MS/MS system originated from Waters Chromatography BV (Etten-Leur, The Netherlands) and included a Waters Acquity UPLC I-Class Binary Solvent Manager connected with a Waters XEVO TQ-S Micro Detector.

2.4.1. Data processing

MassLynx V4.1 SCN945 Software was utilized to collect, process and display data. QuanLynx (as part of MassLynx Software) was used for sample calculations and quantification.

2.4.2. Chromatographic settings

An Acquity UPLC® BEH C18 column 1.7 µm, 50 mm × 2.1 mm, (Waters Chromatography BV) was heated at T = 40 °C to separate analytes. A gradient with a flow rate of 0.400 mL per minute was constructed with mobile phase A (10 mM ammonium formate, having pH 4.5) and mobile phase B (pure acetonitrile acidified by formic acid 0.1%) followed by a 5 µL partial loop injection. For incipient conditioning, separation by a linear gradient was achieved, using 20% to 40% of mobile phase B from 0 to 1.0 min, then 40% to 90% of mobile phase B during 1.0 min and keeping this for 1.0 min before resetting to 20% mobile phase B from minute 3.0 to 5.0. The overall running time for each sample was 5 min. The needle washing solvent consisted of water/acetonitrile/2-propanol/methanol/formic acid (25:25:25:25:0.1, v/v/v/v/v). Column effluent went past the mass spectrometer for monitoring.

2.4.3. Tandem mass spectrometry

The cation electrospray ionization *modus* was used to execute tandem mass spectrometry (MS/MS). Mass transitions in *m/z* were optimized for afatinib, alectinib, crizotinib, osimertinib and the internal standards, by infusing the specific compounds in water/acetonitrile/formic acid (60:40:0.1, v/v/v) through mixed infusion. MS/MS parameters were optimized manually. The ionspray voltage was 3.00 kV. Source and evaporation temperatures were T = 150 °C and T = 650 °C respectively. Nitrogen gas was used for dissolution at 800 mL per hour. The multiple reaction monitoring (MRM) *modus* was used for quantification. Table 1 presents the optimal MS/MS settings for parent and daughter ions. Collision cell Pirani pressure retained ~5e-3 mbar (argon).

2.4.4. Quantification

Calibration curves were constructed by calculating the peak area ratio for every compound, respectively afatinib (486 > 112) to the internal standard afatinib-d6 (492 > 371), alectinib (483 > 396) to the internal standard erlotinib-d6 (400 > 278), crizotinib

Table 1
MS parameters of the analytes.

Analyte	Parent ion	Fragment ion	Cone voltage	Collision energy	Dwell time
	(*m/z*)	(*m/z*)	(V)	(eV)	(ms)
Afatinib	486.4	112.2	20	28	25
	486.4	371.4	20	27	25
Alectinib	483.3	396.4	26	22	100
	483.3	381.4	22	35	100
Crizotinib	450.3	260.4	25	23	10
	450.3	84.2	25	30	10
Osimertinib	500.5	72.2	45	25	100
Afatinib-d6	492.0	371.2	28	27	100
	492.0	118.2	28	26	25
Erlotinib-d6	400.2	339.2	20	24	100
	400.2	278.2	20	35	100
Crizotinib-d5	455.0	265.2	25	22	10
	455.0	89.1	25	35	10

(450 > 260) to the internal standard crizotinib-d5 (455 > 265) and osimertinib (500 > 72) to the internal standard erlotinib-d6 (400 > 278) against the pre-set concentration on a 1/concentration^2 graphic scale.

2.5. Validation

Full validation of this UPLC–MS/MS method was performed *conform* the 'Guidance for Industry, Bioanalytical Method Validation', defined by the Food and Drug Administration (www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf).

2.5.1. Specificity and selectivity

To specifically determine the LLQ, ten different lots of blank human plasma samples were spiked to obtain concentrations of 1.00 ng/mL for afatinib and 10.0 ng/mL for alectinib, crizotinib and osimertinib. All samples were analyzed simultaneously.

2.5.2. Accuracy and precision

The accuracy (ACC), within-run precision (WRP) and between-run precision (BRP) were calculated with quantification of five complementary LLQ and QC sample pools of all compounds. These were processed separately in three consecutive runs, while analyzing calibration curve standards *in duplo*. Calculation of ACC, WRP and BRP for LLQ and QC samples was executed through a unidirectional analysis of variance (*i.e.* one-way ANOVA), using the running cycle as variable factor as reported by Rosing et al. [20].

2.5.3. Recovery and matrix effect

Extraction recovery (RE) was assessed through correlating MS/MS responses of afatinib, alectinib, crizotinib and osimertinib at concentration levels of QC low, QC medium and QC high spiked *in triplo* into blank human lithium heparinized plasma before extraction, with MS/MS responses of the same compounds spiked in blank processed human plasma. Matrix effect calculations were conducted by relating the MS/MS responses of afatinib, alectinib, crizotinib and osimertinib at concentration levels of QC low, QC medium and QC high spiked in triplicate in six lots of blank lithium heparinized plasma, to the MS/MS responses of the same compounds spiked in acetonitrile/water/formic acid (40:60:0.1, v/v/v) as delineated by De Bruijn et al. [21].

2.5.4. Short-term, long-term and (autosampler) storage stability

The stability of afatinib, alectinib, crizotinib and osimertinib in human plasma was researched using sets of plasma at concentrations of QC low, high and diluted which were stored on ice, at ambient temperature and in a water bath (T = 37 °C) for 1, 2, 3, 5 and 24 h. Also three freeze–thaw cycles were tested, wherein the samples completely melted before refreezing for a minimum of 18 h. Subsequent long-term stability tests at T < −70 °C were executed, making use of the earlier specified QC samples. Autosampler stability, *i.e.* the stability of processed samples which are kept in the autosampler for a pre-defined time, was tested in triplicate with QC low, QC high and QC diluted. The QC-samples were repeatedly injected on different time points.

2.6. Clinical applicability

To prove additional applicability of this developed bioanalytical method in clinical practice, multiple blood samples were obtained from five patients who were included in a clinical study that studied afatinib which was orally taken for six weeks after a run-in phase (www.trialregister.nl; Netherlands Trial Register study 6652). During three 24 hour hospital admissions, a total of 36 blood samples were collected in 4 mL lithium heparin blood collection tubes. After centrifuging all samples with 2500 ×*g* at 4 °C for 10 min within 10 min of the blood withdrawal, the separated plasma was isolated and preserved at T < −70 °C until further processing. The study protocol had approval from the institutional review board and was in consonance with the declaration of Helsinki, *cq* patients provided informed consent prior to enrollment. Moreover, blood samples from three patients treated with alectinib, crizotinib and osimertinib were collected and processed similarly in order to measure long-term drug exposure. All samples were analyzed and used for incurred sample reanalysis (Section 2.7). Furthermore, a blood sample from a 60-year old male patient, who was treated with osimertinib for more than six months, was collected approximately four hours after oral intake and transported on ice (T = 0 °C), after which it was centrifuged and preserved similar to the afatinib study samples. Informed consent was obtained prior to collection. Besides demonstration of clinical applicability, this blood was used to perform the stability experiments which are described in Section 2.8.

2.7. Incurred sample reanalysis

To verify the reliability of study sample analyte concentrations, incurred sample reanalysis was performed, for which patient plasma samples from Section 2.6 were reanalyzed in two separate runs. A total of 6 patient samples were analyzed for each compound. The percentage difference of the results between the first measurement and the repeat measurement is determined with the following equation: (repeat measurement − first measurement) ∗ 100 / mean.

2.8. Stability of osimertinib

Additional experiments focusing on the stability of osimertinib were performed to further determine its stability throughout sample handling and processing. Moreover, it could lead to a potential solution for the possible instability. Pooled QC's (QC low, high and diluted) and blood from the patient treated with osimertinib as described in Section 2.6, were stored on ice, at ambient temperature and in a water bath at T = 37 °C, respectively. After 1, 2, 3, 5 and 24 h, QC samples were re-frozen at T < −70 °C. At the same pre-set time points, the blood samples were processed, prior to refreezing them at T < −70 °C. Firstly, the blood samples had to be centrifuged at 2500 ×*g* at ambient temperature for 5 min to obtain the plasmatic supernatant, which was subsequently transferred to a 1.5-mL Safe-lock vial. Hereafter, all pools of plasma samples were processed as specified in Section 2.3.

3. Results and discussion

3.1. Method development

The UPLC-MS/MS conditions to achieve optimal sensitivity and appropriate retention times, were obtained by adding ammonium formate to the water phase. Subsequently, to detect pure peaks, a gradient at a flow-rate of 0.400 mL/min was achieved with mobile phase A, composed of 10 mM ammonium formate (pH 4.5) and mobile phase B, composed of acetonitrile. Chemical structures, fragmentation patterns and ion spectra of the analytes are presented in Fig. 1. The product ions at *m/z* 112, 396, 260 and 72 were chosen to be MRM ions for quantification of afatinib, alectinib, crizotinib and osimertinib respectively and product ions at *m/z* 371, 265 and 278, for the stable isotope labeled internal standards afatinib-d6, crizotinib-d5 and erlotinib-d6 respectively. The primary to secondary ion ratio per analyte (except for osimertinib) showed the quality of each generated peak. For afatinib the ratio of 486.4 > 112.2 to 486.4 > 371.4 was used (ratio 0.9), for alectinib the ratio of 483.3 > 396.4 to 483.3 > 381.4 (ratio 4.0) and for crizotinib the ratio 450.3 > 260.4 to 450.3 > 84.2 (ratio 2.0) was used. For the internal standards afatinib-d6 the ratio 492.0 > 371.2 to 492.0 > 118.3 (ratio 1.2) was used, for erlotinib-d6 the ratio 400.2 > 339.2 to 400.2 > 278.2 (ratio 1.6) and for crizotinib-d5 the ratio 455.0 > 265.2 to 455.0 > 89.1 was used (ratio 1.7). Osimertinib

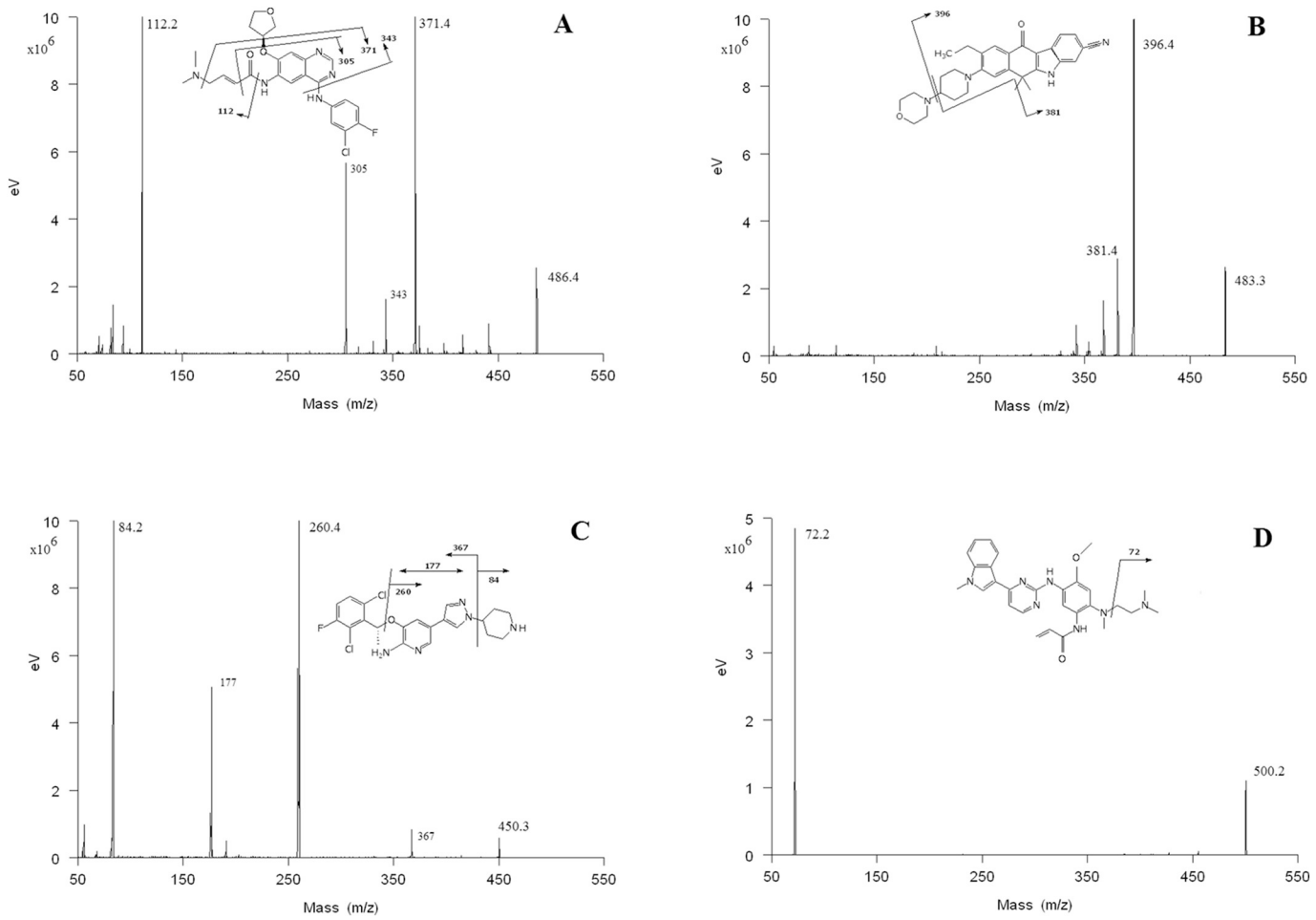

Fig. 1. Selected transitions, proposed fragmentations and ion spectra for afatinib (A), alectinib (B), crizotinib (C) and osimertinib (D).

as parent (500.4 *m/z* in Da) produced only one detectible daughter compound at 72.1 *m/z* (Da). Labeled afatinib-d6 (*m/z* 492 > 371) and crizotinib-d5 (*m/z* 455 > 265) proved to be suitable inter standards for afatinib and crizotinib. Erlotinib-d6 (*m/z* 400 > 278) occurred to be the most suitable internal standard for alectinib and osimertinib, where Heinig et al. used alectinib-d8 [13] and Rood et al. used pazopanib [15] respectively. This was concluded after multiple testing with various MKIs. By using a step gradient, all compounds were segregated from pre-eluting hydrophilic matrix components, which have the potential to suppress compound responses. In Fig. 2 representative chromatograms are presented, showing the relatively brief analysis time of 5 min with retention times of 1.2 min for crizotinib, 1.3 min for afatinib, 1.5 min for the internal standard erlotinib-d6 and 1.6 min for alectinib and osimertinib. A simple deproteinisation step with acetonitrile was sufficient to measure all compounds in plasma in the concentration ranges as earlier described.

3.2. Assay performance

3.2.1. Specificity and selectivity

Method results for all compounds were linear ($r^2 \geq 0.9965$) for the concentration ranges of 1.00 to 100 ng/mL for afatinib and 10.0 to 1000 ng/mL for alectinib, crizotinib and osimertinib in human plasma. There was no interference by the blank plasma samples for any MKI or internal standards. The LLQ's were validated by quantitation of five replicates of a pool of LLQ's in three separate runs at concentrations of 1.00 ng/mL for afatinib and 10.0 ng/mL for alectinib, crizotinib and osimertinib. For afatinib, 14 out 15 samples proved to fall within the defined ACC range of 80–120% with a mean quantitated concentration of 1.01 ± 0.100 ng/mL. For alectinib, 13 out of 15 samples were acceptable with 9.04 ± 0.979 ng/mL as average concentration. For crizotinib, 14 out of 15 samples proved to fall within the acceptable range with an 11.0 ± 1.04 ng/mL mean concentration. All 15 osimertinib samples were acceptable with 10.6 ± 0.932 ng/mL as average concentration. Additionally, blank human plasma from 10 unique subjects was spiked to obtain concentrations of 1.00 ng/mL for afatinib and 10.0 ng/mL for alectinib, crizotinib and osimertinib and analyzed during one run. After measurement of the spiked plasma samples, 8 out of 10 afatinib and alectinib samples proved to fall within the defined ACC range of 80–120% with mean quantitated concentrations of 0.870 ± 0.108 ng/mL and 8.64 ± 0.783 ng/mL respectively. For crizotinib, this was 9 out 10 and for osimertinib 10 out 10, with average measured concentrations of 10.1 ± 1.29 ng/mL and 10.1 ± 0.824 ng/mL respectively.

3.2.2. Accuracy and precision

The BRP, WRP and ACC at five predetermined concentrations (which included LLQ) in human lithium heparinized plasma are shown in Table 2 and proved to fall within the validation limits as specified by the FDA [22].

3.2.3. Recovery and matrix effect

Both RE and ME were calculated after analyzing six unique batches of lithium heparinized plasma spiked with all MKIs at concentrations of QC low, middle and high. No major ME (matrix factor close to 1.0) was observed while the recovery was ≥85% (see Table 3).

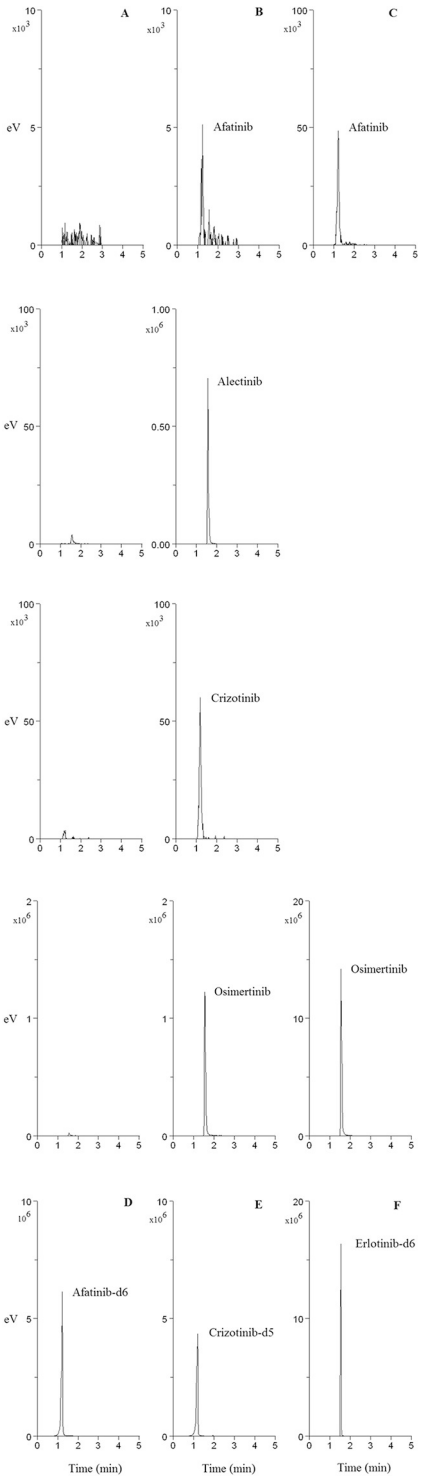

Fig. 2. Representative chromatograms of a double blank processed plasma sample (A), a plasma sample spiked at the concentration of the LLQ (B), a plasma sample collected after afatinib administration containing 11.7 ng/mL afatinib, a plasma sample collected after osimertinib administration containing 167 ng/mL osimertinib (C) and internal standards afatinib-d6 (D), crizotinib-d5 (E) and erlotinib-d6 (F).

3.2.4. Short-term, long-term and (autosampler) storage stability

Concerning stability, all compounds were stable in human plasma following three freeze-thaw cycles and after sample processing for a minimum of 11 h when samples were kept in the self-cooled autosampler at T = 10 °C (see Table 3). Table 4 shows afatinib, alectinib and crizotinib to be stable for at least 24 h on ice and at ambient temperature. Section 3.2.5 focusses especially on the stability of osimertinib. Alectinib and crizotinib were stable in a water bath (T = 37 °C) for at least 24 h, while afatinib concentrations decreased below 80% after 5 h. All compounds were stable in lithium heparinized plasma for minimally 9 months during storage at T < −70 °C (see Table 3).

Table 2
Calculations of the between-run and within-run precisions and the average accuracy of the LLQ and QC samples.[a]

Sample	Spiked (nM)	GM (nM)	ACC (%)	WRP (%)	BRP (%)	n[c]
Afatinib						
LLQ	1.00	1.01	101.0	9.40	3.62	14
Low	3.00	3.04	101.3	6.58	4.00	14
Middle	40.0	39.6	99.0	2.52	4.25	15
High	80.0	79.1	98.9	2.69	4.53	15
Diluted	800	791	98.9	3.44	3.76	15
Osimertinib						
LLQ	10.0	10.6	106.0	6.73	6.69	15
Low	30.0	29.3	97.7	6.94	#[b]	15
Middle	400	372	93.0	6.71	#[b]	15
High	800	806	100.8	4.41	7.22	15
Diluted	8000	7135	89.2	5.20	0.814	13
Crizotinib						
LLQ	10.0	11.0	110.0	8.56	4.66	14
Low	30.0	31.4	104.7	7.67	2.64	14
Middle	400	426	106.5	7.21	5.46	15
High	800	833	104.1	8.33	4.14	12
Diluted	8000	7881	98.5	7.33	#[b]	15
Alectinib						
LLQ	10.0	9.04	90.4	6.50	10.2	13
Low	30.0	31.1	103.7	2.24	3.06	15
Middle	400	384	96.0	5.55	6.02	14
High	800	797	99.6	9.14	7.17	14
Diluted	8000	7344	91.8	5.57	6.63	14

Abbreviations: GM, grand mean; WRP, within-run precision; BRP, between-run precision; ACC, average accuracy;

[a] n = 5 in 3 separate runs.

[b] No additional variation observed by performing the assay in different runs.

[c] Number of individual samples falling within acceptable range of accuracy of 85–115% (80–120% at LLQ).

3.2.5. Stability of osimertinib

In the paper about osimertinib by Rood et al. a full validation was conducted [15]. Focusing hereby on stability tests, only results at room temperature (T = 22 °C) were published, for an unknown length of time (4 or 6 h) though. Albeit this could be considered surmountable when other results underline osimertinib's short term stability in plasma, which the authors claim, other research by Dickinson et al. shows osimertinib to be highly unstable in human plasma after incubation at 37 °C for 6 h [17]. Given the vast discrepancy between the 88.6–92.6% found by Rood et al. and < 1% by Dickinson et al. [15,17], it is improbable to know how osimertinib samples should be handled before analysis. In order to provide a definite answer to this question, extended stability tests were conducted, in which sets of plasma samples (QC low, high and diluted) were stored on ice, at ambient temperature and in a water bath at T = 37 °C. After being exposed to these experimental conditions for 1, 2, 3, 5 and 24 h, all samples sets were analyzed. The results are shown in Table 4. Considering the FDA guidelines [22], all results had to fall in an 85–115% range to be titled as stable. At ambient temperature, QC low and high were stable for 3 h and QC diluted for only 2 h. After that, concentrations decreased further until < 20% after 24 h. Osimertinib is therefore an unstable compound in human plasma at ambient temperature. For all QC samples at T = 37 °C, concentrations decreased so fast that after 1 h none remained > 70% and all were not quantifiable after 24 h (Table 4).

Table 3
Extraction recovery, matrix effect and stability data.

Level	Extraction recovery	Matrix effect	Stability		
			3 F/T	Autosampler (11 h)	Long-term (9 months)
(ng/mL)	(%)	(%)	(%)	(%)	(%)
Afatinib					
3.00	95.3 ± 8.82	125 ± 1.84	116 ± 7.36	99.4 ± 8.72	93.8 ± 3.56
40.0	116 ± 2.66	125 ± 2.91	ND	ND	ND
80.0	100 ± 3.53	121 ± 4.73	99.8 ± 6.19	102 ± 3.05	101 ± 0.773
800.0	ND	ND	97.9 ± 3.03	107 ± 6.80	ND
Alectinib					
30	101 ± 2.45	101 ± 2.03	107 ± 0.908	96.7 ± 0.885	95.6 ± 0.669
400	87.2 ± 1.75	96.3 ± 3.29	ND	ND	ND
800	84.9 ± 0.99	97.9 ± 5.27	101 ± 1.88	103 ± 17.7	103 ± 0.677
8000	ND	ND	100 ± 1.36	97.7 ± 4.55	ND
Osimertinib					
30	89.7 ± 8.41	133 ± 3.15	86.4 ± 12.5	91.5 ± 9.05	108 ± 1.35
400	85.2 ± 1.91	107 ± 3.29	ND	ND	ND
800	85.5 ± 1.28	107 ± 4.60	102 ± 2.29	98.5 ± 3.68	92.9 ± 1.27
8000	ND	ND	97.5 ± 1.30	95.2 ± 2.77	ND
Crizotinib					
30	112 ± 8.11	105 ± 2.98	99.9 ± 5.62	96.2 ± 1.70	105 ± 1.75
400	104 ± 2.41	112 ± 3.06	ND	ND	ND
800	106 ± 4.71	110 ± 4.48	104 ± 7.67	101 ± 2.14	109 ± 2.32
8000	ND	ND	92.2 ± 1.67	98.6 ± 2.56	ND

Abbreviations: ND, not done; F/T, freeze-thaw cycles.

Since osimertinib is a covalently binding MKI, it has the potency to form irreversible Michael adducts with nucleophiles, *e.g.* plasma albumin, which could be an explanation for its instability in (human) plasma. This concept is clearly illustrated by Dickinsons et al. [17]: independent of concentration, only approximately 10% of osimertinib remained measurable in human serum albumin solution after incubation at T = 10 °C for 6 h. This chemical reaction can be overcome by thermic inactivation, reaching T = 0 °C by working on ice: Table 4 shows osimertinib concentrations do not decrease below 90%, thus being stable in plasma for at least 24 h. Moreover, stock solutions remained stable at ambient temperature for 24 h.

Extrapolating these findings to clinical and laboratory practice, stability of osimertinib in blood samples had to be tested additionally, since it will determine how these samples should be handled concerning thermic storage conditions after blood withdrawal. Blood samples were tested after same exposure conditions (on ice, at ambient temperature and in a water bath at T = 37 °C) and at same time points (1, 2, 3, 5 and 24 h) as plasma. The results are presented in Table 5. Osimertinib was unstable in plasma at ambient temperature, as well as in blood and at T = 37 °C. Comparing osimertinib's stability in plasma (Table 4) to whole blood (Table 5), osimertinib is almost 5 times more stable in whole blood at ambient temperature and at T = 37 °C. By centrifuging whole blood, *i.e.* separating plasma from erythrocytes, the plasma concentrations of both osimertinib and nucleophiles (*e.g.* albumin) rise. We hypothesize that this could accelerate the formation of the earlier mentioned Michael adducts and could therefore cause the

Table 4
Stability in plasma samples at different storage conditions.

Time (hrs)	Afatinib (%)			Alectinib (%)			Crizotinib (%)			Osimertinib (%)		
	Ice	Ambient	37°	Ice	Ambient	37°	Ice	Ambient	37°	Ice	Ambient	37°
QC-LOW												
0	100	100	100	100	100	100	100	100	100	100	100	100
1	92.8	115	91.4	103	104	104	99.3	102	95.6	95.6	95.7	54.2
2	99.1	118	90.3	100	102	102	105	94.8	105	92.7	81.3	NQ
3	96.8	108	82.8	98.6	103	99.3	111	102	99.2	90.3	81.2	NQ
5	102	120	82.2	101	107	101	101	109	99.2	93.9	71.7	NQ
24	98.0	95.0	48.3	96.1	109	110	99.5	96.9	106	91.0	12.9	NQ
QC-HIGH												
0	100	100	100	100	100	100	100	100	100	100	100	100
1	103	101	93.9	100	99.3	107	109	106	98.3	102	96.5	61.9
2	104	100	91.2	101	102	110	104	102	105	104	91.7	33.8
3	104	102	91.4	101	107	111	103	114	100	102	91.1	18.7
5	104	95.2	93.0	101	105	113	97.0	92.0	107	103	87.5	4.55
24	104	93.0	67.0	100	116	122	99.1	92.0	104	100	18.3	NQ
QC-DILUTED												
0	100	100	100	100	100	100	100	100	100	100	100	100
1	101	98.0	94.9	100	100	99.3	98.2	92.8	94.7	102	91.3	55.0
2	101	101	93.6	101	100	103	98.6	103	101	102	86.6	28.9
3	98.2	101	92.3	96.0	101	102	90.6	112	96.5	97.9	76.1	12.5
5	98.5	106	89.9	99.3	105	102	89.8	101	97.0	99.9	67.2	4.48
24	102	95.5	70.2	96.7	101	109	83.7	91.8	101	98.5	12.9	NQ

Abbreviation: NQ, not quantifiable.

Table 5
Stability in whole blood at different storage conditions.

Time (hrs)	Osimertinib (%)		
	Ice	Ambient	37°
0	100	100	100
1	101	101	98.8
2	104	100	74.7
3	103	100	64.1
5	105	97.6	46.9
24	108	66.5	18.4

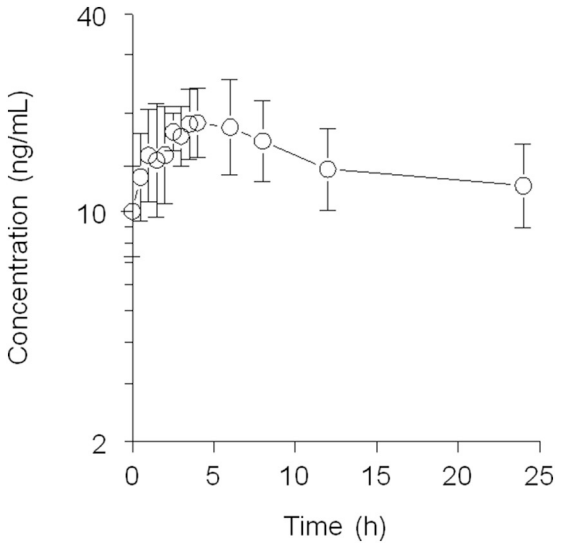

Fig. 3. Mean plasma concentration-time profile of afatinib after an oral dose of 40 mg (n = 4).

Table 6
Incurred sample reanalysis.

Compound	First analysis	Second analysis	Mean	% Difference
	Conc. (ng/mL)	Conc. (ng/mL)	Conc. (ng/mL)	(%)
Afatinib				
1	11.4	10.9	11.2	−4.5
2	19.3	16.8	18.1	−13.9
3	17.4	16.3	16.9	−6.5
4	21.4	19.8	20.6	−7.8
5	16.6	16.6	16.6	0.0
6	14.6	15.2	14.9	4.0
Alectinib				
1	1125	1075	1100	−4.5
2	1065	1016	1041	−4.7
3	1071	1024	1048	−4.5
4	836	832	834	−0.5
Crizotinib				
1	316	295	306	−6.9
2	550	627	589	13.1
3	386	392	389	1.5
4	490	468	479	−4.6
5	588	623	606	5.8
6	396	406	401	2.5
Osimertinib				
1	238	231	235	−3.0
2	153	157	155	2.6
3	130	124	127	−4.7
4	174	171	173	−1.7
5	182	167	175	−8.6
6	266	259	263	−2.7

% Difference: (first analysis − second analysis) / (mean of first and second analysis) ∗ 100.

faster decline of osimertinib concentration in plasma. Similar to plasma samples, storing blood samples on ice (at T = 0 °C) kept osimertinib stable for at least 24 h. Therefore, as strong advice for laboratory and clinical practice, samples should be processed solely on ice.

3.3. Clinical applicability

This validated analytical method was used in adequately quantifying the first samples from five patients enrolled in a pharmacokinetic study in which afatinib was orally administered for six weeks after a run-in phase. A representative chart with the mean plasma concentration-time profile of four patients treated with 40 mg afatinib is presented in Fig. 3. Moreover, the method was used to quantify concentrations of alectinib, crizotinib and osimertinib in patient plasma samples (shown in Table 6). All analytes fell within the measurable concentration range. Furthermore, this analytical method was successfully applied to the osimertinib patient sample that was used for stability experiments and from which results are shown in Table 5.

3.4. Incurred sample reanalysis

The results of the incurred sample reanalysis are presented in Table 6. All of the samples met the acceptance criteria with mean differences between the original and repeated analysis of afatinib, alectinib, crizotinib and osimertinib of −4.8%, −2.0%, 1.9% and −3.0% respectively.

3.5. Assay performance compared to existing methodologies

When comparing the performance results of this newly developed assay to existing methodologies [13–16], this assay has comparable results concerning all FDA validation standards [22]. Only Rood et al. [15] and Heinig et al. [13] reported incurred sample reanalysis. Moreover, our extraction recovery (85–100%) for alectinib was significantly higher compared to Heinig et al. (< 70%) [13]. Furthermore, this assay focused more specifically on short-term stability with different storage temperatures up to 24 h, where Rood et al. [15] and Sparidans et al. [16] only tested short-term stability for 4 or 6 and 8 h respectively. Hence, this is a more efficient assay to quantify the most important MKI's for the treatment of EGFR- or ALK-mutated NSCLC.

4. Conclusion

A sensitive and selective method was developed and validated for the simultaneous analysis of afatinib, alectinib, crizotinib and osimertinib in human lithium heparinized plasma. This method met all the requirements for bioanalytical method validation of the FDA and will be used in present and future (clinical) pharmacokinetic studies. Given osimertinib's instability at ambient temperature in plasma and blood, a strong recommendation for sample handling must be made: to minimize deprivation of osimertinib, both blood and plasma samples should be kept and processed solely on ice (at T = 0 °C). Otherwise, precise measurement of osimertinib concentrations is impossible to be used in Therapeutic Drug Monitoring.

Conflict of interest statement

None.

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