Development and validation of an HPLC-MS/MS method to simultaneously quantify alectinib, crizotinib, erlotinib, gefitinib and osimertinib in human plasma samples, using one assay run

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Development and validation of an HPLC–MS/MS method to simultaneously quantify alectinib, crizotinib, erlotinib, gefitinib and osimertinib in human plasma samples, using one assay run

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Abstract

A liquid chromatography–tandem mass spectrometry method was developed and validated to quantify alectinib, crizotinib, erlotinib and gefitinib. This assay can be combined with our method for osimertinib, allowing quantification of the most used *ALK*- and *EGFR*-tyrosine kinase inhibitors (TKIs) in non-small cell lung cancer with a single-assay setup. Chromatographic separation was performed on a HyPurity® C_{18} analytical column using an elution gradient of ammonium acetate in water and in methanol, both acidified with formic acid 0.1%. Detection and quantification were performed using a triple quad mass spectrometer with an electrospray ionization interface. This method led to robust results, as the selectivity, carryover, precision and accuracy met all pre-specified requirements. The assay was validated over a linear range of 100–2,000 ng/ml for alectinib and erlotinib and 50–1,000 ng/ml for crizotinib and gefitinib. Alectinib, crizotinib, erlotinib and gefitinib were all stable for at least 4 h in whole blood (at room temperature and at 4°C) and for at least 1 month in EDTA plasma when stored at −80°C, while osimertinib proved to be unstable at room temperature. Although high-performance liquid chromatography was used, the run time was short and comparable with other methods using ultra-high performance liquid chromatography.

KEYWORDS
LC–MS/MS analysis, non-small cell lung cancer, tyrosine kinase inhibitors, validation

1 | INTRODUCTION

The prognosis of patients with metastatic non-small cell lung cancer (NSCLC) has improved in recent years, mainly owing to the introduction of tyrosine kinase inhibitors (TKIs) for those with an oncogenic driver and because of the development of immune checkpoint inhibitors for most of the other NSCLC patients (Ferrara, Di Noia, et al., 2020; Ferrara, Imbimbo, et al., 2020).

The oncogenic drivers for which the most TKI options are available are the activating epidermal growth factor receptor (*EGFR*) mutations and the anaplastic lymphoma kinase (*ALK*) gene fusions. For patients with *EGFR* mutations, erlotinib, gefitinib (first-generation TKIs), afatinib, dacomitinib (second-generation TKIs) and osimertinib (third-generation TKI) are available in standard of care. For patients with an *ALK*-gene fusion, crizotinib (first-generation TKI), ceritinib, alectinib, brigatinib and lorlatinib (next-generation TKIs) are available. Those TKIs have proven to be effective in comparison with either chemotherapy or an earlier-generation TKI in clinical trials (Camidge et al., 2018; Maemondo et al., 2010; Mok et al., 2017; Peters et al., 2017; Sequist et al., 2013; Shaw et al., 2013, 2017, 2020; Soria et al., 2018; Wu et al., 2017; Zhou et al., 2011). For all these TKIs, a fixed dose is given, and for example

body weight-based dosing is not considered. As plasma levels can vary between patients, resulting in either undertreatment or toxicity, the interest in therapeutic drug monitoring (TDM) is growing (Verheijen et al., 2017). Many single-drug assays to quantify alectinib, crizotinib, erlotinib, gefitinib or osimertinib in human plasma have been published, but multidrug assays to quantify multiple TKIs simultaneously have been developed and validated less often (Hayashi et al., 2016; Reis et al., 2018; Veerman et al., 2019; Zhou et al., 2021). For research purposes and TDM, we developed and validated a simple and fast high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method for the simultaneous quantification of alectinib, crizotinib, erlotinib and gefitinib in human plasma which can be combined with our previous validated method to quantify osimertinib, allowing quantification of the currently most frequently used TKIs in NSCLC with a single assay setup. Using this assay for TDM may further optimize treatment with these TKIs by revealing under- or overexposure.

2 | MATERIAL AND METHODS

2.1 | Chemicals and reagents

Alectinib (free base purity [FBP] = 98%), crizotinib (FBP = 98%), erlotinib (FBP = 97%), gefitinib (FBP = 98%), crizotinib-D5 (chemical purity 97%, isotopic purity 99,2%), erlotinib-D6 (FBP = 98%) and gefitinib-D3 (chemical purity = 98%, isotopic purity = 99,7%) were purchased from Bio Connect (Huissen, The Netherlands). Methanol (ULC/MS–CC/SFC grade) was purchased from Biosolve (Valkenswaard, the Netherlands). Acetonitrile (≥99.9%), 2-propanol (≥99.8%) and dimethylsulfoxide (DMSO, ≥99.0%) were obtained from Merck (Darmstadt, Germany). Ammonium-acetate (Normapur) was purchased from VWR (Leuven, Belgium). Sodium-citrate plasma (frozen, no additives) was purchased from Sanquin (Amsterdam, the Netherlands). K2-EDTA whole blood, and subsequently, K2-EDTA plasma were obtained from volunteers.

2.2 | Preparation of calibration standards and quality controls

For the calibration standards and quality controls of crizotinib and gefitinib, two separately prepared stock solutions (1 mg/ml) were produced in DMSO. This led to two crizotinib solutions and two gefitinib solutions, one for calibration purposes, and one for quality control purposes. One crizotinib solution and one gefitinib solution were combined and diluted in methanol to 20 μg/ml (solution 1). The same was done for the second crizotinib/gefitinib solutions, which were used for quality control (QC) batches (solution QC_1). For alectinib and erlotinib, a similar approach was taken, as two separate stock solutions (1 mg/ml) were also prepared in DMSO for both drugs, resulting in two alectinib solutions and two erlotinib solutions. Those four solutions were used to form solution 2 and solution QC_2, in a similar way as described for crizotinib and gefitinib. Both solutions (2 and QC_2) were subsequently diluted in methanol to 40 μg/ml. A working solution was produced by combining solution 1 and solution 2, 1:1, to form solution 3 and by combining solution QC_1 and QC_2, forming solution QC_3. The stock solutions of the internal standards (crizotinib-D5, erlotinib-D6 and gefitinib-D3) were prepared, reconstituting approximately 1 mg of each compound separately in 100 ml methanol (10 μg/ml). All stock solutions (solutions 1 and 2, QC_1 and QC_2) were stored at −80°C until analysis. Calibration standards consisted of six different concentrations, a zero sample and a blank sample (Table 1). The calibration standards were prepared by spiking citrate plasma with solution 3. The zero sample only consisted of all internal standards, while the blank sample did not contain any TKI or internal standard. Quality control samples were prepared at five different concentrations from solution QC_3: lowest limit of quantification (LLOQ), QC_{LOW}, QC_{MED}, QC_{HIGH} and upper limit of quantification (ULOQ). QC_{LOW}, QC_{MED} and QC_{HIGH} were used for sample runs. The nominal concentrations of all QCs can be found in Table 1.

2.3 | Instrumentation

For the analysis a Surveyor® Autosampler Plus with a quaternary MS-pump plus and degasser (ThermoFischer, Breda, The Netherlands) as a chromatographic system was used. A TSQ Quantum-Access® triplequad mass spectrometer (ThermoFischer, Breda, the Netherlands) with an electrospray ionization interface combined with Excalibur® software (version 2.2SP1) was used for detection and quantification. Chromatographic separation was performed on a HyPurity® C_{18} analytical column (50 × 2.1 mm, 3 μm, Thermo Fischer Scientific) combined with a drop-in guard (HyPurity® C_{18}, 10 × 2.1 mm, 3 μm).

2.4 | Sample preparation

The solution for deproteinization was made by adding 40 μl of the stock solution of crizotinib-D5, 90 μl of the stock solution of erlotinib-D6 and 20 μl of the stock solution of gefitinib-D3 to 10 ml methanol. A 150 μl aliquot of deproteinization solution was added to a 20 μl plasma sample. The mixture was vortexed for 2 min and centrifuged at 11,300***g*** for 5 min. Subsequently, 100 μl supernatant was mixed with 400 μl mobile phase A to ensure compatibility with the gradient used during chromatography.

2.5 | Chromatographic condition and LC–MS/MS settings

Two mobile phases were used for achieving chromatographic separation. Mobile phase A consisted of 2 mM ammonium acetate in water (+ 0.1% formic acid). Mobile phase B consisted of 2 mM ammonium acetate in methanol (+ 0.1% formic acid). The starting gradient was set at 80% A and 20% B for 0.5 min. After 0.5 min the gradient

TABLE 1 Concentrations for calibration curve and quality controls

Calibration curve Quality controls	Level 1 (ng/ml) LLOQ (ng/ml)	Level 2 (ng/ml) QC_{LOW} (ng/ml)	Level 3 (ng/ml)	Level 4 (ng/ml) QC_{MED} (ng/ml)	Level 5 (ng/ml) QC_{HIGH} (ng/ml)	Level 6 (ng/ml) ULOQ (ng/ml)
Alectinib	100	200	500	1,000	1,500	2,000
Crizotinib	50	100	250	500	750	1,000
Erlotinib	100	200	500	1,000	1,500	2,000
Gefitinib	50	100	250	500	750	1,000

Abbreviations: LLOQ, lower limit of quantification; QC_{LOW}, low quality control; QC_{MED}, middle quality control; QC_{HIGH}, high quality control; ULOQ, upper limit of quantification, ng/ml, nanogram per millilitre.

linearly increased to 100% B in 2.0 min and was maintained for an additional 0.3 min after which the gradient was reset to initial conditions and kept steady for 1 min before a new sample was injected. The flow during the run was maintained at 500 µl/min. The column temperature was set at 60°C. The autosampler temperature was set at 10°C. The divert valve was set in the waste position for the first 1.5 min. A flush/needle-wash solution of water (25%), methanol (25%), acetonitrile (25%) and isopropanol (25%) was used to reduce carryover.

MS detection was in MRM mode with the following settings: spray voltage, 4,500 V; sheath gas pressure, 60; auxiliary gas pressure, 15; capillary temperature, 360°C; collision gas pressure, 1.5 mTorr. Transitions used for the different compounds were as follows: 483.3–396.2 (alectinib), 450.2–260.1 (crizotinib), 394.2–278.0 (erlotinib), 447.2–128.1 (gefitinib), 455.2–265.1 (crizotinib-D5), 400.3–278.0 (erlotinib-D6) and 450.2–128.1 (gefitinib-D3). The collision energy and tube lens value were 23 eV and 85 for alectinib, 22 eV and 87 for crizotinib, 30 eV and 91 for erlotinib, 25 eV and 71 for gefitinib, 24 eV and 88 for crizotinib-D5, 29 eV and 91 for erlotinib-D6 and 23 eV and 88 for gefitinib-D3.

2.6 | Method validation

The validation was based on the most recent guideline of the European Medicines Agency (2011).

2.6.1 | Selectivity and carryover

Selectivity was evaluated by analyzing six different EDTA plasma samples from patients who were not treated with any of the measured TKIs. The largest peaks close to the retention times of the TKIs or internal standards were manually integrated. These values were compared with the lowest response in one of the five LLOQs in the same validation run. The response should be <15% for the QCs of the TKIs and <20% for the LLOQ. For all internal standards, the response should not exceed 5% of the peak area of the internal standard. Carryover was tested by injecting a blank plasma sample after an ULOQ sample. Analysis was carried out in five replicates. The limits for carryover were similar to the limits for selectivity (<15% for QCs of TKIs, <20% for LLOQ of TKIs and <5% for internal standards).

2.6.2 | Lower limit of quantification and linearity

For all TKIs a mean or median plasma trough concentration at steady state ($C_{min}SS$) in the population has been reported: alectinib, 572 ng/ml; crizotinib, 274 ng/ml; erlotinib, 1,010 ng/ml; and gefitinib, 291 ng/ml (Verheijen et al., 2017). In addition, threshold plasma trough concentrations in steady state have been proposed for alectinib (>435 ng/ml) and erlotinib (>500 ng/ml). In our analytical method we used a calibration range of 100–2,000 ng/ml for alectinib and erlotinib, and 50–1,000 ng/ml for crizotinib and gefitinib. Three calibration curves were constructed ($y = a \times x + b$, weighing $1/x$). Calculated concentrations were not allowed to exceed 15% of the nominal value of all QCs, with the exception of the LLOQ, which had to be within 20% of the nominal value.

2.6.3 | Precision and accuracy

Precision and accuracy of the developed method were determined by analyzing five different QCs (LLOQ, QC_{LOW}, QC_{MED}, QC_{HIGH} and ULOQ) on three different days using freshly prepared calibration standards to construct the calibration curve. On each day, five replicates of each QC were analyzed. Precision and accuracy were not allowed to exceed 15% for all QCs except for the LLOQ which should remain within 20% of the nominal value (European Medicines Agency, 2011).

2.6.4 | Matrix effect

The matrix effect was evaluated using QC_{LOW} and QC_{HIGH}. The matrix effect was the ratio between the peak area of spiked blank matrix and the peak area in spiked mobile phase (80% A–20% B).

2.6.5 | Dilution integrity

Dilution integrity is tested by diluting human plasma ($c = 1.5 \times$ ULOQ) 4-fold and 2-fold (European Medicines Agency, 2011). All back-calculated concentrations should be within 15% of the nominal value.

2.6.6 | Stability

Short- and long-term stability of all TKIs were determined in human EDTA and sodium citrate plasma for QC_{LOW}, QC_{MED} and QC_{HIGH} at four different temperatures (room temperature [RT], 4°C, −20°C and −80°C). In addition, short-term stability was evaluated for all TKIs in EDTA whole blood, at RT and at 4°C. For all stock solutions the long-term stability was determined at −80°C. Accuracy was not allowed to exceed 15% of the nominal value. Post-preparation stability in the autosampler was assessed by re-injecting processed QCs and calibration standards (maintained at 10°C in the autosampler for 24 h).

3 | RESULTS AND DISCUSSION

3.1 | Method development

In view of the previously developed assay for the analysis of osimertinib, the new method for the other four TKIs should preferably be similar owing to its simultaneous use in a routine setting in the future. Detailed validation and stability results for osimertinib are published elsewhere, and therefore will only be highlighted when substantially different from the other TKIs (van Veelen et al., 2020).

To minimize differences in sample preparation, we used methanol for protein precipitation. The starting gradient, compared with osimertinib, was altered to ensure an elution time above 1.5 min for alectinib, crizotinib, erlotinib and gefitinib. Using 2 mM ammonium-acetate in the dilution step prior to injection resulted in higher sensitivity for all TKIs, which made it possible to lower the injection volume.

At the start of the method development, deuterated alectinib and M4, the active metabolite of alectinib, were not commercially available and could not be included in this method. Therefore, erlotinib-D6 was used as internal standard for the quantification of alectinib.

The chromatograms for the four TKIs (alectinib, crizotinib, erlotinib and gefitinib) and the three internal standards used (crizotinib-D5, erlotinib-D6 and gefitinib-D3) are shown in Figure 1, where plasma samples were spiked with the LLOQ concentration. Figure 2 shows the chromatograms of the TKIs and the internal standards in blank samples. Because the chromatograms report relative abundance, the absolute abundances are described separately. The absolute abundances were considerably higher for the LLOQ chromatograms, and the ratio between the absolute abundances in the spiked sample compared with the blank sample was 17.4 for alectinib, 55.2 for crizotinib, 44.2 for erlotinib, 45.0 for gefitinib, 423.3 for crizotinib-D5, 718.1 for erlotinib-D6 and 796.7 for gefitinib-D3. For osimertinib we additionally validated the quantification of the active, demethylated metabolite AZ5104 within the same assay. The addition of AZ5104 did not lead to any changes in sample preparation or the detection method. In Figure 3, the chromatograms of osimertinib, its active metabolite (AZ5104) and the internal standard used, osimertinib-C13D3, are shown. As seen with the other TKIs, the absolute abundance was higher for the LLOQ samples; the ratio for osimertinib was 10.1, for AZ5104 it was 15.2 and for osimertinib-C13D3 it was 202.1.

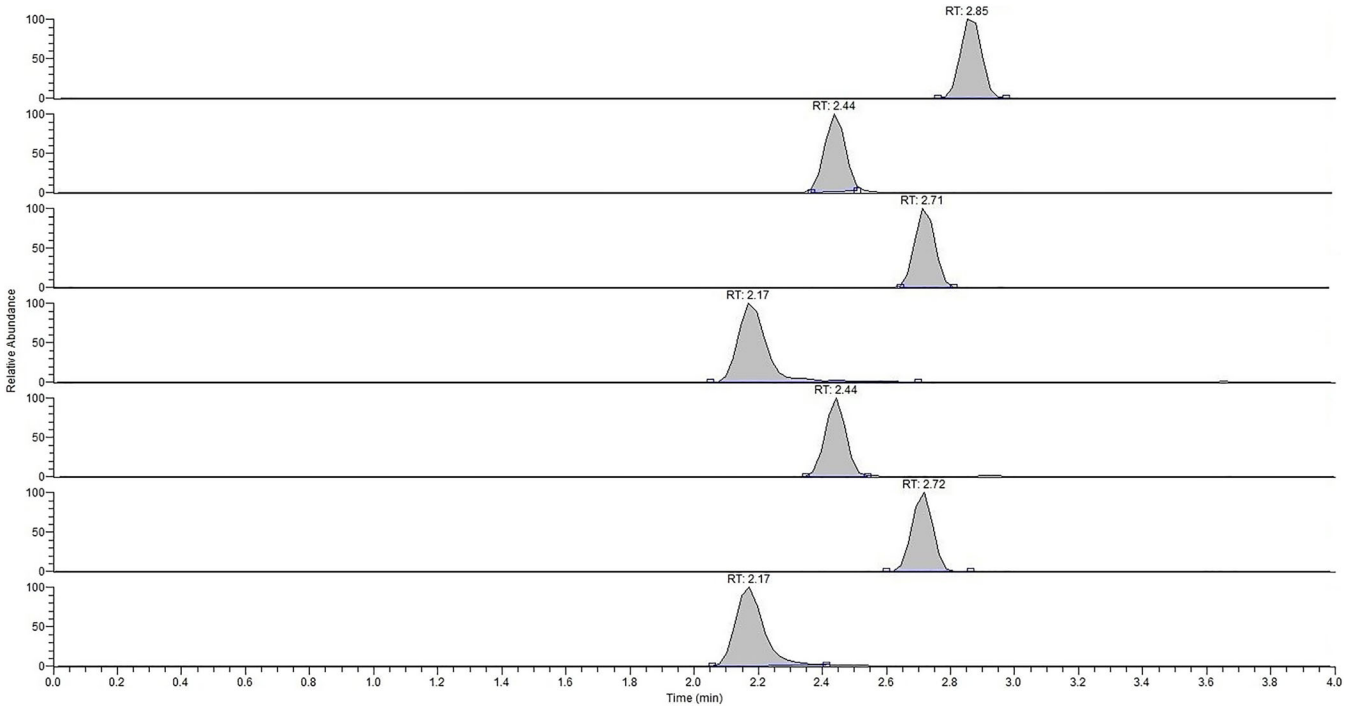

FIGURE 1 Chromatograms of LLOQs of alectinib, crizotinib, erlotinib, gefitinib, crizotinib-D5, erlotinib-D6 and gefitinib-D3 (top-to-bottom); absolute abundances are shown separately

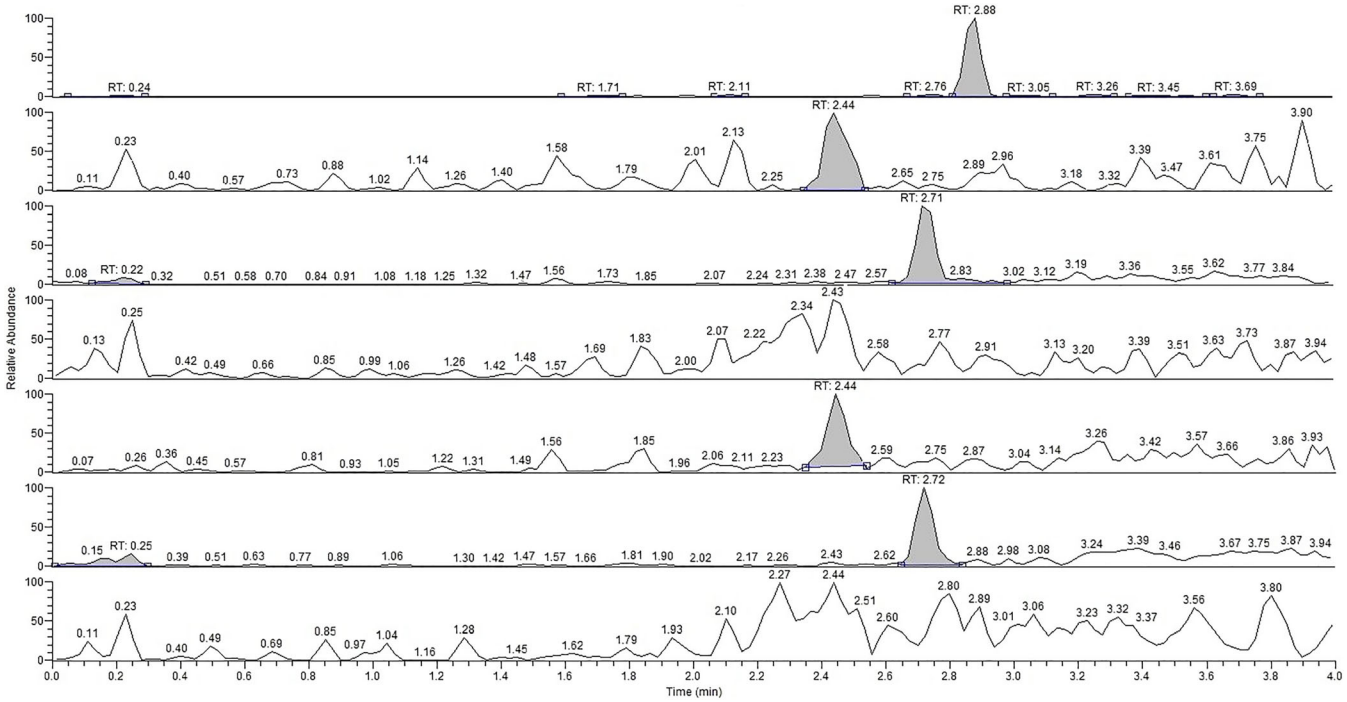

FIGURE 2 Chromatograms of blank samples of alectinib, crizotinib, erlotinib, gefitinib, crizotinib-D5, erlotinib-D6 and gefitinib-D3 (top-to-bottom); absolute abundances are shown separately

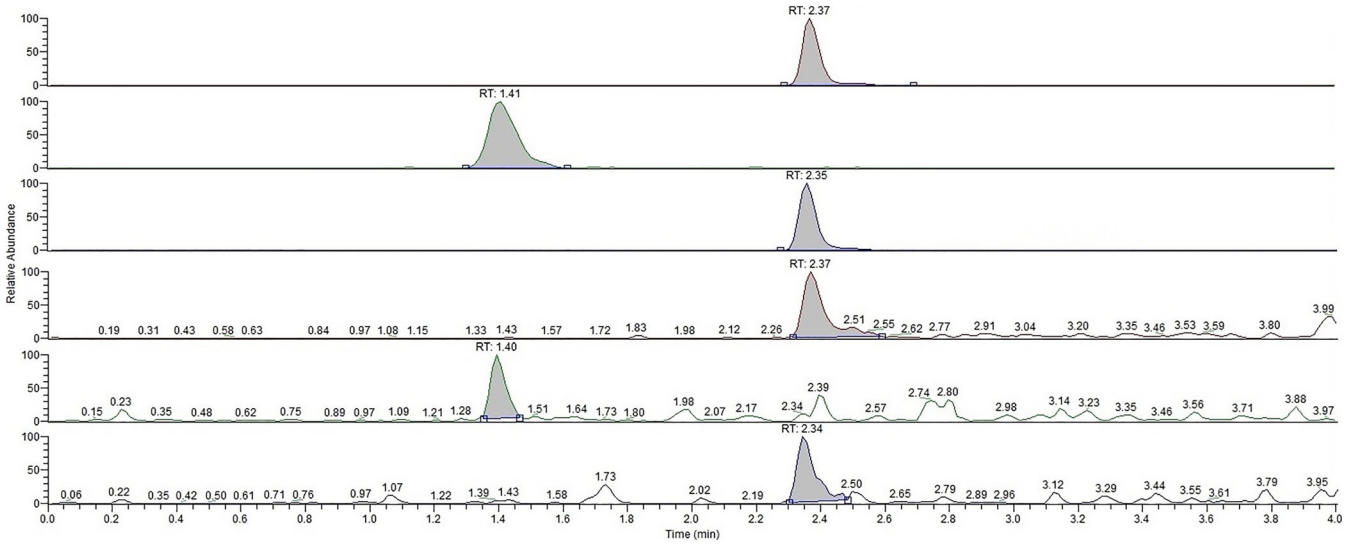

FIGURE 3 Chromatograms of LLOQ and blank samples of osimertinib, its metabolite (AZ5104) and osimertinib-C13D3. From top-to-bottom: osimertinib (LLOQ), AZ5104 (LLOQ), osimertinib-C13D3 (LLOQ), osimertinib (blank), AZ5104 (blank) and osimertinib-C13D3 (blank). Absolute abundances are shown separately

3.2 | Method validation

3.2.1 | Selectivity and carryover

The results for selectivity and carryover are shown in Table 2. For all TKIs six human EDTA plasma samples showed no interfering peaks. Blank TKI responses were <5.6% for all TKIs and were 0.9–1.7% for alectinib, 1.2–5.6% for crizotinib, 0.5–1.4% for erlotinib and 1.0–4.9% for gefitinib. Carryover was <3.9% for all TKIs, 2.6–3.9% for alectinib, 1.5–2.5% for crizotinib, 1.4–2.0% for erlotinib and 0.3–3.4% for gefitinib. For the internal standard blank responses were all <0.3% and carryover was <0.3% for all TKIs. Selectivity and carryover for AZ5104 (metabolite of osimertinib) also met all requirements (Appendix A).

TABLE 2 Selectivity and carryover of erlotinib, gefitinib, crizotinib, alectinib and the internal standard, and accuracy after a two- and four-fold dilution of a high concentration (1.5 × ULOQ)

	Selectivity		Carryover		Dilution	
Drug	Accuracy(%) TKI ($n = 6$)	Accuracy(%) IS ($n = 6$)	Accuracy(%) TKI ($n = 5$)	Accuracy(%) IS ($n = 5$)	Accuracy(%) (two-fold) ($n = 5$)	Accuracy(%) (four-fold) ($n = 5$)
Alectinib	0.9–1.7	0.0–0.2	2.6–3.9	0.1–0.1	111.6 (±3.6)	112.2 (±2.8)
Crizotinib	1.2–5.6	0.0–0.2	1.5–2.5	0.1–0.3	107.3 (±2.1)	111.6 (±3.2)
Erlotinib	0.5–1.4	0.0–0.2	1.4–2.0	0.1–0.1	112.8 (±2.6)	108.2 (±2.3)
Gefitinib	1.0–4.9	0.0–0.3	0.3–3.4	0.1–0.2	106.5 (±1.8)	113.4 (±2.7)

TKI, Tyrosine kinase inhibitor; IS, internal standard; *n*, number of samples.
Selectivity was evaluated in blank samples and compared with the LLOQ of the corresponding TKI. Selectivity should be below 15%.
Carry-over was calculated by running a blank sample directly after the upper limit of quantification (ULOQ) and compare this with the LLOQ. Carry-over should be below 15% the TKI and below 1% for IS.
Dilution integrity should be within 15% from the nominal value (85% - 115%).

TABLE 3 Intra- and inter-day accuracy and precision of alectinib, crizotinib, erlotinib and gefitinib in spiked human plasma samples

Nominal concentration (ng/L)	Mean concentration ($n = 15$; ng/ml)	Intra-day precision ($n = 5$; %)	Inter-day precision ($n = 15$; %)	Intra-day accuracy ($n = 5$; %)	Inter-day accuracy ($n = 15$; %)
$LLOQ_A$ (100.0)	109.1	6.8	5.6	115.7	109.1
QC_{LOW_A} (200.0)	204.8	4.4	0.0	103.7	102.4
QC_{MED_A} (1,000.0)	979.9	3.4	1.5	96.1	98.0
QC_{HIGH_A} (1,500.0)	1,495.1	4.5	1.6	102.4	99.7
$ULOQ_A$ (2,000.0)	2,027.0	3.0	1.7	103.6	101.4
$LLOQ_C$ (50.0)	55.6	4.4	4.0	116.3	111.1
QC_{LOW_C} (100.0)	108.2	5.4	0.0	110.2	108.2
QC_{MED_C} (500.0)	521.9	4.2	1.7	106.8	104.4
QC_{HIGH_C} (750.0)	782.5	2.6	3.0	108.1	104.3
$ULOQ_C$ (1,000.0)	1,035.9	4.2	2.7	106.9	103.6
$LLOQ_E$ (100.0)	106.6	4.0	3.5	111.3	106.6
QC_{LOW_E} (200.0)	206.7	3.6	2.1	106.1	103.3
QC_{MED_E} (1,000.0)	1,000.8	4.0	0.0	101.6	100.1
QC_{HIGH_E} (1,500.0)	1,507.2	4.5	0.0	101.8	100.5
$ULOQ_E$ (2,000.0)	2,012.5	3.7	0.0	101.4	100.6
$LLOQ_G$ (50.0)	55.1	5.5	4.7	115.3	110.2
QC_{LOW_G} (100.0)	107.8	2.7	3.2	110.3	107.8
QC_{MED_G} (500.0)	505.4	1.8	0.5	102.0	101.1
QC_{HIGH_G} (750.0)	753.4	0.9	1.6	102.3	100.5
$ULOQ_G$ (1,000.0)	986.8	2.3	0.0	98.2	98.7

Abbreviations: LLOQ, lower limit of quantification; QC_{LOW}, low quality control; QC_{MED}, middle quality control; QC_{HIGH}, high quality control; ULOQ, upper limit of quantification; *n*, number of samples.
Subscripts: A, alectinib; C, crizotinib; E, erlotinib; G, gefitinib.

3.2.2 | Calibration and linearity

The calibration curves were linear over the examined range for all four TKIs. The coefficients of determination were 0.9906–0.9990 for alectinib, 0.9924–0.9993 for crizotinib, 0.9922–0.9998 for erlotinib and 0.9903–0.9999 for gefitinib.

3.2.3 | Precision and accuracy

Precision and accuracy data for all TKIs are shown in Table 3, and for AZ5104 in Appendix A. Intra- and inter-day precision and accuracy for all TKIs and AZ5104 were within the pre-specified limits of 85–115% for QC_{LOW}, QC_{MED}, QC_{HIGH} and ULOQ, and 80–120% for

LLOQ. Accuracies for the TKIs dilution integrity are shown in Table 2 and were in agreement with requirements mentioned above.

3.2.4 | Matrix effect

Matrix effect was evaluated using QC_{LOW} and QC_{HIGH}. For all TKIs the matrix effect was <5%. The coefficient of variation (CV) was 4.8% (QC_{LOW}) and 2.8% (QC_{HIGH}) for alectinib, 3.9% (QC_{LOW}) and 4.2% (QC_{HIGH}) for crizotinib, 2.0% (QC_{LOW}) and 1.8% (QC_{HIGH}) for erlotinib, and 2.0% (QC_{LOW}) and 1.7% (QC_{HIGH}) for gefitinib. The results for matrix effect are shown in Table 4.

3.2.5 | Stability

Short- and long-term stability

Short- and long-term stability for alectinib, crizotinib, erlotinib and gefitinib in human EDTA and sodium citrate plasma was evaluated at four different temperatures and is shown in Table 5. In citrate plasma, alectinib, crizotinib, erlotinib and gefitinib were stable for at least 24 h at room temperature (20°C). Osimertinib was not stable for 24 h at room temperature and the concentration decreased to <10% after 24 h for all three QCs (van Veelen et al., 2020). A similar trend was seen for AZ5104, which was also not stable for at least 24 h at RT. At 4°C, all TKIs were stable for at least 24 h in citrate plasma and gefitinib showed relatively the best stability at 4°C as it was stable for at least 3 weeks. Osimertinib proved to be less stable at 4°C in citrate plasma, and similar results were seen for AZ5104. However, AZ5104 proved to be stable for 24 h at 4°C in EDTA plasma (Appendix A).

When stored at −80°C, alectinib, crizotinib, erlotinib and gefitinib were stable for at least 1 month in EDTA plasma. When stored at −20°C alectinib, erlotinib and gefitinib were stable for at least 3 weeks in citrate plasma, while alectinib was stable for at least 1 day. Owing to irregularities, the stability of crizotinib after 3 weeks at −20°C could not be determined. Osimertinib was stable at −80°C for at least 6 months (van Veelen et al., 2020). AZ5104 also showed good stability when stored at −80°C, irrespective of the matrix used (Appendix A).

Additionally, the stability of all TKIs was evaluated in EDTA whole blood at room temperature and at 4°C. All TKIs were stable for at least 4 h in whole blood at room temperature. Moreover, alectinib and erlotinib showed even better stability, as they were stable for at least 24 h. When stored at 4°C, all TKIs were stable for at least 24 h (Table 6). AZ5104 was not stable in whole blood when stored at RT, but proved to be stable at 4°C for at least 8 h.

Freeze–thaw stability of the four TKIs is shown in Table 4. The accuracies for the quality controls were 102.6–102.9% for alectinib, 101.6–103.0% for crizotinib, 103.1–105.5% for erlotinib and 102.8–108.5% for gefitinib. Owing to the poor stability of osimertinib at room temperature, freeze–thaw stability was not evaluated for osimertinib.The stability of the processed samples in the autoinjector (10°C) was adequate for at least 1 day for all five TKIs. The stability results of alectinib, crizotinib, erlotinib and gefitinib are shown in more detail in Table 4.

Stock stability

The stock solutions of alectinib, erlotinib and gefitinib were stable for 3 months when stored at −80°C, with accuracies of 99.6% for alectinib, 100.2% for erlotinib and 101.2% for gefitinib. The stock solution of crizotinib was not stable at −80°C, as the accuracy had increased to 125.5% after 3 months. As this was an unexpected result, the stability of the stock solution of crizotinib is currently being reevaluated.

TABLE 4 Matrix effect and freeze–thaw stability of alectinib, crizotinib, erlotinib and gefitinib in human plasma

Drug (ng/ml)	Matrix effect ($n = 6$) Accuracy (variation, %)	Freeze–thaw stability ($n = 6$) Accuracy (variation, %)	Autoinjector stability ($n = 5$) Accuracy (variation, %)
Alectinib – QC_{LOW} (200.0)	51.2 (4.8)	102.6 (3.6)	103.6 (1.4)
Alectinib – QC_{MED} (1,000.0)	—	102.9 (4.3)	97.4 (4.9)
Alectinib – QC_{HIGH} (1,500.0)	55.8 (3.8)	102.8 (3.4)	99.3 (2.3)
Crizotinib – QC_{LOW} (100.0)	96.8 (3.9)	101.6 (1.7)	100.3 (4.2)
Crizotinib – QC_{MED} (500.0)	—	103.0 (1.7)	99.1 (1.6)
Crizotinib – QC_{HIGH} (1,000.0)	104.8 (4.2)	102.5 (1.0)	101.6 (0.7)
Erlotinib – QC_{LOW} (200.0)	101.1 (2.0)	105.3 (1.3)	107.9 (2.5)
Erlotinib – QC_{MED} (1,000.0)	—	105.1 (0.7)	101.0 (3.7)
Erlotinib – QC_{HIGH} (1,500.0)	99.5 (1.8)	103.1 (4.1)	101.5 (2.1)
Gefitinib – QC_{LOW} (100.0)	101.3 (2.0)	108.5 (2.2)	104.7 (2.2)
Gefitinib – QC_{MED} (500.0)	—	102.8 (1.2)	101.5 (2.2)
Gefitinib – QC_{HIGH} (1,000.0)	100.1 (1.7)	104.5 (0.2)	98.7 (2.0)

QC_{LOW}, Low quality standard; QC_{MED}, middle quality standard; QC_{HIGH}, high quality standard; *n*, number of samples.
Accuracy was evaluated compared with the nominal value.

TABLE 5 Stability of alectinib, crizotinib, erlotinib and gefitinib in human plasma at various storage conditions

Temperature (°C)	Drug	Time (days)	Accuracy QC_{LOW} (%)	Accuracy QC_{MED} (%)	Accuracy QC_{HIGH} (%)
−80	Alectinib[a]	1	105.8	98.8	99.4
−80	Alectinib[a]	30	103.9	96.2	103.5
−80	Crizotinib[a]	1	105.1	99.3	103.4
−80	Crizotinib[a]	30	109.6	98.2	96.9
−80	Erlotinib[a]	1	106.7	97.3	99.9
−80	Erlotinib[a]	30	108.4	97.2	100.1
−80	Gefitinib[a]	1	105.6	99.5	101.7
−80	Gefitinib[a]	30	107.3	101.4	99.9
−20	Alectinib[b]	1	103.1	105.9	101.0
−20	Alectinib[b]	21	116.4	105.6	109.6
−20	Crizotinib[b]	1	110.1	109.5	105.3
−20	Crizotinib[b]	21	ND	ND	ND
−20	Erlotinib[b]	1	92.2	101.3	99.2
−20	Erlotinib[b]	21	104.8	106.9	111.7
−20	Gefitinib[b]	1	94.3	94.7	95.9
−20	Gefitinib[b]	21	94.3	93.5	97.5
4	Alectinib[b]	1	101.3	102.0	103.7
4	Crizotinib[b]	1	107.9	106.2	109.5
4	Erlotinib[b]	1	93.3	101.0	103.4
4	Gefitinib[b]	1	93.9	95.3	97.5
RT	Alectinib[b]	1	99.5	100.1	100.5
RT	Crizotinib[b]	1	101.3	107.5	99.9
RT	Erlotinib[b]	1	93.8	101.5	102.5
RT	Gefitinib[b]	1	94.7	93.7	98.0

Abbreviations: RT, room temperature; QC_{LOW}, low quality control; QC_{MED}, middle quality control; QC_{HIGH}, high quality control; ND, not determined.
Results are mean concentration compared with nominal value. All concentrations were analyzed in duplicate.
[a]stability was evaluated in EDTA-plasma.
[b]stability was evaluated in citrate plasma.
For every specific moment one plasma sample was analyzed in duplicate.

3.2.6 | Clinical applicability

The analytical method described here was used to perform two studies evaluating EDTA plasma trough concentrations during steady state of patients who had been treated with one of the TKIs as part of standard patient care (studies approved by the medical ethics review committee of the Maastricht University Medical Center – approval numbers 2019-1080 and 2018-0800). The range of plasma trough concentration measured at steady state was 184.93–783.9 for alectinib ($n = 21$), 216.7–340.4 ng/ml for crizotinib ($n = 4$), 361.8–1,-584.9 ng/ml for erlotinib ($n = 6$) and 101.3–437.6 ng/ml for osimertinib ($n = 43$). Until now, no patients using gefitinib were included. All measured plasma trough concentrations at steady state were within the validated calibration range, except for one osimertinib user who experienced an unusual high trough concentration (measured at 1,130 ng/ml) and we reported this as >500 ng/ml owing to the validated range. Incurred sample reanalyses for alectinib, crizotinib, erlotinib and gefitinib have not been performed yet, but will be performed when more blood samples are collected.

3.3 | Comparison with previous studies

For each TKI several single drug assays have been published, but multidrug assays are less frequently reported. Reis et al. described a method to quantify afatinib, crizotinib, erlotinib, nintedanib and osimertinib in sodium citrate and heparinized plasma (Reis et al., 2018). Another multidrug assay, by Hayashi et al., focused on the quantification of afatinib, erlotinib and gefitinib, but the type of plasma used in the method was not reported (Hayashi et al., 2016). The method described by Veerman et al. was used to analyze alectinib, afatinib, crizotinib and osimertinib in heparinized plasma (Veerman et al., 2019). All of these studies have some similarities with our method, but none simultaneously analyzed alectinib, crizotinib,

TABLE 6 Stability of alectinib, crizotinib, erlotinib and gefitinib in EDTA whole blood at various storage conditions

Temperature (°C)	Drug	Time (h)	Accuracy QC_{LOW} (%)	Accuracy QC_{MED} (%)	Accuracy QC_{HIGH} (%)
RT	Alectinib	4	104.1	100.8	104.5
RT	Alectinib	8	99.7	98.9	107.7
RT	Alectinib	24	110.2	100.0	111.9
RT	Crizotinib	4	87.7	96.7	110.8
RT	Crizotinib	8	80.3	101.8	112.6
RT	Crizotinib	24	87.2	107.3	122.4
RT	Erlotinib	4	106.2	104.9	108.6
RT	Erlotinib	8	105.1	102.2	106.5
RT	Erlotinib	24	99.1	99.3	105.3
RT	Gefitinib	4	99.7	103.3	112.6
RT	Gefitinib	8	104.4	106.4	115.5
RT	Gefitinib	24	98.3	104.9	119.2
4	Alectinib	4	101.4	100.0	105.4
4	Alectinib	8	105.7	94.6	97.6
4	Alectinib	24	106.8	94.4	101.5
4	Crizotinib	4	91.5	108.3	105.7
4	Crizotinib	8	100.3	107.2	102.0
4	Crizotinib	24	99.0	110.5	103.9
4	Erlotinib	4	106.0	105.4	105.5
4	Erlotinib	8	106.1	101.8	98.1
4	Erlotinib	24	106.6	101.0	100.7
4	Gefitinib	4	106.2	109.5	106.8
4	Gefitinib	8	109.5	106.3	104.1
4	Gefitinib	24	109.3	107.0	110.7

Abbreviations: RT, room temperature; QC_{LOW}, low quality control; QC_{MED}, middle quality control; QC_{HIGH}, high quality control. Results are mean concentration compared with nominal value. All concentrations were analyzed in duplicate. For every specific moment one plasma sample was analyzed in duplicate.

erlotinib, gefitinib and osimertinib, which are currently the most commonly used *ALK*- and *EGFR*-TKIs to treat NSCLC patients in our hospital.

In a recent study by Zhou et al. a simultaneous analytical method was developed and validated for the analysis of 12 TKIs, which also included the five TKIs that are presented in this article. Compared with our method, a couple of differences can be indicated. First, a salting-out liquid–liquid extraction (SALLE) was used, while we used a simple protein precipitation method with methanol. Using SALLE could leave residual protein dissolved in the plasma sample, which could be detrimental for the chromatographic column in the long term. Secondly, the run time in the analytical method from Zhou et al. was 6.0 min, while our run time was 3.8 min. Lastly, Zhou et al. used one internal standard (voriconazole) for quantification of each TKI, where ideally a deuterated form is desirable, as both the TKI and the internal standard will be eluted at the same moment, and potential ion-suppression or ion-enhancement will be comparable with both the TKI and the internal standard. However, if the TKI and its internal standard are not eluted at the same time, possible inaccuracies could occur, especially in patients samples, with other, sometimes unknown, substances (Zhou et al., 2021). We used a deuterated form of the TKIs as much as possible, if they were commercially available. Since our new method can be performed with the same equipment as our earlier developed method for osimertinib, it allows us to analyze all five TKIs (alectinib, crizotinib, erlotinib, gefitinib and osimertinib) with a single-assay setup. This workflow is less time consuming. Laboratory technicians can save time, which can be used to perform other analyses for TDM purposes. The amount of (expensive) laboratory equipment and the availability of laboratory technicians is frequently limited, while the intention is to offer a wide range of TDM analysis across various therapeutic fields. Consequently, combining the analysis of multiple TKIs in a single run will be more cost-effective, as the mean preparation time per sample will be lower, compared with a situation where five different runs are performed for all individual TKIs. In addition, in one center only a small number of patients is treated with each individual TKI. Consequently, when single TKI assays are used, only a small number of samples can be evaluated in one run. Otherwise, multiple samples need to be collected over a longer period of time, which could lead to delays in reporting results to physicians. Combining analysis of multiple TKIs in one assay enables us to

perform a run more frequently. Improved reporting efficiency allows quicker dose adjustments when drug concentrations are outside the therapeutic window, for example.

HPLC was used for separation by Reis et al. and resulted in a run time of 11 min (Reis et al., 2018). Hayashi et al. and Veerman et al. used UHPLC which decreased the run time to ~5 min (Hayashi et al., 2016; Veerman et al., 2019). With our HPLC method, we achieved a run time similar to that of published UHPLC methods. In addition, sample preparation was straightforward and efficient, without the need for additional supernatant evaporation using nitrogen as described by Reis et al. and Hayashi et al. The sample preparation of osimertinib was slightly different, as it must be performed on dry-ice owing to the limited stability of osimertinib and its metabolite at room temperature (van Veelen et al., 2020, Appendix A).

We decided to evaluate the stability of all TKIs extensively, to ensure the stability of TKIs throughout the whole process of blood collection until quantification. During daily practice it may be necessary to store a blood sample temporarily at room temperature or in the fridge. All TKIs showed sufficient stability, both in EDTA whole blood (at RT and 4°C) and in plasma (at RT and 4°C) to ensure that all TKIs were stable through the whole process from blood collection to quantification. We evaluated stability in EDTA plasma and EDTA whole blood, while others evaluated stability in heparinized plasma and sodium citrate anticoagulated plasma (Reis et al., 2018; Veerman et al., 2019), while Zhou et al. used unspecified plasma. The stability results are similar, independent of the anticoagulant used. Reis et al. reported that crizotinib and erlotinib were stable for at least 24 h at room temperature and for 60 days at −20°C (Reis et al., 2018). In our study crizotinib and erlotinib were stable for at least 24 h at room temperature and for at least 30 days when stored at −80°C. In the study by Veerman et al. alectinib and crizotinib were stable for at least 24 h when stored at room temperature and for at least 9 months when stored at −70°C (Veerman et al., 2019). These results were similar to our results, as both alectinib and crizotinib were stable for 24 h at room temperature. Furthermore, alectinib and crizotinib were stable for at least 1 month in our study. In the study by Zhou et al. alectinib, crizotinib, erlotinib and gefitinib showed sufficient stability. Furthermore, the limited stability of osimertinib was briefly discussed. This was similar to the stability we have observed, while specific details on osimertinib stability were described previously (Reis et al., 2018; van Veelen et al., 2020; Veerman et al., 2019; Zheng et al., 2018; Zhou et al., 2021).

4 | CONCLUSION

In this study an analytical method was developed and validated to simultaneously quantify alectinib, crizotinib, erlotinib and gefitinib in EDTA plasma. Although HPLC was used for separation, the run time was comparable with UHPLC methods. Furthermore, the assay can be combined with our previously validated method for osimertinib using the same equipment, allowing the simultaneous quantification of the currently most used *ALK*- and *EGFR*-TKIs in the Netherlands among patients with NSCLC with a single-assay setup. Such a setup improves laboratory efficiency and enhances the reporting capabilities. The intended use of this assay in clinical practice for TDM may further support treatment optimization of these TKIs by revealing under- or overexposure, evaluating drug adherence and monitoring drug–drug interactions with co-administered medications.

CONFLICT OF INTEREST

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ENDNOTES

[a] Stability was evaluated in EDTA plasma

[b] Stability was evaluated in citrate plasma

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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APPENDIX A: Validation results for AZ5104, active metabolite of osimertinib

TABLE A1 Selectivity and carryover of AZ5104 and the internal standard

	Selectivity		Carryover	
Drug	Accuracy (%)	Accuracy (%) IS	Accuracy (%)	Accuracy (%) IS
AZ5104	0.0–0.2	0.1–0.3	0.7–1.9	0.1–0.3

IS, internal standard.
Selectivity was evaluated in blank samples and compared with the lower limit of quantitation (LLOQ) of the corresponding tyrosine kinase inhibitor (TKI). Selectivity should be <15%.
Carryover was calculated by running a blank sample directly after the higher limit of quantification (HLOQ) and comparing this with the LLOQ. Carryover should be <15% the TKI and <1% for IS.

TABLE A2 Intra- and inter-day accuracy and precision of AZ5104 in spiked human plasma samples

Nominal concentration (ng/L)	Mean concentration ($n = 15$) (ng/ml)	Intra-day precision ($n = 5$) (%)	Inter-day precision ($n = 15$) (%)	Intra-day accuracy ($n = 5$) (%)	Inter-day accuracy ($n = 15$) (%)
LLOQ (10.0)	9.26	12.2	11.6	82.1	92.6
QC_{LOW} (30.0)	29.27	5.2	2.8	94.5	97.6
QC_{MED} (100.0)	99.59	4.4	0.0	98.7	99.6
QC_{HIGH} (150.0)	154.93	3.9	2.9	107.1	103.3
ULOQ (200.0)	191.62	4.2	3.7	92.1	95.8

Abbreviations: LLOQ, lower limit of quantification; QC_{LOW}, low quality control; QC_{MED}, middle quality control; QC_{HIGH}, high quality control; ULOQ, upper limit of quantification.

TABLE A3 Short-term stability of AZ5104 at room temperature, at 4°C and in the autoinjector in different matrices

Temperature (°C)	Matrix	Time (h)	Accuracy QC_{LOW} (%)	Accuracy QC_{MED} (%)	Accuracy QC_{HIGH} (%)
RT	Whole blood	4	79.0	81.1	84.7
RT	Whole blood	8	75.2	70.3	71.3
RT	Whole blood	24	48.9	38.0	37.2
RT	EDTA plasma	4	98.1	90.6	93.6
RT	EDTA plasma	8	80.5	85.8	84.2
RT	EDTA plasma	24	49.1	57.1	57.4
RT	Citrate plasma	4	62.8	72.4	74.8
RT	Citrate plasma	8	38.2	53.7	58.6
RT	Citrate plasma	24	6.6	14.4	20.7
RT	Serum	4	80.6	82.1	89.3
RT	Serum	8	69.4	64.5	72.1
RT	Serum	24	21.7	27.1	33.2
4	Whole blood	4	90.6	96.2	96.8
4	Whole blood	8	93.6	86.4	89.1
4	Whole blood	24	88.1	78.1	83.3
4	EDTA plasma	4	100.7	97.8	98.4
4	EDTA plasma	8	101.1	94.3	97.3
4	EDTA plasma	24	89.3	90.2	91.2
4	Citrate plasma	4	98.2	101.9	96.1
4	Citrate plasma	8	93.0	93.5	94.6
4	Citrate plasma	24	83.3	79.6	88.0
4	Serum	4	97.3	93.6	95.8
4	Serum	8	95.1	85.5	88.8
4	Serum	24	79.3	81.5	91.5
10	Citrate plasma	24	94.6	101.8	104.0

Abbreviations: RT, room temperature; QC_{LOW}, low quality control; QC_{MED}, middle quality control; QC_{HIGH}, high quality control. Results are mean concentration compared with nominal value. All concentrations were analyzed in duplicate.
[a]Stability in autoinjector for 24 h.

TABLE A4 Long-term stability of AZ5104 at −80°C in different matrices

Temperature (°C)	Matrix	Time (weeks)	Accuracy QC_{LOW} (%)	Accuracy QC_{MED} (%)	Accuracy QC_{HIGH} (%)
−80	EDTA plasma	2	96.0	93.5	96.7
−80	EDTA plasma	4	89.2	92.5	100.4
−80	Citrate plasma	2	96.8	108.4	103.2
−80	Citrate plasma	4	101.4	102.7	99.4
−80	Serum	2	100.8	86.0	98.0
−80	Serum	4	102.0	98.4	101.7

Abbreviations: RT, room temperature; QC_{LOW}, low quality control; QC_{MED}, middle quality control; QC_{HIGH}, high quality control. Results are mean concentration compared with nominal value. All concentrations were analyzed in duplicate.