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RESEARCH ARTICLE

Development and validation of a liquid chromatography–tandem mass spectrometry analytical method for the therapeutic drug monitoring of eight novel anticancer drugs

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Abstract

To support therapeutic drug monitoring of patients with cancer, a fast and accurate method for simultaneous quantification of the registered anticancer drugs afatinib, axitinib, ceritinib, crizotinib, dabrafenib, enzalutamide, regorafenib and trametinib in human plasma using liquid chromatography tandem mass spectrometry was developed and validated. Human plasma samples were collected from treated patients and stored at −20°C. Analytes and internal standards (stable isotopically labeled analytes) were extracted with acetonitrile. An equal amount of 10 mM NH_4CO_3 was added to the supernatant to yield the final extract. A 2 µL aliquot of this extract was injected onto a C_{18}-column, gradient elution was applied and triple-quadrupole mass spectrometry in positive-ion mode was used for detection. All results were within the acceptance criteria of the latest US Food and Drug Administration guidance and European Medicines Agency guidelines on method validation, except for the carry-over of ceritinib and crizotinib. These were corrected for by the injection order of samples. Additional stability tests were carried out for axitinib and dabrafenib in relation to their reported photostability. In conclusion, the described method to simultaneously quantify the eight selected anticancer drugs in human plasma was successfully validated and applied for therapeutic drug monitoring in cancer patients treated with these drugs.

KEYWORDS

anticancer drugs, clinical application, LC-MS/MS, therapeutic drug monitoring, validation

1 | INTRODUCTION

The group of personalized anticancer drugs is an expanding one (US Food and Drug Administration, 2014, 2015, 2016). Most of the recently developed and approved anticancer drugs are destined for oral ingestion. The bioavailability of this drug class is often low and variable leading, usually in combination with other complicating factors, to a variation in drug levels and exposure (Gao et al., 2012; Herbrink, Nuijen, Schellens, & Beijnen, 2015; Lankheet et al., 2014; Terada, Noda, & Inui, 2015; Widmer et al., 2014). This may lead to insufficient efficacy or substantial toxicity (Kato et al., 2016; Verheijen et al., 2017).

For a large number of compounds, a relation between plasma concentration and efficacy of therapy has been established. For others, this remains to be demonstrated. Where such a relation is proven for drugs used in the clinic, the need arises for the integration of this knowledge into clinical practice. With this purpose, therapeutic drug monitoring (TDM) is a useful tool in the therapy of patients with cancer (Yu et al., 2014).

TDM aims to individualize drug dosing by focusing on balancing the therapeutic efficacy and the avoidance of drug toxicity. This is accomplished by quantifying drug concentrations in patient blood plasma or serum and by comparing the results with predetermined guidelines and target levels (Yu et al., 2014).

Our laboratory previously reported a TDM assay for the quantification of 11 oral anticancer compounds for which there was a demand

Abbreviations: DMSO, dimethylsulfoxide; TDM, therapeutic drug monitoring.

from the clinic (Herbrink et al., 2016). The clinical requirement for TDM analyses has grown to entail a larger variety of anticancer compounds. To suit these needs, an additional TDM assay was developed and validated for the quantification of the promising oral drugs afatinib, axitinib, ceritinib, crizotinib, dabrafenib, enzalutamide, regorafenib and trametinib in human plasma. These targeted drugs are indicated for a variety of tumor types (Table 1) and all exhibit variability in pharmacokinetics. The drugs are frequently used and a rapid, robust and adequately ranged combination assay has not been reported in literature as far as we know.

Guidelines and target levels for the TDM of the eight compounds are few in number; however, information on pharmacokinetic targets that may be used in the clinical practice is provided by clinical studies, literature reviews and approval documents (European Medicines Agency, 2015; Falchook et al., 2014; Infante et al., 2012; Locati et al., 2014; Ouellet et al., 2014, 2016; Rini et al., 2015; Scher et al., 2010; US Food and Drug Administration, 2011, 2013a).

The concentration ranges of the assay were chosen to enable a short analysis-evaluation time and to ensure a swift feedback to the clinic whilst taking the plasma trough levels into account. The validation parameters were tested according to the US Food and Drug Administration and European Medicines Agency guidelines (European Medicines Agency, & Use, CHMP., 2011; US Food and Drug Administration, 2013b), with the following modifications: (1) four nonzero calibration standards were used in the calibration curve instead of six to eight; and (2) the quality control low concentration sample was replaced with a QC lower limit of quantification (LLOQ) sample for the determination of the inaccuracy and imprecision. These adaptations made it possible to develop and validate a method that is applicable in time-limited daily clinical routine.

TABLE 1 Trivial names, molecular formulas and molecular masses of the assay analytes

Trivial name	Primary indication(s)	Primary target(s)	Molecular formula	Molecular mass (Da)
Afatinib	NSCLC	EGFR	$C_{24}H_{25}ClFN_5O_3$	485.9
Axitinib	RCC	VEGFR, PDGFR	$C_{22}H_{18}N_4OS$	386.5
Ceritinib	NSCLC after treatment with crizotinib	MEK	$C_{28}H_{36}ClN_5O_3S$	558.1
Crizotinib	NSCLC	MEK	$C_{21}H_{22}Cl_2FN_5O$	450.3
Dabrafenib	Melanoma	B-Raf	$C_{23}H_{20}F_3N_5O_2S_2$	519.6
Enzalutamide	PC	Androgen receptor	$C_{21}H_{16}F_4N_4O_2S$	464.4
Regorafenib	CRC, GIST	VEGFR, PDGFR	$C_{21}H_{17}ClF_4N_4O_4$	482.8
Trametinib	Melanoma, NSCLC	MEK	$C_{16}H_{23}FIN_4O_4$	615.4

B-raf, v-raf murine sarcoma viral oncogene homolog B1; EGFR, endothelial growth factor receptor; GIST, gastro-instestinal stromal tumors; CRC, colorectal cancer; MEK, mitogen activated protein kinase; NSCLC, nonsmall cell lung cancer; PC, prostate carcinoma; PDGFR, platelet derived growth factor receptor; RCC, renal cell carcinoma; VEGFR, vascular endothelial growth factor receptor.

This article describes the development, validation and application of the quantitative analysis of eight anticancer compounds in human plasma for TDM purposes and is the first to do so for these compounds. Special attention was directed to the light sensitivity of axitinib and dabrafenib as reported earlier (Nijenhuis, Haverkate, Rosing, Schellens, & Beijnen, 2016; Sparidans, Iusuf, Schinkel, Schellens, & Beijnen, 2009). Plasma samples were subjected to protein precipitation and were subsequently analyzed using a high-performance liquid chromatography (HPLC) system coupled to a tandem mass spectrometry detection. The method is now routinely used in our laboratory for TDM of patients treated with any of these drugs. Plasma levels are directly reported to hospital pharmacists who relate the levels to pharmacokinetic targets. A case-to-case dosage advice is subsequently discussed with the treating oncologist.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

Afatinib, axitinib, ceritinib, crizotinib, dabrafenib, enzalutamide, regorafenib, trametinib, afatinib-$^{13}C_6$, axitinib-$^{13}C,^2H_3$, ceritinib-$^2H_7$, crizotinib-$^{13}C_2,^2H_5$, dabrafenib-$^2H_9$, enzalutamide-$^2H_6$, regorafenib-$^{13}C,^2H_3$ and trametinib-$^{13}C_6$ were purchased from AlsaChim (Illkirch, France). Acetonitrile and water (HPLC grade), used to prepare mobile phases, along with methanol for stocks and working solutions were obtained from Biosolve Ltd (Valkenswaard, The Netherlands). Dimethylsulfoxide (DMSO) for stocks and ammonium hydrogen carbonate, used for the mobile phases, were obtained from Merck (Darmstadt, Germany). K_2EDTA plasma was obtained from the Medical Center Slotervaart (Amsterdam, the Netherlands).

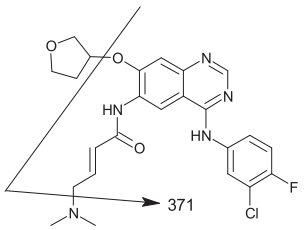
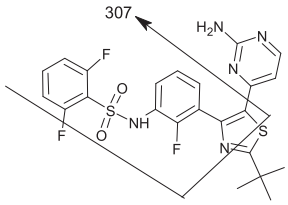
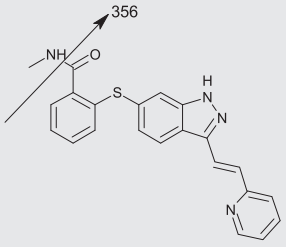
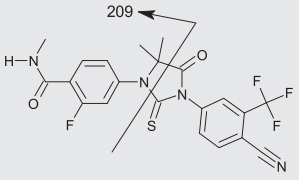
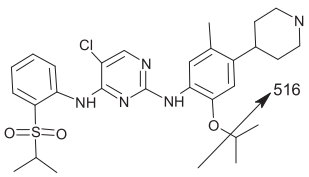
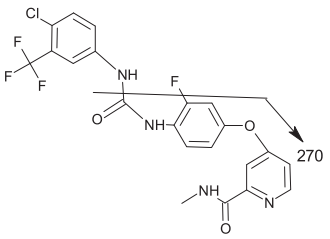
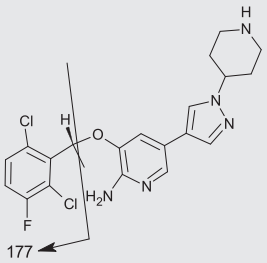
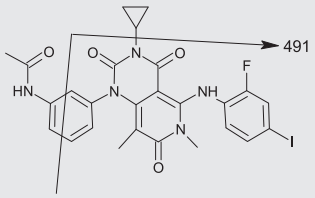
2.2 | Stock solutions, calibrations standards and quality control samples

Stock solutions containing axitinib and dabrafenib were stored in amber-colored containers. The stock of axitinib was prepared in methanol in which it is less soluble than in DMSO, but more stable (Pfizer Inc., 2014; Sparidans et al., 2009). Separate stock solutions for calibration standards and quality control samples were prepared in methanol or DMSO, see Table 3. Stock solutions of the internal standards were prepared in the same medium and concentration as their analyte equivalents. The IS working solution was prepared in methanol at concentrations of 125 ng/mL for afatinib-^{13}C6, axitinib-$^{13}C,^2H_3$, dabrafenib-$^2H_9$ and trametinib-$^{13}C_6$ and 3125 ng/mL for ceritinib-$^2H_7$, crizotinib-$^{13}C_2$, enzalutamide-$^2H_6$ and regorafenib-$^{13}C,^2H_3$. All stock and working solutions were stored at −20°C.

Working solutions were prepared to spike the calibration and quality control plasma samples containing the highest concentration of analytes. This was done to avoid adsorption and stability issues that may occur with axitinib and dabrafenib at lower concentrations in organic solvents. The calibration standard working solution and the quality control sample working solution both contained 4000 ng/mL of afatinib, axitinib, dabrafenib and trametinib and 100,000 ng/mL of ceritinib, crizotinib, enzalutamide and regorafenib.

TABLE 2 Mass spectrometric settings for the analytes and their internal standards and the proposed mass fragmentation pathways

Parameter	Setting
Run duration	8.0 min
Ion spray voltage	5500 kV
Collision gas	6.0 psi
Curtain gas	30 psi
Turbo gas	30 psi
Temperature	500 °C

Analyte Parameter	Setting	Proposed fragmentation pathway	Analyte Parameter	Setting	Proposed fragmentation pathway
Afatinib **Afatinib-$^{13}C_6$** Retention time Collision energy Declustering potential Collision exit potential	486.1 → 371 492.3 → 377.1 4.7 min 37 V 96 V 26 V		**Dabrafenib** **Dabrafenib-$^{2}H_9$** Retention time Collision energy Declustering potential Collision exit potential	520.2 → 307.3 529.2 → 316.3 3.9 min 44 V 116 V 20 V	
Axitinib **Axitinib-$^{13}C,^{2}H_3$** Retention time Collision energy Declustering potential Collision exit potential	387.0 → 327.9 391.3 → 356.1 2.3 min 17 V 50 V 28 V		**Enzalutamide** **Enzalutamide-$^{2}H_6$** Retention time Collision energy Declustering potential Collision exit potential	465.0 → 209.0 471.2 → 215.2 4.1 min 25 V 131 V 14 V	
Ceritinib **Ceritinib-$^{2}H_7$** Retention time Collision energy Declustering potential Collision exit potential	558.2 → 516.1 565.4 → 517.3 5.6 min 25 V 116 V 14 V		**Regorafenib** **Regorafenib-$^{13}C,^{2}H_3$** Retention time Collision energy Declustering potential Collision exit potential	483.1 → 270.1 487.0 → 274.1 4.8 min 30 V 136 V 18 V	
Crizotinib **Crizotinib-$^{13}C_2,^{2}H_5$** Retention time Collision energy Declustering potential Collision exit potential	450.1 → 177.0 457.3 → 177.1 4.4 min 35 V 81 V 20 V		**Trametinib** **Trametinib-$^{13}C_6$** Retention time Collision energy Declustering potential Collision exit potential	615.9 → 490.9 622.2 → 497.0 4.6 min 45 V 90 V 12 V	

The calibration standard containing the highest concentration of analytes was spiked directly after the preparation of the working solutions. A volume of 50 µL of calibration standard working solution was added to 950 µL control human K_2EDTA plasma. The highest standard was subsequently diluted in control human K_2EDTA plasma to obtain the target calibration concentrations as listed in Table 3. The quality control (QC) plasma sample containing the highest concentration of analytes was spiked using a separately prepared working solution. This QC sample (QC high) was further diluted in control human K_2EDTA plasma to obtain QC mid and QC low (Table 3). Both the calibration standards and quality control samples were stored in 50 µL aliquots at −20°C.

2.3 | Sample preparation

Directly after sample collection by venipuncture in the clinic, the blood samples were centrifuged for 10 min at 2000 ***g*** at 4°C. After centrifugation, the plasma was isolated and stored in amber containers at −20°C pending analysis. Prior to processing, the samples were thawed at 20–25°C and a 50 µL aliquot was transferred to an amber-colored

TABLE 3 Concentrations of analytes in stock solutions, calibration and quality control plasma samples

Analyte	Stock (mg/mL)	Calibration (ng/mL)	Quality control (ng/mL)
Afatinib	1.00 (DMSO)	2; 10; 100; 200	2; 100; 200
Axitinib	0.02 (methanol)	2; 10; 100; 200	2; 100; 200
Ceritinib	1.00 (DMSO)	50; 250; 2500; 5000	50; 2500; 5000
Crizotinib	1.00 (DMSO)	50; 250; 2500; 5000	50; 2500; 5000
Dabrafenib	1.00 (DMSO)	2; 10; 100; 200	2; 100; 200
Enzalutamide	1.00 (DMSO)	50; 250; 2500; 5000	50; 2500; 5000
Regorafenib	1.00 (DMSO)	50; 250; 2500; 5000	50; 2500; 5000
Trametinib	0.10 (DMSO)	2; 10; 100; 200	2; 100; 200

DMSO, Dimethylsulfoxide.

container of 1.5 mL. A volume of 20 µL of IS working solution was added to the plasma sample. To precipitate plasma proteins and to extract the analytes from the biomatrix, a volume of 150 µL of acetonitrile was added. The samples were then vortex-mixed and centrifuged (10 min at 20°C and 23,100 ***g***). A 100 µL aliquot was subsequently added to an autosampler vial that contained 100 µL 10 mM ammonium bicarbonate in water. The dilution factor of sample to final extract is 8.8, yielding 0.114 mL sample per milliliter of final extract. The final extract was vortex-mixed and stored at 2–8°C until analysis.

2.4 | Liquid chromatography–tandem mass spectrometry

Chromatographic separation was carried out using an UPLC Acquity I Class binary pump with integrated degasser, an UPLC Acquity FTN autosampler and an UPLC Acquity Column CH-A heater (all Waters, Milford, MS, USA). The autosampler temperature was kept at 4°C and the column heater at 40°C. The mobile phase A consisted of 10 mM ammonium bicarbonate in water and mobile phase B was 10 mM ammonium bicarbonate in methanol–water (1:9, v/v). A gradient program was used at a flow of 0.250 mL/min through a Gemini C_{18} column (5.0 µm, 50 × 2.0 mm i.d.; Phenomenex, Torrance, CA, USA) with an additional Gemini C_{18} guard column (4 × 2.0 mm i.d.). The gradient program was as follows: 40% B, 250 µL/min (0–0.1 min); 40–100% B, 250 µL/min (0.1–2.0 min); 100% B, 250 µL/min (2.0–4.0 min); 100–40% B, 250 µL/min (4.0–4.01 min); 40% B, 500µL/min (4.01–4.5 min); 40% B, 250 µL/min (4.5–5.0 min). The divert valve directed the flow to the mass spectrometer between 2.0 and 5.0 min; during the remainder of the run the eluent was directed to the waste container. Table 2 lists the typical retention times.

The drugs were analyzed using an API5500 quadrupole mass spectrometer (MS) (Sciex, Thornhill, ON, Canada). This instrument is equipped with a turbo ion spray interface, operating in positive ion mode and configured in multiple reaction monitoring (MRM) mode. The LC-MS/MS data were acquired and processed with Analyst™ software version 1.5.2 (Sciex). Table 2 summarizes the MS operating parameters.

2.5 | Validation procedure

The assay was fully validated for calibration model, accuracy and precision, LLOQ, sensitivity and selectivity, dilution integrity, carry-over, matrix effect and stability. The validation was based on the US Food and Drug Administration and latest European Medicines Agency guidelines on bioanalytical method validation.

The accuracy is expressed as the bias and the following equations were used:

$$\text{Intra–assay bias } (\%) = 100\%\times (\text{mean measured concentration per run} - \text{nominal concentration})/(\text{nominal concentration}) \quad (1)$$

$$\text{Inter–assay bias } (\%) = 100\%\times (\text{overall mean measured concentration} - \text{nominal concentration})/(\text{nominal concentration}) \quad (2)$$

The precision is expressed as the coefficient of variation (CV) and the following equations were used:

$$\text{Intra–assay CV } (\%) = 100\%\times (\text{SD of the measured concentration per run})/(\text{mean measured concentration per run}) \quad (3)$$

$$\text{Inter–assay CV } (\%) = \frac{\sqrt{\frac{\left(\frac{s^2_{overall}/\left((n_1+\ldots+n_a-1)-\left((n_1-1)s_1^2+\ldots+\left((n_a-1)s_a^2\right)\right)\right)}{a-1}\right)-\left(\frac{(n_1-1)s_1^2+\ldots+(n_a-1)s_a^2}{n_1+\ldots+n_a-a}\right)}{\left(\frac{n}{a}\right)}}}{\text{mean of runs}} \text{Inter–assay CV } (\%)$$
$$= \frac{\sqrt{\frac{\left(\frac{s^2_{overall}/\left((n_1+\ldots+n_a-1)-\left((n_1-1)s_1^2+\ldots+\left((n_a-1)s_a^2\right)\right)\right)}{a-1}\right)-\left(\frac{(n_1-1)s_1^2+\ldots+(n_a-1)s_a^2}{n_1+\ldots+n_a-a}\right)}{\left(\frac{n}{a}\right)}}}{\text{mean of runs}} \quad (4)$$

where s is the standard deviation, n is the number of replicates and a is the number of runs.

The matrix factor (MF) was determined using the following equation:

$$\text{MF } (-) = [\text{area of processed blank sample spiked with neat solution (matrix present)}]/[\text{area of neat solution(matrix absent)}] \quad (5)$$

$$\text{Resolution } (-) = (\text{difference in retention time})/0.5\times(\text{sum of base widths}) \quad (6)$$

2.6 | Photodegradation of axitinib and dabrafenib

Special attention was given to the light-sensitivity of axitinib and dabrafenib. Sparidans et al. showed that axitinib exhibits light-mediated *trans* to *cis* isomerization (Sparidans et al., 2009). Nijenhuis et al. reported light instability of dabrafenib in organic solvents (Nijenhuis et al., 2016). The stability of these two analytes in plasma in both light and dark environments was investigated. Human K_2EDTA control plasma was spiked at both QC low and QC high levels and was stored at ambient temperature in both light and dark environments for 48 consecutive hours to simulate possible light exposure during transport from the clinic to the laboratory. Samples from both methods were analyzed to investigate whether isomerization or degradation had occurred.

2.7 | Clinical application

The purpose of this assay was to facilitate TDM of afatinib, axitinib, ceritinib, crizotinib, dabrafenib, enzalutamide, regorafenib and trametinib in treated patients with cancer. As part of routine clinical care, K_2EDTA blood samples (4 mL) were collected from patients who were treated with any of the drugs at the Antoni van Leeuwenhoek – the Netherlands Cancer Institute. The plasma samples were collected and processed as described in this report.

3 | RESULTS AND DISCUSSION

3.1 | Development

3.1.1 | Mass spectrometry

Mass spectrometric parameters were optimized for each analyte by performing direct infusion of the analytes. General settings were chosen to maximize the response of trametinib, which was the lowest responder and owing to the fact that it shows the lowest target concentrations. The molecular ions ($M + H^+$) that were observed and used to generate product ion spectra are listed in Table 3. To achieve high selectivity and sensitivity, MRM scan mode was utilized to monitor and select the mass transition for each analyte that yielded the product ion with the highest abundance (Table 3). The optimal settings, however, led to the nonlinearity of the calibration model for ceritinib, dabrafenib and enzalutamide owing to saturation of the detector. To prevent this over-response, the collision energy of the analytes was lowered from 35 to 25, 47 to 44 and 41 to 25 for ceritinib, dabrafenib and enzalutamide, respectively. Figure 1 presents the chromatograms of eight analytes in a single sample, indicating the capability of simultaneous detection.

3.1.2 | HPLC

The chromatographic setup that was used in a previously reported TDM assay was taken as the starting point for this assay. This system used a Gemini C_{18} column and a gradient program that consisted of 10 mM NH_4OH in water and 1 mM NH_4OH in methanol. Afatinib, axitinib and trametinib all responded poorly under these conditions and the chromatographic peaks of crizotinib and regorafenib in the chromatogram were broad and tailing was observed. The poor response of trametinib could be improved by increasing the injection volume to 2 µL. The increase in response of afatinib and axitinib was not satisfactory. The mobile phases were subsequently replaced by the bicarbonate system as mentioned in the 'Chemicals and reagents' section. This resulted in improved peak shapes and sensitivity for afatinib and axitinib.

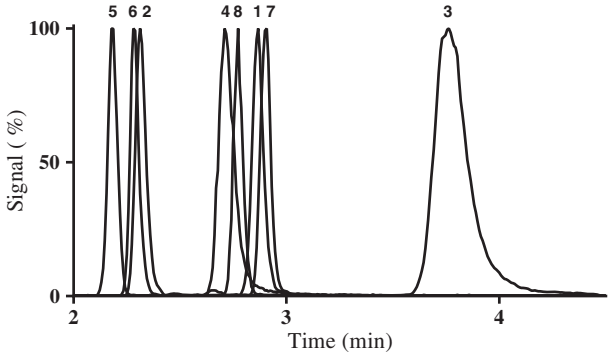


FIGURE 1 Representative normalized multiple reaction monitoring (MRM) chromatograms of spiked human control K2-EDTA plasma at QC mid concentrations: Afatinib (1; 100 ng/mL), axitinib (2; 100 ng/mL), ceritinib (3; 2500 ng/mL), crizotinib (4; 2500 ng/mL), dabrafenib (5; 100 ng/mL), enzalutamide (6; 2500 ng/mL), regorafenib (7; 2500 ng/mL) and of trametinib (8; 100 ng/mL)

3.2 | Validation procedures

3.2.1 | Calibration model

Calibration standards were prepared and analyzed in duplicate in three analytical runs. The linear regression of peak area ratios vs the

TABLE 4 Assay performance data for the analysis of 8 anti-cancer drugs in human plasma

		Intra-assay		Inter-assay		
Analyte	Nominal concentration (ng/mL)	Bias[a] (%)	CV[a] (%)	Bias (%)	CV (%)	Matrix factor (–) (CV, %)
Afatinib	2	±10	≤9.1	3.6	3.9	1.03 (7.9)
	100	±3.5	≤5.7	−1.8	2.1	
	200	±3.7	≤4.3	1.8	1.8	0.85 (5.3)
Axitinib	2	±4.4	≤10.0	−4.2	—[a]	1.01 (7.1)
	100	±2.6	≤2.5	−2.1	—[a]	
	200	±5.4	≤2.0	1.0	3.7	1.05 (8.7)
Ceritinib	50	±16	≤7.5	−3.1	11.4	1.02 (13.3)
	2500	±4.1	≤4.6	0.1	3.7	
	5000	±4.3	≤2.6	4.1	—[a]	1.07 (4.5)
Crizotinib	50.3	±7.8	≤2.2	−3.8	3.6	1.04 (2.7)
	2520	±3.7	≤1.2	1.8	2.1	
	5030	±1.8	≤2.1	−0.6	1.6	0.93 (4.1)
Dabrafenib	2	±10.9	≤11.6	−7.1	—[a]	0.97 (14.5)
	100	±1.0	≤4.9	0.4	—[a]	
	200	±4.4	≤3.8	2.4	1.7	1.12 (10.7)
Enzalutamide	49.9	±6.8	≤5.5	−2.2	5.4	1.13 (8.6)
	2500	±6.5	≤4.9	−3.1	2.7	
	4990	±1.0	≤2.9	0.2	—[a]	1.00 (13.5)
Regorafenib	50.2	±3.9	≤2.3	2.0	2.8	1.05 (8.6)
	2510	±1.3	≤2.0	−0.6	0.9	
	5020	±0.9	≤1.5	0.5	—[a]	0.98 (8.3)
Trametinib	2	±6.7	≤7.5	1.7	3.4	0.95 (7.7)
	100	±1.0	≤2.0	0.2	—[a]	
	200	±6.0	≤2.3	2.1	3.2	0.99 (11.5)

[a]The inter-run precision could not be calculated because there is no significant additional variation owing to the performance of the assay in different runs.

concentration (x) was weighted ($1/x^2$) to obtain the lowest absolute total bias and the most constant bias across the range. The calibration range for afatinib, axitinib, dabrafenib and trametinib was 2.0–200 ng/mL and for ceritinib, crizotinib, enzalutamide and regorafenib it was 50–5000 ng/mL. Calibration curves were accepted if 75% of the nonzero calibration standards, including a LLOQ and an upper limit of quantification standard (ULOQ), had a bias within ±15% of the nominal concentration (±20% for the LLOQ). All calibration curves (n = 3) of the analytes met these criteria and correlation coefficients (r^2) were 0.996 or better.

3.2.2 | Accuracy and precision

To assess the accuracy and precision of the assay, five replicates of QC low (= LLOQ), QC mid and QC high in plasma were analyzed in three analytical runs. Table 4 summarizes the intra- and inter-assay accuracies and precisions of the assay. The biases and CVs were within the acceptance criteria (within ±20% and ≤20%, respectively, at the low level and within ±15% and ≤15% at the other QC levels).

3.2.3 | Lower limit of quantification

The analyte responses at the low were at least 5 times the blank response in three validation runs. The lowest signal-to-noise ratio was 7 for ceritinib. Figure 2 shows representative ion chromatograms of all eight analytes in QC low samples and double blank samples.

3.2.4 | Selectivity and sensitivity

Six different batches of control human K_2EDTA plasma were spiked at the LLOQ level to investigate the selectivity and the sensitivity. Single determinations were performed. The mean deviations from the nominal concentrations from the assay were all within ±20% with CV values ≤20%. There were no peaks observed with areas ≥20% of the LLOQ in double blank samples of these batches for any of the analytes and also

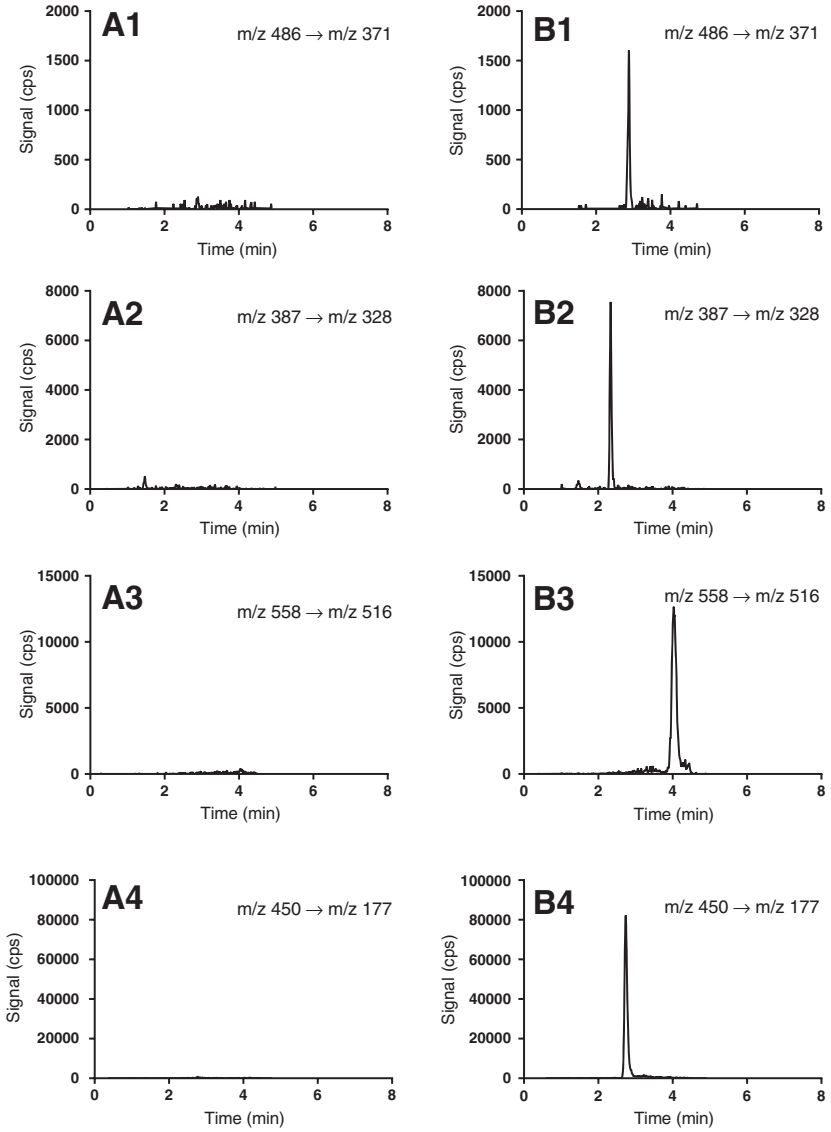


FIGURE 2 MRM chromatograms of a blank sample (A-series) and spiked QC sample at the LLOQ (B-series): Afatinib (1; 2 ng/mL), axitinib (2; 2 ng/mL), ceritinib (3; 50 ng/mL), crizotinib (4; 50 ng/mL), dabrafenib (5; 2 ng/mL), enzalutamide (6; 50 ng/mL), regorafenib (7; 50 ng/mL) and of trametinib (8; 2 ng/mL)

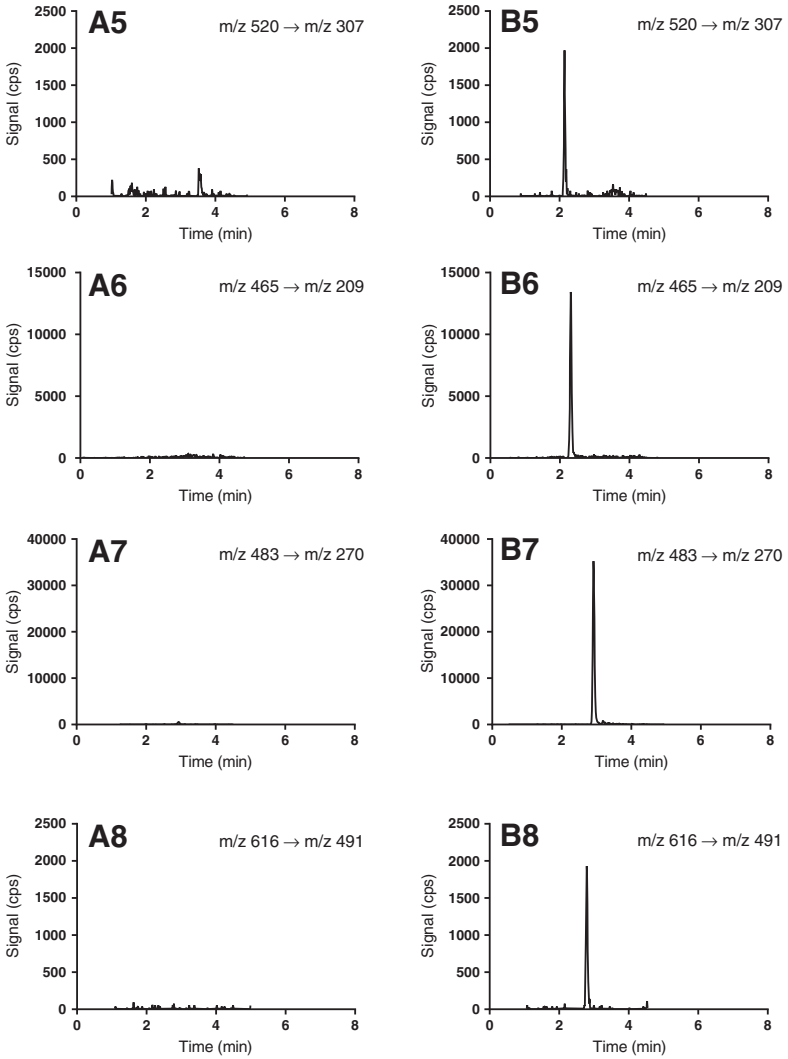


FIGURE 2 Continued.

no interferences were detected at the retention times of the internal standards for both assays. Selectivity was therefore considered acceptable.

Cross-analyte and internal standard interference was tested by spiking control human plasma at ULOQ level with all analytes and IS at nominal concentrations separately. The cross analyte and internal standard interference at the retention times of all analytes was <20% of the peak area of the LLOQ level of the respective analytes. For the internal standard the interference was <5%. For all analytes it was found that the cross analyte and internal standard interference was acceptable.

In order to investigate the photostability of axitinib, the separation between the stereoisomers of axitinib was tested. Figure 3 presents a characteristic chromatogram of a light-exposed axitinib methanolic sample with a resolution of 6. The assay was thus capable of separating the two stereoisomers.

3.2.5 | Dilution integrity

Plasma samples of enzalutamide have to be diluted 10-fold to ensure quantification within the calibration range. Five replicate plasma samples of enzalutamide at QC>ULOQ level were diluted 10-fold with control human plasma (10 µL was added to 90 µL control human matrix). The intra-assay bias and CVs were −4.0 and 4.2%, respectively. The bias and CV were within ±15 and ≤15%, which indicates that the study samples can be diluted 10 times in control matrix with acceptable accuracy and precision.

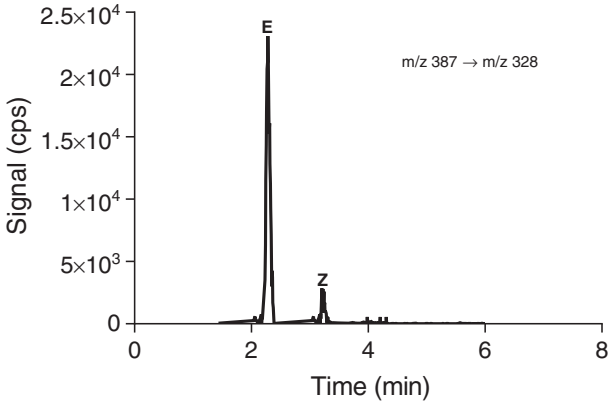


FIGURE 3 MRM chromatogram of an axitinib sample processed from a methanolic solution exposed to light in a transparent container for 2 h: The *E*-isomer (retention time = 2.3 min) and the formed *Z*-isomer (retention time = 3.2 min)

TABLE 5 Recommended doses and plasma concentrations of the analytes in patient samples of patients treated with the drugs (n = 10)

Drug	Recommended dose (mg) (US Food and Drug Administration, n.d.)	Mean plasma concentration (ng/mL)	Minimum – maximum concentration (ng/mL)	Calibration range (ng/mL)
Afatinib	40 o.d.	43.3	10.7–120	2–200
Axitinib	5 b.d.	19.0	7.57–34.9	2–200
Ceritinib	750 o.d.	1195	333–2,630	50–5000
Crizotinib	250 b.d.	227	139–458	50–5000
Dabrafenib	150 b.d.	102	14.1–191	2–200
Enzalutamide[a]	160 o.d.	10,293	7140–12,200	50–5000
Regorafenib	160 o.d.	1118	178–2030	50–5000
Trametinib	2 o.d.	8.40	5.77–11.6	2–200

o.d., once daily; b.d., bi-daily.

[a]Enzalutamide patient samples are diluted 10-fold prior to analysis.

3.2.6 | Carry-over

Carry-over was investigated by injecting two double blank samples after a ULOQ sample in three analytical runs. Eluting peaks with areas >20% of the LLOQ were only observed in blank samples injected after ULOQ samples of ceritinib and crizotinib. However, carry-over into the second double blank samples was ≤20% of the LLOQ level. Therefore, samples containing these analytes should not be grouped. In this way, the carry-over will not have an impact on the integrity of the data.

3.2.7 | Matrix factor

The internal standard-normalized matrix factor was determined in six plasma batches, at QC low and QC high concentrations. Single determinations were performed. Processed blank samples were spiked with working solutions and compared with matrix-free solutions. The CV of the internal standard-normalized matrix factor was ≤15% for all analytes at both concentration levels (Table 4). The acceptance criteria were thus met. The average values of the internal standard-normalized matrix factors are listed in Table 4 and are ~1. These results indicate that the stable isotopes as internal standards are most effective in compensating for matrix effects.

3.2.8 | Stability

All analytes were found to be stable in plasma at −20°C for at least 1 month and after three freeze (−20°C)-and-thaw (20–25°C) cycles. Stability was also demonstrated for all analytes in plasma for at least 48 h at 20–25°C in amber-colored containers. In final extracts, stability was demonstrated for at least 48 h at 4°C. Reinjection reproducibility results showed that an entire analytical run can be reanalyzed after 48 h when kept at 2–8°C. Stock solution are stable for at least 5 months at −20°C and stability tests are still ongoing.

3.2.9 | Photodegradation of axitinib and dabrafenib

The isomerization under influence of light of axitinib was monitored using the chromatographic system described above. Axitinib and dabrafenib were found to be light-stable in plasma at 20–25°C for at least 48 h in transparent containers. Axitinib showed isomerization from the therapeutically active *E*-isomer to the *Z*-isomer under the influence of light in methanolic stock and working solutions (data not shown) as described by Sparidans et al. (2009). The isomerization was not observed in methanolic solutions that were kept protected from light in amber-colored containers. During the sample preparation as presented here, no degradation of dabrafenib was observed.

3.3 | Clinical application

The recommended doses for the drugs are listed in Table 5. To test the applicability of this assay the concentrations were determined in 10 patient samples for each drug of patients treated with the drugs. The results are listed in Table 5. All values are within the validated range of 2.0–200 ng/mL for afatinib, axitinib, dabrafenib and trametinib and 50.0–5000 ng/mL for ceritinib, crizotinib, enzalutamide and regorafenib. These results demonstrate the applicability of this assay for TDM purposes. In clinical practice, plasma level and drug administration data are used in comparison with pharmacokinetic targets to provide an adequate dosage advice to treating oncologists. By doing so, insufficient or excessive exposure is effectively recognized and managed.

4 | CONCLUSION

A sensitive new LC-MS/MS assay for the quantification of afatinib, axitinib, ceritinib, crizotinib, dabrafenib, enzalutamide, regorafenib and trametinib in human plasma was developed and validated. The validated assay ranges from 2.0 to 200 ng/mL for afatinib, axitinib, dabrafenib and trametinib and from 50.0 to 5000 ng/mL for ceritinib, crizotinib, enzalutamide and regorafenib were linear and correlation coefficients (r^2) of 0.996 or better were obtained. Dilution integrity experiments show that samples can be diluted 10-fold for enzalutamide prior to analysis. Both axitinib and dabrafenib are prone to degrade when exposed to light in stock and working solutions but these analytes were shown to be stable in plasma for at least 1 week at 20–25°C.

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