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Validation of an LC-MS/MS assay for rapid and simultaneous quantification of 21 kinase inhibitors in human plasma and serum for therapeutic drug monitoring

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ABSTRACT

Kinase inhibitors have revolutionized cancer treatment in the past 25 years and currently form the cornerstone of many treatments. Due to the increasing evidence for therapeutic drug monitoring (TDM) of kinase inhibitors, the need is growing for new assays to rapidly evaluate kinase inhibitor plasma concentrations. In this study, we developed an LC-MS/MS assay for the rapid and simultaneous quantification of 21 kinase inhibitors. First, a literature search was conducted to ensure that the linear ranges of the analytes were in line with the reported therapeutic windows and/or TDM reference values. Subsequently, the assay was validated according to FDA and EMA guidelines for linearity, selectivity, carry-over, accuracy, precision, dilution integrity, matrix effect, recovery, and stability. The assay was fast, with a short run-time of 2 min per sample. Sample pre-treatment consisted of protein precipitation with methanol enriched with stable isotope-labeled internal standards (SIL-IS), and the mixture was vortexed and centrifuged before sample injection. Separation was achieved using a C18 column (3 µm, 50 × 2.1 mm) with a gradient of two mobile phases (ammonium formate buffer pH 3.5 and acetonitrile). Analyte detection was conducted in positive ionization mode using selected reaction monitoring. The assay was accurate and precise in plasma as well as in serum. Extraction recovery ranged between 95.0% and 106.0%, and the matrix effect was 95.7%-105.2%. The stability of the analytes varied at room temperature and in refrigerated conditions. However, all drugs were found to be stable for 7 days in the autosampler. The clinical applicability of the analytical method (486 analyzed samples between 1 July 2022–1 July 2023) as well as external quality control testing results were evaluated. Taken together, the results demonstrate that the analytical method was validated and applicable for routine analyses in clinical practice.

1. Introduction

The development of kinase inhibitors has changed the therapeutic armamentarium for the treatment of many types of cancer such as haematological and solid malignancies. Kinase inhibitors inhibit uncontrolled cell growth and proliferation by inhibiting protein kinases involved in the proliferation of malignant cells [1]. In recent years, many kinase inhibitors were approved by the European Medicines Agency (EMA) and Food and Drug Administration (FDA) [2,3].

Kinase inhibitors are generally administered in a fixed oral dosing regimen and are known for their high inter- and intra-individual pharmacokinetic variability [4]. High exposure variability of kinase inhibitors can be the result of several factors. First, fixed dosing of kinase inhibitors, primarily based on the maximum tolerated dose (MTD) investigated in early phase I clinical trials [4], can introduce under- or overexposure in individual patients [4]. Second, kinase inhibitors can have variable absorption that depends on fasting state and stomach pH [5,6]. Furthermore, the main metabolic pathway for most kinase

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inhibitors depends on cytochrome P450 (CYP) enzymes, which have several pharmacogenetic polymorphisms and can be affected by a multitude of other drugs. Therefore, variant genotype and drug-drug interactions leading to suboptimal kinase inhibitors blood concentrations are common in clinical practice [5,7,8].

To optimize kinase inhibitor therapy, therapeutic drug monitoring (TDM) can be utilized to tailor the dosing regimen based on blood concentration of the drug in individual patients. An optimal drug blood concentration within the therapeutic window decreases the risk of under- or overexposure, which may potentially lead to therapy failure or toxicity, respectively [9,10].

Although several validated LC-MS/MS analytical methods for kinase inhibitors have been described [11–34], several limitations of these methods remain. For instance, some analytical methods exhibit large matrix effects [26,29], whereas other methods use laborious and time-consuming extraction methods [33,35]. Furthermore, the multiplex analytical methods typically quantify a relatively small number of kinase inhibitors (8–12) or are associated with a relatively long run time of the analysis [32]. Finally, the autosampler stability of the kinase inhibitors is generally not investigated for an extended period of time [23,25,29,33,35]. However, these data are required to substantiate the use of the samples on different days, i.e. during sample retesting or initiating the analysis on a different day than the day of sample preparation.

The objective of this study was to develop and validate a rapid LC-MS/MS analytical method for the simultaneous determination of 21 kinase inhibitors in human plasma and serum. The analytical method must be able to perform TDM of the kinase inhibitors in clinical practice and to aid clinicians in patient-specific dose adjustments and optimizing kinase inhibitors therapy.

2. Methods

2.1. Chemicals and reagents

The following reagents with corresponding suppliers were used in the experiments: afatinib, [$^{13}C_6$] afatinib, dasatinib, [$^{2}H_8$] dasatinib, trametinib, [$^{13}C_6$]trametinib, bosutinib, [$^{2}H_9$] bosutinib formate, ibrutinib, [$^{2}H_5$] ibrutinib, alectinib, [$^{2}H_8$] alectinib, lenvatinib, [$^{2}H_5$] lenvatinib, ponatinib, [$^{2}H_8$] ponatinib, sunitinib, [$^{2}H_{10}$] sunitinib, gefitinib, [$^{2}H_8$] gefitinib, ruxolitinib, [$^{2}H_9$] ruxolitinib, ceritinib, [^{2}H7] ceritinib, osimertinib, [^{13}C,$^{2}H_3$] osimertinib, erlotinib HCl, [$^{13}C_6$] erlotinib HCl, imatinib mesylate, [$^{2}H_8$] imatinib, nilotinib, [$^{13}C_3$,$^{2}H_3$] nilotinib, regorafenib, [^{13}C,$^{2}H_3$] regorafenib, vandetanib, [$^{13}C_6$] vandetanib, sorafenib, [^{13}C,$^{2}H_3$] sorafenib, pazopanib HCl, [^{13}C,$^{2}H_3$] pazopanib HCl, vismodegib, [^{15}C7,$^{2}H_3$] vismodegib (Alsachim, France). DMSO (Merck, Netherlands), Methanol (Biosolve, Netherlands), ammonium formate (Thermo scientific, US). Blank human EDTA plasma was purchased from (BioIVT, US), and serum was purchased from Merck Millipore (S1-liter).

2.2. Drugs, internal standards, and concentration range selection

The choice of kinase inhibitors was made after discussion with medical oncologists, pulmonary oncologists and haematologists. The analytical method was developed to quantify the following 21 kinase inhibitors: afatinib, dasatinib, trametinib, bosutinib, ibrutinib, alectinib, lenvatinib, ponatinib, sunitinib, gefitinib, ruxolitinib, ceritinib, osimertinib, erlotinib, imatinib, nilotinib, regorafenib, sorafenib, vandetanib, pazopanib and vismodegib in clinically relevant concentration ranges for routine TDM. Therefore, the analytical range of every single drug should cover the therapeutic window as well as higher and lower concentrations. The linear range of every drug was based on current guidelines for TDM and pharmacokinetic (PK) studies. Table S1 summarizes the clinically relevant drug concentrations and the rationale for the ranges of the developed analytical method.

The stable isotope labelled on (H) position ([$^{2}H_8$] dasatinib, [$^{2}H_9$] bosutinib formate, [$^{2}H_5$] ibrutinib, [$^{2}H_8$] alectinib, [$^{2}H_5$] lenvatinib, [$^{2}H_8$] ponatinib, [$^{2}H_{10}$] sunitinib, [$^{2}H_8$] gefitinib, [$^{2}H_9$] ruxolitinib [^{2}H7] ceritinib, [$^{2}H_8$] imatinib), on (C) position (afatinib [$^{13}C_6$], [$^{13}C_6$] trametinib, [$^{13}C_6$] erlotinib HCl, [$^{13}C_6$] vandetanib), or on both positions ([^{13}C,$^{2}H_3$] osimertinib, [$^{13}C_3$,$^{2}H_3$] nilotinib, [^{13}C,$^{2}H_3$] regorafenib, [^{13}C,$^{2}H_3$] sorafenib, [^{13}C,$^{2}H_3$] pazopanib HCl, [^{15}C7,$^{2}H_3$] vismodegib) were used for the correcting factor these isotopes have on the matrix effect.

2.3. Literature search for LC-MS/MS methods

A literature search was performed with the search terms ("Tyrosine Protein Kinase Inhibitors"[Mesh] OR TKI [tiab] OR TKIs [tiab] OR tyrosine kinase inhibitor* [tiab]) AND ("Tandem Mass Spectrometry"[Mesh] OR LC-MS/MS [tiab]) to search for published analytical methods for the TDM of kinase inhibitors. The literature search yielded 25 research papers; the method properties, such as the analysed kinase inhibitors, analytical range, extraction/preparation method and time, and sample run time were summarized in Table S2.

2.4. Preparation of stock solutions and working standards

Kinase inhibitor solutions were prepared from stock solutions of the following drug concentrations in dimethyl sulfoxide (DMSO). Afatinib, bosutinib, dasatinib and trametinib: 1,000 mg/l. Pazopanib and vismodegib: 10,000 mg/l. Ibrutinib, alectinib, lenvatinib, ponatinib, sunitinib, gefitinib, ruxolitinib, ceritinib, osimertinib, erlotinib, imatinib, nilotinib, regorafenib, sorafenib, and vandetanib: 5,000 mg/l. kinase inhibitors solutions were then diluted serially in DMSO. Subsequently, the solutions were spiked with plasma to obtain the end concentrations.

The calibration curves had the following ranges. Afatinib, dasatinib, trametinib: 1–100 ng/ml. Bosutinib and ruxolitinib: 1–500 ng/ml. Ibrutinib, alectinib, lenvatinib, ponatinib, sunitinib, gefitinib, ceritinib, osimertinib: 10–2,000 ng/ml. Erlotinib, imatinib, nilotinib, regorafenib, and vandetanib: 100–5,000 ng/ml. Sorafenib: 100–10,000 ng/ml. Pazopanib and vismodegib: 500–50,000 ng/ml.

Stock solutions of the internal standards were diluted in methanol in the following concentrations to obtain the working internal standards (IS): [$^{13}C_6$] afatinib, [$^{2}H_8$] dasatinib, [$^{13}C_6$] trametinib: 5 ng/ml. [^{13}C,$^{2}H_3$] pazopanib HCl and [^{15}C7,$^{2}H_3$] vismodegib: 500 ng/ml. [$^{2}H_9$] bosutinib formate, [$^{2}H_5$] ibrutinib, [$^{2}H_8$] alectinib, [$^{2}H_5$] lenvatinib, [$^{2}H_8$] ponatinib, [$^{2}H_{10}$] sunitinib, [$^{2}H_8$] gefitinib, [$^{2}H_9$] ruxolitinib, [^{2}H7] ceritinib, [^{13}C,$^{2}H_3$] osimertinib, [$^{13}C_6$] erlotinib HCl, [$^{2}H_8$] imatinib, [$^{13}C_3$,$^{2}H_3$] nilotinib, [^{13}C,$^{2}H_3$] regorafenib, [^{13}C,$^{2}H_3$] sorafenib, and [$^{13}C_6$] vandetanib: 50 ng/ml.

2.5. Chromatography

The analytical method consisted of three different chromatographic methods (method 1, 2, and 3), which only differed in the injection volume. Alectinib, bosutinib, ceritinib, erlotinib, gefitinib, ibrutinib, imatinib, lenvatinib, nilotinib, osimertinib, ponatinib, regorafenib, sorafenib, sunitinib and vandetanib were analysed with method 1. Afatinib, dasatinib, ruxolitinib and trametinib were analysed with method 2. Pazopanib and vismodegib were analysed with method 3. A liquid chromatography (LC) system coupled with a C18 column (3 µm, 50 × 2.1 mm, Hypurity, Thermo Fischer Scientific, USA) was used to carry out the chromatographic separation. The autosampler and the column oven temperatures were set at 10 °C and 60 °C, respectively. Elution gradient was performed using the two mobile phases; A consisting of 0.02 mol/L ammonium formate buffer set at pH 3.5 and mobile phase B consisting of acetonitrile. The elution process was as follows: 0.00 min (10.0% B), 0.000–0.001 min (20.0% B), 0.001–1.550 min (60% B), 1.550–1.600 min (95% B), 1.600–1.950 (95% B), 1.950–2.000 min (10.0 % B). The flow rate was 1.000 ml/min for all three methods. The autosampler

injection volume was 0.5, 2.5, and 0.05 µl for methods 1, 2 and 3, respectively. After each injection, the sampler manager was washed with methanol:water (4:1) mixture for 5 s with a wash speed of 50 µl/s before drawing a subsequent sample.

2.6. Mass spectrometry

The mass spectrometry system consisted of a TSQ Quantiva Triple Quadrupole, Vanquish Autosampler, Vanquish Horizon Binairy Pump, Vanquish Column Compartment, and Vanquish Charger (Thermo Fischer Scientific, USA). H-ESI ion source type was utilized with positive polarity and static spray voltage (positive ion 1500 V). The sheath, auxiliary, and sweep gas pressures were 60, 20, and 0 arbitrary units. The ion transfer tube and vaporizer temperatures were 140 °C and 350 °C, respectively. Selected reaction monitoring was used to detect the analytes and their internal standards. The cycle time was 0.15 sec, Q1 and Q3 resolutions were 0.7 Full Width at Half Maximum (FWHM), and collision-induced dissociation (CID) gas had a value of 1.5 m Torr. Collision energies and retention times can be found in Table 1.

2.7. Sample preparation

100 µl EDTA plasma or serum was added in a 1.5 ml screw vial (Fisher scientific). Subsequently, 500 µl IS was added, after which it was vortexed for 1 min. The mixture was then centrifuged for 5 min at 9500 g and the over-pipetted vials were then transferred to the autosampler. The respective amount to the used method 1, 2 or 3 was then transferred from the vial to the LC-MS/MS system for analysis.

2.8. Method validation

Method validation was carried out according to the EMA and FDA [36,37] guidelines for the validation of bioanalytical methods.

2.8.1. Linearity

The back-calculated concentrations of the calibration standards should fall within 15% of the nominal values. For the lowest limit of quantification (LLQ) concentration level, it should be within 20% of the nominal value. It is required that at least 75% of the calibration standards with a minimum of 6 calibration levels comply with the criteria. In the present study, three calibration curves were prepared with 8–12 data points per data set, covering the range from LLQ to the highest limit of quantification (HLQ). The number of data points and the calibration line range for each drug can be found in Table 2. A weighing factor of 1/x was applied for all calibration curves.

2.8.2. Selectivity & carry-over

The selectivity test was carried out by analyzing the interfering signals in 6 independent blank plasma samples. The interfering signals were compared with the response signal of the IS and the analyte

Table 1
LC-MS/MS parameters for quantification of kinase inhibitors in human plasma and serum, including retention time, precursor and product ion *m/z*, collision energy, and minimum dwell time.

Compound	Retention Time (min)	RT Window (min)	Precursor (*m/z*)	Product (*m/z*)	Collision Energy (V)	Min Dwell Time (ms)
Afatinib	0.67	0.25	486.2	371.0	28	5.911
Afatinib [$^{13}C_6$]	0.67	0.25	492.2	377.1	28	5.911
Alectinib	1.01	0.25	483.3	396.2	25	10.151
Alectinib [$^{2}H_8$]	1.01	0.25	491.4	396.2	25	10.151
Bosutinib	0.92	0.25	530.3	141.2	25	10.135
Bosutinib [$^{2}H_9$]	0.92	0.25	539.3	150.2	25	10.135
Ceritinib	1.42	0.25	558.3	433.1	32	16.339
Ceritinib [$^{2}H_7$]	1.42	0.25	565.4	434.1	32	16.339
Dasatinib	0.74	0.25	488.3	401.1	29	5.911
Dasatinib [$^{2}H_8$]	0.74	0.25	496.3	406.1	29	5.911
Erlotinib	0.87	0.25	394.3	336.0	24	10.135
Erlotinib [$^{13}C_6$]	0.87	0.25	400.3	342.0	24	10.135
Gefitinib	0.6	0.25	447.2	128.1	25	10.160
Gefitinib [$^{2}H_8$]	0.6	0.25	455.2	136.1	25	10.160
Ibrutinib	1.4	0.25	441.3	304.1	30	16.339
Ibrutinib [$^{2}H_5$]	1.4	0.25	446.3	309.1	30	16.339
Imatinib	0.67	0.25	494.3	394.1	26	10.160
Imatinib [$^{2}H_8$]	0.67	0.25	502.4	394.1	26	10.160
Lenvatinib	0.61	0.25	427.2	370.0	28	10.160
Lenvatinib [$^{2}H_5$]	0.61	0.25	432.2	370.1	28	10.160
Nilotinib	1.19	0.25	530.2	289.1	29	16.339
Nilotinib [$^{13}C^{2}H_3$]	1.19	0.25	534.3	289.0	29	16.339
Osimertinib	0.92	0.25	500.3	72.1	25	10.135
Osimertinib [$^{13}C^{2}H_3$]	0.92	0.25	504.3	72.1	25	10.135
Pazopanib	0.66	0.25	438.2	357.2	29	10.215
Pazopanib [$^{13}C^{2}H_3$]	0.66	0.25	442.2	361.1	29	10.215
Ponatinib	1.16	0.25	533.3	260.0	30	12.664
Ponatinib [$^{2}H_8$]	1.16	0.25	541.3	260.0	30	12.664
Regorafenib	1.77	0.25	483.2	270.0	33	35.234
Regorafenib [$^{13}C^{2}H_3$]	1.77	0.25	487.2	274.0	33	35.234
Ruxolitinib	0.84	0.25	307.3	186.1	27	10.135
Ruxolitinib [$^{2}H_9$]	0.84	0.25	316.3	186.1	27	5.911
Sorafenib	1.69	0.25	465.2	252.1	33	35.234
Sorafenib [$^{13}C^{2}H_3$]	1.69	0.25	469.2	256.1	34	35.234
Sunitinib	0.82	1.00	399.3	326.1	21	10.135
Sunitinib [$^{2}H_{10}$]	0.82	1.00	409.4	326.0	21	10.212
Trametinib	1.53	0.25	616.1	491.0	34	22.710
Trametinib [$^{13}C_6$]	1.53	0.25	622.1	497.0	34	22.710
Vandetanib	0.7	0.25	475.2	112.2	20	10.135
Vandetanib [$^{13}C_6$]	0.7	0.25	481.2	112.1	20	10.135
Vismodegib	1.22	0.25	421.1	139.0	41	10.199
Vismodegib [$^{13}C^{72}H_3$]	1.22	0.25	431.1	139.0	41	10.199

Table 2
The validation results of the 21 kinase inhibitors analysed with the developed analytical method presented in alphabetical order. The kinase inhibitors were analysed in either human plasma or human serum. Abbreviations: Acc.: Accuracy(expressed as bias%). CC: correlation coefficient. CV: coefficient of variation. LLQ: lower limit of quantification. NC: nominal concentration. Prec.: Precision. QC: quality control sample. RC: regression coefficient.

Analyte	QC level[a]	NC (ng/ml)	Intra-day Acc. (%)			Prec. (CV %)	Inter-day Acc. (%)[c]	Prec. (CV %)[c]	Serum Acc. (%)	Prec. (CV %)	Linearity CC	RC	Range (ng/ml)	Acc. (range %)[d]	Acc. (%). LLQ
			Day 1[b]	Day 2[b]	Day 3[b]										
Afatinib	**LLQ**	1.00	−1.4	5.1	−4.2	3.6	−0.2	4.5	−1.3	1.7	0.999	0.999	1–100; 9 data points	−4.1–3.3	6.4
	L	2.00	−1.7	2.6	−7.8	4.7	−2.3	4.9	−2.8	3.1					
	M	40.0	−4.6	−1.8	−12.0	3.2	−6.1	5.5	1.0	3.0					
	H	80.0	1.6	−1.0	−6.6	1.5	−2.0	4.2	3.1	1.9					
	Dilution	20.0	−1.3	−0.5	−6.3	2.3	−3.0	3.1	5.1	5.0					
Alectinib	**LLQ**	10.0	0.7	4.8	−0.8	2.2	1.6	2.7	−3.0	2.3	1.00	0.999	10–2000; 10 data points	−2.0–1.5	0.6
	L	20.0	−3.1	−0.2	−2.8	1.7	−2.0	1.4	0.7	1.4					
	M	800	1.0	1.5	5.9	3.4	−1.9	3.8	2.3	1.4					
	H	1600	2.4	2.4	−3.4	0.9	0.5	3.3	3.9	1.1					
	Dilution	400	1.3	6.6	0.8	1.0	3.0	3.1	9.3	5.2					
Bosutinib	**LLQ**	1.00	17.8	15.6	6.5	5.3	13.3	4.7	6.6	3.1	1.00	1.00	1–500; 12 data points	−5.7–5.4	6.2
	L	2.00	−1.8	0.5	−3.3	3.3	−1.5	1.3	−0.9	5.1					
	M	200	−2.5	−0.6	−5.5	2.9	−2.9	2.2	−0.1	0.9					
	H	400	3.8	−1.1	−5.6	1.1	−1.0	4.7	0.7	1.6					
	Dilution	100	3.3	2.6	−0.3	1.8	2.0	1.7	8.5	5.2					
Ceritinib	**LLQ**	10.0	−0.3	−1.2	−2.0	4.6	−1.2	0.0	−1.0	1.3	0.999	0.999	10–2000; 10 data points	−4.2–1.6	7.6
	L	20.0	−2.3	−2.1	−5.1	2.9	−3.2	1.1	−3.8	1.7					
	M	800	1.0	1.4	5.9	3.4	−1.2	3.6	0.1	0.9					
	H	1600	4.7	2.4	−2.4	1.0	1.6	3.6	1.8	1.0					
	Dilution	400	5.6	6.5	0.3	1.8	4.0	3.1	7.6	4.7					
Dasatinib	**LLQ**	1.00	−7.2	1.1	5.5	4.1	−0.2	6.2	−1.3	1.7	0.999	0.999	1–100; 9 data points	−4.0–2.3	8.1
	L	2.00	−3.9	−0.8	−3.0	4.0	−2.6	0.0	−2.8	3.1					
	M	40.0	−3.0	−2.5	−9.7	3.2	−5.1	3.0	1.0	3.0					
	H	80	3.2	−0.6	−3.7	2.2	−0.3	3.3	3.1	1.9					
	Dilution	20	−3.0	−1.9	−4.8	2.2	−3.0	1.2	5.1	5.0					
Erlotinib	**LLQ**	100	0.7	0.9	−4.6	2.4	−1.0	3.0	−2.0	4.1	1.00	0.999	100–5000; 8 data points	−2.4–1.9	−0.8
	L	200	−1.6	−0.2	−7.1	2.4	−3.0	3.6	−2.0	2.8					
	M	2000	−3.1	1.3	−5.3	4.0	−2.4	2.9	−0.3	1.6					
	H	4000	1.0	2.9	−4.4	1.3	−0.1	3.8	1.3	1.3					
	Dilution	1000	−0.4	5.5	0.7	2.1	2.0	2.9	5.5	3.8					
Gefitinib	**LLQ**	10.0	0.0	1.8	−6.3	2.6	−1.5	4.2	−3.2	3.7	1.00	0.999	10–2000; 10 data points	−1.8–3.6	−3.4
	L	20.0	1.4	−1.8	−6.0	1.3	−2.1	3.7	0.6	2.8					
	M	800	−2.6	3.9	−6.5	3.5	−1.7	5.1	1.0	2.1					
	H	1600	3.7	2.2	−4.2	1.5	0.6	4.1	2.7	0.4					
	Dilution	400	2.8	6.3	−0.3	1.1	3.0	3.2	8.2	5.3					
Ibrutinib	**LLQ**	10.0	−0.7	1.9	2.0	2.1	1.1	1.2	0.8	2.4	1.00	0.999	10–2000; 10 data points	−5.3–0.8	8.0
	L	20.0	−0.5	−3.9	−2.0	1.5	−2.1	1.6	−1.0	1.3					
	M	800	−0.7	0.8	−6.5	2.6	−2.1	3.7	1.2	1.1					
	H	1600	4.1	1.1	−4.1	1.5	0.4	4.1	0.7	2.0					
	Dilution	400	3.1	6.5	−1.5	1.8	3.0	3.8	6.8	5.6					
Imatinib	**LLQ**	100	−0.2	1.8	2.7	1.8	0.3	2.2	0.0	3.1	1.00	0.999	100–5000; 8 data points	−2.5–1.3	3.3
	L	200	−1.0	−2.4	−5.1	1.8	−2.8	2.0	−3.6	0.7					
	M	2000	−2.9	−0.9	−7.3	3.4	−3.7	3.1	−3.6	0.7					
	H	4000	2.2	1.4	−3.9	1.3	−0.1	3.2	−2.0	2.0					
	Dilution	1000	−2.7	4.0	−1.7	2.3	0.0	3.5	2.5	3.2					
Lenvatinib	**LLQ**	10.0	−3.0	0.6	1.5	3.9	−0.3	1.6	−3.0	3.5	0.999	0.999	10–2000; 10 data points	−7.0–2.3	10.3
	L	20.0	−4.7	−1.1	−0.5	3.3	−2.1	1.8	−3.8	1.6					
	M	800	−1.6	1.9	−4.5	4.1	−1.4	2.7	−1.6	1.2					
	H	1600	4.5	3.3	−2.4	1.7	1.8	3.5	−1.2	1.0					
	Dilution	400	3.3	1.7	1.5	2.3	1.0	3.3	5.2	6.5					
	LLQ	100	−1.0	−0.1	−2.5	1.6	−1.2	1.0	3.9	1.5	1.00	0.999	100–5000; 8 data points	−1.9–1.5	−0.3
	L	200	0.5	1.5	−4.8	1.4	−1.0	3.4	1.9	1.6					
	M	2000	−0.9	1.9	−5.8	3.3	−1.6	3.6	0.7	0.7					
Nilotinib	**H**	4000	3.7	1.9	−2.1	0.8	1.2	2.9	1.2	1.2					
	Dilution	1000	2.3	6.7	0.5	1.2	3.0	3.3	7.8	6.1					
Osimertinib	**LLQ**	10.0	1.5	−3.3	−1.9	2.9	−1.2	2.1	4.4	3.3	0.999	0.999	10–2000; 10 data points	−2.5–1.6	3.9
	L	20.0	−0.4	−2.9	−3.9	2.8	−2.4	1.4	3.0	2.5					
	M	800	−2.9	−2.4	−7.8	3.3	−4.4	2.7	5.5	0.7					
	H	1600	2.7	2.6	−5.5	0.9	−0.1	4.7	5.7	2.0					
	Dilution	400	7.4	10.8	−0.7	1.1	6.0	5.6	13.7	4.1					
Pazopanib	**LLQ**	500	−3.4	1.5	0.1	1.2	−0.6	2.5	1.6	1.1	1.00	0.999	500–50.000; 9 data points	−2.0–1.6	−0.8
	L	1000	−0.6	4.1	−1.7	1.1	0.6	3.0	3.3	1.8					
	M	20,000	−1.8	3.3	−5.9	3.4	−1.5	4.4	0.2	0.8					
	H	40,000	2.1	2.8	−4.2	1.0	0.2	3.9	1.2	0.9					
	Dilution	10,000	0.6	7.8	0.3	1.4	3.0	4.1	7.0	4.5					
Ponatinib	**LLQ**	10.0	0.5	3.0	−1.2	2.2	0.7	1.8	0.3	2.2				−1.0–2.0	−3.1
	L	20.0	0.3	0.5	−1.0	1.2	−0.1	0.6	1.0	1.3	1.00	0.999	10–2000;		
	M	800	−1.8	1.7	−5.9	3.5	−2.0	3.6	−0.4	0.8					

(*continued on next page*)

Table 2 (*continued*)

Analyte	QC level[a]	NC (ng/ml)	Intra-day Acc. (%) Day 1[b]	Day 2[b]	Day 3[b]	Prec. (CV %)	Inter-day Acc. (%)[c]	Prec. (CV %)[c]	Serum Acc. (%)	Prec. (CV %)	Linearity CC	RC	Range (ng/ml)	Acc. (range %)[d]	Acc. (%). LLQ
	H	1600	3.9	2.5	−4.1	0.7	0.8	4.2	0.3	1.6			10 data		
	Dilution	400	2.1	6.4	0.2	1.3	3.0	3.0	5.8	5.0			points		
	LLQ	100	−1.0	−0.1	−1.9	1.1	−1.0	0.8	0.5	2.1	1.00	0.999	100–5000;	−0.9–1.1	−0.1
Regorafenib	**L**	200	0.4	0.9	−5.9	0.9	−1.5	3.8	1.6	0.5					
	M	2000	−1.5	1.1	−4.9	3.0	−1.8	2.8	0.2	0.9					
	H	4000	4.8	1.4	−2.9	0.7	1.1	3.8	1.1	2.5					
	Dilution	1000	2.1	5.6	1.2	1.2	3.0	2.2	7.2	5.0					
	LLQ	1.00	−5.7	−1.4	3.3	2.4	−1.3	4.5	0.6	0.6	1.00	1.00	1–500;	−4.6–0.8	9.2
Ruxolitinib	**L**	2.00	−7.9	−3	−3.9	1.8	−5.0	2.6	−7.2	1.3			8 data points		
	M	200	−1.3	−0.5	−0.7	0.9	−0.8	0.0	−2.1	1.6					
	H	400	−1.9	−2	−0.9	0.7	−1.6	0.5	−2.4	0.4					
	Dilution	100	2.3	−2.8	0.5	1.8	0.0	2.5	−2.7	2.1					
Sorafenib	**LLQ**	100	−0.7	4.4	−1.1	0.7	0.9	3.1	−0.1	1.4	1.00	0.999	10–10.000;	−1.4–2.5	−3.3
	L	200	2.9	5.4	0.2	0.7	2.8	2.5	2.3	2.2			9 data points		
	M	4000	−0.8	1.2	−4.5	3.0	−1.4	2.6	1.1	0.5					
	H	8000	5.1	1.6	−2.5	1.0	1.4	3.7	2.5	1.3					
	Dilution	2000	2.7	5.8	2.7	1.5	4.0	1.6	8.7	5.0					
Sunitinib	**LLQ**	10.0	2.1	2.0	1.3	3.3	1.8	0.0	−8.0	7.1	1.00	0.999	10–2000;	−2.0–2.3	−0.3
	L	20.0	1.4	−2.5	−3.1	2.6	−1.4	2.2	−7.2	4.1			10 data		
	M	800	1.3	1.2	−6.0	3.5	−1.1	3.9	−2.7	1.0			points		
	H	1600	3.9	3.1	−2.7	1.1	1.4	3.5	1.5	1.4					
	Dilution	400	4.8	3.5	−1.2	1.5	2.0	3.0	4.0	2.3					
Trametinib	**LLQ**	1.00	−2.5	1.2	3.5	3.9	0.7	2.5	−0.3	1.5	0.995	0.999	1–100;	−3.9–3.7	7.2
	L	2.00	−5.4	−2.9	−1.3	2.4	−3.2	1.9	−1.4	2.6			9 data points		
	M	40.0	−5.7	−3.4	−9.3	3.5	−6.1	2.8	−0.8	1.1					
	H	80.0	3.0	0.5	−4.4	1.3	−0.3	3.8	1.4	0.8					
	Dilution	20.0	−4.4	−1.4	−4.5	1.3	−3.0	1.8	3.6	5.3					
Vandetanib	**LLQ**	100	0.6	−3.3	−2.6	2.5	−1.8	1.8	−2.0	1.5	1.00	0.999	100–5000;	−2.7–2.5	0.1
	L	200	0.3	0.4	−6.4	1.6	−1.9	3.9	−0.6	2.7			8 data points		
	M	2000	−1.8	0.5	−7.2	3.0	−2.8	3.8	2.6	1.9					
	H	4000	3.4	0.6	−3.8	1.2	0.1	3.6	1.0	1.7					
	Dilution	1000	2.6	4.8	−0.2	1.4	2.0	2.4	7.5	4.0					
Vismodegib	**LLQ**	500	−1.5	4.4	−1.2	0.8	0.5	3.3	3.4	1.6	1.00	0.999	500–50.000;	−1.4–1.5	−1.0
	L	1000	0.5	4.7	−3.0	1.1	0.8	3.8	4.1	1.8			9 data points		
	M	20,000	−1.9	2.5	−6.1	3.0	−1.8	4.2	1.4	0.7					
	H	40,000	2.5	3.4	−4.3	0.7	0.6	4.2	1.7	0.9					
	Dilution	10,000	0.9	8.1	−0.1	1.1	3.0	4.3	8.0	4.5					

[a] : L, M, and H designates the QC samples at the Low, Medium, and High drug concentrations.
[b] : The results are the average of five separate test results.
[c] : The results are the average of fifteen separate test results.
[d] : Linearity bias is the range of all observed biases at all levels except at the LLQ level. The LLQ bias is given in a separate column due to the different acceptance criteria.

response at the LLQ level. The interfering signals should not exceed 20% of the analyte signal at LLQ and should be less than 5% of the IS signal.

Carry-over was assessed by injecting blank samples after the HLQ sample. Eluting peaks on the chromatogram of the blank plasma samples were evaluated at the retention times of each kinase inhibitor. Acceptance criteria for the carry-over test were that the carry-over signals should not exceed 20% of the analyte signal at LLQ and should be less than 5% of the IS peak signal.

2.8.3. Accuracy and precision

Accuracy and precision were determined for four quality control (QC) concentration levels (LLQ, low, medium, and high) in quadruplicate sets repeated on three different days. One-way ANOVA was used to calculate the within-run and in-between run precision. The mean calculated concentration on each day was used to evaluate the within-run bias. The total average for 15 samples was used for the evaluation of the in-between run bias. Bias and CV% were calculated according to the following formulae:

$$Bias(\%) = \frac{(M-N)}{N} \times 100\%$$

$$CV\% = \frac{SD}{M} \times 100\%$$

Where:

M: is the mean value from the tested sample
N: is the nominal value based on the calibration curve.
SD: standard deviation

For the accuracy and precision criteria, the mean concentration of the low, medium, and high QC samples should be within 15% of the nominal values, whereas for the LLQ sample, it should be within 20% of the nominal value.

2.8.4. Dilution integrity

Dilution integrity was assessed by calculating the accuracy and precision for a diluted sample of an initial concentration higher than HLQ. The samples were diluted in plasma with a 10x dilution factor to a concentration in the range of the calibration curve. The accuracy and precision of the diluted sample should be within 15% of the nominal values.

2.8.5. Matrix effect & recovery

The matrix effect and recovery were tested in 6 independent batches at three different concentration levels (low, medium, high) in human serum, using the post-extraction method. The FDA does not state acceptance criteria for the matrix effect, and therefore, the matrix factor

was calculated as proposed by the EMA guideline.

Three sample preparation methods were investigated for the matrix effect and recovery experiments, which resulted in the "spiked matrix" (SM), "spiked precipitation solution" (SPS), and "spiked blank extract" (SBE).

SM was prepared by first spiking blank serum with known concentrations of the kinase inhibitors. Then the sample was treated by adding 500 ul of the IS and precipitation solution, vortexing, and finally centrifuging it, before injecting the extracted layer (see also Section 2.7).

SPS is the solution containing the analyte without the biological matrix. SPS was prepared by first adding 100 ul of ultra-pure water to 500 ul of the IS and precipitation solution, vortexing, centrifuging the mixture, and finally spiking the final solution with known concentrations of the drug before injecting it.

SBE was prepared by adding 100 ul of blank serum to 500 ul of the IS and precipitation solution, then vortexing, and centrifugation the mixture, before spiking the final solution with known concentrations of the drug.

Recovery, and matrix effects of the kinase inhibitors and IS were calculated as follow:

$$\text{Recovery} = \frac{SM}{SBE} \times 100\%$$

$$\text{Matrix factor} = \frac{SBE}{SPS} \times 100\%$$

IS-normalized matrix factor was then calculated by dividing matrix factor of the analyte over the matrix factor of the IS. Reproducibility and stability of the matrix effect is required in EMA guidelines to be assessed by calculating CV of IS-normalised matrix factor, with the proposed criteria CV less than 15%. Similar criteria are set by the FDA for the reproducibility of the recovery, with CV less than 15%.

2.8.6. Stability

All analytes were tested for stability according to the EMA and FDA guidelines under different conditions, including the autosampler, freeze and thaw cycles, refrigerator and room temperature stability tests. Stability at room (20–25 °C) and refrigerator (5 °C) temperatures were carried out at two different concentration levels (high and low) for a period of 7 days. The analyte peak height ratio was evaluated in quintuplicate at nine different time points (T = 0, 2 h, 4 h, 6 h, 1 day, 2 days, 3 days, 5 days, 7 days). The bias was calculated at each timepoint by calculating the deviation from the value at (T = 0).

Freeze-Thaw test was carried out at two different concentration levels in quintuplicate for 5 cycles. Auto sampler test was carried out for 7 days at a temperature of 10 °C.

According to both the EMA and FDA guidelines, the analyte is considered stable at certain period of time when the bias does not exceed 15%.

2.8.7. Matrix comparison

The accuracy and precision (A&P) of the obtained values from both plasma and serum matrices were calculated. A&P values from section (2.8.3) were used for evaluating the assay in human plasma. In serum, A&P for 5 QCs per analyte were calculated based on nominal values from two-point calibration lines. The two-point calibration lines were calculated based on results obtained from human plasma samples.

2.8.8. Clinical application

The clinical feasibility and applicability of the developed analytical method was evaluated during routine clinical practice. In the period between 1 July 2022 and 1 July 2023 our laboratory analyzed 17 out of the 21 kinase inhibitors (486 patient samples). The analyzed patient samples were from our own patients in the University Medical Center (tertiary center) as well as patient sample samples analyzed for other hospitals. The median, range and number of samples were summarized and presented.

In addition, our laboratory participates in a national (The Netherlands) external quality control testing program, namely the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML) to assess the precision and bias of the analytical method. This testing program is carried out by comparing the values obtained in our laboratory with a known reference value from the SKML. Currently, the SKML testing program consist a total of 176 drugs among which the five kinase inhibitors imatinib, lenvatinib, ponatinib, sorafenib, and sunitinib. The test results of this external quality control testing program were summarized and presented.

3. Results

3.1. Linearity

For all calibration curves, the bias between the three replicate datasets was less than 15% at all concentration levels (Table 2). The highest deviation was observed for bosutinib with a range of biases between (-5.7—-5.4%). The bias at LLQ for all kinase inhibitors was less than 20%, with the highest bias observed bias for dasatinib (8.1%).

The correlation coefficients (r^2) and the regression coefficients were all greater than 0.994, thus indicating a strong linear relationship for the analyzed calibration curves. These results show that the method complied with the EMA and FDA linearity criteria.

3.2. Selectivity & carry-over

The peak heights of co-eluting peaks in the six independent lots of blank human plasma samples were less than 20% of the peak heights of the drugs at LLQ and less than 5% of the peak heights of the IS. Figures 1 and 2 show example chromatograms at LLQ concentration level for all kinase inhibitors and their IS, respectively. These results show that the method complied with the EMA and FDA selectivity criteria.

The carry-over effect in the blank plasma samples of all kinase inhibitors was less than 20% at the LLQ level and less than 5% for the IS in all cases. Therefore, the assay complies with the acceptance criteria for carry-over.

3.3. Accuracy and precision

The intra- and inter-day accuracy and precision for all kinase inhibitors are given in Table 2. The intra-day biases for all kinase inhibitors were less than 20% at LLQ concentration level with the highest bias observed for bosutinib (17.8%). For the other QCs, the intra-day biases were less than 15% with the highest bias observed for afatinib (-12.0%) at medium level (40 ng/ml). The inter-day accuracy of the analytical method also complied with the acceptance criteria, with the highest bias again observed for bosutinib (13.3%) at the LLQ level.

The within-run and in-between-run precision complied with acceptance criteria. The highest observed CV for within-run precision was 5.3% for bosutinib at LLQ. The highest observed CV for the in-between run precision was observed for gefitinib (5.1%) at medium level (800 ng/ml). Altogether, these results show that the method complied with the EMA and FDA criteria for accuracy and precision.

3.4. Dilution integrity

The inter-day biases (-6.3%-10.8%), intra-day biases (-3%-4.3%), within-run precisions CV's (1.0%-2.9%), and in-between run precision CV's (1.8%-6.2%) for all 10x diluted samples complied with the EMA and FDA acceptance criteria. These results show that the method complied with the EMA and FDA criteria for dilution integrity for the 10x diluted samples.*3.5. Matrix comparison.*

The results from section 3.3 (Accuracy and Precision) show that the analytical method is accurate and precise for analyzing the kinase

inhibitors in human plasma. The results from the analysis of the kinase inhibitors in human serum are shown in Table 2. The observed serum accuracy biases (-8.0–13.7%) and precision CVs (0.4–6.1%) show that the method complied with the EMA and FDA acceptance criteria. These results show that the method is suitable for analyzing kinase inhibitors in human plasma and serum.

3.5. Matrix effect & recovery

The observed absolute matrix factor (MF) varied between 69.8% (trametinib) and 114.7% (sunitinib) for all kinase inhibitors at the analyzed QC levels. The normalized matrix factor values varied between 95.7% (ruxolitinib) and 105.2% (ceritinib), indicating the enhancing effect of IS on correcting the matrix effect (Table 3). The CVs for all kinase inhibitors were lower than 4.6%, showing a consistent and reproducible matrix effect across all batches. The addition of the IS further decreased the matrix effect (IS-normalised MF), even though the matrix effect was small for the majority of the kinase inhibitors. The non-normalized absolute matrix effect values based on the peak heights were for the majority of the kinase inhibitors within 10%. However, large and moderate absolute matrix effects were observed for trametinib and both dasatinib and sunitinib.

Trametinib's absolute MF was ~ 71%, whereas the MF of dasatinib and sunitinib were ~ 88% and ~ 115% across the three concentration levels, respectively. Adding the respective IS of the kinase inhibitors substantially decreased the MF for all three kinase inhibitors to ~ 100%.

The drug recovery values ranged between 95% and 106%, and all the CVs were 8.6% or lower. The IS recovery values ranged between 96% and 103%, and all the CVs were 8.3% or lower.

Taken altogether, these results show that the matrix effect for most of the kinase inhibitors was small and that large matrix effects could be corrected to an acceptable level by the IS. The recovery CVs of the kinase inhibitors and IS both complied with FDA acceptance criteria (≤15%), demonstrating consistent and reproducible recoveries of the analytical method.

3.6. Stability

The stability results of the kinase inhibitors are shown in Table 4. The results show that the kinase inhibitors stability was dependent on the storage period, storage temperature, and kinase inhibitors concentration. The stability results of ibrutinib and osimertinib stored at room temperature showed that these kinase inhibitors were the least stable (6 h). Moderate stability (1–3 days) at room temperature for several kinase inhibitors (afatinib, bosutinib, ceritinib, and ponatinib) was also observed, whereas good stability (5–7 days) was observed for the majority of kinase inhibitors. Refrigerated storage conditions substantially increased the stability of the low-to-moderate stable kinase inhibitors. For instance, refrigerated storage conditions increased the stability of ibrutinib and osimertinib from 6 h to 2 days and 3 days, respectively. These observations were corroborated by the stability results of the drugs stored in the autosampler at 10 °C. All analytes were stable in the autosampler for 7 days at both concentration levels. All kinase inhibitors were also stable for five consecutive freeze–thaw cycles at the tested low and high concentrations since the CV range was (0.4%-5.2%). Storage at −80 °C should be considered for the long-term storage (months) of kinase inhibitor samples. Currently, long-term stability of the kinase inhibitors was tested for up to 1.5 years at −80 °C and all samples were stable (data not shown). This long-term stability study is currently ongoing.

3.7. Clinical application

Table 5 presents the median, range and the number of patient samples of the kinase inhibitors analyzed during clinical practice. The number of patient samples varied between kinase inhibitors. For instance, 206 patient samples were analyzed for imatinib, while only 1 patient sample was analyzed for lenvatinib. The sample processing and sample run time during clinical practice were similar to the times observed during the validation, which showed the clinical feasibility and applicability of the developed method.

The external quality control test results on precision and bias for the five kinase inhibitors are shown in Table 6. The bias for imatinib, pazopanib, sunitinib, sorafenib, and lenvatinib were 1.1%, 6.9%, 2.0%, 1.4%, and 9.2%, respectively, whereas the CV values were 1.4%, 2.1%, 7.1%, 0.1%, and 2.5%, respectively. Therefore, the quality control test results complied with the SKML acceptance criteria, which showed that the developed analytical method was accurate and precise.

4. Discussion

The present study describes the development and validation of an LC-MS/MS analytical method for the rapid and simultaneous quantification of 21 kinase inhibitors in human plasma and serum. The sample preparation method was fast and easy, and the sample run time was only 2 min. Each kinase inhibitor had a stable isotope labeled internal standard (SIL-IS) to correct for any potential matrix effect. The analytical method complied with the EMA and FDA guidelines on validating bioanalytical methods. Therefore, the analytical method was considered validated and applicable for the routine TDM of kinase inhibitors in clinical practice.

Developing a rapid analytical method for analyzing the investigated kinase inhibitors is challenging for several reasons. First, the physiochemical characteristics of these different kinase inhibitors vary greatly, which may result in different observed matrix effects, drug recoveries, and prolonged range of retention times or irregular peak shapes at early elution times [32]. Second, the dosing regimens of the kinase inhibitors and resulting blood concentrations commonly seen in clinical practice also vary greatly (Table S1). The method should be able to quantify 21 kinase inhibitors in the clinically relevant plasma and serum concentration ranges. For example, the lowest and highest concentrations were 2.5 ng/ml and 45,100 ng/ml for dasatinib and pazopanib, respectively. The validation results showed that the developed method complied with the recovery acceptance criteria, had low matrix effects, and could quantify the kinase inhibitors in their respective clinically relevant blood concentration ranges.

The landscape of anti-cancer drugs is constantly developing. The field of drug discovery is promising for kinase inhibitors. Only 50 of the 500 protein kinases encoded in the human genome have been targeted so far. Thus, the potential of many breakthroughs and discoveries for new kinase inhibitors is promising [38]. Furthermore, new kinase inhibitors are being developed targeting specific mutations of protein kinases [39]. Therefore, it is important to add the element of flexibility to the developed assay so that the analytical method is also applicable for future kinase inhibitors. The current assay uses three different injection volumes to address the large concentration differences between kinase inhibitors. To the best of our knowledge, this study is the first to use such methodology for kinase inhibitors assay. This approach enables future registered kinase inhibitors to be added to our assay. In addition, different injection volumes provide different linear ranges. Therefore, it is also possible to change the linear range by only switching the injection volume.

Ideally, the prepared samples should be stable for an extended period of time, which facilitates flexibility during routine analyses. For instance, sample pre-treatment can be carried out in bulk with analysis at a later time point. The stability results showed that all processed samples were stable up to 7 days in the autosampler. Furthermore, at room temperature, all kinase inhibitors were stable for several days. However, ibrutinib, osimertinib and afatinib were the least stable kinase inhibitors (6 h). Stability of these kinase inhibitors at room temperature has been described to be poor [29–31]. For osimertinib, Veerman et al. proposed that the instability in plasma could be due to irreversible

Table 3
. Matrix effect and recovery for all 21 kinase inhibitors for QC samples in human serum. Abbreviation MF: matrix factor, IS: internal standard. IS-N: Internal standard-normalized.

Analyte	QC[a]	Matrix MF %	CV%	IS-N MF%	CV%	Recovery Analyte	CV%	IS	CV %
Afatinib	L	110.6	3.7	97.2	3.7	99	6.5	99.7	1.7
	M	105.8	1.4	98.4	1.4	99.8	1.7	96.9	1.6
	H	106.7	2.2	98.0	2.2	98	1.5	94.5	1.4
Alectinib	L	98.3	3.7	98.7	3.7	104	2.2	100.2	1.8
	M	97.2	0.9	97.9	0.9	102	1.3	100.9	0.5
	H	96.3	1.4	97	1.4	104	1.5	100.1	1.4
Bosutinib	L	111.9	3.0	100.6	3.0	101.3	2.7	103.2	0.7
	M	107.5	1.6	98.4	1.6	102.8	1.1	101.3	1.4
	H	109.4	1.6	98.2	1.6	102.6	1.7	102.4	1.3
Ceritinib	L	101.4	2.8	105.0	2.8	96.8	1.9	98.5	1.0
	M	99.4	2.0	105.2	2.0	95.3	1.0	99.3	1.7
	H	98.7	1.0	105.1	1.0	96.1	1.1	100.2	1.8
Dasatinib	L	86.8	4.6	99.3	4.6	101.4	6.6	98.4	6.5
	M	86.9	2.6	98.2	2.6	100.3	4.3	97.1	5.3
	H	88.0	3.4	100.1	3.4	100	5.1	97.5	5.0
Erlotinib	L	103.6	2.4	98.6	2.4	104.6	3.5	101.2	3.2
	M	101.9	2.0	98.1	2.0	102.9	2.1	100.8	2.3
	H	100.5	1.9	98.6	1.9	104.6	0.7	102.4	1.7
Gefitinib	L	107.4	3.6	97.3	3.6	106.6	2.0	100.8	1.3
	M	104.3	1.5	98.3	1.5	102.2	1.1	100.3	1.0
	H	101.9	1.5	96.6	1.5	104.6	2.0	99.98	0.8
Ibrutinib	L	92.6	3.2	99.5	3.2	104.6	2.3	101.0	2.8
	M	92.6	1.7	98.0	1.7	103.3	1.3	100.7	0.8
	H	91.0	2.1	98.3	2.1	104.4	1.5	101.2	1.6
Imatinib	L	101.6	2.3	98.0	2.3	105.7	2.5	102.8	2.2
	M	101.2	1.3	97.1	1.3	102.8	1.1	100.3	1.2
	H	101.3	2.1	97.8	2.1	103.3	1.3	101.3	1.5
Lenvatinib	L	95.1	4.6	97.7	4.6	107.7	4.5	100.2	5.68
	M	95.1	1.6	97.3	1.6	102.9	2.4	98.5	2.7
	H	95.3	1.7	99.1	1.7	104.7	3.3	101.8	3.9
Nilotinib	L	105.6	2.7	97.0	2.7	103.6	5.1	98.98	4.0
	M	103.8	1.4	96.7	1.4	103.8	2.5	100.3	2.3
	H	103.0	1.7	97.6	1.7	103.2	2.9	100.8	2.6
Osimertinib	L	112.4	2.4	101.4	2.4	104.5	2.6	100.5	1.1
	M	106.3	1.8	99.0	1.8	101.6	0.9	100.0	1.9

(*continued on next page*)

Table 3 (*continued*)

Analyte	QC[a]	Matrix MF %	CV%	IS-N MF%	CV%	Recovery Analyte	CV%	IS	CV %
	H	105.9	0.9	99.9	0.9	102.8	1.8	101.5	1.3
Pazopanib	L	98.7	2.7	97.5	2.7	103	6.6	98.2	5.6
	M	97.4	0.9	98.3	0.9	107.1	3.6	106.2	3.6
	H	97.1	1.1	98.0	1.1	107	2.3	104.7	2.5
Ponatinib	L	106.1	2.8	99.7	2.8	103.3	2.1	100	1.4
	M	102.9	0.8	97.8	0.8	101.4	0.5	99.6	0.8
	H	101.9	1.3	97.3	1.3	103.7	1.5	99.8	0.7
Regorafenib	L	97.3	2.6	98.1	2.6	102.8	2.1	98.6	1.1
	M	98.9	0.8	98.7	0.8	99.4	1.3	98.2	0.7
	H	98.2	1.9	98.4	1.9	101.2	1.0	98.9	0.6
Ruxolitinib	L	98.2	2.1	97.7	2.1	105	2.7	100.1	2.0
	M	97.8	3.3	95.7	3.3	102.6	1.1	99.8	1.5
	H	97.9	2.6	98.7	2.6	103.6	1.4	102.3	1.2
Sorafenib	L	96.4	2.2	98.5	2.2	100.9	1.2	98.6	0.9
	M	98.1	0.9	98.8	0.9	98.9	1.0	97.7	0.5
	H	98.8	1.3	99.5	1.3	103.7	0.8	98.9	0.4
Sunitinib	L	114.7	2.0	102.2	2.0	104.7	8.6	102.4	8.1
	M	111.7	1.7	99.7	1.7	101.6	7.0	101.6	7.0
	H	113.2	2.2	101.4	2.2	104.1	6.9	104.4	7.0
Trametinib	L	69.8	4.0	99.0	4.0	104.0	5.7	97.3	8.3
	M	70.8	1.6	99.0	1.6	101.3	7.1	99.2	8.2
	H	70.0	1.4	97.1	1.4	103.2	7.2	100.5	8.1
Vandetanib	L	106.6	2.6	99.6	2.6	103.7	2.2	101.2	1.5
	M	105.1	1.6	101.2	1.6	100	1.5	100.6	1.3
	H	103.9	2.4	100.4	2.4	100.8	2.3	100.6	1.8
Vismodegib	L	97.9	2.7	96.6	2.7	100.7	6.5	96.0	5.5
	M	97.0	0.8	98.0	0.8	106.0	3.8	104.0	3.3
	H	96.9	1.0	98.1	1.0	106.0	1.1	103.3	1.7

[a] : L, M, and H designate the QC samples at the Low, Medium, and High drug concentrations.

Michael adducts with nucleophiles such as plasma albumin, which was significantly mitigated at lower temperatures. These observations highlight that ibrutinib, osimertinib, and afatinib should be stored for only a short period of time at room temperature or, alternatively, refrigerated to warrant stability during routine analysis in clinical practice.

Several validated LC-MS/MS methods for analyzing kinase inhibitors have been published previously (Table S2). Compared to the here described method, these methods typically analyze 5–12 kinase inhibitors, have a relatively long run time (5–20 min), may exhibit a large matrix effect (79%), or utilize laborious and time-consuming extraction methods [16,20,21,29,33–35,40].

Endogenous proteins can interfere with the measurement systems and produce undesired matrix effects. Previous studies typically report an IS-normalized matrix effect of 80–120% (Table S2). Several studies aimed to decrease the observed matrix effects. Zhou et al. investigated salting-out assisted liquid–liquid extraction (SALLE) as an extraction method (for 12 kinase inhibitors) since not all endogenous proteins were removed with simple protein precipitation (PPT). The results showed similar matrix effects (80–120%) for PPT and for SALLE. However, the observed variation was significantly lower for SALLE (CV less than 9.49%) compared to PPT (CV less than 16.45%) [33]. Koller et al. also showed that the matrix effect could be decreased by using the micro-solid-phase extraction method (μ-SPE). Compared to PPT, (μ-SPE) resulted in a lower matrix effect (85–118% vs 73–126%, respectively) and slightly higher reproducibility of the matrix effect (CV within 15%

Table 4
. The stability results of 21 kinase inhibitors in human plasma stored either at room temperature (20–25 °C), refrigerated (5 °C), or in the autosampler (10 °C). Abbreviations: QC: quality control sample.

Analyte	QC [a]	Room temperature			Refrigerated			Freeze-thaw [b]		Autosampler [c]	
		Stability	Bias%	CV%	Stability (days)	Bias%	CV%	Bias%	CV%	Bias	CV%
Afatinib	L	1 d	−9.9	7.5	5	−7.9	6.0	0.4	5.2	−3.8	2.8
	H	6 h	−4.3	6.1	3	−5.4	2.0	−1.6	2.5	2.1	1.9
Alectinib	L	7d	1.3	2.7	7	−0.6	1.2	1.2	2.4	−0.5	2.2
	H	7 d	−3.1	1.1	7	0.0	0.9	−0.3	1.1	−1.2	0.3
Bosutinib	L	3 d	−8.4	3.0	7	−5.1	3.3	1.7	3.9	−3.6	4.9
	H	2 d	−5.5	3.0	7	−4.8	1.3	−1.0	1.0	−1.5	0.9
Ceritinib	L	2 d	−5.7	3.3	7	−4.5	2.3	1.4	3.4	−0.2	7.6
	H	3 d	−5.0	3.4	7	−2.3	0.6	1.5	0.9	0.9	1.4
Dasatinib	L	5 d	−9.6	4.3	7	−1.3	3.7	−0.8	3.0	−2.9	2.5
	H	3 d	−9.4	0.7	7	−2.5	2.1	−0.1	0.9	0.4	1.0
Erlotinib	L	7 d	1.9	2.2	7	2.8	1.8	2.8	1.4	0.1	1.4
	H	7d	−0.7	2.1	7	1.2	2.1	0.6	1.7	0.4	1.7
Gefitinib	L	7d	−1.5	1.3	7	−0.4	3.1	5.3	1.3	−2.0	4.0
	H	7d	−6.1	0.9	7	−0.5	0.9	0.7	0.8	−9.8	1.7
Ibrutinib	L	6 h	−7.7	7.7	2	−8.0	3.6	−3.5	1.6	−0.2	2.2
	H	6 h	−5.6	9.6	2	−7.6	2.1	−1.1	0.8	0.5	1.1
Imatinib	L	7d	−4.4	2.3	7	0.1	0.8	2.9	2.0	−0.2	1.6
	H	7d	−2.6	1.2	7	−1.1	0.5	−1.5	0.8	2.9	1.5
Lenvatinib	L	7 d	5.0	4.2	7	−3.3	2.3	−2.5	3.0	−2.3	2.8
	H	7 d	−1.0	2.0	7	0.7	2.7	−1.8	1.8	−7.1	0.8
Nilotinib	L	7d	1.0	2.2	7	0.1	0.9	1.9	1.2	−0.2	0.8
	H	7d	0.7	1.1	7	0.2	1.2	0.6	0.9	0.05	0.6
Osimertinib	L	6 h	−4.4	5.2	3	−5.4	1.7	3.8	1.4	−12.8	2.7
	H	6 h	−3.4	7.1	3	−8.1	1.3	−0.3	1.2	0.9	0.8
Pazopanib	L	7d	3.4	0.7	7	−0.9	2.0	2.0	2.3	4.3	2.3
	H	7d	0.8	0.5	7	−0.4	0.8	1.3	1.4	3.1	0.7
Ponatinib	L	1 d	−3.9	3.8	7	−4.7	0.9	3.3	0.4	−0.3	1.4
	H	2 d	−7.0	3.5	7	−6.2	0.7	0.6	1.3	−0.5	0.8
Regorafenib	L	7 d	2.0	1.3	7	−2.2	2.0	7.0	1.6	−0.6	1.0
	H	7 d	1.6	1.9	7	−1.1	1.0	0.8	1.5	−0.7	0.9
Ruxolitinib	L	7 d	4.4	1.6	7	−0.6	3.5	−0.3	1.7	0.5	4.1
	H	7 d	0.7	0.7	7	0.2	1.2	0.1	0.8	1.6	2.3
Sorafenib	L	7 d	2.3	1.0	7	−0.3	0.6	5.2	1.8	2.4	0.8
	H	7 d	1.2	0.5	7	0.5	1.0	1.0	0.9	0.2	1.2
Sunitinib	L	7 d	−7.7	2.1	7	−1.1	2.0	2.4	4.1	−2.9	4.4
	H	3 d	−6.4	0.4	7	−1.6	0.9	2.0	1.0	1.7	1.0
Trametinib	L	7 d	−3.3	4.3	7	4.7	3.5	−2.6	3.8	−1.0	2.8
	H	7 d	−2.4	1.3	7	−0.3	0.7	−1.0	1.0	−0.2	1.6
Vandetanib	L	7 d	−7.4	2.2	7	−2.2	1.2	2.4	2.3	−2.5	2.4
	H	7 d	−7.5	1.6	7	−1.5	1.4	0.7	1.4	−1.7	1.2
Vismodegib	L	7 d	3.0	0.4	7	−0.6	0.7	1.9	0.6	−2.1	1.1
	H	7 d	2.2	0.5	7	0.2	0.9	0.3	0.5	0.9	0.5

[a] : L and H designate the QC samples at the low and high drug concentrations.
[b] : five thaw-freeze cycles were performed (samples were stored at −20 °C).
[c] : all kinase inhibitors were stable for 7 days in the autosampler.

Table 5
. Median concentration and corresponding range of kinase inhibitors in patients' samples. N.a.: Not applicable.

Kinase inhibitor	Number of samples	Median concentration (ng/ml)	Range (ng/ml)	
			Min	max
Afatinib	1	2	N.a.	N.a.
Alectinib	90	594	154	1468
Bosutinib	3	52	25	68
Dasatinib	36	22	1	315
Ibrutinib	2	17	11	23
Imatinib	206	1425	0.13	12320
Lenvatinib	21	36	11	272
Nilotinib	7	1079	0.337	1605
Osimertinib	8	273	124	602
Pazopanib	35	27920	6070	81200
Ponatinib	3	37	15	73
Regorafenib	25	2588	0.227	5233
Ruxolitinib	16	42	6	218
Sorafenib	5	1954	1147	17435
Sunitinib	25	24	1	60
Trametinib	2	8	7	9
Vandetanib	1	526	N.a.	N.a.

Table 6
. External quality control data on accuracy and precision for imatinib, pazopanib, sunitinib, sorafenib, and lenvatinib.

Kinase inhibitor	Number of samples	Bias (%)	CV (%)
Imatinib	8	1.1	1.4
Pazopanib	8	6.9	2.1
Sunitinib	6	2.0	7.1
Sorafenib	2	1.4	0.1
Lenvatinib	2	9.2	2.5

vs within 17%). Though these studies show that the matrix effect could be decreased by using different pre-analytical work-up protocols, these protocols are laborious and relatively expensive [35]. In the present study, we describe a fast and easy precipitation protocol with good recoveries and low matrix effects.

To the best of our knowledge, there is only one other report on a multiplex analytical method that can quantify more than 17 kinase inhibitors. Guo et al [32] developed and validated an analytical method for quantifying 39 kinase inhibitors in human plasma. The present method could also quantify 19 kinase inhibitors out of the 39 kinase inhibitors and bosutinib and vismodegib, which were not analyzed by

Guo et al. Furthermore, Guo et al. reported a sample centrifugation time and analysis run time of 10 min and 8 min, respectively. This is twice and four times longer, respectively than for the presented method. In addition, the presented method demonstrates a higher sensitivity for afatinib, ceritinib, and trametinib as well as a higher HLQ for alectinib, erlotinib, gefitinib, ibrutinib, lenvatinib, osimertinib, ponatinib, sunitinib, and pazopanib compared to the method described by Ghou et al. Interestingly, Guo et al. used only 17 SIL-IS for the analysis of the 39 kinase inhibitors since one given IS was used for multiple kinase inhibitors that had the same or similar parent nucleus. For example, erlotinib-d6 was used as the IS for the kinase inhibitors afatinib, erlotinib, and vandetanib. In our opinion, this is an elegant and promising approach in view of the practicality and cost reduction of the method. However, for each drug, it has to be demonstrated that adequate correction for the matrix effect is obtained by using a different SIL-IS than the parent compound with different elution characteristics.

Our analytical method was developed based on current guidelines and PK studies for the TDM of the investigated kinase inhibitors. The TDM reference values are based on the maximum blood concentration (C_{max}), trough level (C_{min}), or both (Table S1). The linear range of the analytical method covers a wider range than the therapeutic window of the kinase inhibitors. Strong drug-drug interactions may result in the drug's blood concentration levels that fall outside the therapeutic window[41–44]. However, for these patients, TDM can be crucial in drug-drug interaction management to warrant a safe and effective treatment. The wide linear range of the analytical method facilitates the TDM of the kinase inhibitors during strong drug interactions.

TDM can also be useful for evaluating the pharmacokinetics in special populations with altered pharmacokinetics. Kinase inhibitors have shown variable pharmacokinetics in several populations. These populations include patients with impaired renal or hepatic functions, obsess patients[45,46], pediatric populations[47], and pregnant women [48,49]. For example, TDM can be utilized to optimize kinase inhibitor therapy in adolescent populations with chronic myeloid leukaemia. Adolescents are more prone to poorer adherence, and it has been proposed that optimal treatment outcomes in this population mainly depend on adherence [50]. Renal and hepatic function impairment is another example of potential TDM application, as these impairments have been shown to affect the PK of several kinase inhibitors [46]. For nilotinib, close therapy monitoring is recommended for patients with hepatic impairment since the PK can be altered [51]. Hence, TDM is a valuable tool for determining the optimal kinase inhibitor dose in different patient populations.

5. Conclusion

In conclusion, a rapid and validated LC-MS/MS analytical method was developed for the simultaneous quantification of 21 kinase inhibitors in human plasma and serum. The sample preparation protocol was fast (5 min) and easy, with a rapid sample run time (2 min). The prepared samples were stable for 7 days in the autosampler (10 °C). The linear range of the analytical method was wider than the therapeutic window of the kinase inhibitors. Thus, the analytical method enables routine TDM in monitoring kinase inhibitors in special populations and during strong drug-drug interactions, which may significantly lower or increase the drug blood concentrations.

CRediT authorship contribution statement

Zaid N. Al Shirity: Conceptualization, Formal analysis, Validation, Visualization, Writing – original draft. **Niels Westra:** Conceptualization, Writing – original draft, Writing – review & editing. **Kai van Hateren:** Data curation, Investigation, Software, Validation, Writing – review & editing. **Thijs H. Oude Munnink:** Writing – review & editing. **Jos.G.W. Kosterink:** Writing – review & editing. **Paola. Mian:** Writing – review & editing. **Marjolijn N. Lub-de Hooge:** Writing – review & editing. **Daan J. Touw:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Writing – review & editing. **Bahez Gareb:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2023.123872.

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