Fast and Straightforward Method for the Quantification of Pazopanib in Human Plasma Using LC-MS/MS

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Background: Pazopanib is an angiogenesis inhibitor approved for renal cell carcinoma and soft-tissue sarcoma. Studies indicate that treatment with pazopanib could be optimized by adapting the dose based on measured pazopanib plasma concentrations.

Methods: We describe the validation and clinical application of a fast and straightforward method for the quantification of pazopanib in human plasma for the purpose of therapeutic drug monitoring and bioanalytical support of clinical trials. Stable isotopically labeled $^{13}C,^{2}H_3$-pazopanib was used as internal standard. Plasma samples were prepared for analysis by protein precipitation using methanol and diluted with 10 mmol/L ammonium hydroxide buffer. Chromatographic separation was performed on a C18 column using isocratic elution with ammonium hydroxide in water and methanol. For detection, a tandem mass spectrometer, equipped with a turbo ion spray interface was used in positive ion mode at m/z 438 → m/z 357 for pazopanib and m/z 442 → m/z 361 for the internal standard.

Results: Final runtime was 2.5 minutes. All validated parameters were within pre-established limits and fulfilled the FDA and EMA requirements for bioanalytical method validation. After completion of the validation, the routine application of the method was tested by analyzing clinical study samples that were collected for the purpose of therapeutic drug monitoring.

Conclusions: In conclusion, the described method was successfully validated and was found to be robust for routine application to analyze samples from cancer patients treated with pazopanib.

Key Words: pazopanib, LC-MS/MS, pharmacokinetics, therapeutic drug monitoring, bioanalytical methods

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INTRODUCTION

Pazopanib is a potent and selective multitargeted receptor tyrosine kinase inhibitor of the vascular endothelial growth factor receptor 1 (VEGFR-1), VEGFR-2, VEGFR-3, platelet-derived growth factor receptor α and β, and c-kit.[1] Through blocking the kinase activity of these receptors, pazopanib blocks tumor growth and inhibits angiogenesis.[2] Pazopanib is currently approved by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) for the treatment of renal cell carcinoma and soft-tissue sarcoma.[3,4] A retrospective analysis showed increased tumor shrinkage and longer median progression-free survival in patients with pazopanib plasma trough concentrations ≥20.5 mcg/mL compared with patients with lower concentrations (52.0 versus 19.6 weeks).[5]

Pazopanib pharmacokinetics show large interindividual variability in plasma concentrations. This results in a subset of patients at risk of receiving less than optimal exposure.[6–9] In routine clinical care, this variability may be even greater as these patients are not selected and are likely to have more comorbidities and concomitant medication, may be older, may have impaired renal or hepatic function, and may have suboptimal therapy adherence.

The factors above may influence pazopanib pharmacokinetics, for example, absorption of pazopanib depends on gastric acidity and intake with food,[10] and it is a substrate for various CYP isoenzymes that may be inhibited by concomitant medication and subject to genetic polymorphisms.[11]

Based on the observations above, a prospective study in patients with cancer was conducted to study pazopanib dose individualization based on plasma trough concentrations.[12] This trial found that individualized pazopanib dosing was feasible, safe, and lead to more patients reaching an adequate

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All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.
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pharmacokinetic exposure. Therefore, measurement of pazopanib plasma concentrations in routine care, also known as therapeutic drug monitoring, could be an effective way to estimate the influence of all these factors and assess if patients would require therapy adjustments based on suboptimal exposure.

Previously, methods for the quantification of pazopanib in plasma have been reported.[13,14] However, these methods were only validated for mouse plasma and tissue or suffered from a long runtime (7 minutes per sample) because of the large number of analytes, making them suboptimal for application to routine (eg, weekly) therapeutic drug monitoring measurements in patients with cancer.

We describe the development, validation, and clinical application of a fast and straightforward liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the quantification of pazopanib in human plasma specifically designed for the purpose of therapeutic drug monitoring and bioanalytical support of clinical trials.

MATERIALS AND METHODS

Chemicals

Pazopanib (as hydrochloride) and stable isotopically labeled internal standard (IS) $^{13}C,^{2}H_{3}$-pazopanib, with purities (as free base) of 92.3% and 92.6% were supplied by GlaxoSmithKline (Zeist, The Netherlands). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany), ammonia (Empure 25%), and methanol (HPLC grade) from BioSolve Ltd (Valkenswaard, The Netherlands). Control human EDTA-plasma was obtained from healthy volunteers and used for the preparation of quality control (QC) samples, calibrators, and matrix blanks.

Stock Solutions, Calibrators, and QC Samples

Stock solutions of pazopanib were prepared in DMSO at a concentration of 2 mg/mL. Working solutions were prepared by diluting stock solutions with methanol. Separate stock solutions were used for the preparation of the calibrators and QC solutions. The IS stock solution was prepared in methanol at a concentration of 1 mg/mL. The IS working solution was prepared by further dilution with methanol to a concentration of 0.1 mcg/mL. All stock and working solutions were stored at −20°C, except for the IS working solution that was stored at 2–8°C.

Calibrators and QC samples were prepared by addition of a 10-μL aliquot of working solution to 190 μL of control plasma. Concentrations of 1.00, 3.00, 15.0, and 37.5 mcg/mL were used for the QC samples [lower limit of quantification (LLOQ), low, mid, and high concentrations, respectively]. The concentrations for the calibrators were 1.00, 2.00, 5.00, 10.0, 20.0, 30.0, 40.0, and 50.0 mcg/mL.

Liquid Chromatography-Tandem Mass Spectrometry

All LC-MS/MS experiments were performed using an 1100 series binary pump, degasser, column oven, and autosampler from Agilent Technologies (Santa Clara, CA) and an API 3000 triple quadrupole equipped with Turbo ion spray interface, and Analyst software was used for data analysis (Sciex Framigham, MA). Mass transitions of precursor and product ions, fragmentor voltage, nebulizer, turbo, curtain and collision gases, ion spray voltage, ionization temperature, declustering potential, collision energy, collision cell exit, and entrance potential were optimized. An overview of the optimized mass spectrometer settings is provided in Table 1.

Quantification was performed using selected reaction monitoring in positive ion mode using the m/z 438 → m/z 357 transition for pazopanib and m/z 442 → m/z 361 for $^{13}C,^{2}H_{3}$-pazopanib. Proposed fragmentation patterns of these transitions are provided in Figure 1.

Separation was performed on Gemini C18 Column, 50 × 2.0 mm ID, 5 μm particle size with a Gemini SecurityGuard, 4.0 × 2.0 mm ID guard column by Phenomenex (Torrance, CA) using a 0.2 μm in-line filter.

The column oven was set at 55°C and the tray temperature of the autosampler at 5°C. Isocratic elution was achieved using a mixture of 10 mmol/L ammonium hydroxide in water and 1 mmol/L ammonium hydroxide in methanol (45:55, vol/vol) at a flow of 0.4 mL/min. The total runtime was 2.5 minutes.

Sample Preparation

A 10-μL aliquot of plasma was transferred to an Eppendorf tube of 1.5 mL. A total of 500 μL of methanol containing 0.1 mcg/mL IS were added and the sample was vortex mixed. The corresponding IS concentration is thus 5.00 mcg/mL of plasma. Hereafter, 500 μL of 10 mmol/L ammonium hydroxide in water was added. The samples were briefly mixed and centrifuged for 5 minutes at 23,100*g*. Then, 800 μL of the clear supernatant was transferred to an autosampler vial and 5 μL was injected into the LC-MS/MS system.

Validation

The method was validated in accordance with FDA and EMA guidelines for bioanalytical method validation.[15,16]

The following validation parameters were assessed: calibration model, accuracy and imprecision, LLOQ, dilution

TABLE 1. Optimized Mass Spectrometer Settings

Parameter	Value
Nebulizer gas	7 a.u.
Turbo gas	7 L/min
Curtain gas	15 a.u.
Collision gas	10 a.u.
Ion spray voltage	3000 V
Ionization temperature	400°C
Dwell time	200 ms
Declustering potential	41 V
Collision energy	57 V
Collision cell exit potential	18 V
Entrance potential	10 V

a.u., arbitrary units.

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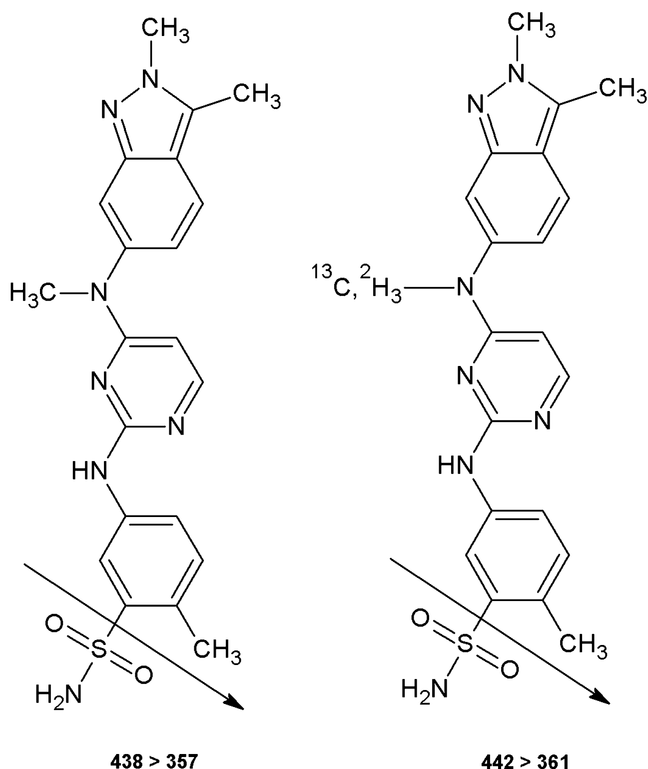

FIGURE 1. Chemical structures of pazopanib and $^{13}C,^{2}H_3$-pazopanib, and the proposed fragmentation patterns.

integrity, selectivity, instrument carry over, matrix factor (MF), and recovery. Furthermore, the stability of pazopanib was studied in various matrices.

Calibration

Weighted linear regression (1/concentration) was applied to fit the calibration plots (area ratio versus concentration). At least 75% of the nonzero standards [including at least 1 LLOQ and an upper limit of quantification (ULOQ)] in each run had to be within ±15% of the nominal value (±20% for the LLOQ). For the LLOQ and ULOQ levels, at least 50% should meet these criteria. The regression coefficient was calculated for each analytical run.

Accuracy and Imprecision

The assay's accuracy and imprecision were determined in 3 separate validation runs on 3 separate days by injecting 5 replicates of QC samples at the LLOQ, low, mid, and high concentrations. Intrarun and overall accuracy were expressed as the bias in %. The intrarun and overall imprecision were calculated as the coefficient of variation (CV) in %. At each concentration, the bias had to be within ±15% and the imprecision is not allowed to exceed 15%. For the LLOQ concentrations, bias had to be within ±20% and the imprecision would not be allowed to exceed 20%.

LLOQ

The LLOQ of the method was evaluated in each analytical run. It was quantified as the ratio of the peak height of the 1.00 mcg/mL calibrators (the signal) to the peak height of a double blank sample (the noise). A predefined limit of ≥5 was set for this ratio.

Dilution Integrity

The dilution integrity was studied by analyzing 5 replicate samples at a concentration of 100 mcg/mL. These were diluted 20 times with blank EDTA-plasma (10 μL sample plus 190 μL matrix) and compared with the nominal concentration. Predefined limits for bias and imprecision were set at ±15% and ≤15%, respectively.

Selectivity

The selectivity of the assay was determined for cross analyte/IS interference. The IS interference was assessed by analyzing a pazopanib ULOQ sample without adding the IS and by spiking the IS separately to a double blank sample at the concentration used in the assay.

The possibility of endogenous interferences was assessed by analyzing double blank samples from 6 different individuals and comparing the peak area in the blank with the peak area of the LLOQ in the same analytical run.

The endogenous and IS interferences were considered acceptable if it was less than or equal to 20% of the response of the LLOQ of the analyte and less than or equal to 5% of the response of the IS.

Carry Over

The instrumentation carry over was tested by injecting 2 double blank samples after a ULOQ sample in each validation run. The carry over was calculated as the ratio (in %) of the peak area in the blanks and the peak area of the LLOQ. The carry over was considered acceptable if the response at the retention time of the analyte (for both pazopanib and the IS) was less than or equal to 20% of the response of the LLOQ in the first blank.

MF

The MF was determined in 6 different batches of plasma spiked at both the QC low and QC high concentrations. The MF was calculated by dividing the pazopanib and IS peak area in the presence of matrix by the peak area ratio at the same concentration in a neat solution. The IS-normalized MF was calculated for 6 different batches of control human plasma and the IS-normalized MF was considered acceptable if the CV was less than or equal to 15%.

Sample Pretreatment Recovery

The pazopanib recovery was determined by dividing the peak area of pazopanib in processed validation samples at QC low and high concentrations (n = 5) by the peak area of pazopanib in the presence of matrix (a double blank sample to which pazopanib was spiked after processing). No specific requirement for recovery was predefined except that it should be reproducible.

Stability

The stability of pazopanib in processed samples (final extract) was determined after being stored at nominally 2–8°C. The stability in plasma samples was assessed at ambient temperatures and at −20°C.

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TABLE 2. Summary of Validation Results

Parameter	Result
Calibration model	Weighted (1/x) linear regression, coefficients all >0.99
Calibration range	1.00–50.0 mcg/mL
Intrarun accuracy (%)	±19.2% (LLOQ) ±4.4% (QC low, mid, and high)
Overall accuracy (%)	±7.8% (LLOQ) ±2.0% (QC low, mid, and high)
Intrarun imprecision (CV)	≤3.8% (LLOQ) ≤8.4% (QC low, mid, and high)
Overall imprecision (CV)	≤9.6% (LLOQ) ≤1.7% (QC low, mid, and high)
LLOQ (S/N)	≥176
Dilution integrity (mean, CV)	bias ±2.2%, CV 2.6%
Selectivity (cross analyte and endogenous interference)	≤0.5% and 0.0%
Instrument carry over	0.7% of the LLOQ
IS-normalized MF (mean, CV)	0.984, 3.1% (QC low) 0.948, 1.5% (QC high)
Recovery (mean, CV)	102%, 3.1% (QC low) 101%, 1.2% (QC high)

All tested parameters met their predefined criteria (predefined acceptance criteria are reported in the text).

S/N, signal to noise ratio.

All these stability analyses were performed in triplicate at the QC low and high concentrations. Samples were considered to be stable if the measured concentration was within ±15% of the nominal concentration. Stability of pazopanib in DMSO (stock) and methanol (working solutions) was studied (also in triplicate) at concentrations of 2 mg/mL and 300 mcg/mL, respectively. For these solutions, the limit for the deviation was set at within ±5%.

Clinical Application

The described method was used to support pharmacokinetic analyses in a clinical trial where pazopanib trough samples were collected (trial registry identifier: NTR3967).[12] Plasma samples were collected weekly for the first 8 weeks of treatment and monthly thereafter. Turn-around time from sample collection to reporting of the result was approximately 1 week. This trial was conducted in accordance with the World Medical Organization Declaration of Helsinki, compliant with Good Clinical Practice and approved by the Medical Ethics Committee of each of the participating medical centers. All patients provided written informed consent before enrollment.

Each analytical run containing patient samples contained 16 calibrators at the 8 different concentrations and 2 QC low, mid, and high standards.

RESULTS

Validation

A complete overview of all the validation results is provided in Table 2. All validated parameters were within their pre-established limits. A representative chromatogram of pazopanib at the LLOQ level, the IS, and a double blank is provided in Figure 2. A full overview of the analytical performance data (accuracy and imprecision) of the method is depicted in Table 3.

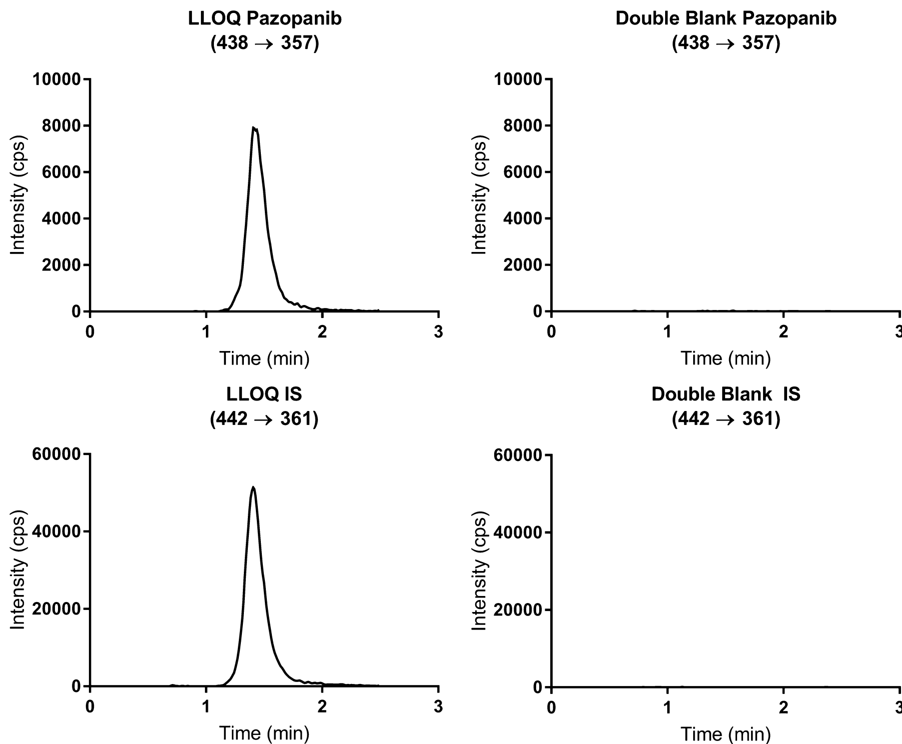

FIGURE 2. Representative LLOQ chromatograms of pazopanib (438 → 357) at 1.00 mcg/mL and the IS $^{13}C,^{2}H_{3}$-pazopanib (442 → 361) at 5.00 mcg/mL (from the same LLOQ sample).

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TABLE 3. Analytical Performance Data, for the LLOQ (1.00 mcg/mL), QC Low (3.00 mcg/mL), QC Mid (15.0 mcg/mL), and QC High (37.5 mcg/mL) Concentration Levels

Run	Nominal Concentration (mcg/mL)	Mean Measured Concentration (mcg/mL)	Accuracy (% Deviation)	Imprecision (% CV)	Replicates (n)
1	1.00	0.808	−19.2	3.2	5
2	1.00	0.973	−2.7	3.1	5
3	1.00	0.983	−1.7	3.8	5
Overall	1.00	**0.922**	**−7.8**	**9.6**	**15**
1	3.00	2.94	−1.9	6.4	5
2	3.00	2.94	−0.2	1.9	5
3	3.00	2.88	−4.0	3.1	5
Overall	3.00	**2.94**	**−2.0**	**4.3**	**15**
1	15.0	15.3	1.9	3.1	5
2	15.0	15.1	0.5	1.3	5
3	15.0	14.7	−2.1	2.3	5
Overall	15.0	**15.0**	**0.1**	**2.8**	**15**
1	37.5	37.3	−0.5	8.4	5
2	37.5	36.4	−2.8	1.7	5
3	37.5	35.8	−4.4	2.2	5
Overall	37.5	**36.5**	**−2.6**	**5.1**	**15**

Stability

Results for the stability measurements of pazopanib in the various matrices are provided in Table 4. All deviations were within ±15% and CVs were also all within 15%.

Clinical Application

After completing the bioanalytical validation, the described method was used to support pharmacokinetic analyses in a clinical trial. Plasma samples were stored at −20°C and then processed and analyzed using the method described in this manuscript. Between September 2013 and January 2014, 22 analytical runs were performed containing patient samples. The CV (%) and bias (%) of the QC samples also contained in these runs were 11.2 and −2.5 for the QC low, 5.6 and −3.5 for the QC mid, and 9.0 and −6.2 for the QC high, respectively.

As displayed in Figure 3, 327 plasma trough samples were measured from patients who received pazopanib dosages ranging from 400 to 1800 mg daily. Of these, 96.9% were within the validated range (0.9% < LLOQ and 2.1% > ULOQ), with a mean of 24.4 mcg/mL and a CV of 49.5%. All samples above the ULOQ were successfully re-analyzed after applying the validated 20-fold dilution.

DISCUSSION

The method described in this manuscript is robust and was fast and easy to implement in routine pazopanib plasma concentration monitoring to support clinical trials and therapeutic drug monitoring. The method was fully validated in accordance with EMA and FDA guidelines and met all pre-established validation criteria (Table 2).

The runtime was only 2.5 minutes and the sample preparation was straightforward consisting only of protein precipitation and dilution. Moreover, chromatographic separation was performed using isocratic elution.

TABLE 4. Stability Data for Pazopanib in Various Matrices

Sample Type	Condition and Interval (d)	Concentration (mcg/mL)	Deviation (% Deviation)	CV (%)
Final extract	70 days at 2–8°C	3.00	−4.0	1.3
		37.5	−4.2	10.4
Plasma sample	5 days at ambient temperatures	3.03	−6.8	0.7
		37.8	−6.0	2.8
Plasma sample	111 days at -20°C	3.00	−3.1	5.2
		37.5	−2.0	3.4
Plasma sample	3 freeze/thaw cycles (−20°C/ambient)	3.00	−0.4	1.5
		37.5	−0.2	3.1
Working solution (MeOH)	38 days at −20°C	300	1.0	8.0
Stock solution (DMSO)	30 days at −20°C	2.00×10^3	1.8	2.6

All analyses were performed in triplicate.
MeOH, methanol.

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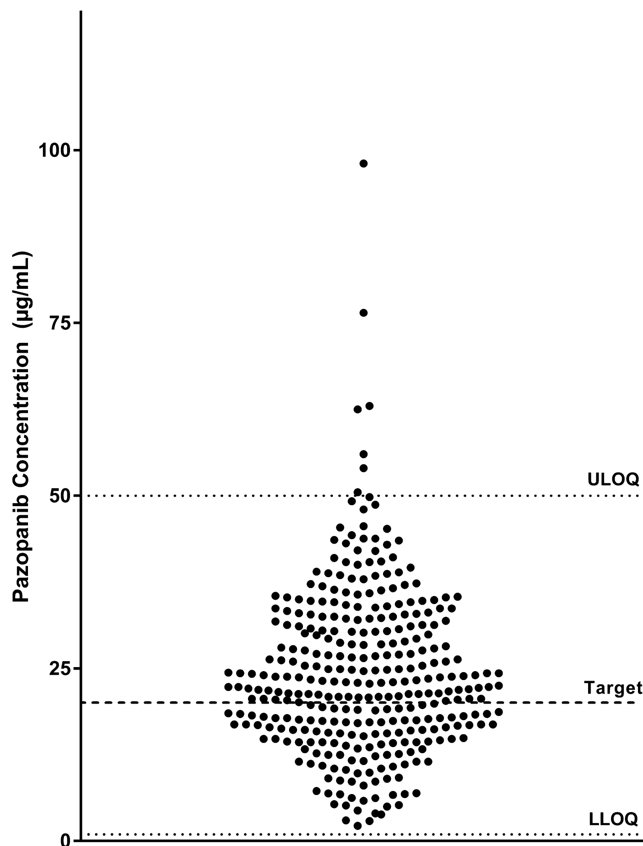

FIGURE 3. Distribution of clinical plasma samples measured using the described LC-MS/MS method. Of all samples (n = 327), only 3 (0.9%) were below LLOQ (not displayed), 7 (2.1%) were above ULOQ. The mean concentration of (non < LLOQ) samples was 24.4 mcg/mL. Dotted lines indicate the LLOQ (1 mcg/mL) and ULOQ (50 mcg/mL), the dashed line indicated the therapeutic target of ≥20.5 mg/L proposed by Suttle et al.[5]

To further reduce analytical workload and facilitate sample handling, extensive stability testing was performed. Pazopanib samples seemed to be very stable (Table 4). Samples were shown to be stable in the final extract for at least 70 days when stored at 2–8°C. This could reduce the need to prepare calibrators for each analytical run, making the method more suitable for application in routine therapeutic drug monitoring. Moreover, concentrations in plasma samples were within specifications for at least 5 days at room temperature, forgoing the need to send samples to the bioanalytical laboratory on ice or dry ice.

Protein precipitation was used as sample pretreatment and despite the limited sample clean-up, the method resulted in reliable quantification, demonstrated by its robust analytical performance (Tables 2 and 3). At our institute, the described method is now routinely applied to support clinical trials and to guide the treatment of patients treated with pazopanib. The described method has been used to quantify a large set of over 300 clinical samples, supporting its clinical applicability.

Pazopanib plasma concentrations have been shown to be a relevant parameter related to treatment efficacy and toxicity.[5,9] Specifically, a pazopanib trough concentration in plasma above 20 mg/L was related to increased progression-free survival.[5] Furthermore, for pazopanib, the intrapatient variability in pharmacokinetics (25%–27%) was considerably lower than interpatient variability (67%–72%).[9] Moreover, a prospective trial in patients with cancer has established the safety and feasibility of individualizing the dose of pazopanib based on plasma concentrations.[12] These considerations all support the further investigation of therapeutic drug monitoring of pazopanib. Ultimately, future randomized clinical studies are needed to show the added value of individualized dosing of pazopanib on a relevant clinical end point such as progression-free survival or overall survival.

CONCLUSION

We describe the bioanalytical validation and clinical application of a fast and straightforward method for the quantification of pazopanib in human plasma using LC-MS/MS. All validated parameters were within the FDA and EMA requirements for bioanalytical method validation. The method has been applied to over 300 clinical samples, demonstrating the clinical applicability of the method.

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