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Short communication

Liquid chromatography–tandem mass spectrometric assay for therapeutic drug monitoring of the tyrosine kinase inhibitor pazopanib in human plasma

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ABSTRACT

A quantitative bioanalytical liquid chromatography–tandem mass spectrometric assay for the tyrosine kinase inhibitor pazopanib was developed and validated. Plasma samples were pre-treated using protein precipitation with acetonitrile containing pazopanib-d_4 as internal standard. The extract was injected into the chromatographic system after dilution with water (1:9, v/v). The system consisted of a sub-2 μm particle, trifunctional bonded octadecyl silica column with isocratic elution using 0.005% (v/v) of formic acid in a mixture of water (76%, v/v) and acetonitrile (24%, v/v). The analyte was quantified using the selected reaction monitoring mode of a triple quadrupole mass spectrometer with a heated electrospray interface. The assay was validated in a 0.1–100 μg/ml calibration range. Within day precisions were 3.6–5.2%, between day precisions 4.0–8.3% and accuracies between 106% and 113% for the whole calibration range. The drug was sufficiently stable under all relevant analytical conditions. The assay has successfully been used to assess drug levels for therapeutic drug monitoring in patients treated with pazopanib.

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1. Introduction

Pazopanib (GW786034; Votrient™; Fig. 1) is an angiogenesis inhibitor which has shown to inhibit the vascular endothelial growth factor (VEGF), platelet-derived growth factor and cytokine receptors [1,2]. The drug has been approved by the US Food and Drug Administration (FDA) (2009) and European Medicines Agency (2010) [2,3] based on the results in a Phase III clinical trial [4]. This study, investigating 800 mg pazopanib given orally once daily compared to placebo, shows an improved progression-free survival and tumor response in advanced and metastatic renal cell carcinoma (RCC). Pazopanib shows similar outcomes compared to other targeted therapies and may be considered for both, first-line therapy and therapy after cytokine failure [2].

The average target drug level in plasma, 15 μg/ml, was obtained using the registered dose (800 mg once daily) 24 h after administration [5]. The maximal plasma concentration (C_{max}) during this exposure was 58 μg/ml [2,3] while the elimination half-life was on average 31 h. Chemical structures of metabolic products of pazopanib have not yet been reported [6]. Hypertension, diarrhea, hair depigmentation and nausea were the most frequently observed drug-related adverse events [2].

Therapeutic drug monitoring (TDM) may be a useful tool to improve pazopanib therapy because the drug has a high target plasma concentration and slow elimination. Further, the elimination is mainly by hepatic cytochrome P450 3A4 (CYP3A4) [2,6], a metabolic enzyme with a high inter-individual variation of activity that also shows interactions with a lot of other drugs [7]. TDM is also relevant to check for compliance of patients during treatment.

For TDM of pazopanib an accurate and precise bioanalytical assay should be available. Numerous validated bioanalytical assays using LC/MS–MS for tyrosine kinase inhibitors (TKIs) have been reported since 2002 after the introduction of the first TKI imatinib [8]. LC–MS/MS assays for multiple components of this class of drugs have also been introduced recently [9–12]. For pazopanib only a short description of a two column turbulent flow-LC–MS/MS method has been reported so far [13]. Further,

Abbreviations: DMSO, dimethylsulfoxide; HESI, heated electrospray ionization; VEGF, vascular endothelial growth factor; LLOQ, lower limit of quantification; PP, polypropylene; SRM, selected reaction monitoring; TDM, therapeutic drug monitoring; TKI, tyrosine kinase inhibitor; QC, quality control.

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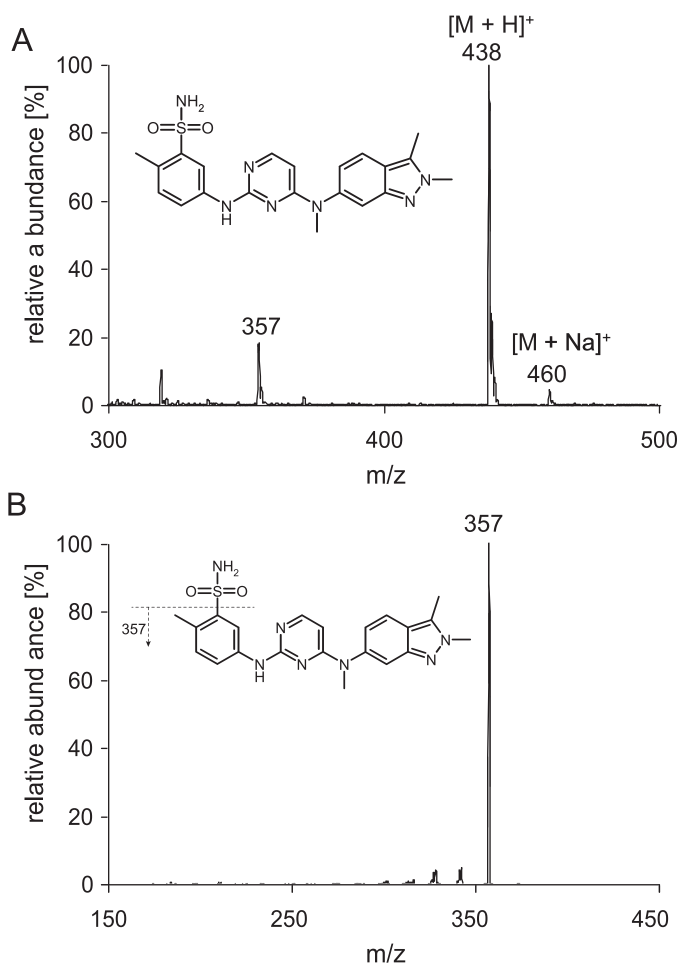

Fig. 1. Electrospray spectrum (A) and product spectrum (B) at m/z 438.2 at −28 V of pazopanib, mass spectrometric conditions are as used in the reported bioanalytical assay without using SRM.

pazopanib was included in a multiple kinase inhibitor LC–UV assay using liquid–liquid extraction assay for calf serum with a low recovery for pazopanib (<50%) [14]. So, a useful assay may also be obtained for pazopanib using less sophisticated equipment, however, it would most likely need a more complex sample treatment and method development compared to the use of LC–MS/MS. On the basis of our good experiences with other kinase inhibitors (axitinib [15], vemurafenib [16]) we decided to develop and validate a bioanalytical LC–MS/MS assay using protein precipitation to obtain a higher recovery and a higher sample throughput for TDM of pazopanib.

2. Experimental

2.1. Chemicals

Pazopanib was obtained from Sequoia Research Products (Pangbourne, United Kingdom) and pazopanib-d_4 (internal standard, IS) from Alsachim (Strasbourg, France). LC–MS grade water, methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were supplied by Biosolve (Valkenswaard, The Netherlands). Water not used as eluent was home-purified by a multi-laboratory reversed osmosis system. Formic acid was of analytical quality from Merck (Darmstadt, Germany) and dimethylsulfoxide (DMSO) was of analytical quality from Acros Organics (Geel, Belgium). Human EDTA disodium plasma (pooled and from individual donors) originated from Sera Laboratories International (Haywards Heath, UK).

2.2. Equipment

The LC/MS/MS equipment consisted of an Accela pump and autoinjector and a TSQ Quantum Ultra quadrupole mass spectrometer with heated electrospray ionization (HESI; Thermo Fisher Scientific, San Jose, CA, USA). Data were recorded and the system was controlled using the Thermo Fisher Xcalibur software (version 2.07).

2.3. LC–MS/MS conditions

Partial-loop injections (1 μl) were made on an Acquity UPLC® BEH C18 column (30 mm × 2.1 mm, d_p = 1.7 μm, Waters, Milford, USA), protected by the corresponding VanGuard pre-column (Waters, 5 mm × 2.1 mm). The column temperature was maintained at 40 °C and the autoinjector sample racks at 4 °C. The eluent comprised a mixture of acetonitrile (24%, v/v), water (71%, v/v) and 0.1% (v/v) formic acid in water (5%, v/v). The whole eluate (0.6 ml/min) was transferred to the electrospray probe, starting at 0.3 min after injection by switching the MS inlet valve, until the end of the analytical run at 1.2 min. The heated electrospray was tuned in the positive ionization mode by introducing the eluent and 5 μl/min of 10 μg/ml pazopanib. A 4000 V spray voltage, 350 °C capillary and vaporizer temperatures and nitrogen sheath, ion sweep and auxiliary gasses set at 50, 8 and 50 arbitrary units, respectively, were used for the HESI to obtain the highest MS response. The skimmer voltage was set off. The SRM mode was used with argon as the collision gas at 1.5 mTorr. The tube lens off set was 130 V for both compounds. Pazopanib was monitored at m/z 438.20 → 357.15, pazopanib-d_4 at m/z 442.20 → 361.15, with −28 V collision energies and a 0.2 s dwell time for pazopanib and 0.1 s for the IS.

2.4. Sample pre-treatment

To a 50-μl plasma sample in a polypropylene (PP) tube 100 μl of 100 ng/ml IS in acetonitrile was added. The tube was closed and shaken vigorously for *ca.* 5 s using vortex-mixing. After centrifugation of the sample at $10 \times 10^3 \times g$ at ambient temperature for 1 min, 100 μl of the supernatant was pipetted into a glass autoinjector vial. Before closing the vial, 900 μl of water was added and finally, after shaking the vial manually shortly, 1 μl of the mixture was injected onto the column.

2.5. Validation

A laboratory scheme based on international guidelines [17–19] was used for the validation.

2.5.1. Calibration

Stock solutions of pazopanib at 5 mg/ml and IS at 1 mg/ml in PP tubes were prepared in DMSO and stored at −30 °C. One 5 mg/ml stock solution of pazopanib was diluted to a 100 μg/ml calibration sample in pooled human EDTA plasma and stored in PP tubes at −30 °C. Additional calibration samples were prepared daily at 30, 10, 3, 1, 0.3 and 0.1 μg/ml by dilution with the blank matrix. All the calibration samples were processed in duplicate for each daily calibration. Least-squares double logarithmic regression was employed to define the calibration curves using the ratios of the peak area of pazopanib and the IS.

2.5.2. Precision and accuracy

Another 5 mg/ml stock solution of pazopanib, with separate weighting, was used to obtain validation (quality control (QC)) samples in pooled human EDTA plasma at 75 (QC-high), 10 (QC-med), 0.3 (QC-low) and 0.1 μg/ml (QC-LLQ). The QC samples were stored in PP tubes at −30 °C. Precisions and accuracies were determined

by sextuple analysis of each QC in three analytical runs on three separate days for all QCs (total: $n = 18$ per QC). Relative standard deviations were calculated for both, the within and the between day precision.

2.5.3. Selectivity and carry-over

Six individual EDTA plasma samples were processed to test the selectivity of the assay. These samples were processed with and without IS and with IS after spiking pazopanib at the LLQ level (0.1 μg/ml). Carry-over by the injector was assessed in duplicate by injection of 3 different blank samples after a QC-high sample.

2.5.4. Recovery

The extraction efficiency was determined in quadruplicate by comparing processed samples (QC-high, -med and -low) with extracts of drug-free human EDTA plasma spiked with the analytes at these levels. Ionization efficiency (ion suppression) was assessed by comparing the spiked blank extracts with reference material solutions in water–acetonitrile (9/1, v/v) at the three validation levels.

2.5.5. Stability

The stability of pazopanib was investigated in QC-high and -low plasma samples stored in PP tubes. Quadruplicate analysis of these samples was performed after storage at ambient temperature (24 h at 23 °C), three additional freeze-thaw cycles (thawing at ambient temperature during *ca.* 1 h and freezing again at for at least 1 day −30 °C), and storage for 6 months at −30 °C, respectively. Furthermore, validation runs were re-injected after additional storage of the extracts for 1 week at 4 °C.

The stability of pazopanib in the 5 mg/ml stock solutions ($n = 2$) was evaluated after storage for 24 h at ambient temperature and storage for 6 months at −30 °C.

2.6. Human plasma samples

A female patient (75 years; 57 kg) received 400 mg pazopanib once daily. The trough level was determined because of cognitive disorders after starting therapy. A male patient (68 years; 70 kg) received 800 mg pazopanib once daily. Because of suspected suboptimal therapy a sample obtained 4.5 h after ingestion was obtained. Plasma samples were assayed using the new method.

3. Results and discussion

3.1. Method development

Because of the expected high selectivity and sensitivity of the MS/MS detection in combination with an LC system using sub 2 μm particles, a simple pre-treatment procedure was explored as the first, and final, option. Protein precipitation with acetonitrile, also used successfully for several other TKI assays [11,12,15,16], showed high extraction recoveries during initial method development. During electrospray ionization, pazopanib formed the corresponding protonated molecule as the most abundant ion, just as has been experienced for pazopanib [13] and all other TKIs [9–12,15,16] previously. The amount of formic acid was optimized to obtain a maximal MS response. Acetonitrile resulted in a higher response than methanol and was therefore chosen as the organic modifier in the eluent. An electrospray mass spectrum and a product spectrum of pazopanib are shown in Fig. 1.

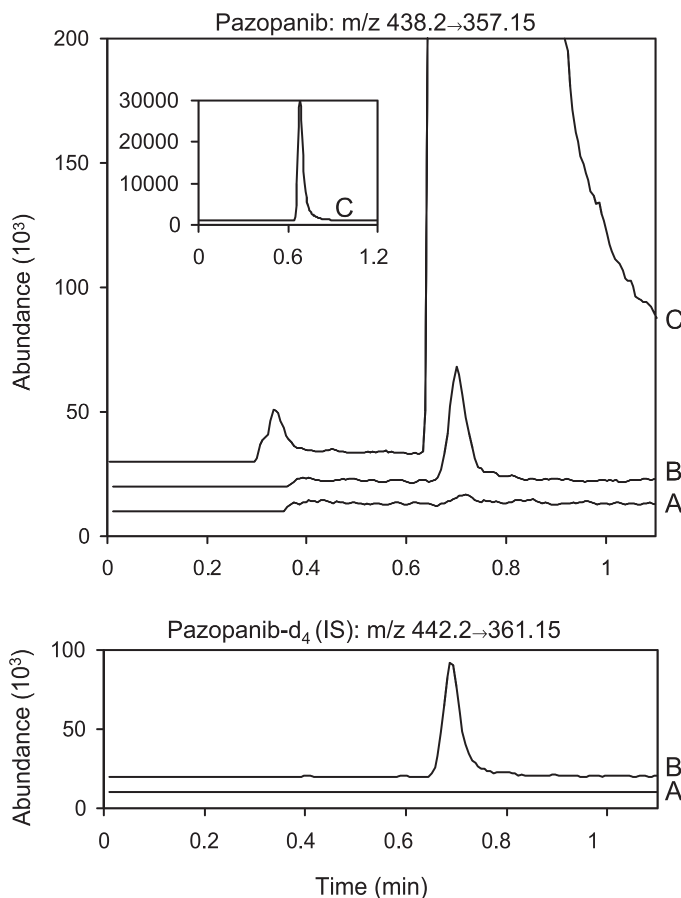

Fig. 2. SRM chromatograms of pazopanib and the IS in plasma extracts: blank plasma (A), plasma spiked with 0.1 μg/ml pazopanib (B) and the plasma sample of the female patient containing 37 μg/ml pazopanib (C). An artificial off set was given to the chromatograms and an inlay of pazopanib in the patient was added.

3.2. Validation

SRM chromatograms of pazopanib and the IS are depicted in Fig. 2, showing chromatograms of blank and LLQ-spiked samples.

3.2.1. Calibration

A 0.1–100 μg/ml range was chosen to trigger expected levels in the range 1–100 μg/ml for the registered 800 mg once daily regimen [2,4]. However, for common TDM use this extended sensitivity will not be necessary. The relative response of pazopanib showed a strongly significant deviation from a linear function ($P = 0.0012$ for a 1-tailed Student's t-distribution of the average double-logarithmic slope ($n = 6$) compared to 1); therefore, the double logarithmic function was used for the assay calibration. For all calibration samples (84 samples in 6 calibrations), the concentrations were back-calculated from the ratio of the peak area (of analyte and IS) using the calibration curves of the run in which they were included; no deviations of the averages of each level higher than 6.7% were observed (Table 1), indicating the suitability of the regression model. The average regression parameters of the double logarithmic regression functions ($n = 6$) were $\log(y) = 0.079(\pm 0.015) + 0.963(\pm 0.006)\log(x)$ with a regression coefficient of 0.9992 ± 0.0004. Here, x is the concentration (μg/ml) and y is the pazopanib response relative to the IS. The functions showed reproducible calibration parameters.

Table 1
Back-calculated concentrations of pazopanib from the calibration samples using double logarithmic calibration ($n = 6$).

Nominal concentration [μg/ml]	Precision [%]	Accuracy [%]
100	3.4	101.4
30	1.8	102.0
10	3.3	100.1
3	4.7	97.1
1	7.2	98.0
0.3	7.7	96.2
0.1	8.2	106.7

Table 2
Assay performance data of pazopanib resulting from 18 validation (QC) samples in 3 analytical runs.

Nominal concentration [μg/ml]	Within day precision [%]	Between day precision [%]	Accuracy [%]
75	3.7	4.6	106.3
10	5.2	5.2	107.5
0.3	3.6	4.0	110.7
0.1	3.6	8.3	113.4

Table 3
Stability data (recovery [%]; ±S.D.; $n = 4$) of pazopanib in human EDTA plasma, reporting the percentage of the initial concentration.

Condition	QC-high	QC-low
24 h at ambient temperature	97.4 ± 3.4	103.5 ± 5.0
3 freeze-thaw cycles	92.3 ± 8.8	107.1 ± 7.6
6 months at −30 °C	85.6 ± 3.6	80.8 ± 5.2

3.2.2. Precision and accuracy

Assay performance data from the validation samples at 4 concentrations are reported in Table 2. Between day variations and deviations of the accuracy lower than 15% were observed for all levels. Therefore, the upper limit of the calibration range could be assigned to the upper limit of quantification, and precisions and deviations of the accuracy met the required ± 15% (± 20% for the LLQ) [17–19].

3.2.3. Selectivity and carry-over

The analysis of six batches of blank samples showed no interfering peaks in the SRM traces for pazopanib and IS in human EDTA plasma. Blank responses could not be distinguished from the detector noise for both, pazopanib and IS. The average responses of the LLQ-spiked blank samples ($n = 6$, ±S.D.) were 0.097 ± 0.003 μg/ml pazopanib. Therefore, the validation was also successful on this point [17–19]. After injection of a QC-high sample the responses from three consecutive blank injections were 0.10%, 0.03% and 0.02%, respectively, of the initial response. Therefore, samples with high pazopanib concentrations should not immediately be followed by samples at the lowest levels.

3.2.4. Recovery

The recovery data showed only small or absent extraction (97%, 94%, 97%) and ionization (74%, 85%, 81%) losses at the three tested levels (QC-high, -med, -low) with small relative standard deviations (1–3%). The negligible extraction losses and small matrix effects could not explain the non-linearity of the calibration and both contributed to a successful validation of the assay [17–19].

3.2.5. Stability

Recoveries of pazopanib in plasma after different storage conditions are shown in Table 3. The recoveries in the range of 81–107% only indicate some significant degradation in human plasma after 6 months storage at −30 °C. However, because storage will usually be not longer than 1 month for TDM the present results can be accepted. The recovery of pazopanib from the stock solutions after storage (ambient temperature for 24 h and −30 °C for 6 months) did not show any losses and were within 96–103% (data not shown). Re-injection of calibration and validation samples after additional storage for 1 week at 4 °C resulted again in successful performances and does therefore allow the storage of diluted extracts long enough before final analysis. Overall, drug instability did not interfere with a successful validation of the assay [17–19].

3.3. Patient samples

Two human plasma samples from different patients were analyzed. The trough level in the female patient was 37 μg/ml (Fig. 2) while 22 μg/ml was observed in the male patient. Both levels are within therapeutic range [2,3]; however, 37 μg/ml is relatively high for the trough level while 22 μg/ml seems rather low 4.5 h after ingestion. Therefore, both results may support suspected over- and under-dosing for the female and male patient respectively and demonstrate the clinical applicability of the presented assay.

4. Conclusions

The first validated assay for pazopanib has now been reported for human plasma samples and its use for TDM has been demonstrated. The LC–MS/MS assay uses an easy method of sample pre-treatment and meets commonly accepted criteria for precision, accuracy, recovery and stability [17–19]. This LC–MS/MS assay can be a valuable tool for pharmacokinetic studies and TDM of pazopanib in the future.

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