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Liquid chromatography–tandem mass spectrometric assay for ponatinib and *N*-desmethyl ponatinib in mouse plasma

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ABSTRACT

Ponatinib is a multi-targeted third generation BCR–ABL1 tyrosine-kinase inhibitor approved for specific types of leukemia. A bioanalytical assay for this drug and its *N*-desmethyl metabolite in mouse plasma was developed and validated using liquid chromatography-tandem mass spectrometric (LC–MS/MS) with liquid-liquid extraction as sample pre-treatment procedure.

After extraction with *tert*-butyl methyl ether of both analytes with their isotopically labeled internal standards and evaporation and reconstitution of the extract, compounds were separated by reversed-phase liquid chromatography under alkaline conditions. After electrospray ionization, both compounds were quantified in the selected reaction monitoring mode of a triple quadrupole mass spectrometer.

The linear assay was validated in the ranges 5–5000 ng/ml for ponatinib and 1–1000 ng/ml for *N*-desmethyl ponatinib. Within-run ($n = 18$) and between-run (3 runs; $n = 18$) precisions were 10% and 12% at the lower limit of quantification for the metabolite, all other precisions were ≤8% for the metabolite and ≤6% for ponatinib. Accuracies were between 92 and 108% for both compounds in the whole calibration range. The drug was sufficiently stable under most relevant analytical conditions, only ponatinib showed more than 15% hydrolytic degradation after storage for 6 h and longer at ambient temperature in mouse plasma. Finally, the assay was successfully applied to determine plasma drug levels and study pharmacokinetics after oral administration of ponatinib to female FVB mice.

1. Introduction

Ponatinib (Iclusig®, AP24524, Fig. 1A) is a multi-targeted third generation BCR–ABL1 tyrosine-kinase inhibitor, being the first in its class. In contrast to the first (imatinib) and second (nilotinib, dasatinib, bosutinib) generation drugs it is also active for the BCR-ABL1 T315I and other mutations [1,2]. Therefore, ponatinib can be highly active in Philadelphia chromosome-positive (Ph+) leukemia [3,4]. Mainly because of serious cardiovascular risks [5], vascular occlusions and heart failure, but also hepatotoxicity, its use remains limited to second-generation drug-resistant and (T351I) mutated patients with Ph+ acute lymphoblastic leukemia (ALL) [6] or as third-line therapy in Chronic Myeloid leukemia (CML) [7].

Bioavailability of ponatinib is unknown, plasma protein binding is high (99%) and plasma half-life ($t_{1/2}$) is *ca.* 24 h in human [8]. The drug is mainly eliminated via feces after Phase I and II

Abbreviations: ALL, acute lymphoblastic leukemia; BCR-ABL, breakpoint cluster region-Abelson murine leukemia viral oncogene homolog; CML, chronic myeloid leukemia; CYP3A4, cytochrome P450 3A4; DMP, *N*-desmethyl ponatinib; ESI, electrospray Ionization; FVB, friend leukemia virus strain B; IS, internal standard; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantification; Ph+, philadelphia chromosome-positive; QC, quality control; SRM, selected reaction monitoring; tBME, *tertiary*-butyl methyl ether; TKI, tyrosine kinase inhibitor.

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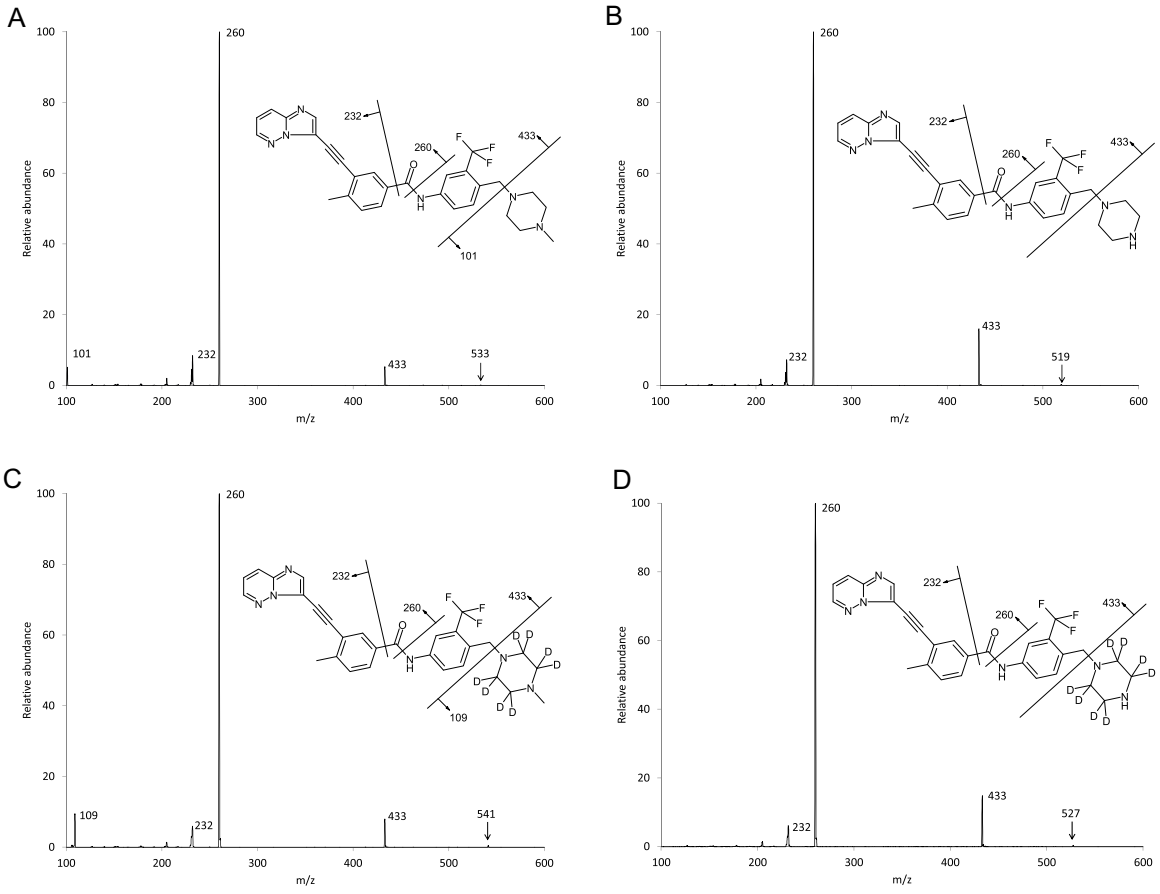

Fig. 1. Chemical structures and product spectra, formed by collision-induced dissociation (−29 V) of the protonated molecules of (A) ponatinib(3-(imidazo[1,2-*b*]pyridazin-3ylethynyl)-4-methyl-N-{4-[(4-methylpiperazin-1-yl)methyl]-3-(trifluoromethyl)phenyl}benzamide); *m/z* 533.2, (B) DMP; *m/z* 519.2, (C) ponatinib-d_8; *m/z* 541.2 and (D) DMP-d_8; *m/z* 527.2. Dissociation pathways of the fragmentation reactions have been proposed.

biotransformation. Extensive metabolic studies of ponatinib have not yet fully been reported but many metabolites could be found in excreta [8] and the main circulating metabolite was found to be the inactive carboxylic acid AP24600 formed by esterase- and/or amidase-mediated hydrolysis [8,9]. An active *N*-desmethyl (DMP, AP24567, Fig. 1B) and *N*−oxide metabolite (AP24734) were additionally formed by Cytochrome P450 (mainly CYP3A4) biotransformation and found in plasma together with a hydroxy metabolite [9]. *N*-oxidation and hydroxylation both take place at an unknown position on the methyl piperazinyl group [8].

Recently, a micellar enhanced spectrofluorometric assay for ponatinib in human plasma and urine was reported [10] that was able to quantify clinical levels of the drug. This method, however, uses a large sample volume (1 ml) and is not able to distinguish the parent compound from the active DMP metabolite and other metabolites. LC−MS/MS will be a more sensitive and selective technique and therefore more suited for mouse studies with low sample volumes. So far, it has been used in several human pharmacokinetic studies to quantify ponatinib in plasma alone [11], with the active DMP metabolite [12], and with the inactive carboxylic acid metabolite [9]. All LC–MS/MS assays use solid-phase extraction, in a 96-wells format with Oasis MCX [9] or unspecified Isolute [11,12] columns, and positive electrospray ionization but reported validation results [8] of the assays were limited. All assays obtained a lower limit of quantification (LLOQ) at 0.5 ng/ml ponatinib using 75 [9] or 150 [11,12] μl of human plasma. Stable isotopically labeled ponatinib (ponatinib-d_3) was used in two of the assays [9,11,12]. For mouse studies we therefore present a new completely validated LC–MS/MS method dedicated to smaller sample volumes with inclusion of the active metabolite.

2. Experimental

2.1. Chemicals

Ponatinib (>98%) was supplied by Sequoia Research Products (Pangbourne, United Kingdom), DMP (97.0%), ponatinib-d_8 (99.5%, isotopic purity >99%, Fig. 1C) and DMP-d_8 (96.8%, isotopic purity >99%, Fig. 1D) were obtained from Alsachim (Illkirch Graffenstaden, France). Water (LC–MS grade) and methanol (HPLC grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Water not used as eluent was home purified by reversed osmosis on a multi-laboratory scale. Ammonium hydroxide (ACS reagent grade) originated from Sigma-Aldrich (Steinheim, Germany) and *tert*-butyl methyl ether (tBME, Lichrosolve quality) from Merck (Darmstadt, Germany). Pooled mouse lithium heparin plasma was supplied by Seralab 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 triple quadrupole mass spectrometer with heated electrospray ionization (HESI), all supplied by Thermo Fisher Scientific, San Jose, CA, USA. Data were recorded and the system was controlled using the Thermo Fisher Xcalibur software (version 2.07).

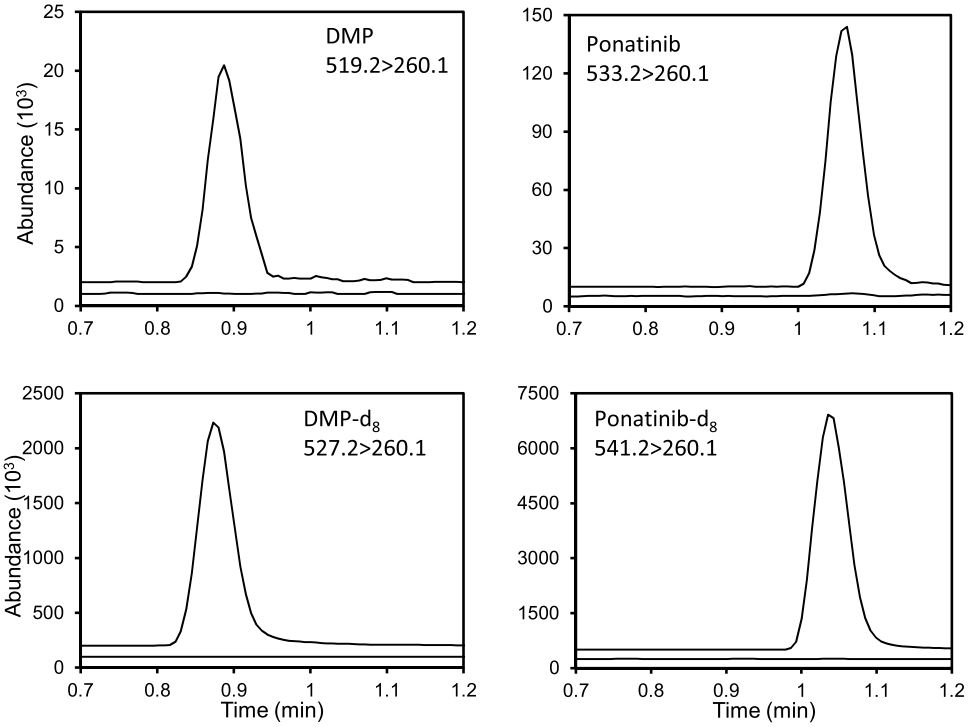

Fig. 2. SRM chromatograms of ponatinib, metabolite and both labeled internal standards at the LLOQ. For each transition the response of the corresponding double blank sample has been added. An artificial off-set was given to all traces.

2.3. Liquid chromatography–tandem mass spectrometry conditions

Partial-loop injections (5 μl) were made on a reversed phase Acquity UPLC® BEH C18 column (30 mm × 2.1 mm, dp = 1.7 μm, Waters, Ireland), protected by the corresponding VanGuard pre-column (Waters, 5 mm × 2.1 mm). The column temperature was maintained at 40 °C and the sample rack compartment of the autosampler at 4 °C. Linear gradient elution was performed at a flow rate of 600 μl/min with solvent A (2% (v/v) ammonium hydroxide in water) remaining constant at 20%; this solvent was supplemented with B (water) and C (methanol): 0 min, 15% A/65% B; 1 min, 0% A/80% B; 1.4 min, 0% A/80% B; 1.41, 15% A/65% B; 2 min, 15% A/65% B. The mobile phase was transferred into the electrospray probe, starting at 0.5 min until 1.88 min after injection by switching the MS divert valve. The heated ESI source was tuned in the positive ionization mode by introducing 0.6 ml/min of a mixture of 0.1% formic acid in water (50%, v/v) and methanol (50%, v/v), 5 μl/min of 10,000 ng/ml ponatinib and analogous for the other compounds to optimize the compound-dependent parameters. The highest response for ponatinib was obtained with a 4000 V spray voltage, a 400 °C capillary temperature, a 385 °C vaporizer temperature, and the nitrogen sheath, ion sweep and auxiliary gasses set at 60, 0 and 35 arbitrary units, respectively; the skimmer voltage was −10 V. The SRM mode was used with argon as the collision gas at 1.5 mTorr. The tube lens off-set was 109 V for ponatinib, 103 V for DMP and 110 V for both labeled internal standards. Selected precursor ions were *m*/*z* 533.2 for ponatinib, 519.2 for DMP, 541.1 for ponatinib-d_8 and 527.2 forDMP-d_8. For all compounds the product ion was monitored at *m*/*z* 260.1 at a −29 V collision energy using 0.1s dwell times. The mass resolutions were set at 0.7 full width at half height (unit resolution) for both separating quadrupoles.

2.4. Sample pre-treatment

A volume of 20 μl mouse lithium heparin plasma was pipetted into a 1.5 ml polypropylene tube, subsequently 10 μl of 500 ng/ml desmethylponatinib-d_8 and 500 ng/ml ponatinib-d_8 in 50% (v/v) methanol in water and then 500 μl of tBME was added to the tubes. The tubes were then closed and mixed using a rotary mixer at 45 rpm for 15 min and were then centrifuged at 10,000*g* for 2 min at 22 °C. Supernatants were transferred to new 1.5 ml polypropylene tubes, tBME was evaporated from the samples at 40 °C using a gentle stream of nitrogen gas. After evaporation 200 μl of 50% (v/v) methanol in water were added to each tube. Tubes were vortex mixed for 15 s and then were centrifuged at 10,000*g* and 22 °C for 2 min. The supernatant was pipetted into a 250 μl glass insert inside an autoinjector vial.

2.5. Validation

A laboratory scheme based on international guidelines was used for the validation procedures [13,14].

2.5.1. Calibration

Stock solutions of ponatinib and DMP were prepared at 0.5 mg/ml in methanol, both labeled internal standards at 0.1 mg/ml. One couple of ponatinib/DMP stock solutions was diluted to 0.1/0.02 mg/ml in 50% (v/v) methanol and further diluted to 5000/1000 ng/ml in plasma to be used as the highest calibration sample. All solutions were stored at −30 °C.

Additional calibration samples were prepared daily in duplicate by further dilution of the highest level (5000/1000 ng/ml ponatinib/DMP) to 1250/250, 500/100, 125/25, 50/10, 12.5/2.5

Table 1
Assay performance data of ponatinib and DMP resulting from four validation (QC, $n = 18$ each) samples in 3 analytical runs.

Nominal concentration (ng/ml)	Within-day precision [%]	Between-day precision [%]	Accuracy [%]
Ponatinib			
4000	4.0	6.2	97.5
250	2.7	5.3	103.2
15	3.8	5.1	108.3
5	3.9	5.6	102.6
DMP			
800	5.2	6.4	96.9
50	5.1	6.1	101.5
3	7.2	8.3	102.6
1	9.9	12.0	91.9

and 5/1 ng/ml to obtain seven non-zero calibrators for both analytes. The linear calibration curves ranged from 5–5000 ng/ml for ponatinib and 1–1000 ng/ml DMP and were constructed by linear least-squares regression using ratios of peak area of analytes and their corresponding labeled internal standards, with the reversed square of the concentration ($1/x^2$) as the weighting factor.

2.5.2. Precision and accuracy

A second couple of ponatinib/DMP stock solutions was diluted to 0.1/0.02 mg/ml in 50% (v/v) methanol and further diluted to obtain Quality Control (QC) samples at the levels 4000/800 (high), 250/50 (med), 15/3 (low) and 5/1 (LLOQ) ng/ml in mouse plasma for both compounds. The QC samples were stored in polypropylene 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). Relative standard deviations were calculated for both the within-day precisions (repeatability) and the between-day precisions (reproducibility).

2.5.3. Selectivity

Six individual mouse plasma samples were processed to test the selectivity of the assay. The samples were processed without analytes and ISs and with both compounds at the LLOQ levels (5 ng/ml ponatinib and 1 ng/ml DMP), supplemented with the ISs.

2.5.4. Recovery and matrix effect

The extraction recovery was determined in sextuple by comparing processed samples (QC-high, −med, −low) with reference solutions in 50% (v/v) methanol at the same three validation levels, diluted 10-fold (analogous to the preparation of plasma samples) in the same solvent.

The matrix effect was determined by a post-column infusion experiment with six individual blank mouse plasma samples. A solution of 2000 ng/ml of both ponatinib and DMP in 50% (v/v) methanol was infused continuously into the electrospray after post-column mixing with the eluent after injecting the blank pretreated samples. No divert valve was used during these runs.

The relative matrix effect was assessed by adding 20 μl of both, a QC−low and −high reference sample in 50% (v/v) methanol, 10 μl of IS solution and 170 μl 50% (v/v) methanol to the extraction residue of six different blank mouse plasma samples. Relative responses were compared to extract-free samples.

2.5.5. Stability

The stability of ponatinib and DMP was investigated in QC-high and QC-low plasma samples stored in polypropylene tubes. Quadruplicate analysis of these samples was performed after three additional freeze-thaw cycles (thawing at 20 °C during *ca.* 30 min and freezing again at −30 °C for at least one day), and storage at −30 °C for 52 days, respectively. Short term stability at 20 °C was investigated after 2, 4, 6, 8 and 24 h storage.

Furthermore, QC samples at the high and low level ($n = 6$) were reanalyzed after storage of the extracts at 4 °C for 8 and 14 days, respectively.

2.6. Pharmacokinetics in mice

Wild-type (FVB [Friend Leukemia Virus, strain B] genetic background) female mice ($n = 5$) were housed and handled as reported previously [15] and were treated with 10 mg/kg ponatinib orally. A 1 mg/ml ponatinib solution was obtained by dissolving the drug in dimethyl sulfoxide to a concentration of 20 mg/ml, followed by 20-fold dilution in 25 mM citrate buffer (pH 2.75) to obtain the solution for administration. Blood samples (≤50 μl) were collected in lithium heparin-containing microvettes via the tail vein at 0.5, 1, 2, 4, and 8 h after oral administration of the drug. After 24 h, mice were anesthetized with isoflurane and blood was drawn by cardiac puncture. After centrifugation at 2100*g* for 6 min at 4 °C, plasma samples were stored at −30 °C prior to analysis.

Pharmacokinetic parameters of ponatinib and DMP were calculated; area under the plasma concentration-time curves ($AUC_{0\text{-}24}$) using the trapezoidal rule for the 24 h of the experiment, the ponatinib terminal half-life ($t_{1/2}$) using the two latest time points and absorption rate (k_a) using curve stripping of the 0.5–4 h time points based on a first-order, one-compartment model.

Sample reanalysis of mouse plasma samples was investigated 14–15.5 months after initial analysis.

3. Results and discussion

3.1. Method development

3.1.1. Tandem mass spectrometry

Positive ESI settings were optimized for both compounds using protonated molecules with a focus on the metabolite because of its lower levels. Product spectra of ponatinib, metabolite and both deuterium labeled internals standards are shown in Fig. 1, all spectra were very similar to the ponatinib spectrum because of the neutral loss of the methyl piperazinyl group that is desmethylated and/or deuterated in the other compounds for most fragmentations. Stable isotope labeled internal standards are favorable in LC–MS bioanalysis because of their optimal ability to correct for variations occurring during sample treatment and ionization. Both internal standards could be commercially obtained. Methanol showed slightly higher responses compared to acetonitrile as well as did ammonium hydroxide compared to formic acid.

3.1.2. Liquid chromatography

A fast reversed-phase chromatographic method was developed using a short column with small particles for an efficient separation. Solvents were chosen for their maximal MS responses and their ratios were optimized to obtain a fast gradient followed by a flushing step to remove remaining strong retaining endogenous

compounds. The desmethyl metabolite initially showed some peak tailing, probably caused by interaction of the desmethylated amine group with the stationary phase. This effect was eliminated by using a relatively high concentration of ammonium hydroxide (0.4% (v/v) throughout the analytical run).

3.1.3. Sample treatment

Initial experiments were started with acetonitrile protein precipitation, previously used for ponatinib in cell lysate [16]. Unfortunately, precisions were insufficient for validation in the low ng/ml range and chromatographic peaks showed additional peak broadening. Therefore, liquid-liquid extraction was investigated as a more efficient alternative. Both ponatinib and DMP could be extracted with tBME, the phase ratio for maximal extraction recovery was relatively high (500 μl tBME to 20 μl plasma and 10 μl IS solution) but still clean blank chromatograms could be obtained using this method (Fig. 2A).

3.2. Method validation

The ponatinib calibration range was chosen based on previously reported levels in human [8,9,11,12] with sufficient margin for potentially higher levels in mice. SRM chromatograms of blank and LLOQ spiked samples are depicted in Fig. 2.

3.2.1. Calibration

Linear responses were observed for both compounds. Data of 19 calibrations were collected resulting in the following calibration parameters: y(ponatinib) = 0.0023 (±0.0015) + 0.0039 (±0.0005) x with regression coefficient 0.996 ± 0.002 and y(DMP) = 0.0049 (±0.0014) + 0.0048 (±0.0005) x with regression coefficient 0.993 ± 0.003. Here, x is the analyte concentration (ng/ml) and y is the drug response relative to the corresponding labeled IS.

3.2.2. Accuracy and precision

Assay performance data in mouse plasma are presented in Table 1. As expected based on the higher concentrations and higher response, variations of ponatinib are lower compared to the metabolite. All precisions, except for DMP at the LLOQ, were ≤8.3%. Furthermore, deviations of the accuracy were all ≤8.3%. The precision and accuracy therefore met the required ±15% variation (±20% for the LLOQ) [13,14].

3.2.3. Selectivity

Average LLOQ levels spiked to independent blank samples (n = 6) were checked as well as blank responses. Average LLOQ levels found were 5.12 ± 0.24 ng/ml ponatinib and 0.93 ± 0.11 ng/ml DMP. Blank responses were ≤1% for ponatinib and ≤2% for DMP compared to LLOQ responses (Fig. 2); blank IS responses were ≤0.01% for ponatinib-d_8 and ≤0.02% for DMP-d_8 compared to normal IS responses. These results demonstrate the applicability of the investigated LLOQ levels [13].

3.2.4. Recovery and matrix effect

Extraction recoveries for both compounds at all QC levels ranged from 68 to 84% (data not shown). Matrix effects could not be observed at the retention times of both components as expected due to the efficient sample clean-up and sample dilution (from 20 to 200 μl). Assessment of the relative matrix effect in six independent blank plasma samples at the QC-low level however showed a small but significant bias (−17.2 ± 4.6% for ponatinib and −18.0 ± 4.5% for DMP). This surprising result could be replicated by a second analyst but could unfortunately not be explained. Fortunately, such a reproducible bias cannot impair the accuracy and precision of the assay and a significant bias was not observed at the QC-high level (3.8 ± 2.9% for ponatinib and 1.6 ± 2.5% for DMP).

Table 2
Stability data (recovery [%]; ±S.D; n = 4) of ponatinib and DMP in murine lithium heparin plasma (and in reconstituted extract), reporting the percentage of the initial concentration (values below 85% in bold).

PLASMA	Ponatinib		DMP	
Condition	QC-high	QC-low	QC-high	QC-low
3 freeze-thaw cycles	89.9 ± 5.8	91.2 ± 4.5	92.8 ± 7.0	88.4 ± 5.9
52 days at −30 °C	89.1 ± 1.4	97.7 ± 9.6	89.6 ± 0.8	89.4 ± 4.2
Ambient temperature (h)				
2	90.9 ± 2.9	92.7 ± 3.8	91.6 ± 2.9	98.0 ± 9.6
4	91.3 ± 10.0	87.9 ± 5.5	96.0 ± 9.2	91.5 ± 3.5
6	**83.8 ± 1.6**	88.2 ± 8.5	93.2 ± 1.8	86.3 ± 11.9
8	**82.9 ± 2.4**	**84.5 ± 4.2**	91.3 ± 3.6	88.3 ± 9.7
24 (n = 3)	**56.4 ± 0.6**	**62.6 ± 0.8**	87.9 ± 1.3	**84.6 ± 7.4**
RECONSTITUTED EXTRACTS				
8 days at 4 °C (n = 6)	90.8 ± 1.7	96.6 ± 2.8	91.0 ± 2.0	96.1 ± 6.1

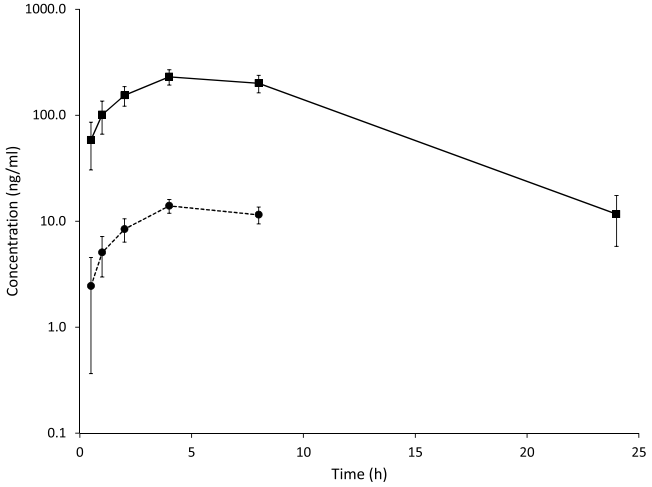

Fig. 3. Pharmacokinetic plots of ponatinib (■; —) and DMP (●; ----) in female FVB mice (n = 5) plasma after oral administration of a single dose of 10 mg/kg ponatinib. Averages ±SD are depicted.

3.2.5. Stability

Stability results in plasma matrix and in reconstituted extracts are listed in Table 2. Both compounds show some degradation in plasma, especially ponatinib, whereas this degradation of ponatinib was not observed in human plasma after 6 [10] and 24 h [8], respectively. Therefore, mouse plasma samples should not be left at ambient temperature for longer than 4 h to remain within ±15% deviations [14]. In additional experiments, degradation products found in mouse plasma (AP24600 [m/z 277.1] and the remaining anilines of both, ponatinib [m/z 274.1] and DMP [m/z 260.1]) clearly indicated amide hydrolysis as a main degradation route. This route has been previously reported *in vivo* [8] and was mediated by esterases and/or amidases [12] that may also be present in plasma. Therefore, additional precautions may be necessary for assays quantifying the AP24600 carboxylic acid metabolite [9].

Finally, Stability in reconstituted extract was shown after 8 days at 4 °C.

3.3. Mouse pharmacokinetics

To complete the validation procedure the new assay was applied in a pharmacokinetic study. Here we report the results for female FVB mice, monitored for 24 after oral administration of 10 mg/kg ponatinib. Levels up to 300 ng/ml ponatinib and 16 ng/ml DMP were measured; pharmacokinetic plots are shown in Fig. 3, parameters

Table 3
Pharmacokinetic data of 5 female FVB mice after oral administration of 10 mg/kg ponatinib (average ±S.D).

	ponatinib	DMP
AUC_{0-24} (ng min ml^{-1})	3126 ± 315	175 ± 15
c_{max} (ng/ml)	239 ± 37	14.1 ± 1.3
t_{max} (h)	6 ± 2	5 ± 2
$t_{1/2}$ (h)	3.8 ± 0.6	not determined
k_a (h^{-1})	0.41 ± 0.11	

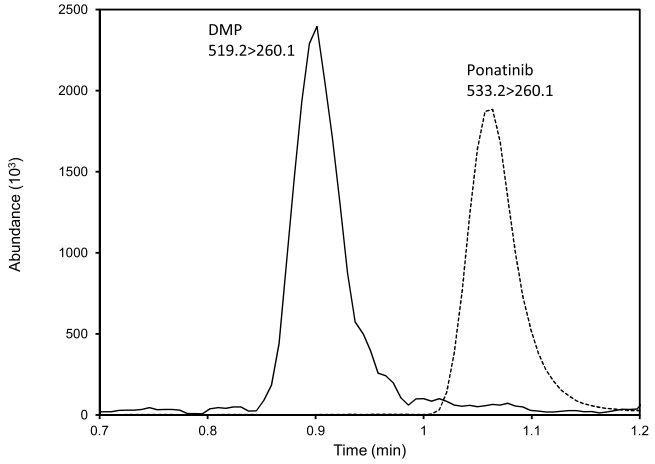

Fig. 4. SRM chromatogram of ponatinib (36.1 ng/ml; ----) and DMP (1.45 ng/ml; —) in a plasma sample. The sample was taken 0.5 h after oral administration of 10 mg/kg ponatinib to a FVB mouse. The metabolite trace was magnified 100 times.

in Table 3. No metabolite could be detected in the 24−h samples. For one of the samples, the SRM chromatogram is shown in Fig. 4. Pharmacokinetics showed results with small variations. Metabolite exposure is 5.6% of the parent compound, $t_{1/2}$ of ponatinib is short compared to 24 h in human [8], $t_{1/2}$ of the metabolite could not be calculated because metabolite levels were below LLOQ at 24 h but seems to be close to the $t_{1/2}$ of ponatinib (Fig. 3). Therefore, metabolite elimination is probably formation rate limited.

Incurred sample reanalysis 14–15.5 months later than initial analysis ($n = 39$) resulted in 3 samples exceeding ±20% for ponatinib and 13 for DMP where 13 is allowed [14]. So even after this long period this requirement could be met, indicating sufficient stability of both compounds during this time.

4. Conclusion

The first fully validated bioanalytical assay for the quantification of ponatinib and DMP has now been reported for mouse plasma. The LC−MS/MS used a fast and simple sample pre-treatment procedure and results showed values for accuracy, precision, recovery and stability which were compliant to international guidelines [13,14]. The new assay was successfully used for the analysis of plasma levels of both compounds after administration of a single oral dose of 10 mg/kg ponatinib to FVB mice.

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