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Short communication

Liquid chromatography–tandem mass spectrometric assay for the mutated BRAF inhibitor dabrafenib in mouse plasma

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ABSTRACT

A quantitative bioanalytical liquid chromatography–tandem mass spectrometric (LC–MS/MS) assay for the mutated BRAF inhibitor dabrafenib was developed and validated. Plasma samples were pre-treated using protein precipitation with acetonitrile containing PLX4720 as internal standard. The extract was directly injected into the reversed-phase chromatographic system after dilution with water. The eluate was transferred into the electrospray interface with positive ionization and the analyte was detected in the selected reaction monitoring mode of a triple quadrupole mass spectrometer. The assay was validated in a 2–2000 ng/ml calibration range with $r^2 = 0.993 \pm 0.002$ for linear regression with quadratic weighting ($n = 5$). Within day precisions ($n = 6$) were 3.3–5.2%, between day (3 days; $n = 18$) precisions 4.7–8.2%. Accuracies were between 95–104% for the whole calibration range. The drug was sufficiently stable under all relevant analytical conditions. Finally, the assay was successfully used to determine drug pharmacokinetics in mice.

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1. Introduction

B-RAF proteins belong to the RAF family of kinases and play a role in regulating the MAP kinase signaling pathway [1]. Vemurafenib (PLX4032) was the first selective, potent and orally bioavailable ATP-competitive inhibitor of the serine/threonine kinase B-RAF protein encoded by the V600E mutated BRAF gene. Several other selective inhibitors of B-RAF enzymes are being developed and tested in order to increase the available therapeutic arsenal of these innovative B-RAF targeted drugs. Dabrafenib (GSK2118436; Fig. 1A) is the latest inhibitor developed against V600E BRAF-mutated metastatic melanoma, it showed no activity for the less frequent V600K BRAF-mutated melanoma [2] and regulatory submissions have been announced in August 2012. In a phase III trial it significantly improved progression-free survival compared to treatment with standard dacarbazine therapy [3]. Currently, the promising combination of dabrafenib with the MEK inhibitor trametinib is under investigation [4].

Dabrafenib is extensively metabolized to mainly GSK2298383 [5]; unfortunately, the identity of dabrafenib metabolites and routes of biotransformation are not yet public knowledge. In addition, as far as we know, analytic methods for the quantification of this drug in biological matrices have not yet been reported hitherto. Therefore, we now report the development and validation of the first bio-analytical assay for dabrafenib in mouse plasma to support our preclinical studies with the agent, using LC–MS/MS and protein precipitation as a simple pre-treatment procedure.

2. Experimental

2.1. Chemicals

Dabrafenib mesylate (GSK2118436B; >99%) was purchased from ChemieTek (Indianapolis, IN, USA) and PLX4720 (>99%, Fig. 1B, internal standard (IS)) from Selleck Chemicals (Houston, TX, USA). Water (LC–MS grade), methanol (HPLC grade) and acetonitrile (HPLC-S grade) were obtained from Biosolve (Valkenswaard, The Netherlands). Water, not used as eluent, was home purified by reversed osmosis on a multi-laboratory scale. Formic

Abbreviations: AUC, area under the plasma concentration–time curve; LLOQ, lower limit of quantification; SRM, selected reaction monitoring; $T_{1/2}$, elimination half-life; QC, quality control.

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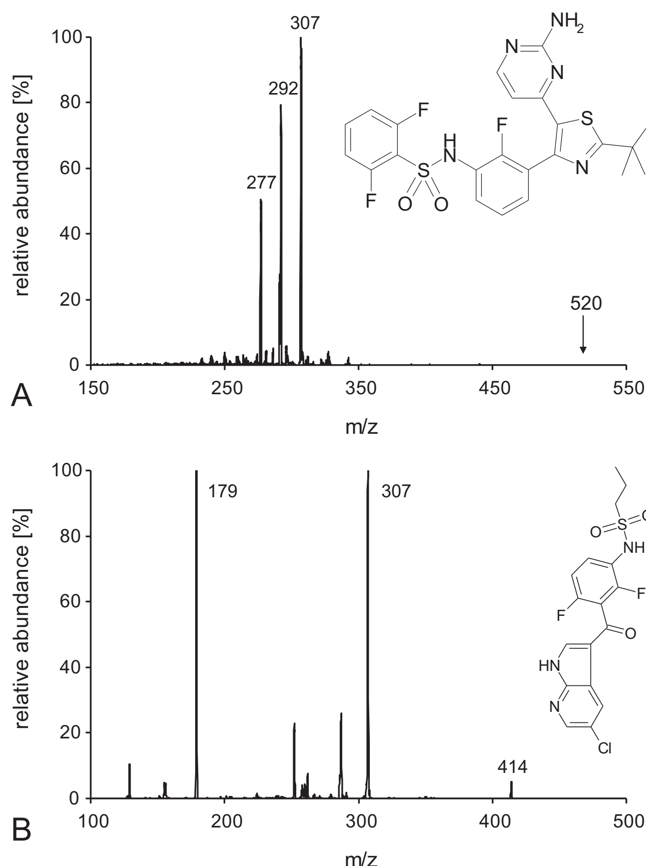

Fig. 1. Chemical structures and product spectra, formed by collision induced dissociation of the protonated molecules of (A) dabrafenib, m/z 420.15 @ −50 V and (B) PLX4720, m/z 414 @ −30 V.

acid was of analytical grade originating from Merck (Darmstadt, Germany). Female mouse lithium–heparin plasma was supplied by Seralab Laboratories (Haywards Heath, UK). Blank, drug-free, human plasma, containing citrate, phosphate and dextrose as anticoagulants, was obtained from the Sanquin Bloedbank (Utrecht, The Netherlands).

2.2. Equipment

The LC–MS/MS equipment consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc autosampler and two LC10-ADvp-μ pumps (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer with electrospray ionization (Thermo Electron, Waltham, MA, USA). Data were recorded on and the system was controlled using the Finnigan Xcalibur software (version 1.4, Thermo Electron).

2.3. LC–MS/MS conditions

Partial-loop injections (5 μl) were made on a Polaris 3 C18-A column (50 mm × 2 mm, d_p = 3 μm, average pore diameter = 10 nm, Varian, Middelburg, The Netherlands) with a corresponding pre-column (10 mm × 2 mm). The column temperature was maintained at 40 °C and the sample rack compartment at 4 °C. A gradient (0.5 ml/min) using 0.1% (v/v) formic acid (A) and methanol (B) was used. After injection, the percentage of methanol was increased linearly from 50 to 70% (v/v) during 1.33 min. Next, the column was flushed with 100% (v/v) methanol for 0.67 min and finally, the column was reconditioned at the starting conditions (50% (v/v) B) for 1 min resulting in a total run time of 3 min. The whole eluate was transferred into the electrospray probe, starting at 0.8 min after injection by switching the MS divert valve until 2.2 min after injection. The electrospray was tuned in the positive ionization mode by introducing 0.5 ml/min of the solvent mixture at 50% (v/v) B and 5 μl/min of 10 μg/ml of dabrafenib. Electrospray settings of the assay were a 3900 V spray voltage, a 391 °C capillary temperature and the nitrogen sheath, ion sweep and auxiliary gasses were set at 41, 8 and 6 arbitrary units, respectively; the skimmer voltage was set at −5 V. The SRM mode was used with argon as the collision gas at 2.0 mTorr. The tube lens off set was 131 V for dabrafenib and 135 V for PLX4720. Dabrafenib was monitored at m/z 520.15 → 277; 292; 307 at −59, −50 and −34 V collision energies, respectively, with 0.15 s dwell times and PLX4720 at m/z 414.1 → 179 at −40 V with an 0.1 s dwell time. Mass resolutions were set at 0.7 full width at half height (unit resolution) for both separating quadrupoles.

2.4. Sample pre-treatment

To a volume of 20 μl of mouse plasma, pipetted into a polypropylene reaction tube, 30 μl of 207 ng/ml PLX4720 in acetonitrile were added. The tubes were closed and shaken by vortex mixing for 5–10 s. After centrifugation of the sample at 10,000 × g at 20 °C for 1 min, 40 μl of the supernatant was transferred to a 250 μl glass insert placed in an autoinjector vial. Before closing the vial, 100 μl of water was added and finally, 5 μl of the mixture was injected onto the column.

2.5. Validation

A laboratory scheme based on international guidelines was used for the validation procedures [6–8].

2.5.1. Calibration

Stock solutions of dabrafenib mesylate at 0.2 and 0.5 mg/ml were prepared in methanol. PLX4720 was prepared at 4 mg/ml in dimethylsulfoxide. One dabrafenib stock solution was diluted to a 2000 ng/ml calibration solution in mouse plasma. All solutions were stored in a 1.5-ml polypropylene tube at −30 °C. Additional calibration samples were prepared daily at 1000, 200, 100, 20, 10, 4 and 2 ng/ml by dilution with blank mouse plasma. The highest and two lowest calibration samples were processed in duplicate for each daily calibration, whereas the levels in between were processed only once. Weighted linear least-squares regression with $1/X^2$ (reversed squared concentration) as the weighting factor was employed to define the calibration curves using the ratios of the peak area of the analyte and the IS.

2.5.2. Precision and accuracy

A second stock solution of dabrafenib was used to obtain validation (quality control; QC) samples in pooled mouse heparin plasma at 1500 (QC-high), 100 (QC-med), 5 (QC-low) and 2 ng/ml (QC-LLOQ). The 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 per QC). Relative standard deviations were calculated for both, the within and between day precisions.

2.5.3. Selectivity

Six individual mouse plasma samples were processed to test the selectivity of the assay. The samples were processed without

dabrafenib and IS and with dabrafenib at the LLOQ level (2 ng/ml), supplemented with the IS.

2.5.4. Recovery and matrix effect

The recovery was determined ($n = 4$) by comparing processed samples (QC-high, -med, -low) with reference dabrafenib solutions in blank plasma extract at the same levels. The matrix effect was assessed by comparing the reference solutions in blank plasma extracts with the same matrix free solutions at the three validation levels. An analogous procedure was used for the internal standard.

2.5.5. Stability

The stability of dabrafenib was investigated in QC-high and -low plasma samples stored in polypropylene tubes. Quadruplicate analysis of these samples from separate tubes was performed after storage at 20 °C (ambient temperature) for 24 h, three additional freeze–thaw cycles (thawing at 20 °C during ca. 2 h and freezing again at −30 °C for at least one day), and storage at −30 °C for 3 months, respectively. Furthermore, analytical runs were re-injected after additional storage of the extracts at 4 °C for four nights to test the stability at the conditions in the autoinjector.

Finally, the responses of dabrafenib from the stock solutions in methanol after 6 h at 20 °C ($n = 2$) and after 8 months at −30 °C ($n = 2$) were compared to fresh stock solutions with LS–MS/MS after appropriate dilution of the samples and adding IS.

2.6. Mouse samples

Wild-type (FVB genetic background) female mice ($n = 5$) were housed and handled as reported previously [9] and were treated with 20 mg/kg dabrafenib mesylate orally. The 2 mg/ml dabrafenib mesylate solution was obtained by dissolving the drug in dimethylsulfoxide (30 mg/ml), followed by 15-fold dilution in 20% polysorbate 80, 13% ethanol and 67% water (v/v/v) vehicle mix and 5% glucose (w/v) in a ratio of 1:2 (v/v). Blood samples (~50 μl) were collected in lithium heparin containing microvettes via the tail vein at 0.25, 0.5, 1, 2 and 4 h after administration of the drug. At 8 h, blood was collected by cardiac puncture. After centrifugation at 2100 × g for 6 min at 4 °C, plasma samples were stored at −30 °C prior to analysis

Plasma samples were diluted with human plasma before final analysis when required because of their high drug level, especially when collected during the 0.25–1 h interval. Human plasma was used for dilution to avoid the use of a relatively excessive amount of mouse plasma for each sample. An additional QC sample at 25,000 ng/ml dabrafenib was processed after dilution, using both, 100-fold (5–500 μl) and 25-fold (5–125 μl) dilution (both $n = 6$, intra-day) to assure the performance of the required sample dilution.

Pharmacokinetic parameters (area under the plasma concentration–time curve (AUC), both for 8 h (AUC_{0-8}) and with extrapolation to infinity ($AUC_{0-\infty}$); elimination half-life ($T_{1/2}$) of dabrafenib melylate were calculated based on a first-order, one-compartment model. For calculation of the (terminal) half-life and extrapolation, 2–8 h time points were used.

3. Results and discussion

3.1. Method development

ESI-MS/MS settings were optimized for dabrafenib to obtain maximal sensitivity. Product spectra of dabrafenib and IS are shown in Fig. 1. Protein precipitation is a simple pretreatment procedure that can be used when separation from interfering plasma compounds, mainly salts at low and phospholipids at high retention under reversed-phase conditions [10], is sufficient. Acetonitrile is the most efficient organic precipitation agent [11]. Strongly retained compounds were removed from the column using a high organic flush in order to prevent long term suppression effects of the ionization. This procedure has earlier successfully been used in our laboratory for several other kinase inhibitors [12–14]. Dabrafenib showed approximately equal MS responses under acidic (formic acid) and alkaline (ammonium hydroxide) conditions while the response was higher using methanol, compared to acetonitrile. Because a stable isotopically labeled analog of dabrafenib was not available, PLX4720, another sulfonamide BRAF inhibitor, showing almost identical retention as dabrafenib in the present assay (Fig. 2) was chosen as internal standard.

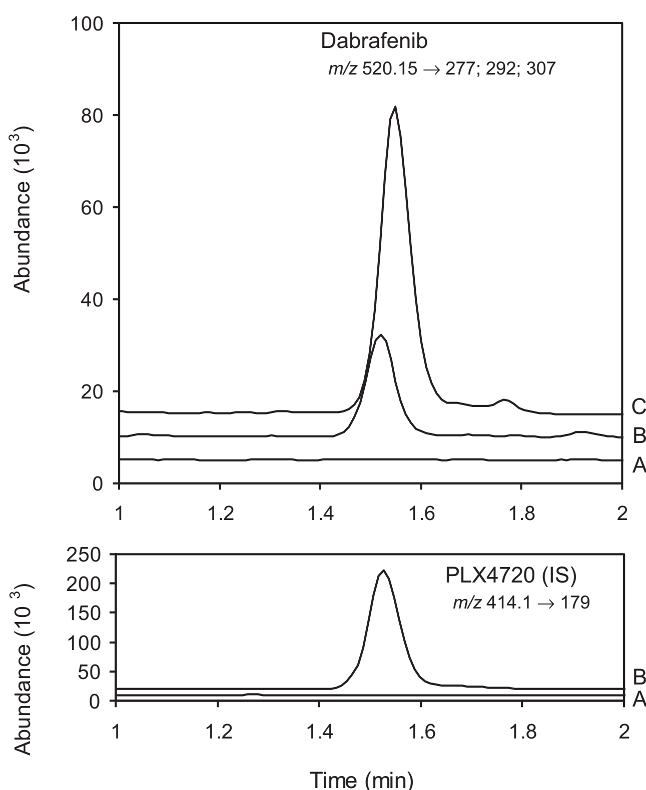

Fig. 2. SRM chromatograms of dabrafenib and the IS in plasma extracts: blank mouse plasma (A), LLOQ (2 ng/ml) spiked plasma (B) and wild type female mouse lithium heparin plasma, containing 10.4 ng/ml of the drug (C), taken 8 h after oral admistration of 20 mg/kg dabrafenib mesylate. An artificial off set was given to the chromatograms.

3.2. Validation

A 2–2000 ng/ml range was chosen because the maximal drug level in human (150 mg BID) was about 1000 ng/ml [5] and mouse data were not available. SRM chromatograms are depicted in Fig. 2, showing chromatograms of blank and LLOQ spiked plasma samples.

3.2.1. Calibration

The relative response of dabrafenib did not show any sign of non-linearity; therefore, a linear function with quadratic weighting was used for the assay calibration. For 5 calibrations (55 samples) the concentrations were back-calculated from the ratio of the peak areas (analyte and IS) using the calibration curves of the run in which they were included. No deviations of the average of each level higher than 6.4% were observed (data not shown), indicating

Table 1
Assay performance data of dabrafenib resulting from four validation (QC, n = 18 each) samples in 3 analytical runs and from a fifth QC sample after additional dilution with human plasma (n = 6) prior to final processing.

Nominal concentration (ng/ml)	Within day precision (%)	Between day precision (%)	Accuracy (%)
25,000 (100-fold)	3.9		104.5
25,000 (25-fold)	6.5		111.7
1500	3.3	5.8	95.8
100	3.3	4.7	94.9
5	5.2	8.2	103.7
2	4.2	8.0	99.7

the suitability of the regression model used. The average regression parameters (n = 5) were $y = -0.0006(\pm 0.0058) + 0.052(\pm 0.009)x$ with a regression coefficient of 0.993 ± 0.002. Here, x is the concentration (in ng/ml) and y is the dabrafenib response relative to the IS.

3.2.2. Precision and accuracy

Assay performance data from the validation samples at four concentrations are reported in Table 1. Within day and between day variations lower than 8.2% were observed and deviations of the accuracies were lower than 5.1%. The precision and accuracy therefore met the required ±15% variation (±20% for the LLOQ) [6–8].

3.2.3. Selectivity

The analysis of six independent blank mouse plasma samples showed no interfering peaks in the SRM traces for dabrafenib and the IS PLX4720. Blank dabrafenib responses were all <5% of the LLOQ response, meeting the required 20% [15], and blank IS responses below 0.1% of the normal response. The signals at the LLOQ level (2 ng/ml) were all distinguishable from blank responses with signal-to-noise ratios in the range 40–100; concentrations found at the LLOQ level (n = 6) were 1.90 ± 0.14 ng/ml, demonstrating the applicability of the investigated LLOQ level [6–8].

3.2.4. Recovery and matrix effect

The extraction recoveries showed no losses for dabrafenib and ranged from 112 to 116% (data not shown), these values slightly exceeding 100% can be explained by solvent contraction caused by mixing different solvents and the formation of protein precipitate. Matrix effects were also not observed; ionization recoveries ranged from 89 to 103% for dabrafenib at the investigated levels. For the internal standard PLX4720 values in the same range, 105.6 ± 4.6% for extraction recovery and 97.3 ± 3.1% (both n = 4) for matrix effect, were found. Overall, the absence of extraction losses and matrix effects contributed to a successful validation of the assay [6–8].

3.2.5. Stability

The stability of dabrafenib in female mouse lithium heparin plasma after different storage procedures is presented in Table 2. Very small losses up to only 8.0% were found without any decline of the precision. Re-injection of calibration and QC samples after additional storage at 4 °C for four nights resulted again in successful performances without any loss of precision and accuracy, so QC failures remained far below a 33% frequency (data not shown) as required [6,15]. Recoveries of dabrafenib in stock solutions were 99.3% (after 6 h at 20 °C; n = 2) and 84.9% (after 8 months at −30 °C; n = 3), respectively. The short term test was therefore considered satisfactory (>95%) for the validation [6–8] but the long term test not. This long term degradation; however, did not interfere with all other validation experiments because for preparation of (stable) plasma samples no methanolic stock solutions older than 1 week were used.

Table 2
Stability data (recovery [%]; ±SD; n = 4) of dabrafenib in female lithium heparin mouse plasma, reporting the percentage of the initial concentration.

Condition	QC-high	QC-low
24 h at ambient temperature	92.0 ± 2.0	97.9 ± 5.3
3 freeze–thaw cycles	92.2 ± 3.1	102.4 ± 7.1
3 months at −30 °C	93.3 ± 1.6	98.2 ± 6.2

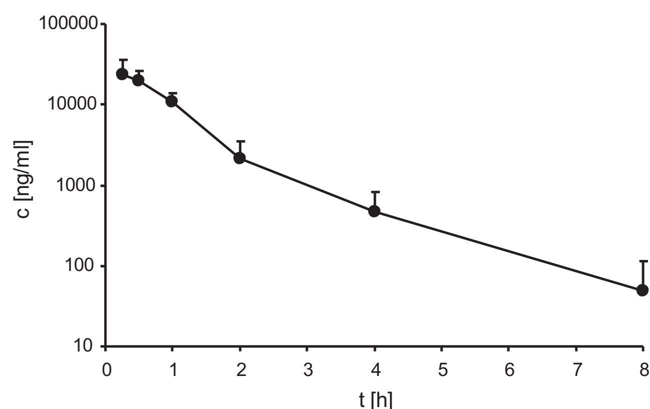

Fig. 3. Pharmacokinetic plot of dabrafenib mesylate in wild type female mouse plasma after oral administration of 20 mg/kg dabrafenib mesylate (n = 5).

3.3. Mouse pharmacokinetics

After the successful validation procedure, the new assay was used to investigate the plasma pharmacokinetics of dabrafenib mesylate in wild type female mice. Successful processing of a QC sample with additional dilution is shown in Table 1, allowing dilution of high concentration samples exceeding the calibration range. Results of the animal experiments (n = 5) at the 20 mg/kg oral dosage are depicted in Fig. 3. Dabrafenib mesylate levels in plasma were in a wide range of 2–38,047 ng/ml and average (±SD) pharmacokinetic parameters were: $AUC_{0-8} = AUC_{0-\infty} = 26 \pm 9$ μg h/ml and $T_{1/2} = 1.1 \pm 0.7$ h. Finally, a short absorption phase was observed ending within approximately 0.5 h after administration of the drug.

4. Conclusions

The first validated assay for dabrafenib has now been fully reported for female mouse lithium heparin plasma samples. The sensitive LC–MS/MS assay includes a fast and simple sample pretreatment method. The results show values of accuracy, precision, recovery and stability allowed by international guidelines [6–8]. The new assay was successfully used for a pharmacokinetic mouse study of the drug and can be used for future pharmacokinetic studies of dabrafenib.

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