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Short communication

Liquid chromatography–tandem mass spectrometric assay for the mutated BRAF inhibitor vemurafenib in human and mouse plasma

Rolf W. Sparidans [a,*], Selvi Durmus [b], Alfred H. Schinkel [b], Jan H.M. Schellens [b,c], Jos H. Beijnen [a,c,d]

[a] Utrecht University, Faculty of Science, Department of Pharmaceutical Sciences, Division of Pharmacoepidemiology & Clinical Pharmacology, Universiteitsweg 99, 3584 CG Utrecht, The Netherlands
[b] The Netherlands Cancer Institute, Departments of Medical Oncology and Molecular Biology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[c] The Netherlands Cancer Institute, Department of Clinical Pharmacology, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
[d] Slotervaart Hospital, Department of Pharmacy & Pharmacology, Louwesweg 6, 1066 EC Amsterdam, The Netherlands

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ABSTRACT

A bioanalytical assay for the mutated BRAF inhibitor vemurafenib was developed and validated. For the quantitative assay, human plasma samples were pre-treated using protein precipitation with water-acetonitrile (1/3, v/v) containing sorafenib as internal standard. The extract was directly injected into the chromatographic system. This system consisted of a sub-2 μm particle, trifunctional bonded octadecyl silica column with isocratic elution using 0.01% (v/v) of formic acid in a mixture of water and methanol. 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 0.1–100 μg/ml calibration range. Within day precisions were 1.6–3.2%, between day precisions 2.7% and 8.2% and accuracies were between 99% and 106% for the whole calibration range. The drug was stable under all relevant conditions. Finally, the assay was successfully used to assess drug levels in a pharmacokinetic mouse study.

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1. Introduction

Vemurafenib (PLX4032, Fig. 1) is the first selective, potent and orally bioavailable inhibitor of the serine/threonine-protein kinase B-Raf protein encoded by the V600E mutated *BRAF* gene [1]. The drug was approved by the FDA in August, 2011 [2]. B-Raf is a frequently mutated protein kinase [3] and mutation of the gene is very common in melanoma [4,5]. Recently, improved survival was observed in an ongoing Phase III clinical study with vemurafenib compared to dacarbazine in patients with previously untreated metastatic melanoma with this mutation [6]. Total metabolite levels in plasma were below 6% compared to the parent drug level [7]. To further improve survival of melanoma patients after a 40 years lack of progress, combination therapies will be explored, especially combinations with immunomodulators are expected to be very active [1].

To support the future clinical investigations with vemurafenib a bioanalytical assay will be indispensable. An LC–MS assay was used by Flaherty et al. [8] to reveal the pharmacokinetic profile of vemurafenib but any details of the method were not given. As far as we know a validated bioanalytical assay for vemurafenib has not been reported hitherto, quantifying metabolites was not expected to be necessary due to their low levels [7]. The drug showed high therapeutic plasma levels (*ca.* 40 ± 20 μg/ml) and a long, 50 h half-life in this Phase I study [8]. The high levels should allow a fast and simple procedure. An LC–MS/MS assay for vemurafenib in plasma was therefore developed and validated in a 96-well format using sub-2 μm LC particles to obtain a high sample throughput.

2. Experimental

2.1. Chemicals

Vemurafenib, sorafenib tosylate and all other kinase inhibitors were obtained from Sequoia Research Products (Pangbourne, UK). 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 acid was of analytical grade and originated from Merck (Darmstadt, Germany) and analytical grade dimethyl sulfoxide (DMSO) from Acros Organics (Geel, Belgium). Sodium EDTA plasma (pooled and from individual donors) was from Innovative Research (Southfield, MI, USA).

Abbreviations: HESI, heated electrospray ionization; LLOQ, lower limit of quantification; SRM, selected reaction monitoring; QC, quality control.

* Corresponding author. Tel.: +31 6 20289970; fax: +31 30 2539166.
E-mail address: R.W.Sparidans@uu.nl (R.W. Sparidans).

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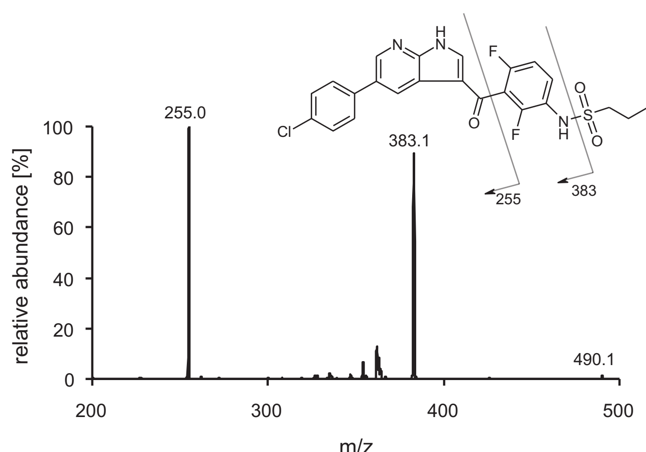

Fig. 1. Chemical structure and product spectrum of vemurafenib (*m*/*z* 490.14@-34 V).

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; 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 (0.5 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 sample rack compartment of the autosampler at 4 °C. The eluent comprised a mixture of 0.1% (v/v) formic acid in water (10%, v/v), water (20%, v/v) and methanol (70%, v/v), that was pumped at 0.6 ml/min. The eluate was transferred into the electrospray probe, starting at 0.4 min after injection by switching the MS divert valve. The HESI 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) and 5 μl/min of 20 μg/ml vemurafenib. HESI settings of the assay were 4000 V spray voltage, a 350 °C capillary temperature and vaporizer temperature and the nitrogen sheath, ion sweep and auxiliary gasses were set at 50, 8 and 50 arbitrary units, respectively; 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 127 V for vemurafenib and 120 V for sorafenib. Vemurafenib was monitored at *m*/*z* 490.1 → 255.05; 383.1 at −41 V and −27 V collision energies, respectively with 0.1 s dwell times and sorafenib at *m*/*z* 465.1 → 252.05; 270.05 at −30 V and −22 V with 0.05 s dwell times. 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 50 μl of human plasma (or 10 μl mouse plasma, supplemented with 40 μl human plasma), pipetted into a 1 ml polypropylene deep 96-well plate, 1 ml of 46 ng/ml sorafenib in water-acetonitrile (1/3, v/v) was added. The wells were then closed with a silicone mat and shaken manually for *ca.* 15 s. After centrifugation of the sample at 2643 × *g* at 20 °C for 5 min the supernatant (0.5 μl) was injected onto the column.

2.5. Validation

A laboratory scheme based on international guidelines was used for the validation procedures [9–11].

2.5.1. Calibration

Stock solution of vemurafenib at 5 mg/ml and sorafenib (IS) at 4.6 mg/ml were prepared in DMSO. All stock solutions were stored at −30 °C. One 5 mg/ml stock solutions of vemurafenib was diluted to a 100 μg/ml calibration sample in pooled human plasma, stored in a polypropylene tube at −30 °C. Additional calibration samples were prepared daily at 20, 5, 1, 0.25 and 0.1 μg/ml [9–11] by dilution with blank pooled plasma. All 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 vemurafenib and the IS.

2.5.2. Precision and accuracy

A second stock solution of vemurafenib was used to obtain validation (quality control; QC) samples in pooled plasma at 80 (QC-high), 5 (QC-med), 0.25 (QC-low) and 0.1 μg/ml (QC-LLOQ). 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) [9–11]. Relative standard deviations were calculated for both, the within day precisions (repeatability) and the between day precisions (reproducibility).

2.5.3. Selectivity

Six individual human plasma samples were processed to test the selectivity of the assay [9,11]. The samples were processed without vemurafenib and IS and with vemurafenib at the LLOQ level (0.1 μg/ml), supplemented with the IS.

2.5.4. Recovery and matrix effect

The recovery was determined in quadruplicate by comparing processed samples (QC-high, -med, -low) with reference solutions in blank plasma extract at the same levels [9]. The matrix effect [11] was assessed by comparing the reference solutions in blank plasma extracts with the same solutions in acetonitrile-water (3:1, v/v) at the three validation levels (QC-high, -med, -low). Recovery and matrix effect of the IS were assessed using an identical procedure at the sorafenib concentration used in the assay.

2.5.5. Stability

The stability of vemurafenib was investigated in QC-high and -low plasma samples [9–11] stored in polypropylene tubes. Quadruplicate analysis of these samples 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 4 months, respectively. Furthermore, a validation run was reinjected after additional storage of the extracts [9,10] at 4 °C for two weeks, both with the original and with freshly prepared calibration samples.

Finally, the responses of vemurafenib from the stock solutions in DMSO after 8 h at 20 °C and after 2 months of storage in the freezer (−30 °C) were compared in duplicate to fresh stock solutions [9,10].

2.6. Mouse samples

Wild-type mice (FVB genetic background, n = 6; housed and handled as reported previously [12]) were treated with 25 mg/kg vemurafinib orally. The vemurafenib solution was obtained by dissolving the drug in DMSO (25 mg/ml), followed by dilution (1:10) with Tween 80–ethanol–water (20:13:67, v/v/v). Blood samples were collected in heparinized tubes *via* the tail vein 0.5, 1, 2, 4

and 8 h after administration of the drug. After centrifugation at 2100 g for 6 min at 4 °C, plasma was stored at −30 °C. Mouse samples (10 μl) were diluted with 40 μl pooled human EDTA plasma before analysis. QC samples (high and med level) in mouse blood were processed analogously ($n = 6$) as a dilution integrity test [10] and a partial validation for the alternative species [9,11].

3. Results and discussion

3.1. Method development

A simple pre-treatment procedure like protein precipitation was investigated as the first option because a high selectivity and sensitivity of the MS/MS detection in combination with an LC system using 2 μm particles and high clinical drug levels were expected. A product spectrum of vemurafenib is shown in Fig. 1. Protein precipitation with acetonitrile showed high extraction recoveries for the analyte. Water was added to the extraction solvent to limit the amount of organic modifier injected onto the reversed-phase column and did still result in an efficient precipitation due to the high volume of acetonitrile. The high dilution factor in the present procedure was applied due to the relatively high drug levels in this assay.

HESI settings previously used in an assay for axitinib, another kinase inhibitor, in human plasma [12] also appeared optimal for vemurafenib and were therefore used in the present assay. Because isotopically labeled vemurafenib was not available, 9 other kinase inhibitors (dasatinib, erlotinib, gefitinib, imatinib, lapatinib, nilotinib, sorafenib, sunitinib, vandatenib) were tested to find a compound with a retention time in the same order as vemurafenib to be used as internal standard. Sorafenib, almost co-eluting with vemurafenib (Fig. 2), turned out to be the only option under the present chromatographic conditions.

3.2. Validation

SRM chromatograms are depicted in Fig. 2, showing chromatograms of blank and LLOQ spiked plasma samples.

3.2.1. Calibration

A 0.1–100 μg/ml vemurafenib range was chosen to assess expected levels up to an average steady-state at 42 μg/ml [8] with the recommended dose of 960 mg twice daily [6,8]. The relative response of vemurafenib showed a small significant deviation from a linear function ($P = 0.03$ for a 1-tailed normal distribution of the average double-logarithmic slope ($n = 4$) compared to 1); therefore, the double logarithmic function was used for the assay calibration. For all calibration samples (48 samples in 4 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 2.3% were observed (data not shown), indicating the suitability of the regression model. The average regression parameters of the double logarithmic regression functions ($n = 4$) were $\log(y) = 0.090(\pm 0.044) + 0.975(\pm 0.009)\log(x)$ with a regression coefficient of 0.9996 ± 0.0002. The functions show reproducible calibration parameters.

3.2.2. Precision and accuracy

Assay performance data from the validation samples at four concentrations are reported in Table 1. Between day variations and deviations of the accuracy lower than 8.2% were observed for all levels. The precision and the accuracy therefore met the required ±15% (±20% for the LLOQ) [9–11].

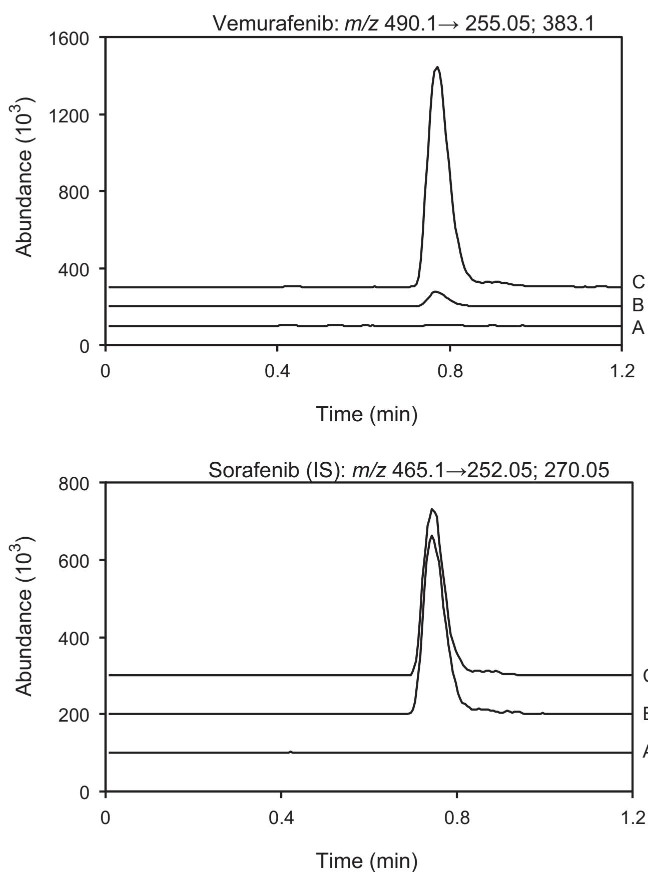

Fig. 2. SRM chromatograms of vemurafenib and the IS in plasma extracts: blank plasma (A), LLOQ (0.1 μg/ml) spiked plasma (B) and mouse plasma (C) taken 0.5 h after administration of 25 mg/kg vemurafinb, diluted 1:5 with human plasma and containing 9.8 μg/ml vemurafenib. An artificial off set was given to the chromatograms.

3.2.3. Selectivity

The analysis of six batches of blank samples showed no interfering peaks in the SRM traces for vemurafenib in human plasma. Blank responses could not be distinguished from the detector noise, they were all <2.5% of the LLOQ response, easily meeting the required 20% [10]. The signals of the LLOQ level (0.1 μg/ml) were very distinguishable from blank responses; concentrations found at the LLOQ level ($n = 6$) were 0.097 ± 0.005 μg/ml, showing the applicability of the investigated LLOQ level [9–11].

3.2.4. Recovery and matrix effect

The extraction recoveries showed no losses for both target compound and IS and ranged from 100% to 109% (data not shown). In addition, no matrix effects were observed, ionization recoveries ranged from 97% to 105% for vemurafenib at the investigated levels and IS. Overall, the absence of extraction losses and matrix effects contribute to a successful validation of the assay [9,11,13]

Table 1
Assay performance data of vemurafenib resulting from 18 validation (QC) samples in 3 analytical runs.

Nominal concentration (μg/ml)	Within day precision (%)	Between day precision (%)	Accuracy (%)
80	1.8	2.7	99.1
5	1.6	3.7	102.1
0.25	2.9	8.2	103.5
0.1	3.2	5.7	106.2

Table 2
Stability data (recovery [%]; ±SD; $n = 4$) of vemurafenib in human EDTA plasma, reporting the percentage of the initial concentration.

Condition	QC-high	QC-low
24 h at ambient temperature	110.5 ± 1.2	92.7 ± 0.5
3 freeze–thaw cycles	109.3 ± 1.5	92.8 ± 2.3
4 months at −30 °C	101.5 ± 2.8	97.3 ± 3.8

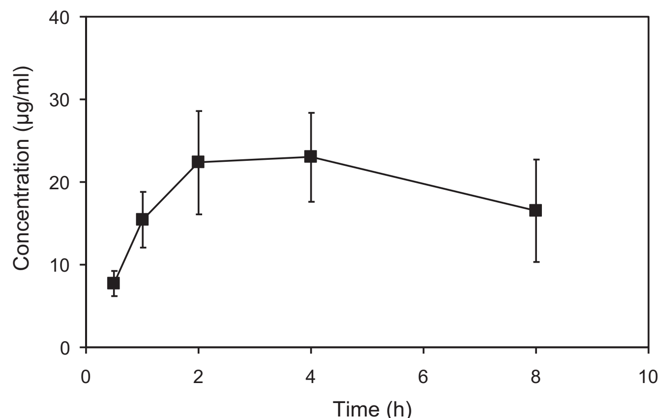

Fig. 3. Pharmacokinetic plot of the average concentrations with standard deviations of vemurafinib in 6 wild type mice after oral administration of 25 mg/kg of the drug.

and can be explained by the high dilution factor of the sample pre-treatment procedure and the small injection volume.

3.2.5. Stability

The stability of vemurafenib in plasma after different storage procedures is shown in Table 2. No losses higher than 7.2% were found with low standard deviations. Re-injection of validation (QC) samples after additional storage at 4 °C for 2 weeks resulted again in successful performances, with both the original and new calibration samples. Additional storage before injection is therefore allowed. Recoveries of vemurafenib in the stock solutions were excellent, 102.0% after 2 months at −30 °C and 98.9% after 8 h at 20 °C, respectively. All stability results can be considered satisfactory for the validation [9,11,13].

3.3. Mouse samples

To show the applicability of the new assay after the successful validation procedure, plasma samples from human pharmacokinetic studies were not yet available. Alternatively, the pharmacokinetics of vemurafenib were investigated in mice. Results of the animal experiments are shown in Figs. 2 and 3, levels in the range 4.5–42 µg/ml were observed. Mouse heparin plasma QC samples ($n = 6$; 10 µl) resulted in 3.6% and 10.3% for the precision and 97.4% and 99.3% for the accuracy at the high and med levels respectively after 1:5 dilution with human EDTA plasma.

4. Conclusions

The first validated assay for vemurafenib has now been fully reported for human plasma samples and can also be used for mouse plasma. The LC–MS/MS assay uses a fast and simple pre-treatment method in 96-well format. The results show values of accuracy, precision, recovery and stability allowed by international guidelines [9,11,13]. The new assay can be applied to clinical vemurafenib studies and for therapeutic drug monitoring applications.

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