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Short communication

Development and validation of a high-performance liquid chromatography–tandem mass spectrometry assay quantifying vemurafenib in human plasma

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ABSTRACT

Vemurafenib is an inhibitor of mutated serine/threonine-protein kinase B-Raf (BRAF) and is registered as Zelboraf® for the treatment of adult patients with BRAF V600 mutation-positive unresectable or metastatic melanoma. To support Therapeutic Drug Monitoring (TDM) and clinical trials, we developed and validated a method for the quantification of vemurafenib in human plasma. Additionally two LC–MS systems with different detectors were tested: the TSQ Quantum Ultra and the API3000.

Human plasma samples were collected in the clinic and stored at nominally −20 °C. Vemurafenib was isolated from plasma by liquid–liquid extraction, separated on a C18 column with gradient elution, and analysed with triple quadrupole mass spectrometry in positive-ion mode. A stable isotope was used as internal standard for the quantification.

Ranging from 1 to 100 μg/ml the assay was linear with correlation coefficients (r^2) of 0.9985 or better. Inter-assay and intra-assay accuracies were within ±7.6% of the nominal concentration; inter-assay and intra-assay precision were within ≤9.3% of the nominal concentration. In addition all results were within the acceptance criteria of the US FDA and the latest EMA guidelines for method validation for both MS detectors.

In conclusion, the presented analytical method for vemurafenib in human plasma was successfully validated and the performance of the two LC–MS systems for this assay was comparable. In addition the method was successfully applied to evaluate the pharmacokinetic quantification of vemurafenib in cancer patients treated with vemurafenib.

1. Introduction

Vemurafenib is a potent inhibitor of mutated serine/threonine-protein kinase B-Raf (BRAF) and is registered (Zelboraf®) for the treatment of adult patients with BRAF V600 mutation-positive unresectable or metastatic melanoma. The drug selectively inhibits mutated BRAF and blocks proliferation in tumours carrying mutant BRAF [1,2]. Currently several clinical trials are conducted to investigate these pathways and the effects of combination therapies [3].

To support clinical trials and Therapeutic Drug Monitoring (TDM) an assay for quantification of vemurafenib is needed. Sparidans et al. [4] reported a validated LC–MS/MS assay for human and mouse plasma and Zhen et al. [5] reported a validated LC-UV assay for human plasma. Both assays use protein precipitation as sample preparation and use another tyrosine kinase inhibitor (TKI) as internal standard. In our hands this sample preparation could only be fitted with a quadratic function and accuracy and precision of the assay was not acceptable when using a TKI as internal standard. The tested TKI's were not able to minimise matrix effects. Therefore we developed a new LC–MS/MS method for quantification of vemurafenib in human plasma with liquid–liquid extraction (LLE) as sample pre-treatment and a stable isotope as internal standard. The use of a stable isotope as IS in this new method minimises matrix

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effects and the used sample preparation improves the robustness of the method. In addition a full validation was performed in compliance with the OECD principles of Good Laboratory Practice (GLP) [6] and according to the FDA and latest EMA guidelines on bioanalytical method validation [7,8]. The applicability of the assay was investigated by analysing plasma samples of patients treated with vemurafenib.

Furthermore we tested two analytical platforms using different MS detectors: the TSQ Quantum Ultra triple quadrupole mass spectrometer and the API3000 triple quadrupole mass spectrometer. This test was performed to investigate the consequences and potential problems of assay transfer between these analytical systems.

2. Experimental

2.1. Chemicals

Vemurafenib was obtained from Sequoia Research Products (Pangbourne, UK) and $^{13}C_6$-vemurafenib, used as internal standard (IS) for the vemurafenib assay, was manufactured by AlsaChim (Strasbourg, France). Methanol (Supra-Gradient grade) was obtained from Biosolve Ltd. (Valkenswaard, the Netherlands). Ammonium acetate (98%) and water (LiChrosolv), used to prepare the mobile phase, and tert-butyl methyl ether (TBME), used for sample preparation, were purchased from Merck (Darmstadt, Germany). Water (distilled), used for sample preparation, originated from B. Braun Medical (Melsungen, Germany). Drug-free control lithium heparinised plasma was from Bioreclamation LLC (Hicksville, NY, USA).

2.2. Stock solutions, calibration standards and quality control samples

Separate 4 mg/ml vemurafenib stock solutions for calibration standards (CAL) and quality control samples (QC) were prepared in DMSO–methanol (20:80, v/v). After storage at −20 °C vemurafenib recrystallised in DMSO–methanol and could not be solubilised any more. The stock solutions were from then on prepared in DMSO. These stock solutions were further diluted with methanol to obtain working solutions.

The stock solution of the IS $^{13}C_6$-vemurafenib was prepared at a concentration of 1 mg/ml in DMSO–methanol (20:80, v/v). The IS working solution contained 50,000 ng/ml vemurafenib in methanol. Stock solutions, working solutions (of CAL/QC stock and IS) were stored at nominally −20 °C.

CAL were prepared freshly before each validation run by adding 20 μl CAL working solution to 380 μl control human plasma to obtain concentrations of 1.00, 2.00, 5.00, 10.0, 20.0, 50.0, 80.0, 100 μg/ml for vemurafenib.

The QC samples were prepared by adding QC working solution (50 μl) to control human plasma (950 μl). Final vemurafenib concentrations at the lower limit of quantification (LLOQ), QC low, QC mid, QC high and QC above the upper limit of quantification (>ULOQ) were 1.00, 3.00, 15.0, 75.0 and 200 μg/ml for vemurafenib. The QC samples were stored in aliquots of 50 μl at nominally −20 °C.

2.3. Sample preparation

After sample collection in the clinic by venipuncture the collection tube was centrifuged for 5 min at 3000 rpm after which the plasma was isolated and stored at nominally −20 °C. Prior to processing the samples were thawed and a 50 μl aliquot was used for analysis. A volume of 20 μl of IS working solution (50,000 ng/ml) was added. Vemurafenib was isolated from plasma by liquid–liquid extraction (LLE) with TBME. A volume of 1 ml of TBME was added to the sample aliquots after which the samples were vortexed, shaken (10 min at 1250 rpm) and centrifuged (5 min at 20 °C and 23,100 g). After snap freezing the samples the organic layer was removed and evaporated under a gentle steam of nitrogen gas (approximately 15 min at 40 °C). The dry extract was then reconstituted with 1000 μl 50% methanol and 0.5 μl of the final extract was injected.

2.4. Liquid chromatography–tandem mass spectrometry

The chromatographic separation was carried out using a LC-20AD Prominence binary pump with a column oven, DGU-20A3 online degasser and a SiL-HTc controller (Shimadzu, Kyoto, Japan). The autosampler temperature was kept at 4 °C and the column oven at 40 °C. The mobile phase A consisted of 10 mM ammonium acetate in water and mobile phase B was methanol. Gradient elution was applied at a flow rate of 0.25 ml/min through a Gemini C18 column (110 Å, 50 × 2.0 mm ID, particle size 5.0 μm; Phenomenex, Torrance, CA, USA) with an additional Gemini C18 guard column (4 × 2.0 mm ID). The following mobile phase gradient was applied: 50–80% B (0.0–0.5 min), 80% B (0.5–2.5 min), 80–95% B (2.5–2.6 min), 95% B (2.6–3.6 min), 95–40% B (3.6–3.7 min), 50% B (3.7–7.0 min). The divert valve directed the flow to the mass spectrometer between 2.2 and 3.2 min and the remainder to the waste container.

The vemurafenib concentrations were analysed on a Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer. This instrument was equipped with an electrospray ionisation (ESI) source (Thermo Fisher Scientific, Waltham, MA, USA), operating in positive mode and configured in multiple reaction monitoring (MRM). The LC–MS/MS data were acquired and processed with LCquan^{TM} software version 2.5.6 (Thermo Fisher Scientific). Table 1 summarises the MS operating parameters.

2.5. Validation procedures

The assay validation was performed in accordance to the OECD principles of Good Laboratory Practice (GLP) [6]. Calibration model, accuracy and precision, selectivity, dilution integrity, lower limit of quantitation, matrix effect, carry-over and stability under various conditions were established according to the US FDA and latest EMA guidelines on bioanalytical method validation [7,8].

To test the performance of the method we also used an API3000 quadrupole mass spectrometer (AB Sciex, Thornhill, ON, Canada). These LC–MS/MS data were acquired and processed with Analyst software (AB Sciex). Table 1 summarises the MS operating parameters. This MS system is coupled to a HP1100 binary pump, a degasser, a HP1100 autosampler and a switching valve (Agilent technologies, Palo Alto, CA, USA). Similar autosampler and column oven temperature, mobile phase, guard column and column were used as described earlier. The gradient was slightly adjusted and the following mobile phase composition and flow rates were applied: 50–80% B, 0.25 ml/min (0–0.5 min); 80% B, 0.25 ml/min (0.5–2.5); 80–95% B, 0.25 ml/min (2.5–2.6 min), 95% B, 0.25 ml/min (2.6–4.6 min); 95% B, 0.25–0.5 ml/min (4.6–4.7 min); 95% B, 0.5 ml/min (4.7–5.2 min); 95–50% B, 0.5 ml/min (5.2–5.3 min); 50% B, 0.5 ml/min (5.3–6.3 min); 50% B, 0.5–0.25 ml/min (6.3–6.4 min). The divert valve directed the flow to the mass spectrometer between 3.2 and 4.7 min and the remainder to the waste container.

3. Results and discussion

3.1. Development

When analysing samples from patients that are treated for cancer one wants to clean the samples from interfering analytes as