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Liquid chromatography-tandem mass spectrometric assay for the PI3K/mTOR inhibitor GSK2126458 in mouse plasma and tumor homogenate

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ABSTRACT

A quantitative bioanalytical liquid chromatography-tandem mass spectrometric (LC-MS/MS) assay for GSK2126458, a dual PI3K/mTOR inhibitor, was developed and validated. Plasma and tumor homogenate samples were pre-treated using protein precipitation with acetonitrile containing dabrafenib as internal standard. After dilution with water, the extract was directly injected into the reversed-phase liquid chromatographic system. The eluate was transferred into the electrospray interface with positive ionization and compounds were detected in the selected reaction monitoring mode of a triple quadrupole mass spectrometer.

The assay was completely validated for plasma in a 4–4000 ng/ml calibration range with $r^2 = 0.9996 \pm 0.0003$ using double logarithmic calibration ($n = 5$). Within-run precisions ($n = 6$) were 2.0–5.3% and between-run (3 runs; $n = 18$) precisions 2.7–5.8%. Accuracies were between 101 and 105% for the whole calibration range. The drug was sufficiently stable under all relevant analytical conditions. Finally, the assay was successfully applied to determine plasma and tumor drug levels after oral administration of GSK2126458 to mice with AMC711T neuroblastoma xenografts.

1. Introduction

The phosphatidylinositol-3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway plays an important tumorigenic role in pediatric cancers, including neuroblastoma, as well as in solid tumors in adults [1,2]. Neuroblastoma is the most commonly diagnosed extracranial tumor type in young children [3] and is responsible for the highest mortality rate in pediatric oncology [4]. Activation of the PI3K/Akt/mTOR axis stimulates the proliferation and survival of neuroblastoma cells and has been shown to confer chemotherapy resistance [5,6]. Targeting the PI3K/Akt/mTOR axis might therefore be beneficial in the future treatment of neuroblastoma patients. Currently, multiple inhibitors are available that target one or more of the key players of the PI3K/Akt/mTOR pathway [7]. Several of these inhibitors have successfully been tested *in vivo* for neuroblastoma treatment [6,8]. The dual PI3K/mTOR inhibitor PI-103, for example, significantly inhibited the *in vivo* growth of neuroblastoma xenografts, supporting the potential benefit of this class of therapeutic agents in neuroblastoma treatment [6,8].

GSK2126458 is a recently discovered dual PI3K/mTOR inhibitor, currently in clinical development for patients with solid tumors or lymphoma [9]. Compared with other dual PI3K/mTOR inhibitors, GSK2126458 exhibits more potent inhibitory activities against PI3K and mTOR [10] and shows more favorable pharmacokinetics [9]. GSK2126458 is slowly cleared from the bloodstream and has a good oral bioavailability [9]. *In vivo* efficacy studies in mice with BT-474 breast cancer xenografts showed that GSK2126458 dose-dependently inhibited tumor growth and that treatment with even 0.3 mg/kg/dose GSK2126458 resulted in a pharmacodynamic response [9]. Its high potency and favorable pharmacokinetic behavior make GSK2126458 a promising drug candidate for neuroblastoma treatment.

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Knight et al. studied the pharmacokinetics of GSK2126458 in blood in mouse, rat, dog and monkey by LC-MS/MS [9]. Unfortunately, detailed information about the analytical conditions used for the LC-MS/MS analysis was missing. Therefore, we started the development and validation of a bioanalytical assay for GSK2126458 in mouse plasma to support our preclinical mouse studies with the agent, using small sample volumes (20 μl), LC-MS/MS and protein precipitation as a simple and fast sample pre-treatment procedure. For tumor homogenate the investigation was extended with a partial validation for this matrix.

2. Experimental

2.1. Chemicals

GSK2126458 (>99%) was obtained from Selleck Chemicals (Houston, TX, USA) and Dabrafenib mesylate (GSK2118436B; >99%) from ChemieTek (Indianapolis, IN, 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 acid was of analytical grade originating from Merck (Darmstadt, Germany). Pooled female CD-1 mouse tripotassium EDTA plasma was supplied by Seralab Laboratories (Haywards Heath, UK), individual blank plasma samples were obtained from female NMRI nu/nu mice (Harlan, Zeist, The Netherlands).

Tumors were obtained from female NMRI nu/nu mice with AMC711T neuroblastoma xenografts. Tumors were homogenized in 4% (w/v) BSA in demineralized water in a final concentration of 0.1 g/ml using the Precellys®24-Dual (VWR, Amsterdam, 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 and processed using the 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 × 2 mm, d_p = 3 μm, average pore diameter = 10 nm, Varian, Middelburg, The Netherlands) with a corresponding pre-column (10 × 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 0.02% (v/v) formic acid in water (A) and methanol (B) was programmed. 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) methanol) for 1 min resulting in a total run time of 3 min per sample. The whole eluate was transferred into the electrospray probe, starting at 0.6 min after injection by switching the MS divert valve until 2.0 min after injection. The electrospray was tuned in the positive ionization mode by introducing 0.5 ml/min of a solvent mixture containing 50% (v/v) of 0.1% (v/v) formic acid and 50% (v/v) methanol and 5 μl/min of 10 μg/ml of GSK2126458. Electrospray settings of the assay were a 4600 V spray voltage, a 399 °C capillary temperature and the nitrogen sheath, ion sweep and auxiliary gasses were set at 49, 36 and 24 arbitrary units, respectively, and 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 120 V for GSK2126458 and 131 V for dabrafenib. GSK2126458 was monitored at m/z 506.15 → 329.0 at −25 V collision energy with a 0.25 s dwell time and dabrafenib at m/z 520.15 → 307.0 at −34 V with a 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 or tumor homogenate, pipetted into a polypropylene reaction tube, 30 μl of 200 ng/ml dabrafenib in acetonitrile were added. 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 into 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 [11–13] with only partial validation (within-run precision, accuracy, selectivity, stability, matrix effect) for the tumor homogenate matrix because of its limited availability.

2.6. Calibration

A stock solution of 0.4 mg/ml GSK2126458 was prepared in dimethylsulfoxide and a dabrafenib stock solution was prepared at 0.5 mg/ml in methanol. Stock solutions were stored in 1.5 ml polypropylene tubes at −30 °C. The GSK2126458 stock solution was diluted to a 4000 ng/ml calibration sample in pooled female tripotassium EDTA mouse plasma, stored in 1.5 ml polypropylene tubes at −30 °C. Additional calibration samples were prepared daily at 1000, 400, 100, 40, 10 and 4 ng/ml by dilution of the 4000 ng/ml calibration solution with blank mouse plasma. All calibration samples were processed in duplicate for each daily calibration. Least-squares double logarithmic linear regression was employed to define the calibration curves using the ratios of the peak area of the analyte and the IS. Plasma calibration was also used for tumor homogenate samples.

2.7. Precision and accuracy

A second stock solution of GSK2126458 at 0.6 mg/ml was used to prepare a 40,000 ng/ml working solution that was further diluted to obtain validation (quality control; QC) samples in pooled mouse female tripotassium EDTA mouse plasma at 3000 (QC-high), 200 (QC-med), 8 (QC-low) and 4 ng/ml (QC-LLOQ). 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-run precisions. Analogous, QC samples at the four levels were prepared in pooled tumor homogenate and processed on one day (n = 6) to obtain within-run precisions and accuracies.

2.8. Selectivity

Six individual mouse plasma and tumor homogenate samples were processed to test the selectivity of the assay. Samples were processed without GSK2126458 and IS and with GSK2126458 at the LLOQ level (4 ng/ml), supplemented with the IS.

2.8.1. Recovery and matrix effect

The recovery was determined ($n = 4$) by comparing processed plasma samples (QC-high, -med, -low; the same samples as used for precision and accuracy) with reference GSK2126458 solutions in blank pooled 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.

Further, inter-lot ($n = 6$) relative matrix effects in plasma were assessed at the QC-low level by processing each sample and by comparing the relative peak area of drug in blank extract with the relative peak area of drug in solvent.

Finally, for both matrices, the matrix effect was assessed using a post-column infusion experiment. A mixture of 6000 ng/ml GSK2126458 and 5000 ng/ml dabrafenib in 50% (v/v) methanol at 5 μl/min was mixed post-column with the eluent while blank samples were injected without using the divert valve. From both matrices six individual blank extracts were injected and responses at het appropriate retention times (1.26 min for GSK2126458 and 1.61 min for dabrafenib) were compared to blank injections of 50% (v/v) methanol ($n = 3$).

2.9. Stability

The stability of GSK2126458 was investigated in QC-high and -low plasma and tumor homogenate samples stored in polypropylene tubes. Quadruplicate analysis of these samples, stored in 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. The long term was only investigated in plasma. Furthermore, an analytical run was re-injected after additional storage of the extracts at 4 °C for four nights to test the stability at these conditions in the autoinjector.

Finally, the responses of GSK2126458 from the stock solutions in dimethylsolfoxide after 6 h at 20 °C ($n = 2$) and after 3 months at −30 °C ($n = 2$) were compared to fresh stock solutions (0.6 mg/ml) with LC-MS/MS after appropriate dilution of the samples and adding IS.

2.10. Mouse samples

Female NMRI nu/nu mice were obtained from Harlan and experiments were performed with permission from and according to the standards of the Dutch animal ethics committee (DEC 102692). Mice with AMC711T neuroblastoma xenografts were treated with a single oral (p.o.) dose of 3 mg/kg GSK2126458. GSK2126458 was formulated in demineralized water containing 40% (w/v) polyethylene glycol 400 and 16% (v/v) 2-hydroxypropyl-β-cyclodextrin to a final concentration of 0.9 mg/ml. Blood samples were collected from the posterior vena cava in tripotassium EDTA vials. Plasma samples were obtained by centrifugation twice at 1150 × *g* for 15 min. Tumor samples were homogenized in 4% (w/v) BSA in demineralized water to a final concentration of 0.1 g/ml using the Precellys®24-Dual. Samples were stored at −80 °C until pretreatment and analysis as described above. All incurred samples (6 plasma, 6 tumor homogenate) were reanalysed after 8 months of additional storage.

3. Results and discussion

3.1. Method development

ESI-MS/MS settings were optimized for GSK2126458 to obtain maximal sensitivity; a product spectrum of GSK2126458 is presented in Fig. 1 and a spectrum of the IS has been reported previously [14]. Simple sample pretreatment was performed by using only protein precipitation with acetonitrile and dilution of the extract as the first investigated and successful option. Acetonitrile is the most efficient organic precipitation agent for plasma [15] and the amount was kept as small as possible to limit sample dilution. Strongly retained plasma constituents were removed from the column using a high organic flush at the end of each analytical run in order to prevent long term suppression effects of the ionization. Because a stable isotopically labeled analogue of GSK2126458 was not available, three potential internal standards (dabrafenib, PLX4720 and GSK269962A) with chromatographic properties in the same range under the present conditions were investigated; the finally chosen dabrafenib showed the best quantitative results in the selectivity and accuracy experiments.

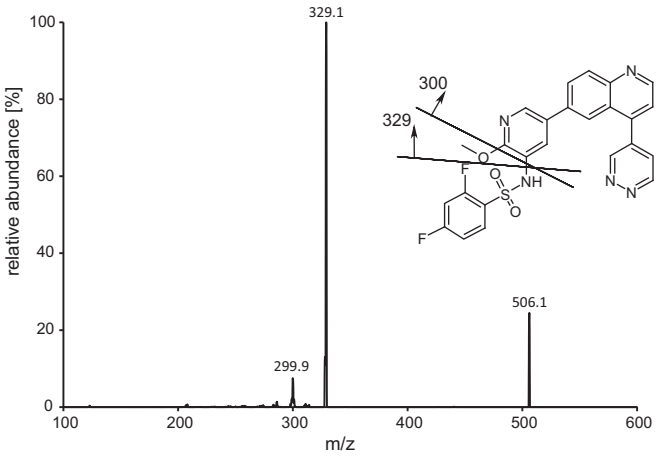

Fig. 1. Chemical structure and product spectrum, formed by collision induced dissociation of GSK2126458, m/z 506.15@−25 V.

3.2. Validation

Because in a preliminary experiment ($n = 3$) maximum plasma levels were observed in the range 800–2700 ng/ml and a range with a magnitude of 1000 was expected to be a suited and achievable range, the 4–4000 ng/ml range was chosen for validation. SRM chromatograms are depicted in Fig. 2, showing chromatograms of blank and LLOQ spiked plasma and tumor homogenate samples.

3.3. Calibration

The relative response of GSK2126458 showed a small but significant deviation from a linear function ($p = 0.04$ for a 1-tailed Student's *t*-distribution of the average double-logarithmic slope ($n = 5$) compared to 1); therefore, the double logarithmic linear function was used for assay calibration [11]. For 5 calibrations (70 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 from the average of each level higher than 2.0% were observed (data not shown), indicating an excellent suitability of the double logarithmic regression model [11,16]. The average of the reproducible regression parameters of the double logarithmic regression functions ($n = 5$) were $\log(y) = -2.77(\pm 0.09) + 0.975(\pm 0.011)\log(x)$ with a regression coefficient of 0.9996 ± 0.0003. Here, x is the GSK2126458 concentration (ng/ml) and y is the drug response relative to the IS.

3.4. Precision and accuracy

Assay performance data of the validation samples at four concentrations are reported in Table 1. Within-run and between-run

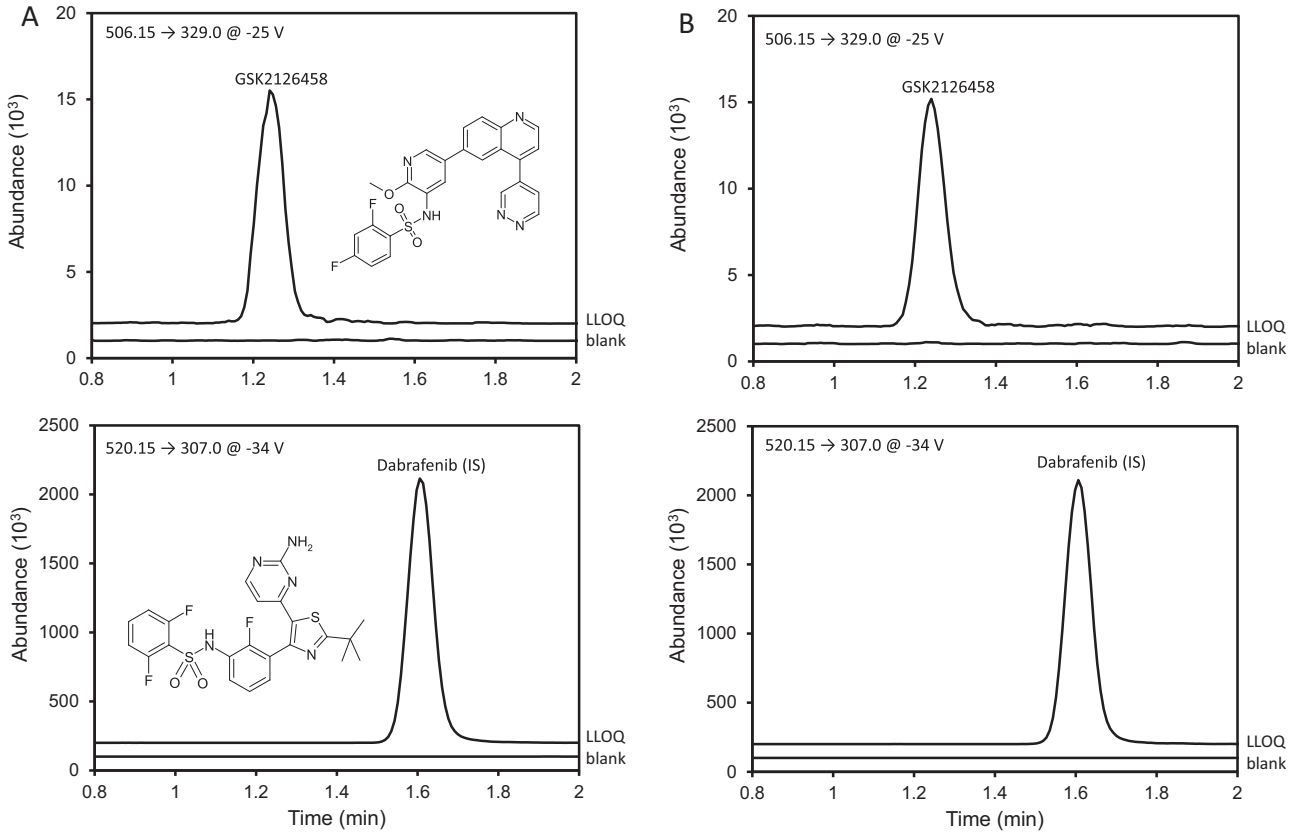

Fig. 2. SRM chromatograms of blank and LLOQ spiked samples (4 ng/ml GSK2126458 with IS): (A) plasma and (B) tumor homogenate. An artificial off set was given to the chromatograms, both chemical structures are shown in Figure A.

variations lower than 6.4% were observed for both matrices and deviations of the accuracies, LLOQ samples excluded, were lower than 3.5% for plasma and 10.4% for tumor homogenate samples. The precision and accuracy therefore met the required ±15% variation (±20% for the LLOQ) [11–13].

3.5. Selectivity

The analysis of six independent blank mouse plasma and tumor homogenate samples showed no interfering peaks in the SRM traces for GSK2126458 and the IS dabrafenib. Blank GSK2126458 responses were all below 1.25% of the LLOQ response, meeting the required 20% [16], and blank IS responses below 0.01% of the normal response. The signals at the LLOQ level (4 ng/ml; $n = 6$) corresponded to 3.86 ± 0.18 ng/ml GSK2126458 for plasma and 3.52 ± 0.22 ng/ml GSK2126458 for tumor homogenate samples, demonstrating the applicability of the investigated LLOQ level for both matrices [11–13].

Table 1
Assay performance data of GSK2126458 resulting from four validation (QC, $n = 18$ each) samples in 3 analytical runs for mouse plasma and 1 run ($n = 6$) for tumor homogenate.

Matrix	Nominal concentration (ng/ml)	Within-run precision (%)	Between-run precision (%)	Accuracy (%)
Plasma	3000	2.0	2.7	101.1
	200	3.3	5.8	101.1
	8	3.7	3.7	103.5
	4	5.3	5.5	104.6
Tumor homogenate	3000	5.2		110.4
	200	6.4		110.2
	8	2.5		107.4
	4	4.5		115.6

3.5.1. Recovery and matrix effect

Extraction recoveries showed no losses for GSK2126458 and IS and ranged from 105 to 114% (data not shown) in plasma. Matrix effects in plasma seemed also negligible or at least very small; the matrix effect ranged from 107 to 117% for GSK2126458 at the investigated levels and the effect was 106.5 ± 1.7% for dabrafenib. The inter-lot ($n = 6$) relative matrix factor in plasma at the QC-low level was 110.9 ± 3.3%. Using the post-column infusion experiment for both matrices, the matrix effects ($n = 6$, inter-lot) in plasma were 112.4 ± 11.7%, 106.1 ± 10.1% and 106.2 ± 10.0%, respectively, for GSK2126458, dabrafenib and GSK2126458 relative to the IS. In tumor homogenate these values were 107.7 ± 7.2%, 108.5 ± 12.6% and 99.7 ± 6.7%, respectively. Overall, the absence of significant extraction losses and matrix effects contributed to a successful validation of the assay for plasma and tumor homogenate samples [11–13].

3.5.2. Stability

The stability of GSK2126458 in female mouse EDTA tripotassium plasma and tumor homogenate after different storage procedures is presented in Table 2. The drug showed sufficient stability under all conditions. Re-injection of extracted plasma 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, thus QC failures remained far below a 33% frequency (1 out of 6 at the QC-H level, none at the other 3 levels) as required [11,16]. Recoveries of GSK2126458 in stock solutions were all

Table 2
Stability data (recovery (%); ±S.D.; $n = 4$) of GSK2126458 in pooled female EDTA tripotassium mouse plasma and tumor homogenate, reporting the percentage of the initial concentration.

Condition	QC-high; 3000 ng/ml		QC-low; 8 ng/ml	
	Plasma	Tumor homogenate	Plasma	Tumor homogenate
24 h at ambient temperature	100.9 ± 2.08	96.1 ± 0.9	102.2 ± 3.9	88.5 ± 10.4
3 freeze-thaw cycles	100.4 ± 0.5	99.9 ± 2.1	98.0 ± 1.4	99.9 ± 6.5
3 months at −30 °C	111.7 ± 7.3		105.9 ± 7.3	

within ±5%: 95.7% (after 6 h at 20 °C; $n = 2$) and 103.3% (after 3 months at -30 °C; $n = 2$), respectively.

3.6. Mouse study

After the successful validation procedure, the new assay was used to investigate GSK2126458 plasma and tumor levels at 1-, 2- and 4 h after administration of a single oral dose of 3 mg/kg to mice with AMC711T neuroblastoma xenografts. Representative SRM chromatograms of plasma and tumor homogenate samples obtained from GSK2126458-treated mice are depicted in Fig. 3. As stated before, Knight et al. already investigated the pharmacokinetics of GSK2126458 in the blood circulation of mice [9]. Although pharmacokinetics were extensively investigated after intravenous as well as oral administration, no information was given on the actual blood GSK2126458 levels obtained. Average plasma GSK2126458 concentrations found in the current study were 1813 ng/ml, 965 ng/ml and 560 ng/ml at 1-, 2- and 4 h after a single oral dose of 3 mg/kg, respectively (Fig. 4). Estimation of the plasma elimination half-life (i.e. $t_{1/2} = 1.9$ h) showed that the half-life observed in the current study after oral administration (Fig. 4) was in the same order as the blood half-life observed by Knight et al. after intravenous administration (i.e. $t_{1/2} = 2.1$ h) [9]. Looking at the tumor GSK2126458 levels, almost equal average tumor levels were observed at 1 and 2 h after administration (i.e. 250 ng/g and 262 ng/g, respectively) (Fig. 4). This could implicate that maximum intra-tumoral GSK2126458 levels after oral administration are reached between 1 and 2 h. From the tumor GSK2126458 levels detected at 2 and 4 h after administration the tumor elimination half-life could be estimated at 1.7 h (Fig. 4), meaning that the elimination rates of GSK2126458 from plasma and AMC711T neuroblastoma xenografts are comparable without accumulation of the drug in the tumor.

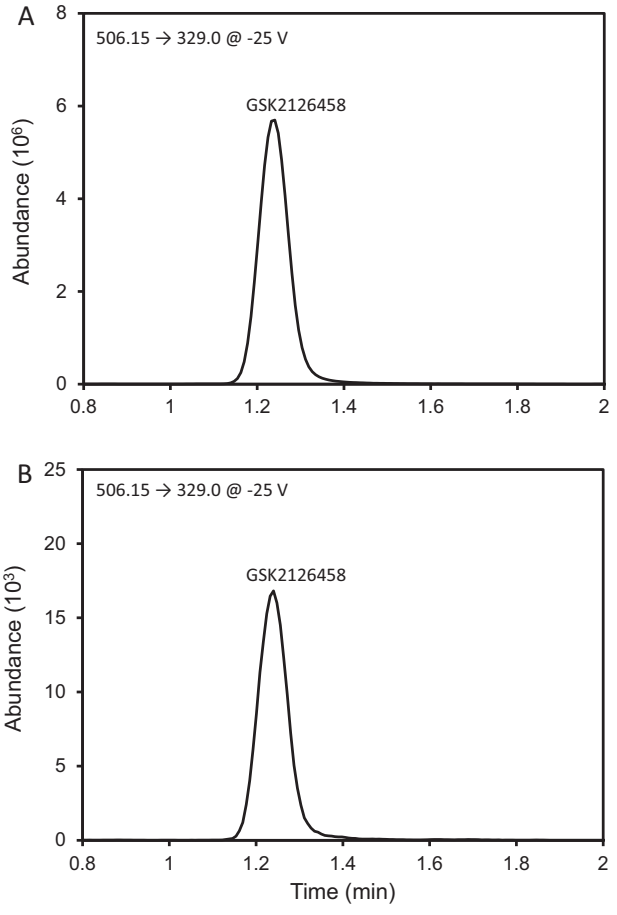

Fig. 3. SRM chromatograms of (A) a plasma and (B) a tumor homogenate sample containing 1534 ng/ml and 21.2 ng/ml (=212 ng/g tumor) GSK 2125468, respectively, from one mouse. The mouse was treated with a single oral dose of 3 mg/kg GSK2126458. Samples were taken at 1 h after drug administration.

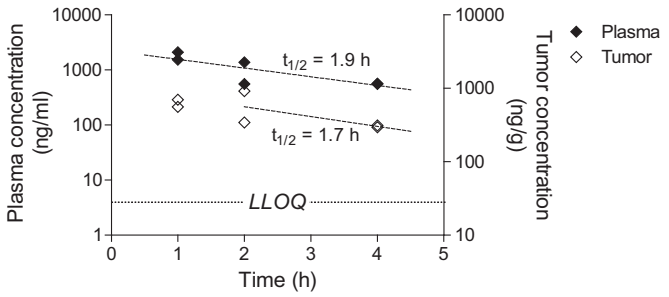

Fig. 4. Plasma (♦) and tumor (◊) GSK2126458 levels at 1, 2 and 4 h after administration of a single oral dose of 3 mg/kg to mice with AMC711T neuroblastoma xenografts ($n = 2$ per time point). Each data point represents an individual mouse. Dotted lines show the straight exponential trend lines used to estimate the plasma and tumor elimination half-lives ($t_{1/2}$). Since almost equal average tumor GSK2126458 levels were observed at 1 and 2 h after administration of the inhibitor, only the last two time points were used to estimate the tumor elimination half-life.

After 8 months of additional storage of the incurred samples at −80 °C, the recovery was 85.6 ± 13.6% for plasma ($n = 6$; 666–2574 ng/ml) and 58.6 ± 14.3% ($n = 6$; 9–41 ng/ml) for tumor homogenate samples. For plasma samples, this is with four out of six samples within the ±20% range acceptable according to EMA guidelines [16]. However, for tumor homogenate samples, having lower drug levels, the investigated storage term is obviously not acceptable and should be omitted in future studies.

4. Conclusions

The current study reports the first fully validated assay for the quantification of GSK2126458 in female mouse EDTA tripotassium plasma samples and was also partially validated for mouse tumor homogenate samples. The sensitive LC-MS/MS assay includes a fast and simple sample pre-treatment method. Results showed values of accuracy, precision, recovery and stability compliant to international guidelines [11–13]. The new assay was successfully used for the analysis of plasma and tumor GSK2126458 levels after administration of a single oral dose of 3 mg/kg to mice with AMC711T neuroblastoma xenografts.

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