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Determination of NVP-BEZ235, a dual PI3K and mTOR inhibitor, in human and mouse plasma and in mouse tissue homogenates by reversed-phase high-performance liquid chromatography with fluorescence detection

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ABSTRACT

NVP-BEZ235 is a novel dual inhibitor of PI3K/mTOR and currently undergoing phase I/II clinical trials for advanced solid tumors. We developed a sensitive and selective reversed-phase high-performance liquid chromatographic (HPLC) assay with fluorometric detection for quantification of NVP-BEZ235 in biological matrices. Liquid–liquid extraction with tert-butyl methyl ether was used for sample pre-treatment, yielding a recovery of >84%. Chromatographic separation of NVP-BEZ235 and the internal standard (IS) NVP-BBD130 was achieved on a GraceSmart C-18 column by isocratic elution with a mobile phase which consisted of acetonitrile, methanol, and milliQ water adjusted with acetic acid to pH 3.7 (20:36:44, v/v/v). Fluorescence detection using excitation and emission wavelengths of 270 and 425 nm, respectively, provided a selectivity and sensitivity allowing quantification down to 1 ng/ml in human plasma and linear calibration curves within a range of 1–1000 ng/ml. The assay was validated for human plasma, mouse plasma and a range of tissues. The accuracy, within-day and between-day precision for all matrices, was within the generally accepted 15% range. NVP-BEZ235 was stable for 72 h in pretreated samples in reconstitution mixture (acetonitrile–water (30:70, v/v)), but unstable in mouse tissue homogenates upon repeated freeze–thaw cycles or long term storage (≥24 h) at room temperature. A pilot pharmacokinetic study in mice demonstrated the applicability of this method for pharmacokinetic purposes. Overall, this assay is suitable for the pharmacokinetic studies of NVP-BEZ235 in mice and in human plasma.

1. Introduction

The phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin inhibitor (mTOR) pathway is frequently deregulated in human malignancies [1,2]. The addiction of tumor cells on the oncogenic state of this pathway provides promising opportunities for anticancer therapeutic intervention. NVP-BEZ235, an imidazo[4,5-c]quinoline derivative (Fig. 1), efficiently blocks the dysfunctional activation of the PI3K/mTOR pathway in cellular and *in vivo* settings, thus inhibiting the growth and proliferation of various cancer cells. Compared to the earlier generation of PI3K/mTOR inhibitors, NVP-BEZ235 has a much better inhibition potency and specificity. Moreover, as a dual PI3K/mTOR inhibitor, NVP-BEZ235 has been reported to have a better antiproliferative activity than the combination of PI3K and mTOR inhibitor [3]. NVP-BEZ235 is currently in phase I/II clinical trials for breast cancer, renal cancer and advanced solid tumors, either as a single agent or in combination with paclitaxel, trastuzumab, BKM120 or BKM162 [4]. To support the clinical and preclinical studies with this targeted therapeutic drug, a bio-analytical method for determining NVP-BEZ235 levels is required. To date, only a very brief description of an HPLC-UV assay for NVP-BEZ235 without validation has been reported by Maira et al. [5]. Moreover, we found that UV absorption at 340 nm did not provide satisfactory sensitivity for this agent. Based on our finding that NVP-BEZ235 has excellent fluorescence properties we now present a novel validated bio-analytical assay for NVP-BEZ235 in human and mouse plasma and in mouse tissue homogenates.

2. Experimental

2.1. Materials

2.1.1. Materials and reagents

NVP-BEZ235 was purchased from LC Laboratories (Woburn, USA). NVP-BBD130 was purchased from Axon Medchem

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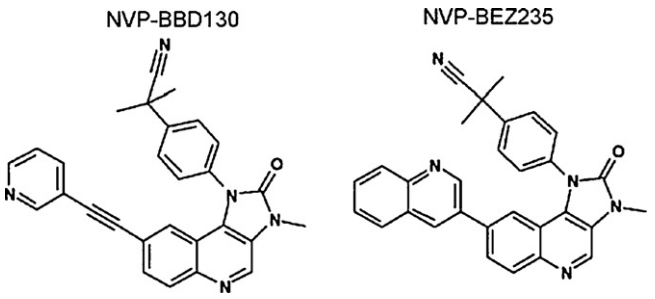

Fig. 1. Structures of NVP-BEZ235 and NVP-BBD130 (internal standard).

(Groningen, The Netherlands). Acetonitrile originated from Biosolve (Valkenswaard, The Netherlands). Water was purified by the Milli-Q Plus system (Millipore, Milford, USA). Drug-free human plasma was obtained from healthy donors from the Central Laboratory of the Blood Transfusion Service (Sanquin, Amsterdam, The Netherlands). All other chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Experimental procedures

2.2.1. Instrumentation and chromatographic conditions

The chromatographic system consisted of a model SRD-3600 Solvent Racks (with in-line degasser), a model DGP-3600A pump, a model WPS-3000TSL autosampler (Dionex, Sunnyvale, CA, USA), and a model FP-1520 fluorescence detector (Jasco, Hachioju City, Japan) operating at excitation and emission wavelengths 270 and 425 nm, respectively. Chromatographic separations were performed using a stainless steel analytical GraceSmart column (2.1 mm × 150 mm) packed with 5 μm C-18 material, preceded by a guard column holding an AJ0-A286 C18 cartridge (Phenomenex, Torrance, CA, USA). The mobile phase consisted of acetonitrile, methanol, and milliQ water adjusted with glacial acetic acid to pH 3.7 (20:36:44, v/v/v). The mobile phase was delivered at a flow rate of 0.2 ml/min. Peak detection and integration was done with a Chromeleon data system version 6.8 (Dionex, Sunnyvale, CA, USA).

2.2.2. Collection of blank murine specimens

Experiments involving laboratory animals were approved by the animal experiment committee of the Netherlands Cancer Institute. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*. Female FVB mice (9–15 weeks of age) were anesthetized with isoflurane flow and whole blood samples obtained by cardiac puncture were collected in heparinized tubes. Next, the mice were killed by cervical dislocation and the following tissues were dissected: brain, liver, kidney, lung, spleen and heart. Blood samples were centrifuged (5 min, 14,000 rpm, 4 °C) to separate the plasma fraction and both plasma and tissue samples were stored at −20 °C.

2.2.3. Drug stock solutions and internal standard

The analyte NVP-BEZ235 and NVP-BBD130 were dissolved in dimethyl sulfoxide (DMSO) to yield stock solutions with concentrations of 1.00 mg/ml. The NVP-BEZ235 stock solution was used to prepare a 10,000 ng/ml calibration stock standard in blank human plasma. The NVP-BBD130 stock solution was diluted in acetonitrile–water (30:70, v/v) to yield an internal standard (IS) working solution with concentration of 250 ng/ml. Calibration stock and IS working solution were aliquoted and stored at −20 °C.

2.2.4. Calibration standards and quality control samples

NVP-BEZ235 calibration standards with concentrations of 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 ng/ml were prepared in blank human plasma for each analytical run in duplicate. Calibration standards with concentrations of 1, 5, 10, 50, 100, 500, 1000 ng/ml were also prepared in blank mouse plasma and tissue homogenates to assess their deviations from the calibration curves prepared in human plasma. Quality control (QC) samples in plasma were prepared by appropriate dilutions of an independently prepared stock solution in human and mouse plasma to final concentrations of 5 ng/ml, 50 ng/ml and 500 ng/ml. QC's were aliquoted and stored at −20 °C.

2.2.5. Sample pre-treatment

Frozen mouse tissues were thawed at 4 °C and homogenized (Polytron PT1200, Kinematica AG, Littau, Switzerland) in 1% (w/v) bovine serum albumin (BSA) in water (3 ml for brain, liver, kidneys and 2 ml for other tissues, respectively). A volume of 50 μl of IS working solution and 1 ml *tert*-butyl-methyl ether was added to 100 μl plasma or tissue homogenate sample. After vigorously mixing for 5 min, the samples were centrifuged at 20,000 × *g* for 2 min to separate the aqueous and organic layers. Next, the lower aqueous layer was frozen by placing the vial in a dry ice/ethanol bath and the upper organic layer was decanted into a 1.5 ml micro tube (Brand, Wertheim, Germany). The organic solvent was evaporated in a Speed-Vac SC210A (Savant, Farmingdale, NY, USA) at 43 °C and the residue was reconstituted in 100 μl acetonitrile–water (30:70, v/v). After brief vortexing and sonication (5 min) the sample was transferred to an HPLC autosampler vial and a volume of 50 μl was injected into an HPLC system.

2.2.6. Selectivity

To assess the selectivity of this analytical assay, drug-free human plasma from six healthy donors and mouse plasma and tissues (brain, liver, kidney, lung, spleen and heart) from untreated mice were processed and analyzed to determine whether endogenous peaks co-eluted with NVP-BEZ235 or the IS. To further characterize the selectivity of this assay, we selected 14 commonly used medications including acetylsalicylic acid, phenytoin, valproate, diazepam, warfarin, ondansetron, simvastatin, pravastatin, triamcinolone acetonide, buprenorphine, fentanyl, fluanisone, sildenafil, and streptomycin, prepared in acetonitrile–water (30:70, v/v) as test solutions (20 μg/ml). All prepared solutions were directly analyzed under the chromatographic condition described above.

2.2.7. Determination of the lower limit of quantification (LLQ)

To determine the LLQ, human plasma spiked with 1, 2, 5 and 10 ng/ml of NVP-BEZ235 were processed and analyzed in 3-fold. The LLQ was accepted when the DEV% of samples with the lowest nominal concentration was within ±20%.

2.2.8. Recovery of sample pre-treatment

The recovery of the sample pre-treatment procedure was calculated by dividing the peak area of NVP-BEZ235 of spiked human plasma sample (5, 50, 500 ng/ml) with those of samples prepared from drug stock solution diluted in acetonitrile–water (30:70, v/v) at the same concentrations.

2.2.9. Linearity and sensitivity

Calibration curves were calculated by weighted linear regression analysis of the peak area ratios of NVP-BEZ235-to-IS *vs.* the concentration of NVP-BEZ235. We first established the most appropriate weight factor as $1/x^2$ (reciprocal of the square of the concentration). The *F*-test for lack of fit ($\alpha = 0.05$) was used to evaluate the linearity of the calibration curves.

2.2.10. Precision and accuracy

To assess the accuracy, within-day precision and between-day precision of the assay, we performed replicate measurements of the QC samples in human plasma in 5, 50, and 500 ng/ml in six different analytical runs. We also assessed the accuracy and within-day precision of the assay in murine matrices, namely, mouse plasma and tissue homogenates spiked with 5, 50, 500 ng/ml NVP-BEZ235 in triplicate for each spiked concentration in one analytical run.

The between-day (BDP) and within-day precision (WDP) was calculated by one-way analysis of variance (ANOVA) for each control sample using the software package SPSS for windows (version 17.0; SPSS, Chicago, IL, USA). The day of analytic runs performed was used as the classification variable. The between-groups mean square ($MS_{between\text{-}day}$), within-groups mean square ($MS_{within\text{-}day}$) and the grand mean (GM) of the observed concentrations across runs were used. The standard deviation of each run (SD_{run}), BDP% (between-day precision) and the WDP% (within-day precision) were calculated using the following formulas:

$$SD_{run} = \left[\frac{MS_{between} - MS_{within}}{n}\right]^{1/2}$$

(n represents the number of replicates within each run).

$$BDP\% = \frac{SD_{run}}{GM} \times 100\%$$

$$WDP\% = \frac{MS_{within\text{-}day}^{1/2}}{GM} \times 100\%$$

The accuracy was calculated by dividing the observed concentration and the nominal concentration and multiplied by 100%. The mean percentage deviation (DEV%) was calculated by:

$$DEV\% = \frac{GM(\text{observed concentration} - \text{nominal concentration})}{\text{nominal concentration}} \times 100\%$$

Values within ±15% for precision (BDP%, WDP%) and 100 ± 15% for accuracy were considered acceptable, except for concentrations at the lower limit of quantitation (LLQ), where 100 ± 20% for accuracy was accepted [6].

2.2.11. Long-term reproducibility

Reproducibility was further established taking the results of QC samples measured in five analytical runs that were performed for the analysis of study samples over a period of about three months. Within-day and between-day precisions were calculated as described based on the one-way ANOVA analysis of 50 and 500 ng/ml QC samples assayed in triplicate within each analytical run for five runs.

2.2.12. Stability of NVP-BEZ235

We examined the stability of NVP-BEZ235 and NVP-BBD130 in pretreated plasma samples reconstituted in acetonitrile–water (30:70, v/v) to assess the appropriate storage conditions while waiting for injection. For this purpose, human plasma was spiked with 500 ng/ml of NVP-BEZ235 and pretreated. The reconstituted samples were pooled and, next, re-aliquoted in sets of triplicates that were analyzed after storage for 72 h at: room temperature with ambient light, room temperature (dark), 4 °C (dark) and at −20 °C (dark).

The stabilities of NVP-BEZ235 and NVP-BBD130 were examined in human plasma samples spiked with 500 and 10 ng/ml of NVP-BEZ235, and murine plasma and tissues samples spiked with 500 ng/ml of NVP-BEZ235. These samples were subjected to 1 or 3 freeze–thaw cycles and analyzed in one run. In addition, the stability of NVP-BEZ235 in human plasma and murine plasma and tissues was checked in samples kept for 0, 4 or 24 h at room temperature or for 4 h at 4 °C before analysis.

Moreover a similar set of pre-treated spiked plasma and tissue samples reconstituted in acetonitrile–water (30:70, v/v) were subjected to direct pre-treatment and analysis (t = 0 h) or kept at room temperature for an additional 24 h prior to injection to confirm the stability of NVP-BEZ235 in pretreated tissue samples while waiting for injection.

2.2.13. In vivo applicability

To assess the applicability of this assay for preclinical pharmacokinetic study purposes, we analyzed murine plasma and tissue samples from mice receiving NVP-BEZ235. A solution of 1 mg/ml of NVP-BEZ235 formulated in DMSO-PEG400 (1:4, v/v) was given orally to an FVB female mouse by oral gavage at dose of 10 mg/kg. After 1 h, cardiac blood and brain, liver, kidney, lung, spleen and heart of this mouse were collected as described in the section "*Collection of blank murine specimens*". Plasma collection, tissue homogenization, sample storage and pre-treatment were executed as described.

3. Results and discussion

3.1. Detection and chromatography

Ultraviolet (UV) and visible absorbance scanning between wavelengths ranging from 200 to 400 nm showed that NVP-BEZ235 has absorption maxima at wavelengths 220 and 270 nm. Since this is in the range where many endogenous compounds absorb UV light, we anticipated that it would be difficult to develop a selective assay using UV detection. We found, however, that NVP-BEZ235 also exhibits strong fluorescence properties, which markedly increased the selective capacity to separate NVP-BEZ235 from endogenous interferences in biological samples. An emission wavelength at 425 nm with excitation wavelength at 270 nm was found to be the optimal for detection.

Next, an appropriate column, mobile phase composition and IS for NVP-BEZ235 chromatographic separation were explored. NVP-BBD130 was selected as IS because of its structural similarity with NVP-BEZ235 (Fig. 1). After some rounds of optimization we established a mobile phase comprised of acetonitrile, methanol and water buffered with glacial acetic acid to pH 3.7 (20:36:44, v/v/v). This mobile phase could successfully separate NVP-BEZ235 and IS from some minor endogenous peaks found in blank plasma and tissue specimens using the relatively economic GraceSmart 5 μm RP18 column (2.1 mm × 150 mm). Under these conditions, the retention times of NVP-BEZ235 and IS were around 16 and 21 min, respectively. Next, we compared the sensitivity of fluorescence detection *vs.* UV detection. When using UV detection at a wavelength of 340 nm, the peak area of a sample containing 50 ng/ml of NVP-BEZ235 in acetonitrile–water (30:70, v/v) was 120-fold lower than by fluorescence detection. A concentration of 5 ng/ml of NVP-BEZ235 was below the detection limit of UV detection (Fig. 2) but still detectable by fluorescence.

3.2. Sample pre-treatment

Liquid–liquid extraction of NVP-BEZ235 using *tert*-butyl methyl ether, yielded high recoveries, ranging from 84.3% to 94.7% for spiked human plasma containing 5, 50, 500 ng/ml NVP-BEZ235. We prepared calibration curves in all matrices in parallel and found that the slopes of the curves deviated from the human plasma calibration curve by 8% or less (Fig. 3). This deviation was considered small

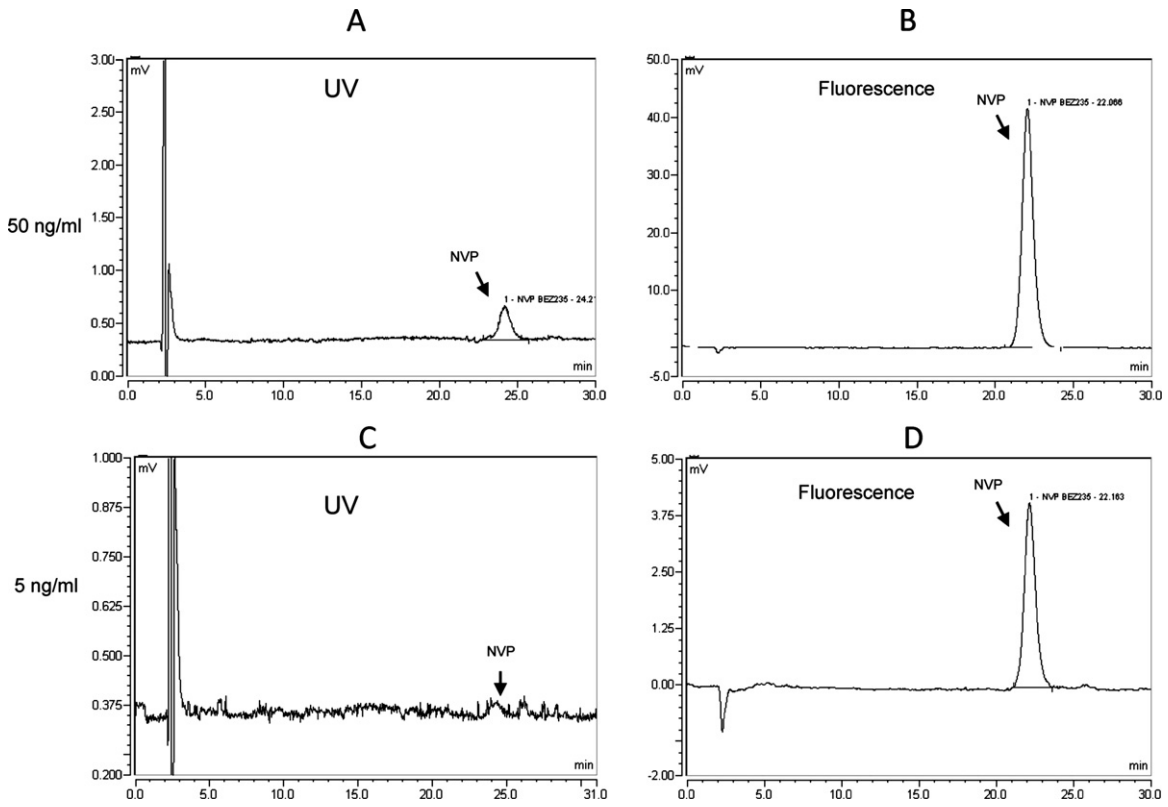

Fig. 2. HPLC chromatograms of 5 and 50 ng/ml NVP-BEZ235 dissolved in acetonitrile–water (30:70, v/v) and detected with UV detection (340 nm, A and C) and fluorescent detection (Ex: 270 nm; Em: 425 nm, B and D).

enough to allow reading of the concentration of unknown specimens in these matrices using a calibration curve constructed in human plasma. In addition, *tert*-butyl methyl ether extracted blank human and mouse plasma were free of endogenous substances that co-eluted with NVP-BEZ235 or IS (Fig. 4A and B), as were all mouse tissue homogenates (data not shown). Representative chromatograms of all spiked tissue (brain, heart, kidney, liver, lung and spleen) homogenates are shown in Fig. 5. We further assessed the selectivity of this assay by testing 14 commonly used medications.

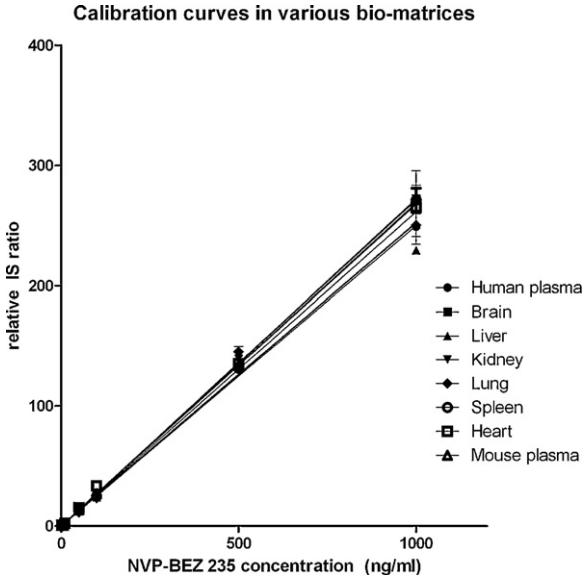

Fig. 3. Calibration curves prepared in different matrices.

No interferences to NVP-BEZ235 or IS elution were observed from above substances.

3.3. Assay validation

To establish the LLQ, we analyzed human plasma spiked at four levels of NVP-BEZ235: 1, 2, 5 and 10 ng/ml. The accuracy and precision of measurements al all these concentrations met the requirements (Table 1) and 1 ng/ml was accepted as the LLQ of this assay. The peak height of NVP-BEZ235 at 1 ng/ml was close to 5 times of that of noise level (Fig. 4C and D).

Calibration curves from 1 ng/ml to 1000 ng/ml were linear based on lack-of fit tests on three calibration curves obtained from three random analytical runs. The optimum weighting factor for curve fitting was $1/x^2$ (reciprocal of the square of the concentration). The r^2 of the calibration curves was always better than 0.99.

For assay validation, we determined the within-day and between-day precisions and accuracy of this analytical assay using spiked blank human plasma. As Table 2 shows, the accuracies were in the range of $100 \pm 15\%$ and within-day and between-day precisions were $\leq$15.0%, thus fulfilling accepted criteria for accuracy and precision of a bio-analytical assay. Next, the accuracy and precision of this assay were assessed in mouse and human plasma and in six mouse tissue homogenates spiked at three concentrations of NVP-BEZ235 and analyzed the same day in a singular analytical run. As a result, both the accuracy and precision were within the acceptable range, with the highest value in 5 ng/ml spiked kidney homogenate but still less than 15% (Table 3). All the above results of accuracy and precision were within the generally accepted ranges for bioanalytical assays [6].

To assess the reproducibility during routine use of this assay, results from the QC samples randomly inserted into five analytical runs were analyzed. The repeatability, namely, the within-day

Table 1
Validation and determination of the lower limit of quantification (LLQ). Accuracy and within-day precision were assessed in human plasma spiked at four low concentrations: 1, 2, 5 and 10 ng/ml.

Specimen	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	Within-run precision (%)
Human plasma	10.0	10.4	104	5.11
	5.00	5.50	110	8.84
	2.00	1.87	93.6	6.62
	1.00	0.87	86.7	11.6

Table 2
Accuracy, within-day precision, and between-day precision of NVP-EZ235 in blank human plasma using three concentrations used in quality control samples. Data was determined using six different analytical runs with triplicate samples assayed in each run.

Specimen	Nominal concentration (ng/ml)	Grand mean	Accuracy (%)	Within-day precision (%)	Between-day precision (%)
Human plasma	500	509	102	1.75	0.96
	50.0	51.2	102	1.66	0.47
	5.00	5.05	101	4.64	2.78

precision of QC 500, and QC 50, was 5.8% and 3.8% respectively. The results of the ANOVA analysis indicated that no statistically significant additional variation was introduced when samples were measured in subsequent runs. Therefore, both repeatability and reproducibility of this assay were acceptable.

3.4. Drug stability

We investigated the stability of NVP-BEZ235 and IS in pretreated samples kept under three different conditions, namely, room temperature with ambient light, room temperature in dark, 4 °C in dark for 72 h. As a result, relative to the same samples analyzed immediately the peak areas of NVP-BEZ235 and IS from all sample groups were not different, confirming both of the two compounds are stable under these conditions.

Daily laboratory analytical practice requires (repeated) freeze–thawing of samples and/or handling and storage of samples at room temperature for some time prior to analysis. To assess the stability of NVP-BEZ235 under these conditions, we analyzed NVP-BEZ235 spiked human plasma and murine matrices after one and three freeze–thaw cycles. The importance of such checks is demonstrated by the fact that freeze–thawing resulted in deviations that exceeded the acceptable range (Table 4). Although still within the acceptable ±15% range, even after one freeze–thaw cycle the concentration in the homogenates was already considerably below the nominal value. After three freeze–thaw cycles, only

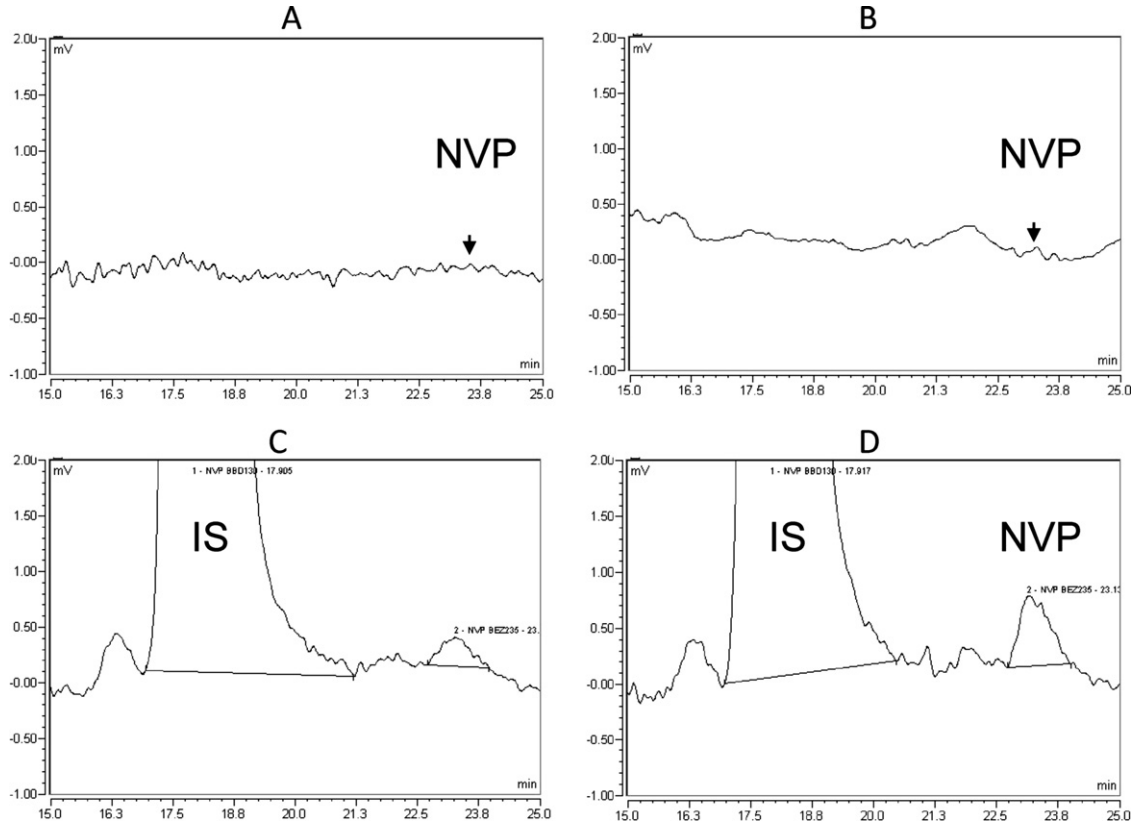

Fig. 4. Representative HPLC chromatograms of blank human plasma (A) and blank mouse plasma (B), human plasma spiked with 1 ng/ml (C) and 2 ng/ml of NVP-BEZ235 (D).

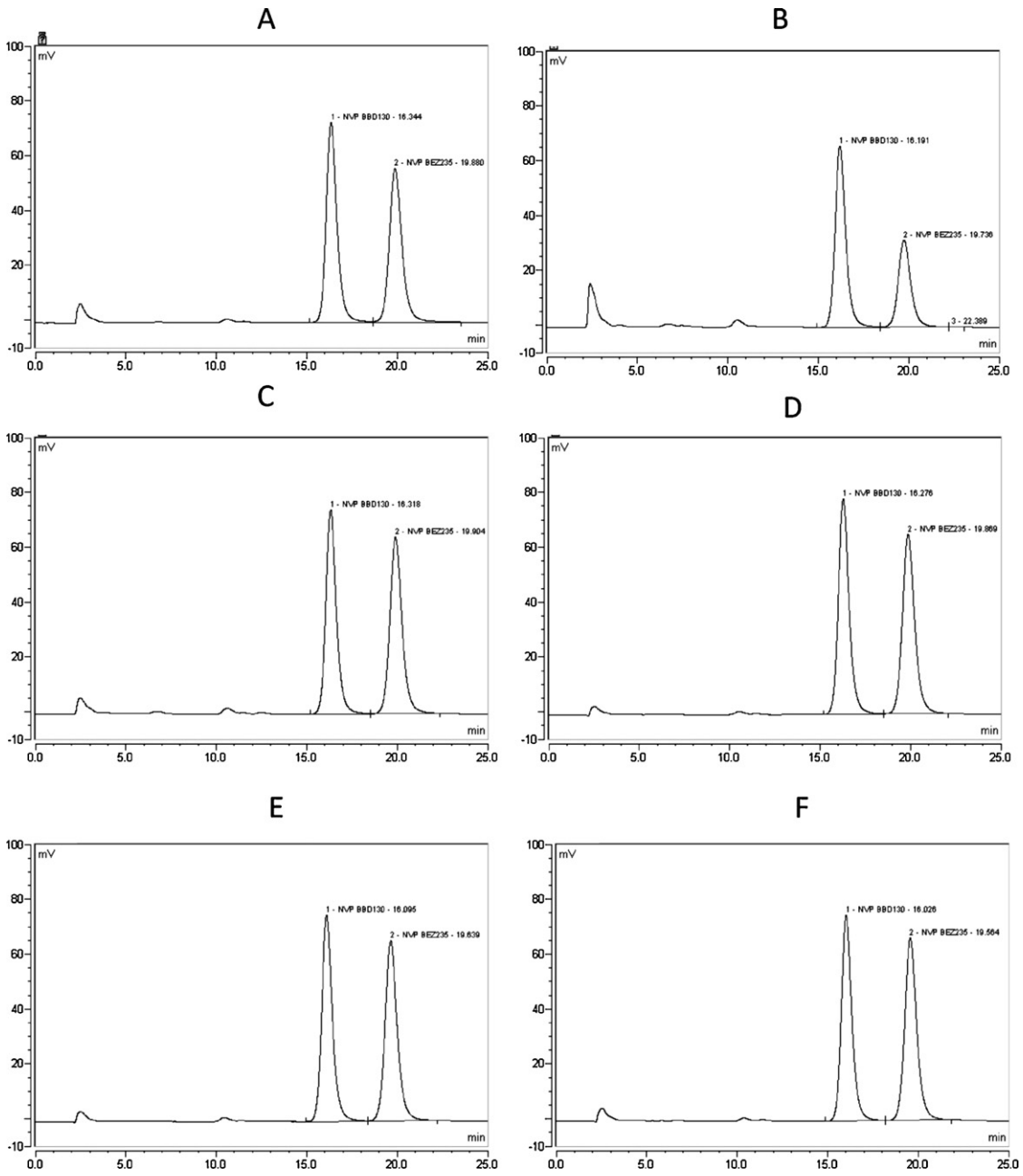

Fig. 5. Representative chromatograms of mouse tissue homogenates spikes with 50 ng/ml of NVP-BEZ235. (A) Brain, (B) liver, (C) kidney, (D) lung, (E) spleen, and (F) heart.

plasma (human and mouse) and brain were within the acceptable range, whereas the levels in liver, kidney, lung, spleen and heart were reduced by 30% or more. No additional peaks emerged in the chromatograms. Thus, NVP-BEZ235 appears to be unstable in mouse tissue homogenates upon repeated freeze–thaw cycles. The reason for this instability in tissue homogenates is not clear, but we have observed similar results with topotecan in heart homogenates [7]. Obviously, repeated freeze–thawing should be avoided by storing aliquots for one-time use rather than storing the sample as a whole.

To assess whether drug degradation also happens during handling for sample pre-treatment, we further tested the stability of NVP-BEZ235 in bio-matrices stored at 4 °C or room temperature for long or short durations prior to analysis. As shown in Table 5, all samples stored both at 4 °C and in room temperature for 4 h did not show substantial decay of the NVP-BEZ235 level with all deviations within the acceptable range, though a trend toward declining drug concentrations in tissue homogenate samples stored at room temperature was noted. However, at 24 h, kidney, lung and heart homogenate samples stored at room temperature showed unacceptable loss of NVP-BEZ235. Consequently, these stability issues demand that samples should be kept on ice during handling to prevent unacceptable degradation.

In line with the finding that NVP-BEZ235 is stable in pretreated plasma samples we also found that NVP-BEZ235 was stable in pretreated tissue samples while waiting for injection into the HPLC system. Leaving these samples at room temperature for an additional 24 h did not notably change the observed concentration (Table 6). Thus, the instability of NVP-BEZ235 in tissue homogenates might be due to degradation catalyzed by enzymes or other factors present in murine tissues but not due to chemical instability.

Table 3
Accuracy and within-day precision of NVP-BEZ235 in murine matrices. Results presented were based on triplicate freshly prepared tissue homogenate samples spiked at three different concentrations.

Specimen	Nominal concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	Within-day precision (%)
Human plasma	5	5.37	107	3.28
	50	49.7	99.4	2.68
	500	509	102	3.29
Mouse plasma	5	5.13	103	5.09
	50	51.2	102	5.78
	500	527	105	1.94
Brain	5	5.29	106	3.71
	50	53.4	107	10.2
	500	542	108	5.04
Liver	5	4.89	97.8	5.88
	50	52.1	104	0.89
	500	520	104	2.30
Kidney	5	5.32	106	13.9
	50	53.6	107	3.27
	500	487	97.3	9.66
Lung	5	5.36	107	3.54
	50	51.2	102	4.45
	500	513	103	3.14
Spleen	5	5.21	104	10.7
	50	52.4	105	0.96
	500	531	106	3.95
Heart	5	5.28	106	3.65
	50	53.5	107	0.36
	500	518	104	6.71

Table 4
Freeze–thaw stability of NVP-BEZ235 in spiked human and mouse matrices in triplicate. The stability was expressed as the mean percentage deviation (DEV%).

Specimen	Nominal concentration (ng/ml)	Measured concentration (ng/ml)		DEV[a] (%)	DEV[a] (%)
		Cycle 1 (mean ± SD)	Cycle 3 (mean ± SD)		
Human plasma (high)	500	458 ± 49	480 ± 22	−8.5	−4.0
Human plasma (low)	10.0	9.60 ± 0.10	9.66 ± 0.37	−4.0	−3.4
Mouse plasma	500	496 ± 75	523 ± 180	−0.9	4.7
Brain	500	434 ± 79	529 ± 76	−13.2	5.7
Liver	500	426 ± 22	346 ± 86	−14.8	−30.7
Kidney	500	428 ± 44	344 ± 15	−14.5	−42.3
Lung	500	463 ± 76	289 ± 19	−7.5	−31.3
Spleen	500	501 ± 56	307 ± 11	0.2	−38.6
Heart	500	517 ± 61	251 ± 44	3.5	−49.8

[a] DEV (%) was calculated as the difference between the concentration of the observed concentration and the nominal concentration. DEV% = (Observed$_{conc.}$ − Nominal$_{conc.}$)/Nominal$_{conc.}$

Table 5
Stability of NVP-BEZ235 in spiked human and mouse matrices in triplicate stored at 4 °C for 4 h, or at room temperature for 4 and 24 h. The stability was expressed as the mean percentage deviation (DEV%).

Specimen	Nominal concentration (ng/ml)	Measured concentration (ng/ml)				DEV[a] (%)	DEV[b] (%)		
		t = 0 h	t = 4 h 4 °C	t = 4 h RT	t = 24 h RT	t = 0 h	t = 4 h 4 °C	t = 4 h RT	t = 24 h RT
Human plasma (high)	500	578 ± 10	538 ± 12	545 ± 8	582 ± 6	15.65	−7.0	−5.7	0.7
Human plasma (low)	10.0	11.2 ± 0.3	10.7 ± 0.1	10.9 ± 0.5	11.3 ± 0.4	12.17	−4.9	−2.9	1.0
Mouse plasma	500	578 ± 10	542 ± 8	546 ± 13	549 ± 11	10.48	−1.9	−1.2	−0.7
Brain	500	526 ± 1	516 ± 7	540 ± 4	533 ± 31	5.27	−2.1	2.6	1.3
Liver	500	487 ± 13	496 ± 8	435 ± 5	448 ± 56	−2.57	1.9	−10.7	−8.0
Kidney	500	512 ± 21	490 ± 7	489 ± 10	363 ± 24	2.42	−4.3	−4.5	−29.0
Lung	500	531 ± 6	516 ± 13	470 ± 2	361 ± 10	6.19	−2.8	−11.6	−32.0
Spleen	500	550 ± 12	538 ± 7	540 ± 21	534 ± 9	10.06	−2.3	−1.9	−3.0
Heart	500	517 ± 61	471 ± 19	440 ± 22	364 ± 62	3.48	−9.0	−14.9	−29.6

[a] DEV (%) was calculated as the difference between the concentration of those freshly prepared and analyzed samples and the nominal concentration. DEV% = (0 h$_{conc.}$ − Nominal$_{conc.}$)/Nominal$_{conc.}$

[b] DEV (%) was calculated as the difference between the observed concentration and concentration of those freshly prepared and analyzed samples. DEV% = (4 or 24 h$_{conc.}$ − 0 h$_{conc.}$)/0 h$_{conc.}$

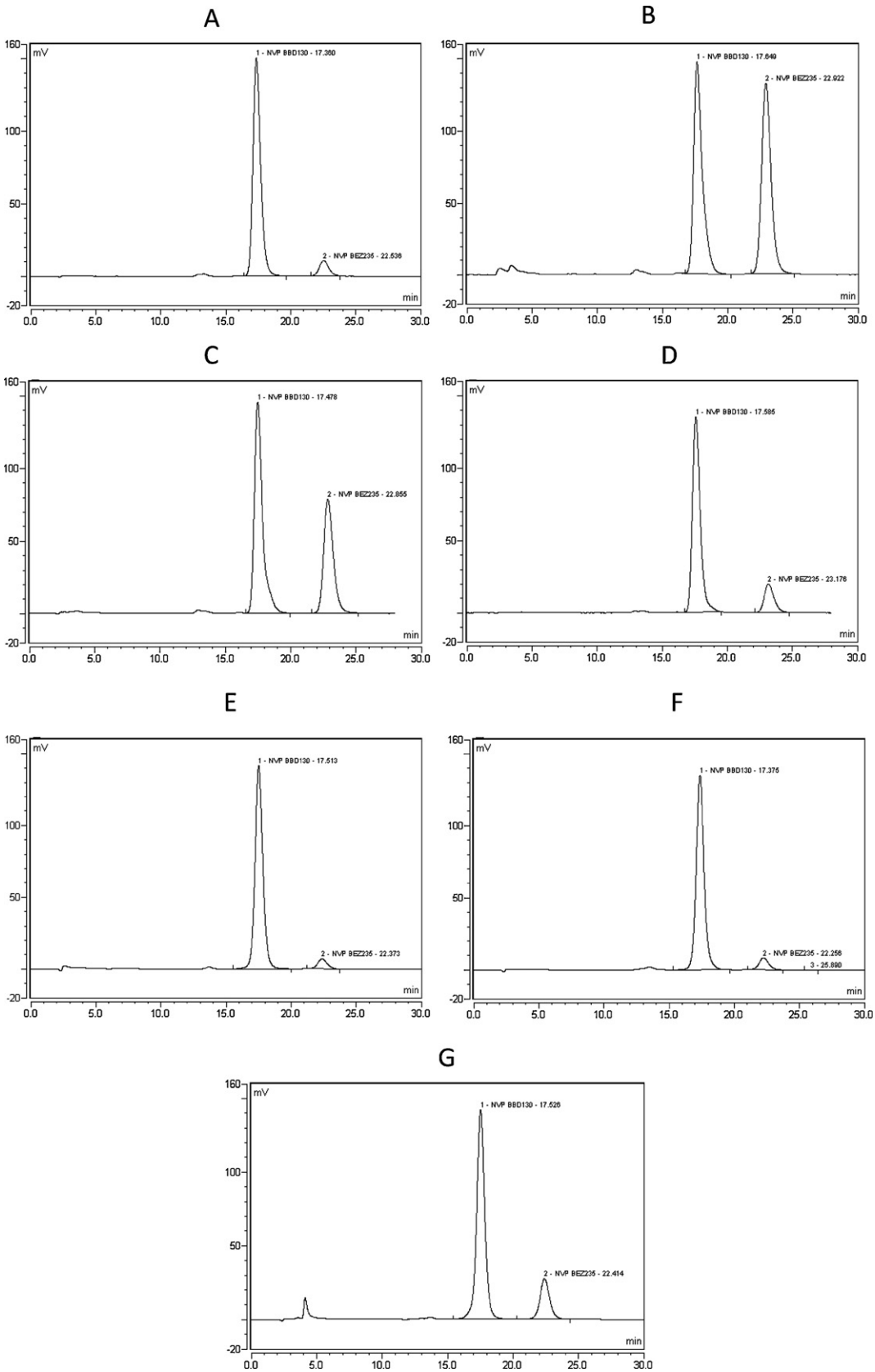

Fig. 6. Representative chromatograms of mouse tissue homogenates obtained from an FVB mouse receiving 10 mg/kg of NVP-BEZ235 orally. The mouse was sacrificed 1 h after administration and plasma and tissues were collected. (A) Brain, (B) liver, (C) kidney, (D) lung, (E) spleen, (F) heart, and (G) plasma.

Table 6
Stability of NVP-BEZ235 in spiked human and mouse matrices after pretreatment stored at room temperature for 24 h. The stability was expressed as the mean percentage deviation (DEV%).

Specimen	Nominal concentration (ng/ml)	Measured concentration (ng/ml)		DEV[a] (%)	DEV[b] (%)
		$t = 0$ h (mean ± SD)	$t = 24$ h (mean ± SD)		
Human plasma (high)	500	502 ± 19	532 ± 42	0.3	6.0
Human plasma (low)	10.0	10.2 ± 2.3	9.83 ± 1.28	1.8	−3.4
Mouse plasma	500	467 ± 10	454 ± 4	−6.7	−2.7
Brain	500	433 ± 60	475 ± 74	−13.5	9.7
Liver	500	460 ± 17	476 ± 31	−7.9	3.4
Kidney	500	450 ± 44	465 ± 64	−10.1	3.4
Lung	500	434 ± 34	435 ± 10	−13.3	0.3
Spleen	500	469 ± 72	498 ± 69	−6.13	6.16
Heart	500	441 ± 37	422 ± 18	−11.8	−4.2

[a] DEV (%) was calculated as the difference between the concentration of those freshly prepared and analyzed samples and the nominal concentration. $DEV\% = (0\,h_{conc.} - Nominal_{conc.})/Nominal_{conc.}$

[b] DEV (%) was calculated as the difference between the observed concentration and concentration of those freshly prepared and analyzed samples. $DEV\% = (24\,h_{conc.} - 0\,h_{conc.})/0\,h_{conc.}$

3.5. *In vivo* applicability

To test the applicability of this assay *in vivo*, we analyzed brain, liver, kidney, lung, spleen, heart and plasma samples from an FVB mouse which received 10 mg/kg NVP-BEZ235 orally. No endogenous interfering peaks have been observed in chromatograms of plasma and all tissue homogenates (Fig. 6). The concentrations of NVP-BEZ235 of all samples were within the dynamic range of the calibration curve.

In conclusion, we have developed and validated a sensitive and selective fluorometric HPLC method to quantify the levels of NVP-BEZ235 in human plasma and mouse plasma and tissue homogenates. We observed that the stability of NVP-BEZ235 in mouse tissues brain, liver, kidney, lung, spleen and heart can be compromised by repeated freeze–thaw cycles and by storage at room temperature for several hours, indicating that frequent freeze–thaw cycles should be avoided during storage, and pretreatment or handling of these samples should be performed at 4 °C. Given the results of the validations in human plasma and mouse matrices, this assay is expected to be suitable for preclinical and clinical pharmacokinetic studies.

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