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Short Communication

Liquid chromatography-tandem mass spectrometric assay for the quantification of galunisertib in human plasma and the application in a pre-clinical study

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ABSTRACT

Galunisertib is an anti-cancer drug currently evaluated in phase I and II clinical trials. This study describes the development and validation of a bioanalytical assay to quantify galunisertib in human plasma using HPLC-MS/MS. Stable isotope labelled galunisertib was added as internal standard and the analyte and internal standard were extracted from the matrix by protein precipitation using acetonitrile-methanol (50:50, v/v). Final extracts were injected onto a C18 column, gradient elution was applied for chromatographic separation and detection was performed using a triple quadrupole mass spectrometer operating in the positive ion mode. The assay was linear over the range 0.05–10 ng/mL, with acceptable accuracy (bias ranging from −6.1 to 3.1%) and precision (below 5.7% C.V.) values. The applicability of the assay was demonstrated in a pharmacokinetic experiment in mice.

1. Introduction

Transforming growth factor-beta (TGF-β) is a key player in several biological processes involved in oncogenesis. Activation of the TGF-β receptor leads to phosphorylation of intracellular proteins; Suppressor of Mothers against Decapentaplegic (SMAD)2 and SMAD3, which subsequently associates with SMAD4. The formed complex translocates into the nucleus, where it can act as a transcription factor, causing induction of nuclear transduction proteins. These proteins induce inflammation, immune escape, angiogenesis and invasive growth and metastasis, which are all hallmarks of oncogenesis. Moreover, the TGF-beta signalling pathway is a key driver of tumor progression by promoting epithelial-mesenchymal transition (EMT) [1].

Galunisertib (LY2157299) (monohydrate) (Fig. 1) is a serine/threonine kinase inhibitor and has been shown to selectively block TGF-β signalling *in vitro*. It inhibits the TGF- β receptor I kinase and specifically down-regulates SMAD2 phosphorylation, leading to reversion of EMT and arrest of tumor progression [2]. Since pre-clinical data and retrospective clinical analyses indicate that EMT leads to resistance to conventional cytotoxic agents, galunisertib may revert the mesenchymal tumors to a more epithelial phenotype and an associated increased response to chemotherapy [3,4].

In animal models galunisertib showed anti-tumor activity in several types of tumors, including colon, lung, breast, glioblastoma and hepatocellular carcinoma [2]. Galunisertib is investigated in patients as monotherapy, but without effective inhibition of apoptosis or proliferation. More potential for galunisertib is seen in combination therapy with standard anti-tumor drugs, such as chemotherapy and checkpoint inhibitors [1].

Different bioanalytical methods have been referred to in literature for the quantification of galunisertib [5,6], but no information or details of these methods has been divulged hitherto. We developed a new, accurate and fast assay to support the upcoming clinical phase I/II trial with galunisertib in combination with capecitabine in patients with colorectal cancer and an activated TGF-beta pathway [NCT03470350] [7].

The assay was fully validated according to the latest guidelines of the European Medicines Agency (EMA) and the US Food and

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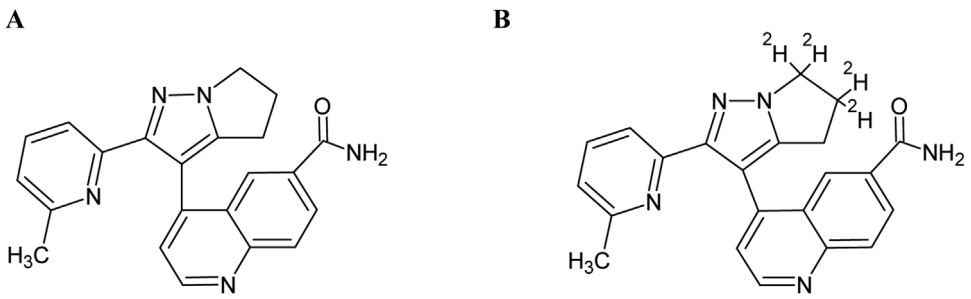

Fig. 1. Chemical structure of galunisertib (A) and galunisertib-d4 (B).

Drug Administration (FDA) [8,9]. We demonstrated the applicability of the validated method by analyzing mouse plasma samples, to support a pre-clinical study.

2. Material and methods

2.1. Chemicals

Reference standards of galunisertib ($C_{22}H_{19}N_5O$) and its stable isotopically labelled internal standard galunisertib-d4 ($C_{22}H_{15}D_4N_5O$; IS) were obtained from AlsaChim (Illkirch-Graffenstaden, France). Water, methanol, acetonitrile and formic acid (>98%) were purchased from Biosolve (Valkenswaard, The Netherlands). Ammonium acetate was obtained from Sigma (Saint Louis, Missouri, USA). Lithium heparinized human plasma originated from Bioreclamation (Hicksville, New York, USA).

2.2. Stock solutions, calibration standards and quality control samples

Stock solutions and working solutions of galunisertib and galunisertib-d4 were prepared by dissolving 1 mg of reference material in 1 mL of methanol and stored at −70 °C. Two sets of working solutions were prepared by diluting two independently prepared stock solutions with methanol. A galunisertib-d4 working solution of 100 ng/mL was also made by diluting the stock solution with methanol. Plasma calibration standards were prepared by diluting the working solutions 20 times in control human lithium heparinized human plasma, obtaining concentrations of 0.05, 0.1, 0.25, 0.5, 1, 5, 8 and 10 ng/mL. Quality control (QC) samples were prepared by spiking the other set of working solutions (obtained from a separate stock solution) to plasma, yielding concentrations of 0.05, 0.15, 0.5 and 7.5 ng/mL (lower limit of quantification [LLOQ], QC Low, QC Mid and QC High concentrations, respectively).

2.3. Sample preparation

A volume of 10 μL of IS working solution of 100 ng/mL was added to a volume of 200 μL of biomatrix yielding a final IS concentration of 5 ng/mL in the biomatrix. The samples were mixed and 400 μL acetonitrile-methanol (50:50, v/v) was added to precipitate plasma proteins. Samples were mixed again and centrifuged for 5 min at 20,000×g. A volume of 250 μL of the clear supernatant and 250 μL of 20 mM ammonium acetate in water were then transferred to an autosampler vial and mixed, before a volume of 10 μL of the final solution was injected into the chromatographic system.

2.4. Instrumentation and operating conditions

2.4.1. Liquid chromatography

Separation of galunisertib from endogenous compounds was carried out using a high performance liquid chromatography (HPLC) Nexera X2 LC-30CE pump (Shimadzu, Kyoto, Japan). A SunFire C18 column (50 mm × 2.1 mm, 5 μm) was kept at 40 °C and samples were injected using a Nexera X2 SIL-30ACMP autosampler (Shimadzu), thermostated at 4 °C. Analytes were eluted by applying a gradient consisting of 20 mM ammonium acetate in water (mobile phase A) and 0.1% formic acid in acetonitrile-methanol (50:50, v/v, mobile phase B). The flow rate was set to 600 μL/min and the following gradient was applied: mobile phase B: 20% (initial-2.5 min), from 20 to 35% (2.5–3.5 min), 35% (3.5–4.5 min), 90% (4.5–5 min), 20% (5–6 min). The 90% between 4.5 min and 5 min were incorporated in the gradient to prevent carry-over, whereas the final 1 min at 20% was added for column equilibration to initial conditions. A divert valve was used to direct the flow to the mass spectrometer from 2.5 to 5.0 min and to the waste for the remainder of the acquisition time to protect the mass spectrometer from contaminants.

Table 1
Mass spectrometry settings.

Parameter	Value
Run time (min)	6
Scan type	MRM
Polarity	Positive
Ion source	Turbo spray
Gas 1 (au) nebulizer gas	35
Gas 2 (au) turbo gas	60
Curtain gas (au)	20
Collision gas (au)	12
Ion spray voltage (V)	2200
Temperature (°C)	500 °C
Dwell time (msec)	150
Declustering potential (au)	101 (galunisertib), 76 (galunisertib-d4)
Collision energy (au)	45
Collision cell exit potential (au)	10
Entrance potential (au)	10

au: arbitrary units.

2.4.2. Mass spectrometry

A Sciex (Framingham, MA, USA) API6500 triple quadrupole mass spectrometer was used with a turbo ion spray (TIS) operated in the positive ion mode. The transition from *m/z* 370 to 284 was selected for galunisertib and from *m/z* 374 to 288 for the internal standard. Detailed mass spectrometry settings are presented in Table 1. Analyst software version 1.6.2. (Sciex) was used for data acquisition and processing.

2.5. Validation procedures

A complete validation of the bioanalytical assay was performed according to the regulatory guidelines [8,9], and included calibration curve, lower limit of quantification, accuracy and precision, carry-over, selectivity, dilution integrity, selectivity, matrix effect, recovery and stability under various conditions.

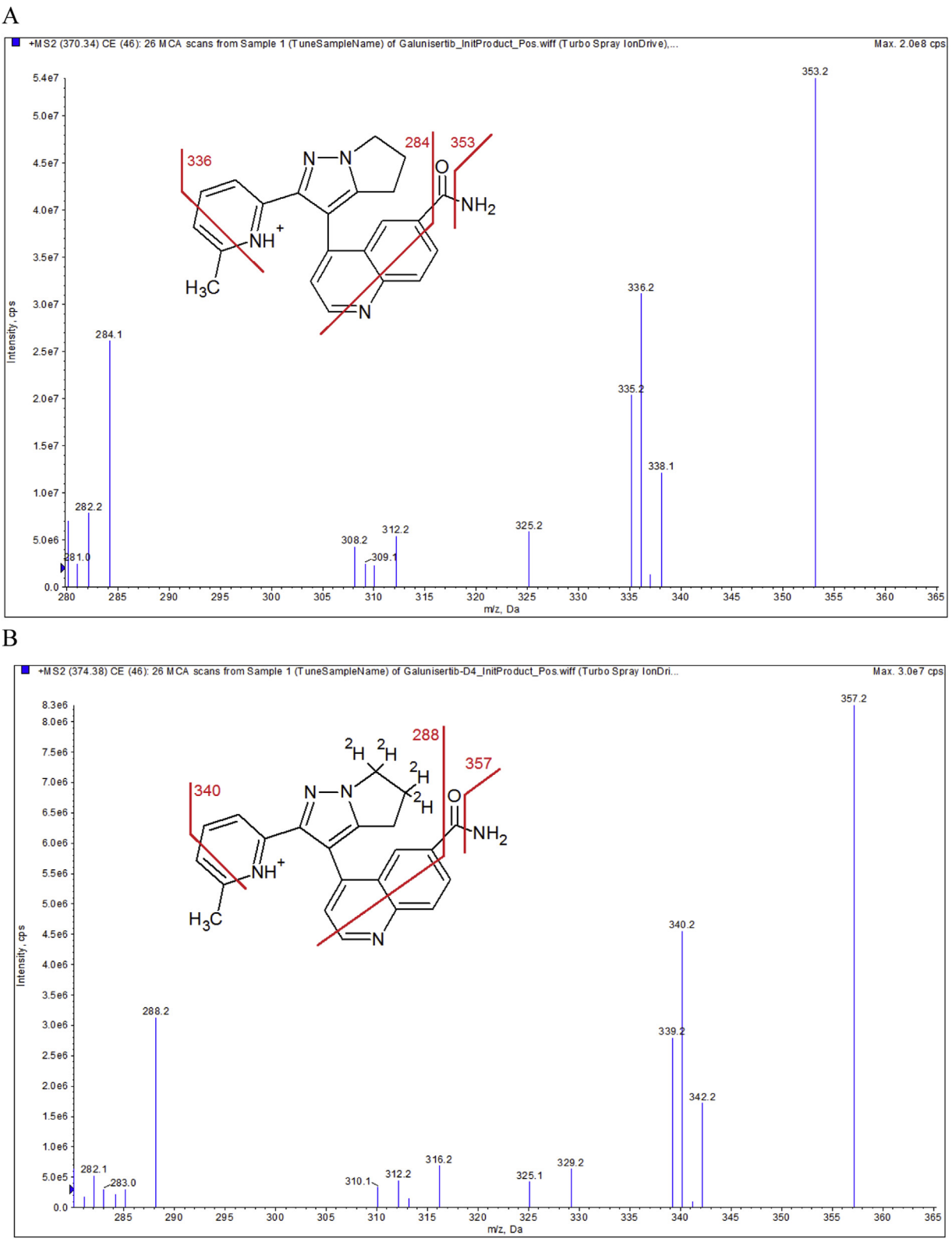

Fig. 2. Product ion spectra of galunisertib (product ions of *m/z* 370, A) and galunisertib-d4 (product ions of *m/z* 374, B) in positive ionization mode.

3. Results and discussion

3.1. Development

Galunisertib (0.5 μg/mL in 0.1% formic acid in methanol:water (80:20, v/v)) was infused into the mass spectrometer and a product ion spectrum was obtained (Fig. 2). Three most abundant product ions were *m/z* 353, 336 and 284. *m/z* 284 was chosen because this ion was more specific than the most abundant ion detected at *m/z* 353 (-17, loss of ammonia), but still had sufficient sensitivity. A compound optimization was performed for galunisertib and those

Table 2
Assay performance data for the quantification of galunisertib in human plasma.

	Intra-run		Inter-run	
Nominal concentration (ng/mL)	Bias (%)	CV (%)	Bias (%)	CV (%)
0.05	−6.1 to 3.1	≤5.7	−4.9	*
0.15	−4.8 to −1.7	≤4.9	−3.2	0.38
0.5	0.4 to 2.9	≤2.7	1.4	1.0
7.5	0.0 to 1.0	≤1.8	0.5	*

*: There is no significant additional variation due to the performance of the assay in different batches. Mean square within groups is larger than mean square between groups.

settings that produced the highest sensitivity by monitoring the parent ion transition were selected (Table 1).

The chromatographic method was an adaption of a method already applied in our laboratory for quantification of an analogous structure [10]. Linearity was tested over a range of 0.01 to 2000 ng/mL in neat solution and revealed that the assay was non-linear at high concentrations. To obtain linearity the collision energy was sub optimized and the upper limit of quantification (ULOQ) was lowered. Initially isocratic elution of galunisertib after injection of clear supernatant that were obtained after protein precipitation was tested, but peak shape was poor showing fronting due to solvent effects. To improve the peak shape the clear supernatants were diluted with an equal volume of 20 mM ammonium acetate in water before injection and furthermore gradient elution was applied. This resulted in sharp symmetric peaks and sufficient selectivity from the endogenous plasma components (Fig. 3).

3.2. Validation

3.2.1. Calibration curve

Eight non-zero calibration standards with a concentration range of 0.05–10 ng/mL were prepared in blank human plasma. The galunisertib area ratio with the internal standard was plotted against the nominal concentration and linear regression with a weighting factor of $1/x^2$ was applied, where x is the nominal concentration of galunisertib. The calibration curves were considered acceptable if 75% of the non-zero calibration standards were within ± 15% of the nominal concentrations, or ± 20% for the LLOQ. Additionally, at least 50% of the non-zero calibration standards at each concentration level should meet these criteria [8,9]. These acceptance criteria were met and thus the calibration curves were accepted.

3.2.2. Lower limit of quantification

The analyte response (as peak height) at the LLOQ level was compared to the double blank response in three validation runs. The mean signal to noise was 23 and was rated as sufficient as it was well above the required value of 5.

3.2.3. Accuracy and precision

Five replicates of QC LLOQ (0.05 ng/mL), QC Low (0.15 ng/mL), QC Mid (0.5 ng/mL) and QC High (7.5 ng/mL) were analysed in 3 analytical runs, to assess accuracy and precision. Accuracy, as intra-run bias and overall bias and precision, as intra- and inter-run coefficient of variation (CV) were calculated according to equations, as reported previously by our group [11].

The acceptance criteria were met if the accuracy was within ±20% for the LLOQ level and within ±15% at the other QC levels, and precision was ≤20% for the LLOQ and ≤15% at the other QC levels. Results are presented in Table 2 and were within the requirements.

3.2.4. Carry-over

Carry-over was assessed by injecting a double blank after the injection of an ULOQ (10 ng/mL galunisertib) sample. It was considered acceptable if the area of the carry-over sample was ≤20% of the LLOQ for galunisertib and ≤5% for galunisertib-d4. The double blank response was ≤14.2% of the mean response of the LLOQ for galunisertib and ≤0.2% for galunisertib-d4. Consequently the carry-over was considered acceptable.

3.2.5. Dilution integrity

Dilution integrity was assessed by spiking galunisertib at 500 ng/mL in control human plasma. Twenty μL of these samples were diluted with 180 μL control human plasma in two successive steps, resulting in a 100-fold diluted sample. The bias and precision were −5.6% and 3.2%, respectively. Hence, samples containing concentrations well above the ULOQ can be diluted 100 times with acceptable accuracy and precision values.

3.2.6. Selectivity

From six different batches of plasma blank samples and spiked samples at the LLOQ were spiked and processed. HPLC–MS/MS chromatograms of the blanks and LLOQ samples were monitored and compared for chromatographic integrity and potential interferences. The LLOQ samples were between -3.0% and 6.0% of their nominal concentrations. No interferences from endogenous material at the retention time of the analyte with areas >20% (or >5% for the internal standards) of the LLOQ areas were observed in the blanks.

To assess the cross-analyte interference of the method, control plasma samples were separately spiked with galunisertib at the ULOQ (10 ng/mL) and galunisertib-d4 at the assay concentration. Additionally a control sample was spiked with capecitabine (2250 ng/mL) and its metabolites 5′-deoxy-5-fluorocytidine (900 ng/mL), 5′-deoxy-5-fluorouridine (900 ng/mL), 5′-fluorouracil (900 ng/mL), 5′-fluorodihydro-pyrimidine-2,4-dione (4500 ng/mL), N-carbamoyl-2-ß-alanine (900 ng/mL) and α-fluoro-ß-alanine (4500 ng/mL). No interferences of galunisertib-d4 at the mass transition of galunisertib and vice-versa, were detected in plasma. Interference of co-administered drugs (capecitabine and metabolites) was ≤20% of the response of a galunisertib LLOQ sample and was found to be acceptable.

3.2.7. Matrix effect

The matrix effect was tested by spiking galunisertib to six different batches of plasma at QC Low and QC High concentrations. The matrix factor (MF) was calculated for both galunisertib and IS (galunisertib-d4) by dividing the peak area in the presence of matrix, by the peak area in absence of matrix. Furthermore, the internal standard normalised (IS-normalised) MF was calculated as reported previously by our group [10]. The matrix effect was considered acceptable if the CV of the IS-normalised MF was ≤15%. The CV of the IS-normalised MF was ≤7.3% and fulfilled the required criterion.

3.2.8. Recovery

The overall recovery (sample pretreatment recovery plus matrix effect) of galunisertib from the plasma matrix was calculated by dividing the peak area of a processed sample by the peak area in absence of matrix. The recovery was 85.6 ± 5.8% for galunisertib and 90.5 ± 2.6% for the internal standard.

3.2.9. Stability

Various storage and processing conditions were tested for plasma, stock and working solutions. Stability in plasma was assessed at QC High and QC Low concentrations by comparing the measured concentration to the nominal concentration. Stability in

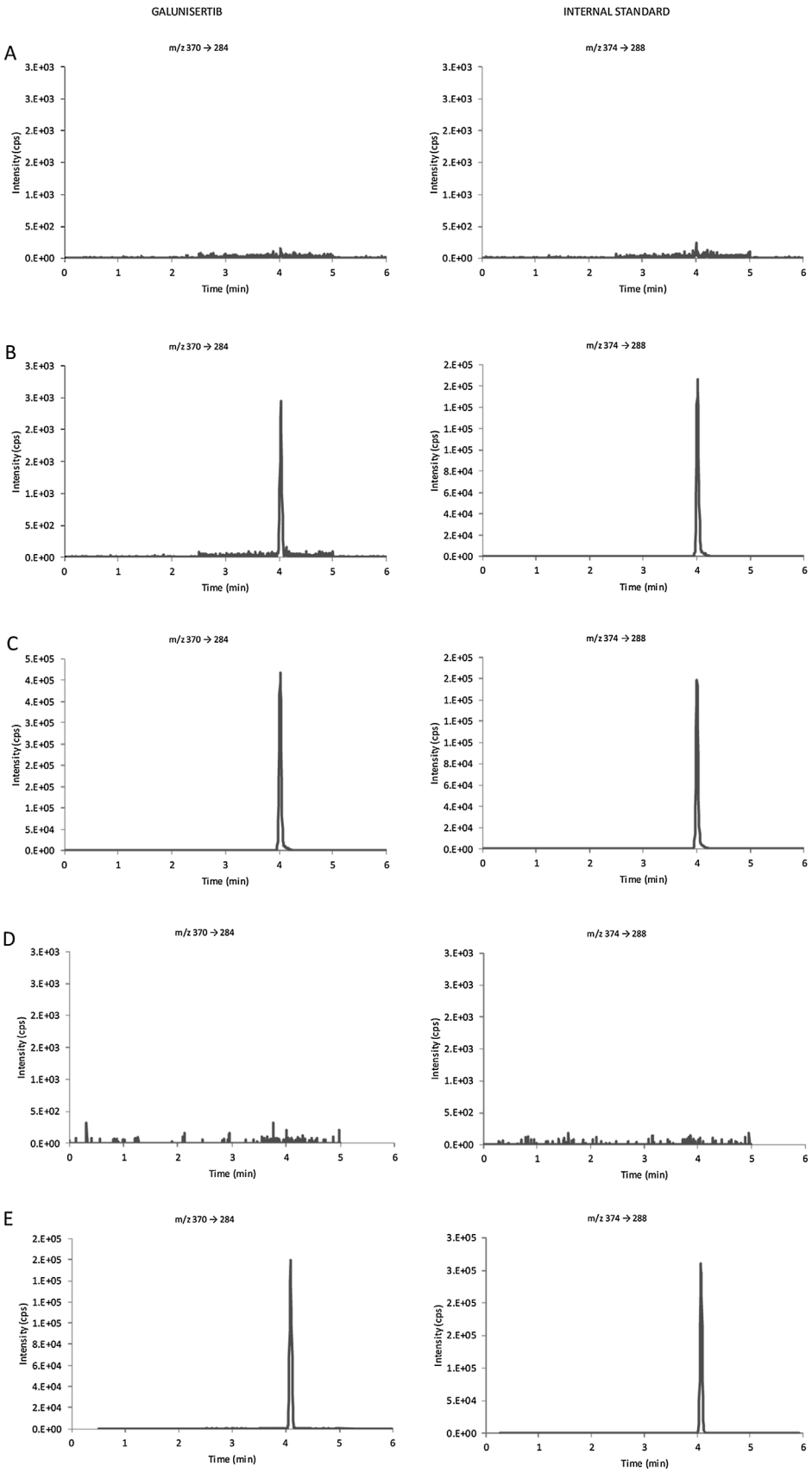

Fig. 3. Representative MRM chromatograms: a blank human plasma sample (A), galunisertib in a human plasma sample spiked at LLOQ level (0.05 ng/mL, B) and at ULOQ level (10 ng/mL, C), a blank mouse plasma sample (D), galunisertib in a diluted mouse plasma sample, 60 min after oral administration of 20 mg/kg galunisertib (367 ng/mL, E). For galunisertib the transition of *m/z* 370-284 was monitored and *m/z* 374-288 for the internal standard (5 ng/mL).

Table 3
Stability data for galunisertib. Analyses were executed in triplicate.

Matrix	Stability conditions	Nominal concentration (ng/mL)	Mean concentration (ng/mL)	Accuracy (% Bias)	Precision (% CV)
Human plasma	RT, 4 h	0.15	0.139	−7.1	5.8
		7.5	7.3	−2.7	0.5
	5 F/T (−20 °C/RT)	0.15	0.151	0.7	0.7
		7.5	7.47	−0.4	0.2
	−20 °C, 32 d	0.15	0.148	−1.3	1.8
		7.5	7.51	0.2	1.7
Final extract	2-8 °C, 4 d	0.15	0.149	−0.4	1.7
		7.5	7.57	0.9	2.1
		Response (t = 0)	Response (t = x)		
Stock in methanol (1 mg/mL)	RT, 4 h	1.23	1.28	6.3	4.5
	−70 °C, 43 d	1.19	1.23	0.6	3.1
	−70 °C, 115 d	1.33	1.35	3.4	0.9
Working solution	RT, 4 h	1.25	1.28	1.4	2.1
	−70 °C, 32 d	1.17	1.25	2.3	7.1
	−70 °C, 104 d	1.33	1.31	2.3	−1.8

RT: Room temperature; F/T: Freeze/thaw cycle; d: days; h: hours.

stock and working solutions was assessed by comparing the measured responses at t = x, to freshly prepared solutions at t = 0.

Stability criteria of ±15% deviation (CV% ≤15%) for the biomatrix and ±5% deviation for the stock- and working solutions were applied.

Galunisertib was stable in plasma at room temperature for at least 4 h, at −20 °C for at least 32 days and at least 5 freeze (−20 °C) / thaw (RT) cycles and final extract stability at 2–8 °C was at least 4 days. Stock and working solutions in methanol were stable for at least 4 h at room temperature, and additionally, stock and working solutions were stable at −70 °C for at least 115 and 104 days, respectively. Stability data are presented in Table 3.

4. Application of the method

The validated method was applied to a pre-clinical in-house study. For this application mouse plasma samples were diluted with control human plasma. Method qualification tests were performed, to show the suitability of the assay for the quantification in this biomatrix. Control mouse plasma samples were spiked with galunisertib and diluted with human plasma (10 times) to yield concentrations of 0.6 and 2 ng/mL. These samples were processed and analysed in triplicate, together with calibration standards in human plasma, to verify accuracy and precision. Accuracy, as bias, was 0.9% and 5.5% and precision, as C.V. was 7.3% and 5.5%, for the 0.6 and 2 ng/mL samples, respectively. Injection of a control mouse plasma sample revealed no endogenous interferences (see Fig. 3). Hence it was concluded that the validated method was also suitable to quantify galunisertib in mouse plasma, after dilution of at least 10 times in control human plasma.

In the pre-clinical study, wild-type male mice (FVB/NRj genetic background) were administered 20 mg/kg galunisertib orally (n = 7). Galunisertib was dissolved in dimethyl sulfate (DMSO) at a concentration of 50 mg/ml and further diluted with pH = 2 (10 mM) hydrochloric acid solution to yield a concentration of 2 mg/ml. Final concentration for DMSO was 4%. Mice were housed and handled according to institutional guidelines complying with Dutch legislation and treated similar to earlier reported protocols. Shortly, mice were 12 to 14 weeks of age and housed in a temperature-controlled environment with a 12-h light/12-h dark cycle. Animals were administered a standard diet and acidified water *ad libitum* and were fasted for 2–3 h before galunisertib was administered by gavage into the stomach, using a blunt-ended needle. At 15, 30 min, 1, 2, and 4 h after administration, blood samples were collected from the tail vein in heparinized capillary tubes (Sarstedt, Germany). After 8 h, isoflurane was used to anaesthetise the mice and a final blood sample was obtained by cardiac puncture. Plasma samples were acquired by centrifugation at 9000×g for 6 min at 4 °C and stored at −30 °C until analysis.

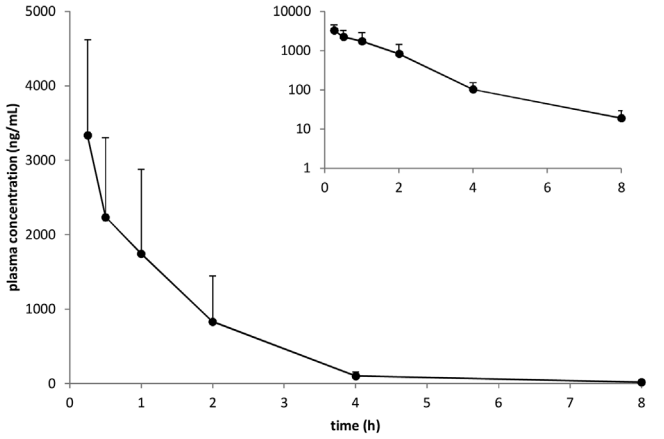

Fig. 4. Plasma concentration-time curve of galunisertib in male FVB/NRj mice (n = 7) after oral administration of 20 mg/kg of the drug. (with semi-logarithmic curve as insert).

The obtained mouse plasma samples were diluted at least 10 times in control human plasma prior to processing and galunisertib concentrations were determined using the validated method. The resulting concentration-time curve is presented in Fig. 4, showing that the validated method is fit-for-use, even for the quantification of galunisertib in mouse plasma.

5. Conclusion

We developed a reliable and sensitive method to quantify galunisertib in human plasma. This is the first reported method to quantify galunisertib in human plasma samples. A simple protein precipitation was used to separate galunisertib from the plasma. The overall recovery was around 90% and the signal-to-noise-ratio at the LLOQ was more than sufficient (>20). Extensive stability testing was executed and revealed no stability issues in the biomatrix and the stock and working solutions. The applicability of the assay was demonstrated in a mouse pharmacokinetic study where plasma samples were diluted at least 10 times with control human plasma before processing.

To conclude, the presented galunisertib LC–MS/MS method has been validated according to the latest guidelines and meets its requirements, and its (pre-)clinical application was demonstrated.

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