Journal of Chromatography B 1125 (2019) 121723

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/jchromb

Development and validation of an LC-MS/MS method with a broad linear dynamic range for the quantification of tivozanib in human and mouse plasma, mouse tissue homogenates, and culture medium

M.A.C. Bruin[a,b,*], H. Rosing[a], L. Lucas[a], J. Wang[b], A.D.R. Huitema[a,d], A.H. Schinkel[b], J.H. Beijnen[a,b,c]

[a] *Department of Pharmacy & Pharmacology, The Netherlands Cancer Institute, Amsterdam, the Netherlands*
[b] *Division of Pharmacology, The Netherlands Cancer Institute, Amsterdam, the Netherlands*
[c] *Division of Pharmacoepidemiology and Clinical Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, the Netherlands*
[d] *Department of Clinical Pharmacy, University Medical Center Utrecht, Utrecht University, Utrecht, the Netherlands*

ARTICLE INFO

Keywords:
Tivozanib
LC-MS/MS
Linear dynamic range
Isotopologue

ABSTRACT

The first bioanalytical assay for tivozanib in human and mouse plasma, mouse tissue homogenates and culture medium was developed and validated over a linear dynamic range from 0.5 to 5000 ng/mL. The extended concentration range will cover the quantification of tivozanib in the majority of study samples, reducing the need for reanalysis which is often not possible due to limited amount of sample in preclinical studies. A simple and fast pretreatment method was used consisting of protein precipitation with acetonitrile followed by dilution of the supernatant. The final extract was injected onto an Ultra-Performance Liquid Chromatography (UPLC) BEH C18 column with gradient elution of formic acid in water and formic acid in acetonitrile mobile phase. Chromatographic separation was followed by detection with a triple-quadrupole mass spectrometer operating in the positive ion-mode. By simultaneously monitoring the sensitive conventional $[M + H]^+$ isotopologue- product transition for quantification of low concentrations and a less abundant $[M + H]^+ + 1$ isotopologue- product transition to reduce the sensitivity for quantification of high concentrations, we were able to extend the overall linear dynamic range up to 0.5–5000 ng/mL. A full validation was performed in human plasma and a partial validation was executed for the other matrices. All results were within the acceptance criteria of the European Medicines Agency (EMA) guidelines and the US Food and Drug Administration (FDA) guidance, except for the carry-over. This was solved by the analysis of extra matrix blanks and by grouping study samples containing a high tivozanib concentration in the sample sequence. In this way carry-over did not impact the data integrity. We demonstrated that by measuring two multiple reaction monitoring (MRM) transitions for tivozanib, the linear dynamic range could be extended from two to four decades. The assay was successfully applied in pharmacokinetic studies in mice and a transport assay.

1. Introduction

Tivozanib is a highly potent and selective tyrosine kinase inhibitor which blocks the vascular endothelial growth factor receptor (VEGFR) -1, -2, and -3. This inhibits angiogenesis and reduces the vascular permeability in tumor tissues. Tivozanib was granted market authorization in the European Union, Iceland and Norway in August 2017 for patients with advanced renal cell carcinoma (RCC) as a first line treatment and for VEGFR and mammalian target of rapamycin (mTOR) pathway inhibitor-naïve patients following disease progression after one prior treatment with cytokine therapy [1]. The pharmacokinetics of tivozanib in human has been investigated in various studies, including a mass balance study in healthy volunteers. The agent has a long half-life of 89.3 ± 23.5 h with no major metabolites circulating in plasma [2,3]. To date, no bioanalytical methods have been described in the literature for the quantification of tivozanib in biological matrices. In order to support pharmacokinetic studies in humans and mice and *in vitro* experiments, to further understand the kinetics of tivozanib, the function of transporters, metabolic enzymes and tissue distribution, a bioanalytical assay is pivotal to monitor tivozanib concentrations.

* Corresponding author at: Department of Pharmacy & Pharmacology, Antoni van Leeuwenhoek – The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, the Netherlands.
E-mail address: ma.bruin@nki.nl (M.A.C. Bruin).

https://doi.org/10.1016/j.jchromb.2019.121723
Received 15 May 2019; Received in revised form 9 July 2019; Accepted 17 July 2019
Available online 18 July 2019
1570-0232/ © 2019 Elsevier B.V. All rights reserved.

It is difficult to predict drug concentrations for first studies in any species. Therefore it is a challenge to determine the concentration range for the development of a liquid chromatography-mass spectrometry (LC-MS/MS) method. Normally the linear dynamic range for triple-quadrupole mass spectrometry (MS) is two to three orders of magnitude depending on the compound to be analyzed [4]. A broader concentration range can be desirable. First, to prevent sample dilution and reanalysis, which can be time-consuming. Second, with limited amount of sample available from preclinical studies, it may not be possible to perform multiple analysis [5]. Therefore, a broad dynamic range is preferred to cover the entire range of study samples.

Extending the dynamic range to four or five orders of magnitude often results in a non-linear calibration curve due to saturation either at the ion source and/or at the MS detector [5]. A quadratic regression model can be used, but this approach is controversial and less desirable since the response is not proportional to the analyte concentration. A stable-isotope-labeled internal standard (SIL-IS) can compensate for ionization variability in the ion source, nevertheless, it cannot compensate for saturation at the MS detector [6]. An approach to overcome MS detector saturation is to monitor a less abundant isotopologue such as $[M + H]^+ +1$ [5,7,8]. The use of the $[M + H]^+ +1$ isotopologue results in a lower analyte response compared to the primary precursor ion $[M + H]^+$, because of the presence of less abundant ^{13}C, ^{2}H, ^{17}O or ^{15}N atoms in the molecule. Measuring a MRM signal corresponding to $[M + H]^+ +1$ will lower the signal and is expected to be less prone to MS detector saturation. Since the signal will be lower, the calibration range can be extended. The combination of the conventional $[M + H]^+$ isotopologue for low concentrations and the less abundant $[M + H]^+ +1$ isotopologue for high concentrations results in a broad overall linear dynamic range. Moreover, a high accuracy and precision around the upper limit of quantification (ULOQ) and sufficient sensitivity for the lower limit of quantification (LLOQ) will be preserved.

Another approach for extending the linear dynamic range is to use a product ion with a lower sensitivity [4]. Although this approach is applicable, there are several advantages of using an less abundant isotopologue over a less abundant product ion. First, the sensitivity can be predicted from the regular transition $[M + H]^+$, second, a less abundant isotopologue is always available in contrast to a suitable product ion with a lower sensitivity, and third, there is no need for optimization of the additional transition and MS parameters can remain unchanged. The use of a less abundant isotopologue for extension of the linear dynamic range has been successfully applied in several bioanalytical methods and this approach has been followed by us for tivozanib analysis in biological samples [5,7,8].

The objective of this study was to develop and to validate an LC-MS/MS method with a broad linear dynamic range from 0.5 to 5000 ng/mL for the quantification of tivozanib in human and mouse plasma, mouse tissue homogenates and culture medium. A full validation was performed in human plasma and a partial validation was executed in the other matrices. A fast and simple protein precipitation was used as a sample pretreatment method prior to analysis by UPLC-MS/MS. Two analyte MRM transitions were measured simultaneously to extend the overall linear dynamic range. The method was set up to support a pharmacokinetic study in mice to investigate the function of transporters, metabolic enzymes and tissue distribution of tivozanib.

2. Materials and methods

2.1. Chemicals

Tivozanib, free base (batch SVI-ALS-16-115) and the SIL-IS $^{13}C_4$, ^{15}N-Tivozanib (batch ALG-ALS16-157P1) were purchased from Alsachim (Illkirch-Graffenstaden, France). Tivozanib, free base (batch BT1603801702) used in the mice- and *in vitro* experiments was purchased from Carbosynth (Berkshire, UK). Acetonitrile, formic acid and water (all UPLC grade) were supplied from Biosolve Ltd. (Valkenswaard, The Netherlands) and dimethylsulfoxide (DMSO), was obtained from Merck (Darmstadt, Germany).

2.2. Blank matrices

Control human K_2EDTA plasma was purchased from Bioreclamations LLC (Hicksville, NY, USA). Mouse plasma and tissue (including brain, kidneys, liver, lung, spleen and small intestine) were obtained from the animal facility of the Netherlands Cancer Institute (Amsterdam, The Netherlands). Culture medium was freshly prepared and contained Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS). Both were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2.1. Tissue homogenates

Tissue homogenates were prepared by adding 4% Bovine Serum Albumin (BSA, Fraction V) obtained from Roche Diagnostics GmbH (Mannheim, Germany) in water. A volume of 3 mL was added to the liver and small intestine, 2 mL to the kidney and 1 mL to the brain, spleen and lung. Subsequently the samples were homogenized by using a homogenizing machine Fast Prep-24™ 5G (MP Biomedicals Inc., Santa Ana, California, USA).

2.3. Stock and working solutions

Stock solutions of tivozanib and tivozanib internal standard were prepared in DMSO at a concentration of 1 mg/mL and stored at −70 °C. From the stock solution, working solutions were prepared in acetonitrile-water (50:50, v/v). An internal standard working solution (WIS) was prepared in acetonitrile-water (50:50, v/v) at a final concentration of 250 ng/mL. Working solutions were stored at −20 °C.

2.4. Calibration standards and quality control samples

Calibration standards and quality control (QC) samples were prepared by adding 50 μL of working solution to 950 μL of human plasma. Ten calibration standards were obtained with concentrations of 0.5, 1, 5, 10, 50 and 80 ng/mL (for the low concentration range (L)), and 50, 80, 100, 500, 1000 and 5000 ng/mL (for the high concentration range (H)). QC samples in human plasma were prepared in the same way obtaining final concentrations of 0.5 (QC LLOQ), 1 (QC L-LOW), 8 (QC L-MID, 66 (QC H&L), 400 (QC H-MID) and 4000 (QC H-HIGH) ng/mL. Aliquots of 50 μL were made for both calibration standards and QC samples in human plasma. QC samples in mouse tissue homogenate (liver, small intestine, spleen, kidney, brain and lung) and culture medium were prepared at three concentration levels of 0.5 (QC LLOQ), 66 (QC H&L) and 4000 (QC H-HIGH) ng/mL. QC samples in culture medium were prepared by spiking 5 μL of working solution directly to 95 μL of culture medium. QC samples in tissue homogenates were prepared by adding 50 μL of working solution to 950 μL of tissue homogenate and subsequently aliquots of 100 μL were made. QC samples in mouse plasma were prepared at three concentration levels: 2.5 (QC LLOQ), 330 (QC H&L) and 80,000 (QC H-HIGH) ng/mL. Aliquots of 10 μL were made. All aliquots were stored at −20 °C until processing and analysis.

2.5. Sample pretreatment

Prior to sample pretreatment, samples were thawed at room temperature. To 10 μL of mouse plasma study sample and QC mouse plasma sample, 40 μL of human plasma was added to obtain a 50 μL sample. The 50 μL samples (Calibration standards, QC samples in human plasma, mouse plasma study samples, QC mouse plasma and culture medium study samples) were mixed with 10 μL of WIS followed by 100 μL of acetonitrile for protein precipitation. The 100 μL samples (QC mouse tissue homogenates, QC culture medium and mouse tissue

homogenate study samples) were mixed with 20 µL of WIS followed by 200 µL of acetonitrile for protein precipitation. Samples were mixed by vortex-mixing, shaken for 10 min and centrifuged at 23,100*g* for 5 min at room temperature. A volume of 140 µL supernatant was transferred to an autosampler vial that contained 140 µL of 0.1% formic acid in water. After mixing the final extract was stored at 2–8 °C until analysis.

2.6. LC-MS system and conditions

2.6.1. LC- triple quadrupole MS settings

A Nexera 2 series liquid chromatography system (Shimadzu Corporation, Kyoto, Japan) was used coupled to a triple quadrupole mass spectrometer API4000 (Sciex, Foster City, CA, USA) for analysis. The chromatographic system was equipped with a binary pump, a degasser, an autosampler, valco valve and column oven (Shimadzu). Chromatographic separation was performed by using a reversed phase Acquity UPLC BEH C18 column (50 × 2.1 mm, particle size 1.7 µm, Waters, Wilmslow, UK). A volume of 2 µL was injected onto the system. The column temperature was maintained at 40 °C and the auto-sampler rack compartment at 4 °C. The mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in acetonitrile (phase B). A block gradient was used followed by a double washing step as described in Fig. 1.

The mass spectrometer was operating in the positive ion mode. Tivozanib (1000 ng/mL in 80% methanol) was infused into the MS to establish analyte-dependent parameter settings of the mass spectrometer. Two MRM transitions were used for the detection of tivozanib and one MRM transition was used for tivozanib internal standard. Table 1 summarizes the settings for the mass spectrometer.

2.6.2. Orbitrap MS settings

An accurate mass measurement was performed by infusion of both reference standards of tivozanib (1000 ng/mL in 80% methanol) into a LTQ Orbitrap Discovery (Thermo Fisher Scientific, Waltham, MA, USA). A full MS spectrum was obtained (average of 42 spectra) for both reference standards. The relative abundance of the different isotopes was calculated.

2.7. Method validation

The method was validated based on the international guidelines for bioanalytical method validation of the United States Food and Drug Administration (FDA) and European Medicines Agency (EMA) [10,11]. A full validation was performed in human plasma (calibration model, accuracy and precision, carry-over, selectivity, overall recovery and stability) and a partial validation was performed for mouse plasma, tissue homogenates and culture medium (accuracy and precision, selectivity and stability). Additional experiments were performed to determine the validity of the approach of using two MRM transitions to quantify tivozanib using a broad linear dynamic range.

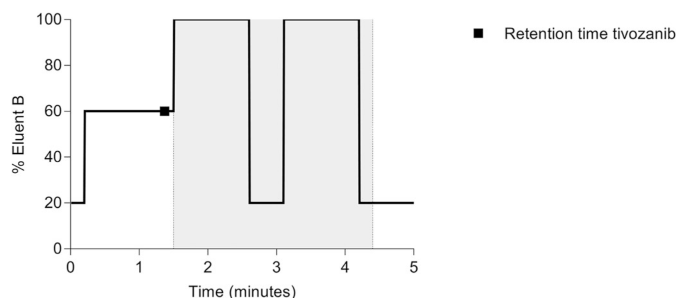

Fig. 1. Gradient profile for the quantification of tivozanib (retention time of tivozanib of 1.35 min is indicated). Eluent A and B consists of 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. After 1.50 min the flow rate was increased from 0.3 mL/min (white area) to 0.6 mL/min (grey area) and decreased again to 0.3 mL/min at 4.41 min to stabilize the system before the next injection.

Table 1
Mass spectrometric settings for the quantification of tivozanib in biological matrices.

Nebulizing gas	55 au
Turbo gas/heater gas	15 au
Curtain gas	10 au
Collision gas	12 au
Ion spray voltage	5500 V
Temperature	650 °C
Dwell time	60 msec

	Parent mass, *m/z*	Product mass, *m/z*
Tivozanib monitored ions (low range)	455.121	341.100
Tivozanib monitored ions (high range)	456.121	341.100
Tivozanib internal standard monitored ions	460.120	341.100
Declustering Potential (DP)	101 V	
Collision Energy (CE)	63 V	
Collision cell Exit Potential	22 V	
Entrance Potential	10 V	
Retention time	1.35 min	

2.7.1. Calibration model

Calibration standards (10) were prepared in human plasma in duplicate at each concentration (see Section 2.4), including a double blank and a zero blank calibration standard and analyzed in each validation run. Calibration standards were divided into two ranges with an overlapping area. For quantification of the low concentration range (0.5, 1, 5, 10, 50 and 80 ng/mL) *m/z* 455 to 341 was used and for quantification of the high concentration range (50, 80, 100, 500, 1000, 5000) *m/z* 456 to 341 was used, which is visualized in Fig. 2. The ratio between peak area analyte/internal standard was plotted against the corresponding concentrations of the calibration standards and least square linear regression was applied. The back-calculated calibration concentrations were determined to establish the best weighting factor. The model with the lowest total- and constant bias across the concentration range was considered to be the best fit. Deviations of the back-calculated concentrations should be within ± 15% and ± 20% for the LLOQ in at least 75% of the calibration standards.

2.7.2. Accuracy and precision

Five replicates of each QC level in human plasma were analyzed in three analytical runs. Five replicates of three QC levels in mouse plasma, mouse tissue homogenate and culture medium were analyzed in one analytical run. QC LLOQ, QC L-LOW and QC L-MID were analyzed using *m/z* 455 to 341, QC H-MID and QC H-HIGH were analyzed using *m/z* 456 to 341 and QC H&L was calculated with both MRM transitions as shown in Fig. 2. The accuracy and precision were calculated as the intra-assay bias (%) and intra-assay coefficient of variation (CV%) respectively. In case of human plasma the inter-assay variability and bias were calculated as well, using analysis of variance (ANOVA). For the bias and precision values 15% acceptance criteria were applied for all QC samples apart from QC LLOQ where 20% was permitted.

2.7.3. Lower limit of quantification (LLOQ)

The LLOQ was evaluated in human plasma in three validation runs by comparing the signal of the LLOQ (0.5 ng/mL) of the low calibration range to the noise in the double blank and comparing the signal of the LLOQ (50 ng/mL) of the high calibration range to the noise in the double blank. A signal-to-noise ratio of at least 5 was found acceptable.

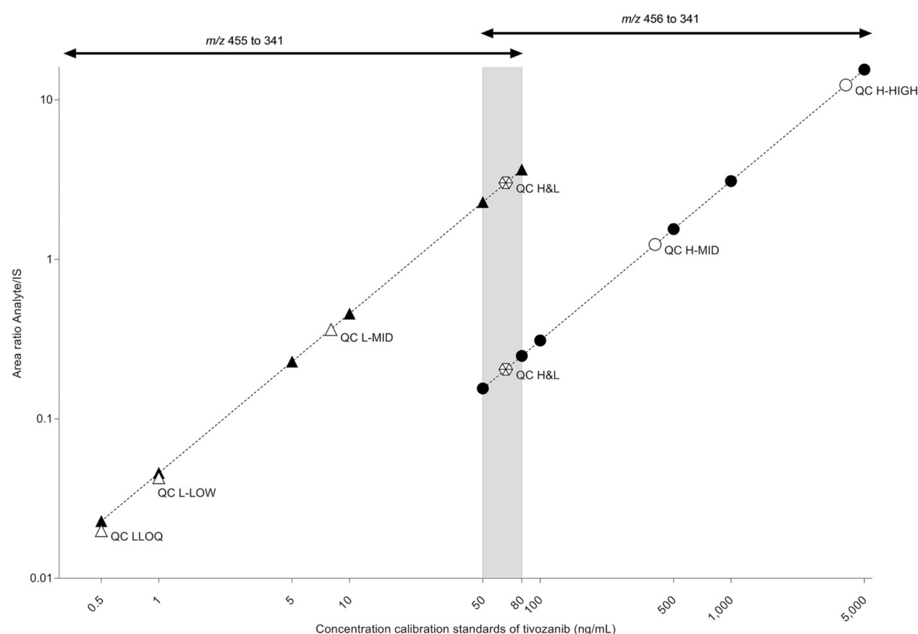

Fig. 2. Tivozanib calibration standards (black) and QC samples (white) measured with two MRM transitions. The grey area shows the overlapping region of both calibration ranges. X- and Y-axis are presented on a logarithmic scale.

2.7.4. Carry-over

Carry-over was determined in three analytical runs by injection of three double blanks after the analysis of the highest calibration level (ULOQ containing 5000 ng/mL in human plasma). The response at the retention time of tivozanib in the first double blank at both MRM transitions was compared with the response of the tivozanib measured in the LLOQ standards of both ranges (0.5 and 50 ng/mL), which should not exceed 20% for tivozanib and 5% for tivozanib internal standard.

2.7.5. Selectivity

Six batches of human K_2EDTA plasma and one pooled batch of mouse plasma were used to prepare LLOQ and double blanks to test selectivity. For culture medium and for each mouse tissue homogenate type a separate pooled batch was used and selectivity was evaluated in triplicate. The response of tivozanib and tivozanib internal standard in the double blank was compared with the response of tivozanib in LLOQ samples. Interfering peaks should not exceed 20% of the LLOQ peak of tivozanib and should not exceed 5% of the LLOQ peak of tivozanib internal standard. The mean measured concentration of the LLOQ should be within ± 20% of the nominal concentration.

2.7.6. Overall recovery

The overall recovery (sample pretreatment recovery plus matrix effects) was determined using 6 different batches of human plasma spiked at QC L-LOW and QC H-HIGH concentrations. The analyte area of processed QC L-LOW and QC H-HIGH samples were compared with matrix absent samples (0.1% formic acid in water) at the same concentration levels.

2.7.7. Stability

The stability of tivozanib was investigated in all matrices at the concentration level QC H&L in triplicate. Short-term stability at room temperature, long-term stability at −20 °C, 3 freeze-thaw cycles (thawing completely at room temperature and freezing at −20 °C for at least 12 h) and stability in the final extract at 4–8 °C was tested. Tivozanib was considered stable in the different matrices when 85–115% of the concentration was recovered. Furthermore stock solution (1 mg/mL in DMSO) stability at −70 °C and working solution (10 ng/mL and 80,000 ng/mL in acetonitrile-water (50:50, v/v)) stability at −20 °C was evaluated. Stock and working solutions were considered stable if 95–105% of the initial concentration was recovered.

2.7.8. Validity of the approach

As described in Section 2.7.2 the tivozanib concentration in the overlapping QC sample (QC H&L) was calculated using both linear calibration models in all matrices. The difference between the calculated concentrations with both MRM transitions was compared to determine the validity of the approach of using two MRM transitions for quantification. A difference of maximally 15% between the calculated concentrations was used as a criterion.

To check the validity of the extended range, calibration standards and QC samples from the high concentration range (containing 66, 80, 100, 400, 500, 1000, 4000 and 5000 ng tivozanib/mL) were diluted 100 times in control human plasma before processing. The processed samples were quantified using the calibration standards from the low calibration range and outcomes were compared to the measured concentrations that were obtained without dilution step using the calibration standards from the high concentration range.

2.7.9. Preclinical application

The bioanalytical assay was developed to support pharmacokinetic *in vitro* studies and *in vivo* studies in mice to investigate the function of transporters, metabolic enzymes and tissue distribution. Transport assays were performed with 2 μM tivozanib, 5 μM zosuquidar (ABCB1 inhibitor) and/or 5 μM Ko143 (ABCG2/Abcg2 inhibitor). A volume of 50 μL of culture medium was taken at different time points from the acceptor compartment. The studies in mice were conducted according to institutional guidelines complying with Dutch and European Union legislation. To minimize variation in absorption upon oral administration, mice were fasted for 3 h before tivozanib (1 mg/kg) was administered by gavage into the stomach, using a blunt ended needle. Blood was collected at different time points from the tail vein. At the last time point blood was taken by cardiac puncture under isoflurane anesthesia. Plasma was obtained using sodium heparin as anticoagulant and the blood was centrifuged for 6 min at 9000*g*, 4 °C. After sacrificing the mice by cervical dislocation, organs were collected and weighted. Tissue homogenates were prepared as described in Section 2.2.1. All samples were stored at −20 °C until analysis.

3. Results and discussion

3.1. Method development

3.1.1. Liquid chromatography

Different gradients and mobile phases in both acidic and alkaline environment were evaluated during method development. The combination of formic acid in water and the organic solvent acetonitrile resulted in symmetric peaks of tivozanib. Carry-over was observed after injection of the highest calibration standard. This was not unexpected, as carry-over is described as a common problem in bioanalytical assays with a broad dynamic range [4,6]. Rinsing of the needle, different wash solutions of the needle and the injection of reagent blanks were tested to reduce carry-over without improvement. The introduction of an extra washing step in the LC gradient reduced the carry-over from approximately 140% to 80% of the LLOQ. Carry-over was completely eliminated after addition of five extra washing steps in the gradient, however, this resulted in a relative long run time of 15 min. To handle the carry-over without prolonging the run time, we chose to add one extra washing step in the gradient profile (Fig. 1), group high concentrated samples during analysis and inject blank matrix samples after samples with expected high concentrations. In this way the carry-over was under control and did not influence the data integrity during the validation. In the routine application of the method, the carry-over was established in each analytical batch and a procedure was in place to evaluate the effect of the carry-over on the determination of the analyte in each sample. As a result, carry-over did not have an impact on the quantification of tivozanib, hence, no re-analysis of study samples was required due to carry-over effects.

3.1.2. Mass spectrometry

The product spectrum of tivozanib is shown in Fig. 3 with the proposed fragmentation pattern for the most abundant product ion (m/z 357) and the selected product ion (m/z 341). Using m/z 455 to 341 for the complete concentration range from 0.5 to 5000 ng/mL resulted in a non-linear calibration model due to detector saturation. To overcome detector saturation, MS parameters could be de-optimized or a less abundant isotopologue could be chosen to reduce the signal. However, this resulted in undetectable signals at low concentrations. The approach of using a less abundant isotopologue for the high concentrations in combination with the conventional isotopologue for the low concentrations was used to overcome this problem. The less abundant fragment ion of tivozanib (m/z 341) was chosen as the conventional isotopologue for low concentrations, because the most abundant

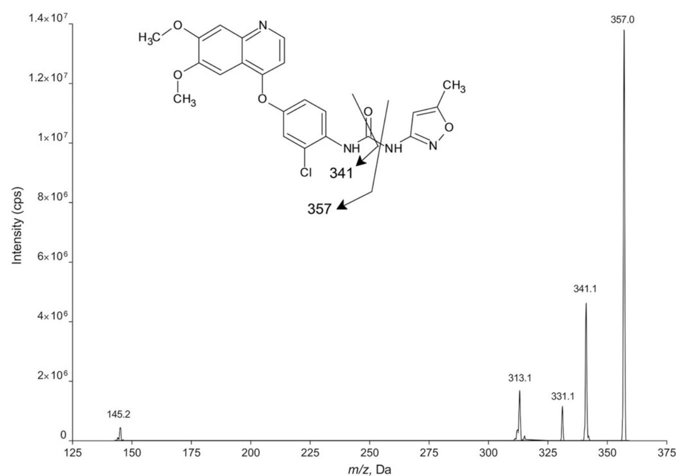

Fig. 3. The chemical structure of tivozanib, the product ion spectrum ($[M + H]^+$ 455) formed by collision induced dissociation (93 V) and the proposed fragmentation pattern to m/z fragments 341 and 357.

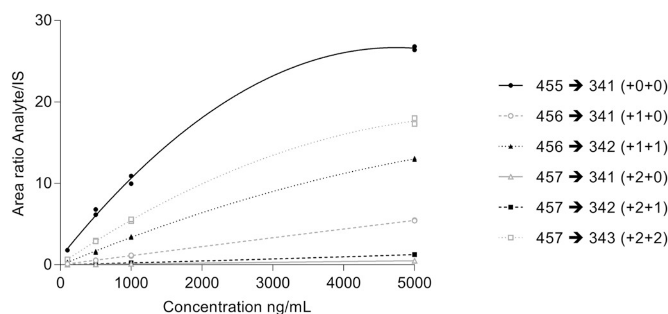

Fig. 4. Tivozanib concentration-response plots using different MRM transitions for the high calibration range (50–5000 ng/mL).

Table 2
Relative abundance of isotopes in tivozanib reference standard obtained from and a reference standard from Carbosynth used in the mouse study.

Mass $[M + H]^+$	Relative abundance (%)		Difference (%)
	Reference standard Alsachim	*Reference standard Carbosynth*	
455	61.89	62.46	0.9
456	14.03	14.17	1.0
457	19.28	18.82	−2.4
458	4.28	4.19	−2.1

Table 3
Accuracy and precision values for tivozanib in human and mouse plasma, mouse tissue homogenates, and culture medium.

Matrix	Nominal concentration (ng/mL)	Intra-assay (n = 5 in 1 run or n = 15 in 3 runs for human plasma)		Inter-assay (n = 15 in 3 runs)	
		Bias (%)	CV (%)	Bias (%)	CV (%)
Human plasma	0.5	± 14.0	≤7.3	−4.0	9.5
	1	± 9.6	≤11.9	−7.4	–[a]
	8	± 8.3	≤12.8	4.7	3.5
	66	± 5.5	≤5.0	1.4	3.1
	400	± 8.3	≤5.6	4.9	2.2
	4000	± 2.4	≤4.0	−0.4	2.1
Mouse plasma	0.5	−16.3	12.7		
	66	−1.9	3.5		
	4000	2.3	3.3		
Liver homogenate	0.5	−11.8	10.0		
	66	−3.3	1.9		
	4000	−3.3	3.7		
Kidney homogenate	0.5	−7.0	9.0		
	66	−2.6	3.3		
	4000	−3.4	2.5		
Spleen homogenate	0.5	−5.6	12.7		
	66	4.6	2.7		
	4000	−4.8	3.3		
Brain homogenate	0.5	−14.4	8.9		
	66	−1.9	3.3		
	4000	−7.2	3.2		
Lung homogenate	0.5	0.4	8.3		
	66	−0.2	6.1		
	4000	−6.0	3.3		
Small intestine homogenate	0.5	−13.8	4.2		
	66	4.4	1.7		
	4000	−1.6	4.4		
Culture medium	0.5	4.8	6.7		
	66	4.6	4.8		
	4000	3.5	2.5		

[a] The inter-assay precision could not be calculated because there is no significant additional variation due to the performance of the assay in different batches.

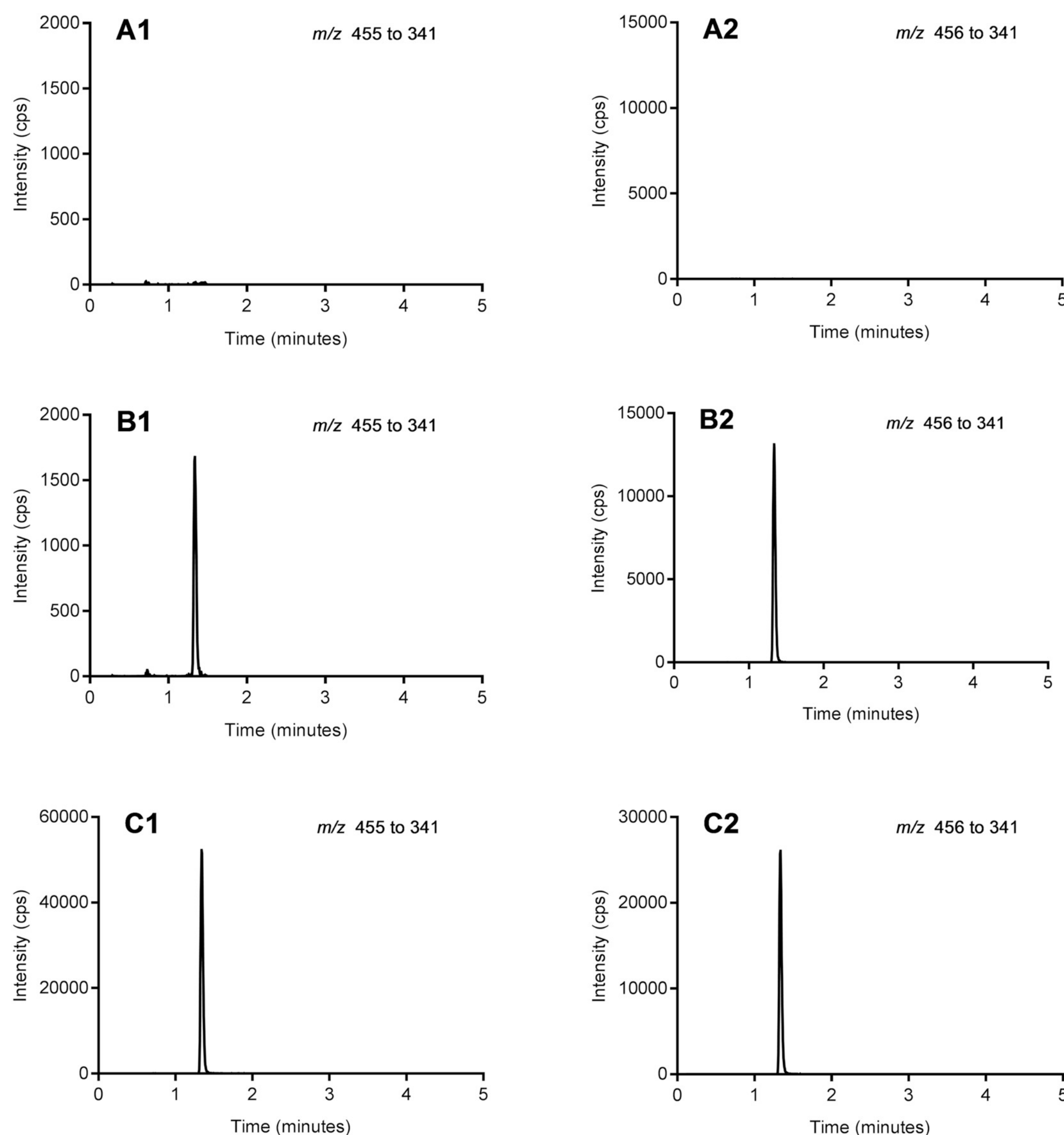

Fig. 5. LC-MS/MS chromatograms of a double blank sample measured with both MRM transitions (A-series), spiked samples at QC LLOQ level in human plasma (B1: 0.5 ng/mL, measured with *m/z* 455 to 341; B2: 50 ng/mL measured with *m/z* 456 to 341) and two mouse plasma study samples (C1: 12.4 ng/mL, measured with *m/z* 455 to 341; C2: 96.8 measured with *m/z* 456 to 341 ng/mL).

fragment (*m/z* 357) already showed a non-linear calibration model in the low concentration range (0.5–80 ng/mL). Different transitions were evaluated for quantification of the high concentration range as shown in Fig. 4. Based on these results, the $[M + H]^+ + 1$ isotope (*m/z* 456) to fragment ion *m/z* 341 was selected for the quantification of tivozanib in the high concentration range.

During the development of the assay, high variation in the internal standard area was observed. Variation in internal standard area was expected considering the different types of matrices, but high variation was observed even within samples of the same matrix. Too much variation could doubt the reliability of the assay, especially if the analyte is affected differently than its internal standard [12]. Accordingly a post-elution infusion test was performed to evaluate the possibility of a co-eluting component at the retention time of tivozanib, which could cause ion suppression or ion enhancement. The results indicated that this was not the underlying cause of the variability in internal standard. By changing the ion spray voltage to a higher voltage, increasing the source temperature and by using fresh eluent, a stable internal standard signal was obtained (Table 1).

As described by Trobbiani et al. the approach of using a less abundant isotopologue may introduce a quantitative error when the reference standard for calibration is from a different source to the analyte in the samples as a result of different composition regarding isotopologues, which was our case [9]. Therefore, we compared the two reference standards and the relative abundance of the different isotopes is shown in Table 2. A relevant major quantitative error was excluded, because of the maximal difference in relative abundance of the selected +1 isotope (*m/z* 456) in the tested batches was only 1.00%.

3.1.3. Sample preparation

A simple and fast sample pretreatment method was desirable, because a large number of samples were expected for analysis. Initially, we started with protein precipitation with different precipitation solutions (methanol, acetonitrile and methanol-acetonitrile (1:1, v/v). No large differences in response and no difference in carry-over were observed between the different solvents. Acetonitrile was selected which was similar to the organic mobile phase. A ratio of 2:1 (acetonitrile:biological sample) was used for precipitation of the samples, since this resulted in a good efficiency of removing endogenous proteins [13]. Aliquots of 50 μL were used for processing for all sample types, which were made out of a large volume of 1 mL. During pre-validation, accuracy and precision values were not acceptable for QC samples in

Table 4
Stability data of tivozanib in human plasma, mouse plasma, mouse tissue homogenates and culture medium (n = 3).

Matrix	Stability conditions	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Accuracy (% Bias)	Precision (% CV)
Human plasma	RT, 5 d	66	63.2	−4.2	6.9
	3 F/T (RT, −20 °C)	66	63.1	−4.4	4.3
	LT, −20 °C, 5 m	66	65.7	−0.4	1.5
Final extract	4–8 °C, 1 m	1.00	0.931	−6.9	3.5
		66	68.8	4.2	6.5
		4000	4113	2.8	2.5
Mouse plasma	RT, 20 h	330	322	−2.4	1.9
	3 F/T (RT, −20 °C)	330	320	−3.0	4.7
	LT −20 °C, 3 m	330	321	−2.7	5.9
Liver homogenate	RT, 20 h	66	60.8	−7.8	11.1
	3 F/T (RT, −20 °C)	66	67.8	2.7	4.7
	LT −20 °C, 5 m	66	66.2	0.4	3.2
Final extract	4–8 °C, 2 m	66	69.1	4.6	2.0
Kidney homogenate	RT, 20 h	66	62.5	−5.3	7.2
	3 F/T (RT, −20 °C)	66	66.0	0.0	1.3
	LT −20 °C, 5 m	66	64.3	−2.6	1.9
Final extract	4–8 °C, 2 m	66	69.4	5.1	0.6
Spleen homogenate	RT, 20 h	66	66.6	1.0	2.0
	3 F/T (RT, −20 °C)	66	66.5	0.8	4.8
	LT −20 °C, 5 m	66	65.1	−1.3	5.0
Final extract	4–8 °C, 2 m	66	69.1	4.6	1.0
Brain homogenate	RT, 20 h	66	62.6	−5.1	4.5
	3 F/T (RT, −20 °C)	66	67.0	1.5	3.3
	LT −20 °C, 5 m	66	66.7	1.0	1.4
Final extract	4–8 °C, 2 m	66	69.7	5.7	2.4
Lung homogenate	RT, 20 h	66	66.4	0.6	1.7
	3 F/T (RT, −20 °C)	66	62.2	−5.8	7.3
	LT −20 °C, 5 m	66	64.9	−1.7	1.2
Final extract	4–8 °C, 2 m	66	67.6	2.4	6.2
Small intestine homogenate	RT, 20 h	66	70.0	6.0	7.5
	3 F/T (RT, −20 °C)	66	68.7	4.1	2.4
	LT −20 °C, 5 m	66	70.2	6.4	3.2
Final extract	4–8 °C, 2 m	66	71.0	7.5	2.8
Culture medium	RT, 20 h	66	68.5	3.7	5.0
	2 F/T (RT, −20 °C)	66	71.6	8.5	2.2
	LT −20 °C, 2 m	66	70.9	7.4	1.1
Final extract	4–8 °C, 1 m	66	68.0	3.0	0.5

RT = room temperature, F/T = freeze-thaw cycles, LT = long term, d = days, m = months.

Table 5
Tivozanib calibration standards and QC samples from the high range quantified using the low calibration range after dilution (100 x) in control human plasma, compared with the back-calculated calibration standard concentrations and QC samples concentrations when using the high concentration range.

Concentration (ng/mL) 100× dilution samples measured with m/z 455 to 341 transition	Concentration (ng/mL) undiluted samples measured with m/z 456 to 341 transition	Relative difference between the concentration from the diluted and undiluted samples (%)
68.8	64.1	−6.8
71.4	63.7	−10.8
85.8	79.5	−7.3
88.0	79.5	−9.7
114	109	−4.4
106	102	−3.8
420	405	−3.6
430	395	−8.1
502	496	−1.2
532	496	−6.8
1000	1020	2.0
1020	932	−8.6
4230	4070	−3.8
3970	3910	−1.5
5370	4990	−7.1
5140	5060	−1.6

culture medium (overall intra-run bias of −34.8%, −16.2% and −28.8% for QC LLOQ, QC H&L and QC H-HIGH, respectively). Probably due to adsorption of the analyte to the Eppendorf tubes or due to the poor water solubility [14]. Direct spiking of working solution (5 µL) to culture medium (95 µL) solved the problem and improved the intra-run bias. To obtain homogenous samples for tissue homogenates, a volume of 100 µL was more practical. The same ratio of 2:1 (acetonitrile:biological sample) was used for precipitation of the 100 µL samples and a double amount of internal standard was added. Therefore these samples could be quantified using one set of calibration standards. Formic acid (0.1% v/v in water) was used to dilute the supernatant (1:1, v/v) before injection, to prevent solvent effects.

3.2. Method validation

3.2.1. Calibration model

The calibration model was linear over both tested ranges. Different weighting factors were evaluated and weighting factor 1/x2 gave a slightly better fit compared to 1/x for both ranges. All criteria were met with a regression coefficient of 0.9966 (± 0.0019) for the low calibration range and 0.9973 (± 0.0009) for the high calibration range.

3.2.2. Accuracy and precision

The accuracy and precision of the method in the different matrices are shown in Table 3. The intra- and inter-run accuracy and precision for human plasma were within ± 15% for all QC samples and within ± 20% for QC LLOQ. For the other matrices the intra-run accuracy and precision were evaluated. All matrices met the criteria as mentioned above. Results demonstrated that calibration standards in human plasma could be used as surrogate matrix to quantify mouse plasma, mouse tissue homogenates and culture medium.

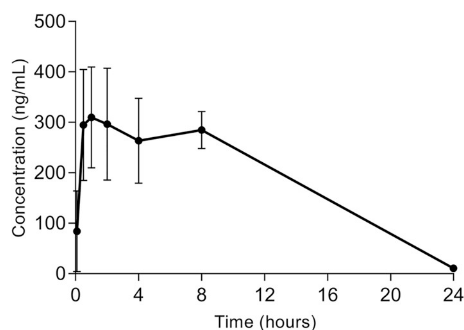

Fig. 6. Plasma curve of tivozanib in wild-type mice ($n = 5$) up to 24 h after oral administration of 1 mg/kg. Data are presented as mean ± SD.

3.2.3. Lower limit of quantification

The LLOQ was evaluated in human plasma for both calibration ranges. The response of the analyte at lowest concentration level was at least 10 times the response in the blank sample in three analytical runs for both ranges. The lowest observed signal-to-noise ratio was 14 for the LLOQ of the low calibration range and 350 for the LLOQ of the high calibration range. Fig. 5A and B shows representative chromatograms of tivozanib in human plasma in a double blank sample and QC LLOQ samples for both calibration ranges.

3.2.4. Carry-over

The carry-over was tested in three separate analytical runs. After injection of the highest calibration standard (5000 ng/mL), a maximum carry-over of 90% was observed for tivozanib in the first double blank and 33% in the second double blank measured with m/z 455 to 341. No carry-over was observed in the double blank measured with m/z 456 to 341. According to the guidelines the carry-over was not within the required limit of ≤20%. Therefore, samples containing high concentrations of tivozanib were grouped in the sample sequence and blank matrix samples were injected after samples with expected high concentrations. By applying these precautionary measures, carry-over was acceptable and did not influence the data integrity during the validation.

3.2.5. Selectivity

The mean measured concentration of the LLOQ in 6 different batches of human plasma and a pooled batch of mouse plasma and other matrices were within ± 20% of the nominal concentration. No response was observed in the blank samples of all matrices for tivozanib and tivozanib internal standard indicating the method to be selective for tivozanib.

3.2.6. Overall recovery

The overall recovery (sample pretreatment recovery plus matrix effects) was ranging between 100 and 123% for QC L-LOW and between 112 and 117% for QC H-HIGH. The CV was 7.3% and 1.9% respectively. This demonstrates that there is no large variation in recovery efficiency. An effect of ion enhancement in the ion source of the MS in matrix present samples could explain the recovering exceeding 100%.

3.2.7. Stability

The stock solution of tivozanib was stable for at least 6 months of storage at −70 °C and working solutions were stable for at least 4 months of storage at −20 °C. Stability in human plasma, mouse plasma, mouse tissue homogenates and culture medium was performed at one QC level since most matrices are not excessively available. The results for stability testing are presented in Table 4. Tivozanib is stable under all tested conditions. Long-term stability assessment in the different matrices was evaluated up to 2 to 5 months and is still ongoing.

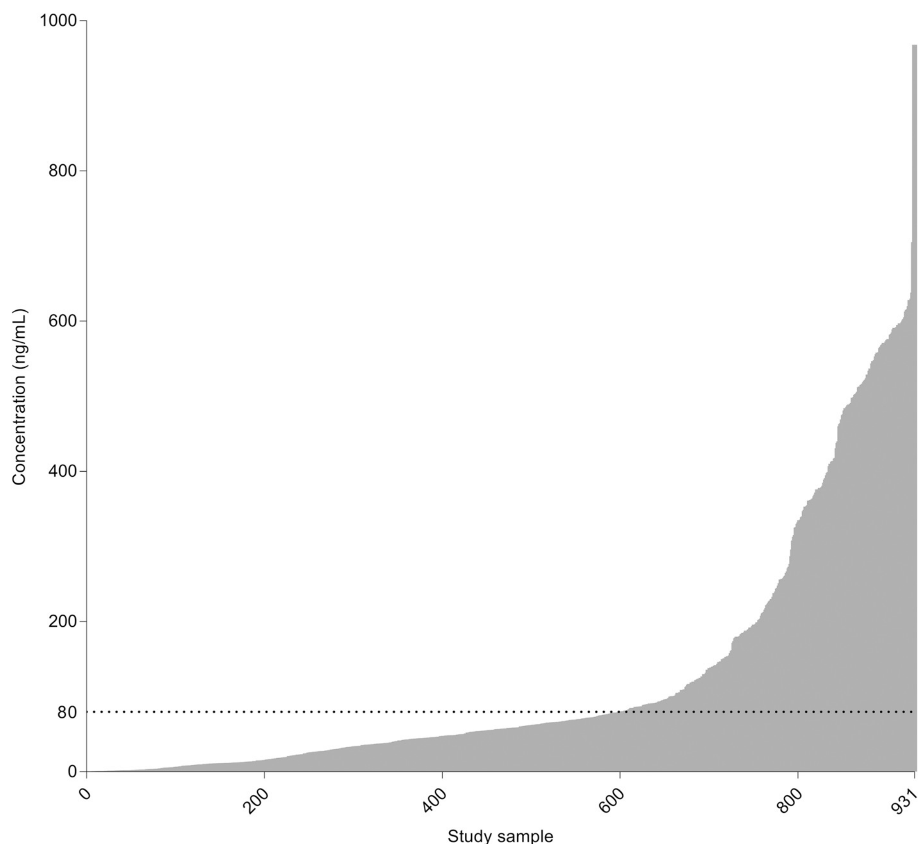

Fig. 7. The measured concentration of tivozanib (ng/mL) in the mouse study samples. Each dot represents a single study sample.

3.2.8. Validity of the approach

The difference between the calculated concentrations of QC H&L using both calibration ranges was ≤15% for all matrices. Accuracy and precision was comparable for QC H&L when this sample was quantified using both calibration ranges. So a clear *a priori* decision based on the relative accuracy and precision as described by Curtis et al. about the used range for study samples in the overlapping region, could not be made [4]. Since an *a priori* decision was still desirable, we decided to use the low calibration range for quantification when study samples fell within the overlapping region of the two ranges (50–80 ng/mL).

Table 5 shows the results for the 100 times diluted samples measured with the low calibration range and the undiluted samples measured with the high calibration range. The data are corrected for the dilution and the relative difference between the measured concentrations is shown. This table demonstrates the feasibility of the method using two calibration ranges, omitting the need of performing re-assays.

3.3. Preclinical application

The bioanalytical assay was applied in mouse pharmacokinetic studies. Two representative chromatograms of tivozanib in mouse plasma study samples are shown in Fig. 5C. An example of the plasma curve of tivozanib in wild-type mice until 24 h after oral administration of 1 mg/kg is shown in Fig. 6. The median time to peak plasma concentration (t_{max}) of tivozanib ranges from 0.5 to 8 h with substantial variability between mice. Multiple peaks suggest that tivozanib undergoes enterohepatic recirculation. This is consistent with data in human, derived from healthy volunteers and oncology patients [2,3]. Fig. 7 shows the measured concentration of the study samples. Out of the 931 measured study samples, 600 samples were quantified using the low calibration range (< 80 ng/mL) and only 5 samples were below the LLOQ (0.5 ng/mL). The remaining samples were quantified using the high calibration range. These results demonstrate the applicability of the presented method.

4. Conclusion

This was the first validated LC-MS/MS assay for the quantification of tivozanib in several matrices. We successfully demonstrated that the use of two MRM channels could increase the linear dynamic range (0.5–5000 ng/mL). The assay was validated and showed results which were compliant to the international guidelines [10,11]. In conclusion, we developed and validated a method for tivozanib where human plasma could be used as a surrogate matrix for the quantification of tivozanib in mouse plasma, mouse tissue homogenates and culture medium. The new assay was applied to measure tivozanib in preclinical studies.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] M. Santoni, F. Massari, F. Piva, F. Carrozza, V. Di Nunno, A. Cimadamore, A. Martignetti, R. Montironi, N. Battelli, Tivozanib for the Treatment of Renal Cell Carcinoma Tivozanib for the Treatment of Renal Cell Carcinoma, Expert. Opin. Pharmacother. 19 (9) (2018) 1021–1025, https://doi.org/10.1080/14656566.2018.1480722.

[2] F.A.L.M. Eskens, M.J.A. De Jonge, P. Bhargava, T. Isoe, M.M. Cotreau, B. Esteves, K. Hayashi, H. Burger, M. Thomeer, L. Van Doorn, J. Verweij, Biologic and clinical activity of tivozanib (AV-951, KRN-951), a selective inhibitor of VEGF receptor-1, -2, and -3 tyrosine kinases, in a 4-week-on, 2-week-off schedule in patients with advanced solid tumors, Clin. Cancer Res. 17 (2011) 7156–7163, https://doi.org/10.1158/1078-0432.CCR-11-0411.

[3] M.M. Cotreau, C.L. Hale, L. Jacobson, C.S. Oelke, A.L. Strahs, R.G. Kochan, M. Sanga, W. Slichenmyer, D.L. Vargo, Absorption, metabolism, and excretion of [14C]-tivozanib, a vascular endothelial growth factor receptor tyrosine kinase inhibitor, in healthy male participants: a phase I, open-label, mass-balance study, Clin. Pharmacol. Drug Dev. 1 (2012) 102–109, https://doi.org/10.1177/2160763X12447303.

[4] M.A. Curtis, L.C. Matassa, R. Demers, K. Fegan, Expanding the linear dynamic range in quantitative high performance liquid chromatography/tandem mass spectrometry by the use of multiple product ions, Rapid Commun. Mass Spectrom. 15 (2001) 963–968, https://doi.org/10.1002/rcm.327.

[5] H. Liu, L. Lam, P.K. Dasgupta, Expanding the linear dynamic range for multiple reaction monitoring in quantitative liquid chromatography-tandem mass spectrometry utilizing natural isotopologue transitions, Talanta 87 (2011) 307–310, https://doi.org/10.1016/j.talanta.2011.09.063.

[6] L. Yuan, D. Zhang, M. Jemal, A.F. Aubry, Systematic evaluation of the root cause of non-linearity in liquid chromatography/tandem mass spectrometry bioanalytical assays and strategy to predict and extend the linear standard curve range, Rapid Commun. Mass Spectrom. 26 (2012) 1465–1474, https://doi.org/10.1002/rcm.6252.

[7] H. Liu, L. Lam, L. Yan, B. Chi, P.K. Dasgupta, Expanding the linear dynamic range for quantitative liquid chromatography-high resolution mass spectrometry utilizing natural isotopologue signals, Anal. Chim. Acta 850 (2014) 65–70, https://doi.org/10.1016/j.aca.2014.07.039.

[8] S.N. Staeheli, M. Poetzsch, T. Kraemer, A.E. Steuer, Development and validation of a dynamic range-extended LC-MS/MS multi-analyte method for 11 different postmortem matrices for redistribution studies applying solvent calibration and additional 13C isotope monitoring, Anal. Bioanal. Chem. 407 (2015) 8681–8712, https://doi.org/10.1007/s00216-015-9023-5.

[9] S. Trobbiani, P. Stockham, T. Scott, Increasing the linear dynamic range in LC–MS: is it valid to use a less abundant isotopologue? Drug Test. Anal. 9 (2017) 1630–1636, https://doi.org/10.1002/dta.2175.

[10] European Medicines Agency (EMA), Guideline on Bioanalytical Method Validation, www.ema.europa.eu/contact, (1922) , Accessed date: 24 April 2019.

[11] US Food and Drug Administration (FDA), FDA, guidance for industry: bioanalytical method validation, (2018) http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ , Accessed date: 4 April 2019.

[12] A. Tan, S. Hussain, A. Musuku, R. Massé, Internal standard response variations during incurred sample analysis by LC–MS/MS: case by case trouble-shooting, J. Chromatogr. B 877 (2009) 3201–3209, https://doi.org/10.1016/j.jchromb.2009.08.019.

[13] C. Polson, P. Sarkar, B. Incledon, V. Raguvaran, R. Grant, Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 785 (2003) 263–275, https://doi.org/10.1016/S1570-0232(02)00914-5.

[14] Committee for Medicinal Products for Human Use (CHMP), European Medicines Agency (EMA), Assessment Report: Fotivda (Tivozanib), www.ema.europa.eu/contact, (2017) , Accessed date: 10 April 2019.