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Validated bioanalytical method for the quantification of RGB-286638, a novel multi-targeted protein kinase inhibitor, in human plasma and urine by liquid chromatography/tandem triple-quadrupole mass spectrometry

Peter de Bruijn[a], Inge M. Ghobadi Moghaddam-Helmantel[a], Maja J.A. de Jonge[a], Thorsten Meyer[b], Mei-Ho Lam[a], Jaap Verweij[a], Erik A.C. Wiemer[a], Walter J. Loos[a,*]

[a] Department of Medical Oncology, Erasmus MC – Daniel den Hoed Cancer Center, University Medical Center, Rotterdam, The Netherlands
[b] GPC Biotech AG, Martinsried/Munich, Germany

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ABSTRACT

A rapid and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method has been developed and validated for the quantitative determination of RBG-286638, a novel multi-targeted protein kinase inhibitor, in 200 μl aliquots of human potassium EDTA plasma with deuterated RGB-286638 as internal standard. The sample extraction and cleaning-up involved a simple liquid–liquid extraction with 100 μl aliquots of acetonitrile and 1 ml aliquots of *n*-butylchloride. Urine was accurately 5- and 10-fold diluted in blank plasma prior to extraction. Chromatographic separations were achieved on a reversed phase C_{18} column eluted at a flow-rate of 0.250 ml/min on a gradient of 0.2 mM ammonium formate and acetonitrile both acidified with 0.1% formic acid. The overall cycle time of the method was 7 min, with RGB-286638 eluting at 1.9 min. The multiple reaction monitoring transitions were set at 546 > 402 (*m*/*z*), and 549 > 402 (*m*/*z*) for RGB-286638 and the internal standard, respectively. The calibration curves were linear over the range of 2.00 to 1000 ng/ml with the lower limit of quantitation validated at 2.00 ng/ml. The within-run and between-run precisions were within 7.90%, while the accuracy ranged from 92.2% to 99.7%. The method was successfully applied to samples derived from a clinical study.

1. Introduction

RGB-286638 is a novel indenopyrazole compound (Fig. 1) with inhibitory activity towards multiple kinases, most notably the cyclin-dependent kinases (CDKs) 1, 2, 4, 5, 7 and 9. Progression through the cell-cycle, in which nuclear DNA is replicated during the S-phase and segregated into two daughter cells during the M-phase, is regulated by activation and inactivation of CDKs [1]. CDKs are serine/threonine kinases that depend on association with regulatory subunits, the cyclin proteins, for proper function. As a complex the catalytic kinase subunit and the activating cyclin subunit can phosphorylate and then trigger downstream processes involved in cell-cycle progression. In tumor cells many genes that directly regulate the cell-cycle were found to be altered resulting in overexpression of cyclins and inactivation or loss of expression of endogenous CDK inhibitors, leading to uncontrolled cell proliferation which is considered the major hallmark of cancer [2]. Several first generation CDK inhibitors, such as flavopiridol and CY-202 are under investigation in late stage clinical trials, however, they only show modest activity. Numerous more potent, second generation, CDK inhibitors are in pre-clinical or early clinical development [3–5].

RGB-286638, as a second generation CDK inhibitor, has been shown in vitro to lead to cell-cycle arrest, through targeting CDKs, and to the induction of apoptosis. RGB-286638 also inhibits other kinases including several receptor (e.g. Abl, Jak, c-Src family members) and non-receptor (e.g. Flt1, Flt3, Flt4) tyrosine kinases as well as serine/threonine kinases such as AMPK, GSK3, PIM1, HIPK1-3 and MAPK that may be important in controlling the proliferation of cancer cells. In several pre-clinical models of both solid and hematological malignancies, tumor regression and increased survival were observed [6–8]. RGB-286638 is currently under clinical evaluation in a phase I study in adult patients with histologically and/or cytologically confirmed relapsed or refractory solid tumors. In view of the evaluation of the pharmacokinetics of RGB-286638 in clinical studies, a bioanalytical method for RGB-286638 in human potassium EDTA plasma and in human urine was developed and validated in agreement with the Guidance for Industry, Bioanalytical Method Validation, as specified by the Food and Drug Administration (http://www.fda.gov/CDER/guidance/4252fnl.htm)

* Corresponding author at: Erasmus MC – Daniel den Hoed Cancer Center, Department of Medical Oncology, Room AS-15, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands. Tel.: +31 10 7041252; fax: +31 10 7041053.
E-mail address: w.loos@erasmusmc.nl (W.J. Loos).

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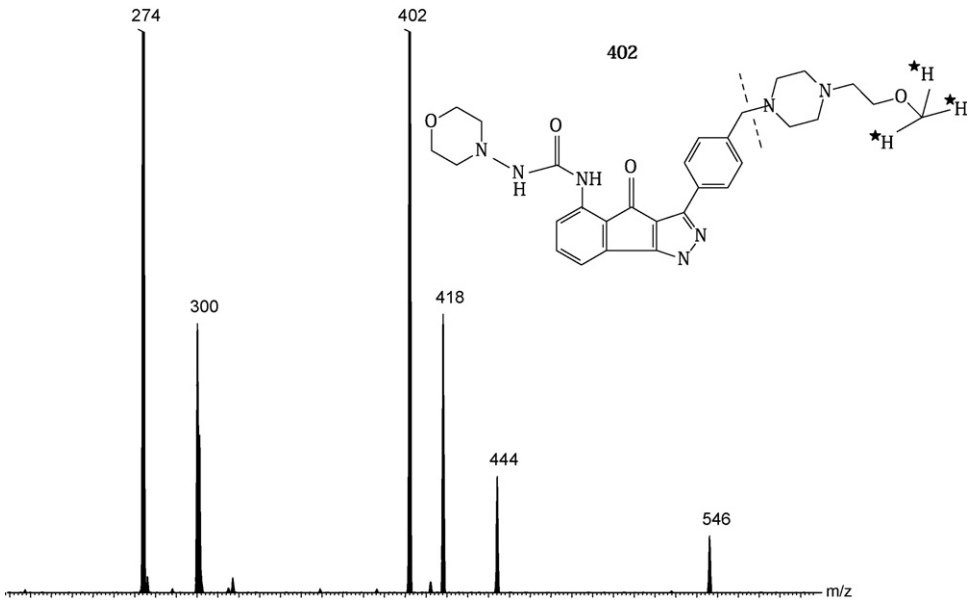

Fig. 1. Mass spectrum and chemical structure of RGB-286638. The asterisks represent the deuterium atoms in the stable labeled internal standard RGB-286638-d3.

and by the Workshop/Conference Report—Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays [9].

2. Experimental

2.1. Chemicals

RGB-286638 dihydrochloride was kindly supplied as ready to use solution (batch FIN 0477 containing 17.7 mg/ml free base and batch 05/768-004 containing 16.9 mg/ml free base) by GPC Biotech AG (Munich, Germany). The deuterated internal standard RGB-286638-d3 dihydrochloride was supplied as powder (lot GCM 1703-1-01, purity 97%) prepared by Bayer HealthCare (Wuppertal, Germany) for GPC Biotech AG. All chemicals were of analytical grade or higher. Acetonitrile, methanol and water were purchased from Biosolve BV (Valkenswaard, The Netherlands). Ammonium formate, dimethylsulphoxide and glycine were from Sigma–Aldrich (Zwijndrecht, The Netherlands) and *n*-butylchloride from Rathburn (Walkerburn, Scotland). Formic acid was obtained from J.T. Baker (Deventer, The Netherlands) and 2-propanol from Merck (Darmstadt, Germany). Blank potassium EDTA plasma was purchased from Biological Specialty Corporation (BSC, Colmar, PA, USA) and drug-free human urine was collected from healthy volunteers.

2.2. Preparation of stock solutions, calibration standards and quality control samples

Two batches of stock solutions of RGB-286638 were provided as ready to use solutions, one of which was used for the preparation of the calibration standards in plasma and the other one was used for the preparation of the pools of quality control (QC) samples in plasma as well as in urine.

The internal standard stock solution was prepared at 1 mg/ml free base in water/formic acid (100:0.1, v/v), which subsequently was aliquoted and stored at $T < -70\,^{\circ}C$. Aliquots of 50 μl were 200-fold diluted in water/formic acid (100:0.1, v/v), resulting in an internal standard solution containing 5 μg/ml RGB-286638-d3 as a free base, which was stored at $T < 8\,^{\circ}C$ for a maximum of 1 month.

Calibration standards were prepared freshly on the day of analysis, in duplicate, by addition of 10 μl aliquots of appropriate dilutions of RGB-286638 stock solution in acetonitrile/DMSO (1:1, v/v) to 190 μl aliquots of human potassium EDTA plasma at the following concentrations: 2.00, 4.00, 10.0, 50.0, 250, 500, 900 and 1000 ng/ml as free bases.

Five pools of QC samples were prepared in human potassium EDTA plasma at concentrations of 1.92 ng/ml (lower limit of quantitation, LLQ), 5.76 ng/ml (QC Low), 384 ng/ml (QC Middle), 768 ng/ml (QC High) and 7680 ng/ml (QC diluted). QC diluted was processed after a 10-fold dilution in blank human potassium EDTA plasma. Pools of QC samples were aliquoted and stored at $T < -70$ and $< -20\,^{\circ}C$ upon processing.

Two pools of QC samples were prepared in human urine at concentrations of 38.4 ng/ml (QC UR1) and 7680 ng/ml (QC UR2). In order to avoid potential precipitation of RGB-286638 in aqueous solution or binding of the component to the container (internal communication from GPC Biotech), both urine pools were acidified with a 1 M glycine–HCl solution pH 2.0. QC UR1 was acidified in a ratio of 100:1 (v/v) and QC UR2 in a ratio of 100:5 (v/v). Pools of QC samples were aliquoted and stored at $T < -70$ and $< -20\,^{\circ}C$ upon processing.

2.3. Sample preparation

Aliquots of 10 μl of internal standard solution were added to 200 μl of plasma samples in 2-ml microcentrifuge tubes. Subsequently 100-μl aliquots of acetonitrile were added, followed by 1-ml aliquots of *n*-butylchloride. Hereafter, the samples were vigorously mixed for 5 min and then centrifuged at 18,000 × *g* at ambient temperature for 10 min. Aliquots of 950 μl of the organic phase were transferred into 4.5 ml glass tubes and evaporated under nitrogen at $T = 70\,^{\circ}C$. The residues were resuspended in 100 μl aliquots of acetonitrile/water/formic acid (10:90:0.1, v/v/v) by ultrasonification. After a brief centrifugation of 30 s at 3000 × *g*, the supernatants were transferred into 350 μl 96-well plates, which were placed into a chilled ($T = 10\,^{\circ}C$) autosampler, from which aliquots of 10 μl were injected onto the HPLC column.

Urine samples were processed after dilution in blank potassium EDTA plasma. QC UR1 was diluted 5-fold and QC UR2 was diluted 2-fold prior to processing as described above.

2.4. Equipment

The LC–MS/MS system was purchased from Waters Chromatography B.V. (Etten-Leur, The Netherlands) and was composed of a Waters 2795 Separation Module coupled to a Quatro micro API Mass Spectrometer. The MassLynx V4.0 SP4, 532 Beta 01 software package was used for the acquisition and processing of data. Quantification was performed using QuanLynx as implemented in the MassLynx software.

2.4.1. Chromatographic conditions

Analytes were separated on an Alltima HP C_{18} column 5 μm, 50 mm × 2.1 mm (Grace, Breda, The Netherlands) thermostated at $T = 40\,^{\circ}C$. A gradient at a flow-rate of 0.250 ml/min was achieved with mobile phase A, composed of 0.2 mM aqueous ammonium formate acidified with 0.1% formic acid and mobile phase B, composed of acetonitrile acidified with 0.1% formic acid. Following a partial loop injection of 10 μl, the step gradient was held for 2 min at 20% mobile phase B, 20–90% mobile phase B in 3 min, 90–20% B in 1 min, which was held for 1 min to re-equilibrate. The overall run time of the assay was 7 min. A pre-column volume of 300 μl was applied and a parallel injection was enabled. The needle wash solvent was composed of acetonitrile/methanol/water/2-propanol/formic acid (25:25:25:25:0.1, v/v/v/v/v).

2.4.2. Mass spectrometry

Tandem mass spectrometry was performed in the positive ion electrospray ionization mode. Mass transitions of m/z were optimized for RGB-286638 and RGB-286638-d3 (IS) by infusion of the respective analytes in acetonitrile/water/0.1% formic acid (40:60:0.1, v/v/v) via a tee union. Optimal MS settings were manually adjusted. The desolvation gas was set at 800 ml/h and the cone gas at 50 ml/h (nitrogen). The ionspray voltage was kept at 3.00 kV and the cone voltage at 40 V for RGB-286638 and 42 V for the IS, with a source temperature of $T = 120\,^{\circ}C$ and desolvation temperature of $T = 350\,^{\circ}C$. The dwell times were set at 200 ms and the inter-channel delay at 100 ms. Multiple reaction monitoring (MRM) mode was applied for the quantitation with the following parameters: m/z 546 > 402, collision energy at 27 V for RGB-28663 and m/z 549 > 402, collision energy at 26 V for the IS (Fig. 1). The collision cell pirani pressure is $\sim 6e^{-3}$ mbar (argon). The column effluent was passed through the mass spectrometer and monitored between 1 and 5 min after the start of MS method, 0–1 min and 5–7 min sent to waste.

2.4.3. Quantitation

Calibration curves were generated using peak area ratios of RGB-286638 to internal standard RGB-286638-d3 versus the known RGB-286638 concentrations with a linear regression equation of 1/concentration.

2.5. Method validation

The quantitative LC–MS/MS method was validated in accordance with the Guidance for Industry, Bioanalytical Method Validation, as specified by the Food and Drug Administration (http://www.fda.gov/CDER/guidance/4252fnl.htm) and by the Workshop/Conference Report—Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays [9].

Potential presence of endogenous contaminating compounds that may interfere with the analytical assay was determined by analyzing blank human potassium EDTA plasma samples of ten different lots. The following substances were investigated for interference with the analytical method: aprepitant, dexamethasone, domperidom, granisetron, lactulose, lorazepam, oxazepam, paracetamol and metoclopramide. All drugs have been dissolved/diluted in water to a concentration of 1 mg/ml followed by a 500-fold dilution in blank human potassium EDTA plasma (final concentrations 2 μg/ml). Aliquots of QC diluted has subsequently been diluted in the plasma containing different drugs to yield final RGB-286638 concentrations of 7.68 and 768 ng/ml (1000- and 10-fold dilutions), which has been processed and compared to equal dilutions of QC diluted in blank human potassium EDTA plasma.

For the determination of the LLQ, blank human potassium EDTA plasma of 10 different donors was spiked at a concentration of 2.00 ng/ml, using the same RGB-286683 stock solution, which was used for the preparation of the calibration standards. The LLQ met the acceptance criteria if at least 8 out of 10 processed LLQ sample concentrations fell within 80–120% of the respective nominal concentrations.

Accuracy (ACC), within-run precision (WRP) and the between-run precision (BRP) were determined by analyzing five replicates of pools of LLQ and QC samples independently over a 3-day period, with the calibration curve standards processed in duplicate. The ACC, WRP and BRP at the level of the LLQ and QC samples were calculated by one-way analysis of variance, using the run day as the variable. The within-group mean square (wgMS), the between-group mean square (bgMS) and the grand mean (GM) of the measured concentrations across run days were calculated. An estimate of the BRP, WRP and ACC was subsequently calculated as presented in Eqs. (1)–(3), respectively.

$$\mathrm{BRP} = \left\{ \frac{((\mathrm{bgMS} - \mathrm{wgMS})/n)^{0.5}}{\mathrm{GM}} \right\} \times 100\% \quad (1)$$

$$\mathrm{WRP} = \left\{ \frac{(\mathrm{wgMS})^{0.5}}{\mathrm{GM}} \right\} \times 100\% \quad (2)$$

$$\mathrm{ACC} = \left\{ \frac{\mathrm{GM}}{\text{nominal concentration}} \right\} \times 100\% \quad (3)$$

In cases where the wgMS is greater than the bgMS, the resulting variance estimation is negative, implying that no significant additional variation was observed as a result of performing the assay on different days.

The stability of RGB-286638 in human potassium EDTA plasma was tested with QC low and QC high at ambient temperature and at $T = 37\,^{\circ}C$ for a period of 6 h as well as following three freeze–thaw cycles, in which the samples were thawed for at least 30 min followed by refreezing for at least 18 h. The stability of RGB-286638 in human urine was tested with QC UR1 and QC UR2 at $T < 8\,^{\circ}C$, ambient temperature and at $T = 37\,^{\circ}C$ for a period of 24 h and following three freeze–thaw cycles, as described for plasma. Long-term stability at $T < -20\,^{\circ}C$ and $<-70\,^{\circ}C$ in human potassium EDTA plasma and human urine has been investigated using the same QC samples as described above. The storage stability of processed samples in the autosampler was tested in triplicate at the concentration of QC low and QC high in plasma. QC samples were processed in triplicate and repeatedly injected on different time points.

The evaluation of the matrix effect for RGB-286638 was tested by comparing the MS/MS response of RGB-286638 at two concentration levels spiked in quintuplicate in acetonitrile/water/formic acid (10:90:0.1, v/v/v) (A, Eq. (4)), to the MS/MS response of RGB-286638 spiked in quintuplicate into extracts of blank human potassium EDTA plasma (B, Eq. (4)). The value obtained according to Eq. (4), is considered as an absolute matrix effect (ME) [10].

$$\mathrm{ME}\,(\%) = \frac{B}{A} \times 100 \quad (4)$$

Extraction recovery (RE) was determined by comparing the MS/MS response of RGB-286638 at the same concentration levels

spiked in quintuplicate into blank human potassium EDTA plasma before extraction (C, Eq. (5)), to the MS/MS response of RGB-286638 spiked in quintuplicate into extracts of blank human potassium EDTA plasma (A, Eq. (5)), corrected for the evaporated volume of organic phase.

$$\text{RE } (\%) = \frac{C}{B} \times \frac{1100}{950} \times 100 \quad (5)$$

2.6. Application of method to clinical samples

To demonstrate the applicability of the validated bioanalytical method, blood and urine samples were collected from a patient enrolled in a phase-I clinical study at the first dose level. RGB-286638 was administered intravenously over 60 min at a dose of 10 mg/day on five consecutive days to a male patient age 72 years diagnosed with a carcinoma of the parotid gland. Blood samples were obtained in the presence of potassium EDTA as anticoagulant after the administration on days 1 and 5 and were processed within 15 min of collection to isolate the plasma, which was stored at $T < -70\,^{\circ}C$ before analysis as described. Urine samples were collected on day 1 from 0 to 8 h and from 8 to 24 h, immediately acidified with 1 M glycine. HCl solution pH 2.0 and stored at $T = 4\,^{\circ}C$. At the end of each collection period, a 3 ml aliquot was stored at $T < -70\,^{\circ}C$ upon processing. The patient gave written informed consent and the local institutional review boards approved the clinical protocol, which was written in accordance with the declaration of Helsinki (see: http://www.wma.net/e/policy/b3.htm).

3. Results and discussion

3.1. LC–MS/MS conditions and method development

RGB-286638 is a basic compound with nitrogen atoms in its structure and therefore, positive ion mode by ESI–MS seems suitable for the determination of the parent drug. The production of positive product ions occurs in solutions acidified by the addition of formic acid. Addition of ammonium formate to the mobile phase increased the production of protonated-molecular ions $[M+H]^+$. Therefore, the mobile phases were composed of 0.2 mM aqueous ammonium formate acidified with 0.1% formic acid and acetonitrile acidified with 0.1% formic acid.

The RGB-286638 product ion spectra (Fig. 1) yield abundant product ions suitable for use in multiple reaction monitoring. The product ion at m/z 402 using collision energy of 27 eV was selected as the MRM ion for quantitation of RGB-286638 and its stable isotope labeled internal standard RGB-286638-d3 (collision energy of 26 eV).

Sample preparation is one of the most important steps in the development of an analytical method, as it directly affects the selectivity, sensitivity, accuracy and precision of the method. For non-volatile compounds, such as RGB-286638, solid–phase and liquid–liquid extractions are initially considered. Solid–phase extraction has, if not automated, a disadvantage over liquid–liquid extraction because of difficulties in reproducibility, standardizing the used vacuum and the variability in drying steps. Moreover, solid–phase extraction is, compared to liquid–liquid extraction, relatively laborious [11]. The applied liquid–liquid extraction with acetonitrile and *n*-butylchloride enables processing of multiple samples in a micro-setting and results in clean extracts.

By applying a steep gradient, RGB-286638 was separated from early eluting hydrophilic, potentially response-suppressing, matrix components, while maintaining a relative short analysis time of 7 min, with RGB-286638 eluting at 1.9 min (Fig. 2).

A minor peak for RGB-286638, with a response <10% of the response at the LLQ, was observed in the blank processed samples including the internal standard RGB-286638-d3 injected prior to each calibration curve. Most likely, this signal resulted from crosstalk of the MS/MS, as the signal increases by decreasing the inter-channel delay (data not shown).

The needle wash solvent was composed of acetonitrile/methanol/water/2-propanol/formic acid to prevent carry-over of RGB-288638 (data not shown).

3.2. Assay performance

The method results were linear ($r^2 \geq 0.9980$) in the concentration range of 2.00–1000 ng/ml RGB-286638 in human potassium EDTA plasma and none of the blank plasma samples showed potential interference for RGB-286638 or the labeled internal standard.

The LLQ was validated at 2.00 ng/ml: the measured concentrations of RGB-286638 for all 10 independently spiked plasma samples fell within the acceptable range of accuracy of 80–100%,

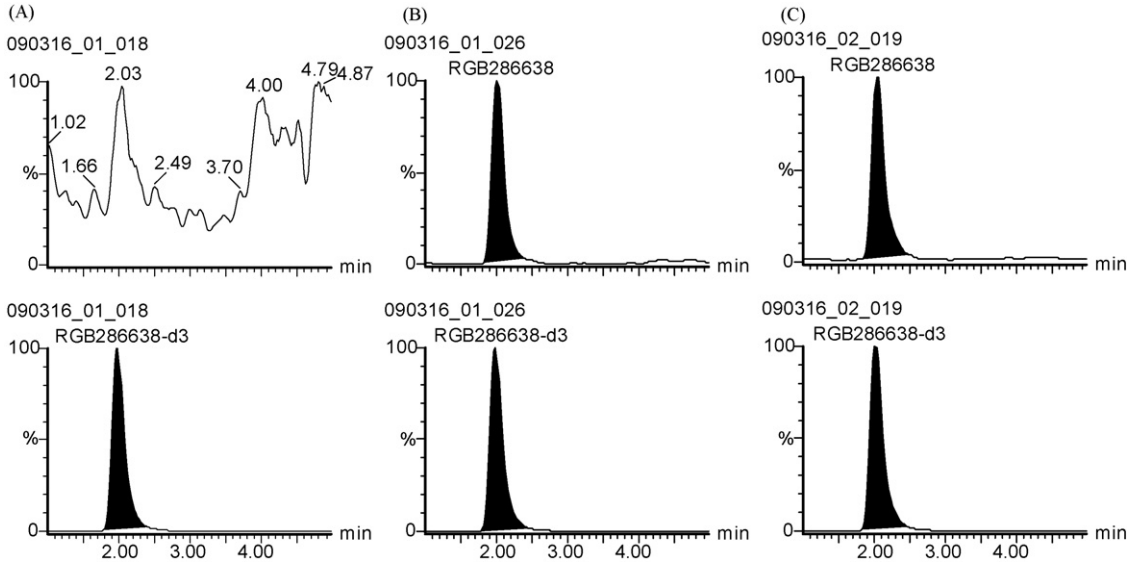

Fig. 2. Representative chromatograms of (A) blank processed plasma sample collected pre-dose prior to the first administration of 10 mg RGB-286638 dihydrochloride, (B) plasma sample taken 4 h after the end of infusion on day 1 containing 2.29 ng/ml RGB-286638 free base and (C) urine sample collected 8–24 h after the first administration, 5-fold diluted in plasma prior to processing, containing 28.5 ng/ml RGB-286638 free base.

Table 1
Calculations of the between-run and within-run precisions and the average accuracy of the LLQ and QC samples.

Matrix	Spiked (ng/ml)	GM (ng/ml)	bgMS	wgMS	WRP (%)	BRP (%)	ACC (%)	*n*
Plasma	1.92	1.79	6.00E−03	2.00E−02	7.90	#[a]	93.2	5
Plasma	5.76	5.74	2.40E−02	4.60E−02	3.74	#[a]	99.7	5
Plasma	384	380	1.69E+02	4.69E+01	1.80	1.30	99.0	5
Plasma	768	7.66	1.18E+02	1.71E+02	1.71	#[a]	99.7	5
Plasma	7680	7516	9.01E+04	1.71E+04	1.74	1.61	97.9	5
Urine	38.4	35.4	1.03E+00	2.64E+00	4.59	#[a]	92.2	5
Urine	7680	7596	5.61E+03	8.95E+04	3.94	#[a]	98.9	5

Abbreviations: GM, grand mean; bgMS, between-group mean square; wgMS, within-group mean square; WRP, within-run precision; BRP, between-run precision; ACC, average accuracy; *n*, number of replicate observations on each analysis day.

[a] No additional variation observed by performing the assay in different runs.

Table 2
Matrix effect and extraction recovery ($n = 5$).

Concentration (ng/ml)	RGB-286638		RGB-286638-d3	
	ME (%)	RE (%)	ME (%)	RE (%)
4.00	146	141	128	158
500	151	96.8	126	100

Abbreviations: ME, matrix effect; RE, extraction recovery.

with an average measured concentration of 2.13 ± 0.128 ng/ml.

The within-run and between-run precisions and the accuracies at five tested concentrations in human potassium EDTA plasma, including at the level of the LLQ, and two tested concentrations in human urine are summarized in Table 1 and all fall within the accepted ranges as specified by the Food and Drug Administration (http://www.fda.gov/CDER/guidance/4252fnl.htm) and by the Workshop/Conference Report—Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays [9].

The extraction recovery (RE) and matrix effect (ME) were determined in five replicates of each of the QC low and QC high samples in EDTA plasma. The mean measured extraction efficiencies for RGB-286638 were 141% and 96.8% at the concentration of QC low and high, respectively. The mean extraction efficiencies for the internal standard (RGB-286638-d3) were 158% and 100% at the concentration of QC low and high, respectively. A significant matrix effect was observed. It has been discussed that the addition of small amounts of ammonium formate to the mobile phase can enhance ionization of analytes in matrix samples [12]. Enhancement of ionization of on average 149% for RGB-286638 and 127% for the internal standard (RGB-286638-d3) was observed. Specific values for recovery and matrix effect are summarized in Table 2.

RGB-286638 was stable in potassium EDTA plasma at ambient temperature and 37 °C for at least 6 h, during three consecutive freeze–thaw cycles and at least 5 months when stored at $T < -20$ °C and <−70 °C. In acidified human urine, RGB-286638 was stable at $T < 8$ °C, at ambient temperature and at $T = 37$ °C for at least 24 h, during three consecutive freeze–thaw cycles and at least 5 months when stored at $T < -20$ °C and <−70 °C. As processed samples, RGB-286638 was stable for at least 19 h in the chilled ($T = 10$ °C) autosampler. Results of the stability experiments are summarized in Table 3.

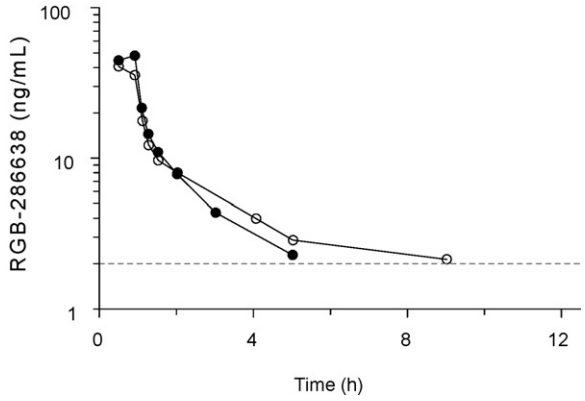

Fig. 3. Plasma concentration–time profile of RGB-286638 after administration of 10 mg by a 1 h infusion to a male cancer patient. The closed symbols represent concentration data measured at day 1 and the open symbols the concentration data measured at day 5. The dotted line indicates the lower limit of quantitation, validated at 2.00 ng/ml.

3.3. Clinical application

As shown in Fig. 3, plasma concentration versus time curves could be readily measured up to 8 h after the end of infusion. The data indicate that the lower limit of quantitation of 2.00 ng/ml is sufficient for monitoring drug-plasma levels in samples obtained from patients treated with RGB-286638, even at a low dose level of 10 mg. A representative chromatogram is shown in Fig. 2B.

In urine sample, RGB286638 was quantifiable, with a total excretion of 1.9% of the dose during the first 24 h after administration. A representative chromatogram is shown in Fig. 2C.

Table 3
Stability of RGB-286638.

Condition	Plasma (% to control)		Acidified urine (% to control)	
	QC Low	QC High	QC UR-1	QC UR-2
$T < 8$ °C (24 h)	ND[a]	ND[a]	93.5	102
Ambient temp (6 h/24 h)[b]	103	96.3	97.5	97.7
$T = 37$ °C (6 h/24 h)[b]	101	103	98.6	102
3 freeze/thaw cycles	100	101	108	102
Processed sample ($T = 10$ °C)	112	105	ND[a]	ND[a]
$T < -20$ °C (5 months)	94.9	99.7	88.9	95.5
$T < -70$ °C (5 months)	90.6	97.6	87.6	96.2

[a] ND = not done.

[b] 6 h for plasma and 24 h for urine.

4. Conclusion

A selective, sensitive, accurate and precise method has been validated for RGB-286638 in human potassium EDTA plasma and human urine, which meets the current requirements of bioanalytical method validation (http://www.fda.gov/CDER/guidance/4252fnl.htm) and by the Workshop/Conference Report—Quantitative Bioanalytical Methods Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays [9]. The method is currently successfully used for the bioanalysis of RGB-286638 concentrations in plasma and urine of cancer patients participating in a clinical study in which RGB-286638 is administered daily intravenously over 1 h during five consecutive days.

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