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A rapid and sensitive method for determination of sorafenib in human plasma using a liquid chromatography/tandem mass spectrometry assay

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Abstract

A rapid, sensitive and specific method was developed and validated using LC/MS/MS for determination of sorafenib in human plasma. Sample preparation involved a single protein precipitation step by the addition of 0.1 mL of plasma with 0.5 mL acetonitrile. Analysis of the compounds of interest including the internal standard ([$^2H_3$ ^{15}N] sorafenib) was achieved on a Waters X-Terra™ C_{18} (150 mm × 2.1 mm i.d., 3.5 μm) analytical column using a mobile phase consisting of acetonitrile/10 mM ammonium acetate (65:35, v/v) containing 0.1% formic acid and isocratic flow at 0.2 mL/min for 6 min. The analytes were monitored by tandem mass spectrometry with electrospray positive ionization. Linear calibration curves were generated over the range of 7.3–7260 ng/mL for the human plasma samples with values for the coefficient of determination of >0.96. The values for both within day and between day precision and accuracy were well within the generally accepted criteria for analytical methods (<15%).

Keywords: Sorafenib; LC/MS/MS; Pharmacokinetics

1. Introduction

Sorafenib is a multikinase inhibitor that decreases tumor cell proliferation in vitro (Fig. 1). Sorafenib inhibited tumor growth of the murine renal cell carcinoma, RENCA, and several other human tumor xenografts in athymic mice. A reduction in tumor angiogenesis was seen in some tumor xenograft models. Sorafenib was shown to interact with multiple intracellular (CRAF, BRAF and mutant BRAF) and cell surface kinases (KIT, FLT-3, VEGFR-2, VEGFR-3, and PDGFR-β). Several of these kinases are thought to be involved in angiogenesis [1–5]. In preclinical studies, sorafenib has demonstrated antitumor activity against a variety of human cancer cell lines, including lung, ovarian, breast, and colon [5–7]. In human xenograft models, sorafenib in combination with standard cytotoxic agents resulted in both delayed tumor growth and tumor regression, leading to enhanced survival [5,7]. Sorafenib is currently in various stages of clinical development in patients with different tumor types and was recently approved by the FDA for the treatment of advanced renal cell carcinoma [8–12].

Sorafenib is absorbed relatively rapidly following a single dose with secondary peaks presumably due to enterohepatic circulation of the glucuronide metabolite [13–17]. Sorafenib oral absorption appears to be saturated above 400 mg, the recommended dose, on a twice daily schedule [13,14,17]. Sorafenib has a long half-life (20–39 h) in cancer patients [13,14,16,17]. Sorafenib is metabolized by two parallel metabolic pathways in man: phase I oxidation mediated by CYP3A4 primarily led to the corresponding N-oxide; and glucuronidation mediated by UGT1A9 [15].

To comprehensively characterize the clinical pharmacokinetic profile of this drug, and to explore the relationship between

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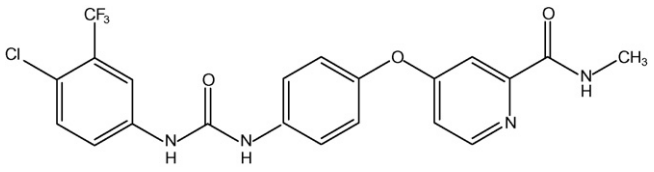

Fig. 1. Chemical structure of sorafenib.

pharmacokinetic parameters and pharmacodynamic effects of sorafenib, a specific, reproducible and accurate method for the quantitation of sorafenib was necessary. The details of one analytical method based on reversed-phase HPLC have been reported for the quantitative determination of sorafenib in mouse plasma [18]. However, the lack of sensitivity and the complex sample preparation procedure and long chromatographic run time prohibited the use of this assay for pharmacokinetic studies. All manuscripts involving the clinical evaluation of sorafenib in cancer patients used validated LC/MS/MS methods with lower limits of quantitation in the range of 1–100 ng/mL for pharmacokinetic evaluations [13,14,16,17]. However, these manuscripts did not describe the analytical methodology nor the calibration range utilized. Recently, details of a validated LC/MS/MS assay quantitating sorafenib and sorafenib N-oxide in plasma over the range of 10–12,000 and 10–2500 ng/mL, respectively were published [15]. While this method is sensitive, the sample preparation includes liquid–liquid extraction and the details of the run time are not described. Here, we describe a rapid, sensitive analytical method for the determination of sorafenib concentrations in human plasma based on LC/MS/MS with electrospray positive ionization after a single protein precipitation with acetonitrile. Based on previous published pharmacokinetic data, the assay range of 7.3–7260 ng/mL should be sufficient to characterize the clinical pharmacology of sorafenib in clinical trials being conducted at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins [13,17,19].

2. Experimental

2.1. Chemical and reagents

Sorafenib (Lot number R 90-3 B, 72.6% pure by HPLC, free drug) and the internal standard ([$^2H_3$, ^{15}N] sorafenib tosylate), were provided by Bayer HealthCare (Wuppertal, Germany). Formic acid (98%, v/v, in water), methanol (HPLC grade) and acetonitrile (HPLC grade) were obtained from EM Science (Gibbstown, NJ, USA). Deionized water was obtained from a Milli-Q-UF system (Millipore, Milford, MA, USA) and used throughout in all aqueous solutions. Drug-free (blank) human plasma from healthy donors originated from Pittsburgh Blood Plasma, Inc. (Pittsburgh, PA, USA).

2.2. Stock solutions, calibration standards, and quality control samples

Stock solutions of sorafenib at a concentration of 1 mg/mL were prepared in duplicate by dissolving 10 mg in 10 mL of methanol and stored in glass vials at −20 °C. The stock solutions were diluted in blank human plasma on each day of analysis to prepare seven calibration standards in duplicate containing sorafenib for human plasma samples at the following concentrations: 7.3, 36, 73, 363, 726, 3630 and 7260 ng/mL. Quality control (QC) samples were prepared independently in blank plasma at four different concentrations for sorafenib including: 7.3 ng/mL, the lower limit of quantitation (LLOQ); 22 ng/mL, the low QC (LQC); 581 ng/mL, the medium QC (MQC); and 5808 ng/mL, the high QC (HQC). A stock solution of sorafenib isotope at a concentration of 100 μg/mL was prepared by dissolving 1 mg in 10 mL of methanol and stored in glass vials at −20 °C.

2.3. Sample preparation

Prior to extraction, frozen samples were thawed in a water bath at ambient temperature. During validation, calibration standards and quality controls were prepared fresh daily except for stability testing. An 0.1 mL aliquot of plasma was added to a borosilicate glass test tube (13 mm × 100 mm) containing 0.5 mL of acetonitrile solution and [$^2H_3$ ^{15}N] sorafenib (50 ng/mL), which was used as internal standard. The tube was mixed vigorously for 10 s on a vortex-mixer, followed by centrifugation at 1200 × *g* for 5 min at ambient temperature. A volume of 100 μL of the top organic layer was transferred to a 250-μL polypropylene autosampler vial, sealed with a Teflon crimp cap, and a volume of 10 μL was injected onto the HPLC instrument for quantitative analysis using a temperature-controlled autosampling device operating at approximately 10 °C.

2.4. Chromatographic and mass spectroscopic conditions

Chromatographic analysis was performed using a Waters Model 2690 separations system (Milford, MA, USA). Separation of the analytes from potentially interfering material was achieved at ambient temperature using Waters X-Terra MS column (150 mm × 2.1 mm i.d.) packed with a 3.5-μM ODS stationary phase, protected by a guard column packed with 3.5 μm RP18 material (Milford, MA, USA) using a temperature-controlled column heating device operating at 50 °C. The mobile phase used for the chromatographic separation was composed of acetonitrile–10 mM ammonium acetate (pH 3.5) (65:35, v/v) containing 0.1% formic acid, and was delivered isocratically at a flow rate of 0.2 mL/min. The column effluent was monitored using a Micromass Quattro LC triple–quadrupole mass spectrometric detector (Beverly, MA, USA). The instrument was equipped with an electrospray interface, and controlled by the Masslynx Version 3.4 software (Micromass), running under Microsoft Windows NT on a Compaq AP200 Pentium III computer. The samples were analyzed using an electrospray probe in the positive ionization mode operating at a cone voltage of 40 V for sorafenib and the internal standard. Samples were introduced into the interface through a heated nebulized probe (350 °C). The spectrometer was programmed to allow the $[MH]^+$ ion of sorafenib at *m/z* 465.1 and that of the internal

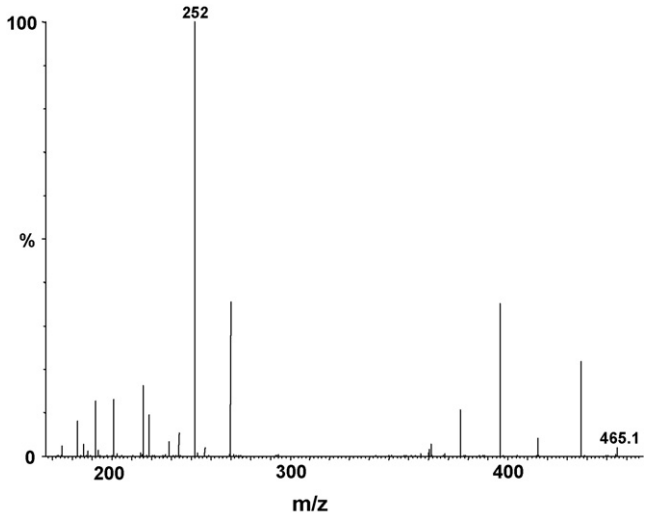

Fig. 2. Daughter mass spectrum of sorafenib with monitoring at *m/z* 465.1 → 252.0.

standard at *m/z* 469.0 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 30 eV for sorafenib and the internal standard. The daughter ions for sorafenib (*m/z* 252.0) (Fig. 2) and the internal standard (*m/z* 256.0) (data not shown) were monitored through the third quadrupole (Q3). Argon was used as collision gas at a pressure of 0.0027 mBar, and the dwell time per channel was 0.5 s for data collection.

2.5. Calibration curves

Calibration curves for sorafenib were computed using the ratio of the peak area of analyte and internal standard by using a weighted (1/[nominal concentration]^2) least-squares linear-regression analysis. The parameters of each calibration curve were used to compute back-calculated concentrations and to obtain values for the QC samples and unknown samples by interpolation.

2.6. Method validation

2.6.1. Pre-study validation

Method validation runs for human plasma calibrator standards and QCs were performed on four consecutive days and included a calibration curve processed in duplicate and QC samples, at four different concentrations, in quintuplicate. The accuracy and precision of the assay was assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$\mathrm{DEV}_{(\mathrm{sorafenib})} = 100 \times \left\{ \frac{[\mathrm{sorafenib}]_{\mathrm{mean}} - [\mathrm{sorafenib}]_{\mathrm{nominal}}}{[\mathrm{sorafenib}]_{\mathrm{nominal}}} \right\}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square ($\mathrm{MS_{bet}}$), the within-groups mean square ($\mathrm{MS_{wit}}$), and the grand mean (GM) of the observed concentrations across runs were calculated using the JMP$^{\mathrm{TM}}$ statistical discovery software Version 4 (SAS Institute, Cary, NC, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$\mathrm{BRP} = 100 \times \left(\frac{\sqrt{(\mathrm{MSbet} - \mathrm{MSwit})/n}}{\mathrm{GM}} \right)$$

where n represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$\mathrm{WRP} = 100 \times \left(\frac{\sqrt{\mathrm{MSwit}}}{\mathrm{GM}} \right)$$

The specificity of the method was tested by visual inspection of chromatograms of extracted human plasma samples from six different donors for the presence of endogenous or exogenous interfering peaks. The peak area needed to be less than 20% than the peak area for sorafenib 7.3 ng/mL in plasma. If not, plasma from six additional donors would be tested. The extraction efficiency of the assay was measured by comparison of the peak area ratio of sorafenib extracted from plasma and an aqueous solution in triplicate at concentrations of the low, middle, and high QCs. The stability of sorafenib in plasma was tested at concentrations of the low and high QCs in triplicate after three freeze–thaw cycles. The long-term stability test in plasma was assessed at the low and high QCs in triplicate at −70 °C at 87 and 179 days. The short-term stability of sorafenib in plasma was assessed in triplicate at room temperature (on the benchtop) for 6 h. Stability of drug in neutral extracts was assessed in single injection on the autosampler at approximately 10 °C.

2.6.2. Cross-validation

Blinded samples that were spiked with varying amount of sorafenib were received from Bayer CRO (West Haven, CT, USA). Samples were analyzed according to the analytical methods described within this manuscript and by Bayer. The Bayer methodology involved isolation of the analytes from plasma by liquid–liquid extraction utilizing methyl-*t*-butyl ether with analysis by reversed-phase HPLC and MS/MS detection [15].

2.7. Pharmacokinetic analysis

The patient studied participated in a phase I study and received a dose of sorafenib of 400 mg administrated orally twice daily for 14 days every 28 days. The drug was formulated as 200 mg immediate-release film coated tablets stored at room temperature. The protocol was approved by the Institutional Review Board of the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (Baltimore, MD, USA), and the patient provided written informed consent.

Blood samples were collected in heparin-containing tubes before drug administration and at 0.25, 0.5, 1, 2, 4, 6, and 8 h after administration of the sorafenib. Additional samples were collected prior to dose administration on days 2, 3, 8, and 15. Blood samples were processed immediately by centrifugation for 10 min at 1000 × g at 4 °C. Plasma supernatant was stored at −70 °C until subsequent analysis. Samples were thawed and processed as described in Section 2.3.

Sorafenib pharmacokinetic parameters were determined by standard noncompartmental methods using steady-state calculations in the program WinNonlin Version 5.0 (Pharsight Corporation, Mountain View, CA). The C_{max} and the time of C_{max} after oral administration were obtained by visual inspection of the plasma concentration–time curve. The area under the plasma concentration–time curve (AUC) value was calculated to the last quantifiable sample (AUC_{last}) by use of the linear up/log down trapezoidal rule.

3. Results and discussion

3.1. Detection and chromatography

The mass spectrum of sorafenib showed a protonated molecular ion ([MH^+]) at *m/z* 465.1. The major fragment observed was at *m/z* 252.0, which was selected for subsequent monitoring in the third quadrupole (Fig. 2). The mass spectrum of the internal standard ([$^2H_3$ ^{15}N] sorafenib) showed a [MH^+] at *m/z* 469.0, and the high collision energy gave one major product ion at *m/z* 256.0 (data not shown).

No peaks were observed in the chromatograms of blank plasma from six donors when monitored for sorafenib (data not shown). Representative chromatograms of blank human plasma and plasma spiked with internal standard and sorafenib are shown in Figs. 3 and 4. The mean (±standard deviation) retention times for sorafenib and the internal standard under the optimal conditions were 4.2 ± 0.2 min with an overall chromatographic run time of 6 min. The selectivity for the analysis is shown by symmetrical resolution of the peaks, with no significant chromatographic interference around the retention times of the analytes and internal standard in drug-free specimens.

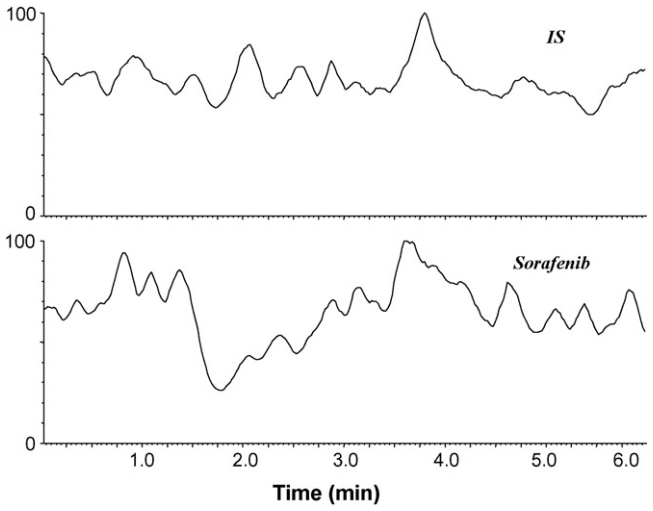

Fig. 3. Chromatograms of blank human plasma.

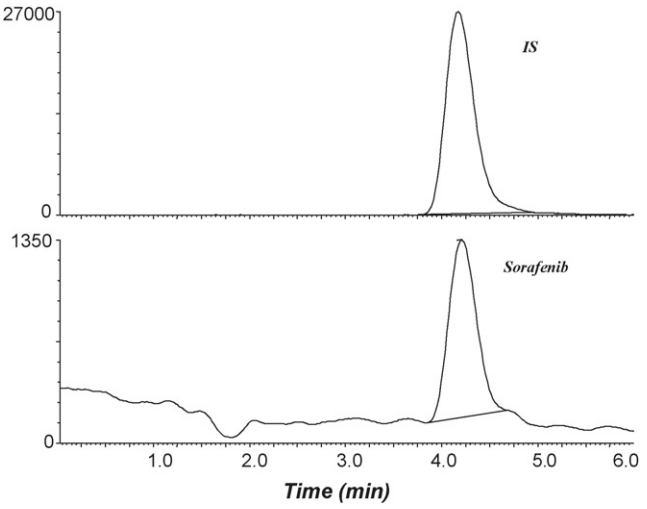

Fig. 4. Chromatograms of plasma spiked with sorafenib (7.3 ng/mL) and the internal standard (IS) (50 ng/mL). The retention times for sorafenib and internal standard were approximately 4.2 ± 0.2 min.

3.2. Linearity of detector responses

The calculated peak area ratios of sorafenib to the internal standard versus the nominal concentration of the analyte displayed a linear relationship in the tested range of 7.3–7260 ng/mL. A weighting factor, which is inversely proportional to the variance at the given concentration level (x^2) was used. This weighting factor was chosen compared to uniform weighting after evaluation of goodness-of-fit by assessment of the R^2 value, intercept closest to a zero value, % recovery of calibrators and QCs, and assessment of residuals. After applying the peak area ratio in combination with a weighting factor of $1/x^2$, a mean least-squares linear-regression correlation coefficient of greater than 0.96 was obtained in all analytical runs. The statistical evaluation of the coefficients of the ordinary least-squares line indicated small bias in the slope and in the intercept, further indicating minor matrix effects and blank problems, respectively [20]. For each point on the calibration curves for sorafenib, the concentrations back-calculated from the equation of the regression analysis were always within 10% of the nominal value (Table 1). A linear regression of the back-calculated concentrations versus the nominal values provided a unit slope and an intercept not significantly different from zero (data not shown). The slope (0.0056 ± 0.0001, mean ± standard deviation, $n = 4$) and *y*-intercept (0.0015 ± 0.0019, mean ± standard deviation, $n = 4$) were calculated for each calibration curve. The distribution of the residuals showed random variation, was normally distributed, and centered on zero (data not shown).

The LLOQ for sorafenib was established at 7.3 ng/mL for human plasma, which was associated with a signal-

Table 1
Back-calculated concentrations from calibration curves over the concentration range of 7.3–7260 ng/mL[a]

	Nominal concentration (ng/mL)						
	7.3	36	73	363	726	3630	7260
Accuracy (%)	100.2	99.4	98.3	102.1	98.5	101.9	99.5
Concentration (ng/mL)	7.28 ± 0.22	36.07 ± 1.48	71.38 ± 1.10	370.65 ± 20.07	715.43 ± 21.62	3698.24 ± 74.66	7225.12 ± 529.50
Precision (%)							
Within-run	4.0	3.3	2.0	0.7	2.4	1.9	1.7
Between-run	[b]	2.7	[b]	5.8	2.0	0.7	7.7
No. of samples	8	8	8	8	8	8	8

[a] Performed in duplicate on 4 separate days.
[b] No significant variation was observed as a result of performing the assay in different runs.

Table 2
Assessment of accuracy, precision, and recovery

	Nominal concentration (ng/mL)			
	7.3	22	581	5808
Accuracy (%)	102.3	95.9	103.9	104.8
Concentration (ng/mL)	7.42 ± 0.33	20.88 ± 0.71	603.56 ± 27.07	6086.08 ± 123.28
Precision (%)[a]				
Within-run	3.3	3.1	1.8	1.5
Between-run	3.3	1.6	4.6	1.6
Recovery (%)[b]	[c]	96.9	97.0	95.8
No. of samples	20	20	20	20

[a] Performed in quintuplicate on 4 separate days.
[b] Performed in triplicate on 1 day.
[c] Not done.

to-noise ratio greater than 71.5 (mean value = 209) from 20 observations.

3.3. Accuracy, precision, and recovery

For QC samples prepared by spiking human plasma with sorafenib, the within-run and between-run variability (precision), expressed as the percentage relative standard deviations, was less than 6%. Likewise, the mean predicted concentration (accuracy) was less than 5% of the nominal value (Table 2). The recovery of sorafenib from human plasma was greater than 95% at low QC, middle QC, and high QC concentrations.

3.4. Analyte stability

QC samples prepared in human plasma undergoing three freeze–thaw cycles showed no significant degradation (<10%) for sorafenib. Plasma spiked with sorafenib stored at room temperature for up to 6 h indicated that sorafenib was stable during this time period (Table 3). In neutral extracts, sorafenib was stable up to 7 h on the autosampler without any significant degradation, allowing for more than 70 samples to be analyzed simultaneously within a single chromatographic run. Long-term stability studies from 179 days demonstrate sorafenib is stable in plasma (see Table 3).

Table 3
Assessment of stability in human plasma[a]

Condition	Sorafenib	
	22 ng/mL	5808 ng/mL
Freeze–thaw stability (−70 °C)[b]		
Cycle 1	97.5	97.1
Cycle 2	93.4	100.3
Cycle 3	91.2	98.7
Short-term stability (room temperature)[b]		
Time = 0.5 h	91.1	99.8
Time = 1 h	91.4	101.7
Time = 2 h	92.2	104.3
Time = 4 h	91.3	101.3
Time = 6 h	93.4	102.4
Long-term stability (−70 °C)[b]		
Time = 87 days	106.2	107.0
Time = 179 days	108.9	107.1
Autosampler stability (10 °C)[c]		
Time = 2 h	100.6	106.7
Time = 7 h	101.2	105.8

[a] Expressed as the mean percentage change from time zero (nominal concentration).
[b] Performed in triplicate.
[c] Performed repeatedly for 7 h with one sample.

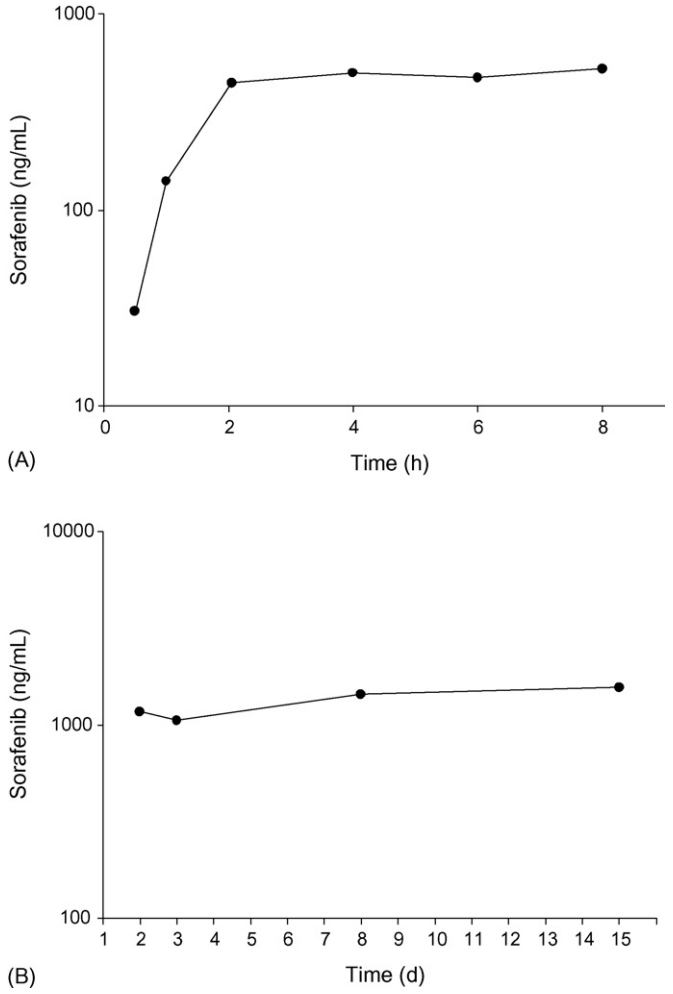

Fig. 5. (A) Sorafenib plasma concentration–time profile on day 1 and (B) pre-treatment trough concentrations on days 2, 3, 8, and 15 (B) in a patient receiving an oral dose of 400 mg twice daily.

3.5. Cross-validation samples

This LC/MS/MS method was applied to the quantitation of sorafenib in plasma samples from Bayer. The results from both analytical methodologies were within 15% precision and accuracy. Both analytical methods result in similar concentrations without bias.

3.6. Plasma concentration–time profile

This LC/MS/MS method was applied to the quantitation of sorafenib in plasma samples from a patient who has received sorafenib administered at a dose of 400 mg as a single dose. Fig. 5 shows a sorafenib plasma concentration–time profile on day 1 (panel A) and pre-treatment trough concentrations on days 2, 3, 8, and 15 (panel B). Following a single oral dose of sorafenib 400 mg, the initial maximum plasma concentration achieved was 500.4 ng/mL and occurred at 4 h. The concentration–time profile plateaued after 4 h and the final concentration obtained at 8 h was 525.3 ng/mL. The AUC_{last} was 3250 ng h/mL. Overall, the pharmacokinetic profile is consistent with previous reports with the exception of a prominent secondary peak due to enterohepatic recirculation [13,17,19].

4. Conclusion

In conclusion, we have developed and validated an assay for measuring sorafenib in human plasma. In comparison to the published method with an assay lower limit of quantitation of 80 ng/mL in mouse plasma, the current assay is 10 time more sensitive, the sample preparation procedure is much simpler and faster, and the chromatographic run time is reduced from 35 to 6 min. These characteristics allow this assay to be easily applied to the quantitation of sorafenib in a large number of plasma samples. The sensitivity is similar to those previously described in human plasma (1–100 ng/mL) but the specific details of these assays have not been published [13,17]. The described method for quantitation over the concentration range of 7.3–7260 ng/mL is sufficient to allow plasma pharmacokinetic monitoring of sorafenib during daily, continuous administration. This method is being used to characterize the plasma pharmacokinetics and pharmacodynamics of sorafenib as a single agent or in combination therapy in cancer patients.

Acknowledgments

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