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Development of a rapid and sensitive LC–MS/MS assay for the determination of sorafenib in human plasma

Lokesh Jain [a,b], Erin R. Gardner [c], Jürgen Venitz [a], William Dahut [d], William D. Figg [b,*]

[a] *Department of Pharmaceutics, School of Pharmacy, Virginia Commonwealth University, Richmond, VA 23298, USA*
[b] *Clinical Pharmacology Program, Medical Oncology Branch, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA*
[c] *Clinical Pharmacology Program, SAIC-Frederick Inc., NCI-Frederick, Frederick, MD 21702, USA*
[d] *Medical Oncology Clinical Research Unit, Center for Cancer Research, National Cancer Institute, Bethesda, MD 20892, USA*

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Abstract

A rapid and sensitive liquid chromatography/tandem mass spectrometric (LC/MS/MS) assay was developed for the quantitative determination of sorafenib in human plasma. Sample pretreatment involved simple protein precipitation by the addition of 0.5 mL acetonitrile, containing internal standard ([$^2H_3$, ^{15}N] sorafenib), to 50 μL of plasma sample volume. Separation was achieved on a Waters SymmetryShield RP8 (2.1 mm × 50 mm, 3.5 μm) column at room temperature using an isocratic elution method with acetonitrile/0.1% formic acid in water: 65/35 (v/v) at a flow rate of 0.25 mL/min. Detection was performed using electrospray ionization in positive ion multiple reaction monitoring (MRM) mode by monitoring the ion transitions from *m/z* 464.9 → 252.0 (sorafenib) and *m/z* 469.0 → 259.0 (internal standard). Calibration curves were linear in the concentration range of 5–2000 ng/mL. The accuracy and precision values, calculated from three different sets of quality control samples analyzed in quintuplicate on six different days, ranged from 92.86% to 99.88% and from 1.19% to 4.53%, respectively.

Keywords: BAY43-9006; Kinase inhibitor

1. Introduction

Sorafenib (Fig. 1) is an orally administered multi-kinase inhibitor that prevents tumor growth by anti-angiogenic, antiproliferative and/or pro-apoptotic effects [1,2]. It directly blocks the autophosphorylation of cell surface receptor tyrosine kinases, namely VEGFR1, 2 and 3, PDGFRβ, c-Kit and RET and also targets the downstream Raf kinase isoforms (wild type C-Raf, and B-Raf and mutant B-Raf). Sorafenib is found to induce apoptosis in several human cancer cell lines by downregulating the levels of the anti-apoptotic protein MCL 1 (myeloid cell leukaemia sequence 1) [3].

Sorafenib (Nexavar®) is US FDA and EMEA (European Medicines Agency) approved for renal cell carcinoma. It is currently in phase III clinical trials as a single agent treatment for hepatocellular carcinoma and in combination with chemotherapy for malignant melanoma and non-small cell lung cancer.

There is a need for development of a rapid and sensitive assay to characterize the pharmacokinetics of sorafenib, both as a single agent and in combination with other anti-cancer agents. The only published detailed LC–MS–MS method by Zhao et al. for quantification of sorafenib uses 100 μL of plasma and has a chromatographic run time of 6 min with LLOQ of 7.3 ng/mL [4]. Here, we describe a rapid, sensitive and specific LC–MS–MS assay for the determination of sorafenib in human plasma which offers the advantages of shorter run time (4 min) and lower LLOQ (5 ng/mL) with a decreased plasma volume requirement (50 μL).

* Corresponding author at: Clinical Pharmacology Program, Medical Oncology Branch, National Cancer Institute, 9000 Rockville Pike, Building 10, Room 5A01, Bethesda, MD 20892, USA. Tel.: +1 301 402 3622; fax: +1 301 402 8606.
E-mail address: wdfigg@helix.nih.gov (W.D. Figg).

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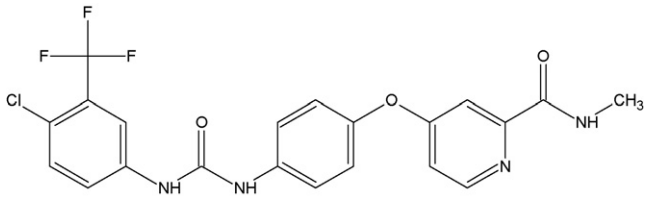

Fig. 1. Chemical structure of sorafenib (BAY43-9006).

2. Methods

2.1. Materials and reagents

Sorafenib and the internal standard ([$^2H_3$, ^{15}N] sorafenib) were provided by Bayer Health Care (New Haven, CT, USA). Acetonitrile (Optima grade) and formic acid (purity ≥ 98%) were purchased from Fisher Scientific and Sigma–Aldrich, respectively. Deionized water was generated with a Hydro-Reverse osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Millipore, Billerica, MA, USA). Drug-free heparinized human plasma was obtained from the National Institutes of Health Clinical Center Blood Bank (Bethesda, MD, USA).

2.2. Stock solutions and standards

Stock solution of sorafenib was prepared by dissolving drug in acetonitrile/water: 90/10 (v/v) at a concentration of 1 mg/mL and stored in glass tubes at −20 °C. Serial (working) dilutions were prepared from this stock solution for the preparation of calibration and quality control (QC) samples. The internal standard (IS) master stock and working stock were prepared respectively at concentrations of 1 mg/mL and 5 μg/mL in acetonitrile/water: 90/10 (v/v). Both the master and working internal standard stocks were stored at −20 °C.

QC samples were prepared in batch, by addition of plasma to the required amount of working solution in a volumetric flask, to obtain three different final concentrations of 8, 160 and 1600 ng/mL. The 50 μL aliquots of prepared QCs were stored at −20 °C.

3. Sample preparation

The calibration curve samples were prepared by spiking 45 μL of blank human plasma in polypropylene microcentrifuge tubes with 5 μL of the appropriate sorafenib working solution. Patient samples were allowed to thaw at room temperature, vortex mixed for 30 s, and a volume of 50 μL of each sample was aliquotted into a microcentrifuge tube. The QC samples were also thawed at room temperature. Next, 0.5 mL of acetonitrile containing internal standard (concentration 50 ng/mL) was added to each sample. All tubes were then vortexed for 30 s, followed by centrifugation for 10 min at 13,063 × *g*. Finally, the supernatant was transferred to a glass vial and a volume of 25 μL of this solution was then injected onto the column.

3.1. HPLC–mass spectrometry apparatus and conditions

Chromatographic separation was performed with a Waters® 2695 Alliance separation module (Milford, MA, USA) using a Waters SymmetryShield RP8 (2.1 mm × 50 mm, 3.5 μm) column, maintained at 35 °C. The autosampler was maintained at 4 °C. Samples were eluted using an isocratic mixture of acetonitrile/0.1% formic acid in water: 65/35 (v/v) at the flow rate of 0.25 mL/min with for a total run time of 4 min.

The HPLC system was coupled with a Waters® Micromass Quattro micro API triple quadrupole mass spectrometer equipped with an electrospray ionization source operating in positive mode. Analysis was performed in multiple reaction monitoring (MRM) mode by monitoring the ion transitions from *m/z* 464.9 → 252.0 (sorafenib) and *m/z* 469.0 → 256.0 (IS). The MS/MS conditions were as follows: capillary voltage, 3.5 kV; cone voltage, 45.0 V; source temperature, 130 °C; desolvation temperature, 410 °C; desolvation gas flow, 610 L/h; collision energy, 33 eV. The instrumentation was controlled and the data were collected using MassLynx software (Waters®).

3.2. Data evaluation

QuanLynx, a component of MassLynx, was used for generation of each calibration curve. The output was based on a least-squares linear regression analysis, with appropriate weighting factor, of the peak area ratio of sorafenib and the internal standard against the nominal drug concentration. The least-squares regression line was not forced through the origin (0, 0), and the blank (zero concentration) samples were not included in the calibration curve. The concentrations of the QC and unknown samples were determined by back calculation (interpolation) using the standard calibration curve.

3.3. Validation procedures

Validation was carried out on six different days, following the guidelines for Bioanalytical Method Validation published by FDA [5]. On each day of analysis, calibration standards were prepared in duplicate at 5, 10, 25, 100, 500, 1000 and 2000 ng/mL. QC samples were prepared independently in batch before validation at concentrations of 8, 160 and 1600 ng/mL. Along with calibrators, five QC samples at each concentration were thawed and analyzed each day. Each validation run included two blank (zero concentration) samples and two samples containing only IS, along with the calibrators and QC samples. The accuracy and precision were calculated using the formulas published elsewhere [6]. To enable the analysis of samples for which plasma concentrations were found to be above the upper limit of quantification (ULOQ) in initial analysis, sample dilution was validated. QC samples of concentration 10,000 ng/mL (5 μL) were diluted 10-fold with human blank plasma (45 μL), then processed as normal. Resulting concentration values were multiplied by the dilution factor, and accuracy and precision were calculated using these data. The limit of detection (LOD) was determined as the concentration having a signal of three times the noise in blank plasma samples across the retention window of sorafenib. The

lower limit of quantitation (LLOQ) was determined as the lowest concentration for which sorafenib spiked in five different sources of plasma resulted in measurements with acceptable accuracy and precision.

4. Results and discussion

4.1. Specificity

Blank plasma samples from six different lots of human plasma were evaluated for the presence of any interference across the retention window of sorafenib and IS. No interferences were observed in any of these samples. Typical chromatograms for blank plasma and plasma spiked with 5 ng/mL of sorafenib are shown in Fig. 2.

4.2. Calibration curve

The lowest bias over the concentration range 5–2000 ng/mL was obtained following regression analysis of the data to a quadratic fit with a weighting factor of $1/x$ (x being the nominal concentration) for the ratio of the peak area of sorafenib and the IS against the nominal concentration. The mean correlation coefficient for regression equations, generated for six different days, was 0.9995 (S.D.: ±0.0005; range: 0.9988–0.9998).

The percentage deviations from nominal (% DEV) determined for mean back calculated concentrations (Table 1), for each standard, ranged from −4.33 to 3.08. This indicated a good fit of the data to the weighted quadratic regression equation.

4.3. Limits of detection and quantitation

The limit of detection (LOD) was assessed with plasma sample spiked with sorafenib to the final concentration of 1 ng/mL. The LOD was determined as 0.2 ng/mL, which provides the signal-to-noise (S/N) ratio of approximately 3:1.The lower limit of quantitation (LLOQ) was determined to be 5 ng/mL with acceptable percent relative standard deviation (% R.S.D.) of 7.45% and % DEV of −4.33%. Five different lots of human plasma were spiked with sorafenib to the final concentration of 5 ng/mL and analyzed. The % R.S.D. and % DEV were found to be 4.80% and 2.00%, respectively. This further confirmed that the analytical method was able to quantify the LLOQ in an accurate and reproducible manner.

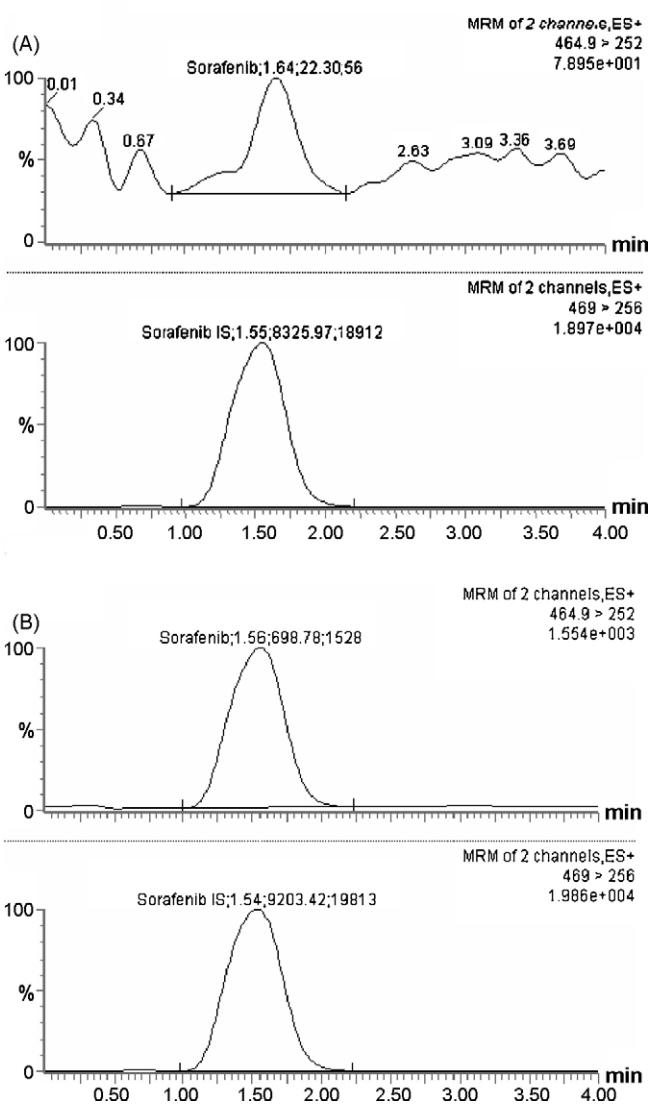

Fig. 2. Typical chromatogram showing 464.9 → 252.0 m/z (sorafenib) and 469.0 → 269.0 m/z (IS) transitions for (A) blank human plasma with IS added; (B) a calibrator sample spiked with 5 ng/mL sorafenib with IS (LLOQ).

4.4. Accuracy and precision

The back calculated concentration values for QC samples, run in quintuplicate at each concentration level on six different days, were used to assess the accuracy and precision of the assay. The results are shown in Table 2. The assay was found to be

Table 1
Back calculated concentrations from calibrators run in duplicate on six different days

Nominal concentration (ng/mL)	N	Mean (ng/mL)	S.D. (ng/mL)	R.S.D. (%)	DEV (%)
5	12	4.78	0.36	7.45	−4.33
10	12	10.31	0.56	5.42	3.08
25	12	25.14	1.15	4.59	0.57
100	12	101.58	3.41	3.35	1.58
500	12	495.96	16.22	3.27	−0.81
1000	12	1002.67	20.82	2.08	0.27
2000	12	1999.48	34.49	1.72	−0.03

Table 2
Intra-run and inter-run quality control accuracy and precision

	Sorafenib concentration (ng/mL)			
	8 ($N=30$)	160 ($N=30$)	1600 ($N=30$)	10,000 (dilution 10×) ($N=20$)
Grand mean (ng/mL)	7.99	159.46	1485.79	9618.00
S.D. (ng/mL)	0.47	7.60	86.38	41.88
R.S.D. (%)	5.93	4.77	5.81	4.35
DEV (%)	−0.12	−0.34	−7.14	−3.82
Deviation range (%)	−10 to 15	−7.44 to 13.75	−14.99 to 3.90	−13.13 to 1.83
Precision				
Within-run (%)	4.53	2.64	1.19	3.98
Between-run (%)	1.68	1.75	2.50	[a]

S.D., standard deviation; R.S.D., relative standard deviation; DEV, deviation from the nominal value; *N*, total number of observations during validation.
[a] No additional variability.

Table 3
The recovery, matrix effect and process efficiency

	Sorafenib concentration (ng/mL)	
	10	2000
A: Mean extracted response ratio ($n=5$)	0.2635	55.13
R.S.D. (%)	3.80	1.17
B: Mean unextracted response ratio ($n=5$)	0.2590	51.92
R.S.D. (%)	3.93	1.32
C: Mean response ratio in pure solvent ($n=3$)	0.2627	51.86
R.S.D. (%)	1.95	1.21
Recovery, $A/B \times 100$ (%)	101.74	106.18
Matrix effect, $(1 - B/C) \times 100$ (%)	1.41	−0.12
Process efficiency, $A/C \times 100$ (%)	100.30	106.31

Response ratio = peak area for sorafenib/peak area for IS. R.S.D., relative standard deviation.

accurate with the percentage deviation from nominal value for all the individual measurements being less than 15% on each validation day at all three concentrations. The between-run and within-run precision values were less than 5%, indicating good precision. The results for dilution analysis are also reported in Table 2. The accuracy and precision were 3.82% and 3.98%, respectively. This established that the samples of concentration above the calibration range can be diluted 10-fold with blank human plasma to reduce its concentration to the level that would lie within the assay calibration curve range for analysis.

4.5. Recovery, matrix effect and process efficiency

Recovery, matrix effect and the process efficiency were calculated at two concentrations, 10 and 2000 ng/mL, and the results are shown in Table 3. The sorafenib stock was added either pre-extraction or post-extraction, and the IS was added post-extraction in all the samples. Recovery was calculated as the response ratio (sorafenib peak area/IS peak area) measured in pre-extraction sorafenib spiked samples ($n=5$), as a percentage of that measured from post-extraction sorafenib spiked samples ($n=5$). The matrix effect was determined by comparison of response ratio in post-extraction sorafenib spiked samples ($n=5$) with that of response ratio observed in pure solvent, i.e., acetonitrile/water: 90/10 ($n=3$). The process efficiency, defined as the overall extractability of the assay method, was estimated as the sorafenib response observed after extraction as compared to the sorafenib response observed in pure solvent. The observed variability (% R.S.D., percent relative standard deviation) for a set of observations at any concen-

Table 4
Freeze–thaw stability

Sorafenib concentration (ng/mL)	Back predicted mean concentration (% R.S.D.) [percentage of corresponding mean concentrations at Cycle 0[a]]			
	Cycle 0	Cycle 1	Cycle 2	Cycle 3
8	7.99	7.74 (7.34%) [96.87%]	7.7 (7.40%) [96.37%]	7.58 (7.57%) [94.87%]
160	159.46	153 (4.55%) [95.95%]	153.82 (1.80%) [96.46%]	151.54 (2.88%) [95.03%]
1600	1485.79	1463.12 (1.21%) [98.47%]	1479.46 (0.71%) [99.57%]	1438.94 (1.17%) [96.85%]

[a] Cycle 0 indicates the mean back calculated concentrations reported in Table 2.

Table 5
Autosampler stability

Sorafenib concentration (ng/mL)	Mean response (% R.S.D.) [percent change from mean response at 0 h]		
	0th (h)	7th (h)	24th (h)
8	8.44 (0.02%)	8.36 (0.02%) [−0.95%]	8.36 (0.03%) [−0.95%]
160	172.36 (0.03%)	171.80 (0.03%) [−0.32%]	171.88 (0.03%) [−0.28%]
1600	1650.46 (0.01%)	1652.90 (0.01%) [0.15%]	1645.06 (0.01%) [−0.33%]

tration level was within 4%, which was within the acceptable limits.

4.6. Freeze–thaw stability

QC samples at nominal concentrations of 8, 160 and 1600 ng/mL were subjected to three freeze-and-thaw cycles with each freeze cycle lasting at least 12 h, in quintuplicate for each cycle. All the samples were analyzed on the same day and the results were compared with the calculated mean concentrations reported in Table 2. The results are presented in Table 4. After three freeze–thaw cycles, the mean observed concentrations deviated less than 6% at the three concentrations. This indicates that repeated freeze–thaw cycles do not affect the sample integrity of sorafenib in human plasma.

4.7. Re-injection and short term stability

An entire set of samples (two calibration curves plus QC samples in quintuplicate) were left in the autosampler after the initial sample run. These samples were re-analyzed after remaining in the autosampler at 4 °C for 7 h and then again after 24 h. The results are presented in Table 5. The percent change from the initial analysis was less than 1% at all three concentration levels. This indicated that the processed sorafenib samples were stable at 4 °C upon standing in the autosampler tray for at least 24 h. The short term bench-top stability of sorafenib working stock in ACN/water: 90/10 was assessed by leaving sorafenib 10 and 2000 ng/mL samples at room temperature for 6 h. The observed percent change in sorafenib response was −8.82% and −1.79%, respectively for 10 and 2000 ng/mL.

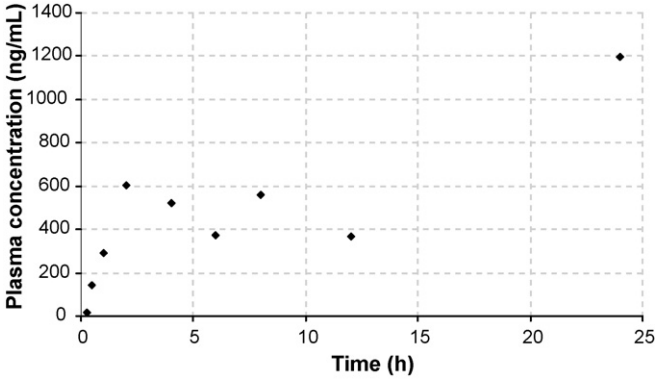

Fig. 3. Plasma concentration time profile of sorafenib in a cancer patient treated with 400 mg b.i.d. oral dose, administered at 12 h interval.

5. Application

After completion of the validation process, the assay was used for determination of sorafenib concentration in plasma samples obtained from a patient with cancer treated with 400 mg oral dose administered twice daily at 12 h interval. The observed concentration–time profile for this patient is shown in Fig. 3. The existence of secondary peaks in this particular profile is in accordance with sorafenib's disposition by entero-hepatic recycling [7].

6. Conclusion

The method described here is a rapid and sensitive method for the determination of sorafenib in human plasma. The method allows quantification of sorafenib with only a small volume of plasma and in a short run time of 4 min. It was found to meet or exceed all FDA guidelines for bioanalytical method validation. The selected calibration model was found to be appropriate and the results for specificity, accuracy and precision, stability following freeze–thaw cycles and at room temperature were found to be within the acceptable limits. Currently, the method is being used for analysis of plasma samples from patients treated with sorafenib at 400 mg/q12 h and at 200 mg/q12 h in combination with other chemotherapeutic agent.

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