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Short communication

Validation of an HPLC-UV method for sorafenib determination in human plasma and application to cancer patients in routine clinical practice

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ABSTRACT

Sorafenib, a new oral multikinase inhibitor with antiangiogenic properties, has demonstrated preclinical and clinical activity against several tumor types. The aims of this study were to validate a method for the measurement of sorafenib in plasma from cancer patients, then to test this method in clinical practice. Following liquid–liquid extraction, the compounds were separated with gradient elution (on a C18 ultrasphere ODS column using a mobile phase of acetonitrile/20 mM ammonium acetate), then detected at 255 nm. The calibration was linear in the range 0.5–20 mg/L. Intra- and inter-assay precision was lower than 7 and 10%, respectively, at 0.5, 3 and 20 mg/L. Plasma sorafenib concentrations were measured in 22 cancer patients (99 samples). The mean trough sorafenib concentration (C_{min}) and concentration at peak were 4.3 ± 2.5 mg/L ($n = 68$, CV = 57.5%) and 6.2 ± 3.0 mg/L ($n = 31$, CV = 47.5%), respectively. Mean sorafenib C_{min} in eight patients who experienced grade 3 drug-related adverse events was approximately 1.5-fold greater than that observed in the remaining patients (7.7 ± 3.6 mg/L *vs.* 4.4 ± 2.4 mg/L, $P = 0.0083$). In conclusion, the method was successfully used in routine practice to monitor plasma concentrations of sorafenib in cancer patients. Finally, large interindividual variability and higher exposure in patients experiencing severe toxicity support the need for therapeutic drug monitoring to ensure an optimal exposure to sorafenib.

1. Introduction

Sorafenib (BAY 43-9006) is a multikinase inhibitor initially developed to inhibit the Raf1-kinase pathway [1]. However, besides the RAF/MEK/ERK pathway, sorafenib targets receptor tyrosine kinases like VEGFR-2 and -3, PDGFR-β, FLt-3 and c-KIT [1,2]. In several clinical and preclinical trials sorafenib proved to be a promising anti-cancer therapeutics that negatively regulates tumor growth, cell proliferation and angiogenesis [3,4], and additionally induces apoptosis in tumor cells [5]. Sorafenib has demonstrated preclinical and clinical activity against several tumor types, either in monotherapy or in combination with other anti-cancer agents [6]. Sorafenib at 400 mg twice daily (bid) was recently approved for the treatment of patients with advanced renal cell carcinoma or unserectable hepato-carcinoma.

Several factors may contribute to a large interindividual variability of sorafenib pharmacokinetics. The mean relative bioavailability is 38–49% for the tablet form, when compared to that of an oral solution. With a high-fat meal, bioavailability is reduced by 29% compared to administration in the fasted state [7]. In cancer patients, the median time to reach peak concentration (T_{max}) is approximately 3 h (range 0–24 h). Sorafenib oral absorption may

Abbreviations: C_{min}, trough sorafenib concentration; bid, twice daily; T_{max}, time to reach peak concentration; CYP3A4, cytochrome P4503A4; UGT1A9, uridine diphosphate glucuronyl transferase 1A9; $T_{1/2}$, terminal half-life; AUC_{0-12}, area under the plasma concentration–time curve over 12 h; LC–MS/MS, liquid chromatography with tandem mass spectrometry; HPLC-UV, high performance liquid chromatography with ultraviolet detection; TDM, therapeutic drug monitoring; IS, internal standard; DMSO, dimethylsulfoxyde; IQC, in-house quality control; LOQ, limit of quantitation; C_{max}, peak concentration.

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be saturated above 400 mg [8]. Sorafenib is 99.5% bound to plasma proteins, and mainly to albumin. Sorafenib is metabolized primarily in the liver and undergoes oxidative metabolism mediated by cytochrome P4503A4 (CYP3A4), as well as glucuronidation mediated by uridine diphosphate glucuronyl transferase 1A9 (UGT1A9). Sorafenib undergoes enterohepatic circulation. The terminal half-life ($T_{1/2}$) varies between 20 and 39 h in cancer patients. A large interindividual variability of the values of the sorafenib area under the plasma concentration–time curve over 12 h (AUC_{0-12}) was observed in the different phase I monotherapy trials [4,9–13]. However, results in such settings may substantially differ from those observed in routine clinical practice.

Several analytical methods using liquid chromatography with tandem mass spectrometry (LC–MS/MS) have been reported to determine sorafenib concentrations in human plasma [14–16]. As such equipment is not available in all clinical laboratories, high performance liquid chromatography with ultraviolet detection (HPLC-UV) may be used instead. To our knowledge, only one HPLC-UV method has been reported for the determination of sorafenib concentration in mouse plasma [17]. However, since the specificity of that method has not yet been evaluated in human plasma it cannot be used for sorafenib therapeutic drug monitoring (TDM). In addition, its long runtime, due mainly to the long elution of the internal standard, represents a major drawback for its use in the clinical laboratory.

The aims of this study were firstly to optimize the chromatographic conditions for the measurement of sorafenib in human plasma and to validate the method, then to apply this method in routine clinical practice for the TDM of sorafenib.

2. Materials and methods

2.1. Chemicals

Sorafenib and erlotinib (internal standard, IS) were purchased from LC laboratories (Woburn, USA), ethyl acetate, acetonitrile and dimethylsulfoxide (DMSO) from VWR (Fontenay-sous-Bois, France). Ammonium acetate was purchased from Sigma (St. Louis, MO, USA). Deionised purified water was prepared in the laboratory using an ELGA system (Veolia, Le Plessis Robinson, France). Fig. 1 presents the chemical structure of sorafenib.

2.2. Equipment and chromatographic conditions

The chromatography system (Dionex Ultimate 300) consisted of a gradient pump with degas option and gradient mixer, an autosampler, a UV detector, and a Chromeleon® chromatography workstation (Dionex Corporation, Sunnyvale, CA, USA). Chromatographic separation was achieved on a C18 ultrasphere ODS column (250 mm × 4.6 mm, 5 μm; Beckman Coulter, Fullerton, USA) associated with a guard column packed with the same phase. The composition of the mobile phase at time zero (the time of injection) was 40% ammonium acetate (20 mM) and 60% acetonitrile. The percentage of acetonitrile was increased to 72% over 9 min. Then, the composition was changed back to ammonium acetate–acetonitrile (40:60; v:v) within 6 s. Finally, the chromatographic system was equilibrated during 5 min before the next injection. The flow rate was of 1 mL/min throughout the 14-min run. Chromatography was performed at 40 °C. The eluent was monitored at a wavelength of 255 nm.

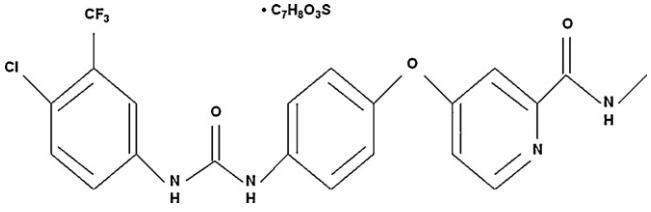

Fig. 1. Chemical structure of sorafenib.

2.3. Stock solutions, standards and quality controls

Stock solutions containing 1000 mg/L of erlotinib and 1000 mg/L of sorafenib were prepared in DMSO. Stock solutions of erlotinib and sorafenib were stored at −20 °C in the dark. Each day, working solutions of sorafenib (100 and 5 mg/L) were freshly prepared with drug-free plasma for a set of calibrating standards at 0, 0.5, 1, 2.5, 5, 10 and 20 mg/L; in-house quality controls (IQC) at 0.5, 3 and 20 mg/L were prepared using a different stock solution of sorafenib; finally, a working solution of IS (25 mg/L) was also freshly prepared with deionised purified water.

2.4. Sample preparation

Sample preparation was adapted from the previously published HPLC-UV method validated in mouse [17]. First, 100 μL of IS at 25 mg/L were added to 400 μL of plasma (calibration standard, IQC or plasma sample). After 10 s of mixing, 500 μL of acetonitrile was added to precipitate proteins. The tubes were vortexed-mixed for 20 s, and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred into a glass tube containing 1 mL of deionised purified water. Then, 3 mL of ethyl acetate was added for the extraction of sorafenib and IS. After an agitation of 10 min with a mechanical shaker, the tubes were centrifuged for 5 min at 4000 rpm. The supernatant was transferred in a glass tube and evaporated to dryness at 25 °C under a nitrogen stream. The residue was dissolved in 120 μL of mobile phase and 50 μL of each sample was injected into the chromatographic system.

2.5. Validation procedures

The method validation was carried out according to the recommendations for bioanalytical method validation [18].

2.5.1. Linearity

Complete calibration curves were analyzed on 6 separate days. The three levels of IQC were assayed thrice with each standard curve. A linear regression was used to plot the peak area ratio (y) of sorafenib to IS *vs*. sorafenib concentration. The model homoscedasticity was assessed by the Levene test. Best weighting factor for linear regression was determined according to the result of the Levene test and the evolution of variance with respect to concentration. Slope, intercept and correlation coefficient were calculated for each standard curve.

2.5.2. Accuracy and precision

Intra- and inter-day accuracy and precision were evaluated at 0.5, 3 and 20 mg/L. Six replicates of each level of IQC were assayed in one run for the intra-day experiment. Three replicates of each level of IQC were assayed within 6 different days for the inter-day experiment. Accuracy was evaluated by calculating the bias that was determined as ((measured concentration − theoretical concentration)/(theoretical concentration)) × 100. Precision was evaluated as the coefficient of variation (CV). Acceptance criteria for accuracy and precision were: bias within ±15% and CV lower than 15%. The limit of quantitation (LOQ) was determined as the lowest concentration of sorafenib that could be determined with acceptable accuracy and precision (<15% for each criterion).

2.5.3. Recovery

Recovery of sorafenib was evaluated at concentration levels corresponding to those of the IQCs (0.5, 3 and 20 mg/L). Recovery after liquid–liquid extraction was determined by comparing the peak area of the extracted plasma with that of the identical concentration of sorafenib prepared in the mobile phase without extraction. The analysis was carried out on 6 samples for each level.

2.5.4. Selectivity and specificity

The selectivity and specificity were investigated in plasma samples from patients receiving neither erlotinib nor sorafenib. The selectivity was evaluated by analyzing plasma samples from 10 cancer patients and 10 patients who exhibited severe hepatic or renal impairment. The specificity was assessed in plasma samples from 10 cancer patients and 30 other hospitalized patients who were not cancer patients. Overall, this analysis allowed testing potential interferences of 72 drugs with sorafenib and erlotinib (Table 1).

2.5.5. Stability

The stock solutions of sorafenib and IS stored in the dark at −20 °C were compared monthly to a freshly made stock solution during a 3-month period. Freeze–thaw, short-term, autosampler and long-term stabilities were evaluated by the difference between observed and theoretical concentration. Freeze–thaw stability of sorafenib was determined by assaying the three levels of IQC in triplicate over three freeze-thawing cycles. The stability of sorafenib in plasma at 4 °C and room temperature was investigated by comparing in triplicate the three concentration levels of IQC stored in these conditions to the freshly extracted ones. The stability of sorafenib in plasma at −20 °C was also evaluated by assaying weekly in triplicate the three concentration levels of routine IQC samples stored at −20 °C for a 2-month period. Sorafenib and IS stability after extraction was evaluated by keeping in triplicate the three extracted IQC samples in the autosampler at 4 °C for 24 h. For all stability studies, the solution was considered stable if the difference with the reference concentration was not greater than 15%.

Table 1
Drugs tested for the specificity.

DCI	DCI
Acebutolol	Imipenem
Aciclovir	Insulin
Folic acid	Irbésartan
Ursodesoxycholic acid	Lansoprazole
Alfacalcidol	Levothyroxine
Alfuzosin	Loperamide
Allopurinol	Magnesium sulfate
Alprazolam	Morphine sulfate
Mitomycin	Mycophenolate mofetil
Amikacin	Nefopam
Amlodipine	Noradrénaline
Aspirin	Omeprazole
Atorvastatin	Acetaminophen
Bisoprolol	Paroxetine
Bromazepam	Phloroglucinol
Captopril	Prednisolone
Cefotaxim	Prednisone
Ceftazidime	Propranolol
Potassium chloride	Ramipril
Cladribine	Rifabutin
Clindamycin	Rifampicin
Clofibrate	Serenoa repens
Clonazepam	Seropram
Cyclosporin A	Spironolactone
Digoxin	Sulfamethoxazole
Domperidone	Iron sulfate
Enalapril	Tacrolimus
Enfuvirtide	Terbutaline
Enoxaparin	Ticarcillin
Entecavir	Tramadol
Escitalopram	Trimethoprim
Flécainide	Valganciclovir
Fluconazole	Vancomycin
Furosemide	Vitamin K
Gentamicin	Zolpidem
Hydroxyzine	Zopiclone

2.6. Sorafenib therapeutic drug monitoring

The method was applied to determine the plasma concentrations of sorafenib in cancer patients treated with twice daily administration. From April 2008 to July 2008, cancer patients above 18, who attended routine clinical follow-up, were studied. To be eligible, patients had to have been treated by sorafenib monotherapy for at least 2 weeks, the time necessary to reach the sorafenib steady state. Patients initially received the recommended daily dose of sorafenib on a twice daily schedule (400 mg bid). Subsequently, doses were adjusted based on efficacy and adverse events. During the sorafenib treatment period, physical examination, complete blood cell count, serum chemistry and urinalysis were performed twice monthly. Adverse events were graded according to the National Cancer Institute Common Toxicity Criteria 3.0. At the same time, blood samples were drawn into 5-mL lithium heparinized Vacutainer tubes to determine sorafenib plasma concentrations. After centrifugation at 3000 rpm for 5 min at 4 °C, plasma was transferred to propylene tubes and stored at −20 °C until analysis. All patients were informed and approved the protocol and the sampling in compliance with the ethical principle of the revised Declaration of Helsinki [19] and according to French regulations.

2.7. Statistical method

Results are reported as means ± standard deviation (S.D.) or median and range, depending on data types. For clinical sample analysis, two subgroups were defined according to the sampling time after sorafenib intake. Considering the pharmacokinetic characteristics of sorafenib (T_{max} and $T_{1/2}$), sorafenib concentrations measured from samples drawn between 0–6 h and 6–12 h after drug administration were arbitrarily defined as peak (C_{max}) and trough (C_{min}) concentrations, respectively. The interindividual variability of plasma sorafenib exposure was assessed in both these groups by calculating CV. The difference in mean values of sorafenib C_{min} in patients with or without grade 3 drug-related adverse events was statistically examined with unpaired *t*-test. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using Medcalc® software, version 7.3.0.1 (Mariakerke, Belgique).

3. Results

3.1. Chromatographic conditions

Representative chromatograms of plasma samples from cancer patients treated or not with sorafenib are shown in Fig. 2. Retention times of IS and sorafenib are 4.75 and 7.85 min, respectively. The method was found to be selective as no interference was observed with biological compounds in plasma from 10 cancer patients and 10 patients with severe renal or hepatic impairment. An excellent chromatographic specificity was also observed, with no interference due to drugs in plasma from 30 hospitalized patients and 10 cancer patients.

3.2. Validation

3.2.1. Linearity

For linearity assessment, the Levene statistic test showed a significant difference ($P < 0.05$) between variances of each con-

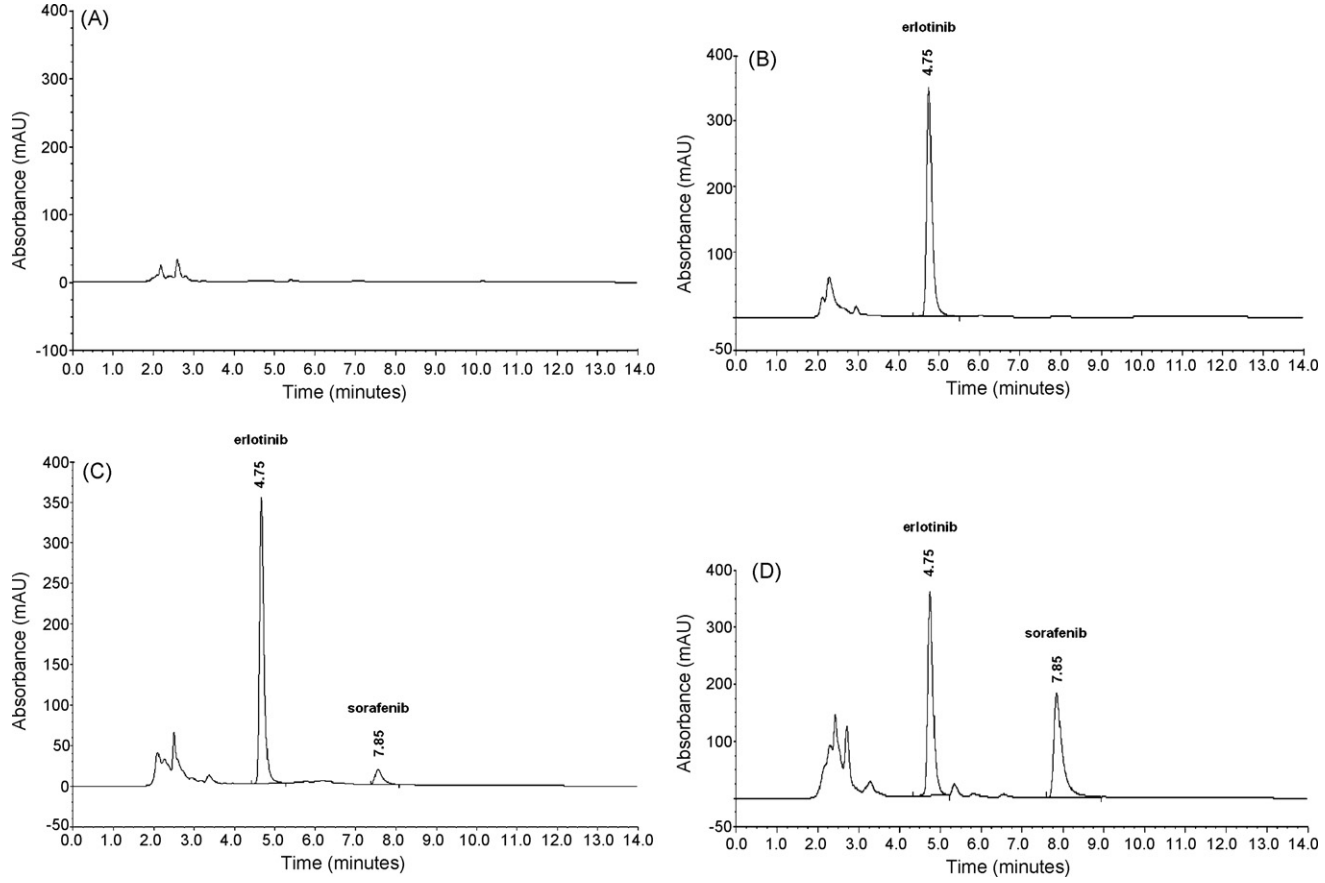

Fig. 2. Representative chromatograms. (A) Blank human plasma from cancer patient, (B) blank plasma with internal standard, (C) limit of quantitation (0.5 mg/L) and (D) plasma from cancer patient treated with sorafenib (concentration of sorafenib: 3.8 mg/L).

centration standard. As the variance grew proportionally to the concentration, the best weighting factor was 1/(peak area ratio). The six standard curves were linear over a concentration range of 0.5–20 mg/L, with a mean slope of 10.38 ± 0.87 (CV = 8.4%) and 0.94 ± 0.34 as intercept. The average coefficient of correlation was 0.997 ± 0.002.

3.2.2. Accuracy, precision and limit of quantitation

Intra- and inter-assay precision and accuracy are reported in Table 2. At all levels, intra- and inter-assay precision was lower than 7 and 10%, respectively. The intra- and inter-assay accuracy ranged from −2.7 to 2% and from −3.1 to 4.0%, respectively. The LOQ was set at the lowest calibration standard value (0.5 mg/L, CV = 9.6%). The next concentration assayed, 0.25 mg/L, could not be determined with acceptable accuracy and precision (24 and 20.8%, respectively).

3.2.3. Recovery

The absolute recoveries of sorafenib at 0.5, 3 and 20 mg/L were 68.1 ± 6.1%, 81.8 ± 3.9% and 87.6 ± 5.5%, respectively. The absolute recovery of the IS was 87.9 ± 4% at the concentration used (5 mg/L).

3.3. Stability

The stock solutions of IS and sorafenib stored for 3 months at −20 °C were comparable to the freshly made ones. The biases for freeze–thaw stability were lower than 12%. IQCs samples stored in a freezer at −20 °C remained stable for at least 2 months. Other results met the criterion set up for stability (Table 3).

3.4. Analysis of patient samples

Plasma sorafenib concentrations were analyzed in 22 cancer patients under sorafenib monotherapy, whose baseline characteristics are summarized in Table 4. All patients were initially treated with the recommended daily dose (400 mg bid). Two patients required dose escalation to stabilize tumor progression. By contrast, a reduction to 200 mg bid ($n = 6$) or treatment withdrawal ($n = 2$) was necessary in eight patients due to grade 3 drug-related adverse events.

Ninety-nine plasma samples were analyzed. Whatever the sorafenib daily dose, mean sorafenib C_{max} and C_{min} were 6.2 ± 3.0 mg/L ($n = 31$, CV = 47.5%) and 4.3 ± 2.5 mg/L ($n = 68$, CV = 57.5%), respectively. Fig. 3 presents the distribution of sorafenib C_{min} measured in patients treated with the recommended daily dose (400 mg bid). There was a wide interindividual variability of sorafenib C_{min} ($n = 51$; CV = 53.4%), and the median C_{min} (interquartile range) was 4.2 (2.8–5.6) mg/L. To explore the effect of drug exposure on the grade 3 drug-related adverse event occurrence, sorafenib concentrations measured during the previous month of each grade 3 toxicity episode were pooled. Sorafenib C_{min} in patients exhibiting grade 3 toxicity was significantly greater than that observed in the remaining patients (7.7 ± 3.6 mg/L *vs.* 4.4 ± 2.4 mg/L, $P = 0.0083$). Finally, the two patients who required dose escalation to 1600 mg daily exhibited sorafenib C_{min} of 3.1 and 3.3 mg/L. The potential saturation of sorafenib oral absorption could not be evaluated in these patients because no sorafenib C_{min} was available before the beginning of dose escalation. Nevertheless, dose escalation allowed stabilizing disease progression in those two patients.

Table 2
Accuracy and precision of the determination of sorafenib concentration in human plasma.

Theoretical concentration (mg/L)	Observed concentration (mg/L)	Bias[a] (%)	Precision (%)
Between-day (n = 18)			
0.5	0.52	4.0	9.6
3	2.95	−1.7	9.9
20	19.39	−3.1	7.6
Within-day (n = 6)			
0.5	0.51	2	6.2
3	2.92	−2.7	3.9
20	20.38	1.9	3.4

[a] Accuracy is expressed as the bias.

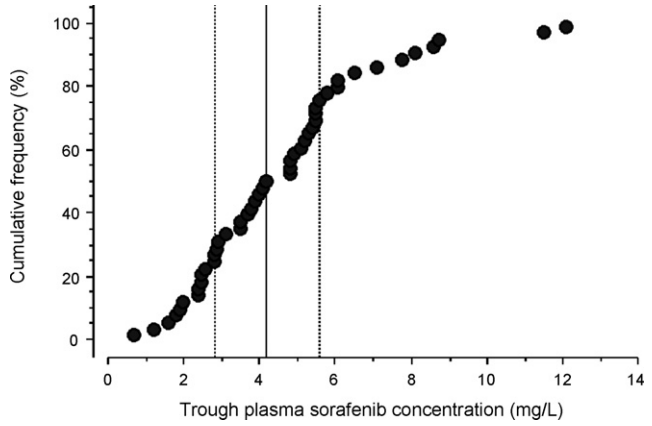

Fig. 3. Distribution of sorafenib trough concentration measured in patients treated with the recommended daily dose (400 mg bid). The solid line represents the median. The dotted lines represent 25th and 75th percentiles.

4. Discussion

We have optimized and validated an HPLC-UV method to determine sorafenib plasma concentrations. Afify et al. initially developed an HLPC-UV method to measure sorafenib plasma concentration in mice [17]. However, the very long runtime (a 35 min linear gradient not including equilibration step between two runs) is a major drawback of this technique in the context of TDM in routine practice laboratories. In the present work, chromatographic conditions were optimized. To shorten the runtime ammonium acetate was substituted for 0.2% acetic acid solution (pH 4.0) in the mobile phase. Then, the heating of the column was increased from 25 °C to 40 °C to decrease the viscosity of the mobile phase and to increase the mass transfer coefficient. Finally, erlotinib was used as internal standard instead of tolnaftate because of its shorter elution time. In addition, erlotinib is not used in combination with sorafenib in clinical settings. The optimization of chromatographic conditions allowed reducing the time of analysis (including the equilibration step between two runs) by a factor 2.5, as well as solvent waste. Overall, our analytical method is more convenient for routine TDM of sorafenib in cancer patients.

The present method has a satisfactory accuracy, reproducibility and specificity to perform TDM of sorafenib in cancer patients. The LOQ of our method (0.5 mg/L) is approximately 50- to 70-fold higher than those previously reported with the LC–MS/MS method [14–16]. However, phase I studies carried out in patients with refractory solid tumors and treated with the recommended daily dose of 800 mg or less [4,9–13] showed that a LOQ of 0.5 mg/L

Table 3
Stability of sorafenib under various storage conditions (n = 3 for each value).

Storage conditions	Theoretical amount (mg/L)	Bias (%)	Precision (%)
Freeze–thaw cycle 1	0.5	−8.0	6.1
	3	−7.3	2.5
	20	−1.7	2.7
Freeze–thaw cycle 2	0.5	−2.0	4.1
	3	−4.5	5.0
	20	4.7	2.4
Freeze–thaw cycle 3	0.5	−12.0	8.3
	3	−7.3	6.1
	20	−10.9	7.4
Autosampler at 4 °C (24 h)	0.5	1.8	6.6
	3	3.1	9.9
	20	3.3	7.0
Before extraction at 4 °C (24 h)	0,5	−4.4	3.2
	3	−8.2	2.6
	20	7.5	3.0
Before extraction at room temperature (24 h)	0.5	−2.2	4.5
	3	−8.4	0.8
	20	3.1	4.4
Before extraction at −20 °C (30 days)	0.5	−16.0	8.2
	3	−1.1	7.3
	20	2.4	6.8
Before extraction at −20 °C (60 days)	0.5	−7.7	6.9
	3	−11.9	3.5
	20	−14.2	4.7

Table 4
Characteristics of patients with sorafenib treatment.

Characteristic	$n = 22$
Sex (male/female)	16/6
Age in years mean (range)	64 (48–78)
ECOG[a] 0–1/≥2	20/2
Cancer (number of patients)	
Hepato-carcinoma	9
Thyroid cancer	4
Melanoma	4
Renal cell carcinoma	4
Neuro-endocrine tumor	1
Pretreatment (number of patients)	
1 lines/≥2	7/15
Chemotherapy	5
Chemo-embolization	4
Anti-angiogenic treatment	7
IFN+ IL-2	1
Sorafenib treatment	
Treatment duration in days mean (range)	119 days (20–253)
Cause of change or interruption	
Disease progression	2
Toxicity grade III	8
Grade 3 sorafenib-related adverse events	
Hand–foot skin reactions	5
Diarrhea	1
Asthenia	1
Gastro-intestinal hemorrhage	1

[a] ECOG = Eastern Cooperative Oncology Group performance status; IFN = interferon; IL = interleukin.

is clinically relevant for a TDM based on sorafenib C_{min} measured at steady state. Our data support this assumption since sorafenib C_{min} was always greater than 0.5 mg/L even in patients receiving 200 mg bid. Overall, these different elements show the reliability of the analytical method to perform sorafenib TDM in cancer patients.

In this study, interindividual variability (approximately 50%) is in accordance with that previously reported in Phase I clinical trials [8]. This large interindividual variability may result from the analytical variability (∼9%), the wide range of tumor types and pharmacokinetic factors. Indeed, frequent diarrhea occurrence under sorafenib therapy [6] and the lack of restriction on high-fat meal intake before drug administration [7] could contribute in this study to the absorption variability. As sorafenib is mainly eliminated by glucuronidation via UGT1A9 [16] and undergoes enterohepatic circulation [7], the extent of the latter and pharmacogenetic variability of UGT1A9 could also account for interindividual variability.

Regarding safety, this investigation is the first to show a correlation between plasma exposure to sorafenib and grade 3 drug-related adverse event occurrence. Indeed, mean sorafenib C_{min} in patients experiencing grade 3 toxicity episode was approximately 1.5-fold greater than that observed in the remaining patients. This would suggest that severe toxicity occurrence might be related to high drug exposure. In this context, TDM may be particularly helpful to prevent severe drug-related adverse events. Currently, an appropriately powered trial is underway to assess the relationship between clinical outcomes (tumor response, survival rate and toxicity) and drug exposure.

5. Conclusions

A simple, specific and accurate HPLC-UV method has been validated to determine sorafenib concentration in plasma from cancer patients. The method was successfully implemented in routine clinical practice for the TDM of sorafenib. Finally, large interindividual variability and higher exposure in patients experiencing severe toxicity support the need of TDM to ensure an optimal exposure to the drug in cancer patients.

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