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Short communication

A routine feasible HPLC analysis for the anti-angiogenic tyrosine kinase inhibitor, sunitinib, and its main metabolite, SU12662, in plasma

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ABSTRACT

Sunitinib is an oral inhibitor of multiple tyrosine kinase receptors with antitumor activity in metastatic renal cell carcinoma. So far, published methods for analysis of sunitinib and its active metabolite (SU12662) in plasma are exclusively based on mass spectrometry. In the context of a large-scale feasibility pharmacokinetic analysis, we developed an original, simple, high-performance liquid chromatography (HPLC) assay with UV detection. A stability study of sunitinib and SU12662 in different light exposure conditions is presented. Due to photo-instability of the compounds, blood sampling and the whole handling procedure have to be performed quickly and with minimal light exposure (6–7 lx). Following single organic extraction with tert-butyl methyl ether, HPLC analysis was performed on an ODS column and UV detection was monitored at 369 nm (run time 15 min). This assay was selective and sensitive enough (limit of detection approximately 1 ng/ml) to quantify minimal concentrations at steady state (Css min) of sunitinib and SU12662 in treated patients.

1. Introduction

Renal cell carcinoma (RCC) represents almost 3% of all cancers and, until recently, few innovative treatment strategies were offered to patients suffering from this disease [1]. Recent experimental and clinical data have shown that angiogenic factors are promising therapeutic targets in this disease [2]. Sunitinib malate is a potent oral inhibitor of multiple tyrosine kinase receptors, including VEGF receptors 1, 2 and 3 and PDGF receptor [3]. Sunitinib has demonstrated antitumor activity in metastatic RCC in second-line therapy, a situation where no effective therapy was recognized [4]. More recently, sunitinib was compared to the current standard treatment, interferon, in a phase 3 trial conducted in first-line treatment of RCC [5]. Progression-free survival was longer and response rates higher in patients receiving sunitinib [5]. Sunitinib is thus currently considered as a major treatment in RCC and studies are ongoing to determine its activity in other solid tumors [3].

Sunitinib (SU11248) is metabolized by cytochrome P450 3A4 to an active N-desethyl metabolite, SU12662 (Fig. 1), which is further transformed to an inactive moiety [3]. As for other oral tyrosine kinase inhibitors, the pharmacokinetics of sunitinib is characterized by wide inter-subject variability, with coefficient of variations of C_{max} and $AUC_{0-24\,h}$ ranging between 30% and 60% [6]. Interestingly, a pharmacokinetic–pharmacodynamic study conducted on metastatic RCC patients receiving the conventional 50 mg daily dosage has shown that greater systemic exposure to sunitinib plus SU12662 was significantly associated with improved clinical response and longer time to tumor progression [7]. Moreover, individual patient exposure to sunitinib and SU12662 could be predicted from sparse sampling points using population PK analysis [7]. Such results open up the possibility of a pharmacokinetically based dose adaptation for sunitinib, as already performed for the tyrosine kinase inhibitor imatinib in chronic myeloid leukemia [8].

So far, published techniques of sunitinib analysis are based on high-performance liquid chromatography–tandem mass spectrometric (LC/MS–MS) methods [6,9–11]. Routine feasibility of sunitinib analysis is thus limited by the need for the laboratory to dispose of such a specific equipment. In the context of a large-scale pharmacokinetic feasibility analysis, we thus developed an original, simple, high-performance liquid chromatography (HPLC) assay with UV detection, allowing simultaneous measurement of sunitinib and SU12662 to be performed in plasma, after a single organic extraction. This simple and robust analytical assay could be helpful in the setting of pharmacokinetically based dose adaptation for sunitinib. In addition, a stability study of sunitinib and SU12662 in different light exposure conditions is presented.

2. Materials and methods

Sunitinib and SU12662 being sensitive to and degraded by light (see Section 3.3), special care is required for blood sampling and

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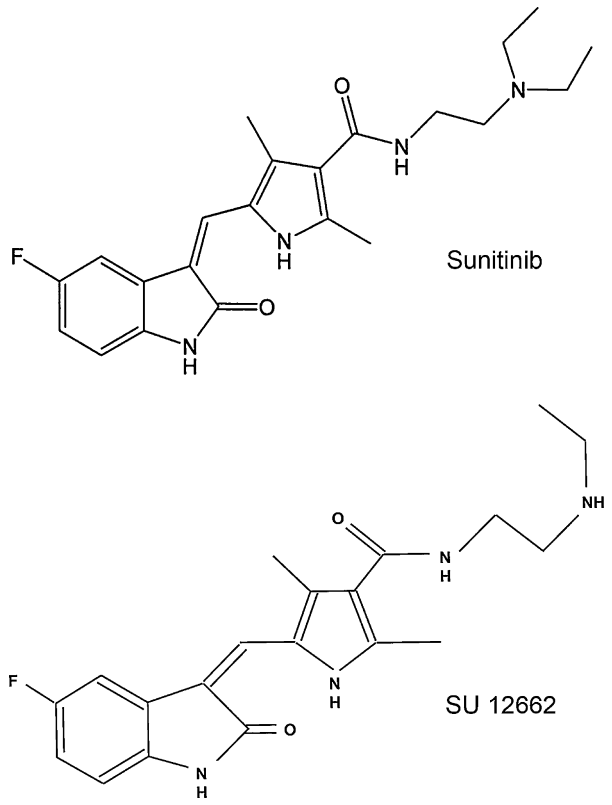

Fig. 1. Chemical structure of sunitinib and its active metabolite SU12662 (N-desethyl-sunitinib).

for the whole handling procedure. To this end, all tubes must be opaque and all manipulations have to be conducted quickly and with minimal light exposure. The laboratory handling procedure is performed with no artificial light and is lit naturally through closed shutters with luminosity measured between 6 and 7 lx (Roline Digital Lux Meter RO1332).

2.1. Chemicals

Sunitinib (99.3% purity) and SU12662 (purity not documented) were kindly provided by Pfizer (Groton, CT, USA). The internal standard (IS), vandetanib, was kindly provided by Astra Zeneca (UK) (purity not documented). For each compound, 100 μl aliquots at 1 mg/ml were made in methanol and stored at −20 °C in 1.5 ml black tubes. Methanol and formic acid were from Prolabo (France). Ammonium formate was from Fluka (France), acetonitrile from Merck (France) and tert-butyl methyl ether from Sigma (France). Water was of injectable preparation quality.

2.2. Material

1.5 ml black polypropylene tubes (Treff ref. 96.7514.0.21), 5 ml black polypropylene tubes (ref. 442077) and 15 ml black polypropylene tubes (Greiner Bio-One ref 188280) were from Dutscher, France. The WISP 717 Waters was set at +4 °C and equipped with light shield and tinted microvials (Interchim, France ref. 5183-4433). HPLC precolumn RP18 was from Merck (ref. 1 50957 0001) and HPLC column Superiorex ODS 5 μm, 4.6 mm × 250 mm was from Shiseido (ref. 41504 Interchim). A Waters 2487 UV detector and a Hewlett Packard 3396 Integrator were used.

2.3. Blood sampling procedure

After obtaining approval of the institutional board and written informed consent, plasma from one patient receiving a conventional oral daily dose of 50 mg and from 2 patients receiving a lower oral daily dose of 37.5 mg was taken at steady state (day 21–day 27 after starting treatment), just before the morning intake. Blood sampling was done on a 5 ml EDTA tube previously wrapped in aluminum foil in order to totally protect it against light. The tube was then immediately placed in a stoppered opaque bottle containing crushed ice and water, and quickly dispatched to the laboratory (<10 min). The tube was centrifuged (+4 °C, 10 min, 2500 rpm) and as soon as the centrifuge was opened (out of the light), the plasma was immediately recovered in a black 5 ml tube, and stored at −80 °C until analysis.

2.4. Preparation of working solutions

All solutions were prepared in 5 ml black polypropylene tubes. The working solution of vandetanib (2.5 μg/ml) was prepared in methanol. Working solutions of sunitinib plus SU12662 in mixture were prepared in methanol at 50,000, 5000, 500 and 50 ng/ml.

2.5. Preparation of quality control samples (QC)

Quality controls (QC) at low concentration (5 ng/ml sunitinib + 5 ng/ml SU12662) and high concentration (100 ng/ml sunitinib + 100 ng/ml SU12662) were prepared in blank plasma and stored at −20 °C in black polypropylene tubes. In each series of analyses, the low and high QCs were analyzed, each in duplicate (each one at the beginning and at the end). The mean of each QC was then computed.

2.6. Preparation of the calibration curve

The calibration curve was prepared in blank plasma in 10 ml glass tubes that were placed in 15 ml black polypropylene tubes. Final concentrations were 0–2.5–5–10–25–50–100–250 ng/ml for sunitinib and SU12662 (500 μl per point).

2.7. Extraction procedure

The entire process has to be performed under minimal light exposure (6–7 lx). To this end, plasmas (500 μl) were placed in 10 ml glass tubes that were inserted in 15 ml black polypropylene tubes. Samples were spiked with 20 μl of the vandetanib solution at 2.5 μg/ml. Four millilitre of tert-butyl methyl ether was then added and tubes were capped and vortexed for 30 s. After 10 min centrifugation (+4 °C, 2500 rpm), the resulting organic phase was placed in a 5 ml glass tube which was inserted in a 15 ml black propylene tube and evaporated to dryness at +37 °C under a nitrogen stream (out of the light). The residue was reconstituted with 200 μl of the HPLC mobile phase (vortexed for 10 s and centrifuged for 10 min at +4 °C, 2500 rpm) and immediately placed in tinted microvials.

2.8. HPLC analysis

Injected volumes were comprised between 5 and 80 μl. The mobile phase was composed of 60% ammonium formate 20 mM pH 3.25 (adjusted with formic acid) and 40% acetonitrile. The flow rate was 0.8 ml/min. UV detection was performed at 369 nm and integration was performed according to peak height. Concentrations were computed from the calibration curves, normalized by the IS, as follows: Concentration (ng/ml) = Slope × [peak height of interest/peak height of IS] + Constant.

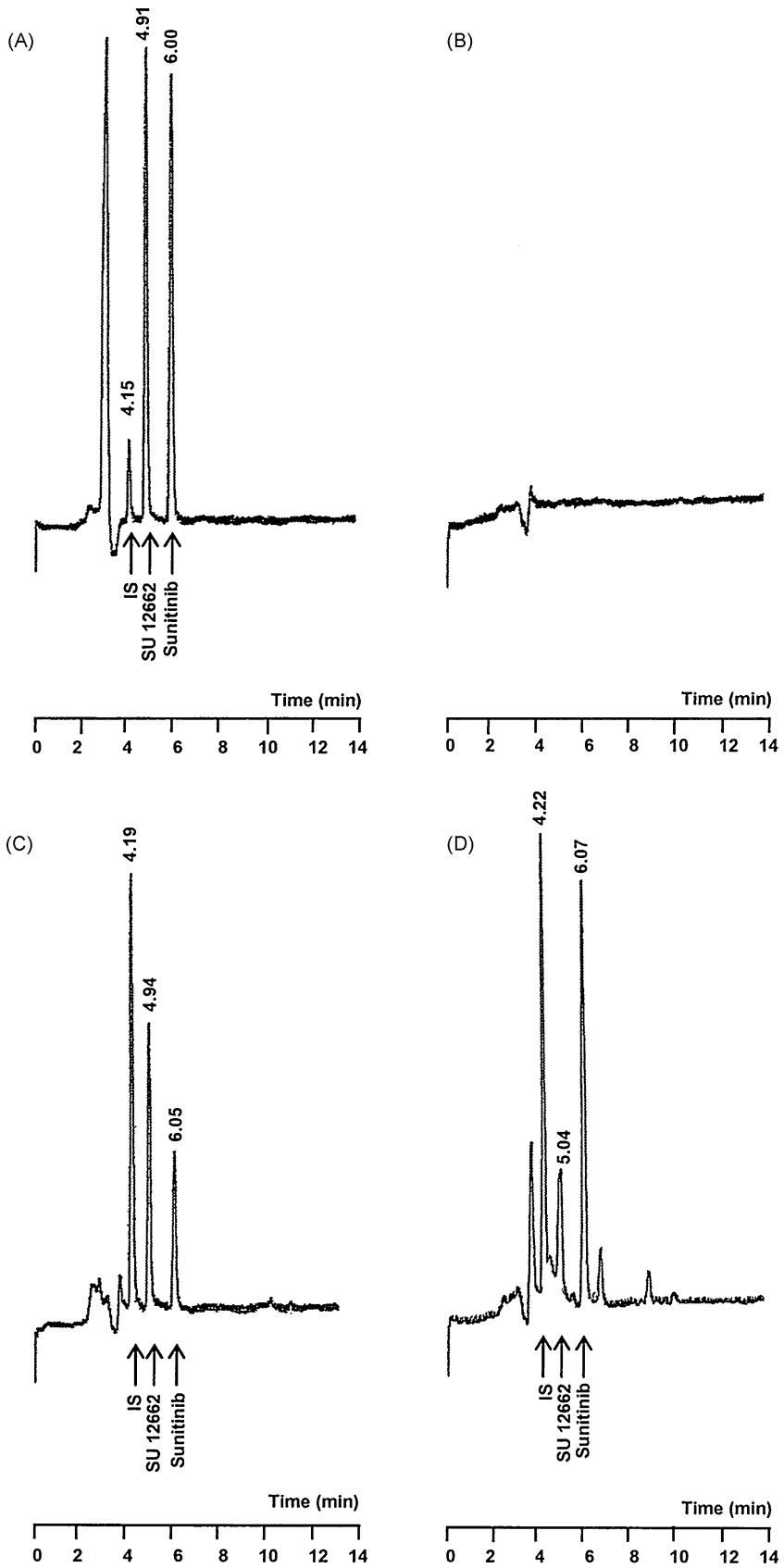

Fig. 2. (A) HPLC profile of pure standard of sunitinib, SU12662 and IS, each at 250 ng/ml, in methanol/HPLC mobile phase (50/50, v/v). 10 μl was injected. (B) HPLC profile of a blank plasma (60 μl injected). (C) HPLC profile of a blank plasma spiked with 10 ng/ml sunitinib, 10 ng/ml SU12662 and 100 ng/ml IS (60 μl injected). (D) HPLC profile of a plasma sample obtained at steady state before the morning intake (Css min) in a patient receiving *per os* 50 mg daily of sunitinib, spiked with IS (100 ng/ml final concentration). 60 μl was injected. The calculated concentrations were 29 ng/ml for sunitinib and 4.7 ng/ml for SU12662. Optical density was monitored at 369 nm.

Table 1
Summary of the validation of the assay.

	Sunitinib	SU12662
Recovery		
At 5 ng/ml	63.2%	92.8%
At 100 ng/ml	59.0%	80.4%
Sensitivity		
Limit of detection	1.4 ng/ml	0.8 ng/ml
Limit of quantification	5 ng/ml	2.5 ng/ml
Linearity		
r^2 computed from 8 concentrations between 0 and 250 ng/ml	0.9931	0.9998
Intra-assay reproducibility ($N = 5$)		
CV at 5 ng/ml	13.5%	3.4%
CV at 100 ng/ml	2.4%	2.9%
Inter-assay reproducibility ($N = 8$)		
CV at 5 ng/ml	14.5%	10.0%
CV at 100 ng/ml	6.4%	8.3%

3. Results

3.1. HPLC profiles

As illustrated in Fig. 2, retention times are close to 4 min for the IS (vandetanib), 5 min for SU12662 and 6 min for sunitinib. Total run time was fixed at 15 min, allowing 4 samples to be analyzed per hour. Peak resolution and blank plasma profile were satisfactory.

3.2. Validation of the assay (Table 1)

3.2.1. Specificity

Fig. 1 illustrates the satisfactory specificity of the assay, as shown by the blank plasma profile.

3.2.2. Recovery rates

Recoveries were comprised between 59.0% and 63.2% for sunitinib (at 100 and 5 ng/ml, respectively), and between 80.4% and 92.8% for SU12662 (at 100 and 5 ng/ml, respectively). For the IS (vandetanib), recovery rate was 85% on average.

3.2.3. Sensitivity

The limit of detection, defined as the concentration corresponding to 3-fold the background noise, was 1.4 ng/ml for sunitinib and 0.8 ng/ml for SU12662.

The limit of quantification, corresponding to the lowest concentration resulting in ±20% accuracy and ±20% precision, was 5 ng/ml for sunitinib and 2.5 ng/ml for SU12662. For expressing final results (ng/ml), the figures have been rounded without any decimal after the point.

3.2.4. Linearity

The mean square of the linear regression coefficient (r^2) computed from 3 calibration curves between 0 and 250 ng/ml (8 concentrations) was 0.9931 for sunitinib and 0.9998 for SU12662.

3.2.5. Reproducibility

Intra-assay reproducibility at low concentration (low QC at 5 ng/ml) gave coefficients of variation at 13.5% and 3.4% for sunitinib and SU12662, respectively ($N = 5$). For the high concentration QC (100 ng/ml), coefficients of variation were 2.4% and 2.9% for sunitinib and SU12662, respectively ($N = 5$).

The inter-assay reproducibility, computed from both low and high QC from 8 independent series of analyses, gave a mean coefficient of variation of 14.5% and 6.4% for the sunitinib at 5 and 100 ng/ml, respectively. Coefficients of variation for SU12662 were 10.0% and 8.3% at 5 and 100 ng/ml, respectively.

3.3. Stability study

3.3.1. Light stability

Stability of sunitinib, SU12662 and vandetanib were tested separately over 5 h (T0, 5 min, 15 min, 30 min, 1 h, 2 h, and 5 h) in the 3 following light conditions: complete darkness (black tubes), normal light exposure (~1200 lx), minimal light exposure as defined in the present procedure (6–7 lx). Vandetanib was stable up to 5 h in the 3 tested conditions. Results for sunitinib and its metabolite are summarized in Fig. 3. Both analytes were very stable in the complete light-free conditions up to 5 h. In contrast, in normal light exposure, sunitinib and SU12662 concentrations decreased by 40% as quickly as 5 min after light exposure and this 40% decrease in concentration

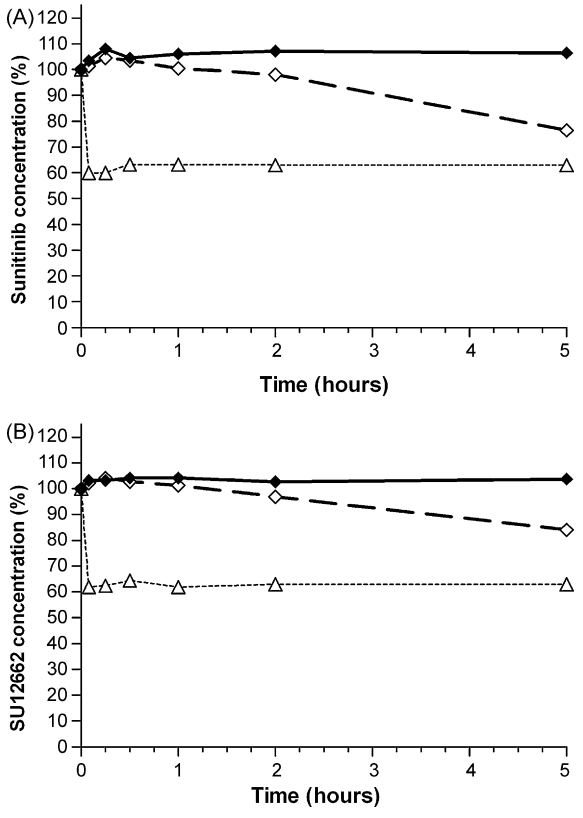

Fig. 3. Stability of sunitinib (50 ng/ml) and SU12662 (50 ng/ml) tested separately over 5 hours, in the 3 following light conditions: complete darkness (black tubes —◆—), normal light exposure (~1200 lx, ···△···), minimal light exposure as defined in the present procedure (6–7 lx, - ◇ -).

Table 2
Application of the assay to measurement of Css min in patients.

Patient no.	Daily oral sunitinib dose (mg)	Sunitinib concentration (ng/ml)	SU12662 concentration (ng/ml)
1	50	29	5
2	37.5	37	8
3	37.5	46	9

then remained stable for 5 h. In minimal light exposure, sunitinib and SU 12662 were very stable up to 2 and 5 h, respectively.

3.3.2. Stability after freeze–thaw cycle

After a freeze–thaw cycle (24 h at −80 °C) on 3 plasmas spiked with sunitinib and SU12662 at 10, 50 and 100 ng/ml, respectively, the concentrations of sunitinib and SU12662 were similar to those measured on fresh spiked plasmas.

3.4. Application of the assay to a patient sample

A typical HPLC profile of a plasma sample obtained in a patient receiving a conventional daily oral dose of 50 mg of sunitinib is shown in Fig. 1D. This plasma was obtained at steady state, just before the morning intake (Css min). Table 2 summarizes the Css min obtained in 3 patients (one patient receiving the conventional daily dose of 50 mg and 2 patients receiving a lower daily dose of 37.5 mg). These data confirm the clinical applicability of the present analytical method.

4. Discussion

The presently validated HPLC assay is clearly applicable to large-scale routine analysis of sunitinib and its main active metabolite, SU12662, in plasma. So far, analytical methods developed for pharmacokinetic investigations of sunitinib were based on liquid chromatography–mass spectrometry methods (LC–MS or LC–MS–MS), and required costly MS equipment, most often available in centralized laboratory structures [6,9–11]. The advantage of LC–MS method is its high sensitivity, around 0.1 ng/ml for sunitinib and SU12662. Such a high sensitivity is necessary to obtain maximum knowledge of the elimination profile of the drug at very distant times after drug administration. Even though the limit of detection of the present HPLC assay is 10-times higher, i.e. approximately 1 ng/ml, this concentration is well above the minimal concentrations of sunitinib and SU12662 observed at steady state (Css min) for the approved dose of 50 mg daily. In fact, at this dose, median Css min values at 41 ng/ml and 17 ng/ml have been reported for sunitinib and its metabolite, respectively [6]. These figures concur with the sunitinib concentrations measured in the presently studied patients (Table 2). Pre-clinical studies have shown that a sunitinib concentration of 50 ng/ml is able to inhibit VEGFR-2 and PDGFR-β phosphorylation, and can thus be considered as an active target plasma concentration [12]. The presently validated HPLC assay is thus feasible for routine pharmacokinetic follow-up of sunitinib and SU12662 in treated patients.

As shown in Fig. 3, an important feature that must be kept in mind, irrespective of the analytical method, is the photo-instability of the compounds which requires blood sampling and the whole handling procedure to be performed quickly and with minimal light exposure. From a practical point of view, the relative short run time of the present assay strengthens the routine applicability of the method. The clinical interest of pharmacokinetic follow-up for sunitinib has been recently supported by data showing a significant relationship between steady state systemic exposure of sunitinib plus metabolite and clinical response in metastatic RCC [7,13].

5. Conclusions

In total, the method described herein is selective and sensitive enough for the quantification of sunitinib and SU12662 in treated patients. This method could be extremely useful in routine pharmacokinetic surveys and pharmacokinetic–pharmacodynamic study programs.

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