Journal of Chromatography B, 877 (2009) 1982–1996

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Therapeutic Drug Monitoring of the new targeted anticancer agents imatinib, nilotinib, dasatinib, sunitinib, sorafenib and lapatinib by LC tandem mass spectrometry

A. Haouala[a,c], B. Zanolari[a], B. Rochat[b], M. Montemurro[d], K. Zaman[d], M.A. Duchosal[e], H.B. Ris[c], S. Leyvraz[d], N. Widmer[a], L.A. Decosterd[a,*]

[a] Division de Pharmacologie clinique, Département de Médecine, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland
[b] Quantitative Mass Spectrometry Facility, Chromatography–Mass Spectrometry Plateform, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland
[c] Divisions of Thoracic and Vascular Surgery, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland
[d] Multidisciplinary Oncology Centre, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland
[e] Service of Haematology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Lausanne, Switzerland

ARTICLE INFO

Article history:
Received 3 November 2008
Accepted 29 April 2009
Available online 13 May 2009

Keywords:
LC–MS/MS
Anticancer targeted therapy
Tyrosine kinase inhibitors
Imatinib
Nilotinib
Dasatinib
Sunitinib
Sorafenib
Lapatinib
Plasma

ABSTRACT

The treatment of some cancer patients has shifted from traditional, non-specific cytotoxic chemotherapy to chronic treatment with molecular targeted therapies. Imatinib mesylate, a selective inhibitor of tyrosine kinases (TKIs) is the most prominent example of this new era and has opened the way to the development of several additional TKIs, including sunitinib, nilotinib, dasatinib, sorafenib and lapatinib, in the treatment of various hematological malignancies and solid tumors. All these agents are characterized by an important inter-individual pharmacokinetic variability, are at risk for drug interactions, and are not devoid of toxicity. Additionally, they are administered for prolonged periods, anticipating the careful monitoring of their plasma exposure *via* Therapeutic Drug Monitoring (TDM) to be an important component of patients' follow-up. We have developed a liquid chromatography–tandem mass spectrometry method (LC–MS/MS) requiring 100 μL of plasma for the simultaneous determination of the six major TKIs currently in use. Plasma is purified by protein precipitation and the supernatant is diluted in ammonium formate 20 mM (pH 4.0) 1:2. Reverse-phase chromatographic separation of TKIs is obtained using a gradient elution of 20 mM ammonium formate pH 2.2 and acetonitrile containing 1% formic acid, followed by rinsing and re-equilibration to the initial solvent composition up to 20 min. Analyte quantification, using matrix-matched calibration samples, is performed by electro-spray ionization–triple quadrupole mass spectrometry by selected reaction monitoring detection using the positive mode. The method was validated according to FDA recommendations, including assessment of extraction yield, matrix effects variability (<9.6%), overall process efficiency (87.1–104.2%), as well as TKIs short- and long-term stability in plasma. The method is precise (inter-day CV%: 1.3–9.4%), accurate (−9.2 to +9.9%) and sensitive (lower limits of quantification comprised between 1 and 10 ng/mL). This is the first broad-range LC–MS/MS assay covering the major currently in-use TKIs. It is an improvement over previous methods in terms of convenience (a single extraction procedure for six major TKIs, reducing significantly the analytical time), sensitivity, selectivity and throughput. It may contribute to filling the current knowledge gaps in the pharmacokinetics/pharmacodynamics relationships of the latest TKIs developed after imatinib and better define their therapeutic ranges in different patient populations in order to evaluate whether a systematic TDM-guided dose adjustment of these anticancer drugs could contribute to minimize the risk of major adverse reactions and to increase the probability of efficient, long lasting, therapeutic response.

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1. Introduction

A new era of cancer therapy is emerging, with targeted therapies characterized by unique mechanisms of action and high specificity for single or multiple key biological pathways responsible *per se* or implicated in the cancer process. The first prominent example of this approach, imatinib, a tyrosine kinase inhibitor

* Corresponding author at: Division of Clinical Pharmacology – Laboratory, BH18-Lab 218–226, Centre Hospitalier Universitaire Vaudois, CH-1011 Lausanne, Switzerland. Tel.: +41 21 314 42 72; fax: +41 21 314 42 88.
E-mail address: LaurentArthur.Decosterd@chuv.ch (L.A. Decosterd).

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doi:10.1016/j.jchromb.2009.04.045

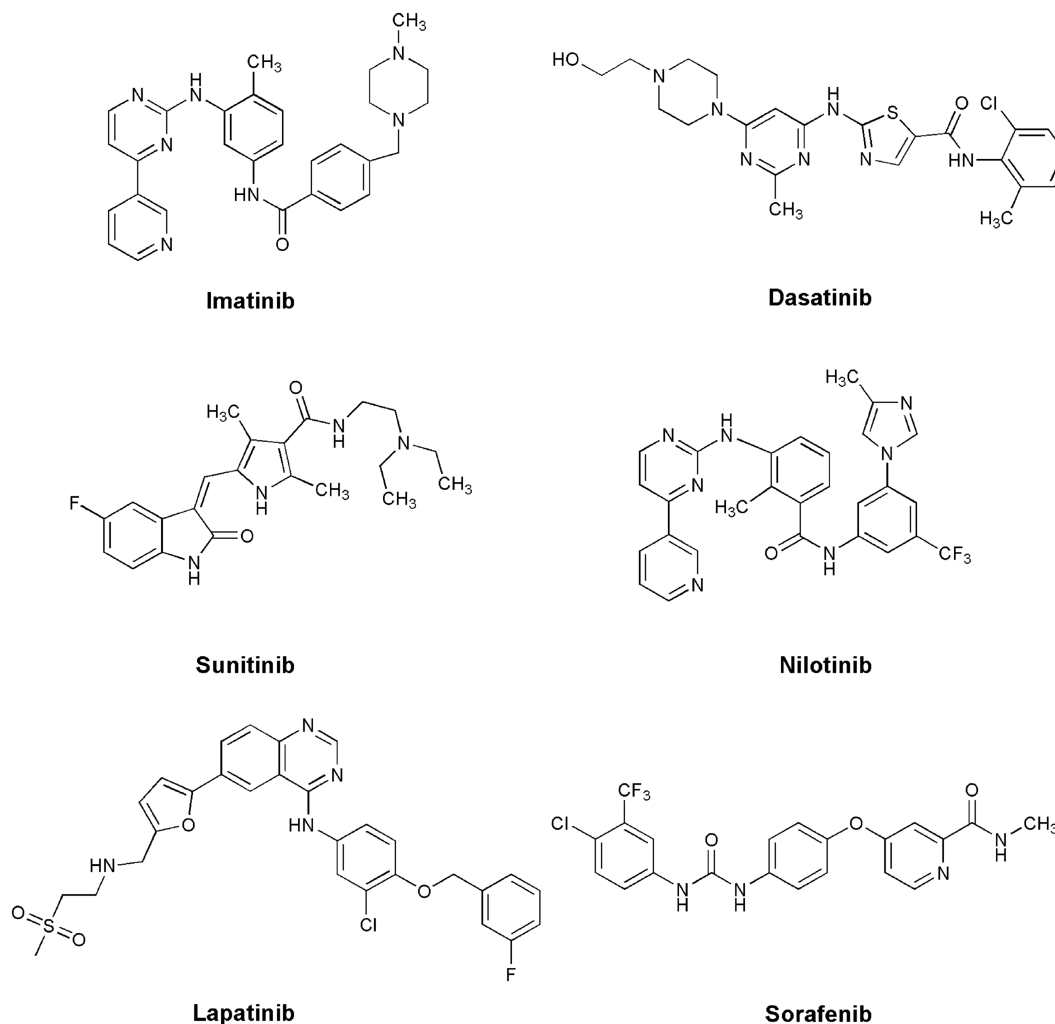

Fig. 1. Chemical structures of the six TKIs studied.

(TKI), has revolutionized the treatment and prognosis of chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST) [1,2]. However, imatinib treatment is not devoid of toxicity and resistance occurs. It is becoming increasingly recognized that the response is influenced not only by the genetic heterogeneity of drug target determining the tumor's sensitivity (BCR-ABL for CML, and c-KIT for GIST), but also by patient's genetic background and environmental factors that influence drug disposition in the body [3,4]. Indeed, imatinib drug exposure was found to be a predictor in clinical response in CML [5,6] and in GIST [7–9].

Following imatinib, several other TKIs, including sunitinib (SU11248), nilotinib (AMN107), dasatinib (BMS 354825), sorafenib (BAY 43-9006) and lapatinib (GW 572016) (Fig. 1) have been developed and are now used for treating of various hematological malignancies, solid tumors including GIST [10], advanced renal cell carcinoma (RCC), hepatocellular carcinoma (HCC) and breast cancer [11], and have shown promising activity in other tumors as well [12].

While most standard anticancer chemotherapy regimens are administered through short *i.v.* cycles, targeted drugs such as TKI imatinib and the more recent TKIs are orally administered and must be taken for a prolonged period, if not indefinitely. Moreover, they are metabolized mostly by cytochromes P450 3A4, whose activity is known to present a large inter-individual variability and influenced by environmental factors (i.e. drug-induced interactions, food). Moreover, some of them are substrates of drug transporters, such as efflux pumps (e.g. P-glycoprotein, P-gp) or uptake pumps (e.g. human organic cation transporter 1, hOCT1) [7,11,13–16]. Finally, as they are extensively bound to circulating proteins in plasma (such as for instance, imatinib on α1-acid glycoprotein [15]), and only a small free fraction is likely to enter cells to exert its pharmacological action. A given dose can therefore yield very different circulating concentrations between patients, favoring the selection of resistant cellular clones in case of sub-therapeutic drug exposure, or increasing the risk of adverse drug reactions at excessive plasma levels.

During the past decades, it has been established that the therapeutic use of selected drugs could be optimized by an individualization of their dosage, based on blood concentrations measurement. Such a feedback strategy, termed Therapeutic Drug Monitoring (TDM), is now current practice for drugs such as antibiotics, antiepileptics, immunosuppressive drugs, and more recently, anti-HIV drugs [17]. TDM has been proved to definitely improve the treatment benefit and has been shown to be cost-effective for many drugs. Suitable drugs for a TDM program are mainly those with large inter-individual but low intra-individual pharmacokinetic variability, with both consistent concentration–efficacy and concentration–toxicity relationship [18,19]. Such characteristics are notably met by imatinib [5,8], and probably also by newer targeted agents calling for further extensive evaluation of TDM in well-conducted clinical trials.

Among these new agents, TDM appears for imatinib to present some clinical benefit for the optimal management of anticancer targeted therapy [5,8,9,20]. Whether TDM is also beneficial for the other TKIs remains to be established, but can be anticipated considering their metabolic pathways (CYP3A) and the drug interactions potential. Interestingly, TDM has been only recently recognized as having potential clinical importance for imatinib, leading to recommendations on target levels to reach in plasma for optimal clinical response [5,20]. Moreover, based on preclinical data, a target plasma concentration of 50 ng/mL was defined for sunitinib [21], even though no formal TDM study has, to the best of our knowledge, been initiated yet for this latter drug. Finally, in a recent-provocative article [22], Ratain and Cohen have suggested that a lower dose of lapatinib could be administered if taken with food, to take advantage of the increased absorption of lapatinib in the presence of high fat meals, or if taken with grapefruit juice a known CYP3A inhibitor, which should result in an overall reduction in treatment cost. However they did strongly recommend that this approach should not be done without a formal pharmacokinetic assessment.

Most analytical methods published to date using liquid chromatography coupled to triple quad mass spectrometry (LC–MS/MS), have focused on the assay in biological fluids of a single tyrosine kinase inhibitor, namely imatinib [23–26], sorafenib [27,28], lapatinib [29] and sunitinib [30]. The only reported assay for nilotinib analysed in various biological fluids (plasma, urine, cells) either alone [31] or with imatinib [32], uses HPLC coupled to UV detection. So far, extensive studies on dasatinib metabolism have been performed using LC–MS/MS [33,34], but no validated method has been yet published for the quantification of dasatinib in human plasma.

However, triple quad mass detection qualifies for the simultaneous measurement of arrays of structurally unrelated anticancer targeted agents in a single analytical run, resulting in an overall reduction in analytical time and costs. Thus, the development and validation of an enhanced throughput method using simple extraction method followed by LC–MS/MS technology is of high interest for the simultaneous analysis of every major anticancer targeted agent which in the future may possibly be also used, following the HIV treatment model, in combination therapy [35].

We describe here a sensitive LC–MS/MS method for the simultaneous analysis in a small volume of plasma of the six major TKIs currently used imatinib, nilotinib, dasatinib, sunitinib, sorafenib and lapatinib. This assay is notably applied for plasma levels monitoring of TKIs in some specific clinical situations (toxicity, questionable compliance, managing drug interactions and less than optimal clinical response) where information on drug plasma exposure may be useful for optimizing patient treatment management.

2. Experimental

2.1. Chemicals and reagents

Imatinib mesylate and nilotinib (base) were kindly provided by Novartis Pharma AG (Basel Switzerland). Dasatinib base was generously provided by Bristol–Myers Squibb (Baar, Switzerland). Sunitinib malate (Sutent™, Pfizer), sorafenib tosylate (Nexavar™, Bayer) and lapatinib ditosylate (Tyverb™, GlaxoSmithKline) pharmaceutical preparations were kindly provided by the Pluridisciplinary Center for Clinical Oncology (Pr Serge Leyvraz Head, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland).

The internal standard (IS) imatinib-D_8 was provided by Novartis Pharma AG (Basel Switzerland) and was used initially as IS for all TKIs before stable-isotope-labeled IS of additional TKIs have become available. The IS sunitinib-D_{10} and sorafenib-^{13}C D_3 were purchased at Alsachim (67400 Illkirch, France).

Chromatography was performed using Lichrosolv® HPLC-grade acetonitrile (MeCN) purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q® UF-Plus apparatus (Millipore Corp., Burlington, MA, USA). Ammonium formate was purchased from Fluka (Buchs, Switzerland). Formic acid (98%) and methanol for chromatography Lichrosolv® (MeOH) were purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

Different sources of blank plasma used for the assessment of matrix effects and for the preparation of calibration and control samples were isolated (1850 × *g*, 10 min, +4 °C, Beckman Centrifuge, Model J6B) from outdated blood donation units from the Hospital Blood Transfusion Centre (CHUV, Lausanne, Switzerland) or from blood withdrawn from patients with Vaquez Disease, at the occasion of their phlebotomy.

2.2. Equipment

The liquid chromatography system consisted of a Rheos 2200 quaternary pumps, equipped with an online degasser and a HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) controlled by Janeiro-CNS software (Flux Instruments, AG, Thermo Fischer Scientific Inc, Waltham, MA). Separations were done on a 2.1 mm × 50 mm XTerra® dC18 5 μm analytical column (Waters, Milford, MA, USA) placed in a thermostated column heater at 25 °C (Croco-Cil, Cluzeau Info Laboratory, Courbevoie, France). The chromatographic system was coupled to a triple quadrupole (TSQ) Quantum Discovery mass spectrometer (MS) from Thermo Fisher Scientific, Inc. equipped with an Ion Max electrospray ionization (ESI) interface and operated with Xcalibur 2.0 software (Thermo Fischer Scientific Inc, Waltham, MA).

2.3. Solutions

2.3.1. Mobile phase solutions

The mobile phase used for chromatography was 20 mM ammonium formate in ultrapure water adjusted to pH 2.2 with formic acid (FA) 98–100% (solution A), and acetonitrile with 1% FA (solution B). A solution of 20 mM ammonium formate (adjusted to pH 4) was used for the dilution of plasma supernatents prior to their LC–MS analysis. Solvents were regularly prepared for each series of analysis.

2.3.2. TKIs stock and working solutions, internal standard, calibration standards and quality controls (QCs) solutions

Stock solutions of imatinib and nilotinib at 1 mg/mL and dasatinib at 0.05 mg/mL were prepared by dissolution of 59.7 mg imatinib mesylate, 50 mg nilotinib base and 10 mg dasatinib base in 50.0 mL, 50.0 mL and 200.0 mL MeOH, respectively. Sunitinib, sorafenib and lapatinib stock solutions were prepared from Sutent™ 50 mg capsules (Pfizer) Nexavar™ 200 mg tablets (Bayer) and Tyverb™ 250 mg tablets (GlaxoSmithKline), respectively. Sunitinib stock solution (1 mg/mL) in MeOH was obtained by extraction of Sutent™ capsules with MeOH in which sunitinib is reported to be freely soluble (2 mg/mL) [36]. One Sutent™ capsule (containing 50 mg sunitinib base) was carefully opened to collect the solid content into a volumetric flask. The empty capsule shell was thoroughly rinsed with MeOH (ca 5 mL) which was collected into the same flask. The resulting suspension was sonicated for 5 min and after allowing to equilibrate at room temperature (RT), was completed to the volume with MeOH, before being filtrated through a filter paper (150 mm Ø, Schleicher and Schuell, MicroScience, Dassel, Germany). Sorafenib and lapatinib stock solutions were prepared similarly after careful grounding in a mortar and extraction with MeOH of one Nexavar™ 200 mg tablet and one Tyverb™ 250 mg.

Table 1
Preparation of working solutions.

Drug	Stock solution concentration (solvent = MeOH)	Working solution concentration (solvent = MeOH)	Calibration range (ng/mL)	Qcs controls (ng/mL)
Imatinib	1 mg/mL	100 μg/mL, 1 μg/mL	1–10000	3, 2000, 8000
Dasatinib	50 μg/mL	5 μg/mL, 0.5 μg/mL	1–200	3, 60, 150
Sunitinib	1 mg/mL	25 μg/mL, 25 μg/mL, 0.5 μg/mL	1–500	3, 80, 400
Sorafenib	1 mg/mL	50 μg/mL	100–15000	300, 1500, 6000
Nilotinib	1 mg/mL	250 μg/mL, 25 μg/mL, 0.25 μg/mL	1–5000	3, 800, 3000
Lapatinib	1 mg/mL	250 μg/mL, 25 μg/mL, 2.5 μg/mL	5–5000	15, 800, 4000

Table 2
Gradient elution program.

Time (min)	Flow rate (mL/min)	Solvent A (%)	Solvent B (%)
0.00	0.30	95	5
11.00	0.30	50	50
12.00	0.30	10	90
14.00	0.30	0	100
14.01	0.50	0	100
17.00	0.50	0	100
17.01	0.30	95	5
20.00	0.30	95	5

Solvent A: 20 mM NH_4 acetate pH 2.2 (addition of formic acid). Solvent B: acetonitrile +1% formic acid. Temperature (°C): 20. Injection volume (μL): 20.

The resulting MeOH suspension was filtrated and completed to the volume of 200 and 250 mL of MeOH, respectively.

The stock solutions were stored at 4 °C in glass flasks, with caps tightly wrapped with Parafilm®. Stock solutions were diluted with MeOH as indicated in Table 1. A stock solution of imatinib-D_8 (internal standard) at 0.4 μg/mL in MeOH: 1% formic acid in H_2O (1:1, v/v). The IS sunitinib-D_{10} and sorafenib-^{13}C D_3 were diluted with MeOH and MeOH: 1% formic acid in H_2O (1:1, v/v) respectively, to obtain stock solutions at 0.2 μg/mL. All stock IS solutions were combined to give a single IS solution containing imatinib-D_8, sunitinib-D_{10}, sorafenib-^{13}C D_3 at 20 ng/mL. Plasma calibration samples and three plasma quality control (QC) samples were prepared by adding the appropriate volume of each working solution diluted 1:50 with blank plasma, in accordance with the recommendations on bioanalytical methods validation stating that total added volume must be ≤10% of the biological sample [37]. The calibration standard and control plasma samples were stored at −20 °C prior to analysis.

2.4. LC–MS/MS conditions

The mobile phase was delivered using the following stepwise gradient elution program (reported in detail in Table 2): 5% of B at 0 min, 50% of B at 11 min, 100% of B at 14 min with a flow rate of 0.3 mL/min. The second part of the run includes 3 min of intensive rinsing (100% B with 0.5 mL/min) and re-equilibration step to the initial solvent up to 20 min (at 17.01 with 0.3 mL/min). The thermostated column heater was set at 25 °C and the autosampler was maintained at 10 °C.

The MS conditions were as follows: ESI in positive mode, capillary temperature: 350 °C, in source induced dissociation: 10 V, tube lens voltages range: 86–129 V, spray voltage: 4 kV and sheath and auxiliary gas (nitrogen) flow-rate: 60 psi and 5, respectively. The Q2 collision gas (argon) pressure was 1 mTorr (0.13 Pa). MS is acquired in selected reaction monitoring (SRM). The determination of optimal potential settings and MS/MS transitions were chosen by direct infusion into the MS/MS detector of a solution of each TKI drug solution at a concentration of 1 μg/mL in 50:50 of solution A buffer/MeOH. The selected m/z transitions and the collision energy for each analyte are reported in Table 3.

The first (Q1) and third (Q3) quadrupoles full width half maximum was set at the unity. Scan time was set 0.05 s. MS acquisitions were done in centroid mode. One single segment of data acquisition was programmed in the positive mode, during the entire analytical run. The injection volume was 20 μL.

Chromatographic data acquisition, peak integration and quantification were performed using the Xcalibur LC-Quan software package (version 2.0) (ThermoQuest, San Jose. CA, USA).

2.5. Blood samples collection for TKI drugs determination: general procedure

Blood samples (5 mL) were collected at random time after last drug intake in Monovettes® (Sarstedt, Nümbrecht, Germany, with K-EDTA as anticoagulant) from patients treated with one of the six considered TKIs, at the occasion of their regular medical visit. The exact time of blood sampling, the daily regimen, as well as the dose, the date and the exact time of the last TKI drug intake were reported on a dedicated drug dosage request form. All relevant information useful for the TDM interpretation was accurately recorded on the form: clinical response, any signs suggesting a clinical toxicity as well as concomitant treatments.

Blood samples were centrifuged (1850 × g (3000 rpm), +4 °C, 10 min) (Beckmann Centrifuge, Model J6B) without delay and the

Table 3
Instrument method for the LC–MS/MS analysis for TKIs with imatinib-D_8, sunitinib-D_{10} and sorafenib-^{13}C D_3 as internal standards.

Drug	Precursor ion (m/z)	Product (m/z)	CE (eV)	Tube lens (V)	Mean RT (min)
Imatinib	494.3	394.1	36	103	4.1
Dasatinib	488.1	401.0	38	115	6.5
Sunitinib	399.1	283.0	34	86	5.4 and 7.6 (Z/E isomers)
Sorafenib	465.1	252.0	47	102	12.7
Nilotinib	530.0	289.0	38	129	7.4
Lapatinib	581.1	364.9	40	127	8.5
Imatinib-D_8 (IS)	502.3	394.1	36	103	4.0
Sunitinib-D_{10} (IS)	409.3	283.0	36	105	5.4 and 7.6 (Z/E isomers)
Sorafenib-^{13}C D_3 (IS)	469.2	256.0	46	135	12.7

The IS imatinib-D_8 was used for the assay of nilotinib, dasatinib and lapatinib. CE: collision energy, Mean RT: retention time determined with the seven standard samples of the calibration curves analysed in duplicate (n = 14). MS acquire time (min) = 18. Q2 Collision gas pressure (mTorr) = 1.00.

plasma was separated and transferred into polypropylene test tubes before storage at −20 °C prior to analysis.

2.6. Plasma sample extraction procedure

A plasma sample aliquot (100 μL) is mixed with a 100 μL of IS solution. The resulting sample is subjected to protein precipitation with acetonitrile (600 μL) and carefully vortex-mixed. The mixture is centrifuged at 4 °C for 10 min at 20,000 × g (14000 rpm) on a benchtop Hettich® Centrifuge (Benchtop Universal 16R centrifuge, Bäch, Switzerland). A 200 μL aliquot of the supernatant and 400 μL of buffer ammonium formate 20 mM pH adjusted to 4.0 with formic acid, are introduced into 1.5 mL glass HPLC microvials which are, after secure closing with aluminium crimp seals, finally vortexed-mixed. Processed samples are maintained at +10 °C in the thermostated autosampler rack during the entire LC–MS/MS analysis.

2.7. Quantification

2.7.1. Calibration curves

Quantitative analysis of the six TKIs in plasma was performed using the IS method. Imatinib-D_8, sunitinib-D_{10} and sorafenib-^{13}C D_3 were used as IS for their target analyte, whereas imatinib-D_8, was also found to be suitable IS for nilotinib, dasatinib and lapatinib. Each level of the calibration curve was measured with two sets of calibrators: one at the beginning and the second at the end of the run. Calibration curves were established with calibration standards prepared with plasma isolated from different batches of outdated transfusion blood (see below Section 2.8.4 matrix effects).

Six-point calibration standard curves have been calculated and fitted by $1/x^2$ weighted quadratic regression of the peak-area ratio of TKIs to IS, versus the concentrations of the respective TKIs in each standard. To determine the best weighting factor, concentrations were back-calculated and the model with the lowest total bias across the concentration range was considered the best suited. The calibration for the six TKIs was established over the range reported in Table 1. The standard curve was chosen to cover the clinically relevant range of concentrations expected in most patients.

2.8. Analytical method validation

The method validation was based on the recommendations published on-line by the Food and Drugs Administration (FDA) [37] as well as on the recommendations of the Conference Report of the Washington Conference on "Analytical methods validation: Bioavailability, Bioequivalence and Pharmacokinetic studies" [38], the Arlington Workshop "Bioanalytical Methods Validation – A revisit with a Decade of Progress" [39]. More recent recommendations form Matuzewski et al. were also considered [40,41].

2.8.1. Accuracy and precision

Replicate analysis ($n = 6$) of quality control samples at the three concentrations (low, medium, high) was used for the intra-assay precision and accuracy determination. The three concentrations were chosen to encompass the whole range of the calibration curve corresponding to the drug levels anticipated to occur in most patient samples. Of importance, the concentration selected for the low (L) QC sample corresponds to three times the respective lower limits of quantification (i.e. the lower calibration level) kept in the finalized method used for the routine analysis of clinical samples, in accordance to the FDA recommendations [37]. Inter-assay accuracy and precision were determined by repeated analysis performed on six different days. The concentration in each sample was determined using calibration standards prepared on the same day. The precision was calculated as the coefficient of variation (CV %) within a single run (intra-assay) and between different assays (inter-assays), and the accuracy as the bias or percentage of deviation between nominal and measured concentration.

During the routine analysis of patient samples, duplicate control samples at three concentration levels (low (L), medium (M) and high (H)) were assayed. The analytical series were considered valid and accepted only if the percentage of deviation (bias) between theoretical and back-calculated (experimental) concentrations for each calibration level and quality control samples were less than ±15%, and less than 20% at the limit of quantification (defined as the lowest calibrator).

2.8.2. Limit of quantification and limit of detection

The lowest levels chosen for calibration curves were selected initially to reflect the lowest clinically relevant concentrations expected to occur in patients, based on published pharmacokinetic data. However, it was observed that our LC–MS/MS instrument was able to attain far higher sensitivity levels. Thus, for the sake of analytical method validation, LOQ values have been determined by establishing calibration curves using serial dilutions (3/4 and 1/2) of the low standard samples of our first calibration curves and were analysed in triplicate (i.e. those diluted samples were used and *integrated* for the establishment of the calibration curves). Back-calculated values of the lowest calibration samples with a bias and CV% below ±20%, enabled the determination of LOQ values, in accordance with the documents mentioned above [37]. The LOQ concentrations were finally established by analysing a series ($n = 3$) of QC samples containing all TKIs at their LOQ level (i.e. those samples were *not integrated* in the calibration curves). Back-calculated values of these QC samples with the established calibration curves enable the formal determination of the LOQ values. The limit of detection (LOD) was defined as the concentration that produced a signal three times above the noise level of a blank preparation.

2.8.3. Stability of TKIs

Stability studies of TKIs included:

(a) Stability of plasma and blood spiked with TKIs kept at room temperature (RT) and in the fridge at +4 °C: their concentrations were measured immediately after preparation and after being left at RT and at +4 °C up to 48 h. TKIs concentrations variations were expressed as a percentage of the initial concentration measured at $T = 0$. Analyses were performed in triplicate.
(b) Stability plasma samples after multiple freeze–thaw cycles: QCs at low, medium and high levels of TKIs underwent two freeze–thaw cycles. Frozen samples were allowed to thaw at room temperature and were subsequently refrozen during approximately 2 h. TKIs levels were measured in aliquots from the two consecutive freeze–thaw cycles.
(c) Stability of plasma samples kept frozen at −20 °C: The response of freshly prepared plasma calibration and QC samples was compared to those of frozen calibration and QCs samples stored during 5 months at −20 °C.

2.8.4. Matrix effects, extraction yield and overall recovery

In the initial step of method validation, the matrix effects were examined qualitatively by the simultaneous post-column infusion of the six TKIs and IS into the MS/MS detector during the chromatographic analysis of six different blank plasma extracts. The standard solution of all analytes at 100 ng/mL and dasatinib and sunitinib at 50 ng/mL, was infused at a flow-rate of 10 μL/min during the chromatographic analysis of blank plasma extracts from six different sources. The chromatographic signals of each selected MS/MS transition were examined to ascertain that no signal perturbation (drift

or shift) of the MS/MS signal was present at the analyte's retention time.

Subsequently, the quantitative determination of the matrix effects was also assessed. Three series of QC samples at L, M, H in duplicates were prepared as follows:

(A) Pure standard solutions samples in the reconstitution solvent (MeOH/buffer 1:1) directly injected onto column.
(B) Plasma extract samples from six different sources, spiked *after* extraction with drugs and IS, as follows: solution of analytes and IS in MeOH were evaporated to dryness into test tubes under a nitrogen flux, prior to addition of the blank plasma extracts.
(C) Plasma samples from six different sources (same as in B) spiked with drug standard solutions and IS *before* extraction.

The recovery and ion suppression/enhancement of the MS/MS signal of drugs in the presence of plasma matrix (i.e. matrix effects) was assessed by comparing the absolute peak areas of analytes either in the reconstitution solvent: MeOH-buffer (ammonium formate 20 mM, pH adjusted to 4.0 with formic acid) 1:2 (A), or added after (B) and before (C) extraction of six different batches of plasma, based on the recommendations recently proposed by Matuszeski et al. [40,41].

The *extraction yield* of TKIs and IS were calculated as the absolute peak-area response in processed plasma samples spiked with drugs *before* extraction (C) expressed as the percentage of the response of the same amount of drugs added into blank plasma extracts *after* the extraction procedure (B) (C/B ratio in %). The matrix effects were assessed as the ratio of the peak areas of analytes added into blank plasma extracts *after* the extraction procedure (B) to the peak areas of analytes solubilized in MeOH–ammonium formate 20 mM pH 4.0 1:2 (A) (B/A ratio in %). The *overall recovery* of TKIs and IS was calculated as the ratio of absolute peak-area response of TKIs either in processed plasma samples spiked with drugs *before* extraction (C) – such as calibration and control samples – to the peak areas of analytes solubilized in MeOH–ammonium formate 20 mM pH 4.0 1:2 (A) (C/A ratio). Recovery studies were done with plasma from six different sources spiked with drugs at concentrations reported in Table 1 (20 ng/mL for Imatinib-D_8). The results normalized with the signal of Imatinib-D_8 (i.e. B2 and C2), used as an index of actual injection volume are also reported in Table 4.

2.9. Selectivity

The assay selectivity was assessed by analysing extracts from 10 batches of blank plasma from different sources.

2.10. Application of the LC–MS/MS method

The proposed method is currently used for the measurement of TKIs levels in patients samples for whom monitoring of total plasma levels is requested by physicians as part of patient's medical follow-up. Blood samples are collected from patients during the medical visit (i.e. at unselected time after drug intake) and are received in our laboratory as part of our TDM Service for TKIs. Otherwise, written informed consents are obtained from patients in case of samples collected within the frame of a research protocol approved by the Institutional Ethics Committee of our University Hospital. This study focuses on the validation of our population pharmacokinetic model [42] and on the extrapolation of imatinib C_{trough} concentration from plasma levels measured in samples taken from patients at random time during the whole dosing interval.

Table 4
Matrix effects, extraction yield, overall recovery and process efficiency of TKIs.

Component	Nominal conc. (ng/mL)	Mean peak area			Mean peak area ratio		ME (%)	CV (%)	Ext RE (%)	CV (%)	Analysis RE (%)	Mean	CV (%)	PE (%)	CV (%)
		A (n = 2)	B (n = 6)	C (n = 6)	B2	C2	B/A		C/B		C2/B2			C/A	
Imatinib	3	36853	33631	35022	0.014	0.014	91.3	8.5	104.1	9.1	102.0	101.8	1.8	95.0	6.2
	2000	18663688	16650076	17591817	6.706	6.942	89.2	5.5	105.7	6.6	103.5			94.3	3.6
	8000	61159272	58706056	59835852	23.646	23.611	96.0	6.2	101.9	9.9	99.9			97.8	6.7
Dasatinib	3	44140	41508	42336	0.017	0.017	94.0	5.5	102.0	6.8	99.9	100.6	1.2	95.9	9.8
	60	499394	426249	434814	0.172	0.172	85.4	9.6	102.0	8.4	99.9			87.1	4.6
	150	1007123	966215	1005524	0.389	0.397	95.9	5.8	104.1	12.6	102.0			99.8	3.5
Nilotinib	3	111888	100763	102052	0.041	0.040	90.1	9.5	101.3	8.4	99.2	102.0	4.4	91.2	3.6
	800	25004912	24413019	24838201	9.833	9.801	97.6	4.0	101.7	4.6	99.7			99.3	6.3
	3000	82221237	72094169	78761157	29.039	31.079	87.7	6.2	109.2	12.7	107.0			95.8	3.6
Lapatinib	15	45637	35966	36871	0.014	0.015	78.8	2.9	102.5	8.6	100.4	101.3	0.9	80.8	11.3
	800	6062505	5581211	5828465	2.248	2.300	92.1	9.5	104.4	6.6	102.3			96.1	6.4
	4000	25202424	24906456	25706626	10.032	10.144	98.8	5.1	103.2	9.1	101.1			102.0	9.7
Sunitinib	3	87028	77510	84219	0.031	0.033	89.1	7.6	108.7	6.6	106.4	99.4	6.1	96.8	8.7
	80	2901178	2720650	2649395	1.096	1.045	93.8	6.6	97.4	5.6	95.4			91.3	9.6
	400	12478227	12128477	11931737	4.885	4.708	97.2	4.1	98.4	8.6	96.4			95.6	6.4
Sorafenib	300	1753082	1901568	1826413	0.766	0.721	108.5	2.3	96.0	4.5	94.1	97.5	3.3	104.2	3.0
	1500	7459456	7040456	7233319	2.836	2.854	94.4	5.0	102.7	5.0	100.7			97.0	5
	6000	20997800	20493582	20448616	8.255	8.069	97.6	3.7	99.8	2.9	97.8			97.4	3.8
Imatinib-D8(IS)	20	2716583	2482691	2534216	1.000	1.000	91.4	9.2	102.1	3.4	100.0	100.0		93.3	8

3. Results

3.1. Chromatograms

The proposed method enables the simultaneous quantification of all major, current and newly introduced TKIs in 100 μL-plasma aliquots by liquid chromatography coupled with tandem MS. A typical chromatographic profile of the highest calibrator sample containing all TKIs is shown in Fig. 2a in the positive ionization mode and using the selected reaction monitoring (SRM) detection mode and the proposed gradient program given in Table 2. Fig. 2b shows the chromatogram of a sample containing TKIs at their lowest limit of quantification. The respective retention times and mass spectrometry conditions for TKIs and the IS imatinib-D_8, sunitinib-D_{10} and sorafenib-^{13}C D_3 are reported in Table 3. There is a satisfactory separation for all considered analytes.

Even if all TKIs were eluted within 14 min, a relatively prolonged rinsing step of 3 min with 100% of acetonitrile +1% formic acid at a flow rate of 0.5 mL/min was introduced to efficiently eliminate the *memory effect* observed in the initial setting-up of the analytical method. This rinsing step was followed by the column conditioning

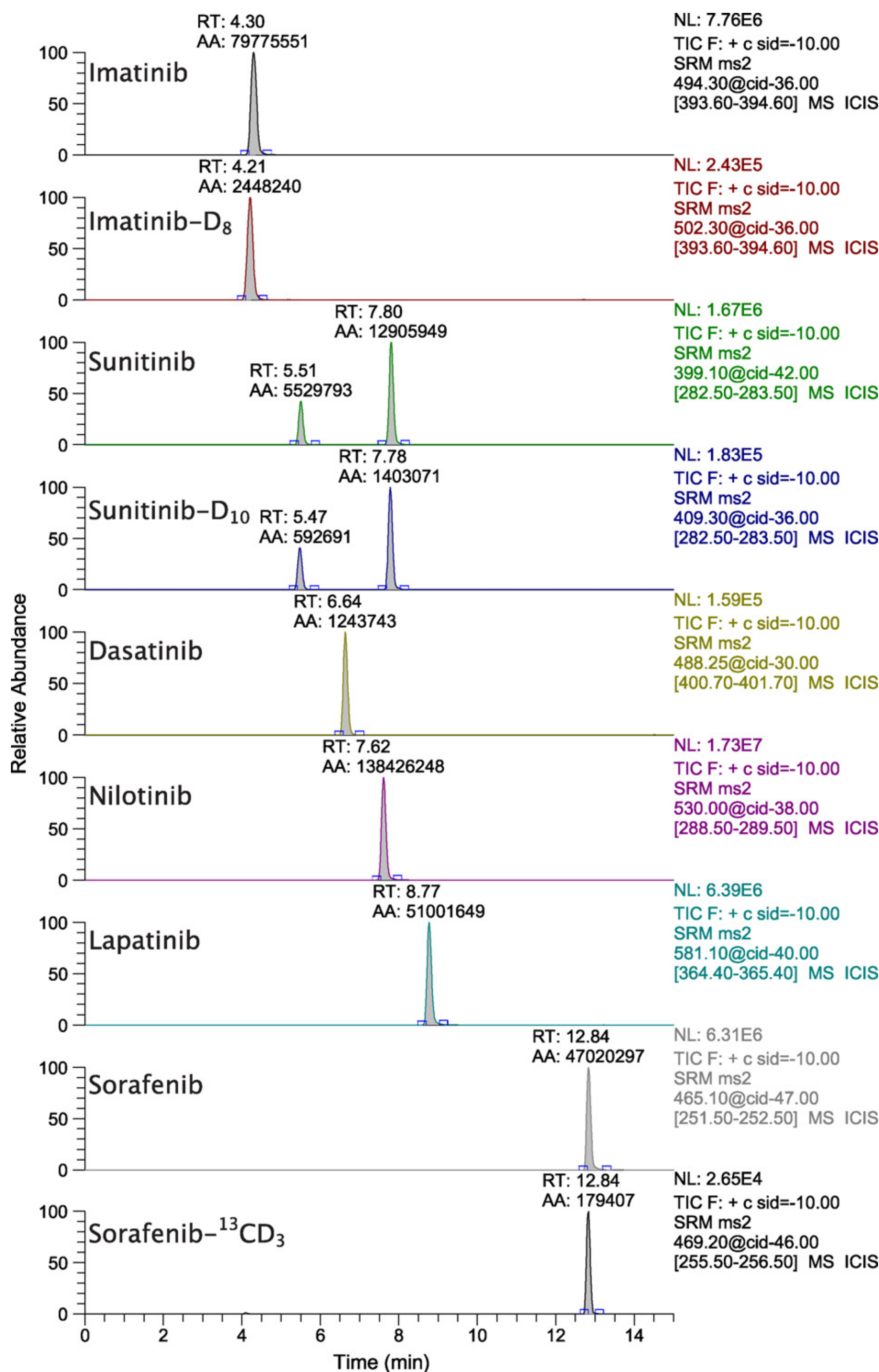

Fig. 2. Chromatogram of (a) the highest and (b) lowest (at LOQ) calibrator sample containing each TKI at concentrations reported in Table 1 (20 ng/mL of IS), in the positive mode. The two signals observed for the transition of sunitinib, corresponds to the *E* and *Z* isomers of sunitinib (details in the text [46]).

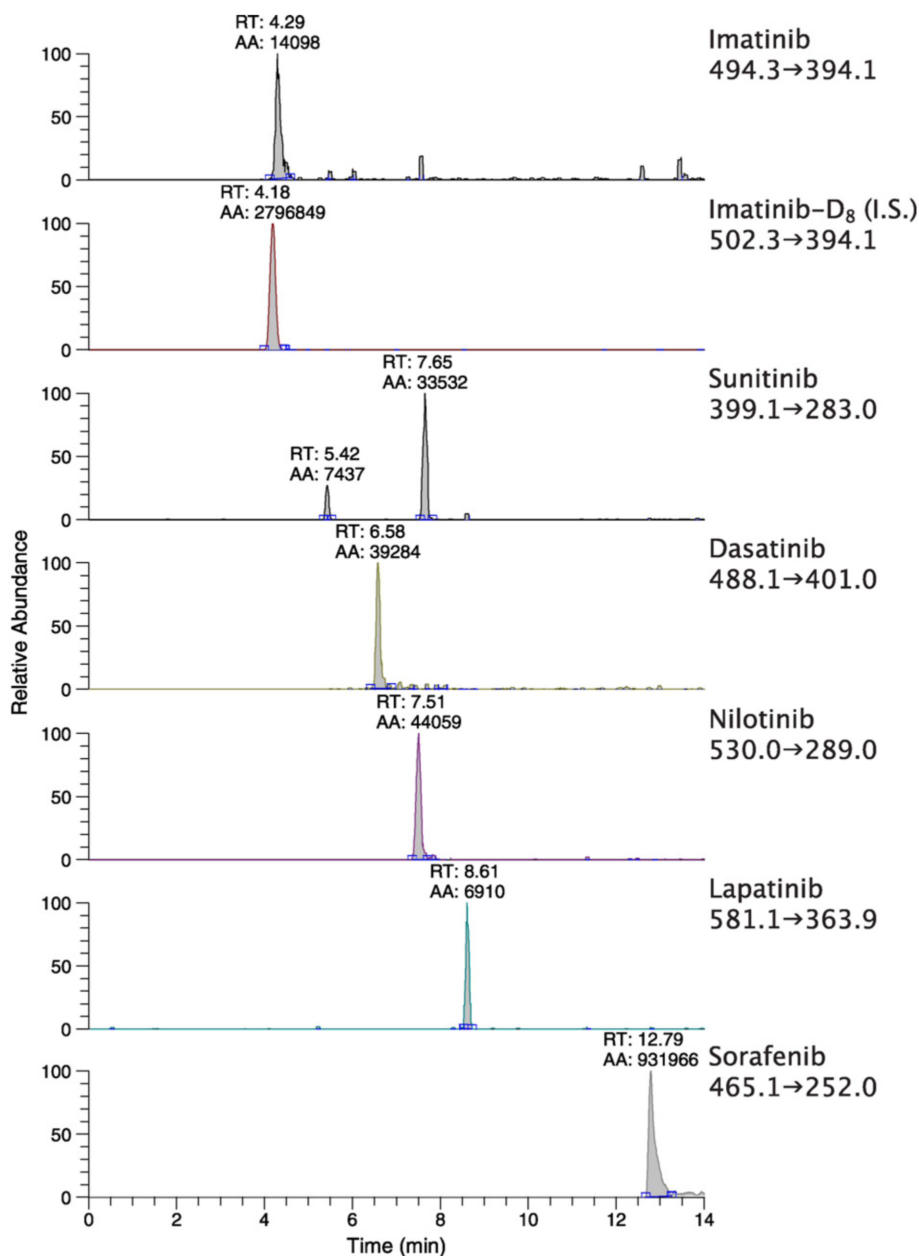

Fig. 2. (*Continued*)

step with the initial solvent composition (95/5 ammonium formate 20 mM pH 2.2/acetonitrile +1% formic acid) at a flow-rate of 0.3 mL/min.

Fig. 3 shows the signals at all selected *m*/*z* transitions when a solution containing all TKIs and IS was continuously infused post-column directly into the MS/MS detector during the chromatographic analysis of six different blank plasma extracts. The signals at the *m*/*z* transition showed a remarkably similar pattern, with all traces being essentially superimposable. No noticeable matrix effects (no drifts or shifts of the signals) could be observed at the respective retention time of the TKIs and IS in this experiment, and no significant matrix effects were found as shown in the experiments reported below (see Section 3.5). Of note, sorafenib is eluted at 12.7 min just before an increase in the selected transition signal appearing between 12.8 and 13.0 min which was therefore not affecting sorafenib signal intensity, as verified in Section 3.5 and in Table 4.

The convenience of having a single analytical method for the simultaneous assay of several TKIs is demonstrated in Fig. 4a–d.

Fig. 4a shows the chromatographic profile of plasma obtained at steady-state from a GIST patient receiving a regimen of imatinib 400 mg once a day (QD). The plasma concentration measured 21 h after drug intake was 803 ng/mL. This concentration appears therefore to lie below the target trough level of 1100 ng/mL 24 h post-dose proposed by Demetri et al., for achieving an optimal response in GIST patients [9,20].

Fig. 4b shows the chromatographic profile of plasma obtained at steady-state from a GIST patient receiving dasatinib 70 mg twice a day (BID). The plasma concentration measured 2.5 h after drug intake was 24 ng/mL. There are no therapeutic intervals nor target plasma concentrations that have been defined for dasatinib yet. However, by comparison with dasatinib pharmacokinetic curves established at steady state in a small number GIST and other solid

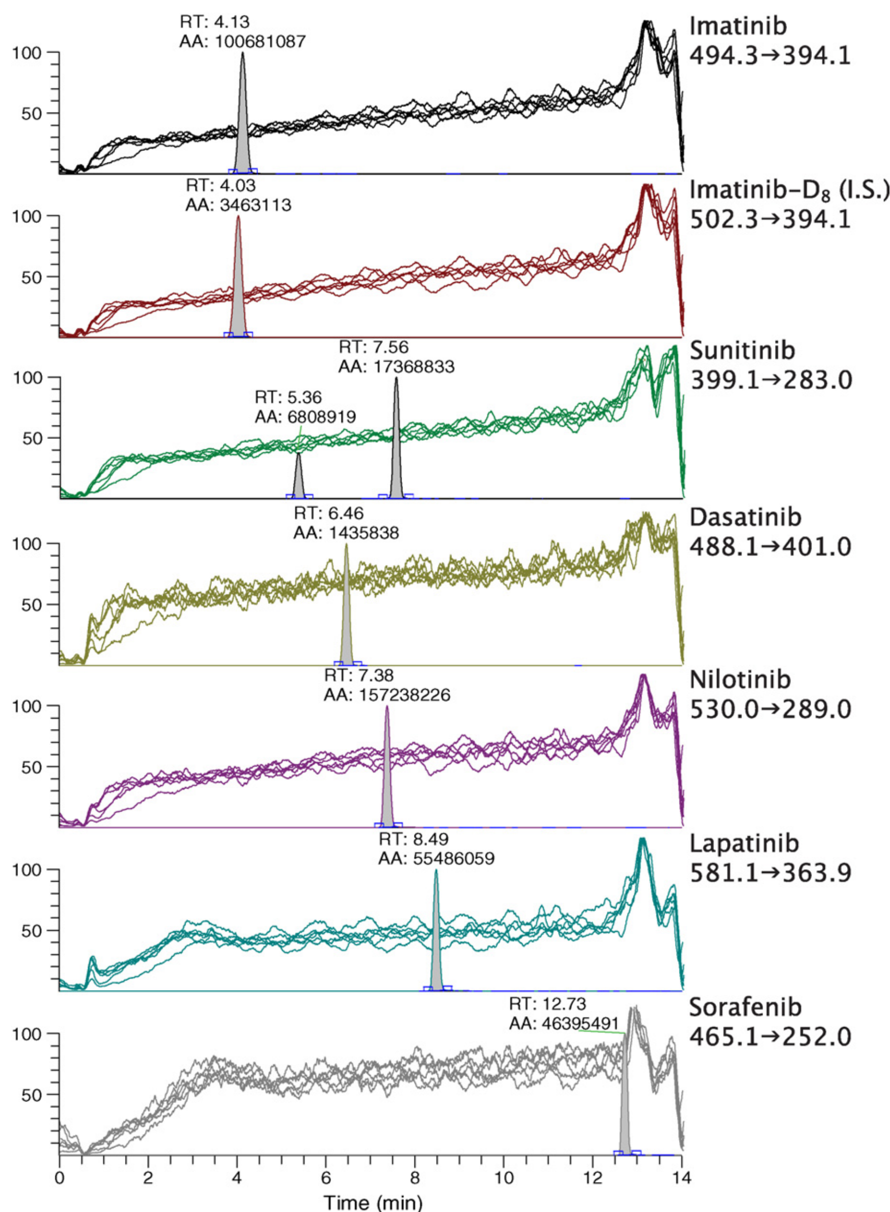

Fig. 3. Chromatogram of six blank extracts with post-column infusion of a calibration sample at 100 ng/mL of each analysed TKI and 20 ng/mL of imatinib-D_8.

tumors patients [43], this concentration seems to be within the range of, or slightly below, the concentrations that have been reported for dasatinib under the 70 mg BID. Of note, dasatinib plasma concentration encountered in patients lie mostly within 1–200 ng/mL, implying that an assay allowing the direct measurement of dasatinib in diluted plasma supernatants constituted an analytical challenge that has been indeed successfully met thanks to the ultimate sensitivity provided by LC tandem MS.

Fig. 4c shows the chromatographic profile of plasma obtained at steady-state from a GIST patient receiving sunitinib 37.5 mg once a day QD. The plasma concentration measured 18 h after drug intake was 65 ng/mL. By comparison with sunitinib pharmacokinetic curves established at steady state in patients advanced refractory malignancies [44], this concentration seems to be overall within the range of concentrations reported for sunitinib under a dosage of 50 mg daily for 2 weeks followed by one week off period. Of note, there are no therapeutic intervals defined for sunitinib yet, but in the sunitinib labeling information [21], a target plasma concentration of 50 ng/mL has been proposed based on preclinical data.

Of note, the observation of the presence of 2 peaks with the same molecular mass/signal transition for sunitinib had never been reported before [45]. The phenomenon was known however [46], and is due to the *Z*–*E* isomerization reaction. Previous studies by sunitinib manufacturer have shown that *E* isomer can be generated from the *Z* isomer in a reversible manner in solution [46]. The rate of inter-conversion between the *Z*–*E* configurations in solution is dependent on a number of factors, most notably exposure to light. In our studies, we found that both stereoisomers could be detected in the pharmaceutical preparation (tablet) at ratios of about 1:2, as well as in patient's plasma samples (variable ratios). Sunitinb calibration curves have been established therefore using the summation of the peak areas of both isomers. This was also applied for the deuterated IS.

Fig. 4d shows the chromatographic profile of plasma obtained at steady-state from a GIST patient receiving sorafenib 400 mg BID. The plasma concentration measured 6 h after drug intake was 6351 ng/mL. There are also no therapeutic intervals defined for sorafenib yet. However, by comparison with sorafenib pharmacokinetic curves established at steady state in patients with advanced

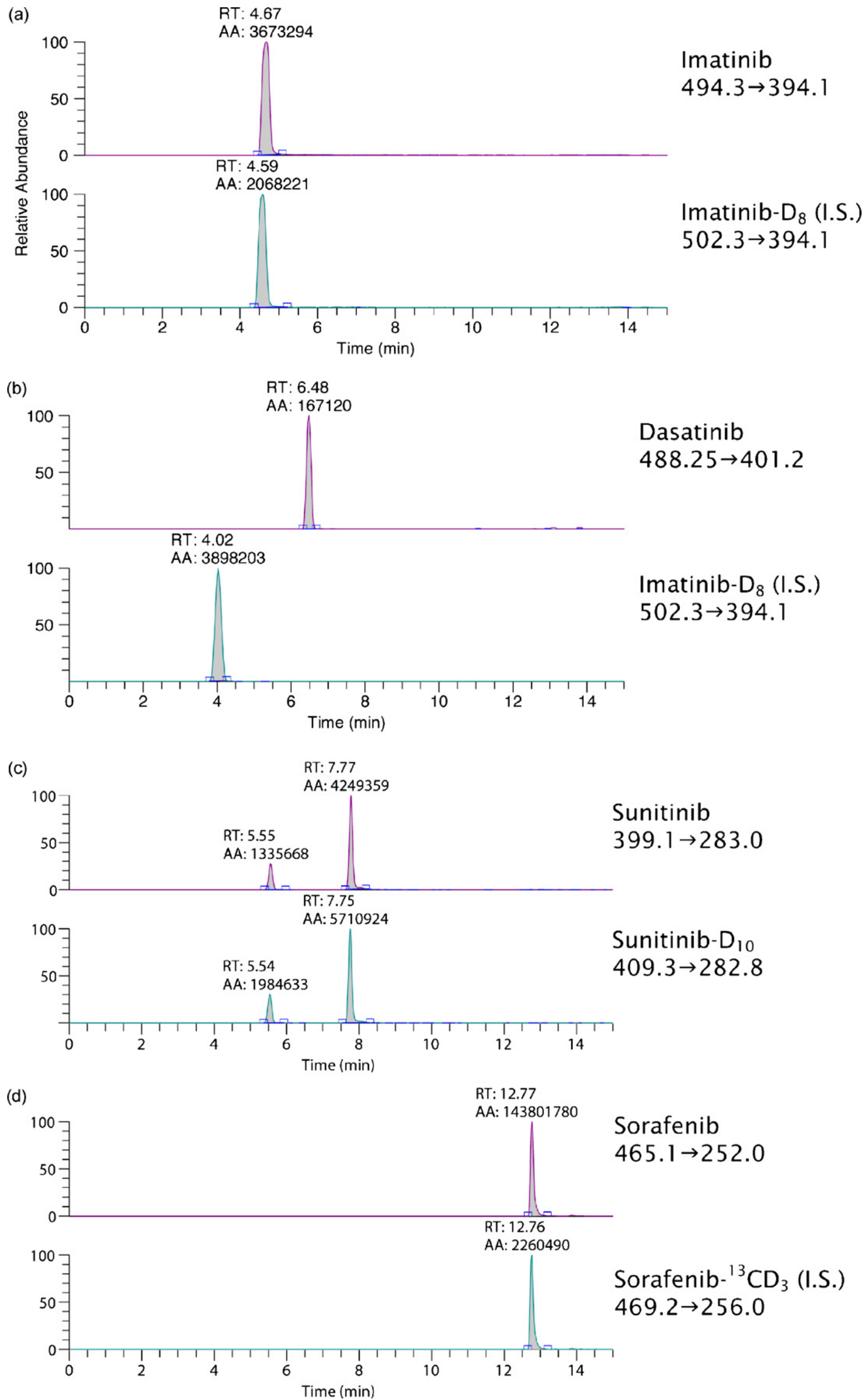

Fig. 4. (a) Chromatographic profile of plasma from patients receiving various regimen of (b), (c) and (d) the TKIs imatinib, dasatinib, sunitinib and sorafenib, respectively (details in the text).

refractory solid tumors [47], this concentration seems to be within the range of concentrations that have been reported for sorafenib under 400 mg BID.

3.2. Internal standard and calibration curve

The choice of the IS is a critical aspect of the method development, because it influences repeatability, reproducibility and accuracy, particularly important aspects when using electrospray mass spectrometry. Ideally, deuterated analogues of the respective TKIs drugs would be the first-choice standards: stable-isotope-labeled analogues for three of them (i.e. imatinib-D_8, sunitinib-D_{10}, sorafenib-^{13}C D_3) and have been therefore introduced during our analytical method validation procedure. The IS lapatinib-^{13}C D_7 (Alsachim, 67400 Illkirch, France) was not available to us at the time we have initiated this analytical development but should certainly be considered as the first choice IS for this drug at present. We have not been able to find any commercial sources for stable-isotope-labeled IS for nilotinib and dasatinib. For all these latter drugs, imatinib-D_8 was used as IS with a satisfactory chromatographic profile, with a negligible memory effect.

Calibration curves over the entire ranges of concentrations delineated in Table 1 were satisfactory described by $1/x^2$ weighted quadratic regression of the peak-area ratio of TKI to their IS, versus the concentrations of the respective TKI in each standard sample. Over the considered concentration range regression coefficient r^2 of the calibration curves were always greater than 0.99 with back-calculated calibration samples within ±15% (±20% at LLOQ).

Of note for sorafenib, the intensity of its IS sorafenib-^{13}C D_3 added to samples was unexpectedly found to steadily decrease with increasing sorafenib calibration levels. The phenomenon of signal suppression of the stable-isotope-labeled IS by increasing concentration of the co-eluting target analyte has been already described [48,49]. This phenomenon of signal suppression effect was described by invoking Enke's model of electrospray generation [48,49]. This model suggests that there is a mutual competition between sorafenib and its IS at the ionization step. However, this reciprocal ionization competition was found not to affect the linearity of calibration curves of sorafenib using the internal standard method, as previously observed for other types of drugs [48,49]. This indicates that sorafenib quantification should not be affected by the variation in the peak area of the IS, as testified by the very low deviation (bias) between nominal and back-calculated (experimental) concentrations for each calibration level and quality control samples (see Table 5) across the entire concentration range.

There was originally some concern that the calibration samples prepared with citrated plasma collected from blood from outdated transfusion bag or from Vaquez patients may not fully reflect the plasma matrix from patients collected on EDTA. However, getting blood on EDTA from volunteers solely for the purpose of calibration samples preparation would be unpractical and difficult to justify from an ethical point of view. For the sake of validation, the cross-validation has been performed between two series of the three levels of QC and two series of calibration samples analysed in duplicate (citrate versus EDTA). Head-to-head comparison shows that the anticoagulant does not influence significantly the analytical results for any TKIs. No statistically significant differences in concentrations were found for QCs samples prepared in EDTA plasma using calibration curves established with citrated plasma samples, or reciprocally (p = 0.77, 0.84, 0.36, 0.72, 0.35 and 0.14, for imatinib, dasatinib, sunitinib, sorafenib, nilotinib and lapatinib, respectively (Student t-test)).

3.3. Precision, accuracy, LOQ and LOD

Precision and accuracy determined with the L, M and H control samples are given in Table 5. The levels of control samples were selected to reflect low, medium and high range of the calibration curves. They were chosen for encompassing the clinically range of concentrations found in patients' plasma. The mean intra-assay precision was similar over the entire concentration range and always less than 9.4%. Overall, the mean inter-day precision was good, with CVs within 1.3% and 9.4%. The intra-assay and inter-assay deviation (bias) from the nominal concentrations of QCs for each considered TKI was comprised between −8.7% and +7.2%, and −9.2% and +9.9%, respectively.

The results of the assessment of LOQ and LOD of TKIs in plasma are given in Table 6 and compare well with those previously reported for these TKIs. By analyzing in triplicate plasma

Table 5
Precision and accuracy of L, M and H QC samples determined by repeated analysis performed on six different days (inter-assays) and within the same day (intra-assay).

	Nominal conc. (ng/mL)	Intra-assay (n = 6)				Inter-assay (n = 6)			
		Concentration found (ng/mL)	SD±	Precision CV (%)	Accuracy bias (%)	Concentration found (ng/mL)	SD±	Precision CV (%)	Accuracy bias (%)
Imatinib	3	3.2	0.2	6.8	7.2	3.2	0.2	6.1	6.5
	2000	1848.1	81.9	4.4	−7.6	1795.8	66.7	3.7	−9.2
	8000	7848.7	320.9	4.1	−1.9	7770.2	370.3	4.8	−2.9
Dasatinib	3	2.9	0.3	9.4	−2.1	3.1	0.3	9.4	2.4
	60	54.7	1.7	3.2	−8.7	56.8	4.4	7.7	−5.3
	150	149.3	7.6	5.1	−0.5	148.2	3.4	2.3	−1.2
Nilotinib	3	3.2	0.1	3.7	5.2	3.1	0.1	4.7	4.2
	800	816.7	48.5	5.9	2.1	832.1	44.3	5.3	4.0
	3000	3054.0	268.3	8.7	1.8	3296.9	271.5	8.2	9.9
Sunitinib	3	2.7	0.04	1.5	−8.7	2.8	0.1	5.1	−5.4
	80	78.9	1.5	1.9	−1.4	79.1	4.8	6.1	−1.2
	400	400.3	8.0	2.0	0.1	398.9	5.2	1.3	−0.3
Lapatinib	15	14.9	1.0	6.8	−0.2	15.7	0.7	4.4	4.5
	800	741.4	8.7	1.2	−7.3	744.2	14.9	2.0	−7.0
	4000	3952.4	171.5	4.3	−1.2	4087.1	195.5	4.8	2.2
Sorafenib	300	276.4	2.4	0.9	−7.8	280	5.4	1.9	−6.6
	1500	1587.6	33.5	2.1	5.8	1584.1	28.6	2.1	5.6
	6000	5540.5	82.8	1.5	−7.7	5513.2	99.8	1.8	−8.1

Table 6
Limit of detection (LOD) and limit of quantification (LOQ) of TKIs. Performance of the analytical method at the LOQ is expressed by the precision and accuracy established after analyses of plasma samples ($n = 3$) containing TKIs at their LOQ levels, at a single occasion.

	LOD (ng/mL)	LOQ (ng/mL)	Accuracy at LOQ (% bias)	Precision at LOQ (CV %)	Minimum quantifiable amount of drug on column (pg)	Lowest reported LOQ (ng/mL)	References
Imatinib	0.3	1	+6.7	4.1	20	10	Titier et al. [26]
Dasatinib	0.3	1	+1.1	9.9	20	1	Dai et al. [34]
Nilotinib	0.4	1	+1.4	6.3	20	5	Pursche et al. [31]
Sunitinib	0.1	1	−1.0	8.1	20	0.6	Minkin et al. [30]
Sorafenib	1	10	−1.9	4.0	200	5	Jain et al. [27]
Lapatinib	0.4	5	−6.3	3.4	100	15	Bai et al. [29]

LOD: Limit of detection, LOQ: Limit of quantification.

Table 7
Evolution of TKIs concentrations in plasma (triplicate analysis).

Drug	Imatinib	Dasatinib	Nilotinib	Sunitinib	Sorafenib	Lapatinib
Nominal concentration (ng/mL)	2000	60	800	80	1500	800
Stability in plasma at RT						
0 h	0	0	0	0	0	0
1 h	−2	−4	7	4	4	8
2 h	8	−11	15	−8	13	13
4 h	6	4	14	13	11	−14
8 h	−15	−3	12	5	12	−15
24 h	−5	−11	13	−1	10	−47
48 h	−7	−15	12	−16	9	−79
Stability in plasma at 4 °C						
0 h	0	0	0	0	0	0
1 h	1	−2	11	10	11	14
2 h	−5	−6	−9	11	3	11
4 h	3	4	3	2	2	1
8 h	3	−11	14	−12	14	10
24 h	6	−7	15	−14	9	1
48 h	−7	−14	13	8	5	−14

samples spiked with decreasing concentrations of TKIs, the lowest achievable LOD among the considered TKIs was 0.1 ng/mL, obtained for sunitinib. The lowest LOQ was obtained for imatinib, dasatinib, sunitinib and nilotinib at 1 ng/mL, corresponding to an amount of 20 pg of drug into the 20 μL-injection volume. Finally, the LOQ for sorafenib was 10 ng/mL using a dynamic range of calibration comprised within 10–5000 ng/mL. However, since the expected concentrations in patient plasma are expected to lie mostly between 100 and 10,000 ng/mL, the lowest calibrator for sorafenib was set at 100 ng/mL [47,50]. Overall, the precision and the accuracy of the lower calibration sample (LOQs) were, for all TKIs, comprised within the ±20% limit recommended by the FDA [37] and the Washington and Arlington Conference Report [39].

3.4. Stability of TKIs in plasma

(a) The stability of TKIs in human plasma samples was ascertained with medium QC samples left at room temperature up to 48 h. The variations over time of each drug are shown in Table 7, and are mostly comprised within the ±15% of starting concentrations indicating that TKIs can be considered stable at RT with the notable exception of lapatinib which shows at room temperature a marked decrease of −47% and −79% after 24 and 48 h, respectively, and −16% for sunitinib after 48 h (Table 7). By contrast, all TKIs in plasma samples left during the same period of time in the fridge at 4 °C were found remarkably stable (Table 7). This indicates that after blood centrifugation without delay, plasma can be conveniently stored temporary at +4C °C

Table 8
Evolution of TKIs concentrations in plasma and in plasma after their addition in anticoagulated whole blood (triplicate analysis).

Drug	Imatinib	Dasatinib	Nilotinib	Sunitinib	Sorafenib	Lapatinib
Nominal concentration (ng/mL)	2000	60	800	80	1500	800
Stability in whole blood at RT						
0 h	0	0	0	0	0	0
1 h	−12	−11	12	−5	−4	11
2 h	−5	−11	14	11	1	11
4 h	−7	−13	9	−4	−6	10
8 h	−2	−11	12	3	−5	6
24 h	−14	−8	11	1	−1	4
48 h	−6	−15	13	3	1	−14
Stability in whole blood at 4 °C						
0 h	0	0	0	0	0	0
1 h	−6	−7	14	−4	−6	13
2 h	−10	−9	2	−7	−11	11
4 h	−7	−11	10	4	−6	13
8 h	−11	−4	6	−4	−8	9
24 h	−13	−12	13	1	−10	15
48 h	−3	−15	10	−14	1	11

Table 9
Stability upon freezing/thawing cycles.

Drug	Imatinib			Dasatinib			Nilotinib		
Nominal concentration (ng/mL)	3	2000	8000	3	60	150	3	800	3000
Freeze–thaw									
Cycle 0	−3.4	−4.4	−2.2	6	10.7	2	5.8	8.9	9.9
Cycle 1	−5.3	−1.1	−3.5	3	0.2	2.5	6.6	− 4.7	10.2
Cycle 2	−4.8	−1.1	−6.1	11	9.9	5.5	5.6	11.1	10

Drug	Sunitinib			Sorafenib			Lapatinib		
Nominal concentration (ng/mL)	3	80	400	300	1500	6000	15	800	4000
Freeze–thaw									
Cycle 0	10	14	12	10.3	10.9	9.1	1	−4	−12
Cycle 1	14	10	11	6.7	8.7	6.6	8	−10	−12
Cycle 2	15	13	14	11.3	4.4	2.3	−8	−11	−11

up to 48 h prior to final storage at −80 °C. During the analysis of patient samples in the laboratory, plasma samples were never allowed to stay more than 1 h at room temperature prior to extraction, indicating that the stability of TKIs in plasma at room temperature is such that the accuracy is not likely to be notably affected.

The evolution of plasma levels after the addition of TKIs at medium QCs concentration to citrated whole blood samples left at room temperature and at +4 °C was ascertained up to 48 h. The evolution over time of the medium QCs concentrations in plasma collected from blood is reported in Table 8. Some decrease in imatinib and dasatinib levels (mean −8% and −11.5%, respectively) could be noticed in plasma from whole blood stored at RT and at 4 °C, without however exceeding the 15% allowance. Conversely, plasma levels of nilotinib at RT and at 4 °C, and lapatinib at 4 °C appear to slightly increase during blood storage for 48 h (mean +10.5% and +12%, respectively). Of note, it is not known at present whether this concentration change observed in the early phase of these *in vitro* experiments may only reflect the evolution of TKIs distribution in and out red blood cells (RBC), as previously observed by our group for the antiviral agent ganciclovir [51,52].

(b) Table 9 shows the variation of TKIs concentrations after two freeze–thaw cycles. For all TKIs the variation was always less than −15% after two cycles.

(c) Calibration and quality control samples are prepared in batches, distributed as 200 μL-aliquots and stored at −20 °C in 1.5 mL-Eppendorf vials for use in our TKIs monitoring facility. The last available series of calibration aliquots have been prepared on September 22, 2008. Samples from this batch have been reanalysed on February 25, 2009 using fresh standards. The results of this comparison are given in Table 10 and confirm the good stability of TKIs in plasma stored frozen at −20 °C after at least 5 months. However, slightly decreased levels were noticeable in the medium and high QC samples for imatinib and sorafenib after 5 months (mean: −8.7% and −6.3%, respectively) but this variation remains always less than the ±15% allowance and should not affect to a clinically relevant extent plasma levels determination.

3.5. Matrix effects and recovery

The extraction recovery obtained using spiked samples is not necessarily an accurate indicator of the true recovery, some solvent mixture may be more appropriate for denaturing cellular proteins, maximizing the analytes extraction at a more favorable pH, resulting in more reproducible analytical results. Among the solvent mixtures tested, the best overall recovery for the six TKIs was obtained after acetonitrile precipitation of plasma, followed by dilution of the supernatant 1:2 with ammonium formate 20 mM pH 4.

The matrix effects were examined by the simultaneous post-column infusion of TKIs and IS into the MS/MS detector during the chromatographic analysis of six different batches of blank plasma extracts from blood donors. As exemplified in Fig. 3, no significant drifts or shifts of the selected transition signals were apparent during the chromatography of blank matrices, at the retention time of the six drugs. As previously mentioned, the transition signal selected for sorafenib shows an increase in intensity between 12.80 and 13.00 min, just after sorafenib elution (Fig. 3), without how-

Table 10
Long-term stability of plasma samples stored at −20 °C.

Drug	Storage duration at −20 °C (months)	Percentage of concentration measured at T_0			Mean percentage of concentrations measured at T_0 (%)
		QC low (%)	QC medium (%)	QC high (%)	
Imatinib	0	100	100	100	100
	5	101.0	92.8	89.9	94.6
Dasatinib	0	100	100	100	100
	5	98.4	98.7	97.3	98.2
Sunitinib	0	100	100	100	100
	5	104.1	98.9	95.9	99.6
Sorafenib	0	100	100	100	100
	5	99.4	94.7	92.7	95.6
Nilotinib	0	100	100	100	100
	5	102.0	94.1	99.8	98.6
Lapatinib	0	100	100	100	100
	5	102.2	97.4	94.9	98.2

ever affecting sorafenib signal intensity, since the mean determined matrix effects with three QCs samples were $100 \pm 7\%$ (Table 4). Moreover, this signal increase was reproducible between the six plasma batches (Fig. 3) and, because all calibrations are prepared in plasma, would be corrected for any possible matrix effects.

The assessment of the matrix effects is reported in Table 4. A value above or below 100% for the matrix effects indicates an ionization enhancement or suppression, respectively. The results indicate that co-eluting plasma matrix components appear not to change the ion intensity for the six TKIs. Moreover, the extraction recovery (B/C) was not affected and results in an excellent sensitivity. Overall, the matrix effects have, to the best of our knowledge, never been investigated to such an extent for all TKIs drugs.

The *analytical recovery* was calculated similarly, considering through drugs response/IS ratio instead, before (C2) and after (B2) the extraction procedure (ratio C2/B2). Taking into account IS response in the calculation enables to correct for the occurrence of any variation over time in the MS/MS spectrometer performance. As indicated in Table 4, the extraction recovery for the IS imatinib-D_8 was essentially identical and above 91.4%. The *analytical recoveries* achieved were always higher than 95.4%. Finally, the overall *recovery* also given in Table 4 takes into account the extraction yield and the matrix effects: for example, imatinib-D_8 has a mean matrix-mediated ionization decrease of 8.6% leading to an overall signal close to 91.4% (column ME), which combined with an extraction yield of 102.1% (Ext RE) gives finally an overall recovery (PE) around 93.3%. Overall, these results indicate that even though no apparent matrix effects could be observed in the infusion experiment (Fig. 2), matrix components do influence the overall process efficiency, which requires therefore the preparation of calibration and control samples in plasma matrix that would reflect at best the composition of the samples to be analysed. Most importantly, this is not so much the matrix effects, but rather its variability that must be reduced to the maximum. As shown in Table 4, the variability of the matrix effects of six different plasma matrix never exceeds 9.6%, which indeed demonstrates that the proposed extraction procedure is able to if not eliminate, at least normalize these matrix effects.

3.6. Selectivity

No peaks from endogenous compounds were observed at the drugs retention time in any of the blank plasma extracts evaluated. The product ion monitoring was chosen, given its relative abundance, while avoiding possible structural analogies with the other drugs or fragments analysed. Every channel was simultaneously observed, and we never notice any selectivity problem as well as any *crosstalk* signal between acquisition channels.

4. Discussion and conclusion

LC–MS/MS has become the method of choice for the measurements of a large range of drugs, some of their metabolites, as well as several biomarkers in biological fluids, offering the best sensitivity, selectivity and enhanced throughput capability. In the present method, the issue of ion suppression and potential matrix effects, and the extend of its variability between samples has been thoroughly investigated (see Table 4 and Fig. 3) based on the recommendations of Matuszewski et al. [40,41].

The proposed method is characterized by a very low limit of quantification (ranging from 1 ng/mL to 10 ng/mL), well below the clinically relevant range of concentration encountered in patients, and required plasma volume as low as 100 μL. The stringent workup for preparation of calibration and QCs plasma samples containing six different drugs is counterbalanced by a simplified extraction step and time sparing offered by the establishment of simultaneous calibration curves. Since experience in the simultaneous analysis of a large number of structurally unrelated TKIs is at present limited, special attention has been given to describe in detail the preparation of calibration and quality control (QCs) samples we used.

This assay reaches the required level of sensitivity, specificity, and reproducibility for routine and large-scale clinical research applications. In that context, the European Leukemia Net (ELN) (www.leukemia-net.org), has decided within the frame of the 'European Treatment and Outcome Study' (EUTOS) initiative (www.eutos.org) to organize a "blood levels monitoring" program for imatinib, and soon for the 2nd generation TKIs nilotinib and dasatinib, all currently used for the targeted treatment of Chronic Myeloid Leukemia (CML). In that context, there is certainly a high interest in the development and validation of an enhanced throughput method using simple extraction method followed by LC–MS/MS technology allowing the simultaneous analysis of all targeted agents (e.g. imatinib, nilotinib and dasatinib) currently used against CML.

Moreover, these drugs are also used in other types of cancer (i.e. GIST), and conversely, a number of additional newer TKIs have been recently introduced in the clinics for various other malignancies, and many more are in the pipe-line. Since all these drugs, because of their mutual pharmacokinetic characteristics (see Section 1), are potential candidates for TDM, it has been therefore decided to develop a general methodology aiming at being applied not only for imatinib, nilotinib and dasatinib used in CML, but for all other major anticancer TKIs, and possibly in the future, combinations thereof. At present, this method is being mostly applied routinely for the monitoring of imatinib, allowing notably to verify whether patients plasma levels lie above those currently proposed for optimal response in CML (i.e. 1000 ng/mL trough levels) [5] and GIST (1100 ng/mL) patients [9,53]. It can also be used to evaluate patient short-term adherence to daily oral therapy known to decrease over time [54,55], and can be used for the identification of drug–drug interactions, and more generally, for clinical studies on the pharmacokinetic/pharmacodynamic relationships of TKIs. In fact, with the notable exception of imatinib, no target values have been defined yet for an optimal clinical response to latest TKIs, except for the target plasma concentration of 50 ng/mL suggested for sunitinib, based on preclinical data [21]. At present, estimation of patients exposure to the more recent TKIs is thus only based on comparison with steady-state pharmacokinetic curves established during phase I or II studies.

In addition, it must be acknowledged that at present, *total* ("bound" plus "free") plasma concentrations is being used for the monitoring of imatinib levels [5,20] and other TKIs. However, only the free (unbound) levels (representing for imatinib only *ca* 1% of total plasma concentration) are expected to penetrate into cells to exert intracellular activities. Since imatinib is principally bound to α-1-acid glycoprotein (AGP) in plasma, any variation of AGP levels – known to be increased during inflammation and advanced state of disease – does influence imatinib pharmacokinetics (clearance and distribution volume [42]) and clinical response [42,56]. Altered free fractions (free to total plasma levels ratios) would thus impact on total concentration–effect relationships and would in some instances compromise the interpretation of TDM results. Thus, unbound drug concentration determination of imatinib (and other TKIs) may therefore provide a more accurate direct indicator of "effective" drug exposure and bring relevant additional information for optimizing the individualization of TKIs dosage regimens. In that perspective, the determination of free plasma concentration for imatinib as well as for other TKIs is currently being developed using a methodology recently published by our laboratory for antiretroviral agents [57].

The method described in this paper covers the major currently in use TKIs, and constitute an improvement over previous methods

in terms of convenience, sensitivity, selectivity and throughput. It may contribute to filling the current knowledge gaps in the pharmacokinetics/pharmacodynamics relationships of the latest TKIs developed after imatinib.

Note added in proof

While in press, an analytical method by HPLC-MS for the simultaneous assay of the antileukemia drugs imatinib, dasatinib and nilotinib in human plasma has been recently published [58].

Acknowledgment

The Swiss National Science Foundation (Switzerland) (grant no. 118222/1) has supported in part the salary of AH. LAD has received a REQUIP grant no. 326000-121314/1 from the Swiss National Science Foundation for the acquisition of a new LC-MS/MS instrumentation. The program *B*iomedizin – *N*aturwissenschaft – *F*orschung (BNF, Universität Bern, Switzerland) is acknowledged for having supported part of the salaries of BZ. Novartis Switzerland has provided support for this project as part of the 'European Treatment and Outcome Study' (EUTOS) initiative of the European Leukemia Net (ELN). We are indebted for the Swiss Group for Clinical Cancer Research (SAKK) for their collaboration within the frame of the clinical project SAKK 56/07.

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