A New HPLC–UV Validated Method for Therapeutic Drug Monitoring of Tyrosine Kinase Inhibitors in Leukemic Patients

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Abstract

Development and validation of simple, rapid, and reliable high-performance liquid chromatography (HPLC)–UV method for quantification of major tyrosine kinase inhibitors, imatinib, dasatinib, and nilotinib, in human plasma is presented. Chromatographic separation of the drugs is achieved on an RP-C^{18} column at flow rate of 0.9 mL/min at 35°C; eluate is monitored at 267 nm. Mean intra-day and inter-day precision for all compounds are 2.5 and 13.3%; mean accuracy is 13.9%; extraction recovery ranges within 40.24 and 81.81%. Calibration curves range from 10 to 0.005 µg/mL. Limits of detection are 10 ng/mL for imatinib and nilotinib, 50 ng/mL for dasatinib; limits of quantitation are 50 ng/mL for imatinib and nilotinib, 100 ng/mL for dasatinib. Although this method allows the detection of dasatinib, levels found in patients plasma are close to the limit of detection, then below the limit of quantitation. Quantification with HPLC–mass spectrometry, then, is required for dasatinib to give a correct evaluation. In conclusion, the sensitivity of this new method is sufficient to perform therapeutic monitoring and pharmacokinetic studies of imatinib and nilotinib but not dasatinib in CML patients.

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder (1) characterized by the presence of the Philadelphia chromosome, a consequence of a reciprocal translocation between the arms of chromosomes 9 and 22, producing a fusion oncogene referred to as BCR-ABL. Current frontline therapy for CML is imatinib (Gleevec™, STI-571), a 2-phenylaminopyrimidine-type competitive inhibitor of Bcr-Abl kinase that competitively inhibits the binding of ATP to the ATP binding pocket of Bcr-Abl (2–4).

Although most patients show excellent responses to imatinib treatment, clinical resistance may occur in approximately 15–20% of chronic phase cases and in a higher percentage in more advanced phases of the disease. Resistance, mainly caused by point mutations, leads to a reduced affinity of imatinib for the ATP binding domain of the Bcr-Abl protein and to a reactivation of the Bcr-Abl kinase activity (5,6). Two potent second generation Bcr-Abl inhibitors, dasatinib and nilotinib, both active against most of the imatinib resistant Bcr-Abl mutants (7), have recently been registered for the treatment of CML in imatinib acquired resistance and intolerance cases. Dasatinib (Sprycel™, BMS-354825) is a structurally distinct drug which has a more potent activity than imatinib (8,9). It also inhibits Src kinases, proteins that play a critical role in the development, growth, progression, and metastasis of a number of human cancers (10).

Nilotinib (Tasigna™, AMN107) is a close analog of imatinib

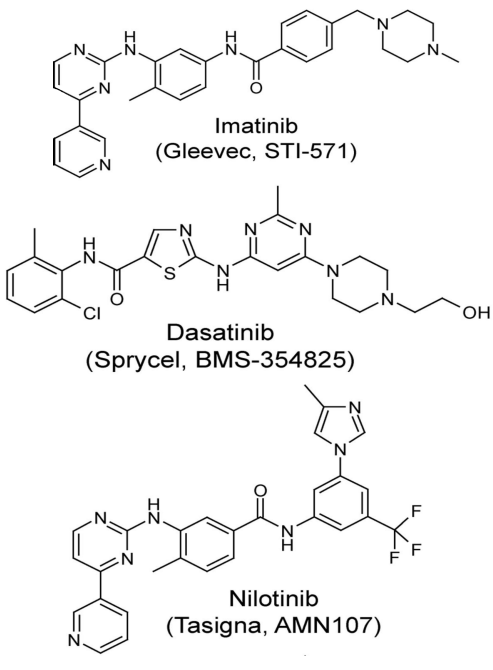

Figure 1. Chemical structures of imatinib, dasatinib, and nilotinib.

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with a higher potency regarding Bcr-Abl kinase inhibition in vitro and in vivo (11,12). Nilotinib is an aminopyrimidine as is imatinib and was rationally designed using structural information from the x-ray structure of the imatinib–Abl complex, with a key feature being the replacement of the *N*-methylpiperazinyl group of imatinib. Imatinib, dasatinib, and nilotinib structures are shown in Figure 1.

In the last few years, numerous laboratories reported the use of liquid chromatography tandem mass spectrometry (LC–MS–MS) instrumentation for the quantification of imatinib. Bakhtiar (3) was the first in 2002 to report an assay capable of fast, sensitive, and robust analyses of imatinib mesylate and its metabolite (CGP 74588) in blood, using a solid-phase extraction (SPE) procedure coupled with LC–MS–MS. The sample pretreatment procedure applied included either an SPE procedure (3) or a protein precipitation step (13). A paper by Titier (14) later reported a method suitable for imatinib quantification in human plasma by high-pressure liquid chromatography (HPLC) tandem MS. By an LC–MS–MS method in 2008, Rochat (15) established quantification of imatinib and the profiling of its metabolites in the plasma of treated patients. Other LC assays using detection methods not based on MS have been published (16–18). Velpandian (16) developed an HPLC method coupled with ultraviolet (UV) detection for the estimation of imatinib in patient's plasma. The assay was conducted using a C^8 column under isocratic elution. Another sensitive method has been developed by Widmer (17) for the assay of imatinib in human plasma, employing an off line SPE followed by HPLC with UV diode array detection. An isocratic online enrichment HPLC assay was then developed by Schleyer (18) that permitted the analysis of imatinib and its main metabolite in plasma, urine, cerebrospinal fluid, culture media, and cell preparations in various concentrations, using UV detection. For the purpose of cellular studies, Guetens (19) described more recently a reverse phase (RP) HPLC–UV method for the simultaneous determination of imatinib and nilotinib. A paper by Pursche (20) reported finally an HPLC–UV method for the quantification of nilotinib in plasma, urine, culture medium, and cell preparations. Based on validated HPLC methods, the pharmacokinetics of imatinib has been well investigated, while only rudimentary informations on the pharmacokinetics of nilotinib, even less for that of dasatinib, are available at present. Kamath (9) developed an LC–MS–MS assay in order to characterize the pharmacokinetics and the metabolism of dasatinib in mouse, rat, dog, and monkey plasma. In a paper by Luo (21), dasatinib plasma concentrations were determined using LC–MS–MS. Herein, the aim of the present study was the development and validation of a simple, sensitive, rapid, and reliable HPLC–UV method, suitable for antileukemia drugs quantification in human plasma. Because MS facilities are not always available in standard hospital laboratories, the reported validation results herein of an inexpensive method for the assay of these drugs are broadly applicable to clinical routine.

Materials and Methods

Chemicals and sample preparation procedures

Imatinib and nilotinib were kindly supplied by Novartis Pharma AG (Basel, Switzerland); dasatinib was purchased from Sequoia Research (Pangbourne, United Kingdom). Acetonitrile (HPLC grade), methanol (HPLC grade), and triethylamine were purchased from VWR International (Milan, Italy). HPLC-grade water was produced with Milli DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Blank plasma from healthy donors was kindly supplied by the Blood Bank of S. Luigi Hospital (Orbassano, Italy). Stock solutions of drugs were prepared at a final concentration of 1 mg/mL in methanol and stored at –20°C for a maximum of 3 months. In dasatinib and imatinib evaluations, the internal standard (IS) was nilotinib; while in the nilotinib evaluation, the IS was imatinib. IS solutions were made at 50 µg/mL in methanol and used immediately. The highest calibration standard (STD8) and the highest quality control (QC5) were prepared by adding a determined volume of the stock solutions to blank plasma; the other STDs and QCs were prepared by serial dilution with blank plasma. The calibration range and QCs concentrations chosen were the same for all drugs (STDs: 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 µg/mL; QCs: 0.05, 0.5, 5 µg/mL). The STDs and QCs were stored at –20°C until analyses, avoiding more than one freeze thaw cycle, and not longer than 3 months. Blood samples were prepared after the separation of the plasma from red cells by 10 min of centrifugation at 1,500 g at 4°C. The drugs were extracted by C18 SPE columns (Grace, Italy); 500 µL of acetonitrile was added to 500 µL of plasma aliquots, spiked with 50 µL of IS. The samples were vortexed and centrifuged at 12,000 g for 15 min; 800 µL of the supernatant were transferred onto C^{18} SPE columns, eluted twice with 250 µL of methanol, and re-suspended in 200 µL of mobile phase after evaporation to dryness.

Chromatographic system and conditions

HPLC was performed with a VWR Hitachi system (LaChrom Elite) equipped with an autosampler, a spectrophotometer, and a heated column compartment. System management and data acquisition were performed with the EzChrom Elite software. The separation was achieved with a C18 RP column (LiChroCART 250-4 LiChrospher 100 RP 18, 5µ, VWR) preceded by a guard column (LiChroCART 4-4 LiChrospher 100 RP 18, 5µ, VWR). The mobile phase consisted of 40% solvent A, 20% methanol, and 40% acetonitrile. Solvent A consisted of water (72.5%), methanol, (25%), and triethylamine (2.5%). The analysis was carried out at the constant flow rate of 0.9 mL/min at 35°C in an isocratic condition. The eluate was monitored at 267 nm.

Method validation

In the validation procedure, each drug was added to blank plasma and extracted using the protocol described herein. Linearity, intra- and inter-day variability, accuracy, and recovery were measured over 3 days. Calibration curves were built with the spike height ratios of each STD and IS, and fitted using linear regression. Intra-day variability was defined as relative standard deviation (RSD), calculated from the values measured from 3 samples performed in duplicate at concentration of 0.05, 0.5, and 5 µg/mL, respectively. Inter-day variability was defined as the RSD calculated using the values measured from 9 different samples (3 samples/day), performed in duplicate at a concentration

of 0.05, 0.5, and 5 µg/mL, respectively. Accuracy was calculated as the medium percent deviation from the nominal concentration from 9 samples performed in duplicate at concentration of 0.05, 0.5, and 5 µg/mL. The percent recovery was obtained from the spike height ratio between the extracted sample and the drug methanol solution at an equal concentration. The limit of detection (LOD) in plasma was defined as the concentration that yielded a signal-to-noise ratio of 3/1; the lowest concentration levels that could be determined with a percent deviation from the nominal concentration, and an RSD < 20% was considered the lowest limit of quantification (LOQ), as requested by international guidelines (22). Interference from endogenous compounds was investigated by analysis of different blank plasma samples.

Table I. Daily Doses of Imatinib, Dasatinib, and Nilotinib Administered to Patients*

Drug	QD (range, mg)	BD (range, mg)	Patients (NS)
Imatinib	200–800	100–400	31 (61)
Dasatinib	40–140	40–70	14 (44)
Nilotinib	200-800	200-400	11 (49)

* QD: once a day; BD: twice a day; NS: number of samples.

Table II. Patients Pharmacokinetic Data*

Drug	Mean C_{trough}* (µg/mL)	Lowest C_{trough} (µg/mL)	Mean C_{max}† (µg/mL)	Upper C_{max} (µg/mL)
Imatinib	1.226 (n:31)	0.274 (n:31)	1.743 (n:17)	4.273 (n:17)
Dasatinib	0.078 (n:14)	0.000 (n:14)	0.215 (n:12)	1.567 (n:12)
Nilotinib	0.586 (n:11)	0.000 (n:11)	0.919 (n:10)	1.374 (n:10)

* C_{trough}: drug concentration measured 24 h (once daily administration) or 12 h (twice daily administration) after last drug intake.
† C_{max}: maximum concentration among several measurements at steady state; *n*: number of patients.

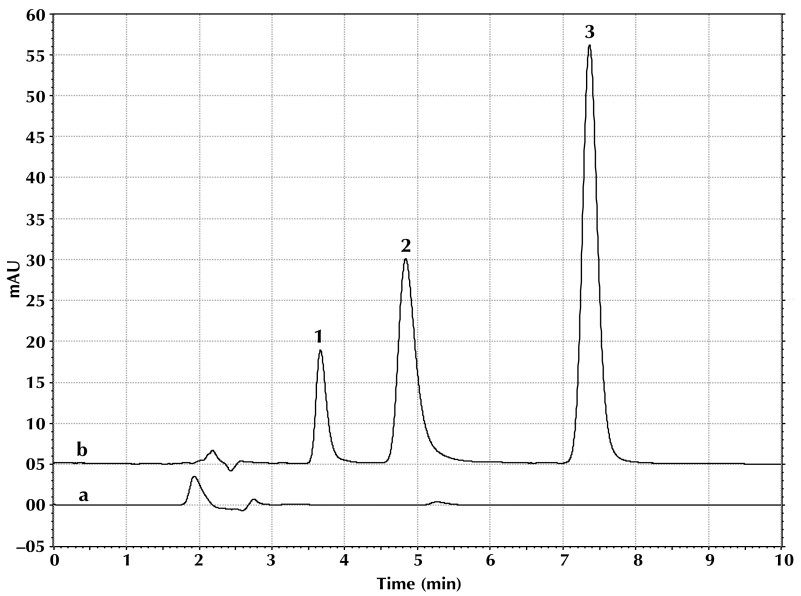

Figure 2. Chromatograms of mobile phase injection (a) and mixture of 5 µg/mL dasatinib, 1; imatinib, 2; and nilotinib, 3 (b). Analyte retention times were 5.4 for imatinib, 3.8 for dasatinib, and 7.9 for nilotinib.

Leukemic patients

The blood samples were obtained from the CML patients, followed by this study's institution, and treated at different drug doses, as shown in Table I. The pharmacokinetic data of the patients are reported (Table II) as the mean and the lowest C_{trough} (the drug concentration measured 24 h after the last drug intake in a once a day administration and 12 h after the last drug intake in a twice a day administration) and the mean and upper C_{max} (the maximum concentration among several measurements at a steady state).

Results

Method validation

The time of the analytical run was chosen as 10 min, according to the retention times of the substances and their good separation. Representative chromatograms are shown in Figures 2 and 3. In Figure 2, chromatograms are shown of the mobile phase injection (A) and the mixture of 5 µg/mL dasatinib (1), imatinib (2), and nilotinib (3) (B). In Figure 3, chromatograms are shown of the blank human plasma (A) and 10 µg/mL dasatinib (1), imatinib (2), and nilotinib (3) extracts from human plasma (B). In spite of the low wavelength employed, no significant interference were observed in the baseline and blank chromatograms for each specified drug detected. Over the concentration range from 0.005 to 10 µg/mL, the regression analysis indicated a good linearity for each drug (imatinib: $r > 0.999$, dasatinib: $r > 0.999$, nilotinib: $r > 0.998$). Validation data (intra- and inter-day variability, accuracy, and recovery measurements) are summarized in Table III. The LOD was 10 ng/mL for imatinib and nilotinib and 50 ng/mL for dasatinib; the LOQ was 50 ng/mL for imatinib and nilotinib and 100 ng/mL for dasatinib.

Leukemic patients

The pharmacokinetic data of the patients are listed in Table II. Values obtained for all drugs resulted in the expected range of concentrations according to the data present in the literature (11,14,18,23).

Discussion

Therapeutic drug monitoring (TDM) has become an essential tool for the management of CML patients. The measurement of antileukemic drug plasma concentrations, in fact, can be useful to evaluate patient adherence to daily oral therapy, potential drugs interactions, treatment efficacy, and severe drug related adverse events (24–26).

Regarding drugs interactions, in vitro studies have shown that imatinib is mainly metabolized by the cytochrome P450 3A4 (CYP3A4), and in addition, it also competitively inhibits 2C9 and 2D6 isoforms. Even if only limited information for dasatinib and nilotinib are available to date, these drugs are also extensively metabolized in the liver by CYP3A4. This enzymatic complex, consequently, is implicated not only in different drugs kinetics, but it is also responsible for various drugs interactions. Being susceptible to induction or inhibition by numerous co-medications, environmental, and dietary constituents, its activity can lead to a large inter-individual variability. In light of these observations, pharmacokinetics/pharmacodynamics correlation studies on CYP3A4 affecting drugs gain importance.

Drugs such as imatinib, dasatinib, and nilotinib must be taken daily and for years. Thus, quantification of these drugs concentrations in plasma treated patients can be a useful tool in clinical CML patient management. At present, some reports show that the imatinib C_{trough} should be more than 570 ng/mL to insure efficacy (27).

More recently, Picard established a higher threshold (about 1000 ng/mL) for achievement of major molecular response (28). The possibility to quantify accurately imatinib drug concentration reached from CML patients, then, can allow for safer treatment, in terms of adverse events control and therapy efficacy maintaining. If a threshold for the efficacy of treatment with imatinib is defined, for dasatinib and nilotinib this information has not been yet achieved. Very few studies are available up to now about nilotinib pharmacokinetics, but the debate for a hypothetical threshold is still open (12). Even less information is available in literature for dasatinib. This observation suggests that the possibility to analyze dasatinib and nilotinib concentrations reached from CML patients can be useful to obtain information about the pharmacokinetic/pharmacodynamic relationship and to assess the concentration response to the relationship. In order to strictly monitor the behavior of these drugs, a method that allows a simple and reproducible assay, which is easily applicable in many laboratories for routine clinical use, should be used. Several reports describe methods using HPLC–MS, but this technology is not yet widely available. The work herein shows that HPLC–UV methodology can be usefully applied instead of HPLC–MS for monitoring of imatinib and nilotinib plasma concentration, but that is not applicable for dasatinib quantification.

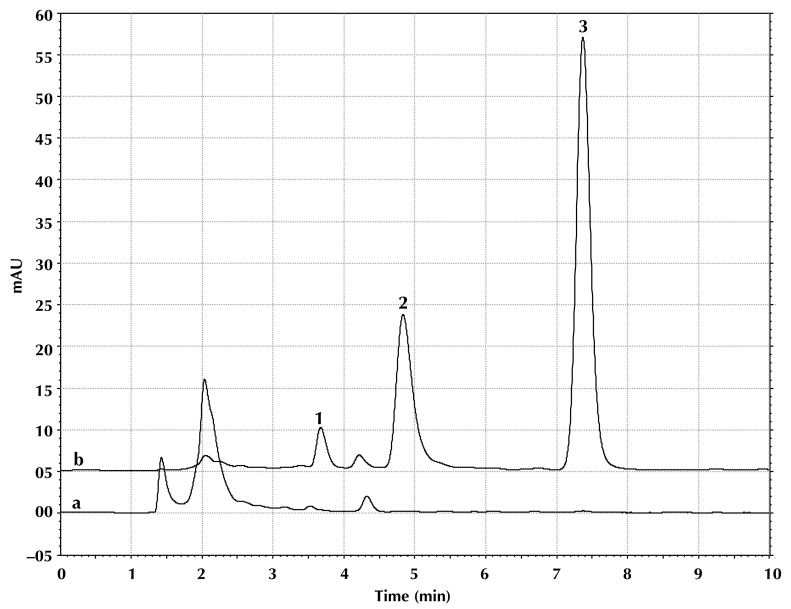

Figure 3. Chromatograms of blank human plasma (a) and 10 µg/mL dasatinib, 1; imatinib, 2; and nilotinib, 3 extracts from human plasma (b). Analyte retention times were 5.4 for imatinib, 3.8 for dasatinib, and 7.9 for nilotinib.

Conclusions

The reliability of this method has been demonstrated for all drug concentrations; linearity, intra- and inter-day variability, accuracy, and recovery indicate the good performance of the developed method. The absence of the interfering peaks at the analyte retention times allowed for the accurate measurement of the plasma levels of the drugs. The LOQ (50 ng/mL) for imatinib and nilotinib makes this method convenient to perform TDM in CML patients. A different consideration should be done for dasatinib. Although this method allows the detection of the drug, levels found in patients plasma were close to the LOD (50 ng/mL), and then below the LOQ (100 ng/mL), so quantification with HPLC–MS is required for dasatinib to give a correct evaluation. In conclusion, the sensitivity of this new method is sufficient to perform TDM of imatinib and nilotinib, but not dasatinib in CML patients.

Table III. Validation Data

	0.05 µg/mL			0.5 µg/mL			5 µg/mL			
Drug	Variability Intra-day (*n* = 6)	(RSD%) Inter-day (*n* = 18)	Accuracy % (*n* = 18)	Variability Intra-day (*n* = 6)	(RSD%) Inter-day (*n* = 18)	Accuracy % (*n* = 18)	Variability Intra-day (*n* = 6)	(RSD%) Inter-day (*n* = 18)	Accuracy % (*n* = 18)	Recovery % (*n* = 3)
Imatinib	8.57	19.87	23.33	1.34	12.39	11.23	0.62	10.45	7.05	76.41
Dasatinib	3.97	18.47	14.00	1.97	12.45	10.50	0.26	5.92	6.51	40.24
Nilotinib	4.39	14.43	30.50	0.54	16.12	13.43	0.80	9.68	8.23	81.81

* *n*: number of samples

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Manuscript received May 11, 2010;
revision received August 19, 2010.