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A new HPLC-UV validated method for therapeutic drug monitoring of tyrosine kinase inhibitors in leukemic patients.

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Keywords: imatinib; dasatinib; nilotinib; HPLC UV.

1 Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative disorder [1] characterized by the presence of the Philadelphia chromosome, consequence of a reciprocal translocation between 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 with higher potency regarding Bcr-Abl kinase inhibition \textit{in vitro} and \textit{in vivo} \cite{11,12}. Nilotinib is an aminopyrimidine as imatinib is 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 years, numerous laboratories reported the use of Liquid Chromatography tandem Mass spectrometry (LC-MS) instrumentation for the quantification of imatinib. Bakhtiar \cite{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. Sample pretreatment procedure applied included either an SPE procedure \cite{3} or a protein precipitation step \cite{13}. A paper by Titier \cite{14} reported later a method suitable for imatinib quantification in human plasma by High Pressure Liquid Chromatography (HPLC) tandem MS. By an LC MS/MS method Rochat \cite{15} in 2008 established quantification of imatinib and the profiling of its metabolites in plasma of treated patients. Other LC assays using detection methods not based on MS have been however published \cite{16-18}. Velpandian \cite{16} developed an HPLC method coupled with ultraviolet (UV) detection for estimation of imatinib in patients plasma. The assay was conducted using a C8 column under isocratic elution. Another sensitive method has been developed by Widmer \cite{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 \cite{18}, permitting 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 \cite{19} described more recently a reverse phase (RP) HPLC-UV method for the simultaneous determination of imatinib and nilotinib. A paper by Pursche \cite{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, pharmacokinetics of imatinib has been meanwhile well investigated, while only rudimentary informations on the pharmacokinetics of nilotinib, even less for that of dasatinib, are available at present. Kamath \cite{9} developed an LC-MS assay in order to characterize pharmacokinetics and metabolism of dasatinib in mouse, rat, dog, and monkey plasma. In a paper by Luo \cite{21} dasatinib plasma concentrations
were determined using LC-MS/MS. Herein, aim of 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.

2 Materials and Methods

2.1 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 final concentration of 1 mg/ml in methanol and stored at −20°C maximum for 3 months. In dasatinib and imatinib evaluation internal standard (IS) was nilotinib, in nilotinib evaluation 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 adding a determined volume of stock solutions to blank plasma; the others STDs and QCs were prepared by serial dilution with blank plasma. 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). 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 separation of plasma from red cells by 10 min centrifugation at 1,500 g, 4°C. Drugs were extracted by C18 SPE columns (Grace, Italy); 500 μl of acetonitrile were added to 500 μl of plasma aliquots spiked with 50 μl of IS. Samples were vortexed and centrifuged at 12,000 g for 15 min; 800 μl of supernatant were transferred onto C18 SPE columns, eluted twice with 250 μl of methanol and re-suspended in 200 μl of mobile phase after evaporation to dryness.
2.2 Chromatographic system and conditions

HPLC was performed with a VWR Hitachi system (LaChrom Elite) equipped with autosampler, spectrophotometer, and heated column compartment. System management and data acquisition were performed with the EzChrom Elite software. 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). Mobile phase consisted of 40% solvent A, 20% methanol, 40% acetonitrile. Solvent A consisted of Water (72.5%) Methanol (25%) and Triethylamine (2.5%). Analysis was carried out at the constant flow rate of 0.9 ml/min at 35°C in isocratic condition. The eluate was monitored at 267 nm.

2.3 Method validation

In validation procedure each drug was added to blank plasma and extracted using the protocol described above. 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, 5 μg/ml, respectively. Inter-day variability was defined as RSD calculated using the values measured from 9 different samples (3 samples/day) performed in duplicate at concentration of 0.05, 0.5, 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, 5 μg/ml. Percent recovery was obtained from the spike height ratio between extracted sample and drug methanol solution at equal concentration. Limit of detection (LOD) in plasma was defined as the concentration that yields signal to noise ratio of 3/1; lowest concentration levels that could be determined with a percent deviation from the nominal concentration and 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.

2.4 Leukemic patients

Blood samples were obtained from CML patients, followed by our institution, treated at different drug doses,
as shown in table I. Pharmacokinetic data of patients are reported (table II) as mean and lowest Ctrough (drug concentration measured 24h after last drug intake in once a day administration and 12h after last drug intake in twice a day administration) and mean and upper Cmax (maximum concentration among several measurements at steady state).

3 Results

3.1 Method validation

Time of analytical run was chosen as 10 min, according to the retention times of substances, and their good separation. Representative chromatograms are shown in figures 2 and 3. In figure 2 are shown chromatograms of mobile phase injection (a) and mixture of 5 µg/ml dasatinib (1), imatinib (2) and nilotinib (3) (b). In figure 3 are shown chromatograms of 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 baseline and blank chromatograms for each specified drug detected. Over the concentration range from 0.005 to 10 µg/ml 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. LOD was 10 ng/ml for imatinib and nilotinib, 50 ng/ml for dasatinib; LOQ was 50 ng/ml for imatinib and nilotinib, 100 ng/ml for dasatinib.

3.2 Leukemic patients

Pharmacokinetic data of patients are listed in table II. Values obtained for all drugs resulted in the expected range of concentrations according to data present in literature [11, 14, 18, 23].

4 Discussion

Therapeutic drug monitoring (TDM) has become an essential tool for the management of CML patients. Measurement of antileukemic drugs 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, also these drugs are extensively metabolized in 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 as imatinib, dasatinib and nilotinib must be taken daily and for years. Thus, quantification of these drugs concentrations in plasma treated patients can be useful tool in clinical CML patients management. At present some reports showed that the imatinib C\text{trough} should be more than 570 ng/ml to insure efficacy [27]. More recently Picard established an 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 to treat them more safely, in terms of adverse events control and therapy efficacy maintaining. If a threshold for 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 an hypothetical threshold is still open [12]. Even less informations are 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 informations about pharmacokinetics/pharmacodynamics relationship and to assess concentration response relationship. So, in order to monitor strictly behaviour of these drugs, a method which allows a simple and reproducible assay, 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. Our work 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.

5 Conclusions

Reliability of our method has been demonstrated for all drug concentrations; linearity, intra- and inter-day variability, accuracy and recovery indicate the good performances of method developed. Absence of interference peaks at the analyte retention times allowed accurate measurement of drugs plasma levels. The
LOQ (50 ng/ml) for imatinib and nilotinib makes our 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 are close to LOD (50 ng/ml) and then below 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.

References


Table 1

Daily doses of imatinib, dasatinib and nilotinib administered to patients*

<table>
<thead>
<tr>
<th>Drug</th>
<th>QD (range, mg)</th>
<th>BD (range, mg)</th>
<th>Patients (NS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>200-800</td>
<td>100-400</td>
<td>31 (61)</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>40-140</td>
<td>40-70</td>
<td>14 (44)</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>200-800</td>
<td>200-400</td>
<td>11 (49)</td>
</tr>
</tbody>
</table>

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

Table 2

Patients pharmacokinetic data

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Mean Ctrough * (µg/ml)</th>
<th>Lowest Ctrough (µg/ml)</th>
<th>Mean Cmax † (µg/ml)</th>
<th>Upper Cmax (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib</td>
<td>1.226 (n:31)</td>
<td>0.274 (n:31)</td>
<td>1.743 (n:17)</td>
<td>4.273 (n:17)</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>0.078 (n:14)</td>
<td>0.000 (n:14)</td>
<td>0.215 (n:12)</td>
<td>1.567 (n:12)</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>0.586 (n:11)</td>
<td>0.000 (n:11)</td>
<td>0.919 (n:10)</td>
<td>1.374 (n:10)</td>
</tr>
</tbody>
</table>

* Ctrough: drug concentration measured 24 h (once daily administration) or 12 h (twice daily administration) after last drug intake.
† Cmax: maximum concentration among several measurements at steady state;

n: number of patients.

Table 3

Validation data

<table>
<thead>
<tr>
<th>Drugs</th>
<th>0.05 µg/ml Variability (RSD %)</th>
<th>Accuracy (%)</th>
<th>0.5 µg/ml Variability (RSD %)</th>
<th>Accuracy (%)</th>
<th>5 µg/ml Variability (RSD %)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday (n=6)</td>
<td>Interday (n=18)</td>
<td></td>
<td>Intraday (n=6)</td>
<td>Interday (n=18)</td>
<td></td>
</tr>
<tr>
<td>Imatinib</td>
<td>8.57</td>
<td>19.87</td>
<td>23.33</td>
<td>1.34</td>
<td>12.39</td>
<td>11.23</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>3.97</td>
<td>18.47</td>
<td>14.00</td>
<td>1.97</td>
<td>12.45</td>
<td>10.50</td>
</tr>
<tr>
<td>Nilotinib</td>
<td>4.39</td>
<td>14.43</td>
<td>30.50</td>
<td>0.54</td>
<td>16.12</td>
<td>13.43</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n=3)</td>
</tr>
<tr>
<td>Imatinib</td>
</tr>
<tr>
<td>Dasatinib</td>
</tr>
<tr>
<td>Nilotinib</td>
</tr>
</tbody>
</table>

* n: number of samples
Figure 1

Chemical structures of imatinib, dasatinib and nilotinib
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.
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.