A SIMPLE AND RAPID METHOD FOR THE QUANTIFICATION OF IMATINIB MESYLA TE AND DESMETHYL IMATINIB IN HUMAN PLASMA USING LC-MS/MS AND ITS APPLICATION TO ROUTINE THERAPEUTIC DRUG MONITORING

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ABSTRACT

Introduction: Imatinib mesylate is the standard of care in the treatment of chronic myeloid leukemia. Therapeutic drug monitoring (TDM) can be used as a tool to cross-check treatment adherence or toxic manifestations to imatinib mesylate in chronic myeloid leukemia patients. The study aimed to develop a simple, cost effective and rapid method for quantification of imatinib mesylate and desmethyl imatinib in human plasma using LC-MS/MS. Methods: The biological samples were prepared by a simple protein precipitation method and separated using a XTerra MS C18 column. Imatinib and its metabolite desmethyl imatinib was quantified using multiple reaction monitoring (MRM) with a triple quadrupole tandem mass spectrometer working in the positive electrospray ionization (ESI+) mode using verapamil as internal standard. RESULTS: The method was validated and found to be precise and accurate within the dynamic range of 5ng-8µg/mL for the drug and 5ng-1µg/mL for the metabolite. The method was then applied to routine therapeutic drug monitoring of CML patients and the mean±SD trough level concentration was found to be 1437±459 ng/mL.
CONCLUSION: This simple and rapid LC-MS/MS method was validated to be sensitive, specific, precise and accurate for the quantification of imatinib and its metabolite in human plasma. The method was found to be cost effective for implementing in routine TDM process.

KEY WORDS: Therapeutic drug monitoring; Imatinib mesylate; Liquid chromatography-Mass spectrometry.

1. INTRODUCTION
Imatinib mesylate is a tyrosine kinase inhibitor which has profoundly improved the prognosis of chronic myeloid leukemia (CML)[1]. Regardless of its excellent success, some subset of patients did not respond to the treatment. Pharmacokinetic variability is considered to be one of the reasons for this inter patient variability[2]. Therapeutic drug monitoring (TDM) is warranted in patients with treatment failure and suboptimal response. The dose of imatinib can be modified in case of poor response based on the observations made from routine therapeutic drug monitoring data[3, 4]. Many bio-analytical methods have been published regarding the quantification of imatinib and its metabolite desmethyl imatinib in human plasma. Most of the methods use deuterated imatinib as internal standard whose cost is high[5-10]. The application of the methods involving d-8 compound as internal standard and lengthy extraction methods are of much difficulty in day to day processing of patient samples. The bio-analytical method for imatinib and its metabolite described in the current article was developed with the intention to i) identify a substitute for Imatinib d-8 which is cost effective and easily available ii) develop an easier extraction procedure from plasma samples. The method was then validated according to the FDA guidelines[11]. Further the method was successfully applied to routine therapeutic drug monitoring for chronic myeloid leukemia patients.

2. Experimental
2.1 Chemicals and reagents
Pure form of imatinib mesylate was supplied by Naprod Life sciences Pvt. Ltd, (Mumbai, India) and desmethyl imatinib was procured from Synfine Research Inc., Canada. Verapamil (Torrent Pharmaceuticals, India) was selected as the internal standard based on its physicochemical properties and low cost availability. The chemicals like acetonitrile, formic acid and methanol were procured from Sigma Aldrich (Bangalore, India). Milli-Q water (Millipore Corporation) was used for the entire process.
2.2 Instrumentation
The LC-MS/MS analysis was performed with Waters e2695 high-performance liquid chromatography (HPLC) system connected to a Acquity TQD triple quadrupole mass spectrometer with an electrospray ionization (ESI) source operated in positive ion mode (Waters, Milford, MA, USA). Masslynx software Version 4.0 (Waters, USA) was used for instrument control and quantitation analysis.

2.3 LC-MS/MS conditions
The separation of compounds was performed on an X Terra MS C18 column (3.9mmx150 mm, 5µm; Waters, Milford, MA, USA). The mobile phase used for the separation was 0.1% formic acid in MilliQ water as (A) and methanol alone (B) at 0.6 mL/min with the gradient flow as given in Table 1. The column temperature was 40 °C and the injection volume was 20 µL. The mass spectrometer was operated in positive electrospray ionization (ESI+) mode. Nitrogen was used as the desolvation gas at a flow rate of 800 L/h and a temperature of 350 °C. The source temperature was set at 120 °C. The drug, metabolite and the internal standard were detected and quantified using Multiple reaction monitoring (MRM) functions.

Table 1: Gradient flow of mobile phase used for the separation

<table>
<thead>
<tr>
<th>Time</th>
<th>A</th>
<th>B</th>
<th>Flow (mL/min)</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 min</td>
<td>75</td>
<td>25</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>0.1 min</td>
<td>25</td>
<td>75</td>
<td>0.6</td>
<td>1</td>
</tr>
<tr>
<td>2.5 min</td>
<td>75</td>
<td>25</td>
<td>0.6</td>
<td>11</td>
</tr>
</tbody>
</table>

*A – 0.1% Formic acid in MilliQ water & B- Methanol (100%)

2.4 Solutions and standards
The stock solutions of imatinib mesylate, desmethyl imatinib and verapamil (IS) were prepared in methanol to yield a concentration of 1.0 mg/mL. The working solutions for calibration curve were prepared over a range of 5 ng/mL to 8 µg/mL and 5 ng/mL to 1 µg/mL for imatinib and desmethyl imatinib respectively by serial dilutions from the stock solution. The quality control (QC) working solutions were 10 ng/mL, 150 ng/mL, 7.50 µg/mL and internal standard concentration was 100 ng/mL in 50% methanol. Stocksolutions were kept at -80 °C when not in use.

2.5 Sample preparation
The sample preparation from plasma was carried out using a simple one step protein precipitation method using acetonitrile. To 500 µL of plasma 50 µL of internal standard
working solution (100 ng/mL) was added and vortexed (30 s), followed by the addition of 1000 µL of acetonitrile. The whole solution was then vortexed for 30 s and centrifuged at 13,500 rpm for 5 min. After centrifugation, 100 µL of supernatant layer was transferred into LC vials for analysis and 20 µL was injected to the system.

### 2.6 Method validation

The bio-analytical assay developed was validated according to the US FDA guidelines. Calibration curves were constructed using linear regression analysis of imatinib, desmethyl imatinib and verapamil (IS) versus concentration. The intra-day and inter-day precision and accuracy were assessed by analyzing QC samples. The QC samples were also subjected for bench-top stability (27°C, 6 h) and freeze/thaw stability (-20°C, 3 freeze/thaw cycles, 48 h) in plasma by comparing samples before and after the stability tests.

### 2.7 Application to routine TDM

The bio-analytical method developed was then applied for routine therapeutic drug monitoring of chronic myeloid leukemia patients who were on imatinib therapy during the time period from March, 2012 to February, 2014 at Regional Cancer Centre (JIPMER, Puducherry, India). The samples were taken at trough level as this was associated with response of the patients [12].

### 3. RESULTS

#### 3.1 Bio-analytical method development using LC-MS/MS

The separation of imatinib and its metabolite was achieved using LC-MS/MS using verapamil as the internal standard. Verapamil was used as the internal standard because of its similarity in physicochemical properties with imatinib and its metabolite. Also verapamil is widely available and cost is less compared to deuterated imatinib. A mobile phase system containing water and MeOH was used with X Terra C18 column to separate Imatinib and its metabolite from the matrices by reversed-phase liquid chromatography. The retention time of imatinib, desmethyl imatinib and verapamil were 3.3 min, 3.3 min and 3.5 min respectively. A runtime of 5 min was set to avoid carry over effect of biological matrices. The MRM transitions, cone voltage and collision energy of the analytes and IS are presented in Table 2. A representative chromatogram of all three compounds after extraction of a patient sample are given in Fig. 1.
Table 2: Table showing MRM transitions, cone voltage and collision energy of analytes and internal standard.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MRM</th>
<th>Dwell (seconds)</th>
<th>Cone voltage (V)</th>
<th>Collision Energy (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imatinib mesylate</td>
<td>494.24&gt;394.1</td>
<td>0.025</td>
<td>46</td>
<td>26</td>
</tr>
<tr>
<td>Desmethyl imatinib</td>
<td>480.29&gt;394.1</td>
<td>0.025</td>
<td>40</td>
<td>26</td>
</tr>
<tr>
<td>Verapamil</td>
<td>455.27&gt;165.1</td>
<td>0.025</td>
<td>44</td>
<td>30</td>
</tr>
</tbody>
</table>

Fig 1. A representative chromatogram of all three compounds after extraction of a patient sample

3.2 Method validation

3.2.1 Linearity and Sensitivity

The calibration curves were plotted by spiking blank human plasma over a wide range of 5 ng/mL – 8 µg/mL for imatinib and 5 ng/mL – 1 µg/mL for desmethyl imatinib. The R² value was >0.99 for both the analytes over this wide range. The lower limit of quantification (LLOQ) was 5 ng/mL which defined the sensitivity of the method.

3.2.2 Precision and accuracy

The accuracy and precision was calculated for the QC samples at 10 ng/mL, 150 ng/mL, 7.5 µg/mL for imatinib and 10 ng/mL and 150 ng/mL for desmethyl imatinib. The accuracy of the present method was close to 100% over this range. The intra-day and inter-day precision were within limit of 15% which are the generally accepted criteria.
3.2.3 Specificity and stability
The analytical run of blank samples without IS and with IS in plasma showed no cross interference of individual compounds. The clear background in the analyte run channel indicated that there was no endogenous contamination of the analytes. This was suggestive of the good specificity of the current method for quantification of imatinib and its metabolite over a wide range. The stability test was performed using three sets of QC samples for benchtop stability and freeze thaw stability. The first set of QC samples were analyzed immediately after preparation. The second set was kept at 27°C and was analyzed after 6 h for measuring bench top stability. The freeze thaw stability was calculated by keeping the third set of QC samples at -20°C and was analyzed at 24th h and 48th h. The response deviation of second set and third set samples were less than 10% from the first set of QC samples which indicated the stability of drug and metabolite in human plasma.

3.3 Application to routine therapeutic drug monitoring
The bio-analytical method developed was applied in monitoring the trough level concentration of chronic myeloid leukemia patients who were on imatinib mesylate during the time period from March, 2012 to February, 2014. A total of 119 patients were diagnosed with chronic myeloid leukemia and were started on imatinib mesylate. The trough level concentration was obtained for 85 patients who achieved steady state drug concentration. The blood sample was taken once steady state achieved and prior to next dose. The samples were quantified using the developed bio-analytical method. The mean±SD trough level concentration was found to be 1437±459 ng/mL (range from 781.7–2940.4 ng/mL).

4. DISCUSSION
The therapeutic drug monitoring of imatinib mesylate is considered to be an essential tool in optimizing the therapy in chronic myeloid leukemia patients. Various bio-analytical methods using LC-MS/MS were published for the measurement of imatinib mesylate alone or along with its metabolite. Most of the methods use imatinib d-8 as the internal standard; other compounds which were used as internal standards were risperidone, quinoxaline, tamsulosin and trazodone[13-16]. The method demonstrated in the current study used verapamil as the internal standard. The bio-analytical method developed in the current article had many advantages like use of relatively cheaper and commonly available internal standard, simple mobile phase system and sample extraction procedure. The current method also showed a good linearity and stability over a dynamic range; so that it could be applied to
pharmacokinetic studies over a varied range of measurements. The therapeutic drug monitoring of 119 CML patients who were on imatinib treatment was carried out using the developed bio-analytical method. The mean trough level concentration was found to be 1437±459 ng/mL (range from 781.7–2940.4 ng/mL) for 85 patients who achieved steady state concentration after initiation of the therapy. These values were in accordance with the previous published results which stated that the trough level concentration of Imatinib should be more than 1000 ng/mL [12, 17]. The method could thus be easily applied for routine therapeutic drug monitoring of Imatinib mesylate in clinical settings.

5. CONCLUSION
A simple and rapid bio-analytical assay was developed and validated for the quantification of imatinib and desmethyl imatinib in human plasma using verapamil as internal standard. The low cost of internal standard, simple one step extraction method and shorter run time makes the method easier for implementing TDM in routine patient care. This method can be used as a tool to cross-check adherence to therapy or toxic effects.

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Conflict of Interest
The authors declare no conflict of interest.

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