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Imatinib Quantification by Liquid Chromatography Ultraviolet Detection for Monitoring of Plasma Levels

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Abstract. Imatinib is a new anticancer agent which acts by selectivity inhibiting the Abl tyrosine kinase and has a striking antitumor activity in patient with chronic myelogenous leukemia (CML). In this study a rapid and sensitive High Pressure Liquid Chromatography (HPLC) method has been developed with UV detection for estimation of Imatinib from the plasma of CML patients. Samples were prepared in a simple and single step by precipitation of plasma proteins with methanol. Calibration plots in spiked plasma were linear in a concentration range of 500-4000 ng/mL. Inter and intra-day coefficient of variation (precision) and bias (accuracy) were <10%. Limit of detection (LOD) and limit of quantification (LOQ) of Imatinib were 125.41 ng/mL and 380 ng/mL, respectively. Mean recovery of Imatinib ranged from 93.84 % to 109.68 %. The cross validation showed that the number of samples within a 20% difference from reference value was 19 out of 24 samples. Method developed is flexible and may be useful for analysing clinical samples containing Imatinib.

Keywords: Imatinib, liquid chromatography, plasma level monitoring

Introduction

Chronic myeloid leukaemia treatment has been revolutionised by the development of the tyrosine kinase inhibitor (TKI) Imatinib (STI571, Glivec®), which has demonstrated significant clinical efficacy in this type of cancer. Imatinib is used as first line therapy in the treatment of chronic myeloid leukaemia (Cohen *et al.,* 2002a; 2002b). The mechanism of action of Imatinib is based on the specific inhibition of tyrosine kinases. This selectivity of inhibition is not unfortunately absolute, causing many side effects. Many of these effects are dose dependent, and hence, can be controlled by dose reduction. Imatinib is administered as oral drug; it has a rapid and complete oral bio-availability (98%) and a proportional dose-exposure relationship (Peng *et al.,* 2005; 2004). Its bio-availability presents high variability. Indeed, several studies have shown no correlation between doses and plasma concentrations of the drug but the presence of a correlation between the cytogenetic and haematological response and plasma concentrations has been demonstrated. According to these studies Imatinib may be toxic even at low dose. (Cortes *et al.,* 2009; Larson *et al.,* 2008). The analysis of pharmacokinetic/pharmacodynamic relationships indicated that the initial haematological response depends on the administered dose for patients with CML (Peng *et al.,* 2004). The measurement of Imatinib plasma levels has been shown to be useful clinically by various teams and allows to locate plasmatic concentrations compared to the efficacy threshold in CML patients (Larson *et al.,* 2008; Picard *et al.,* 2007). CML patients absolutely need to be monitored to avoid Imatinib related toxic events. Monitoring of drug plasma levels is important to enhance efficacy and avoid toxicity in order to optimize disease treatment. Most of the methods used for Imatinib quantification utilize liquid chromatographic (LC) -mass spectrometric assay (Awidi *et al.,* 2010; Katerina *et al.,* 2010; Roth *et al.,* 2010; Chahbouni *et al.,* 2009; De Francia *et al.,* 2009; Haouala *et al.,* 2009; Lankheet *et al.,* 2009; Parise *et al.,* 2009; Titier *et al.,* 2005; Bakhtiar *et al.,* 2002a; 2002b). This technique is quite expensive and yet inaccessible for many of emerging countries' investigators and hence cannot be used for routine TDM. An LC method is easier and more convenient to be used routinely. Several UV-HPLC methods for Imatinib quantification in human plasma have been described (Oostendorp *et al.,* 2007; Schleyer *et al.,* 2004; Velpandian *et al.,* 2004). The

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devised method is simpler, using only protein precipitation using methanol for sample pre-treatment. Indeed, the solubility of mesylate salt of Imatinib in aqueous medium is pH dependent and is marked at pH 5.8. However, it is insoluble to slightly soluble in organic solvents (Novartis Pharma, 2001). Methanol volume is twice that of plasma. The method reported in this article and which has been already cross-validated is based on a protein precipitation extraction procedure which can be easily adaptable in any laboratory.Therefore, an assay that detects Imatinib in biological samples routinely would be of a great benefit. In consideration of this, the present study was undertaken for developing and validating a simple and rapid HPLC method with UV detection, which could be useful for therapeutic drug monitoring (TDM) of Imatinib in routine setting.

Materials and Methods

Chemicals and reagents. Pure compound of Imatinib was purchased from Avantix (Newark, DE). The internal standard (IS) Clozapin (Leponex®) was provided by Novartis Pharmaceutical, UK Ltd. (Horsham, GB) for Novartis Pharma AG (Basle, Switzerland). Acetonitrile EHPLC grade, Methanol Lichrosolv, Water Lichrosolv EHPLC grade, K_2HPO_4 buffer HPLC grade were obtained from Scharlau (Tunis, Tunisia).

Preparation of solutions. Imatinib solution (IS) (1 mg/mL) was prepared from accurately weighed 25 mg pure powder dissolved in water and stored at -20 °C. Clozapin solution (250 µg/mL) was prepared in water and stored at -20 °C. Standards (STD) of Imatinib were prepared by dilution in human blank plasma to make concentrations: 0.25, 0.5, 1, 1.5, 2 and 4 µg/mL. IS solution used in extraction (25 µg/mL) was prepared by diluting in plasma. These solutions were freshly prepared before each day of experiment. Drug free plasma for analytical development and validation was obtained from a Blood Bank (H.U.C Charles Nicolle, Tunisia).

HPLC Equipment. HPLC system manufactured by Varian (Australia) used for the present study consisted of a Varian Prostar 240 pump, a Prostar 410 Autosampler, Varian Prostar 325 LC Detector and a Varian Prostar 510 Column Oven. For the data acquisition and integration, Galaxie chromatography data system version 1.9.3.2 software operated by Pentium 4HT was used.

Chromatography conditions. Imatinib was separated using A Lichocart cartridge column (250 x 4 mm) filled with Lichrospher 1000 RP-8, having a 5µm practical size (Merck, France) maintained at 25 °C. The samples were eluted in a mobile phase consisting of 0.02 M dipotassic hydrogen phosphate buffer and acetonitrile (73:27, V/V) at a flow rate of 1 mL/min. The mobile phase was filtered through a 0.22 µm filter (Millipore) and degassed under vacuum prior to use. The detector wavelength was set at 265 nm.

Assay Procedures. ***Sample extraction.*** Aliquots of 300 µg/mL plasma samples (blank, standard or patient sample) and 30 µL of IS solution were vortexed for 1 min. 600 µL methanol was added to the mixture and re-vortexed for 3 min. Each sample was centrifuged at 4 °C (3000 g x 12 min), and then the supernatant was separated into another tube and evaporated under nitrogen at 50 °C. The residue was dissolved in 150 µL of the mobile phase solvent and then 50 µL of aliquot were injected into the HPLC column for quantification.

Method validation. The validation study was performed according to FDA guidelines for bioanalytical methods (US,FDA, 2001).

Linearity. The calibration curve was prepared with five increasing concentrations of Imatinib (STD 500, 1000, 1500, 2000 and 4000 ng/mL) and was built with the spike area ratios of each STD and IS, and fitted using linear regression.

Precision. Replicate analysis (n=5) of control samples (QCs) at three concentrations (low, medium, high) was carried out for the precision and accuracy determination. All the three concentrations were chosen to encompass the whole range of the calibration curve corresponding to the drug levels frequent in the majority of patients samples. Inter-assay accuracy and precision were determined by repeated analysis performed in three different days. The concentration in each sample was determined using calibration curve prepared on the same day. The precision was calculated as the coefficient of variation (CV %) within a single run (intra-day) and between different assays (inter-days). 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 samples were less than 10%.

Accuracy. Accuracy was measured as the percentage difference between measured value and nominal value, according the equation:

$$\text{Bias \%} = \frac{C_{measured} - C_{theoretical}}{C_{theoretical}} \times 100\%$$

where, $C_{measured}$ and $C_{theoretical}$ are the determined (observed) concentration of Imatinib with the present method and their theoretical concentration in the spiked plasma sample, respectively. Extractions were repeated three times for each concentration.

Determination of LOD and LOQ. LOD was calculated using the equation LOD=3.3(S.D./b) and LOQ was calculated using the equation LOQ=10(S.D./b), where, S.D. is the standard deviation and b is the slope of the calibration curve. The aim was to make the bias and CV % less than 10% for all quality control standards except for the LOQ where, less than 20% was accepted (US, FDA, May 2001).

Cross laboratory validation. Cross validation was performed using samples prepared in another laboratory. Analysis of results was made by comparing the Imatinib concentrations found using this technique and those by another technique in another pharmaceutical laboratory. The difference % is expressed by concentration found using this technique/true concentration of laboratory that prepared samples (reference sample).

Statistical analysis. Validation data and the equation of the calibration curve were calculated by regression analysis using the software-package Pythagore Biostat (Version 2.0). Data were given as mean ± standard deviation (S.D).

Application in patients with CML. Sixty eight Tunisian CML patients receiving Imatinib treatment underwent blood drawing for measurement of plasma drug concentrations. Patients with CML who were on a daily dose of 100 mg (n=1), 200 mg (n=2), 300 mg (n=9), 400 mg (n=39), 600 mg (n=9), 800 mg (n=8) of the drug were included in this study. There were 27 females and 41 males. The mean age ± S.D. was 44.85 ± 12.87 years and the mean body weight was 72.03 ± 17.32 kg. Blood samples were taken 24 h after and immediately prior to taking the oral dose of Imatinib. Samples were collected in lithium heparin tube (5 mL); plasma was obtained after centrifugation at 1400 x g 10 min at 4°C.

Results and Discussion

Representative chromatogram of blank plasma extracted of Imatinib is shown in Fig. 1A. Representative chromatogram of Imatinib standard plasma (medium calibration standard, 1000 ng/mL) extracted and Clozapin is shown in Fig.1B. Time of analytical run was set as 15 min, according to the retention times of substances, which showed a clean separation (Fig. 1B). Analyte

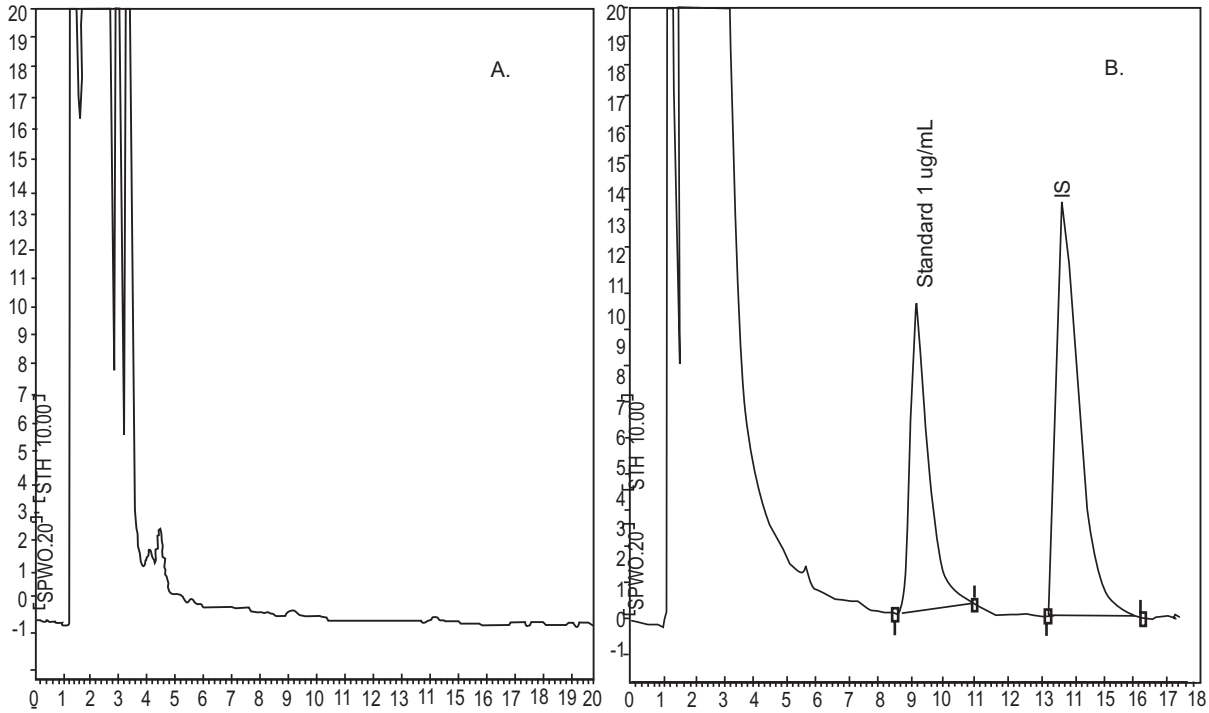

Fig. 1AB. Representative chromatograms of blank and Imatinib spiked plasma. (A) Representative chromatogram of blank plasma extracted of Imatinib. (B) Representative chromatogram of Imatinib standard plasma (medium calibration standard 1000 ng/mL) extracted.

retention times were 8.67 min ± 0.49 min for Imatinib and 13.28 min ± 0.66 min for the IS. The peak was well resolved with λ max at 265 nm. Imatinib plasma sample did not show any interfering peaks. Mean regression coefficient (r^2) of all Imatinib calibration curves was higher than 0.9998 (data not shown). The analytical method presented a linear response over the concentration range from 500 to 4000 ng/mL. The regression coefficient (r^2) was 0.9993 (data not shown). The precision and accuracy of data obtained from QCs have been presented in Table 1. The within day (intra-assay) coefficient of variation of the assay varied from 1.84 % at 500 ng/mL, 5.78 % at 1000 ng/mL, and 3.49 % at 4000 ng/mL. The intra-day accuracy of estimation in the seeded samples of low, medium and higher concentrations of Imatinib in plasma was found to be 4.27, 0.49, and 9.68%, respectively.

Between a day (inter-assay) coefficient of variation was 8.06% at 500 ng/mL, 8.23% at 1000 ng/mL and 6.76% at 4000 ng/mL. The inter-day accuracy of estimation in the seeded samples of low, medium and higher concentrations of Imatinib in plasma was found to be 6.16, 0.82, and 8.25 %, respectively. These results suggest good precision and accuracy.

The LOD and LOQ of Imatinib were 125.41 ng/mL and 380 ng/mL, respectively (data not shown).

Table 1. Precision and accuracy of Imatinib QCs in human plasma.

Intra - day* Added Imatinib concentration (ng/mL)	Observed concentration (Mean±S.D. ng/mL)	Accuracy (%)	Precision (%)
500	521.4 ± 9.6	4.27	1.84
1000	981.5 ± 56.8	0.49	5.78
4000	4387.3 ± 153.2	9.68	3.49
Intra - day** **Added Imatinib concentration (ng/mL)**	**Observed concentration (Mean±S.D. ng/mL)**	**Accuracy (%)**	**Precision (%)**
500	469.2 ± 37.86	6.16	8.06
1000	991.7 ± 81.6	0.82	8.23
4000	4330.3 ± 293	8.25	6.76

*n=5 assays in the same day for each concentration;**n=5 assays per day during 3 days for each concentration.
Daily (intra-day) and day to day (inter-day) precisions are represented as mean values ± S.D. of five different assays for each concentration.

Multiple aliquots (n=5) at each of three QCs were assayed and mean recovery of Imatinib ranged from 93.84 % to 109.68 %, showing a good recovery which was within±7.28%. As shown by various chromatograms (chromatogram for the blank sample and chromatogram for 720 ng/mL of Imatinib in plasma from a patient treated with 400 mg /day of Imatinib (Fig. 2)), the present HPLC/UV method seems sufficiently sensitive to be used for trough Imatinib plasma level determination. This simple separation method uses isocratic mobile phase conditions making the assay accessible to any laboratory or institute as it can be performed by those unfamiliar to basic HPLC techniques. The cross validation shows that the number of samples within a 20% difference from reference value was 19 out of 24 samples (the mean difference ± S.D was 6.8 ± 19.4). Indeed, 2/3 of the samples must have a difference less than 20%.

This method is flexible and may prove to be useful to analyze clinical samples containing Imatinib alone. Much cannot be said about the analyses of Imatinib in patients taking concomitant drugs as the population under study did not present this condition. Cases of two patients were excluded from the interpatient variability

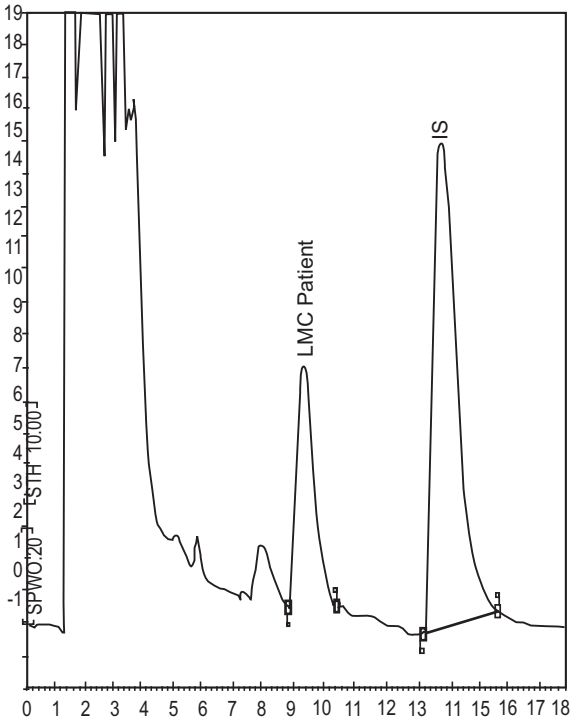

Fig. 2. Representative chromatogram for 720 ng/mL of Imatinib in plasma from a patient treated with 400 mg/day of Imatinib.

because one patient was receiving 100 mg of Imatinib daily (491 ng/mL) and the other was receiving a dose of 200 mg of Imatinib daily (627.4 ng/mL), and the patients who received a dose of 300 mg and higher were studied. Indeed, a large inter-patient variability was observed as previously reported (Larson *et al.,* 2008). Plasma trough levels of Imatinib at steady state were comparable in both males and females (1926.09 ± 826.91 ng/mL *vs*. 1811.13 ± 974.7 ng/mL, respectively) whereas, a difference between plasma levels of Imatinib at steady state in both the sexes was previously noted (Larson *et al.,* 2008) suggesting that this difference could be correlated with body weight. Due to limited sampling pharmacokinetic estimates could not be performed but increasing the number of patients and the continuous monitoring of its plasma levels will help to understand more about its efficacy as well as its safety.

Conclusion

In this study, a simple isocratic HPLC method with UV detection has been developed and validated to evaluate Imatinib levels from biological samples in CLM patients.

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