ANALYSIS OF IMATINIB IN BONE MARROW AND PLASMA SAMPLES OF CHRONIC MYELOID LEUKAEMIA PATIENTS USING SOLID PHASE EXTRACTION LC-ESI-MS

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ABSTRACT

The LC-ESI-MS was developed and validated for the analysis of imatinib in plasma and bone marrow samples using deuterated imatinib (D_8-IM) as an internal standard. The biological samples were extracted using Strata-X-C SPE cartridges and separated on C_8 column (50 x 3 mm, 3 µm), and methanol: 0.1% formic acid (70:30) was delivered at the rate of 0.7 ml/min as a mobile phase. Imatinib was quantified in samples by monitoring the ions m/z 494.3 for imatinib and 502.3 for D_8-imatinib on mass spectrometer. The method was linear in the concentration range of 1-1500 ng/250 µl in spiked human plasma samples and limit of quantification was 5 ng/mL. Inter-day and intra-day variations in spiked human plasma spiked with 50, 250 and 500 ng /mL were less than 3.16%. The repeatability and reproducibility and other parameters of the methods were also validated. The method was employed for the analysis of the imatinib in human plasma and bone marrow samples. The drug levels in bone marrow and plasma samples were correlated to the degree of cytogenetic response. No significant difference of imatinib level between blood and bone marrow in IM-treated patients dosed to steady state was observed.

Keywords: Bone marrow, Imatinib, Leukaemic, LC- ESI-MS, Human plasma.

INTRODUCTION

The selective Abl tyrosine kinase inhibitor, imatinib mesylate (IM; Gleevec® or Glivec®) has become the gold standard treatment of Chronic Myeloid Leukaemia (CML) (Druker *et al*., 2001; Druker *et al*., 2001; Saito and Morioka, 2010). Discovered from a rational drug design program, IM blocks the activity of BCR-ABL, the oncogenic tyrosine kinase created by the Philadelphia (Ph) chromosome translocation diagnostic of CML. Although clinical studies in the three sequential stages of the disease chronic phase (Kantarjian *et al*., 2002) accelerated phase (Talpaz *et al*., 2002) and terminal blast crisis (Kantarjian *et al*., 2002; Sawyers *et al*., 2002) confirm the substantial activity of the drug in vivo, responses are not sustained in advanced phases. Further, the majority of patients in the early chronic phase do not achieve the deepest level of response, i.e. molecular remission that is polymerase chain reaction (PCR) negativity for BCR-ABL (Hughes *et al*., 2003). Studies showed the patients with higher CYP3A responds better to imatinib (Green *et al*., 2003).

Knowing that CML is a clonal disease of stem cell origin, it has been postulated that low stem cell turnover *in vivo* may facilitate survival of 'protected' quiescent stem cells able to maintain and repopulate the disease (Elrick *et al*., 2005; Jørgensen *et al*., 2005). Physiologically, bone marrow is the major compartment for white cell production, and this is the compartment in which quiescent CML stem cells are presumed to reside. In the face of emerging resistance it would be of interest to know the level of IM achieved in patient marrow. We sought to assess whether inadequate patient response was in part related to failure to achieve a sufficiently high IM level in bone marrow as compared to peripheral blood.

Several methods have been reported for the analysis Imatinib using LC-ESI-MS (Parise *et al*., 2003; Bakhtiar *et al*., 2002; Guens *et al*., 2006; Klawitter *et al*., 2009) and HPLC with UV detection and (Rosasco *et al*., 2005; Chahbouni *et al*., 2009; Roth *et al*., 2010) methods for the analysis of imatinib in plasma or pharmaceutical preparations. These methods are commonly based on the liquid extraction followed tandem mass spectrometry (Hsieh *et al*., 2009; Ma *et al*., 2009; De Francia *et al*., 2009). The aim of the present study was to develop a validated method for the analysis of the IM in plasma and bone marrow samples using ESI Ion Trap mass spectrometry using solid phase extraction and to evaluate the drug levels in plasma and bone marrow samples of the CML patients.

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MATERIALS AND METHODS

Reagents and solutions

Methanol, acetonitrile, Water (HPLC), formic acid (98% v/v) and ammonia solution (35% v/v, analytical reagent grade) were obtained from BDH Laboratory Supplies (Poole, UK). IM and the internal standard D_8-IM were kind gifts from Novartis Pharma, Basle, Switzerland. Stock IM and internal standard D_8-IM solutions at 1mg/mL were prepared in methanol: 1.0 % w/v formic acid (50:50) and stored at +4ºC until analysis.

Clinical samples

Peripheral blood was collected into glass tube containing ethylene diamine tetra-acetic acid (EDTA) after obtaining the informed consent from CML patients or healthy volunteers according to the Declaration of Helsinki. Bone marrow aspirate, excess to clinical diagnostic requirements, was also harvested from the CML patients. CML marrow was sampled at 3 or 4 months follow-up post-initiation of IM. The plasma supernatant was separated by centrifugation of whole blood at 1200 rpm for 10 minutes and stored frozen at -20°C until required. Glasgow Royal Infirmary Local Research Ethics Committee approved the use of human tissue in this study.

Solid phase extraction (SPE) method for plasma and bone marrow samples

Frozen peripheral blood plasma or bone marrow samples were thawed at room temperature; 0.25 mL was transferred to a 1 mL plastic vial and spiked with imatinib (250 ng/mL) and appropriate volume of internal standard equivalent 10 ng D_8-IM and vortex for 30 second. Then 0.25 ml 0.1M phosphate buffer pH 3.0 was added and vortex for further 30 seconds then transferred to a Strata-X-C column (Phenomenex, Macclesfield, UK) previously washed with 1ml of phosphate buffer. The column was washed with 1 mL water, 1 mL methanol, and then twice eluted with 1 Ml of ammonium hydroxide (2M) in methanol. The eluent was collected, evaporated under a stream of nitrogen and dissolved in formic acid (0.1% w/v) and injected into LC-ESI-MS.

Extraction of Samples with Methanol or Acetonitrile

Plasma (0.25 mL) was spiked with imatinib (250 ng/mL), mixed with methanol (1.5 mL) or acetonitrile (1.5 mL) and vortexed for 30 seconds then centrifuged at 3600 g for 10 min. Then supernatant was collected and evaporated under the stream of nitrogen. The residues were re-dissolved into formic acid (0.1%).

High pressure liquid chromatography mass spectrometry (LC-ESI-MS) for analysis of IM in biological fluids

IM analysis of plasma or bone marrow samples was performed using a Spectra Systems HPLC equipped with a P2000 pump, AS1000 autosampler with 20 µl fixed injection loop and UV2000 detector (ThermoSeparations, Hemel Hempstead, Herts, UK). Chromatographic separation was conducted at ambient temperature using an ACE® 3 µm C8 column (50 x 3 mm internal diameter; HiChrom, Reading, UK). Mobile phase (methanol:formic acid 0.1% w/v) in the ratio of 70:30 was delivered in isocratic mode at a flow rate of 0.7 mL/min over 10 minutes. Samples (20 µl) were injected into LC-ESI-MS (LCQ Finnigan Automass LC-ESI-MS system) via the autosampler without splitting at a flow rate of 0.7 mL/min. The interface was operated in positive ion electrospray mode at 4.5 kV. The capillary temperature was adjusted to 270°C. The MS data were acquired under selected ion monitoring mode by monitoring for the IM ion at m/z 494.3 and at 502.3 for D_8-IM. The mass spectrums of the IM and D_8-IM are shown in Fig 1.

Linearity

Linearity of the method was studied over the concentration range of 1-1500 ng/250 µl in spiked human plasma in triplicate by employing the standard calibration curves at least 8 points (non-zero standards). However, the spiked plasma samples with only internal standard and blank plasma sample (non-spiked) was also analysed with each batch just to confirm the absence of interferences. The method exhibits the linear response in the above range with correlation coefficient of 0.998 – 0.9991 (CV 0.05%) and the regression equation was, $y = -0.019 + 0.001x$.

Intra- and inter-day accuracy and precision

The intra-day precision and accuracy of the assay was measured by analysing the three different concentrations of spiked human plasma samples at concentration range of 50, 250 and 500 ng/250 µl. The intra-day accuracy of the method was ranged from 90.2-96.3% and precision was (CV) ranged from 2.7-6.9%. The inter-day accuracy and precision was determined over three days by analysing three spiked human plasma samples at concentration range of 50, 250 and 500 ng/250 µl. The precision was expressed as the coefficient of variation (% CV).

Recovery

The recovery was evaluated by comparing the peak areas of the extracted spikes human plasma samples with the un-extracted pure standard solutions at the three concentration level i.e. 50, 250 and 500 ng/ml (n=3 for each concentration).

Specificity

Specificity of the assay was demonstrated by obtaining ion-chromatograms for blank pooled human plasma samples and blank human plasma spiked with only the internal standard.

Freeze–thaw stability

In freeze–thaw stability studies, samples of imatinib and CGP 74588 (at three concentrations) were subjected to three freeze–thaw cycles and subsequently analyzed in duplicate. Plasma samples were stored at −20 °C for 24 h and thawed unassisted at room temperature. This cycle of thawing and freezing was repeated two more times followed by LC–ESI–MS analysis on the third cycle.

Limits of detection and limit of quantification

Limit of detection and quantification were determined from the calibration curve of the plasma spiked with imatinib, using the following formula:

$$LOD = 3.3\ \frac{\delta}{S}$$

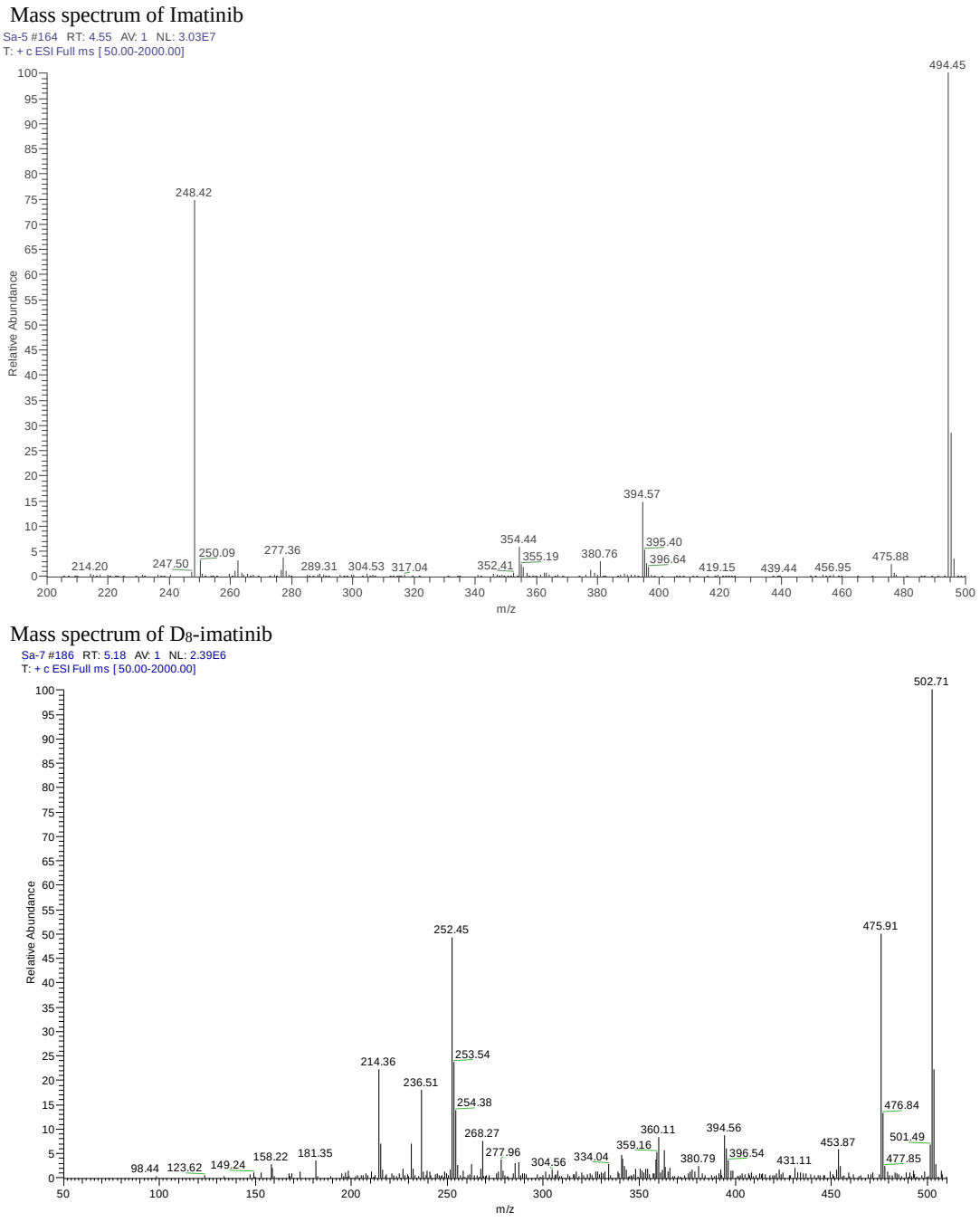

Fig. 1: Mass spectrum of imatinib and D_8-imitinab under ESI-Ion Trap Mass Spectrometer.

$$LOQ = 10\,\frac{\delta}{S}$$

Where δ is the residual standard deviation of the regression line and S is the slope of the regression line.

Stability

The stability of imatinib in human plasma was evaluated over 3 days at room temperature and stored at –20°C for three weeks at three concentrations in duplicate.

STATISTICAL ANALYSIS

Statistical analysis of the data in this study was performed using manitab-8.

RESULTS

The mass spectrum of imatinib and D_8-imitinab, used as internal standard, under ESI mode of ionozation gave the ion $[M+1]^+$ i.e. m/z 494 and m/z 502, respectively as a

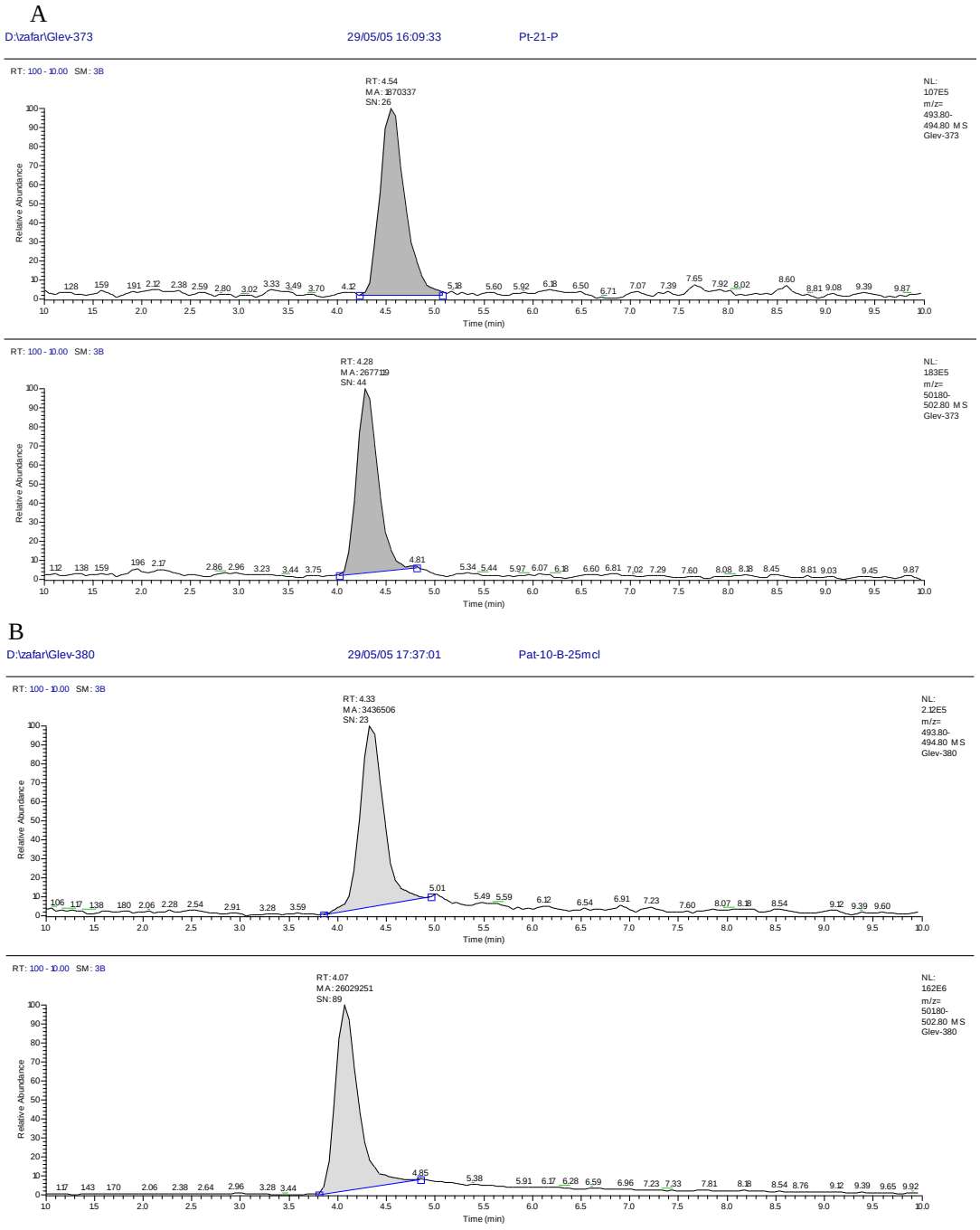

Fig. 2: The typical spectrum of the imatinib and D_8-imatinib extracted from Human plasma (A) and bone marrow (B)

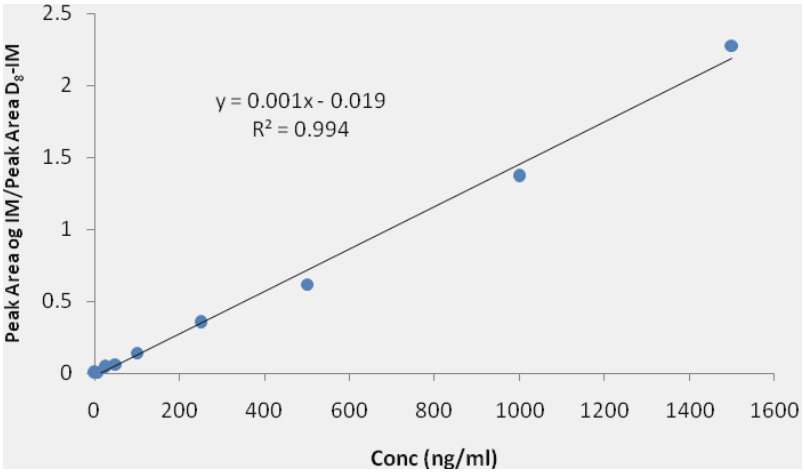

Fig. 3: Calibration curve of imatinib in spiked plasma samples.

Table: Concentration of IM in matched bone marrow and plasma samples (ng/ml) from treated CML patients at haematological remission (3 or 4 months).

Sample	n	IM Concentration (μmolar)	
		Peripheral blood	*Bone marrow*
All patients	10	967.1 ± 41.28	1261.96 ± 47.16
CCR	5	1049.7 ± 123.8	1267.85 ± 70.76
No CCR	5	878.65 ± 70.76	1256.06 ± 141.53

base peak. The figs. 1 and 2 show the typical mass spectrum of the imitinab and D8-imitinab.

Extraction

Extraction was carried out using the latter methodology, the poorest result was achieved with acetonitrile and methanol as the extraction solvent with only 68% and 73%, respectively drug recovery in comparison to 89 % ± 5% IM recovery by SPE (IM at 250 ng in 0.250 ml, n = 5).

Recovery

The recovery of the imatinib was evaluated by comparing the quantitative results of the spiked human plasma samples with the aqueous standard solution of imatinib in three different concentrations i.e. 50, 250 and 500 ng/ml (*n* = 3 per concentration) in both medium. The mean recovery was 87.1% ± 5.84, 89.1% ± 4.57 and 90.3% ± 3.84, respectively.

Linearity

Linearity of the method was studied for the concentration range of 1-1500 ng/250 µl in spiked human plasma in triplicate by employing the standard calibration curves at least 8 points (non-zero standards). The method demonstrated the linear response in the above concentration range with correlation coefficient of between 0.998 – 0.9991 (% CV = 2.9) and the regression equation was, $y = -0.0206 + 0.00145x$. The calibration curve is shown in fig. 3.

Sensitivity

The limit of detection measures from the calibration curve was 0.30 ng/mL and limit of the quatification of the method was 0.7 ng/ mL

Precision and accuracy

The precision and accuracy (expressed as co-efficient of variation, % CV) of the intra- and inter-samples were studied at three different concentrations i.e. 50, 250 and 500 $ng.ml^{-1}$ and was 3.35, 2.59 and 2.49, respectively.

DISCUSSION

Both imitinab and D8-imitinab ions were used for the selected ion monitoring (SIM). This method produced high-abundance $[M+1]^+$ ions with minimum fragmentation of analyte. The MS and HPLC conditions for the analysis of imatinib in plasma and bone marrow samples were optimized and validated under different instrumental conditions. The optimization of the present LC and ESI conditions gave peaks at about 4.5 min and yielded high selectivity and sensitivity without interference from any other component of the plasma or bone marrow.

Extraction of the imatinib using different methods showed that solid phase extraction produce better results compared with the solvent extraction. The plasma samples were extracted using STRATA Cartridges (SPE), methanol and acetonitrile. Extraction of IM from biological samples using an SPE method proved to be superior to the solvent extraction based approach of existing methods (Druker *et al*., 2001; Parise *et al*., 2003; Widmer *et al*., 2004).

The ion-chromatograms for blank pooled human plasma samples and blank human plasma spiked with only the internal standard was free from any interfering endogenous substances.

The results indicate that the method is sensitive enough to quantify the imatinib in plasma and particularly in bone marrow samples.

The mean intra-assay precision and accuracy was within close limits. The statistical analysis showed that the difference between inter-day or intra-day sample is not significant ($p > 0.1$). Results were entirely satisfactory with intra-day accuracy in the range 89% to 98%, and inter-day precision (% CV) ranging from 0.6% to 3.5%. This indicates that following extraction of the imatinib from the biological samples it is stable when stored at 4°C.

Studies of Imatinib concentration in blood and bone marrow samples

Based on our group's research interest in persistent quiescent CML stem cells within the bone marrow compartment, we postulated that truly successful chemotherapeutic disease eradication may only be achieved if local drug delivery is optimal. An investigation into the correlation of peripheral blood and bone marrow plasma drug levels was therefore planned, for which a sensitive, precise and accurate analytical methodology had to be developed.

From a cohort of 10 CML patients enrolled in a clinical trial studying IM in chronic phase (Novartis studies 0106 and 0113), matched peripheral blood and bone marrow aspirate samples were processed to determine drug levels. The average IM level in peripheral blood samples taken at 3 or 4 months post-initiation of IM therapy (table) was in keeping with published human pharmacokinetic data dosed to steady state (Peng *et al*., 2003). At this time, all patients had achieved a haematological remission (to < 10 x 10^6/mL). However, only half had achieved the next level of response that is a complete cytogenetic response (CCR). None of the patients were PCR-negative for *BCR-ABL* at this stage.

On analysis of total data from all patients, there was no significant difference in the average drug concentration between peripheral blood and bone marrow plasma. This suggests that 400 mg IM daily is sufficient to sustain equilibrium between peripheral blood and bone marrow plasma at a therapeutic level (approximately 2 μM). When the levels measured in the cytogenetically. responding patients were compared with the non-responders, there were still no significant differences between sub-groups in peripheral blood or bone marrow.

In summary, using a sensitive assay for measurement of IM in biological samples, we have found that the IM concentration in peripheral blood and bone marrow plasma from CML patients dosed to steady state is in equilibrium. Despite the relatively limited patient numbers studied, these data were nonetheless consistent demonstrating good distribution of the drug into this previously un-assayed tissue. However, as we believe that higher intracellular levels of IM are required to kill stem cells than mature leucocytes in vitro, due to over-expression of BCR-ABL protein and/or multi-drug resistance pumps (Copland *et al*., 2006; Jordanides *et al*., 2006) we now hypothesise that more effective intracellular stem cell drug loading may be required within the bone marrow compartment.

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