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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 (D8-IM) as an internal standard. The biological samples were extracted using Strata-X-C SPE cartridges and separated on C8 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 D8-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)[1,2]. 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[3], accelerated phase[4], and terminal blast crisis[5,6]) 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[7].

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[8,9]. 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.

The aim of the present work was to relate drug levels to the clinical status of patients at the time of sampling using a suitably sensitive, precise and accurate method for the analysis of IM in peripheral blood and matched bone marrow plasma samples.

**DESIGN AND METHODS**

*Reagents and solutions*

Methanol, acetonitrile, high pressure liquid chromatography (HPLC) water, 98% v/v formic acid and 35% v/v ammonia solution (analytical reagent grade) were all 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 50:50 methanol:1% w/v formic acid and stored at +4ºC until use. The stock solution was diluted for HPLC analysis with 1% w/v formic acid.

*Clinical samples*

Peripheral blood was drawn into ethylene diamine tetra-acetic acid (EDTA) tubes with 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 1200rpm 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 or bone marrow plasma samples were thawed at room temperature. 0.25mL was transferred to a 1mL plastic vial to which was added 0.25mL 0.1M phosphate buffer pH3.0 with an appropriate volume of internal standard solution. The solution was vortex mixed for 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 1mL water, 1mL methanol, and then twice eluted with 1mL of 2M ammonium hydroxide in methanol. The eluent was collected, evaporated under a stream of nitrogen and dissolved in 0.1% w/v formic acid.

*High pressure liquid chromatography mass spectrometry (LC-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 20µL fixed injection loop and UV2000 detector (ThermoSeparations, Hemel Hempstead, Herts, UK). Chromatographic separation was at ambient temperature using an ACE® 3µm C8 column (50 x 3mm 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 0.7mL / min over 10 minutes.

Analytes were detected using an LCQ Finnigan Automass LC-MS system (ThermoSeparations). Samples at 20µL were injected via the autosampler into the mass spectrometer without splitting at a flow rate of 0.7mL / 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.

**RESULTS AND DISCUSSION**

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.

Extraction of IM from biological samples using an SPE method proved to be superior to the solvent based approach of existing methods.[11-13] Using the latter methodology, the poorest result was achieved with acetonitrile as the extraction solvent with only 68% drug recovery in comparison to 89% ± 5% IM recovery by SPE (IM at 200ng in 250µL, n = 5).

The LC-MS analytical methodology proved to be both specific without interference from other peripheral blood or bone marrow plasma components, and sensitive with a limit of detection (LOD) of 5ng / 250µL of IM. Method linearity was proven by direct injection of standards in the range 1ng to 500ng / 250µL mobile phase (7 data points) described by the regression equation y = -0.0245 + 0.00203x ($r^2$ = 0.998). Method precision (expressed as co-efficient of variation, % CV) and accuracy were studied at IM concentrations of 50, 250 and 500ng / 250µL. Results were entirely satisfactory with intra-day accuracy in the range 89% to 98%, and intra-day precision (% CV) ranging from 0.6% to 3.5%. These results were reproducible in inter-day studies.

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 1) was in keeping with published human pharmacokinetic data dosed to steady state[10]. At this time, all patients had achieved an haematological remission (to $< 10 \times 10^6$ / 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 400mg 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[14,15], we now hypothesise that more effective intracellular stem cell

drug loading may be required within the bone marrow compartment.

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**TITLES AND LEGENDS**

**Table 1.** Concentration of IM in matched bone marrow and plasma samples from treated CML patients at haematological remission (3 or 4 months).

**Table 1.** Concentration of IM in matched bone marrow and plasma samples from treated CML patients at haematological remission (3 or 4 months)

| Sample | n | IM Concentration (μmolar) | |
| --- | --- | --- | --- |
| | | *Peripheral blood* | *Bone marrow* |
| All patients | 10 | 1.64 ± 0.07 | 2.14 ± 0.08 |
| CCR | 5 | 1.78 ± 0.21 | 2.15 ± 0.12 |
| No CCR | 5 | 1.49 ± 0.12 | 2.13 ± 0.24 |