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Quantification of an anti-cancer drug (imatinib) in human plasma

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1 ABSTRACT

Imatinib mesylate (Gleevec® or Glivec®) is the first of a new class of drugs developed for cancer treatment that selectively eliminates cancer cells instead of destroying all the rapidly dividing cells. It eliminates cancer by competitively inhibiting tyrosine kinase bcr-abl that is responsible for chronic myelogenous leukemia (CML). Imatinib also inhibits c-Kit tyrosine kinases responsible for the gastro-intestinal stromal tumours (GISTs) and platelet-derived growth factor receptor (PDGFR) implicated in the pathogenesis of the hypereosinophilic syndrome.

Therapies with imatinib have shown significant inter-individual variability in pharmacokinetics, demanding a need for Therapeutic Drug Monitoring (TDM) in order to achieve an optimal response in CML therapy and to minimize adverse side effects. Two methods for determination of imatinib level in plasma have been evaluated and presented in this thesis. The first method is liquid chromatography coupled to mass spectrometry (LC-MS) technique after direct precipitation of protein; and the second technique is direct analysis in real time (DART) mass spectrometry. Both techniques are comparable in precision and accuracy.

The linearity of the LC-MS method was evaluated over the imatinib concentration range of 30 – 10000 ng/mL in human plasma with correlation coefficient (R^2) of ≥ 0.999 and a mean coefficient of variation (CV) of 2.89 % (n=3). The associated limit of detection (LOD) and limit of quantification (LOQ) were 3 and 10 ng/mL respectively. The average instrument precision was 2.25 % and the inter/intra-day accuracy and precision (CV) ranges were 98 – 106 % and 1.8 - 4.4 % respectively. The recoveries determined at low, medium and high concentration levels varied from 95 – 107 % and within the acceptable limits.

The developed DART method was partially evaluated. This method showed a great potential in rapid analysis of imatinib in human plasma. Results indicated that the linearity of the calibration curve stretched over the range of 30 – 10000 ng/mL with R^2 ≥ 0.995 and a mean CV of 8.3 % (n=3). LOD was found to be 5 ng/mL and LOQ of 16 ng/mL. Its precision was 11 %. The use of DART method for imatinib quantification is novel. To our knowledge, this is the first evidence of a successful application of this method for the analysis of imatinib in plasma.

Keywords

Imatinib, Gleevec, Glivec, tyrosine kinase, liquid chromatography, TOF mass spectrometry, quantification, human plasma

2 ABBREVIATIONS

AccuTOF	Accurate time of flight
AP	Accelerated phase
APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photoionization
CML	Chronic myelogenous leukemia
CP	Chronic phase
CV	Coefficient of variation
DART	Direct analysis in real time
ESI	Electrospray ionization
FDA	Food and drug administration
GISTs	Gastro-intestinal stromal tumours
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MALDI	Matrix assisted laser desorption ionization
MS	Mass spectroscopy
PDGFR	Platelet-derived growth factor receptor
QC	Quality control
RT	Retention time
SD	Standard deviation
TIC	Total ion chromatogram
TKI	Tyrosine kinase inhibitor

3 INTRODUCTION

The central theme of this study is measurement of imatinib in human plasma using in-house instrumentation. In the first part of the thesis, a brief introduction of imatinib and theoretical background of the instruments that have been used in this study is presented. The second part of the thesis describes the methodology, results and discussion.

Chronic myelogenous leukemia (CML) is a form of cancer that leads to uncontrolled multiplication of white blood cells in the bone marrow, and a consequently increasing amount of white blood cells in blood. The Philadelphia chromosome, named after the city where it was first discovered in 1950s, is the main cause for CML [1]. Nearly 95 % the CML patients have shown this abnormality [2]. The reciprocal translocation between chromosome 9 and 22 makes up the Philadelphia chromosome. The result is that part of bcr gene from chromosome 22 is fused with part of the abl gene of chromosome 9. This swapping of genetic material provides DNA codes for mutant tyrosine kinase, known as bcr-abl. Normal functioning of the tyrosine kinase requires activation by other cellular messaging proteins before initiating cell division. The fused bcr-abl protein transcript is constitutively active and it is not required to be activated by other cellular messaging proteins as illustrated in Figure 1A. The bcr-abl activates a number of cell cycle controlling proteins and enzymes thereby speeding up cell division [1, 3, 4].

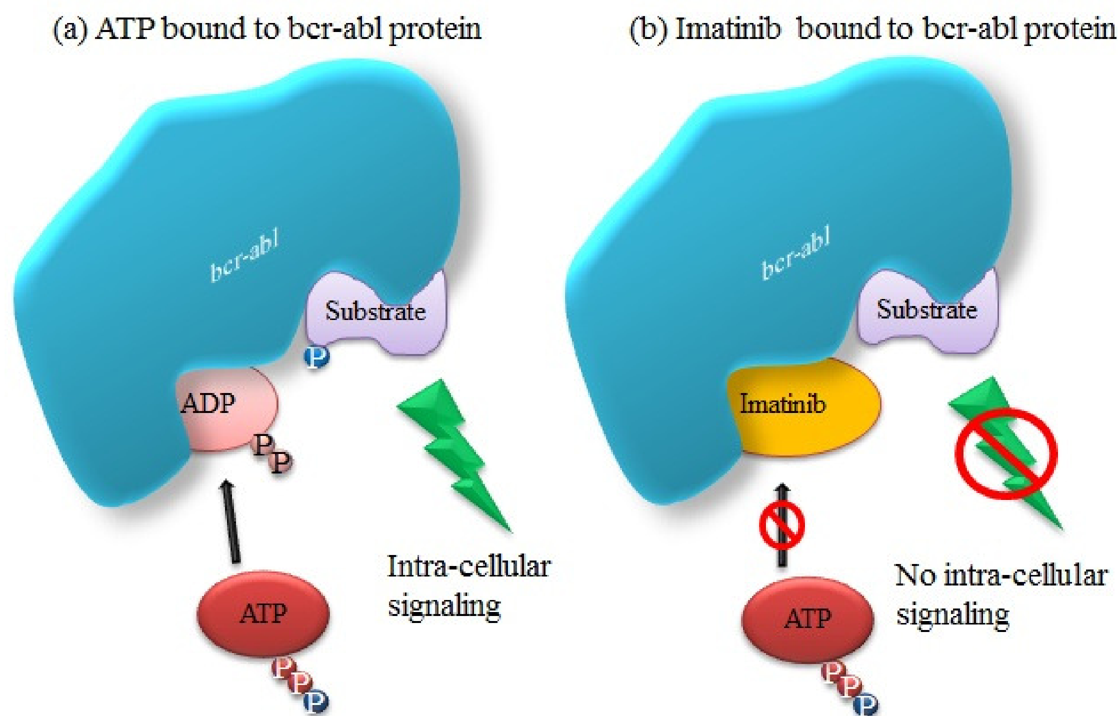


Figure 1. A simplified representation of the mechanism of action of imatinib showing the binding to the kinase activation domain of bcr-abl oncoprotein occupying adenosine triphosphate (ATP).

Diagnosis of CML usually takes place in the initial chronic phase (CP) and if left untreated it progresses to an accelerated phase (AP) in three to five years [5]. According to the statistics published by the United States (US) National Cancer Institute [6], most of the CML patients are diagnosed in the latter part of their life with an average of 64 years of age and an incidence rate of 1.6 per 100000/year. Statistics also shows that CML tends to be more common in men than women. Chemotherapy, bone marrow transplantation, immunotherapy (interferon) and tyrosine kinase inhibitors (imatinib, dasatinib and nilotinib) are among the treatments for CML [3].

Until the introduction of imatinib, interferon alfa was widely regarded as the best treatment for CML patients who cannot be treated with stem cell transplantation in the chronic phase of the disease. This drug is known to cause a number of side effects, especially in older patients. Stem cell transplantation can only be used for a selected number of patients and may still cause serious complications or even death [3].

Imatinib (Figure 2) is chemically designated as 4-[(4-methyl-1-piperazinyl)methyl]-N-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl]amino]-phenyl] benzamide. The molecular formula of imatinib is $C_{29}H_{31}N_7O$ and its monoisotopic molecular weight is 493.259 g/mol. Imatinib is highly soluble in water at low pH (≤ 5.5), but is poorly soluble or insoluble at neutral and alkaline pH [7]. Imatinib is readily soluble in most of polar organic solvents like methanol and ethanol, however it is less soluble or insoluble in low polar solvents.

Figure 2. Chemical structure of imatinib.

Imatinib acts by blocking the ATP binding site of bcr-abl, thereby competitively inhibiting the enzyme activity of the protein as illustrated in Figure 1. In addition, imatinib is also known for inhibiting c-kit tyrosine kinase responsible for the gastro-intestinal stromal tumours (GISTs) and platelet-derived growth factor receptor (PDGFR) involved in the pathogenesis of the hypereosinophilic syndrome. Imatinib, commercially available as Gleevec® or Glivec®, is therefore regarded as the first drug for cancer treatment that selectively eliminates cancer cells rather than destroying all the rapidly dividing cells [8]. It was first clinically tested for treating CML

patients in 1998 and received FDA and EU approval in 2001 [2]. There are two second generation drugs of imatinib; namely Nilotinib and Dasatinib used for treating imatinib resistant CML patients having more than 1002 ng imatinib per mL of plasma [9].

Imatinib is taken orally (400-600 mg per day) and its concentration in blood has to be within a certain range (500 – 1000 ng/mL) in order to achieve an optimal clinical response [4]. Pharmacokinetic studies of imatinib show that orally administrated imatinib is well absorbed and demonstrated 98% imatinib bioavailability irrespective of oral dosage [7]. It has been reported that the half-life of imatinib is approximately 18 hours and about 95% of imatinib bound to plasma proteins, mainly to albumin. Elimination of imatinib from the human body is mostly via faeces as metabolites and approximately 81% of the drug is eliminated in 7 days. However, the role of drug metabolizing enzyme activity in the fate of imatinib in cancer cells remains to be investigated [7]. Nausea, changes in blood cell counts, fluid retention and muscle cramps are some of the side-effects that imatinib treated CML patients may experience, which are however considered manageable and much less severe than that of the interferon therapy [3]. The imatinib concentration in cancer patients treated within the recommended dosage does vary depending on several factors: such as individuals' drug metabolism, body weight, sex, etc. Picard et al. [10] suggested that monitoring imatinib concentration in CML patients is useful for grasping the reason for treatment failure, to monitor the suboptimal response of patients receiving recommended dosage for individual dosage adjustment. According to Götze et al. [11], therapies with thyrosine kinase inhibitors (TKIs) have shown significant inter-individual variability in pharmacokinetics, which demand a need for Therapeutic Drug Monitoring (TDM) in order to achieve optimal response in CML therapy and thereby to minimize adverse side effects. According to another report, there is a slight age dependency of the imatinib distribution in plasma, which accounts for about 12% of CML patients younger than 65 years [7].

3.1 Aim of the study

The general aim of this research was to develop a rapid and sensitive method for quantification of imatinib. The LC-MS (TOF) and DART-MS (TOF) methods have been explored to quantify imatinib in human plasma samples.

3.2 Quantification of imatinib

The most commonly used method for determination of imatinib concentration in plasma is high performance liquid chromatography (HPLC) with either an ultra violet (UV) detector [12-15] or tandem mass spectrometry (MS) [16-21]. LC-MS has widely been applied as a reliable technique for quantification of imatinib in human plasma. The main difference among the reported LC-MS methods is the ionization technique, i.e. ESI or APCI. The reported methods describe various sample preparation techniques such as protein precipitation [15, 18] and liquid-liquid extraction [19] prior to the separation of analytes with reverse phase chromatography. In some protocols, use of solid phase extraction columns for the enrichment of the

analyte was discussed [14-16, 22]. There are some reports describing simultaneous quantification of imatinib and its main metabolite, CGP74588 [13, 16, 18, 23]. Although most of the published methods were initially on the quantification of single tyrosine kinase inhibitor (TKI), recent publications focus more on simultaneous TKIs analysis [9, 20, 21, 24] as improvement of the clinical outcome may be achieved through combination therapies according to recent studies [25, 26]. Use of ultra performance LC-MS-MS for quantification of 9 TKIs was also reported [22].

Recently imatinib in plasma has been quantified by capillary electrophoresis coupled to MS (CE-MS) [27]. However, CE-MS is laborious due to both sample preparation and instrumentation.

3.2.1 Liquid chromatography (LC)

Liquid chromatography is an analytical technique applied for separating analytes that are dissolved in a solvent. It was developed in the late 1960's and made a significant impact on biological research. The differential partitioning of analyte molecules between the stationary phase and mobile phase is the basis of the chromatography. The mobile phase is a liquid which is pumped through a solid phase. The most commonly used solid phase is silica particles covered with covalently bound molecules with desired properties. Due to different distribution of compounds between the phases, constituents of a sample introduced to the system will gain distinct velocities and thus be separated from each other (Figure 3).

Depending upon their specific chemical nature, analytes will be more or less strongly associated with either of these phases, and the difference in this degree of affinity for the stationary phase or mobile phase allows separating molecules.

In the first study of the project LC was employed with reverse phase (RP) mechanism. Here a commercially available C18 column (see method section for more detail) was used with gradient elution of the mobile phase. In RP chromatography the retention mechanism is dependent on hydrophobic interaction of the solute with the non-polar stationary phase [28]. The elution strength of the mobile phase is controlled by adjusting the type and amount of organic modifier. Retention can also be influenced by pH adjustments or by adding a counter ion to the mobile phase (pairing the solute to a counter ion). In this study, formic acid (0.1%) was added to the mobile phase to enhance retention interaction between the column's stationary phase and analytes and to improve the peak shapes since any silanol activity is suppressed under acidic conditions [28]. Moreover, in positive ion mode mass spectrometry, enhancement of ionization efficiency was previously observed in electro spray ionization (ESI) as formic acid has the necessary acidity and volatility to provide protons for this purpose [29].

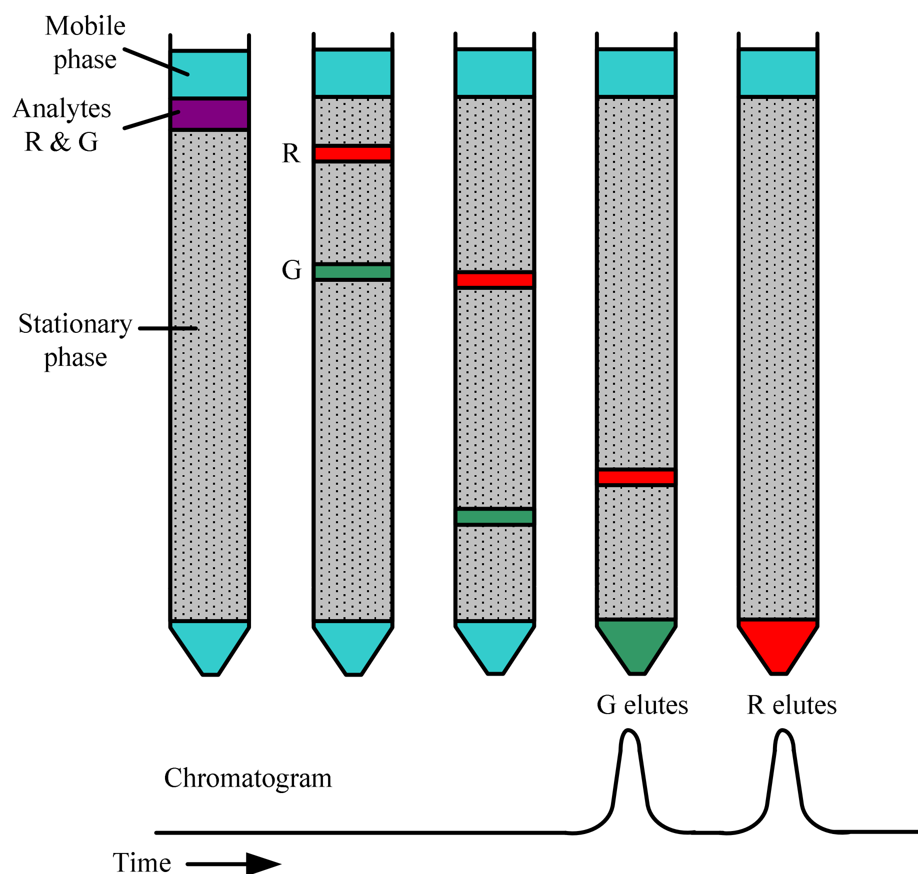


Figure 3. Schematic representation of chromatographic separation technique.

Liquid chromatography is often used prior to mass spectrometry (MS) or tandem mass spectrometry (MS-MS) analysis in order to pre-concentrate and separate the analytes and to minimize suppression effects on analyte. Combination of modern chromatography and mass spectrometry enables identification and quantification of analytes with very low limit of detection and better selectivity. Figure 4 depicts a flow diagram of a LC system coupled with a mass spectrometer.

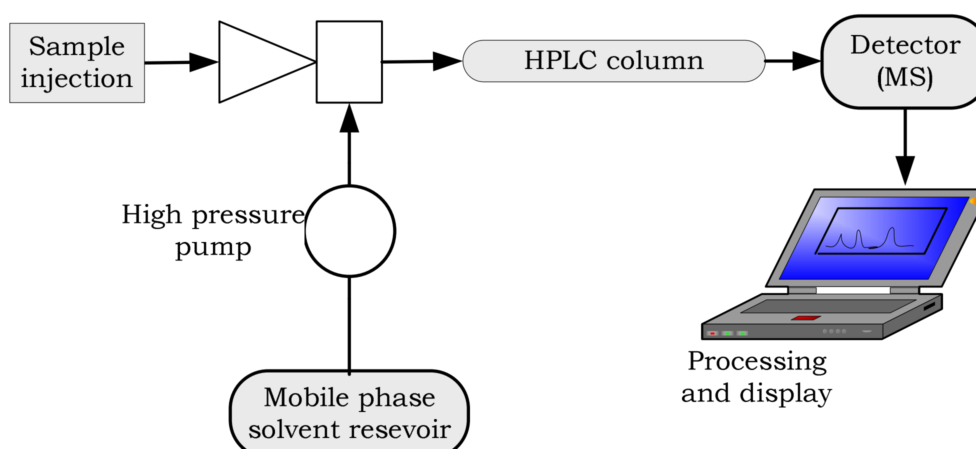


Figure 4. A flow diagram of a liquid chromatography system.

3.2.2 Mass spectrometry (MS)

A mass spectrometer is an analytical instrument designed to separate ions according to their mass- to- charge (m/z) ratio and record their relative intensities. An MS system consists of an ion source, a mass analyser and a detector as schematically illustrated in Figure 5.

In order to separate and detect molecules in a mass spectrometer, they need to be present as ions in gas phase. Electro-spray ionization (ESI) is the most frequently used ionization method [30] and can be directly coupled with HPLC allowing analysis of natural products, drugs and drug metabolites, proteins, nucleic acids, etc. In ESI, molecular species are dissociated in liquid phase and get desorbed into the gas phase when high voltage is applied in between the sample outlet and the mass spectrometer inlet. The LC-MS system employed in this study used ESI.

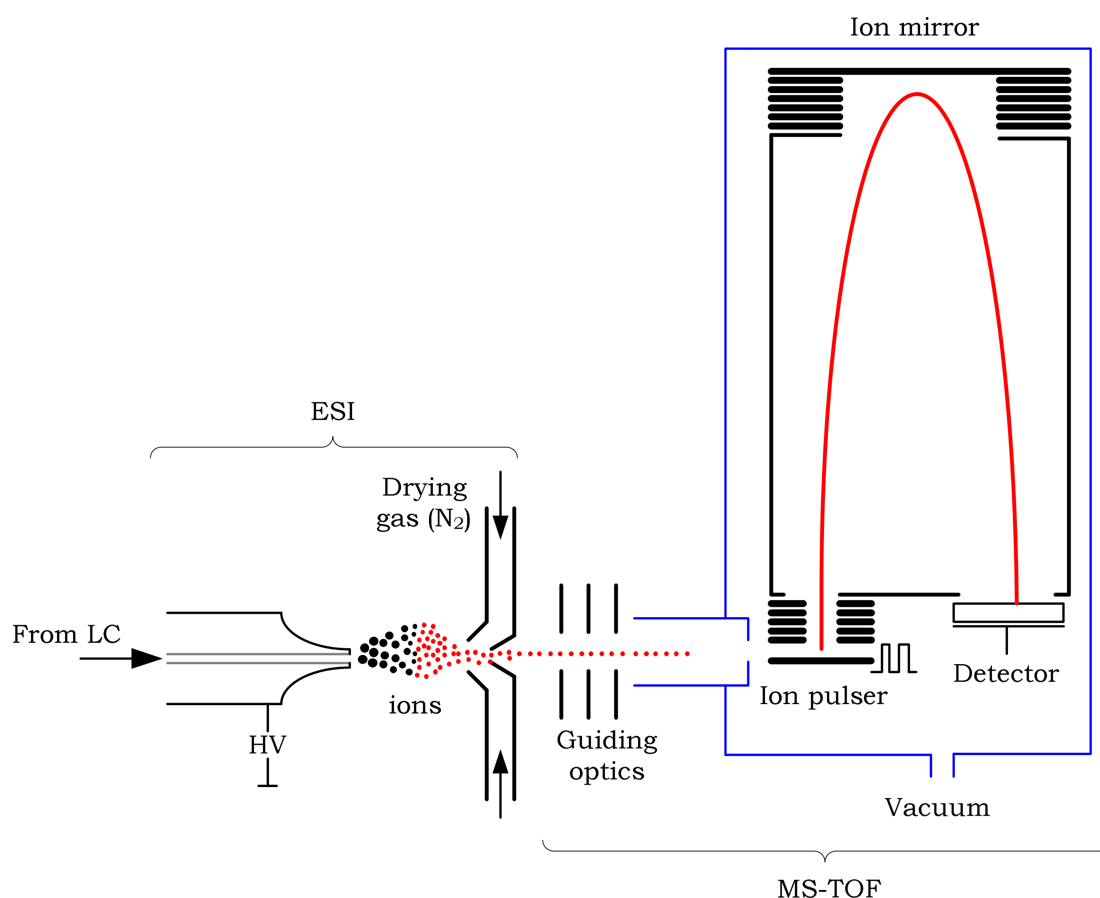


Figure 5. Schematic diagram of mass spectrometer.

In this method development we used a time of flight mass spectrometer (TOF) coupled with a liquid chromatography (LC) and a rather new DART. The basic principle of a TOF is to accelerate ions to a certain kinetic energy, measure the time of flight between two given points in a field free space, and thereby calculate the m/z ratio [31].

MS-TOF continuously registers ions over the complete mass range and thus this MS is the ideal choice for the investigations of imatinib, since its ability to acquire wide range of masses.

3.2.3 Direct Analysis in Real Time

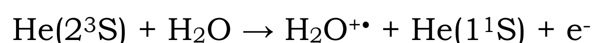
Direct Analysis in Real Time, developed by Laramee and Cody in 2005 [32, 33], is an ion source operated at atmospheric pressure allowing direct introduction of solid and liquid samples into mass spectrometry system. This feature eliminates the need of conventional sample preparation and separation conducted by liquid chromatography. DART is literally an atmospheric pressure ionization technique among others, and performs the ionization through collisions between the analyte (A) and electronically excited atoms or vibronically excited molecules (or metastables M*) [32].

The DART source is usually coupled with an atmospheric pressure ionization time of flight (TOF) mass spectrometer (MS) allowing high-resolution and accurate mass detection. The DART-MS (TOF) offers analysis of solid samples in original state and ionization takes place directly on the sample surface in open air (e.g., currency bills and drugs). In case of liquid samples, a one end closed melting point glass tube (Merck, Eurolab, Stockholm, Sweden) is used for sample introduction into the DART ion stream as illustrated in Figure 6. A gas (typically helium or nitrogen) flows through a chamber where an electrical discharge produces ions, electrons and excited-state atoms and molecules (metastables). The needle electrode on high potential produces He metastable ions. The function of grounded electrode and perforated disk electrodes is to remove most of the charged particles as the gas passes through. Only the metastable and neutral gas molecules enter the gas heating chamber. A grid electrode at the exit supports ion drift towards the orifice of the mass spectrometers' atmospheric pressure interface.

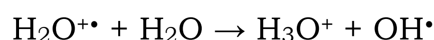
When nitrogen or neon used in positive ion mode, the ionization process of the analyte with the interaction of metastable ions can simply be described by the Penning ionization mechanism where transfer of energy takes place from the excited gas to an analyte which has an ionization potential lower than that of the excited gas [34]. Consequently, an electron is released from the analyte molecule producing a radical cation. The molecular cations then drift off from the sampling surface towards the mass analyser along with the gas stream.

$M^* + A \rightarrow A^{+\bullet} + M + e^-$

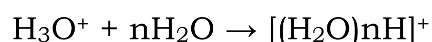
When He is used as the carrier gas in positive ion mode, the ionization process can be described by the following mechanism. Metastable helium interacts with atmospheric water molecules to form hydronium clusters, which in turn transfer proton to the sample molecule.



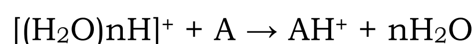
$He(2^3S) + H_2O \rightarrow H_2O^{+\bullet} + He(1^1S) + e^-$



$H_2O^{+\bullet} + H_2O \rightarrow H_3O^+ + OH^\bullet$



$H_3O^+ + nH_2O \rightarrow [(H_2O)nH]^+$



$[(H_2O)nH]^+ + A \rightarrow AH^+ + nH_2O$

Desorption and ionization of the analyte (solid, liquid or gas) takes place by direct interaction of excited species with the sample.

The absolute signal intensities in a repeated set of DART-MS measurements of a particular sample can vary significantly, which is more pronounced for manual sampling as the positioning of the sample in the DART gas stream largely determines the absolute response produced by the mass spectrometer. In order to alleviate such variations, an internal standard can be used to normalize the analyte responses. [33].

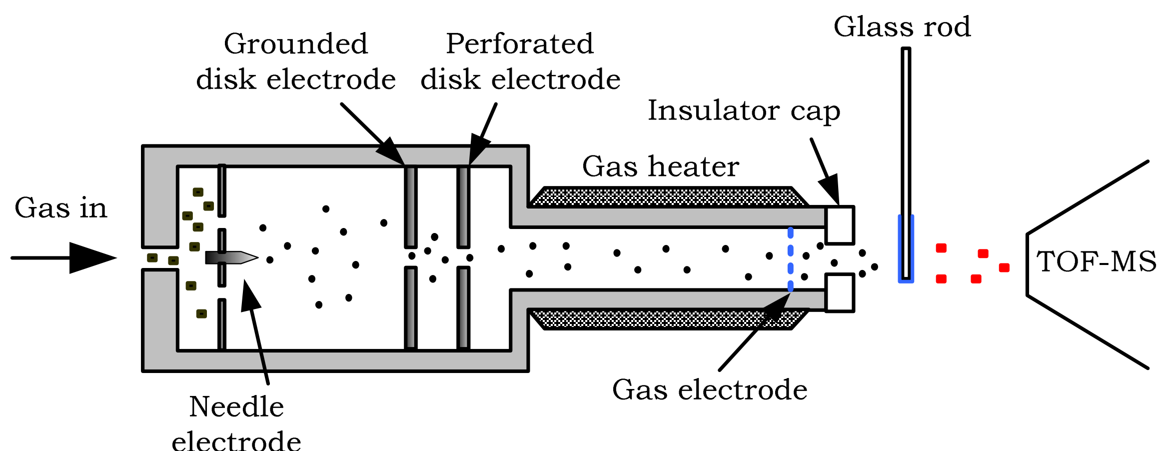


Figure 6. Schematic representation of (DART) ion source coupled to a TOF mass spectrometer.

Due to DART's distinctive advantage in high throughput chemical detection, analysis and quantification, the number of publications presented based on the application of DART in a wide range of fields such as forensics, pharmaceuticals, natural products, food and beverages has become significant in recent years. Most studies reflect DART's ability to identify different compounds, [35-39], whereas DART's quantitative capability has only been reported in a relatively low number of publications.

Nilles et al. [40] used DART in the identification and quantification of chemical warfare agents obtaining linear calibration curves with R^2 = 0.99. While mentioning DART's advantages over LC-MS-MS method in terms of simplicity and speed of operation, Zhao et al. [41] pointed out its lower sensitivity and inability to solve matrix effect in some particular cases. Yu et al. [42] reported DART's usefulness for the quantification of drugs in biological matrices. They found that the DART method complies with the general requirements of pharmaceutical analysis and is capable of delivering comparable reproducibility, sensitivity and linearity to those of LC-MS-MS, especially without sample clean up or chromatographic separation.

The method described previously by Wang et al. [43] on quantification of imatinib in dried blood spots on paper using a paper spray method has not been validated. The detection limit was as low as 30 ppb and the calibration curves in plasma were linear over the therapeutic range.

In this report, a sensitive method for the assay of imatinib in human plasma is introduced using HPLC coupled with a time of flight mass spectrometer. This assay has the level of sensitivity and reproducibility required for routine clinical application. In this project, I also explored DART for developing an imatinib quantification method. To the best of my knowledge, no comprehensive analysis of imatinib using DART-MS has been reported. The new method was partially evaluated and very promising results have been obtained.

3.3 Quantification

In order to study imatinib in plasma, quantification of imatinib is a necessity. The measurements can be either absolute or relative. The performance of any quantitative method has to be confirmed by validating it. A set of parameters are used to define how close the measurements are to the true values [44].

Calibration method

In order to correlate the measured response with imatinib concentration, a calibration curve is required. Two different methods are mainly used for the plotting of the calibration curve using external and internal standards [44].

External standard (ES)

The calibration curve is prepared by plotting the responses versus the known concentrations of the analyte. The mathematical description of the curve (a linear equation in the form of $y = ax+b$ which is determined from linear regression analysis) can be used to determine the unknown concentrations of samples by substituting the ordinate (the responses - y) of the linear equation [44].

Internal Standard (IS)

Internal standard can be used to minimize the instrumental variations such as matrix effects or variations in sample administration and preparation. It is added as a fixed amount to all samples. The response ratio between the analyte and the IS is used for generating a calibration curve. In order to have the same reactions as the analyte, the chosen IS exhibits similar physical and chemical properties as the analyte [44].

3.4 Method validation

The main objective of method validation is to demonstrate the reliability of a particular method for the determination of the concentration of one or more analyte(s) in a specific biological matrix, such as blood, serum, plasma, urine, or saliva. The applicability of an analytical method is evaluated by performing a series of validation tests according to FDA recommendations [44]. Method validation proves the suitability of the protocol for the particular measurements.

The main characteristics of a bioanalytical method that are essential to ensure the acceptability of the performance and the reliability of analytical

results are: selectivity, lower limit of quantification, the response function and calibration range (calibration curve performance), accuracy, precision, matrix effects, stability of the analyte(s) in the biological matrix and stability of the analyte(s) and of the internal standard in the stock and working solutions and in extracts under the entire period of storage and processing conditions.

According to the FDA guidelines for industrial bioanalytical method validation, validation of an analytical procedure should include these parameters: accuracy, precision, repeatability, reproducibility, specificity and selectivity, limit of detection (LOD), limit of quantification (LOQ), linearity, and range.

The definition of accuracy of an analytical procedure is that the closeness of mean value found by the method to the accepted reference value (concentration) of the analyte. Accuracy is assessed by replicate analysis of samples with minimum of three concentrations. The concentration should cover the range of expected concentrations. The mean value within 15% of the actual value is regarded as acceptable.

FDA describes precision as the closeness of agreement (degree of scatter) between a series of measurements obtained by applying the procedure repeatedly to multiple aliquots of a single homogeneous sample. FDA requires precision to be tested from a minimum of three concentrations in the range of expected concentration. A 15% coefficient of variation (CV) is the maximum accepted value for the precision estimated at each concentration except for the LOQ, where a maximum of 20% CV is acceptable.

According to FDA definitions recovery is the extraction efficiency of an analytical process. Recovery experiments are performed by comparing the detector response obtained for extracted samples with the detector response obtained for the unextracted samples representing 100% recovery.

The specificity of the assay methodology is the extent to which a particular method can determine analytes under given conditions in the presence of interferences from other substances in the matrix under the stated conditions of the method.

Selectivity of a method refers to the extent to which a bioanalytical method differentiates and quantifies the analyte in the presence of other components in the sample including impurities, metabolites, or other matrix components.

Limit of quantification (LOQ) is the lowest analyte concentration that can be quantitatively determined with acceptable precision and accuracy. The recommended precision at the LOD is ≤20% with accuracy in range of 80-120%.

Limit of detection (LOD) is the lowest concentration of an analyte that the bioanalytical procedure can distinguish from the absence of that substance (a blank value). The value is expressed in units of concentration (or amount).

The definition of linearity is the ability of the method to elicit test results that are directly proportional to the concentration of analyte in the sample.

Range is the interval between the upper limit of quantification (ULOQ) and the lower limit of quantification levels of analyte (inclusive) that have been demonstrated to have an acceptable level of precision, accuracy and linearity using the defined method. For assay tests, the minimum specified range is required to be 80 to 120 % of the expected concentration of the analyte.

The developed imatinib quantification procedure based on LC-MS was validated for all the characteristics listed above, except the reproducibility which is supposed to be performed in case of standardization of an analytical procedure. Due to time limitations and unavailability of the DART instrument, a comprehensive assessment of all validation parameters was not performed and only the linearity, LOD and LOQ and instrument precision were determined for the method developed with DART.

4 MATERIALS AND METHODS

4.1 Materials

All chemicals and reagents used in this study were of analytical grade. An internal standard trazodone ($C_{19}H_{22}ClN_5O$) was obtained from Sigma Aldrich (Stockholm, Sweden). All other chemicals and reagents were purchased from Merck, Eurolab (Stockholm, Sweden) unless otherwise stated. Water was deionized and osmosed with a Milli-Q purification system (Millipore, Bedford, MA, USA). The imatinib was kindly donated by Novartis (Basel, Switzerland). Human plasma samples from healthy blood donors and the plasma samples of imatinib treated CML patients were obtained from Karolinska institute, Solna, Sweden. The reference samples for validation were received from reference Laboratory in Bordeaux, France.

4.2 Sample preparation

Sample preparation procedure consisted of four steps: adding IS to human plasma, protein precipitation, enrichment and introduction to LC-MS or DART systems as illustrated in Figure 7.

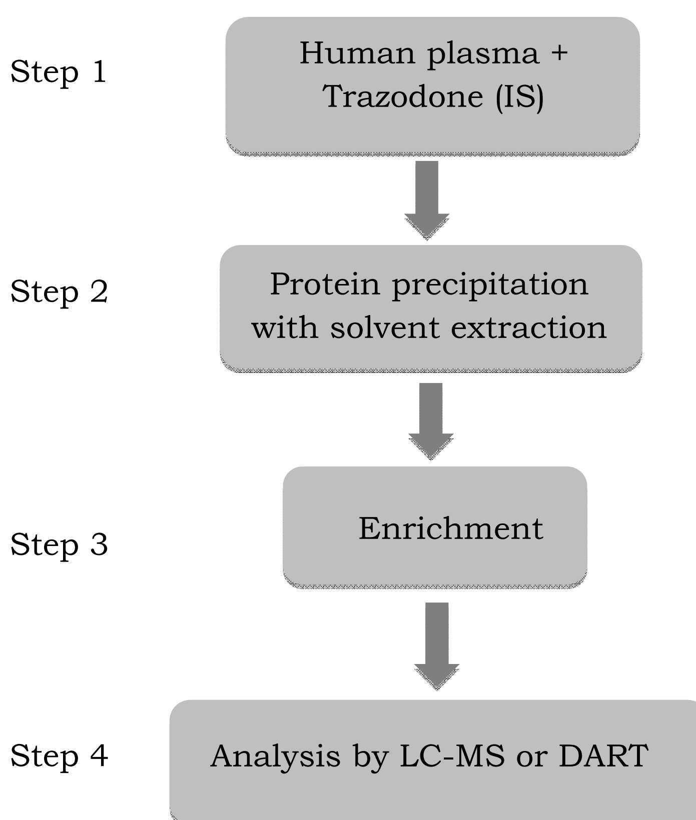


Figure 7. A flow diagram showing the basic steps in sample preparation.

4.2.1 Sample preparation

The sample preparation procedure described in this section is the same for LC-MS and DART except the sample enrichment designated as the step 3 in Figure 7.

Stock solutions, working solutions, internal standard, calibration standards and quality controls (QC) were prepared. Stock solutions of imatinib and trazodone (IS) were prepared by dissolving an accurately weighed amount in 100% methanol to yield 1 mg/mL. The stock solutions were kept in storage at -20 °C, in tightly capped (wrapped with Parafilm®) glass flasks. In order to obtain the working, calibration and QC solutions at different concentrations, the Stock solution was diluted with MeOH (Table 1). The concentrations for QC were selected to cover the expected imatinib concentration range in the patients' plasma.

Table 1. Imatinib concentrations of different solutions.

Stock solution concentration	Working solution concentration (μg/mL)	Calibration range (ng/mL)	QC (ng/mL)
1mg/mL	100, 10, 1 & 0.1	30-10000	100, 1000, 5000

The collection of blood was performed by venipuncture, using standard EDTA tubes (VACUETTE®, Greiner Bio-One). Plasma was obtained by centrifugation at 2500g for 10 min. Subsequently, plasma was aliquoted and stored at -80° C until analysis.

In all cases except in the patient plasma samples, an appropriate amount of imatinib solution (100, 10, 1 or 0.1 μg/mL working solutions) was added to 0.2 mL of blank human plasma in 1.5 mL Eppendorf tubes to yield the required imatinib concentration. Each sample was spiked with 50 μL of trazodone (10 μg/mL). For protein precipitation, 1 mL of methanol was added to each tube and vortexed for 15 min and then centrifuged at 4 °C for 14 min at 14,000g. The resulting supernatants were transferred to borosilicate glass tubes (DURAN 12 x 100 mm with Teflon screw-cap) for evaporation to dry the sample under a gentle stream of nitrogen at room temperature. The dried residue was redissolved in 100 μL of methanol and distilled water mixture (20:80, v/v for LC-MS and 50:50, v/v for DART) and vortexed briefly. The final solution was then transferred to the HPLC auto sampler vials which injected 10 μL into the LC–MS system. Samples were introduced into the DART system by dipping the closed end of a glass melting point tube in the sample and placing between the dart source and the mass spectrometer.

The method validation procedure applied in this work is based on FDA recommendations. The samples were prepared accordingly to yield the appropriate imatinib concentration levels and the number of replicates demanded by each test (linearity, precision, accuracy, selectivity, sensitivity, stability, limit of detection etc.).

Linearity

For the linearity check, nine calibration samples at 30, 100, 300, 1000, 2000, 3000, 5000, 7000 and 10000 ng/mL imatinib concentrations were prepared and run in triplicate.

Precision and accuracy

QC samples were used for precision and accuracy determinations on intra-day and inter-day basis.

Limit of detection and limit of quantification

For the determination of the LOD and LOQ, a separate series of samples with low concentrations (1 – 40 ng/mL) was run in triplicates.

Recovery

For determination of absolute recoveries of imatinib and internal standard (trazodone), along with QC samples unextracted reference solutions containing the same QC concentrations were prepared in the mobile phase (methanol : water, – 20:80,v/v).

Patient plasma samples

In case of patient plasma sample analysis, a 0.2 mL of human plasma was spiked with 50 µL of trazodone (10 µg/mL) and followed the rest of the sample preparation procedure described above.

4.2.2 Direct Analysis in Real Time (DART)

The DART measurement system consists of a DART ion source (control software ver. 1.55, Ion Sense Inc., Saugus, MA, USA) and an Accurate Time of Flight (AccuTOF) mass spectrometer (JEOL JMS-T100LC, Tokyo, Japan), which operates in positive-ion mode and controlled by “Mass Center Main” software (ver. 1.3.7, JEOL Ltd., Akishima, Japan). Helium is used as the ionization gas of the DART ion source. All the measurements were performed with the orifice 1 voltage 15 V, gas heater temperature of 400 °C, discharge electrode needle at 3000 V, electrode voltage at 177 V, and grid electrode voltage at 384 V. Internal mass calibration was achieved using a dilute solution of polyethylene glycol (PEG) 600 in methanol sampled within each data file. Mass to charge ratio (m/z) range of 350-550 was used in recording mass spectra. The recorded mass spectral data was processed with the Mass Center software (JEOL, Tokyo, Japan); Background subtraction and intensity centralising were carried out in peak detection.

4.2.3 LC-MS

The LC system used in this project has three main units: an Agilent model 1100 Auto-sampler (Agilent Technologies, Palo Alto, CA, USA), an Agilent 1100 Quaternary pump, and an Agillent ZORBAX C18 (5 µm, 2.1x50 mm) reversed-phase analytical column controlled by Agillent ChemStation software. The chromatographic system was coupled to a time of flight (TOF) mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped

with an electrospray ionization (ESI) interface controlled by Agilent MassHunter software. Analysis of the mass spectra was performed by means of Analyst QS software provided with the instrument.

10 µL of final solution was injected into the LC–MS system by the LC auto-sampler and the mobile phase was delivered according to the developed stepwise gradient elution program given in Table 2. The mobile phase consisted of 0.1% formic acid in water (buffer A) and 0.1% formic acid in methanol (buffer B). The total run time was 14 minutes.

The instrument settings for the mass spectrometer were as follows: the ion source operated at 3.8 kV, the nitrogen gas flow rate was 7 L/min and nebulizing gas pressure of 15 psi at a temperature of 300 °C. MS operated in ESI positive ion mode and fragmentor, skimmer and octopole guides were set at 225V, 60V and 250V respectively.

Extraction of ion chromatogram for imatinib and trazodone were done with *m/z* ranges 494.15 – 494.3 and 372.1 – 372.2 respectively.

Table 2. LC-MS gradient elusion program.

Time (min)	Flow rate (mL /min)	Solvent A (%)	Solvent B (%)
0	0.2	80	20
6	0.2	40	60
7	0.4	0	100
10	0.4	80	20
13	0.2	80	20
14	0.2	80	20

5 RESULTS AND DISCUSSION

5.1 LC-MS (TOF)

Imatinib is a multi-target inhibitor with a monoisotopic mass of 493. 25 Da (Figure 9). The MS detection of imatinib showed abundant singly charged molecular ions $[M+H]^+$ at *m/z* of 494.266 providing evidence of the analyte's molecular weight. It also showed the doubly charged ion of imatinib $[M+2H]^{2+}$ at *m/z* of 247.638. The major fragmented imatinib ion was seen at *m/z* of 395.174 (Figure 8), which could be useful confirming the analyte. Marull et al. [45] have shown two other fragments with very low abundance (< 3 %). Trazodone used in the measurements showed the singly charge ion at *m/z* of 372.162 as it can be seen in Figure 10. Quantification of imatinib was accomplished by monitoring the molecular ions (*m/z* of 494.266 for imatinib and *m/z* of 372.162 for the trazodone). Imatinib eluted with an average retention time of 8.30 ± 0.10 min and the internal standard trazodone with a retention time of 8.03 ± 0.11 min.

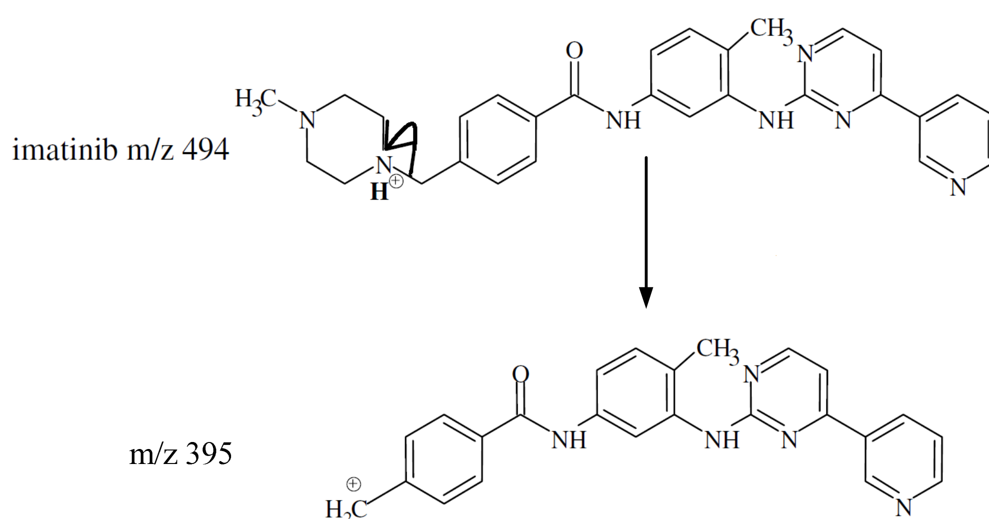


Figure 8. Major fragmentation of imatinib at m/z 395 [45].

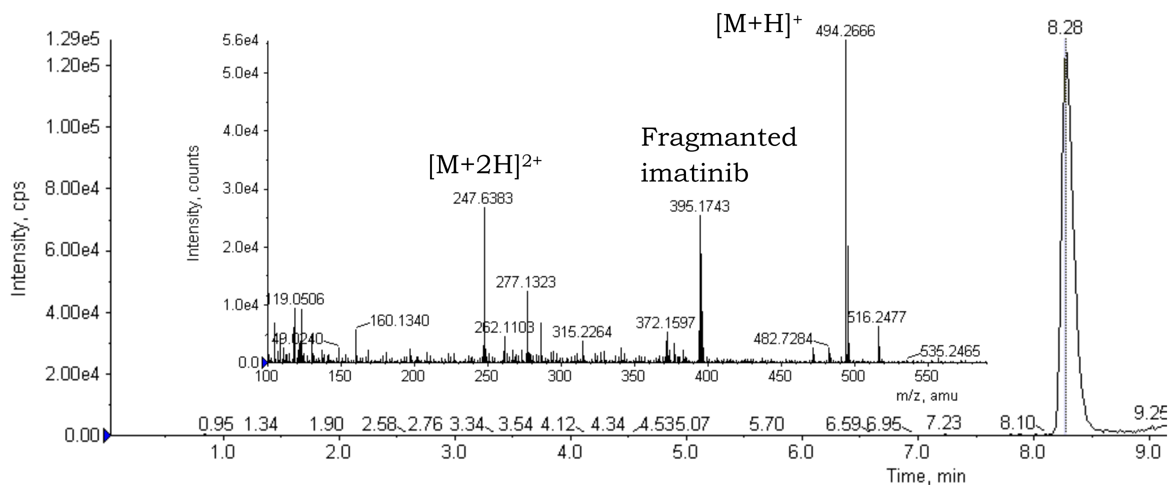


Figure 9. Representative extracted ion chromatogram of human plasma with 2000 ng/mL imatinib following LC-MS. m/z range used for extraction was 494.15 – 494.3. Retention time of imatinib is 8.28 min.

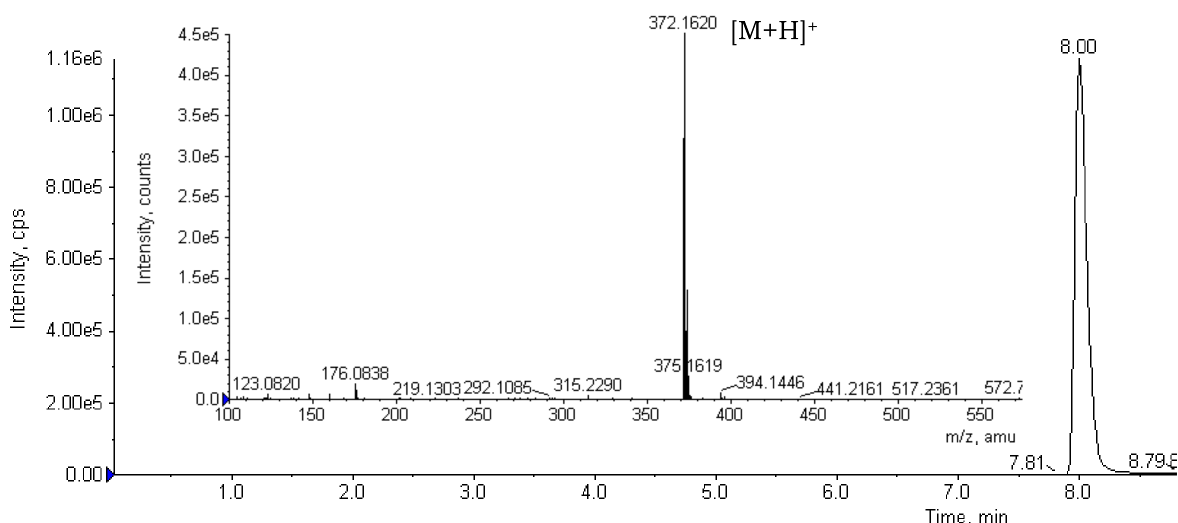


Figure 10. Representative extracted ion chromatogram of human plasma with 2000 ng/mL trazodone following LC-MS. m/z range used for extraction is 372.1 – 372.2. Retention time of trazodone is 8.00 min.

The linearity of the calibration curve was established in order to cover the clinically relevant range of imatinib concentrations expected in most patients. In order to eliminate various sources of error arising from different steps of the assay, the ratio between the drug and its internal standard, instead of the absolute signal intensity of the imatinib, was utilized to determine the concentration of imatinib in plasma, i.e., the ratios of areas under the peaks of imatinib and trazodone were plotted against the initial concentration of the trazodone and imatinib spiked plasma samples (Figure 11). The quality of the linear fit was scrutinized by means of several statistical analysis methods as presented in the following section.

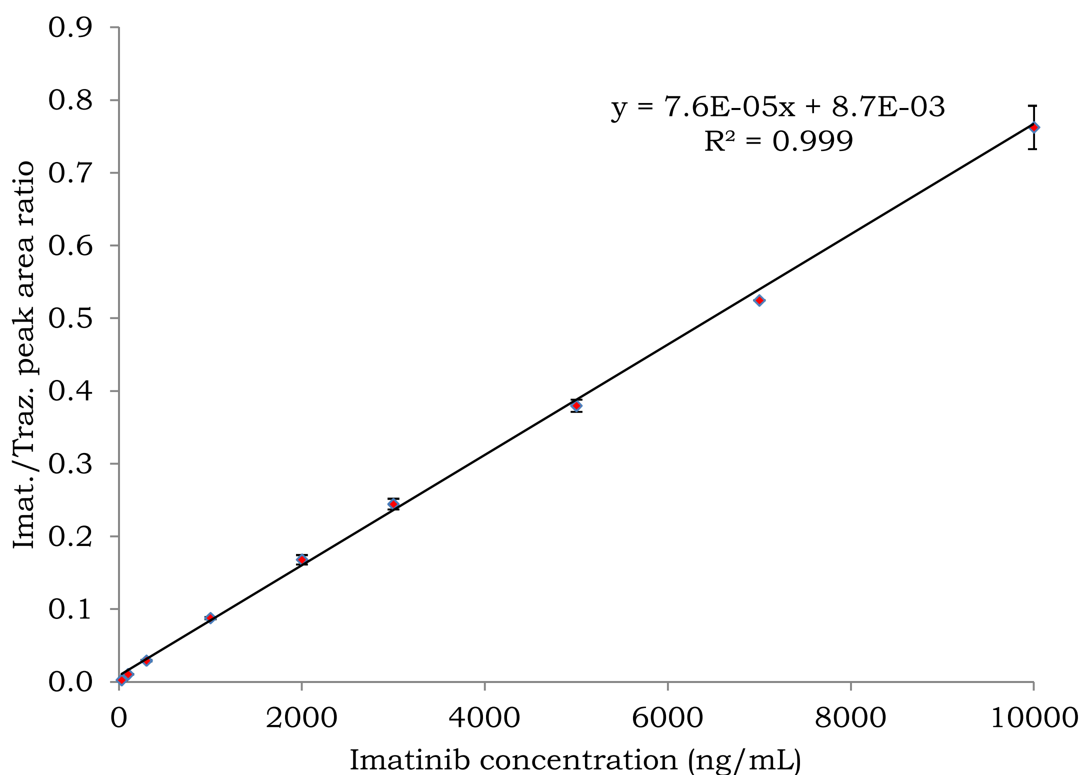


Figure 11. Calibration curve for LC-MS, showing average (n=3) imatinib to trazodone peak area ratio versus concentration.

5.1.1 Method validation

Linearity

The linearity of the method was evaluated over the range of 30 – 10000 ng/mL. The linearity was determined by linear regression analysis and correlation coefficient of linear regression (R^2) was of 0.9994 (n = 3). The quality of the linear fit was further analysed by estimating coefficient of variation (CV %) of data at each concentration that was run in triplicate and presented in Table 3. The maximum CV (4.8 %) occurs at 30 ng/mL which is acceptable according to the FDA recommendations.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ were determined as the concentrations producing signal-to-noise ratio of 3 and 10 respectively, resulting in LOD of 3 ng/mL and LOQ of 10 ng/mL.

Accuracy and precision

The instrument precision was determined by multiple injections of the QC samples (100, 1000 & 5000 ng/mL), and summarized in Table 4. The CV values are lower than 2.4 %.

Table 3. Mean, standard deviation, and coefficient of variation of imatinib/trazodone peak area ratio at each concentration.

Nominal concentration	Peak area ratio (n=3)	
(ng/mL)	Mean±SD	CV (%)
30	0.0042±0.0002	4.8
100	0.0107±0.0003	2.8
300	0.0295±0.0011	3.7
1000	0.0880±0.0015	1.7
2000	0.1680±0.0064	3.8
3000	0.2450±0.0074	3.0
5000	0.3800±0.0084	2.2
7000	0.5250±0.0002	3.8
10000	0.7630±0.0298	3.9

Table 4. Instrument precision at three different concentration levels (n= 3).

Nominal concentration (ng/mL)	Estimated concentration	
	Mean±SD (ng/mL)	CV (%)
100	106±2	2.2
1000	1006±24	2.4
5000	4953±106	2.1

The intra-day precision and accuracy were determined at three different concentrations run in triplicate. The samples in triplicate at QC concentrations were analysed on six different days for the inter-day precision and accuracy determination, and the results are presented in Table 5. Inter- and intra-day precisions (estimated as CV) exhibit a maximum value of 4.4 % for 100 ng/mL inter-day assay, which can be regarded as acceptable (< 15 %) [44]. The accuracy is estimated as the percentage ratio between the estimated mean and the nominal concentrations, which ranges from 98 % to 106 %.

Recovery

The recovery of imatinib was estimated as the ratio between the area under the peaks of the extracted samples (QC) and those of unextracted samples (prepared in mobile phase). The estimated extraction recoveries determined for imatinib ranged over 95 – 107 % and met the acceptance criteria.

Table 5. Inter-day and intra-day accuracy and precision (n=9).

	Nominal concentration (ng/mL)	Mean concentration±SD ng/mL	CV (%)	Accuracy (%)
Intra-day	100	106±3	2.4	106
	1000	1020±18	1.8	102
	5000	4952±102	2.1	99
Inter-day	100	102±5	4.4	102
	1000	1007±20	2.0	101
	5000	4893±98	2.0	98

Specificity and selectivity

The specificity and selectivity of the assay were investigated by comparing total ion chromatograms of blank human plasma samples and blank plasma samples spiked with 1000 ng/mL imatinib and 1000 ng/mL trazodone. It was observed that the other substances in the blank plasma did not significantly interfere with the total ion chromatogram around the retention time of imatinib; and for trazodone as well.

5.1.2 Validation samples from a reference laboratory

Two sets of 15 plasma samples containing different amounts of imatinib received from a reference laboratory in Bordeaux, France were analysed by the newly developed method and presented in Table 6. Some of the results show mixed agreements with the values provided by the reference laboratory; deviation ranging between -0.3 and 22.3 %. There was no information available regarding the particular quantification method that the reference laboratory used in obtaining those reference values. Thus it is not possible to infer any further about this discrepancy except the possibility of aggregated statistical errors on both measurement occasions.

5.1.3 Patient sample analysis

The purpose of the imatinib quantification method development in this study is eventually to estimate the imatinib concentration of patient plasma samples. The method was successfully employed to measure levels of imatinib in cancer patient plasma. Estimated imatinib concentration of 10 CML patient plasma samples is presented in Table 7. Here one notices that the patient #10 has a rather high imatinib concentration in clinical terms and it was reported that this patient showed severe imatinib side-effects. The measured imatinib concentrations varied over a wide range (400 – 4500 ng/mL) where 8 patients exceeded the upper limit of the therapeutic imatinib plasma concentration[27].

Table 6. Comparison of the measured imatinib concentration with the proposed method and the values provided by the reference laboratory.

Code	Measured concentration mean±SD (ng/mL)	Reference value (ng/mL)
E1008	1047±44	1280
E1014	705±20	850
F1004	1285±32	1250
F1007	247±2	200
F1008	3827±68	4000
F1009	1372±26	1250
F1013	1204±18	1200

Table 7. Estimated imatinib concentration of the CML patient plasma samples (n=3).

Patient No	Imatinib concentration mean±SD (ng/mL)
1	439±9
2	1555±30
3	2371±57
4	2010±41
5	917±23
6	2014±41
7	1454±28
8	1504±69
9	1977±5
10	4417±6

5.2 DART

In the beginning of the DART method development, dried protein precipitated plasma samples were redissolved in 100 % methanol, which resulted in inconsistent results in imatinib detection (LOD was about 3000 ng/mL). After several trials, a mixture of methanol and water (50:50, v/v) was found to be a better solvent which improved the sensitivity by an order of magnitude.

The total ion chromatogram (TIC) obtained from DART instrument is shown in Figure 12. A series of samples are introduced in a single run, and each sample produced a separate signal/peak. The mass spectrums of such peaks are shown in Figure 13. The MS detection of imatinib showed singly charged molecular ion $[M+H]^+$ at *m/z* of 494.36 providing evidence of the analyte's molecular weight. Trazodone used in the measurements showed the singly charge ion at *m/z* of 372.218. The spectrum in Figure 13(a) is crowded with peaks due to the presence of lots of other compounds such as proteins in human plasma. However, after removal of proteins by protein precipitation one can yield a less crowded spectrum as presented in Figure 13(b), which may alleviate the matrix effect [42]. Conversely, before introducing samples the instrument should be calibrated with polyethylene glycol (PEG).

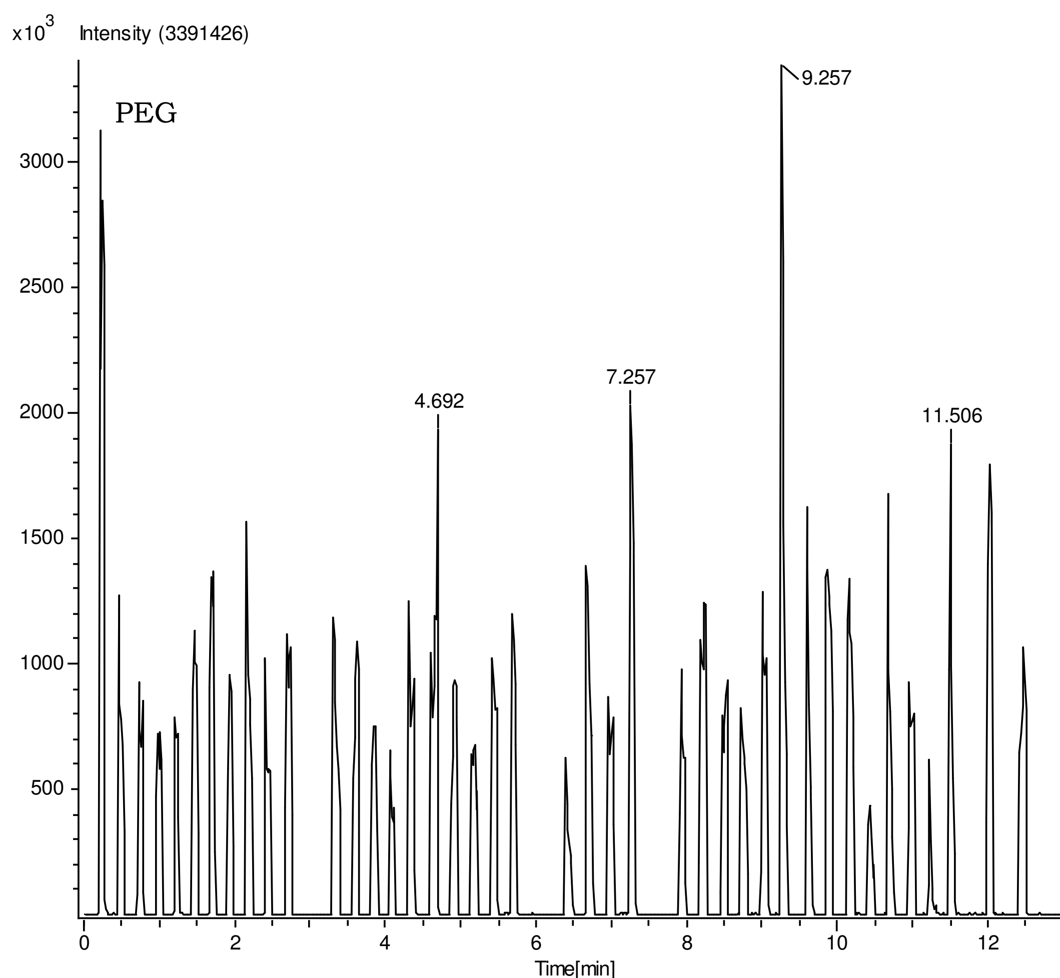
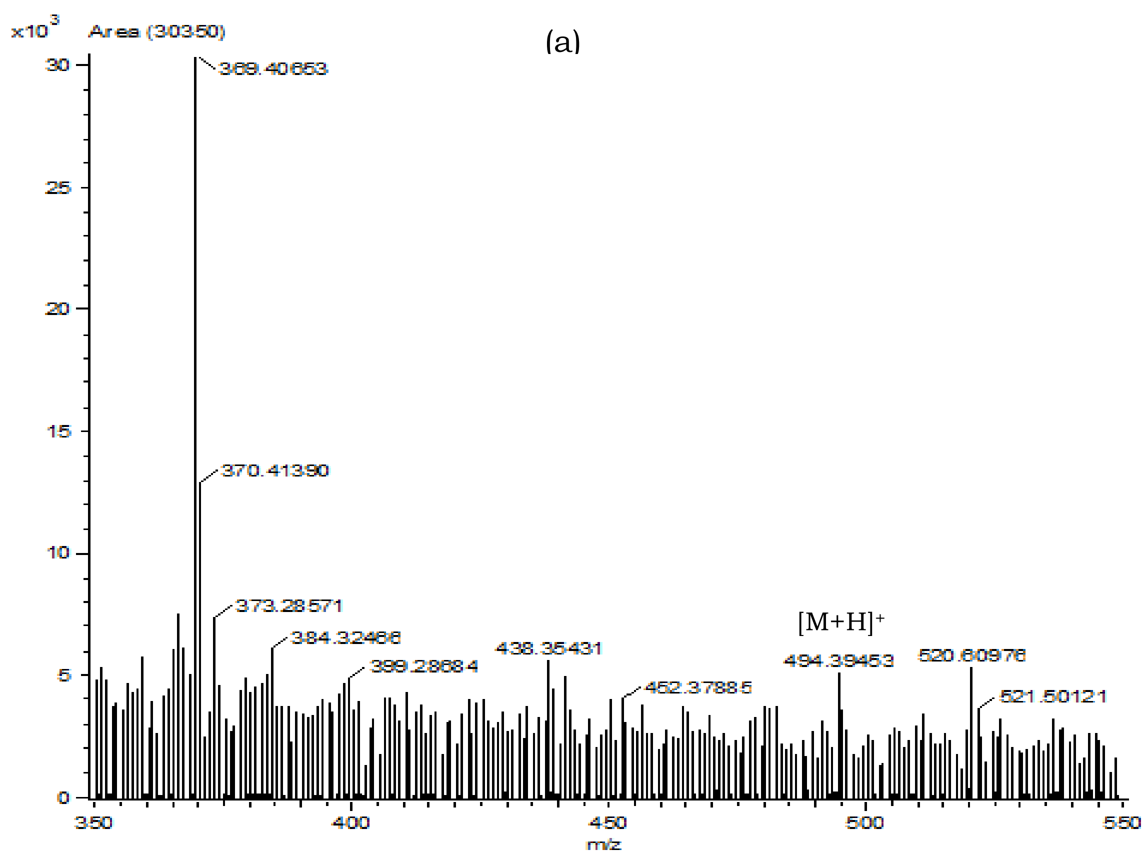


Figure 12. The total ion chromatogram obtained from DART.



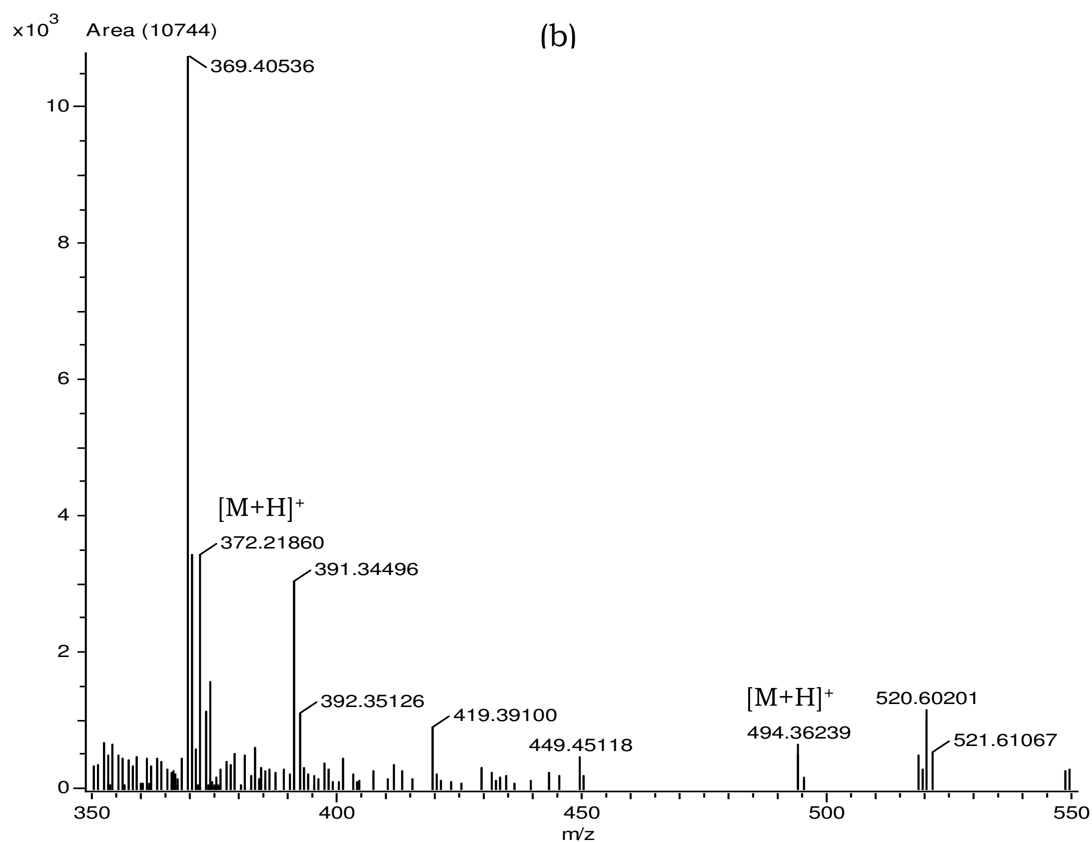


Figure 13. Mass spectrum of imatinib spiked plasma following DART: (a) without protein precipitation and (b) after protein precipitation.

The ratios of areas under the peaks of imatinib and trazodone were plotted against the initial concentration of imatinib (Figure 14).

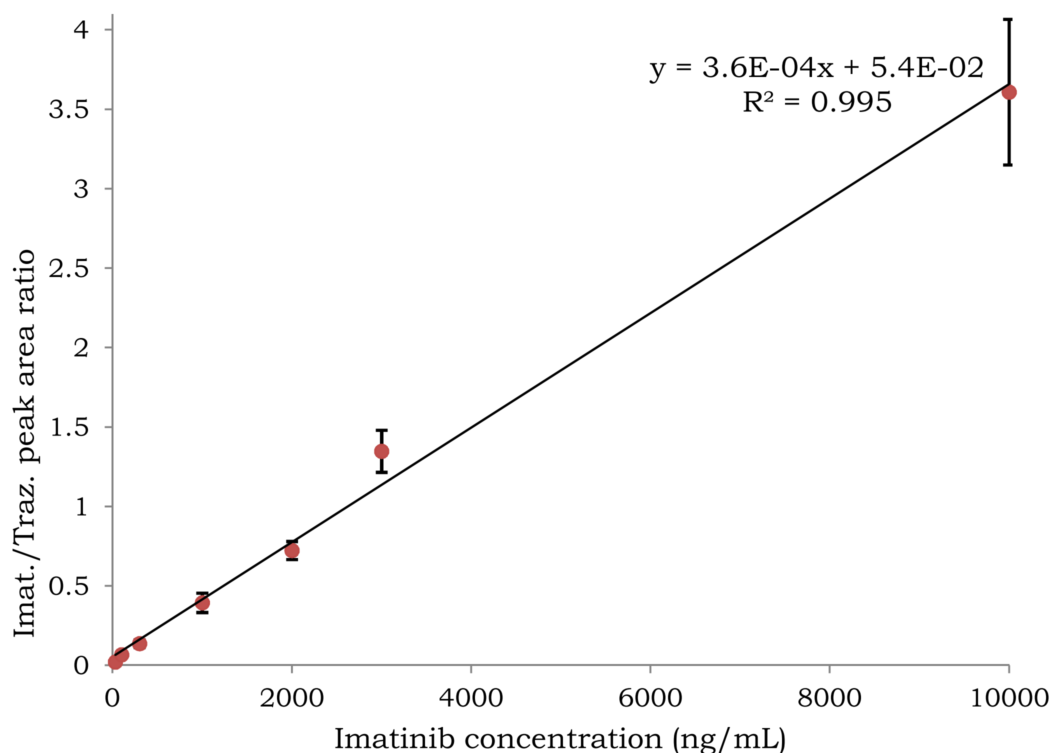


Figure 14. Calibration curve for DART with IS, showing average (n=3) imatinib to trazodone peak area ratio versus initial imatinib concentration.

5.2.1 Method validation

The DART method was evaluated partially for linearity, precision and repeatability, LOD and LOQ.

Linearity

The linearity of the method was evaluated over the concentration range of 30–10000 ng/mL in human plasma (n=3). The method exhibits acceptable linearity with coefficients of correlation ≥ 0.99. The coefficient of variation was examined for each standard concentration (Table 8). CV was less than 15 % and can be acceptable as per the FDA requirements.

Table 8. Mean, standard deviation, and coefficient of variation of imatinib/trazodone peak area ratio at each concentration of the data presented in Figure 14.

Nominal concentration (ng/mL)	Peak are ratio (n = 3)	
	Mean±SD	CV (%)
30	0.0217±0.0003	1.7
100	0.0676±0.0001	0.1
300	0.1604±0.0237	14.8
1000	0.4760±0.0613	12.9
2000	0.8907±0.0572	6.4
3000	1.3480±0.1321	9.8
10000	3.6082±0.4583	12.7

Precision and repeatability

Instrument accuracy in terms of CV was found to be 11 % at 1000 ng/mL, which is consistent with comparable studies reported by Zeo et al. [41] and Yu et al. [42] for several drugs in dog plasma.

Limit of detection (LOD) and limit of quantification (LOQ)

The Limit of detection and the limit of quantification were determined as described for LC-MS. LOD was found to be 5 ng/mL and LOQ of 16 ng/mL.

5.3 Comparison of LC-MS and DART methods

Having developed two imatinib quantification methods for human plasma, the next step would naturally be to compare the estimated imatinib concentrations of the samples by both methods. The following table shows a comparison of both methods in terms of selected reference and patient samples.

The imatinib concentrations determined by DART and LC-MS can be considered as satisfactory (Table 9). The cumulative effect of statistical variations, mainly in DART method, could be one of the reasons for the discrepancies of two methods.

Table 9. Comparison of estimated imatinib concentration in patients and reference samples by LC-MS and DART methods.

	Sample ID	Imatinib concentration (ng/mL)	
		LC-MS	DART
patients	1	439	420
	5	918	1006
	11	1978	1779
Reference	E1008	1047	1102
	F1008	3827	3216
	F1007	247	138

6 CONCLUSIONS

A rapid and sensitive method for the analysis of imatinib in plasma is necessary in conducting therapeutic drug monitoring and pharmacokinetic studies. The developed LC-MS (TOF) method is robust and reached the level of sensitivity and reproducibility demanded by clinical patient sample analysis. Hence, the method can be readily incorporated into the day-to-day routine testing of imatinib.

The proposed DART-MS method is capable of delivering analytical results in seconds. It suits for fast quantification of imatinib and has a potential to be even faster and robust for routine analysis.

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