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RESEARCH ARTICLE

Bioanalytical Method Development and Validation for Simultaneous Estimation of Imatinib and Its Metabolite Desmethyl Imatinib in Human Plasma Using Liquid Chromatography and Mass Spectrometric Detection

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ABSTRACT

An isocratic online-enrichment HPLC-assay was developed allowing for the simple and fast separation and quantitation of STI-571 and its main metabolite N-desmethyl-STI (N-DesM-STI) in plasma, urine, cerebrospinal fluid (CSF), culture media, and cell preparations in various concentrations using UV-detection at 260 nm. The analytical procedure consists of an online concentration of STI-571 and N-DesM-STI in the HPLC system followed by the elution on a ZirChrom-PBD analytical column. Time of analysis is 40 min including the enrichment time of 5 min. The detection limit is 10 ng/mL in plasma, CSF, culture medium (RPMI), and 25 ng/mL in urine for both STI-571 and N- DesM-STI. The intra-day precision, as expressed by the coefficient of variation (CV), in plasma samples ranges between 1.74 and 8.60% for STI-571 and 1.45 and 8.87% for N- DesM-STI. The corresponding values for urine measurements are 2.17–7.54% (STI-571) and 1.31–9.51% (N-DesM-STI). The inter-day precision analyzed over a 7-month time period was 8.31% (STI-571) or 6.88% (N-DesM-STI) and 16.45% (STI-571) or 14.83% (N-DesM-STI) for a concentration of 1000 ng/mL in plasma and 750 ng/mL in urine, respectively. Moreover, we demonstrate that with an alternative, but more time and labor consuming sample preparation and the implementation of electrochemical detection, a detection limit <10 ng/mL can be achieved. The method described was used to perform pharmacokinetic measurements of STI-571 and N-desmethyl-STI in patient samples and for kinetic measurements of intracellular STI-571 and N-DesM-STI following *in vitro* incubation.

Keywords: Bioanalytical method, Imatinib, Metabolite desmethyl imatinib

INTRODUCTION

Introduction to bio-analytical method development

Bio-analytical methods are widely used to quantitate drugs and their metabolites in physiological matrices, and the methods could be applied to studies in areas of human clinical pharmacology and non-human pharmacology/toxicology. Bio-analytical method employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic (PK), and toxic kinetic studies. The major bio-analytical services are,

- Method development
- Method validation
- Sample analysis (Method application).

HPLC and LC-MS/MS are used for the analysis of drugs in plasma. Each of the instruments has its own merits and demerits. HPLC coupled with

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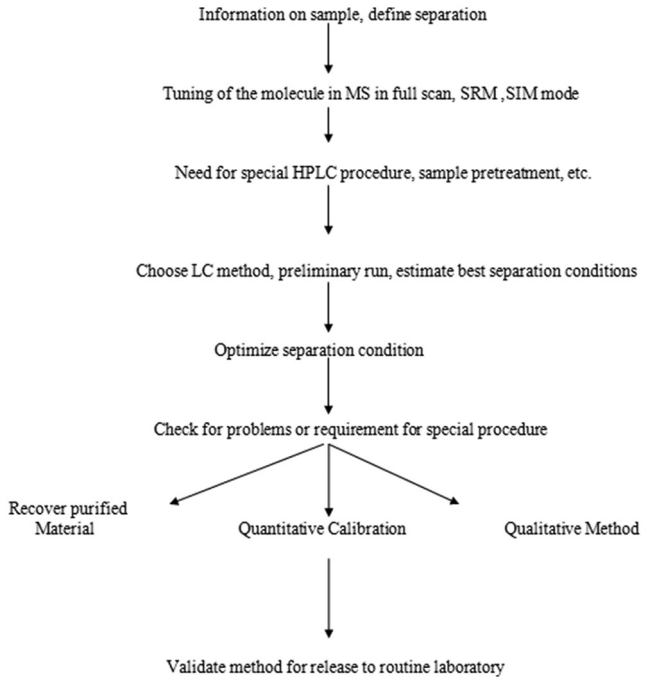
UV, PDA, or fluorescence detector can be used for estimation of many compounds but it does not give the high sensitivity, low dose drugs, and lacks selectivity.

The key advantages of using LC/MS methods over HPLC methods include:

- Selectivity: Co-eluting peaks are isolated by mass selectivity and are not constrained by chromatographic resolution
- Peak assignment: A chemical fingerprint for the compound of interest is generated and ensuring correct peak assignment in the presence of complex matrices
- Molecular weight information: Confirmation and identification of known and unknown compounds
- Structural information: Controlled fragmentation enables structural elucidation
- Rapid method development: It gives easy identification of eluted analytes without retention time validation
- Sample matrix adaptability: Reduces the sample preparation time
- Quantitation: Quantitative and qualitative data are obtained easily with limited instrument optimization.

Steps involved in LC-MS/MS method development



Requirements for developing effective LC/MS methods

Mobile-phase requirements

Mobile-phase degassing is an important component in LC/MS experiment and it can be accomplished through on-line membrane or vacuum devices, sonication, helium purging, or as a part of mobile-phase filtration. Degassing will eliminate pump cavitation, ensure reproducible retention time, and minimize possible sputtering from the ion source.

Organic components

Acetonitrile and methanol are exclusively chosen in the LC/MS methods as organic mobile-phase components. Methanol has a greater gas-phase acidity, polarity, and volatility than acetonitrile and may be preferred for separations. In positive ion mode, methanol has been shown to deliver 10–50% better sensitivity than acetonitrile, while in the negative ion mode little difference in sensitivities for most analytes. A typical mobile phase for the experiments can be 90/10 mixtures of MeOH/H_2O or ACN/H_2O and can be varied until the desired capacity factor is achieved. Although there is a small chance for methanol analyte adducts formation, methanol is often the solvent of choice in LC/MS because higher percentages of this solvent in the mobile phase will give equivalent chromatographic capacity relative to the same percentage of acetonitrile. Higher organic composition is desirable in LC/MS due to improved effluent evaporation at a given temperature, thereby decreasing the back-ground.

Ethanol, 2-propanol, and tetrahydrofuran are less used as mobile-phase components. Toluene, hexane, benzene, cyclohexane, dichloromethane, and carbon tetrachloride are used for normal-phase separations although many of these organic solvents require addition of more polar solvents for good ionization. Normal-phase HPLC solvents are compatible with LC/MS and generally pose no threat of fire or explosion in the nitrogen atmosphere that blankets the ion source. These solvents are often more effective when used with APCI rather than electrospray because as a solvent-

mediated ionization process, electrospray will only produce an ion beam if preformed ions are present.

Aqueous components

An appropriate acid or buffer help to get success in the LC/MS experiment. Non-volatile aqueous components whether salts, acids, bases, or buffers are greatly decrease and prevent the detection of analyte ions. These non-volatile buffers can also foul ion sources and vacuum regions of mass spectrometers. Non-volatile phosphate or citrate buffers are strongly discouraged for both ionization and practical reasons. Although many instrument manufacturers developed ion sources rugged enough to tolerate deposition of non-volatile components, frequent cleaning is often necessary. Ion suppression and decreased sensitivity will also be observed when non-volatile buffers are used. In most cases, volatile acids or buffers can be substituted for more traditional HPLC choices.

Buffers

Ammonium acetate or formate buffers are used with concentrations ranging from 2 to 50 mM, although a maximum concentration of 10–20 mM is recommended to avoid ion suppression. The acceptable buffer concentration that does not adversely affect ionization is observed to be highly compound dependent. A useful rule of thumb is to use as low a concentration of buffers to give reasonable chromatographic performance. Buffers should be chosen such that the pH of the mobile phase falls within the buffer's natural pKa range for maximum buffering capacity. For example, the pKa of acetate buffer is 4.8, with a range 3.8–5.8; the useful pH range for formate buffers is 2.8–4.6. Buffer capacity ranges are limited, particularly at lower concentrations. N-methylmorpholine may be used at higher pH ranges and has shown utility in improving sensitivity and chromatographic peak shape for negative ion APCI analysis. Ammonium adducts can be observed in positive ion mode and formate or acetate adducts in negative mode at higher buffer concentrations. Adduct formation is detrimental only in that it can cause greater variability and loss of detection sensitivity for the analyte. These adducts can be destroyed by increasing the source temperature, voltage or both.

Acids and bases

Formic acid or acetic acid concentrations of 0.1–1% (v/v) are recommended for preparing low pH mobile phases for the ionization in electrospray. Trifluoroacetic acid is used for protein and peptide separations but should be avoided when negative ion mode is utilized. Ammonium hydroxide, or, in rare cases, triethylamine, are recommended for high pH mobile phases. For basic compounds, 0.1 % acid should be mixed with the organic component, whereas water or neutral buffer should be used for neutral or acidic species. The previously discussed buffers are used as the aqueous component to improve peak shape and resolutions by providing greater control of pH.

Mobile-phase components that are not recommended

Certain types of traditional LC mobile phase additives should be avoided due to non- volatility and ion suppression effects. Mobile-phase related ion suppression will not depend on the analyte proximity to the solvent front or capacity factor. These additives include detergents, surfactants, and ion pairing agents, inorganic acids such as sulfuric, phosphoric, hydrochloric and sulfonic acids; non-volatile salts are phosphates, citrates, and carbonates; strong bases; and quaternary amines. Complete suppression of ionization as well as interferences in both positive and negative ion mode will occur when these agents are utilized. Tetrahydrofuran usage is discouraged as a major mobile-phase component in LC/MS. Safety concerns due to flammability and peroxide formation restrict usage of this solvent in heated nebulizer ion sources.

In addition, mobile phase solvents containing high percentages of tetrahydrofuran will cause peek tubing, commonly used in LC/MS plumbing, to swell with extended usage and often degrade polymeric components in HPLC seals and valves. However, tetrahydrofuran is commonly used as a mobile phase for HPLC and has been coupled with

mass spectrometric detection. In some experiments, the dilute solution of NaI in methanol is added post column to allow analyte cationization. Common sense should prevail when tetrahydrofuran is considered as a mobile-phase component; up to 10% is used, with care to effectively alter chromatographic selectivity.[1-10]

Useful HPLC column dimensions and parameters

Flow rates

The initial impulse of an analyst conducting an LC separation with mass spectrometric detection is to use the analytical column, mobile-phase and flow rate conditions from an LC/UV method. As discussed above, mobile-phase components should be carefully considered in light of their mass spectrometric compatibility. In addition, the flow-rate requirements of electrospray versus APCI should be considered. Atmospheric pressure chemical ionization can be operated at flow rates from 0.1 to 2 mL/min and provide optimum sensitivity at ~ 1 mL/min, electrospray is better suited to flow rates in the range of 10–500 μL/min and is optimal at ~ 200 μL/min. Flow-rate limits for electrospray are defined by source design; some instruments operate well up to 1 mL/min, provided nebulizer gas temperatures; and gas flow rates are proportionally increased. Optimized flow rates can be achieved by splitting the LC effluent before introduction into the mass spectrometer or by decreasing overall flows concurrent with decreased column dimensions. Splitting is accomplished by diverting part of the LC flow to an additional detector or waste.[11-19]

Column manufacturers have developed the wide range of column bore size, length, and column packing particle size to accommodate the increased demand for smaller and more efficient columns. The column bore size will dictate the appropriate flow rate and can be tailored to meet the analyst's needs.

Column dimension trade-offs

Decreasing the size of the column inner diameter will increases sensitivity compared with analytical columns when other parameters are keep constant and the system is not limited by extra column band broadening. The injection volume should be proportionally decreased to minimize band-broadening effects. Because overall sensitivity is increased, interfering as well as analyte peaks will increase in intensity. The extra column volume within an HPLC system will have greater effect on band broadening for smaller bore columns, which decrease resolution between peaks. One disadvantage to using smaller columns is loss of ruggedness because smaller columns will clog more rapidly from "dirty" samples. Use of a guard column before the analytical column will help reduce column replacement costs and prolong column lifetimes. For greater chromatographic capacity and resolution, the column length should be increased at the price of longer run times.

One of the more impressive advantages of LC/MS is the less resolution and selectivity is required from the LC separation. For the assay methods involving complex matrices such as plasma, the separation of analyte from matrix components is not critical, so the separation should be much shorter. Assay methods requiring 15–20 min with LC/UV or fluorescence are shortened to only a few minutes. Methods that previously required large numbers of theoretical plates and long (25 cm) analytical columns can be now implemented with less resolution and shorter (1–15 cm) columns.[20-23]

Simple strategies for choosing an appropriate HPLC column

The first step in selecting a column for LC/MS analysis, once the column size has been determined, to consider the analyte molecule's characteristics, such as hydrophobicity or hydrophilicity, pKa(s), or salt form. For hydrophobic compounds, the traditional C18 column allows the selectivity for polar versus non-polar compounds. Shorter chain packing's such as C8 or C4 present fewer hydrophobic surfaces for the interaction with analyte molecules. Aromatic compounds are may be well suited for analysis by phenyl columns, which allow separation through π–π* interactions.

Cyanopropyl columns allow the greatest degree of separation for polar compounds, although they generally are less rugged and reproducible from column to column than other reverse-phase columns. Assay sensitivity can often be an issue, even with LC/MS. Some ways to improve sensitivity in LC/MS center on the composition of the mobile phase. A mobile phase is high in organic content will be readily volatilized and pumped away in the atmospheric pressure region, which lessens the mass spectral background and improves the s/n ratio and also the chromatographic peaks that are rapidly eluted from the column (k' of 1–3) are much narrower than those with longer retention. From this perspective, it is therefore useful to run mobile phases high in organic content. To gain adequate retention with the high organic mobile phases, highly retentive columns should be selected initially. These columns are characterized by high carbon loads and are usually C18-sorbent material. In many cases, the quality of the column is dictated by the combination of manufacturing factors such as silica particle size, batch reproducibility, and surface pore size.[23-29]

Silanol effects and solutions

Although bonded silica-based chromatographic media are the most efficient and versatile packing materials currently available, they are not without problems. One important feature of these materials is the presence of silanol functional groups in the silica backbone. Because these silanol groups are present in all silica (Monomeric, polymeric) and because it is not possible to deactivate all of them through end capping, silanol activity is dramatically affect separations. In reverse-phase separations this is so-called secondary interaction primarily occurs in the form of mixed mechanism retention, a combination of reverse-phase partitioning and ion exchange. If controlled and utilized, silanol-derived cation exchange is allow greater chromatographic retention and selectivity for amine containing analytes. If it is not correctly controlled, silanol effects will lead to peak tailing, variable retention, and loss of chromatographic efficiency.

The number and nature of unreacted surface silanols affects the character of the stationary phase. Initially, free geminal or associated silanols are minimized through the process known as end capping, which bonds various species to the residual silanols. Hydrophilic end caps or bulky steric end caps that separate the hydrocarbon chains and prevent analyte interaction with the silica surface is used. If residual silanols are left unreacted, the analyte will be separated based on the combination of interactions with both the reverse-phase support and the highly polar silanol groups. Increased retention, changes in elution order, and tailing are result for basic compounds.

An approach to modulate silanol effects through mobile-phase pH control. At pH is below 4, most silanol groups will be protonated and less available as cation exchange sites. Addition of acetic or formic acid to the mobile phase is useful for this purpose. Buffer concentrations of about 25 mM will promote retention of buffer cations rather than analyte molecules, but can promote ion suppression.

Silanol effects are also manifest as columns "age." At low pH is (<2), the bonded phase and endcapping is hydrolyzed from silica support, leaving behind free silanols. At higher pH is (>8), the silica itself can slowly dissolve. This hydrolysis limits the useful mobile – phase pH range to 2–8. Greater column stability is observed as a function of chain length, with C18 columns exhibiting longer lifetimes than C8 or phenyl columns. This will be useful when greater retention of amines is required. Polymeric stationary phases that may not use silica can be a useful choice if silanol effects are problematic, but are generally not as chromatographically efficient or technically well developed.

Retaining ionic compounds

A significant challenge for users of API LC/MS is achieving adequate retention for ionic compounds when using the limited mobile phase additives are compatible with this technique. This is much less of an issue with other detection techniques such as UV absorbance, fluorescence, or amperometry because ionic compounds will be readily retained in reverse-phase mode using

ion-pair reagents or through the use of ion-exchange chromatography. Because each of these approaches typically requires non- volatile mobile phase additives, they are not generally useful with API LC/MS. A few approaches have been adapted for use with API LC/MS and these are described below. Chromatographic ionization suppression is useful for increasing the retention of organic acids in reverse-phase systems. The apparent pH of the mobile phase is decreased by the addition of acetic or formic acid. Acetic acid (0.1% by volume) will give an apparent mobile-phase pH of slightly less than 3 and this is capable of protonating most carboxylic analytes. In this way, the organic acid analytes become neutral molecules and are better retained under reverse-phase conditions. It is reported that there is little or no loss in sensitivity for carboxylic acids when using electrospray ionization under acidic conditions. It is possible to suppress the ionization of basic drug molecules by elevating the mobile phase pH above its pKa. For amines, this would be pH 10 or higher. To do this effectively, a special HPLC sorbent such as polymer or a surface-modified zirconium could be used. Chromatographic ionization suppression is not practical for compounds that are unstable under acidic and/or basic conditions.

The majority of ion-pair reagents is either non-volatile and will quickly lead to fouling of the ion source and the vacuum region of the mass spectrometer or will lead to ion suppression in mass spectrometer source and is unsuitable. Although this approach is not as rugged, reproducibility of chromatographic retention, as addition of the ion-pair reagent to the mobile phase, it does achieve adequate analyte retention and also improves assay sensitivity.

Although weak cation-exchange separations, such as those obtained on the cross-linked benzoic acid/divinyl benzene or silica, used for many years with more conventional HPLC detectors, they are starting to gain some attention as alternative separations for API LC/MS. This ion-exchange separations are advantages for small zwitter ionic compounds such as amino acids that cannot be retained by chromatographic ionization suppression or by addition of an ion-pair reagent to the injection solvent. In an ion- exchange mode, a cationic analyte is exchanged on the active silanol sites of silica or on the cation exchange sites of other materials. The separation parameters are limited because the highest useable concentrations of counter ions are 25–50 mM and the practical selection of counter ions is limited to volatile components such as H^+ or $NH4^+$.

Sample preparation

In LC-MS/MS sample preparation is key to success and is focused on concentrating the analyte and removing compounds that can suppress ionization. Common matrices for bio-analytical studies contain different components that need to remove. Thus, sample preparation is often specific to sample matrix. For example, blood plasma major constituents include proteins, sugars, salts, lipids and numerous peptides, and small molecules. Urine is a less complex matrix, comprised primarily of salts and urea. Sample preparation has three major goals: Removal of protein-related materials, elimination of endogenous compounds, such as phospholipids, that cause ion suppression/enhancement, and concentration to increase assay sensitivity. Three common sample preparation techniques are,

Protein precipitation

- Solid-phase extraction (SPE)
- Liquid-liquid extraction (LLE).

Protein precipitation

Many drugs, particularly hydrophobic drugs, are bound by plasma albumin protein by 99% or higher. Although this is not a covalent linkage, the hydrophobic forces are enough to form a very tight bond, in some cases to specific sites on the albumin protein. HPLC analysis of plasma samples requires the removal of plasma protein. Proteins will be removed by precipitation and centrifugation by adding reagents that reduce the solubility of the proteins. Acetonitrile is first choice of solvent for protein precipitation because of its complete precipitation of proteins and methanol is the second choice of organic

precipitant provided the solubility of the analyte in these solvents. Protein precipitation is performed by simply adding a large proportion of acetonitrile to the sample, usually a ratio of three to four volumes of acetonitrile to plasma. A lower ratio is result in insufficient protein precipitation and a larger ratio may over-dilute the sample. After protein precipitation the supernatant obtained will be injected directly in to the HPLC or it will be evaporated and reconstituted with the mobile phase and further cleanup of the sample can be carried out using micro centrifuge at very high speed. Protein precipitation, however, only addresses the removal of proteins. Phospholipids and other matrix contaminants that are remain may cause ion suppression or enhancement, resulting the inconsistencies and inaccuracies in the detection and quantitation of the analyte. Despite these shortcomings, precipitation remains the popular method for the sample cleanup because it is relatively fast and it is inexpensive.

SPE

SPE is the sample preparation technique that is derived from the liquid chromatography technology and is applied extensively to the analysis of biological samples and pharmaceutical products. It is the step gradient technique in which the analyte, dissolved in a weak solvent, is retained on the stationary phase and subsequent additions of various moving phases of increasing solvent strength results in the selective and controlled elution of the interferences and the analytes.

In this most common mode, a small (about 0.1–1 g) open-bed column of octadecyl- bonded silica is used to retain the analyte from an aqueous sample while allowing interferences to pass through. Interferences are washed from the column using an appropriate solvent mixture so that the analyte remains on the column. The analyte is eluted, typically using a common organic HPLC solvent such as acetonitrile or methanol. The final eluate is then analyzed directly or exposed to further treatment such as evaporation to concentrate the analyte or derivatization. Alternatively SPE is used in a reverse mode, that is, to retain (filter) an interference while passing a sample through the column. In this case, the solvent strength of the sample must be maximized with respect to the analyte and minimized with respect to the interferences.

SPE is most commonly used with reversed-phase packing's, but the technique also works using other packing's, such as in normal-phase or ion-exchange methods, or in combination with other techniques such as LLE, to achieve the necessary levels of sample clean-up. A significant advantage that SPE offers over LLEs is the potential for automation of a routine procedure. The steps of adding solvent and samples to a small column are simpler to automate than in LLE, where mixing of tube contents and removing of solvent at a liquid-liquid interface is required. The transfer of the eluate from a solid-phase extraction to the chromatographic system can be automated. Another advantage of SPE is that it generally uses smaller volumes of organic solvents compared to more conventional methods such as LLE.

LLE

LLE is generally reserved for these more complex samples because it offers the poor precision than other techniques. It is the most commonly used for the preparation of biological samples in which less precise methods will be tolerated occasional. However, an extraction is necessary for the determination of a water-insoluble compound in water-soluble matrix, such as the analysis of the fat-soluble vitamins in tablets or menthol in pharmaceutical lozenges. In these cases, the water-soluble matrix must be treated with water to gain access to the analytes, but the solvent cannot be made sufficiently nonpolar to dissolve the analytes by adding water-miscible solvent.

As discussed previously, in partitioning systems, there are some critical parameters that must be controlled. Selectivity and recovery can be manipulated by solvent selection. For ionizable drugs, the pH must be controlled to optimize the recovery. In general, when the drug is in the ionized form, it is more soluble in aqueous phase. Likewise, when the drug is uncharged state, it will

be generally partition into the organic layer, which is often exploited in the sample clean-up steps called back-extractions. After the analyte is transferred to organic solvent, a back-extraction technique is used to take the sample back to an aqueous phase by adjusting the pH of the aqueous phase. Using sequential extractions and back-extractions, a high degree of selectivity is achieved. However, care must be taken to maintain reproducible and high recovery.

To overcome the poor extraction efficiencies, organic fraction from sequential extractions is pooled. Liquid extractions are also used for trace enrichment of the analyte by evaporating the organic layer to dryness and then reconstituting into the smaller volume of mobile phase.

Internal standard (IS)

To obtain sufficient accurate results with good precision, an IS must be used in quantitative bio-analysis using LC/MS either an isotropically-labeled IS (ILIS) or an analogue IS (ANIS) can be applied. The ILIS is the ideal is, because its physicochemical properties are (almost) identical to those of the analyte. In selecting an ILIS, adequate separation between the isotopic patterns is required. In addition, the ILIS selected should have an isotopic purity with respect to the non-labeled compound (D0) of better than 99.9%. The labels must be resistant towards exchange in the chemical environments they have to exist in with respect to ANIS, LC- MS does not pose additional selection rules compared to other techniques. The ANIS should be as much alike as possible.

Therefore, a small difference in aliphatic substitution is generally preferred over changes in conjugation, or changes in the number or nature of the polar groups. With an ANIS, proper attention should be paid to the possibility of common fragment or adduct ions. Almost co-elution of ANIS and analyte provides the best results. It has been frequently demonstrated that an ILIS provides (somewhat) better precision than ANIS. It is also generally believed that ion suppression by matrix effect can always be corrected for by an ILIS.

Developing an effective LC/MS method

Choosing between APCI and electrospray

Analyte volatility, thermal stability and molecular weight

APCI and electrospray are suitable for a wide range of compounds, with complementary advantages for each technique. APCI is best suited for less polar compounds with molecular weights below 1000 Da, whereas electrospray should be chosen for mid to high molecular weight polar or ionic analysts. Volatility and thermal stability are more important factors in APCI than electrospray because APCI is a gas-phase ionization technique. In fact, the molecular weight limit of approximately 1000 is defined by the compound volatility. At molecular weights greater than approximately 1000 Da, the molecule will not volatilize well and thus cannot be readily ionized in the gas phase. Increased analyte volatility and thermal stability will increase the ease of ionization for either technique.

In general, APCI is better suited for heteroatom-containing molecules and electrospray for ionic compounds. Atmospheric pressure chemical ionization is also less prone to matrix effects and performs better at higher flow rates (> 0.5 mL/min), which has led to its reputation as a more robust technique than electro-spray.

Ionizability

The ability of a given analyte to form a gas-phase ion plays a critical role in mass spectrometry. The presence of highly polar functional groups such as carboxylic acid groups or primary amines indicates high ionizability. Molecular gas-phase acidity or basicity can generally be predicted from general considerations of acidic or basic groups within the molecule. Other functional groups such as hydroxyls, thiols, ethers, sulfones, or amides are also easily ionized. More ionic/polar species are amenable to electrospray due to their ability to accept or donate a proton from/to themolabile phase during the ionization process. Atmospheric pressure chemical ionization requires donation or acceptance of a proton during gas-phase reaction with a mobile phase ion but less polar compounds with no readily ionizable functional group have also been determined by this technique.

Table 1: Recommendations for LC/MS mobile-phase preparation

Mobile-phase	Mobile-phase preparation	Recommended/not recommended/highly discouraged
Organic solvents	Acetonitrile, methanol, ethanol, isopropanol, dichloromethane normal phase: toluene, hexane, benzene, cyclohexane carbon tetrachloride	Tetrahydrofuran
Buffers	Ammonium acetate, ammonium formate, triethylammonium acetate (10 mM)	Phosphate, citrate, carbonate
Acids	Acetic acid, formic acid, trifluoroacetic acid (positive ion mode only)	Sulfuric acid, perchloric acid, phosphoric acid, hydrochloric acid, sulfonic acids
Bases	Ammonium hydroxide	Quaternary amines, strong bases, generally triethylamine
Other components	--	Detergents, surfactants, ion pairing agents, non-volatile salts

Table 2: Column and flow rate parameters

Column I.D. (mm)	Flow rate
Capillary	<10 mL/min
1.0	40–50 µL/min
2.1	0.1–0.5 mL/min
3.0	0.5 mL/min
4.6	1.0 mL/min

Table 3: Typical liquid-liquid extraction solvents (In order of polarity)

Solvent	Dielectric constant
Water	78.54
Methyl ethyl ketone	18.5
Isobutyl alcohol	15.8
Methylene chloride	9.8
Ethyl acetate	6.02
Chloroform	4.806
Diethyl ether	4.335
Toluene	2.379
Carbon tetrachloride	2.238
Benzene	2.284
Cyclohexane	2.023
Hexane	1.890

Aqueous content of the mobile phase

For reverse-phase separations, either APCI or electrospray ionization can be routinely used for aqueous or organic contents ranging from 10 to 90%. Chiral separations using normal-phase chromatography can be conducted with APCI and completely organic mobile phases such as acetonitrile, tetrahydrofuran, methanol, chloroform, and others. Some general considerations include the ease of mobile-phase vaporization (thus, limiting a completely aqueous mobile phase) and surface tension effects that will interfere with electrospray ionization. As discussed above, the ionic strength of the aqueous component should be minimized to avoid ion suppression. The use of ion pair reagents and surfactants should also be avoided if at all possible due to ion suppression and interference with the vaporization step.

Tables 4 and 5 show the gas-phase acid-base scale in both positive and negative ion modes for LC/MS mobile-phase solvents. Particularly for APCI, the mobile phase components should be considered gas-phase reagents to promote either protonation or deprotonation of the analyte ion. In this manner, appropriately volatile mobile-phase components can be tailored to the assay.

Sample cleanliness

APCI has been shown to be more readily influenced by the presence of negative or positive species from salts or buffers which will prevent protonation or deprotonation by competition with the analyte molecules. Salts are sometimes deliberately added to samples to be assayed by electrospray to increase analyte ionization through cation adduct formation ((M + Na)+ or (M + K)+). Buffers are not necessary for APCI, although they can be required for adequate chromatographic separation of sample components.

Gradient use

Gradients can be used with equal ease for either ionization technique. In most cases, cycle time for system re-equilibration (determined by the overall system dead volume) provides the practical limitation to their usage. If, for example, a particular HPLC pump/auto sampler combination has 1.0 mL of dead volume (or dwell volume, the volume of all plumbing between where the solvents are mixed and the column head) and is operating at a flow

Table 4: Gas phase acid-base scale for positive ions

Reagent ions	Neutral reagent ion molecules
Strong acid	Weak base
CH_5^+	Methane
N_2OH^+	N_2O
$C_2H_5^+$	Formaldehyde
$C_2H_5^+$	Water
$CH_3OH_2^+$	Formic acid
CH_3CNH^+	Methanol
$C_4H_9^+$	Benzene
NH_4^+	Acetonitrile
Weak base	Acetic acid
C_5H_5NH	Acetone
	Phenol
	Ethyl acetate
	Diethyl ether
	Ammonia
	Methylamine
	Pyridine
	Strong base
	Trimethyl amine

Table 5: Gas-phase acid-base scale for negative ions

Reagent ions	Neutral reagent ions molecules
Strong base	Weak acid
NH_2^-	Ammonia
OH^-	Water
$C_6H_5CH_2^-$	Toluene
CH_3O^-	Methanol
NCH_2C^-	Ethanol
$CH_3COH_2C^-$	Acetonitrile
CH_3S^-	Acetone

Table 6: Comparison of APCI versus electrospray

	APCI	Electrospray
Advantages	Neutral molecules ionize Less ion suppression Wider dynamic range 103–104 Mass sensitive detection Flow 0.5–2.0 mL/min Higher buffer cone, (up to 20 mM)	Wide range of compounds No problems with thermal stability/ volatility Higher MW Concentration sensitive detection Better sensitivity
Disadvantages	Thermal stability necessary (130–150°C) Volatility necessary More sensitive for non-volatile components	Mobile-phase ion suppression Lower dynamic range LC flow <200–300 µL/min Multimer formation

Table 7: Working standards

S. No.	Materials	% Purity
1	Imatinib	99.2
2	Desmethyl imatinib	98.7
3	Nicotinib	97.2

Table 8: Chemicals and reagents

S. No.	Materials	Manufacturer
1	Methanol	Merck
2	Acetonitrile	Merck
3	Sodium hypochlorite	Merck
4	Milli Q water	Merck
5	Ortho phosphoric acid	Merck
6	Strata-X-cartridges	Phenomenex

Table 9: Description of LC-MS/MS instrument

S. No.	Equipment/Software	Model
1	HPLC Degasser Pump Auto sampler Column oven	SIL - HTc Shimadzu Shimadzu Shimadzu Shimadzu
2	MS	TSQ Quantum Thermo Scientific
3	Software	LC Quan 2.5.6

Table 10: List of the other equipments

S. No.	Equipment	Manufacturer
1	Analytical balance	Saetories
2	Micro balance	Sartories
3	pH meter	Thermo Scientific
4	Milli Q water system	Merck
5	Pipettes	Eppendorf
6	Vortexer	Spinix
7	SPE	Eppendorf
8	Nitrogen Evaporator	Takahe analytical
9	Refrigerator	LG
10	Deep freezer (–20±5)	Sanyo
11	Deep freezer (–70±15)	Sanyo
12	Pipette tips	Torson

Table 11: m/z values of parent ions

Compound	Molecular weight (g/mol)	m/z value (amu) of parention
Imatinib	493.603	454.081
Desmethyl imatinib	479.58	457.256

Table 12: m/z values of product ion

Compound	m/z value (amu) of Product ion
Imatinib	457.256
Desmethyl imatinib	288.139

rate of 1.0 mL/min (typical for APCI), then the lag time between when the gradient is initiated and when the correct solvent composition reaches the pump head is 1 min (1.0 mL/min)). If the flow rate is only 0.2 mL/min (typical for electrospray),

Table 13: Optimized chromatographic conditions

Parameter	value
	ISTD: 1.0±0.50 min
Runtime	2.2±0.5 min
Auto sampler temperature	10°C
Detector	Mass
Rinsing volume	500 µL
Column oven temperature	35°C
Purge time	1.0 min

Table 14: Mass parameters

Parameter	Imatinib	Desmethyl imatinib
m/z values	454.081 (m/z) 284.909 (m/z)	457.256 (m/z) 288.139 (m/z)
Detection	Positive mode	Positive mode

Table 15: Source-dependent parameter

Parameters	Value
Spray voltage	4500 V
Sheath gas pressure	30 psi
Auxiliary gas pressure	20 psi
Capillary temperature	350°C
Capillary offset	35

Table 16: Compound-dependent parameters

Parameters	Imatinib	Desmethylimatinib
Turbulence offset	85	100
Skimmer offset	10	10
Collision energy	15	15
Collision pressure	1.5 psi	
Resolution	0.70 min	

then the lag time will be 5 min. This means that a typical gradient run would require 5 min to initiate re-equilibration plus whatever time is required for elution and final re-equilibration (usually 10–20 column volumes). This is clearly an unacceptable time delay.

Two solutions to this problem are feasible; one is to purchase a low-dead volume pump. Second solution is to construct and introduce a splitter before the column or the ion source. In this way, the pump can be operated at 1.0 mL/min and the lag time shortened to 1 min. Using a 5:1 split, the ion source will be exposed to 0.2 mL/min, a typical flow rate for an electrospray source.

Gradient separations in LC/MS offer three distinct advantages. First, poorly shaped chromatographic peaks can be sharpened. Tailing and fronting can be eliminated to a considerable extent. Second, the time is similar analytes can be shortened extensively. Early eluting compounds can exhibit retention while late eluting compounds can be more quickly eluted. Third, gradients can serve as a rudimentary sample cleanup technique, especially for samples with high salt content but low amounts of proteins, cellular components, or lipids.

Generic gradient methods for compound classes

Depending on sample mixture complexity, a gradient method is easily tailored to affect separation of closely and widely spaced chromatographic peaks. For basic compounds, a gradient consisting of 10–90% of 0.1% formic or acetic acid with acetonitrile can be used over any run time desired. Basic or neutral compounds can be separated using water/organic gradients in the same manner.

Establishing the mass spectral properties

Choosing ionization polarity

Ionization polarity should be chosen in conjunction with the mobile phase because pH can influence ease of ionization in positive or negative ion mode. Basic or neutral compounds are readily ionized in positive ion mode at a pH below 7. Because many potential drug molecules contain amine moieties, the majority of LC/MS methods are conducted in positive ion mode. Acidic compounds are most amenable to negative ion mode unless the molecule exists as a zwitterion. Negative ion mode provides an additional advantage of selectivity because chemical background is lower compared to positive ion mode. In some cases, the LC/MS experiment can be conducted in both positive and negative ion mode. If, for example, the analyte is best suited for positive ion mode analysis but its corresponding hydroxylated metabolite is best analyzed in negative ion mode, both polarities can be monitored in tandem. The LC conditions should be developed to sufficiently separate the positive and negative components to allow polarity switching. The first portion of the chromatogram may be measured in positive ion mode, with the second portion in negative, or vice versa. Rapidly

cycling between the two modes during the entire chromatographic acquisition is possible with some instruments. However, stabilization of the electronics after switching requires significant delay times. Insufficient delay times between polarity switching leads to extensive electronic noise and reduced sensitivity.

Precursor and product ions

For quantitative determinations, the precursor and product ions should be chosen to minimize the possibility of interference and maximize reproducibility. Interference generated by selection of non-specific precursor – productions will increase the background noise and thus decrease sensitivity. In general, the molecular ion (either (M + H)+ or (M – H)-) is preferred as a precursor although (M +Na)+ or (M + K)+ can be more effective under certain circumstances. Selection of a molecular fragment as a precursor should only occur in cases in which fragmentation is unavoidable, even at low cone/orifice voltages. Frequently, higher molecular weight conjugates such as metabolites or degradation products will produce fragments identical to those observed for the parent compound. The production chosen should represent a significant loss (i.e., greater than 50 Da) from the precursor molecule. Loss of water or carbon dioxide is generally less compound specific than losses of phenyl groups or rings. Very low molecular weight product ions such as NH_4^+, COO^-, or Br^- should also be avoided based on increased potential for interferences. Despite careful attention to the selection of precursor and productions, a real potential exists for interference from higher molecular weight metabolites, such as glucuronides.

Compound profiling techniques

Before the sample preparation and chromatographic separation, a clean solution of the compound to be determined will be profiled by the analytical chemist to determine the appropriate precursor product masses, polarity and ionization mode (electrospray versus APCI). The appropriate mass spectrometric parameters such as orifice or cone voltage, collision energy, lens voltages, and nebulizer gas flows will also be optimized. Solution concentrations of 500–1000 ng/mL should be used to avoid contaminating the source with the analyte (thus increasing background in subsequent experiments). Ideally, the sample should be dissolved in mobile phase to mimic experimental HPLC conditions, but neat organic solvents often perform equally well. Profiling should be conducted in high resolution mode to precisely determine mass and verify precursor isotopic distributions of M + 1 and M + 2 isotopic patterns where appropriate. Excessively high standard concentrations will interfere with isotopic distribution verification and accurate mass determination if the signal for the molecular ion is saturated. Once the precursor and product ion masses have been determined, the resolution can be lowered for SRM/ MRM experiments to increase sensitivity.

Infusion versus loop injection

To determine the correct mass spectrometer operational conditions for a given compound, a clean standard solution can be introduced into the ion source by either syringe pump infusion at a low flow rate (10–30 μL/min) or by multiple loop injections. An actual loop (50–100 μL) can be partially filled with solution and a Rheodyne valve manually switched by the analyst to inject the sample. Alternatively, small-volume (~1 μL) loop injections can be introduced by an auto sampler while mobile phase is pumped into the mass spectrometer ion source. The choice of continuous infusion versus loop injection depends on both the type of instrument and analyst preference. Infusion techniques require larger volumes of solution but provide additional time for parameter optimization. The maximum practical flow rates provided by the syringe pump typically limit this type of introduction to electrospray sources because APCI requires flow rates of 500 μL/min or greater. If APCI conditions are to be obtained in this manner, the analyte can be added to the mobile phase and delivered by the HPLC pump or a simple mixing tee. While profiling with loop injections is not as precise as continuous infusion, it can provide great time saving and higher throughput.

Profiling multiple components simultaneously

To gain higher throughput in signal profiling, as it pertains to development of quantitative methods, several compounds can be combined in a multi component mixture and profiled simultaneously. The multicomponent mixture can contain eight or more compounds that have molecular ions separated by several atomic mass units and, ideally, ionized by the same mode (such as electrospray positive ion). As this solution is introduced into the ion source, either by infusion or loop injection, each of the components to be profiled is sequentially optimized by a process similar to that described in the preceding section.

Although some of the results obtained by this type of multiplexed procedure can be less than optimal, the resulting throughput for compound profiling is improved.

Bio-analytical method validation

Bio-analytical method validation is a procedure employed to demonstrate that an analytical method used for quantification of analytes in a biological matrix is reliable and reproducible to achieve its purpose: To quantify the analyte with a degree of accuracy and precision appropriate to the task. Validation data, through specific laboratory investigations, demonstrate that the performance of a method is suitable and reliable for the analytical applications intended. The quantitative approach used in bio-analytical methods involves the use of a standard curve method with IS. In this approach, the analyte concentration can be assigned by referring the response to other samples, called calibrators or calibration standards. In addition to the samples of unknown concentration, the bio-analytical set includes the calibration standards, and samples containing no analyte, called blanks, to assure that there are no interferences in the matrix. Accuracy and precision of the method are calculated using the back-calculated concentrations of samples of known composition called quality control (QCs) samples. The calibrator standards and QC samples should be prepared in the same matrix as the actual samples.

Selectivity

The analytical method should be able to differentiate the analyte of IS from endogenous components in the matrix (i.e., blood, plasma, and urine) or other components in the sample. Selectivity should be proven by using at least six sources of the appropriate blank matrix, which are individually analyzed and evaluated for interference.

Absence of interfering components is accepted where the response is less than 20 % of the lower limit of quantitation (LOQ) for the analyte.

Matrix effect

Matrix effects should be investigated when using mass spectrometric methods, using at least 6 lots of matrix including hemolysed, hyperlipidemic and if applicable, sample matrix from special populations, such as renally or hepatically impaired populations. For each analyte and the IS, the matrix factor (MF) should be calculated in each lot of matrix, by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked with analyte at a concentration of maximum 3 times the lower limit of quantification [LLOQ] after extraction), to the peak area in the absence of matrix (pure solution of the analyte). The IS normalized MF should also be calculated by dividing the MF of the analyte by the MF of the IS. The coefficient of variation (CV) of the IS-normalized MF calculated from the 6 batches of matrix should not be greater than 15%.

If this approach cannot be used, for instance, in the case of online sample preparation, the variability of the response from batch to batch should be assessed by analyzing at least six batches of matrix in triplicate, spiked at a concentration of a maximum of 3 times the LLOQ. The validation report should include the peak areas of the analyte and of the IS and the calculated concentration for each individual sample. The overall CV calculated for the concentration should not be greater than 15%.

The mean concentration should be within 15% of the nominal concentration. The mean concentration should also be reported for each individual batch of matrix; a deviation of this mean from the nominal concentration of more than 20% in any individual batch of matrix should lead to additional investigations. If the matrix is difficult to obtain, less than six different batches of matrix may be

used, but this should be justified. However, matrix effects should still be investigated.

Calibration/standard curve

A calibration (standard) curve is the relationship between instrument response and known concentrations of the analyte. A calibration curve should be generated for each analyte in the sample. A sufficient number of standards should be used to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended by spiking the matrix with known concentrations of the analyte. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without IS), a zero sample (matrix sample processed with IS), and six to eight non-zero samples covering the expected range, including LLOQ.

LLOQ

The lowest standard on the calibration curve should be accepted as the limit of quantification if the following conditions are met:

- The analyte response at the LLOQ should be at least 5 times the response compared to blank response
- Analyte peak (response) should be identifiable, discrete, and reproducible with a precision of 20 % and accuracy of 80–120%.

Calibration curve/standard curve/concentration-response

The simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. The following conditions should be met in developing a calibration curve:

- 20% deviation of the LLOQ from nominal concentration
- 15% deviation of standards other than LLOQ from nominal concentration.

At least four out of six non-zero standards should meet the above criteria, including the LLOQ and the calibration standard at the highest concentration. Excluding the standards should not change the model used.

Accuracy

The accuracy of an analytical method describes the closeness of the determined value obtained by the method to the true concentration of the analyte (expressed in percentage). Accuracy should be assessed on samples spiked with known amounts of the analyte, the QC samples.

During method validation accuracy should be determined by replicate analysis using a minimum of five determinations at a minimum of four concentration levels which are covering the calibration curve range: The LLOQ, within 3 times the LLOQ (low QC), around 50 % of the calibration curve range (medium QC), and at about 75 % of the upper calibration curve range (high QC).

The QC samples are analyzed against the calibration curve, and the obtained concentrations are compared with the nominal value. The accuracy should be reported as percent of the nominal value. Accuracy should be evaluated for the values of the QC samples obtained within a single run (the within-run accuracy) and in different runs (the between- run accuracy). The latter will support the accuracy over time.

To enable evaluation of any trends over time within one run, it is recommended to demonstrate accuracy of QC samples over at least one of the runs with a size equivalent to a prospective analytical run.

Within-run accuracy

For the validation of the within-run accuracy, there should be a minimum of five samples per concentration level at LLOQ, low, medium, and high QC samples in a single run. The mean accuracy value should be within 15 % of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

Between-run accuracy

For the validation of the between-run accuracy at least five determinations per concentration per run at LLOQ, low, medium, and high QC samples from three runs analyzed on at least two different days should be evaluated. The mean accuracy value should be within 15% of the nominal values for the QC samples, except for the LLOQ which should be within 20% of the nominal value.

Precision

The precision of the analytical method describes the closeness of repeated individual measures of analyte. Precision is expressed as the CV. The statistical method for estimation of the precision should be predefined and calculated according standard practice. Precision should be demonstrated for the LLOQ, low, medium, and high QC samples, within a single run and between different runs, that is, using the results generated for demonstration of accuracy.

Within-run precision

The within-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

Between-run precision

The between-run CV value should not exceed 15% for the QC samples, except for the LLOQ which should not exceed 20%.

Recovery

The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Recovery of the analyte need not be 100%, but the extent of recovery of analyte and of the IS should be consistent, precise, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. The procedure should also include an evaluation of analyte stability in stock solution. All stability determinations should use a set of samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, and interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at known concentrations.

Freeze and thaw stability

Analyte stability should be determined after three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at the intended storage temperature for 24 h and thawed unassisted at room temperature. When completely thawed, the samples should be refrozen for 12–24 h under the same conditions. The freeze-thaw cycle should be repeated two more times, and then analyzed on the third cycle. If an analyte is unstable at the intended storage temperature, the stability sample should be frozen at –70°C during the three freeze and thaw cycles.

Short -term temperature stability

Three aliquots of each of the low and high concentrations should be thawed at room

temperature and kept at this temperature from 4 to 24 h (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed.[30-32]

Long-term stability

The storage time in a long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. The volume of samples should be sufficient for analysis on three separate occasions. The concentrations of all the stability samples should be compared to the mean of back-calculated values for the standards at the appropriate concentrations from the 1st day of long-term stability testing.

Stock solution stability

The stability of stock solutions of drug and the IS should be evaluated at room temperature for at least 6 h. If the stock solutions are refrigerated or frozen for the relevant period, the stability should be documented. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions.

Post-preparative stability

The stability of processed samples, including the resident time in the auto sampler, should be determined. The stability of the drug and the IS should be assessed over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards.

Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits for evaluation of analyte(s) stability in a biological matrix can be used. SOPs should clearly describe the statistical method and rules used. Additional validation may include investigation of samples from dosed subjects.

EXPERIMENTAL

Materials and Methods

Stock solutions

Imatinib and desmethyl imatinib stock solution

Weighed accurately, about 5.0 mg of Imatinib working standard and transferred to a 5 mL clean glass volumetric flask, dissolved in methanol and made up the volume with the same. Corrected the above concentration of Imatinib solution accounting for its potency and the actual amount weighed. The stock solution was stored in refrigerator at 2–8°C and used for maximum of 3 days.

The stock solutions were diluted to suitable concentrations using a mixture of methanol and Milli Q water (diluent) in the ratio of (80:20 v/v) for spiking into plasma to obtain calibration curve (CC) standards, QC samples, and DIQC samples. All other final dilutions (system suitability dilutions, aqueous mixture, etc.) were prepared in mobile phase.

Nicotinib stock solution (IS)

Weighed accurately, about 1.0 mg of Nicotinib is transferred to a 5 mL volumetric flask, dissolved in HPLC grade methanol and made up the volume with the same. Corrected the above concentration of Nicotinib accounting for its molecular weight, potency, and the actual amount weighed. The stock solution was stored in refrigerator 2–8°C and used for maximum of 3 days. The stock solution was diluted to suitable concentration using diluent for IS dilution.

Biological matrix

Six lots of K_2EDTA human plasma, one hemolytic, and one lipemic plasma were screened for selectivity test. All eight human plasma lots including hemolytic and lipemic plasmas were found free of any significant interference for analyte and IS. After bulk spiking, aliquots of 300 µL for CCs and 300 µL for QCs of spiked plasma samples were pipetted out into a pre labeled polypropylene RIA vials and then all the bulk spiked samples were stored to deep freezer at below –70°C, except

12 replicates each of LQC and HQC, which were stored in deep freezer at –20°C for generation of stability data at –20°C.

Calibration curve standards and QC samples

Calibration curve standard consisting of a set of nine non-zero concentrations ranging from 251.298 to 0.508 µg/mL of imatinib was prepared. Prepared QC samples consisted of concentrations ranging from 178.340 to 0.508 µg/mL for Nicotinib. These samples were stored at –70°C until use. Twelve sets of LQC and HQC were stored at –20°C deep freezer to check –20°C stability. From these, six sets each of 2 times dilution and 4 times dilution were performed.

Solutions

5 mM Ammonium acetate

This solution is used for preparation of mobile phase and reconstitution solution. Weigh approximately 0.3854 g of ammonium acetate transfer into a 1000 mL reagent bottle containing 500 mL of water, mix well, and add remaining amount of water, mix well, sonicate, filter, and label. Provide expiry of 2 days from the date of preparation. Store the solution at ambient temperature.

5mM Ammonium acetate:ACN (20:80)

This solution used as reconstitution solution and mobile phase. Transfer 200 mL of 5 mM ammonium acetate into a 1000 mL reagent bottle containing 800 mL of ACN mix well, sonicate, and label. Provide expiry of 3 days from the date of preparation. Store the solution at ambient temperature.

MeOH:Water (80:20)

A mixture of HPLC grade Methanol and Milli Q water was prepared in the volume ratio of 80:20 as dilution solvent. It was then sonicate in an ultrasonicator for 3–5 min. The solution was stored at room temperature (20 ± 5°C) and used within a period of 7 days of preparation. This solution is used for diluent and rinsing solution.

5% MeOH

This solution is used as washing solution. Transfer 50 mL of methanol into a 1000 mL reagent bottle and add 950 mL of HPLC grade water, mix well, sonicate, and label. Provide expiry of 2 days from the date of preparation. Store the solution at ambient temperature.

2% OPA

This solution is used as treatment solution. Transfer 2 mL of OPA into a 100 mL reagent bottle containing 98 mL of water, mix well, sonicate, and label. Provide expiry of 2 days from the date of preparation. Store the solution at ambient temperature.

System suitability solution

A mixture of analyte and ISs was prepared for system suitability test. The concentration of analyte corresponds to highest concentration of calibration range (251.298 µg/mL for Imatinib) and that of ISs corresponded to working concentration used for spiking (500 ng/mL for desmethyl imatinib).

Method development

Tuning of MS/MS

- Electro spray ionization source and positive ion mode were selected
- Injected a concentration of 100 ng/mL in full scan mode
- The m/z value of the parent ion was selected based on the molecular weight of the compound at Q1
- Injected a concentration of 100 ng/mL in product ion mode
- The most prominent and stable daughter ion was selected by altering various parameters at Q3
- Injected 100 ng/mL concentration of analyte to selected parent and daughter ion in Multiple Reaction Monitoring (MRM) mode
- The parameters were optimized to give maximum stable response.

Mobile phase selection trails

Aqueous standards at HQC level were prepared and injected to check the retention time, peak shape,

and reproducibility with different mobile phases.

Trial 1

Samples were reconstituted with 0.1 % formic acid:ACN (70:30) and analyzed.

Column: 5 µ Gemini 5 µ C18 (50 × 4.6 mm, 5 µm), 100 Å.

Result: Peak shape was not good. The samples failed to meet the acceptance criteria.

Trail 2

Samples were reconstituted with methanol:2mM ammonium formate (80:20) and analyzed.

Column: Thermo Hypersil Gold (100 × 4.6 mm, 5 µm).

Result: The peak shape and reproducibility were not good. The samples failed to meet the acceptance criteria.

Trail 3

Samples were reconstituted with 2 mM ammonium acetate:ACN (20:80) and analyzed.

Column: Inertsil ODS3 (100 × 4.6 mm, 5 µm).

Result: The peak shape and reproducibility were not good. The samples failed meet the acceptance criteria.

Trail 4

Samples were reconstituted with 2mM Ammonium acetate:ACN (20:80) and analyzed.

Column: Thermo Hypersil Gold (100 × 4.6 mm, 5 µm).

Result: Peak shape and reproducibility were good. The samples met the acceptance criteria.

Sample extraction trails

Trail 1

Using solid phase extraction technique.

Extraction procedure:

980 ml of plasma+20 ml spiking solution

↓

300ml of spiked plasma

↓

50µl of IS vortex

↓

300 µL of 2%OPA solution

↓

Solid Phase Extraction:

Condition	:	1 mL of methanol
Equilibrate	:	1 mL Water
Sample Loading	:	Load the prepared sample
Wash1	:	1 mL of water
Wash2	:	1mL of 5% methanol
Drying	:	Dry the cartridge
Elution	:	Elute with 0.500 mL methanol twice

↓

Evaporated at 40^0c

↓

Reconstituted with 0.300 ml of with0.1 % Formic acid (70: 30) Vertex Analyzed in LC-MS/MS.

Less recovery, peak shape was not good.

Trail 2

Using solid phase extraction technique.

Extraction procedure:

980 mL of plasma+20 mL spiking solution

↓

300 mL of spiked plasma

↓

50 µL of ISvertex

↓

300 µL of 2%OPA solution

Solid Phase Extraction:

Condition : 1 mL of methanol
Equilibrate : 1 mL Water
Sample Loading : Load the prepared sample
Wash1 : 1 mL of water
Wash2 : 1mL of 5% methanol
Drying : Dry the cartridge
Elution : Elute with 0.500 mL methanol twice

Evaporated at 40^0c

Reconstituted with 0.300 mL of 2mM ammonium acetate:MeOH (20:80) Vertex

Analyzed in LC-MS.

Recovery not improved, peak shape was good

Parameter	Description
Column	Thermo Hypersil Gold, 100×4.6 mm, 5 µ
Mobile phase	2 mM Ammonium acetate: ACN (20:80)
Flow rate	0.8 mL/min
Injection volume	10.0 µL
R_t	Drug: 1.0±0.50 min

Optimized bio-analytical conditions

Sample preparation

980 ml of plasma+20 ml spiking solution

300ml of spiked plasma

50µl of ISvertex

300 µL of 2%OPA solution

Solid Phase Extraction:

Condition : 1 mL of methanol
Equilibrate : 1 mL Water
Sample Loading : Load the prepared sample
Wash1 : 1 mL of water
Wash2 : 1mL of 5% methanol
Drying : Dry the cartridge
Elution : Elute with 0.500 mL methanol twice

Evaporated at 40^0c

Reconstituted with 0.300 ml of 2mM Ammonium acetate: ACN (20:80) Vertex

Recovery and precision was good.

Method validation

Selectivity

Six lots of K_2EDTA human plasma, one hemolytic, and one lipemic plasma were screened for selectivity test.

System suitability test

System suitability was performed by injecting six consecutive injections of Imatinib and desmethyl imatinib at higher concentration (251.298 µg/mL) of calibration range and Nicotinib at working concentration (0.500 µg/mL).

The results are presented in Table 17.

Auto sampler carry over test

Carry over test was performed in the following sequence.

RS⇨AQS STD H⇨RS⇨AQS STD H⇨RS⇨AQS STD L⇨AQS STD L⇨BLNK⇨EXT STD H⇨BLNK⇨EXT STD H⇨BLNK⇨EXT STD L⇨EXT STD L
The results are presented in Table 18.

Sensitivity

The sensitivity was determined in terms of LLOQ. 6 LLOQ and Calibration curve samples were prepared using same stock solutions. The LLOQ concentrations were back calculated from Calibration curve data. The lowest limit of reliable quantification for Imatinib and desmethyl imatinib in human plasma was set at the concentration of the LLOQ 0.508 µg/mL.
The results are presented in Table 19.

Matrix effect

Six sets of LQC (1.513 µg/mL) and HQC (178.340 µg/mL) samples in triplicates were prepared using different batches of blank matrices. All the QC samples were analyzed along with freshly spiked calibration curve standards. The QC concentrations were calculated using from calibration curve data.
The results are presented in Table 20a and b.

Linearity

A regression equation with a weighting factor of 1/(concentration ratio) 2 of drug to IS concentration was judged to produce the best fit for the concentration-detector response relationship for Imatinib and desmethyl imatinib in human plasma. The representative calibration curve for regression analysis is illustrated in Table 21.

Precision and accuracy

To assess precision and accuracy of the developed analytical method, five distinct concentrations (QC's) in the range of CC were prepared, processed, and evaluated using six replications per QC concentrations.

Table 17: System suitability

S. No.	Standard	Precision	
		Area ratio	Retention time
1	Drug 1	0.6	0.0
	Drug 2	2.1	0.0
	ISTD	NA	0.5
2	Drug 1	1.9	0.0
	Drug 2	1.4	0.9
	ISTD	NA	0.0

Table 18: Sampler carry over test

Sample ID	Response (area count)	% Carry over	Response (area count)	% Carry over
	Imatinib		Nicotinib	
RS/MP/Elution	0		0	
Unextracted highest	3739639		365162	
RS/MP/Elution	0	0.0	0	0.0
Unextracted highest	3740969		360061	
RS/MP/Elution	0	0.0	0	0.0
Unextracted lowest	7310		401141	
Unextracted lowest	7232		393660	
Average of unextracted lowest STD	7271.0		397400.5	
Extracted STD	0		0	
Extracted highest	1695808		213252	
Extracted STD	277	7.3	0	0.0
Extracted highest	2190966		253127	
Extracted STD	0	0.0	0	0.0
Extracted lowest	4335		257497	
Extracted lowest	3204		257806	
Average of extracted lowest STD	3769.5		257651.5	

Table 19a: Sensitivity for imatinib

LOQ Concentration (ng/mL)	
45.362	36.20
	54.434
001	42.961
002	43.408
003	41.465
004	45.138
005	40.165
006	43.104
Mean	42.7068
±S.D.	1.71042
% CV	4.0
%	94.1

Table 19b: Sensitivity for desmethyl imatinib

LOQ Concentration (ng/mL)	
18.774	15.019
	22.529
001	22.917
002	19.862
003	18.784
004	19.112
005	17.773
006	15.847
Mean	19.0492
±S.D.	2.34851
% CV	12.3
%	101.3

Table 20a: Matrix effect for imatinib

Blank matrices post spiked with Drug I and ISTD						
Unextracted LQC	**1**	**2**	**3**	**4**	**5**	**6**
Area Ratio						
0.975	0.809	0.870	0.824	0.840	0.877	0.835
0.999						
0.993	0.840	0.812	0.842	0.860	0.808	0.847
0.997						
0.961	0.829	0.836	0.846	0.864	0.839	0.858
0.965						
Mean area ratio						
0.982	0.839	0.839	0.837	0.855	0.841	0.847
Matrix effect for individual matrix lot						
NA	0.841	0.855	0.853	0.871	0.857	0.862
Matrix factor	Mean		±S.D		% CV	
	0.8566		0.00977		1.1	

Table 20b: Matrix effect for desmethyl imatinib

Blank matrices post spiked with Drug II and ISTD						
Unextracted LQC	**1**	**2**	**3**	**4**	**5**	**6**
Area ratio						
0.698	0.639	0.642	0.703	0.703	0.747	0.714
0.673						
0.680	0.707	0.682	0.670	0.713	0.667	0.685
0.715						
0.690	0.988	0.661	0.661	0.689	0.714	0.687
0.635						
Mean area ratio						
0.682	0.678	0.662	0.678	0.702	0.709	0.694
Matrix effect for individual matrix lot						
NA	0.994	0.970	0.994	1.029	1.040	1.018
Matrix factor	Mean		±S.D		% CV	
	1.0077		0.02596		2.6	

Intra-batch precision and accuracy

Intra-batch precision and accuracy were determined by calculating CV % and % accuracy, respectively, at each QC level of a bio-analytical batch.

The results are presented in Table 22a.

Inter-batch precision and accuracy

Inter-day precision and accuracy were determined by calculating CV% and % accuracy respectively, at each QC level of all bio-analytical batches run in a day.

The results are presented in Table 22b.

Recovery

Recovery was evaluated by comparing the analytical results of extracted samples at four distinct levels (LQC, MQC2, MQC1, and HQC) with unextracted QC samples at each level that represent comparison samples.

The results are presented in Table 22.

Stabilities

Short term stock solution stability

Stock solution of Imatinib and desmethyl imatinib and Nicotinib was prepared. Stored in refrigerator at 2–8°C. An aliquot of these stock solution were kept on bench at room temperature. After 23 h 39 min and h 01 min, the fresh stock solution of analyte and IS was prepared, respectively. All the stock solutions of analyte and IS (keep it on bench as well as fresh stock) were diluted to get appropriate concentration. Comparison was done against area.

The results are presented in Table 22.

Short-term dilution stability

Dilutions of Imatinib and desmethyl imatinib and Nicotinib were prepared. An aliquot of these stock solution were kept on bench at room temperature and remaining dilution was stored in refrigerator at 2–8°C. After 23 h 38 min and 23 h for analyte and ISTD, respectively, fresh dilutions of analyte and ISTD were made from freshly prepared stock solution. All the stock dilutions of analyte and IS

Table 21: Linearity

Imatinib				Back calculated concentration (ng/mL)			Calibration standards accuracy and precision			
STD ID	Nominal conc.(ng/mL)	Acceptance range		P&A II	P&A III	P&A V	Mean	±S.D.	% CV	% Accuracy
		Minimum	Maximum							
STD 1-1	45.362	36.290	54.434	44.292	44.454	44.775	44.5070	0.2458	0.6	98.1
STD 1-2	45.362	36.290	54.434	45.260	47.204	47.874	NA	NA	NA	NA
STD 2	103.095	87.631	118.559	106.261	106.247	104.547	105.6850	0.9856	0.9	102.5
STD 3	303.221	257.738	348.704	330.765	324.996	324.209	326.6567	3.5796	1.1	107.7
STD 4	606.442	515.476	697.408	588.498	575.232	572.849	578.8597	8.4317	1.5	95.5
STD 5	1212.883	1030.951	1395.815	1254.136	1265.977	1284.614	1268.2423	15.3648	1.2	104.6
STD 6	1810.273	1538.732	2081.814	1610.846	1653.408	1701.853	1655.3690	45.5352	2.8	91.4
STD 7	2413.698	2051.643	2775.753	2455.666	2432.991	2429.983	2439.5467	14.0405	0.6	101.1
STD 8-1	3017.122	2564.554	3469.690	2988.120	3029.152	2953.849	2990.3737	37.7021	1.3	99.1
STD 8-2	3017.122	2564.554	3469.690	2924.385	3028.476	2897.703	NA	NA	NA	NA
r2				0.9956	0.9966	0.9971				
Desmethyl imatinib				**Back calculated concentration (ng/mL)**			**Calibration standards accuracy and precision**			
STD ID	Nominal conc.(ng/mL)	Acceptance range		P&A II	P&A III	P&A V	Mean	±S.D.	% CV	% Accuracy
		Minimum	Maximum							
STD 1-1	18.774	15.019	22.529	17.791	18.357	18.130	18.0927	0.2848	1.6	96.4
STD 1-2	18.774	15.019	22.529	18.936	16.130	17.860	NA	NA	NA	NA
STD 2	42.667	36.267	49.067	46.910	43.839	44.778	45.1757	1.5736	3.5	105.9
STD 3	125.492	106.668	144.316	134.884	138.097	138.726	137.2357	2.0607	1.5	109.4
STD 4	250.983	213.336	288.630	250.649	235.243	243.919	243.2703	7.7235	3.2	96.9
STD 5	501.967	426.672	577.262	511.287	523.538	518.537	517.7873	6.1598	1.2	103.2
STD 6	749.204	636.823	861.585	668.903	717.884	796.231	704.3393	30.9713	4.4	94.0
STD 7	998.939	849.098	1148.780	991.273	974.743	959.644	975.2200	15.8199	1.6	97.6
STD 8-1	1248.674	1061.373	1435.975	1218.308	1223.801	1179.430	1207.1797	24.1883	2.0	96.7
STD 8-2	1248.674	1061.373	1435.975	1202.003	1201.174	1134.877	NA	NA	NA	NA
r2				0.9944	0.9964	0.9960				

(keep it on bench as well as fresh stock) were diluted to get appropriate concentration. Comparison was done against area.
The results are presented in Table 23.

Long-term stock solution stability

Stock solution of Imatinib and desmethyl imatinib and Nicotinib was prepared. Stored in refrigerator at 2–8°C. An aliquot of these stock solution were kept on bench at room temperature. After a period of 3 days 21 h 58 min for analyte and 3 days 21 h 8 min for ISTD were prepared. The fresh stock solution of analyte and IS was prepared respectively All the stock solutions of analyte and IS (keep it on bench as well as fresh stock) were diluted to get appropriate concentration. Comparison was done against area.
The results are presented in Table 24.

Long-term dilution stability

Dilutions of Imatinib and desmethyl imatinib and Nicotinib were prepared. Dilution was stored in refrigerator at 2–8°C. After a period of 3 days 21 h 58 min for analyte and 3 days 21 h 8 min, the fresh dilutions of analyte and ISTD were made, respectively, from freshly prepared stock solution. All the stock dilutions of analyte and IS (keep it on bench as well as fresh stock) were diluted to get appropriate concentration. Comparison was done against area.
The results are presented in Table 25.

Auto sampler stability

In assessing the auto sampler stability of Imatinib and desmethyl imatinib six sets of LQC and HQC were processed and placed in the auto sampler. These

Table 22a: Intra-batch precision and accuracy

SET ID	QC ID	HQC	MQC 1	LQC		MQC 3	LOQQC
				Actual concentration (ng/mL)			
		3562.752	2208.906	30.218		265.069	10.159
Minimum limit		3028.339	1877.570	25.685	Minimum limit	225.309	8.127
Maximum limit		4097.165	2540.242	34.751	Maximum limit	304.829	12.191
P&A II	007	3494.941	2239.737	31.239	007	266.898	11.335
	008	3439.999	2114.950	33.064	008	266.398	11.614
	009	3624.961	2263.897	33.046	009	241.288	12.653
	010	3367.583	2247.328	36.543	010	271.776	12.882
	011	3638.278	2303.402	29.059	011	275.352	10.866
	012	3568.257	2165.114	30.605	012	262.776	12.475
	Mean	3522.3365	2222.4047	32.2593	Mean	264.0813	11.9708
	±S.D.	107.29542	69.32891	2.59369	±S.D.	12.00647	0.81265
	% CV	3.0	3.1	8.0	% CV	4.5	6.8
	% Accuracy	98.9	100.6	106.8	% Accuracy	99.6	117.8
P&A	019	3488.663	2270.125	25.046	013	253.507	11.433
SET ID	**QC ID**	**HQC**	**MQC 1**	**LQC**		**MQC 3**	**LOQQC**
				Actual concentration (ng/mL)			
		3562.752	2208.906	30.218		265.069	10.159
Minimum limit		3028.339	1877.570	25.685	Minimum limit	225.309	8.127
Maximum limit		4097.165	2540.242	34.751	Maximum limit	304.829	12.191
III	020	3661.905	2216.756	32.246	014	259.601	12.420
	021	3654.645	2292.084	31.201	015	264.680	13.731
	022	3606.460	2247.604	33.947	016	268.441	11.319
	023	3663.579	2300.608	32.591	017	265.711	10.847
	024	3652.332	2280.266	34.966	018	263.792	9.657
	Mean	3621.2640	2267.9072	31.6662	Mean	262.6220	11.5678
	±S.D.	68.29926	31.11329	3.50216	±S.D.	5.31444	1.38971
	% CV	1.9	1.4	11.1	% CV	2.0	12.0
	% Accuracy	101.6	102.7	104.8	% Accuracy	99.1	113.9
P&A V	031	3640.369	2304.776	34.300	025	285.485	8.432
	032	3778.793	2224.993	30.213	026	254.767	10.755
	033	3717.130	2129.451	33.141	027	250.981	11.061
	034	3593.499	2293.430	28.138	028	259.764	12.820
	035	3829.343	2146.596	30.167	029	266.040	8.980
SET ID	**QC ID**	**HQC**	**MQC 1**	**LQC**		**MQC 3**	**LOQQC**
				Actual concentration (ng/mL)			
		3562.752	2208.906	30.218		265.069	10.159
Minimum limit		3028.339	1877.570	25.685	Minimum limit	225.309	8.127
Maximum limit		4097.165	2540.242	34.751	Maximum limit	304.829	12.191
	036	3714.073	2280.065	25.449	030	249.109	9.668
	Mean	3712.2012	2229.8852	30.2347	Mean	261.0243	10.2860
	±S.D.	86.52692	76.43643	3.23349	±S.D.	13.46898	1.59791
	% CV	2.3	3.4	10.7	% CV	5.2	15.5
	% Accuracy	104.2	100.9	100.1	% Accuracy	98.5	101.3

Table 22b: Inter-batch precision and accuracy

QC ID	Actual Conc. (ng/mL)	Mean Values			Mean	±S.D.	% CV	% Accuracy
		P&AII	P&A III	P&A V				
HQC	3562.752	3522.3365	3621.2640	3712.2012	3618.60056	94.960352	2.6	101.6
MQC 1	2208.906	2222.4047	2267.9072	2229.8852	2240.06567	24.399822	1.1	101.4
MQC 3	265.069	264.0813	262.6220	261.0243	262.57589	1.529022	0.6	99.1
LQC	30.218	32.2593	31.6662	30.2347	31.38672	1.040858	3.3	103.9
LOQ QC	10.159	11.9708	11.5678	10.2860	11.27489	0.879789	7.8	111.0

Table 23: Recovery

QC ID	Drug area		ISTD area	
	Aqueous	Extracted	Aqueous	Extracted
HQC	1851426	1955887	407517	402094
	1850785	2032638	412962	413998
	1891877	2004963	417125	423010
	1842826	1995031	409243	410389
	1808407	2030252	406545	417535
	1854414	1992738	420191	415385
Mean	1849955.8	2001918.2	412263.8	413735.2
QC ID	**Drug area**		**ISTD area**	
	Aqueous	**Extracted**	**Aqueous**	**Extracted**
±S.D	26673.01	28306.76	5501.30	7070.59
% CV	1.4	1.4	1.3	1.7
% Recovery	108.2		100.4	
MQC1	1163921	1308801	420681	426506
	1132235	1291463	423643	434944
	1153177	1262569	432826	428522
	1150562	1254806	434010	433646
	1133300	1261316	432131	430639
	1154788	1266760	418726	420518
Mean	1147997.2	1274285.8	427002.8	429129.2
±S.D	12629.69	21109.02	6768.57	5251.58
% CV	1.1	1.7	1.6	1.2
% Recovery	111.0		100.5	
LQC	17050	15533	450212	450843
	16410	18152	441319	434137
	16147	19516	429616	455757
	18138	17949	450137	456198
	14596	16755	447144	457050
	16073	19956	449436	459906
Mean	16402.3	17976.8	444644.0	452315.2
±S.D	1172.06	1660.29	8090.55	9376.30
% CV	7.1	9.2	1.8	2.1
% Recovery	109.6		101.7	
Global Statistics				
Mean	109.60		100.85	
± S.D.	1.393		0.738	
% CV	1.3		0.7	

samples were injected after a period of 36 h and were quantified against freshly spiked calibration curve standards of concentration range equivalent to that used for calculation of precision and accuracy.
The results are presented in Table 26.

Bench top stability

Bench top stability of Imatinib and desmethyl imatinib using six sets each of LQC and HQC was kept at room temperature (25±3°C) for 18 h 17 min. These QC samples were quantified against the freshly spiked calibration curve standards.
The results are presented in Table 27.

Freeze-thaw stability

The stability of Imatinib and desmethyl imatinib in human plasma was determined during fours freeze-thaw cycles. Six replicates, each of LQC and HQC were analyzed after three freeze-thaw cycles. The freeze-thaw QC samples were quantified against the freshly spiked calibration curve standards of concentration range equivalent to that used for the calculation of precision and accuracy.
The results are presented in Table 28.

Ruggedness

One precision and accuracy batch (PA BATCH) was processed and analyzed using different column of same make and different solutions.
The results are presented in Table 29a and b.

Data processing

The chromatograms were acquired and processed by peak area ratio method using the Analyst 2.5.6

Table 24a: Short term stock solution stability (room temperature i.e., 25±3°C)

Duration	**00.00 h**	**7.28 h**	**00.00**	**7.28**	**00.00**	**7.35**	**00.00**	**7.35**
Concentration	**HQC**		**LQC**		**ISTD**		**ISTD**	
	3562.938	**3561.263**	**30.733**	**30.271**	**0.203**	**0.203**	**0.203**	**0.203**
1	2706474	2352863	23925	23377	420620	361255	374842	394466
2	2218381	2394111	23970	24395	339051	357192	388238	385119
3	2281550	2396001	22296	23919	354692	349899	386444	381063
4	2325640	2379450	21615	21158	361635	366750	383120	394533
5	2347899	2385857	21798	24099	357693	344457	390116	381226
6	2345524	2375865	22692	23165	359552	369086	398785	388572
Mean	2370911.3	2380691.2	22716.0	23352.2	365540.5	358106.5	386924.2	387496.5
±S.D.	171509.09	15750.61	1026.26	1167.61	28163.21	9588.33	7915.48	6092.69
% CV	7.2	0.7	4.5	5.0	7.7	2.7	2.0	1.6
% Stability	100.5		104.4		98.0		100.1	

Table 24b: Short-term dilution stability (room temperature, i.e., 25±3°C)

Duration	**00.00 h**	**07.60 h**	**00.00**	**7.60**	**00.00**	**7.67**	**00.00**	**7.67**
Concentration	**HQC**		**LQC**		**ISTD**		**ISTD**	
	3552.938	**3561.263**	**30.733**	**30.271**	**0.203**	**0.203**	**0.203**	**0.203**
1	2446373	2427475	22487	23382	369727	366377	411268	409084
2	2460337	2429711	27324	23314	378659	382745	403346	400867
3	2473407	2423115	24103	22546	362863	369564	410668	386269
4	2439644	2443711	23560	19832	370054	375993	415448	389781
5	2408577	2443948	22309	23470	377760	371031	413493	399480
6	2463972	2454241	26918	18634	373639	370811	417711	403204
Mean	2448718.	2437033.	24450.	21863.	372117.	372753.	411989.	398114.
±S.D.	23135.98	12059.90	2177.1	2098.2	5874.73	5794.78	4978.64	8549.12
% CV	0.9	0.5	8.9	9.6	1.6	1.6	1.2	2.1
% Stability	99.3		90.8		100.2		96.6	

Table 25a: Long-term stock solution stability imatinib (2–8°C)

Duration	**00.00**	**7.06**	**00.00**	**7.06**	**00.00**	**7.04**	**00.00**	**7.04**
Concentration	**HQC**		**LQC**		**ISTD**		**ISTD**	
	3577.187	**3561.263**	**30.406**	**30.271**	**0.221**	**0.203**	**0.221**	**0.203**
1	3161860	3138179	27987	28860	637616	616356	622696	672972
2	3165598	3094216	27697	29797	620472	633847	656377	652228
3	3125932	2997687	27704	28224	635570	603605	648863	645746
4	3194108	2970612	28252	26558	635204	592240	664896	655234
5	3132914	2960840	27946	26143	626277	591=948	669110	653174
6	3118581	2918874	26307	28289	633552	589476	651279	650696
Mean	3149832.	3013401.	27648.	27978.	631448.	604578.	652203.	655008.
±S.D.	28940.14	84707.09	688.82	1387.6	6645.02	17528.0	16413.7	9361.90
% CV	0.9	2.8	2.5	5.0	1.1	2.9	2.5	1.4
% Stability	96.1		101.6		104.0		109.1	

Table 25b: Long-term dilution stability desmethyl imatinib (2–8°C)

Duration	00.00	7.03	00.00	7.03	00.00	7.03	00.00	7.03
Concentration	HQC		LQC		ISTD		ISTD	
	3577.187	3561.263	30.406	30.271	0.221	0.203	0.221	0.203
1	3099167	3057353	26879	30522	609166	605159	656060	638060
2	2988068	3024597	26987	29512	609539	600776	653147	642181
3	3012534	3107416	30533	29475	605876	607818	652459	637372
4	3080724	3094610	26279	27733	619080	607451	664052	665289
5	3063129	3053430	26144	26891	596887	601247	644020	638695
6	3102253	3076598	28817	28592	603663	591524	647608	640432
Mean	3057645.	3069000.	27606.	28787.	607368.	602329.	652891.	643671.
±S.D.	47228.44	30130.03	1723.0	1323.1	7361.00	6077.89	6949.35	10732.8
% CV	1.5	1.0	6.2	4.6	1.2	1.0	1.1	1.7
% Stability	100.8		104.7		107.8		107.1	

Table 26: Auto sampler stability

S. No.	At 0.0 h		1.85 days	
	HQC	LQC	HQC	LQC
	Actual concentration (ng/mL)			
	3562.963	30.220	3562.752	30.218
Minimum	3028.519	25.687	3028.339	25.685
Maximum	4097.407	34.753	4097.165	34.751
QC IDs	Back calculated concentration (ng/mL)			
121	3704.701	27.245	3570.678	30.739
122	3698.425	28.916	3579.918	28.731
123	3577.543	33.598	3601.864	23.403
124	3597.692	32.059	3598.934	31.198
125	3594.554	30.961	3647.935	27.858
126	3519.214	33.764	3554.225	32.541
Mean	3615.3548	31.0905	3592.2590	29.0783
± S.D.	72.52701	2.60408	32.56149	3.25761
% CV	2.0	8.4	0.9	11.2
% Stability			99.4	93.5

Table 27: Bench Top Stability (Room temperature, i.e., 25±3°C)

S. No.	At 0.0 h		9.00 h	
	HQC	LQC	HQC	LQC
	Actual concentration (ng/mL)			
	3562.963	30.220	3562.752	30.218
Minimum	3028.519	25.687	3028.339	25.685
Maximum	4097.407	34.753	4097.165	34.751
QC IDs	Back calculated concentration (ng/mL)			
097	3514.873	27.960	3507.838	26.162
098	3440.930	29.101	3433.676	29.662
099	3607.151	27.697	3361.749	27.786
100	3410.066	26.246	3487.447	26.962
101	3575.107	27.831	3368.871	29.678
102	3398.867	33.060	3429.900	23.387
Mean	3491.1657	28.6492	3431.5802	27.2728
± S.D.	87.95522	2.34460	59.58592	2.37430
% CV	2.5	8.2	1.7	8.7
% Stability			98.3	95.2

software. The concentration of the unknown was calculated from the following equation using regression analysis of spiked standard with the reciprocal of the square of ratio of the drug to IS concentration as a weighting factor [1/(concentration ratio) 2].

$y = mx+c$

where, y = Peak area ratio of Imatinib to internal standard m = Slope of the calibration curve

x = Area ratio of desmethyl imatinib to IS ng/ml

c = Y-axis intercept of the calibration curve.

RESULTS AND DISCUSSION [TABLES 1-3]

System suitability test [Tables 6-16]

The precision for retention time of Imatinib and desmethyl imatinib.

Acceptance criteria

% CV for the area ratio should be <5% and retention time should be <2%.

Table 28: Freeze-thaw stability

S. No.	At 0.0 h		FT Cycle V	
	HQC	LQC	HQC	LQC
	Actual concentration (ng/mL)			
	3562.963	30.220	3562.752	30.218
Minimum	3028.519	25.687	3028.339	25.685
Maximum	4097.407	34.753	4097.165	34.751
	Back calculated concentration (ng/mL)			
175	3506.540	30.312	3525.477	33.020
176	3646.355	31.289	3662.122	27.928
177	3641.850	30.357	3657.493	35.290
178	3721.967	31.099	3983.097	31.045
179	3682.249	31.405	3772.635	28.419
180	3890.409	27.361	3693.213	31.098
Mean	3681.5617	30.3038	3715.6728	31.1333
± S.D.	125.45866	1.51516	153.42220	2.77446
% CV	3.4	5.0	4.1	8.9
% Stability			100.9	102.7

Conclusion

Tabulated results indicate that acceptance criteria.

Auto sampler carry over test

No significant auto sampler carry over was observed for Imatinib, desmethyal imatinib, and IS.

Acceptance criteria

Response of RS/M.P/Extracted standard blank should not be exceed 2% of the response of preceding injection of aqueous/extracted highest standard.

Conclusion

The carryover of Imatinib, desmethyl imatinib, and the IS Nicotinib was within the acceptance criteria NMT 2%.

Table 29a: Linearity-ruggedness

Analyzed with CC 07					
STD ID	**Nominal Conc.(ng/ml)**	**Acceptance Range**		**Back calculated concentration (ng/mL)**	**% Accuracy**
		Minimum	**Maximum**		
STD 1	10.159	8.127	12.191	9.478	93.3
STD 2	20.318	17.270	23.366	23.158	114.0
STD 3	50.795	43.176	58.414	50.216	98.9
STD 4	101.591	86.352	116.830	102.430	100.8
STD 5	406.363	345.409	467.317	387.824	95.4
STD 6	1231.031	1046.376	1415.686	1189.764	96.6
STD 7	2462.062	2092.753	2831.371	2418.626	98.2
STD 8	3517.231	2989.646	4044.816	3703.447	105.3
STD 9	5024.616	4270.924	5778.308	4895.170	97.4
r2				0.9951	

Table 29b: Precision and accuracy-ruggedness

SET	QC ID	HQC	MQC 1	LQC		MQC 3	LOQQC
		Actual concentration (ng/mL)					
Nominal conc.		**3562.752**	**2208.906**	**30.218**	**Nominal conc.**	**265.069**	**10.159**
Maximum limit		3028.339	1877.570	25.685	Minimum	225.309	8.127
Minimum limit		4097.165	2540.242	34.751	Maximum	304.829	12.191
Ruggedness	037	3733.934	2229.934	26.286	031	276.440	10.873
	038	3668.399	2107.975	31.732	032	262.385	11.868
	039	3717.541	2151.257	27.521	033	258.571	11.331
	040	3406.779	2169.830	30.440	034	259.372	11.810
	041	3781.267	2155.199	29.475	035	249.721	11.124
	042	3483.550	2179.650	34.247	036	261.712	10.575
	Mean	3631.9117	2165.6408	29.9502	Mean	261.3668	11.2635
	±S.D.	151.04647	39.97172	2.87856	±S.D.	8.66923	0.51276
	% CV	4.2	1.8	9.6	% CV	3.3	4.6
	%	101.9	98.0	99.1	% Accuracy	98.6	110.9

Sensitivity

The precision and accuracy six samples at LLOQ level were processed as per extraction method and injected along with a precision and accuracy batch. LOQ was 45.362 ng/mL for Imatinib and 18.774 ng/mL desmethyl imatinib.

Acceptance criteria

1. The analyte response at the LLOQ should be at least 5 times the response compared to blank response
2. Accuracy should be within 80–120% of nominal concentration and precision should be less than or equal to 20%.

Conclusion

The lowest standard 45.362 ng/mL for Imatinib and 18.774 ng/mL for desmethyl imatinib proved to be accepted as the lower LOQ of the method with required accuracy and precision.

Matrix effect

Matrix effect was evaluated by injecting three replicates of two distinct concentrations (high and low) using four different lots of screened matrices other than those used for validation comparison was done against QC samples of precision and accuracy batch.

Acceptance criteria

Accuracy at LQC and HQC levels of at least three different lots (75% screened lots) should be 85–115% and precision should be less than ±15%.

Conclusion

All lots should be no significant matrix effect the results should be with in acceptance criteria.

Linearity

To define the relationship between concentration and response, calibration curves containing eight non zero standards for imatinib and desmethyl imatinib were prepared.

Acceptance criteria

At least 75% non-zero standards fall within ±15% except for LLOQ

When it should be ±20% for nominal value.

Conclusion

Results should be within acceptance criteria.

Precision and accuracy

Recovery

The mean overall recovery of Imatinib and desmethyl imatinib was 109.60% with a precision 1.3%.

The mean recovery of IS Nicotinib was 100.85% with a precision 0.7 %.

Stabilities

Short-term stock solution stability

To evaluate the short-term stock solution stability of Imatinib, desmethyl imatinib and Nicotinib, stock solutions were prepared as per method SOP. An aliquot of these stock solutions were kept on bench at room temperature (25 ± 3°C). After a period of 7.28 h for analyte at HQC and LQC level and a period of 7.35 h for ISTD, stock solutions were diluted to get the HQC and LQC level concentrations for analyte and working concentration for IS, respectively. For comparison, freshly prepared stocks of analyte and IS were diluted to get the concentration of HQC and LQC for analyte and working concentration for IS.

Both the solutions were injected onto the chromatographic system. Short-term stock solution stability was evaluated by comparing the mean area obtained from the stability stock solution against the freshly prepared stock solution (0.0 h).

The results are shown in Table 24a.

Short-term dilution stability

To evaluate the short-term dilution stability, dilutions of Imatinib, desmethyl imatinib, and Nicotinib were prepared. An aliquot of these dilutions were kept on bench at room temperature

(25 ± 3°C). After a period of 7.60 h at HQC, LQC level for analyte and a period of 7.67 h for ISTD, fresh dilutions of HQC, LQC for analyte, and working concentration for IS were made from fresh stock solution respectively.

Both the solutions were injected onto the chromatographic system. Short-term dilution stability was evaluated by comparing the mean area obtained from the stability dilution against the freshly prepared dilution (0.0 h). The results are shown in Table 23.

Acceptance criteria

% stability of analyte and IS should be 90–110%.

Long-term stock solution stability

To evaluate the long-term stock solution stability of Imatinib, desmethyl imatinib, and Nicotinib stock solutions were prepared and stored in refrigerator maintained at 2–8°C. After a period of 7.06 days for at HQC and LQC level for analyte and 7.04 days for ISTD, stock solutions were retrieved from refrigerator and were diluted to get HQC, LQC for analyte and working concentration for IS, respectively. For comparison, freshly prepared stocks of analyte and IS were diluted to get concentration of HQC, LQC for analyte, and working concentration for IS on the day of evaluation of the stability.

Both the solutions were injected onto the chromatographic system. Long-term stock solution stability was evaluated by comparing the mean area obtained from the stability stock solution against the freshly prepared stock solution (0.0 h). The results are shown in following Table 24.

Long-term dilution stability

To evaluate the long-term dilution stability, dilutions of Imatinib, desmethyl imatinib and Nicotinib were prepared from stock solution and stored in refrigerator maintained at 2–8°C. After a period of 7.03 days for at HQC and LQC level for analyte and ISTD, fresh dilutions of HQC, LQC for analyte and working concentration for IS were made from fresh stock solution on the day of evaluation of the stability.

Both the solutions were injected onto the chromatographic system. Long-term dilution stability was evaluated by comparing the mean area

S. No.	Parameter	Table No.		Units	Limits		Results	
					Lower	Higher	Lower	Higher
1	Selectivity	1	No significant interference at the retention time of DRUG and ISTD observed under controlled condition					
2	Linearity	2	Range: 10.159–5024.616 ng/ml		R^2<0.99		0.9923–0.9978	
3	Precision	3a	Intra batch (LQC, MQC2, MQC1, HQC)-P&A I	%	≤15		3.0	8.0
			Intra batch (LOQ QC) P&A I		≤20		6.8	
			Intra batch (LQC, MQC2, MQC1, HQC)-P&A II		≤15		1.4	11.1
			Intra batch (LOQ QC) P&A II		≤20		12.0	
			Intra batch (LQC, MQC2, MQC1, HQC)-P&A III		≤15		2.3	10.7
			Intra batch (LOQ QC) P&A III		≤20		15.5	
		3b	Inter batch (LQC, MQC2, MQC1, HQC)		≤15		0.6	3.3
			Inter batch (LOQ QC)		≤20		7.8	
4	Accuracy	3a	Intra batch (LQC, MQC2, MQC1, HQC)-P&A I	%	85	115	98.9	106.8
			Intra batch (LOQ QC) P&A I		80	120	117.8	
			Intra batch (LQC, MQC2, MQC1, HQC)-P&A II		85	115	99.1	104.8
			Intra batch (LOQ QC) P&A II		80	120	113.9	

(Contd...)

(Continued)

S. No.	Parameter	Table No.		Units	Limits		Results	
					Lower	Higher	Lower	Higher
			Intra batch (LQC, MQC2, MQC1, HQC)-P&A II		85	115	98.5	104.2
			Intra batch (LOQ QC) P&A III		80	120	101.3	
		3b	Inter batch (LQC, MQC2, MQC1, HQC)		85	115	99.1	101.6
			Inter batch (LOQ QC)		80	120	111.0	
5	Matrix effect	4a	The coefficient of variation of IS normalized MF for HQC	%	≤15		0.9	
		4b	The coefficient of variation of IS normalized MF for LQC				3.0	
6	Sensitivity	5	Precision	%	≤20		12.2	
			accuracy	%	80	120	101.4	
7	Recovery	6	Analyte	%	-	-	109.60	
			ISTD	%	-	-	100.80	
8	Stability	7	Short term stock solution stability of drug-7 h 28 min		90	110	100.5	
			Short term stock solution stability of ISTD-7 h 35 min				100.1	
		9	Short term dilution stability of drug-7 h 60 min				99.3	
			Short term dilution stability of ISTD-7 h 67 min	%			100.2	
		11	Long-term stock solution stability of drug- 7 days 6 h 58 min				101.6	
			Long-term stock solution stability of ISTD- 7 days 4 h 8 min				109.1	
		13	Long-term dilution stability of drug- 7 days 3 h 58 min				104.7	
			Long term dilution stability of ISTD- 7 days 3 h 08 min				107.8	
		14	Auto injector stability-HQC-1 day 11 h 18 min	%	85	115	99.4	
			Auto injector stability-LQC-1 day 11 h 18 min				93.5	
		15	Bench top stability-HQC-18 h 17 min				98.5	
			Bench top stability-LQC-18 h 17 min				95.2	
		16	Freeze thaw-HQC-cycle				100.9	
			Freeze thaw-LQC- cycle				102.7	
9	Ruggedness	17a	Linearity	%	R^2>0.99		0.9978	
		17b	Precision (LQC, MQC2, MQC1, HQC)		≤15		0.6	3.3
			Accuracy (LQC, MQC2, MQC1, HQC)		85	115	99.1	103.9
			Precision (LOQQC)		≤20		7.8	
			Accuracy (LOQQC)		80	120	111.0	

Figure 1: LC-MS/MS

Figure 2: Thermo ion source

obtained from the stability dilution against the fresh dilution (0.0 h). The results are shown in Table 25.

Acceptance criteria

- % Stability of analyte and IS should be within 90–110%
- % CV should not exceed 10.

Auto sampler stability

To evaluate the post-processing stability, six aliquots of each QC sample at two distinct levels (HQC and LQC) were kept in auto sampler at 10°C immediately after preparation. The stability of replicate concentrations of high and low QC samples was evaluated by comparing the mean of back calculated concentrations of the samples stored in auto sampler at specified temperature for 1.85 days against freshly prepared and processed

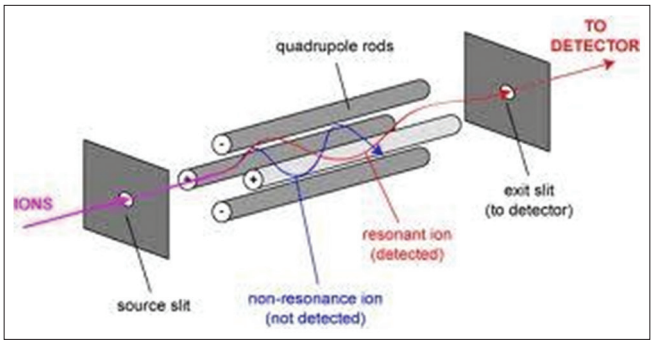


Figure 3: Mass spectrometer

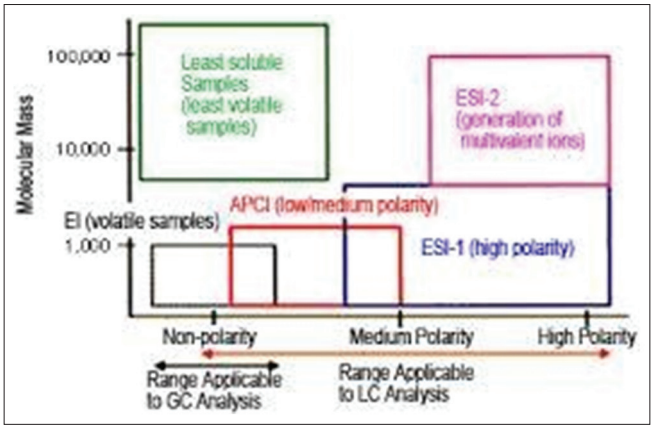


Figure 4: Comparison of APCI and electrospray applicability to analyte molecular weight as a function of polarity

QC samples (HQC and LQC). The results are shown in Table 26.

Acceptance criteria

- Precision at each level should be ≤15%
- % Stability of the analyte should be within 85–115%.

Bench top stability

To evaluate the bench top stability, six aliquots of each QC sample at two distinct levels (HQC and LQC) were frozen, thawed and maintained, at room temperature (25 ± 3°C) for 9.00 h, which exceeds the expected duration that samples could be maintained at room temperature after thawing until they are analyzed [Figures 1-4]. The stability of replicate concentrations of high and low QC samples were evaluated by comparing the mean of back calculated concentrations of the samples kept on the bench at room temperature against

freshly prepared calibration standards and QCs samples (HQC and LQC). The results are shown in Table 27.

Acceptance criteria

% Stability of analyte should be within 80–110%.

Freeze-thaw stability

Samples were quantified after the five cycles together with the freshly spiked calibration curve and QC samples. The results are shown in Table 28.

Acceptance criteria

- Precision at each level should be ≤15%
- % Stability of the analyte should be within 85–115%.

Ruggedness

Ruggedness was carried out, by performing precision and accuracy batch on a different column and analyst.

Acceptance criteria

- Accuracy for all CC standards should be within 85–115% and for LOQ should be within 80–120%
- Precision at each concentration level should not exceed 15% and for LOQ it should be 20%.

Acceptance criteria

- At least 75% of non-zero standards (including ULOQ and LLOQ) when back calculated should fall within ±15%, except for LLOQ when it should be ±20% of the nominal value
- Regression coefficient should be greater than 0.99
- Precision-At each concentration level should not exceed 15% of CV except at LLOQ where it should not exceed 20%
- Accuracy – The mean value of samples at each concentration level should be within 85–115% of the nominal value except for LLOQ where it should be within 80–120%.

VALIDATION SUMMARY

CONCLUSION

The detection limit is 10 ng/mL in plasma, CSF, culture medium (RPMI), and 25 ng/mL in urine for both STI-571 and N- DesM-STI. The intra-day precision, as expressed by the coefficient of variation (CV), in plasma samples ranges between 1.74 and 8.60% for STI-571 and 1.45 and 8.87% for N- DesM-STI. The corresponding values for urine measurements are 2.17–7.54% (STI-571) and 1.31–9.51% (N-DesM-STI). The inter-day precision analyzed over a 7-month time period was 8.31% (STI-571) or 6.88% (N-DesM-STI) and 16.45% (STI-571) or 14.83% (N-DesM-STI) for a concentration of 1000 ng/mL in plasma and 750 ng/mL in urine,respectively. Moreover, we demonstrate that with an alternative, but more time and labor consuming sample preparation and the implementation of electrochemical detection, a detection limit <10 ng/mL can be achieved. The method described was used to perform pharmacokinetic measurements of STI- 571 and N-desmethyl-STI in patient samples and for kinetic measurements of intracellular STI-571 and N-DesM-STI following in vitro incubation.

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