Validation of an HPLC Method for Determination of Imatinib Mesylate in Rat Serum and Its Application in a Pharmacokinetic Study

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Abstract

A simple, selective, and sensitive liquid chromatographic method has been developed and validated for quantitative determination of Imatinib mesylate in rat serum. Efficient chromatographic separation has been performed on a Zorbax Extend (5 µm, 4.6 × 250 mm) double end-capped C_{18} column using a mobile phase consisting of methanol and aqueous triethyl amine (pH 10.5; 1%, v/v) (60:40, v/v) in an isocratic mode at a flow rate of 1 mL/min. Simple and effective liquid–liquid extraction technique has resulted in consistent and high recoveries (90.32–95.86%) at all concentrations studied. The method has demonstrated linearity from 25 to 1600 ng/mL with a regression coefficient of 0.9995. Accuracy of the method is acceptable with intra-batch %bias between –2.34 to 3.42 and inter-batch %bias between –2.17 to 3.45. The method has demonstrated high sensitivity with lower limit of quantification of 25 ng/mL and excellent stability of Imatinib mesylate in serum. The method is found to be rapid, reliable, and suitable for in vivo pharmacokinetic study.

Introduction

Imatinib mesylate (IM), a phenylaminopyrimidine derivative (Figure 1), represents the first of new generation of molecularly targeted chemotherapy for cancer treatment (1). Deregulated tyrosine kinase activity has been reported to be the central pathogenic event in a number of human malignancies (2). IM is a selective tyrosine kinase inhibitor that has been rationally designed to selectively intervene the key signal transduction pathways (3). It has been approved in the treatment of chronic myeloid leukemia and gastrointestinal stromal tumors as first line therapy. Although IM represents a new class of rationally designed targeted chemotherapeutic agents (4), the chronic oral administration may induce over-expression of drug transport pumps, which may limit oral bioavailability and efficacy of IM (5). Like several other cancer drugs, IM develops resistance leading to subtherapeutic concentrations and poor bioavailability. Important underlying causes of clinical resistance of IM are principally attributed to efflux mechanisms (6–13). Thus, clinical use of efflux inhibitors for enhancing therapeutic efficacy of IM may be a promising strategy for management of chemotherapy. In order to investigate the effect of various efflux modulators on IM bioavailability, the accurate determination of pharmacokinetic parameters in a small experimental animal model expressing these proteins is essential. Although few high-performance liquid chromatographic (HPLC) methods have been published for the determination of IM, there is no single reported bioanalytical method for determination of IM in small laboratory animals like rats.

Amongst the reported bioanalytical methods, only a few include use of UV detector for quantification (14–16) while other methods use sophisticated analytical instruments such as liquid chromatography–tandem mass spectrometry, making them unsuitable for routine analysis (17–23). The bioanalytical method reported by Widmer et al. used higher sample volume of 750 µL with gradient elution system. Moreover, the calibration range was 100 ng/mL to 10 µg/mL, and the drug extraction was carried out using a solid-phase system with a total run time of 45 min (14). Similarly, the method reported by Oostendorp et al. used a costly ion-paring agent, 1-octane sulfonic acid, resulting in increased cost of analysis (15). In the method reported by Velpandian et al., the authors have mentioned intra- and inter-day variation resulting from analytical standards, prepared in

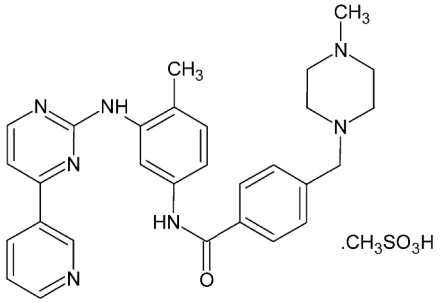

Figure 1. Chemical structure of Imatinib mesylate.

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solvent system instead of plasma calibration standards, prepared in biomatrix as per the standard bioanalytical method validation guidelines (16). Moreover, the stability of drug in biomatrix under various processing and storage conditions, which is a critical component of validation, has not been studied. Therefore, the reported HPLC–UV methods were found to have limitations such as high sample volume, complex extraction protocols, gradient elution, and use of ion paring agents, etc., leading to increased time and cost of analysis (14–16). Thus, extensive literature survey did not reveal any simple bioanalytical method suitable for routine analysis IM in rat serum.

The objective of the present study was to develop a simple, sensitive, accurate, and reproducible bioanalytical method for determination of IM in rat serum samples. Method was validated as per standard validation guidelines for bioanalytical methods using suitable statistical tests (24–27). Further, the method was employed for analysis of serum samples collected during in vivo pharmacokinetic studies in the rat.

Experimental

Materials and reagents

IM (assay 99.95%) was obtained as a gift sample from Cipla (Mumbai, India). HPLC-grade acetonitrile, methanol, triethylamine, *n*-hexane, and methylene chloride were purchased from Spectrochem (Mumbai, India). Sodium dihydrogen orthophosphate and disodium hydrogen phosphate were purchased from S.D. Fine Chemicals (Mumbai, India). HPLC-grade water was prepared using a Millipore Milli-Q water purification system (Mosheim, France). All other chemicals and reagents were HPLC- or analytical-grade. Drug-free serum pool was obtained from a few healthy male Wistar rats, and it was stored at –80°C in sealed cryovials.

Chromatographic system and conditions

A Shimadzu HPLC system (Kyoto, Japan) consisting of a pump system (LC10AT VP), autosampler (SIL HTA), and UV detector (SPD-10A VP) was used. Data acquisition and analysis was done using 21 CFR part 11 compliant LCSolutions workstation (Shimadzu).

Optimized mobile phase consisted of methanol and aqueous triethylamine (pH 10.5; 1%, v/v) (60:40, v/v), and aqueous phase pH was adjusted with 1 M hydrochloric acid. Mobile phase was delivered in isocratic elution mode at a flow rate of 1 mL/min. The chromatographic separation was performed on a Zorbax Extend-C_{18} (5 µm, 80 Å, 4.6 × 250 mm) double end-capped column (Agilent, Santa Clara, CA) with a guard column (4.6 × 12 mm) of the same material. Quantification was carried out at 285 nm with a 50 µL injection volume. Analysis was performed at ambient temperature (25°C) after baseline stabilization for at least 60 min.

Animals

Healthy male rats with average weight of 250 ± 25 g (12–15 weeks old) were obtained from Central Animal Facility, BITS, Pilani (New Delhi, India). Selected animals were placed in standard plastic cages and maintained in controlled environmental conditions. Animals were acclimatized to the study environment for five days prior to commencement of work, and they were provided standard laboratory pellet food with water ad libitum. Study protocols (Protocol No. IAEC/RES/05/05-06) were approved by the Institutional Animal Ethics Committee (IAEC), BITS Pilani prior to commencement of work. All experimental procedures including euthanasia and disposal of carcass were in accordance with the guidelines set by the IAEC, BITS Pilani.

Stock solutions and standards

A primary stock solution of 1.0 mg/mL was prepared in reconstitution solution consisting of methanol–water (60:40, v/v). A series of seven working stocks containing 1, 2, 4, 8, 16, 32, and 64 µg/mL of IM was prepared by serial dilution of primary stock in reconstitution solution. Seven analytical standards containing 25, 50, 100, 200, 400, 800, and 1600 ng/mL of IM were prepared fresh by diluting 250 µL of each working stock to 10 mL with reconstitution solution in triplicate on three different days of validation.

Seven serum standards containing 25, 50, 100, 200, 400, 800, and 1600 ng/mL of IM were prepared fresh by spiking 25 µL of each working stock in 975 µL of blank rat serum. Each serum standard was vortex-mixed for 1 min and allowed to equilibrate with the drug. The serum standards were prepared fresh in three replicates on three different days of validation. Similarly, four quality control (QC) standards were prepared at lower limit of quantification (LLOQ = 25 ng/mL), low (LQC = 100 ng/mL), medium (MQC = 400 ng/mL), and high (HQC = 1600 ng/mL) concentration levels of calibration curve. The QC standards were prepared fresh in five replicates on each day of validation. Serum and QC standards were processed as described in the "Sample preparation" section, and they were analyzed by the proposed method.

Sample preparation

Aliquot of serum sample (100 µL) was transferred to a clean glass tube, and 1.5 mL of methylene chloride was added to it. Drug was extracted by vortex mixing for 1 min, and methylene chloride was separated by centrifugation (10,000 rpm, 5 min, 20°C). The upper aqueous layer was removed by aspiration, and organic layer was transferred to a fresh tube. Separated organic layer was dried using a vacuum concentrator, and dry residue was reconstituted in 100 µL of the reconstitution solution by vortex-mixing for 1 min. Resultant solution was centrifuged (10,000 rpm, 2 min, 4°C), and the clear supernatant was transferred to autosampler microvials.

Method development

Successful analysis of an analyte in biological fluids relies on the optimization of sample preparation, chromatographic separation, and interference-free detection. Each of the steps was optimized for developing a sensitive, selective, and reproducible method. For selectivity purpose, the principle metabolite of the drug was synthesized by *N*-demethylation of IM as reported elsewhere (28). Briefly, IM was dissolved in dry 1,2-dichloroethane, and drug solution was refluxed with four equivalents of α-chloroethyl chloroformate for 24 h under nitrogen. The obtained

carbamate intermediate was hydrolyzed by treatment with methanol at 50°C for 2 h (29). The separation and purification was carried out using thin-layer chromatography over a distance of 10 cm with mobile phase consisting of toluene, acetone, ethanol, and ammonia (45:45:06:04, v/v). After separation and drying, the *N*-desmethyl derivative was extracted with solvent phase consisting of methylene chloride, propranol, and ammonia (95:04:01, v/v).

For sample clean up, various techniques such as simple one-step precipitation with methanol or acetonitrile, single and multi-stage liquid–liquid extraction with organic solvents were investigated. In addition, acidification and basification of serum were studied for enhancement of recovery. Considering the physicochemical properties of drug such as ionization coefficient, partition coefficient, and solubility in aqueous and organic solvents, various extraction solvents such as *n*-hexane, diethyl ether, chloroform, methylene chloride, and ethyl acetate were studied individually and in combinations. Finally, injection volume and wavelength of detection were optimized for better sensitivity and selectivity.

Method validation

The developed liquid chromatographic method was validated with respect to various validation parameters viz. selectivity, linearity, range, recovery, accuracy, precision, sensitivity, and drug stability in biological matrix. As part of validation, intra- and inter-batch variability were studied by repeating the analysis on three different occasions. Method was also applied for the determination of IM in real world serum (test) samples in order to study in vivo pharmacokinetics in rats.

Selectivity

Selectivity of the method was studied by investigating the interference from various endogenous proteins and other impurities present in the bio-matrix. Blank rat serum samples collected from six different rats were processed independently and analyzed by the proposed method. Obtained chromatograms of blank serum samples were compared against analytical and calibration standards for investigating interference in determination.

Linearity and range

Linearity and range of the method was assessed by three separate series of seven serum standards (25–1600 ng/mL) prepared and analyzed on three consecutive days. Average peak area at each level was plotted against concentration, and the curves were subjected to linear regression analysis by the least square method. Calibration equation was used to calculate the corresponding predicted concentrations. One-way analysis of variance (ANOVA) was performed on each replicate response obtained at seven concentration levels. Analytical range of the proposed method was established by analysis of residuals, and a test of the intercept was carried out using t-statistic (25).

Recovery studies

Recovery studies were conducted at four QC levels using LLOQ (25 ng/mL), LQC (100 ng/mL), MQC (400 ng/mL), and HQC (1600 ng/mL) standards prepared in five replicates on three consecutive days. All QC standards were processed as described in the "Sample preparation" section. Absolute recovery at each QC level was calculated by comparing the peak area obtained from QC and analytical standard.

Accuracy and precision

Accuracy and precision of the method was determined by analyzing QC standards prepared at LLOQ (25 ng/mL), LQC (100 ng/mL), MQC (400 ng/mL), and HQC (1600 ng/mL) levels. Each QC standard was processed and analyzed in five replicates, and analysis was repeated on three different occasions to study intra- and inter-batch accuracy and precision. Concentration of IM in QC standards was calculated from the calibration equation. Accuracy was expressed as %Bias, which was calculated using Equation 1:

$$\%\text{Bias} = 100 \times \frac{(\text{observed conc.} - \text{nominal conc.})}{\text{nominal conc.}} \qquad \text{Eq. 1}$$

Precision was determined as intra and inter-batch variations, and it was expressed as percent relative standard deviation (%RSD).

Sensitivity

Sensitivity of the method is defined as the lowest concentration of IM, which can be estimated with acceptable accuracy and precision (%RSD < 20), and it was expressed as a LLOQ. The QC standards were prepared at LLOQ concentration (25 ng/mL) in pentaplates and analyzed by the proposed method on three different occasions. Concentrations of IM in QC standards were calculated from calibration equation, and parameters such as mean calculated concentration, %Bias, and %RSD were determined.

Stability studies

In order to investigate the integrity of drug under storage and different operational conditions of the proposed method, short-term, long-term, dry-residue, and freeze-thaw stability studies were carried out at four QC levels in triplicate.

For short-term stability studies, prepared QC standards were kept at room temperature and each set of QC standards was analyzed at 0, 1, 3, 6, 12, and 24 h of post-spiking. Long-term stability of IM in rat serum was investigated over a period of 90 days. Prepared QC standards were stored at –20°C for 0, 7, 15, 30, 60, and 90 days, and they were processed and analyzed. Post extraction stability of IM in dry residue was investigated for 15 days. All QC standards were prepared and processed (without reconstitution) for drug extraction immediately after spiking. Processed standards (dry residue) were stored at –20°C, and one set of QC standards (n = 3) was analyzed on day 0, 1, 3, 5, 10, and 15.

Freeze-thaw stability studies were conducted to investigate the integrity of drug after exposing it to alternate freezing (at least 24 h) and thawing (at least 2 h) cycles. For this purpose, prepared QC standards were stored in sealed glass tubes at –20°C. Upon completion of required freeze-thaw cycles, the respective set of QC standards were processed and analyzed in a similar manner as described earlier.

The stability results were calculated from fresh serum standards, and it was expressed as accuracy in terms of %Bias.

Over-curve dilution integrity

To study the over-curve dilution integrity, three dilution integrity (DI) standards were prepared in serum at higher (over-curve) concentrations. Before extraction, these standards were diluted in rat serum to bring the concentrations within the calibration range. Five series of DI standards were prepared in rat serum at 5, 10, and 15 µg/mL concentrations, and they were diluted 5, 10, and 15 times, respectively. The DI standards were vortex mixed for 5 min and processed as described earlier to determine IM content.

In vivo pharmacokinetics of IM

Selected rats were randomly divided into three groups and were fasted overnight (12–15 h) before start of the study with water ad libitum. On the day of study, freshly prepared aqueous solution of IM was analyzed for the drug assay by the HPLC method. Studies were performed in triplicate as per standard experimental design described elsewhere (30). Briefly, each animal received a single oral dose of IM solution equivalent to 50 mg/kg of body weight, and blood samples were collected between 0 and 48 h after dosing. All dosing and sampling volumes were in accordance with the standard guidelines and were within the ethical limits (31). At each time point, a single blood sample was collected from three separate animals (n = 3) by the cardiac puncture technique. From each animal, approximately 0.5 mL of blood sample was withdrawn under general anesthesia, and it was transferred to a fresh centrifuge tube. Blood was allowed to clot, and serum was separated by centrifugation (3000 rpm, 15 min, 4°C). Separated serum samples were transferred to labeled cryovials. Samples were sealed and stored at –80°C until analysis. All samples were processed and analyzed within seven days of sample collection. For pharmacokinetic and statistical analysis, serum concentration versus time data were plotted and analyzed by non-compartmental analysis method using WinNonlin (Pharsight, Mountain View, CA) software.

Results and Discussion

Method development

In order to develop a selective and sensitive bioanalytical method, a primary objective was to obtain high height-to-area ratio with better peak symmetry. In general, a sharper peak generates a higher signal-to-noise ratio, resulting in improved sensitivity, and lower peak-width provides better resolution from undesirable components. Considering the hydrophilic and highly basic nature of the drug, the analytical method with proper retention and separation from endogenous impurities were major concerns during the method development. In preliminary studies, peak properties and response function were optimized by changing type of organic modifiers, organic to aqueous phase ratio, buffer type, buffer strength, and pH (32). The use of ion pairing agents showed improved peak symmetry (Tf ≈ 1.35 to 1.65) over simple buffer systems (Tf > 1.85). Amongst the studied ion pairing agents, triethylanime showed advantages of peak symmetry and resolution from the metabolite. The ion-paring agents like triethylamine are known to suppress the silanophilic interactions to increase the retention of polar compounds and reduce the peak tailing (33,34). However, adjusting aqueous phase pH to acidic side resulted in peak tailing (Tf ≈ 1.65, pH 4.5), making it unsuitable for analysis due to reduced resolution between the metabolite and the drug. Interestingly, at alkaline pH of aqueous phase, IM demonstrated highest resolution with good peak symmetry.

A doubled endcapped reversed phase column with bidentate silane (Zorbax Extend-C_{18}, Agilent) was selected for enhanced stability, which is reported to protect silica from dissolution at extreme alkaline conditions (35–37). Mobile phase was optimized to methanol and aqueous triethylamine (pH 10.5; 1%, v/v) (60:40, v/v), which was found to provide adequate drug retention (R_t ≈ 8.0 ± 0.25 min) with better chromatographic properties such as tailing factor 1.14 ± 0.005, retention factor 2.65 ± 0.002, and number of plates 3975 ± 52.31. In addition, this system has shown high sensitivity and injection repeatability (%RSD ≤ 0.77).

The purification and characterization (H^1-NMR) of *N*-demethylated derivative of IM further confirmed the selective *N*-demethylation of IM (yield *ca* 48%). The H1-NMR spectrum indicated the absence of singlet at δ 2.27 ppm corresponding to –CH3 proton, indicating the formation of *N*-desmethyl derivative of IM. Pelander et al. have reported that the nor-metabolites synthesized obtained from this selective procedure can directly be used as qualitative standards for analytical purpose without extensive purification (38). The purified product was used to prepare standards of the *N*-desmethyl IM, which showed retention time of 5.67 ± 0.29 min, indicating clear peak resolution from the drug in optimized mobile phase.

A simple one-step precipitation with methanol or acetonitrile led to a higher protein load and inefficient sample clean up. It was observed that even a double extraction with *n*-hexane, diethyl ether, ethyl acetate, and their combination showed poor recovery of drug. Further, basification of serum showed only a marginal improvement in drug recovery; however, there was significant interference from matrix components. Chloroform and methylene chloride showed high extraction efficiency with reproducible and consistent recovery (>90%). However, extraction with chloroform showed a little interference due to endogenous component of serum in near vicinity of drug retention time. Single extraction with 1.5 mL methylene chloride resulted in high extraction efficiency. Optimized injection volume (50 µL) led to sensitive method with interference-free determination at 285 nm. Thus, optimized extraction protocol showed consistent and high recovery at all concentration levels without any interference from endogenous components and impurities.

Method validation

Selectivity

Chromatograms of six blank samples revealed that there was no peak present in the elution window of IM. A lack of response in blank biological matrix originating from endogenous components confirmed the selectivity of the method. Further, test samples obtained from oral pharmacokinetic studies proved that there was no interference from metabolites or degradation products in the near vicinity of drug peak. Comparison of chromatograms of blank, spiked, and test serum samples indicate

selectivity of the method (Figure 2). Thus, the proposed method was found to be selective in the determination of IM from spiked as well as test samples.

Linearity and range

The linear regression analysis indicated linear relationship between the average peak area and concentration over the range 25–1600 ng/mL with high regression coefficient ($R^2 \geq 0.9995$). The best-fit linear equation was average peak area (µV s) = 263.98 × concentration (ng/mL) + 44.51 with low standard error of estimate 3.57. Mean responses obtained for individual concentrations are indicated in Table I. Further, 95% confidence interval and standard error (SE) of the slope was found to be 258.26 to 269.72 (SE ± 2.23). The standard deviation of peak area was significantly low across the analytical range, and %RSD was less than 10.5. In homoscedasticity test, analysis of residuals indicated that the residuals are normally distributed around the mean observed response with uniform variance across all concentrations, suggesting homoscedastic nature of the data. In addition, selected linear model with univariant regression showed acceptable %Bias, indicating the goodness of fit which was further supported by low values of standard error of estimate and mean sum of squared residuals. Test of the intercept revealed that intercept was not significantly different from zero as $t_{df,5}$ value was 0.0286 (tabulated 2.57) at 0.05 significance level. Finally, one-way ANOVA was performed for peak area obtained at individual concentration levels, and F-value (calculated $F_{8,54} = 7.21 \times 10^{-3}$) was found to be less than theoretical F-value (critical $F_{8,54} = 2.115$) at 0.05 significance level.

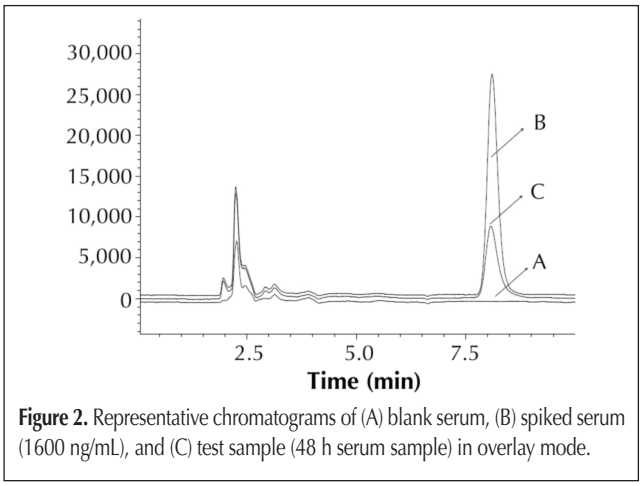

Figure 2. Representative chromatograms of (A) blank serum, (B) spiked serum (1600 ng/mL), and (C) test sample (48 h serum sample) in overlay mode.

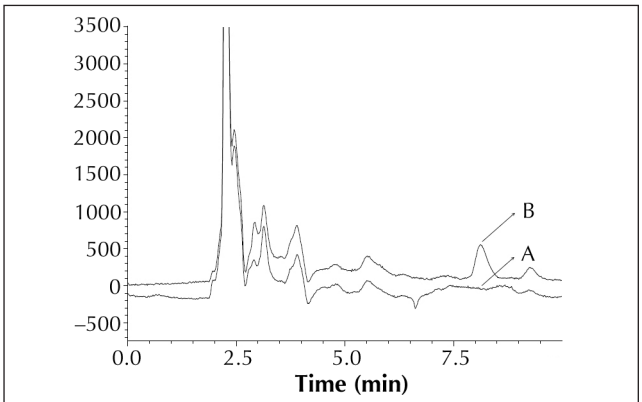

Figure 3. Representative chromatograms of (A) blank and (B) spiked serum standards at LLOQ.

Recovery studies

The proposed method showed high and consistent recovery of IM from rat serum at all concentrations studied and use of internal standard was not necessary. Mean absolute recovery values were ranged from 90.32 to 95.86% over the calibration range (Table II). In addition to this, %RSD was below 3.72 at all QC levels except at LLOQ (≤ 7.71). Simple liquid–liquid extraction procedure with methylene chloride was found to be efficient and reproducible. The high and consistent recovery results indicated that the method was sensitive and precise for quantitative analysis of IM in rat serum.

Accuracy and precision

The obtained results confirmed the accuracy of the proposed method as the percent deviation was significantly less. At all QC levels, intra-batch %Bias ranged from –2.34 to 3.42, and inter-batch %Bias ranged from –2.17 to 3.45. Results obtained for %Bias and %RSD at each QC level is presented in Table III. The method was found to be precise with %RSD not exceeding 8.53 and 8.01 at LLOQ for intra- and inter-batch, respectively.

In addition, testing of the intercept along with homoscedasticity test suggested that there was no significant interference from matrix components in analysis of IM. Consistent and high recovery observed at four QC levels was in agreement with the previous findings. Results of accuracy and precision study were

Table I. Calibration Data for Serum Standards of IM

Conc. (ng/mL)	Mean peak area* (µVs)	Predicted conc. (ng/mL)	%RSD	%Bias
25.0	6506.14 ± 681.03	24.48 ± 2.58	10.45	–2.09
50.0	12670.52 ± 885.36	47.83 ± 3.35	7.01	–4.34
100.0	26518.98 ± 933.17	100.29 ± 3.53	3.52	0.29
200.0	53788.21 ± 2498.48	203.58 ± 9.46	4.65	1.79
400.0	109138.74 ± 3919.95	413.25 ± 14.85	3.59	3.31
800.0	205575.40 ± 6995.69	778.56 ± 26.50	3.40	–2.68
1600.0	424281.28 ± 19418.47	1607.02 ± 73.56	4.58	0.44

* Each value is mean of nine (n = 3 on three occasions) independent determinations.

Table II. Mean Absolute Recovery of Imatinib From Quality Control Standards

Quality control standard*	Mean absolute recovery† (%)	%RSD
LLOQ (25 ng/mL)	90.32 ± 6.97	7.71
LQC (100 ng/mL)	92.20 ± 3.43	3.72
MQC (400 ng/mL)	95.86 ± 3.34	3.48
HQC (1600 ng/mL)	93.75 ± 3.36	3.58

* LLOQ = lower limit of quantitation, LQC = low quality control, MQC = medium quality control, and HQC = high quality control.

† Each value is mean of fifteen independent determinations (n = 5 on three occasions); Recovery = [(Peak area of serum standard / peak area of analytical standard) × 100].

in acceptable limits and indicated that the method was accurate and precise (Table III).

Sensitivity

Serum standards prepared at LLOQ showed quantifiable amount of IM when analyzed in pentaplates on three different days (Figure 3). Further, it confirmed that the method was precise and accurate at LLOQ with %RSD less than 8.53 and %Bias not exceeding –2.34. The method was found to be sensitive with a high signal-to-noise ratio at 285 nm detection wavelength. It can be suggested that the present method is suitable for various pharmacokinetic investigations in rats, which demand high sensitivity.

Table III. Intra- and Inter-batch Accuracy and Precision of the Proposed Method

Quality control standard	Accuracy (%Bias)		Precision (%RSD)	
	Intra-batch*	Inter-batch†	Intra-batch*	Inter-batch†
LLOQ (25 ng/mL)	–2.34	–2.17	8.53	8.01
LQC (100 ng/mL)	0.42	–0.19	3.55	3.76
MQC (400 ng/mL)	3.42	3.45	4.19	3.49
HQC (1600 ng/mL)	0.87	1.43	3.29	3.59

* $n = 5$. † $n = 15$.

Table IV. Pharmacokinetic Parameters for Imatinib Mesylate in Rats

Pharmacokinetic parameters	Value
Area under the curve (AUC)	200 µg h/mL
Maximum drug concentration in serum (C_{max})	14.65 µg/mL
Time to reach maximum drug concentration (T_{max})	3 h
Elimination rate constant (k)	0.057 /h
Half-life ($T_{1/2}$)	12.3 h
Volume of distribution / F (V_d/F)	4.12 L/kg
Clearance / F (Cl/F)	0.24 L/h/kg
Mean residence time (MRT)	11.2 h

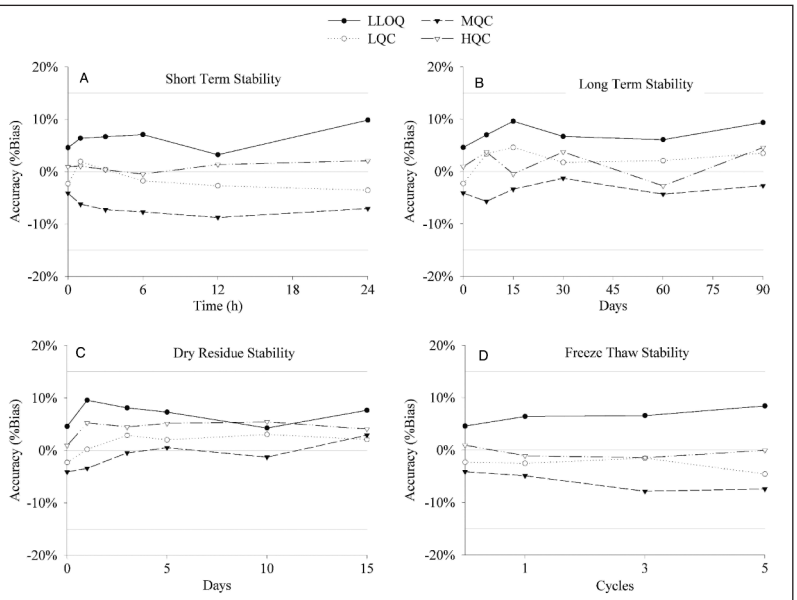

Figure 4. Stability study of Imatinib mesylate in rat serum (A) short-term stability, (B) long-term stability, (C) dry-residue stability, and (D) freeze-thaw stability.

Stability studies

Results obtained for short-term stability studies at all QC levels demonstrate that IM was stable in rat serum under bench top conditions. IM did not show significant change (%RSD < 5) in response up to 24 h at room temperature when compared with the response obtained from fresh standards (Figure 4A). Similarly, in long-term stability study, IM was found to be stable in rat serum at –20°C for all QC levels as there was no significant difference between the response of standards at zero time and during 90 days. The maximum deviation observed was within acceptable limits at all QC levels (Figure 4B). In dry-residue stability study, obtained results have indicated that IM was stable under post-extraction storage conditions up to 15 days at –20°C (Figure 4C).

There was no significant degradation detected in QC standards prepared at all four QC levels up to five freeze-thaw cycles. Results are expressed as accuracy in terms of %Bias, which was within ± 10% at LLOQ and ± 8% at other concentration levels. And the deviation observed during five freeze-thaw cycles was within acceptable limits (Figure 4D). Thus, the drug was found to be stable for five freeze-thaw cycles, making it suitable for sub-zero storage conditions. Percent deviation calculated for all stability samples were well within the acceptance range of ± 20% at LLOQ and ± 15% at other concentration levels, demonstrating that IM was stable under processing and storage conditions as stated in the method.

Over-curve dilution integrity

The dilution integrity of the method was found to be acceptable with accuracy (%Bias) of –2.34, 3.18, and 0.61 for respective 5, 10, and 15 µg/mL concentration levels. Precision for 5, 10, and 15 times dilution was within acceptable limits with %RSD 4.55, 4.62, and 3.61, respectively. Thus, the results demonstrated that the method was suitable for over-curve dilution up to 15 times in rat serum.

In vivo pharmacokinetics of IM

The mean serum concentration-time profile is shown in Figure 5. IM has shown rapid absorption with maximum drug concentration in serum (C_{max}) of 14.65 µg/mL reaching at around (T_{max}) 3.0 h. The summary of pharmacokinetic parameters obtained by non-compartmental analysis is presented in Table IV. The drug has shown slow elimination from the systemic circulation with detectable concentration levels up to 48 h of post-dosing. This was further indicated by a high mean residence time (MRT = 11.2 h), which represents the time for 63.2% of the administered dose to be eliminated from the system, according to statistical moment theory. Volume of distribution (V_d/F) was found to be 4.12 L/kg, indicating rapid and extensive distribution of the drug in tissues, which was in accordance with its calculated partition coefficient value (LogP = 2.48 ± 0.73, ACD/Labs). Moreover, it has shown elimination half-

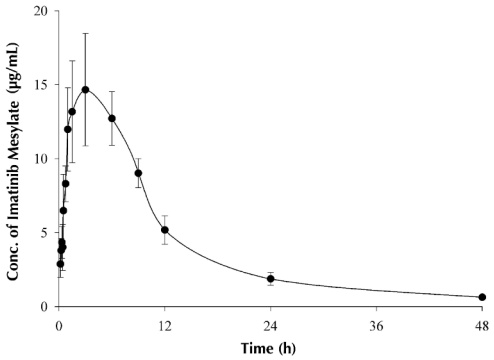

Figure 5. Mean serum concentration-time profile of Imatinib mesylate after single oral dose in male Wistar rats.

life ($T_{1/2}$) of 12.3 h with clearance (Cl/F) of 0.24 L/h/kg. In literature, there was no report available that describes the in vivo pharmacokinetic parameters of IM in detail. The validated method was successfully applied to study pharmacokinetic disposition of IM in rats.

Conclusions

A new, simple, and sensitive reversed-phase HPLC method has been successfully developed and validated for determination of IM in rat serum. Simple liquid–liquid extraction technique provided consistent and high recovery with selective determination of IM from rat serum. The method was found to be accurate, precise, and reproducible with good stability of IM under various processing and storage conditions. In addition, the method was successfully employed for in vivo pharmacokinetic investigations of pure drug.

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