

IMATINIB QUANTIFICATION IN RAT PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRA VIOLET DETECTION - AN APPLICATION TO PRECLINICAL PHARMACOKINETIC STUDY.

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Summary

A simple and robust method for quantification of imatinib in rat plasma has been established using high performance liquid chromatography with UV detection. Mizolastine was used as an internal standard (IS). imatinib and internal standard in plasma sample were extracted using simple protein precipitation technique. The samples were injected into a C₁₈ reverse-phase phenyl column and mobile phase used was acetonitrile – phosphate buffer (pH3.2; 25.0 mM) (24:76%, v/v) at a flow rate of 1.0 mL min⁻¹ using ultraviolet detector. imatinib and internal standard were detected without any interference from rat plasma. Detection of imatinib in rat plasma by the high performance liquid chromatography method was accurate and precise with a quantitation limit of 20.0 ng mL⁻¹. The proposed method was validated with linearity range of 20.0 – 10000.0 ng mL⁻¹. Reproducibility, recovery and stability of the method were evaluated. This method has been successfully applied to pharmacokinetic evaluation of imatinib liposome formulation.

Keywords: Imatinib, high performance liquid chromatography, rat plasma, pharmacokinetic.

Introduction

Imatinib(IMA) mesylate is a new chemotherapy drug indicated for the treatment of patients with chronic myeloid leukemia (CML) in blast crisis accelerated phase, or in chronic phase after failure of interferon- α therapy. It is the first of its kind developed to fight cancer by turning off an enzyme that causes cells to become cancerous and multiply. Food and Drug Administration (FDA) has approved imatinib mesylate for treatment of patients at any of the three stages of CML. Imatinib mesylate designated chemically as 4-[(4-Methyl-1-piperazinyl) methyl]-n-[4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl] amino]-phenyl] benzamide methane sulfonate.

IMA is a protein kinase inhibitor which potently inhibits the abelson tyrosine kinase in *in-vitro* and *in-vivo* studies. Oral imatinib is indicated as first-line therapy in newly diagnosed patients with Philadelphia positive chronic myeloid leukemia in blast crisis¹⁻⁵.

Several high performance liquid chromatography (HPLC) methods for estimation of IMA in human and monkey plasma have been reported⁶⁻¹⁰. These methods involve the extraction of IMA by solid phase extraction and protein precipitation techniques. Different types of analytical techniques such as liquid chromatography coupled to tandem mass spectrometry (LC-MS)¹¹⁻¹⁸ and capillary electrophoresis^{19,20} for estimation of IMA and its metabolite in biological fluids have been reported. These methods are sensitive and have low quantitation limits. However, these methods require sophisticated instrumentation and are expensive.

The preclinical studies are the preliminary studies for novel drug delivery systems like liposomes, microspheres and nanoparticles. Although several published methods are available for clinical pharmacokinetic evaluation, a small number of reports are available for IMA in animal models. A sensitive, simple and reliable method with wide calibration range is a prerequisite for pharmacokinetic and toxicokinetic evaluation of IMA novel drug delivery systems. This work was aimed at developing a sensitive HPLC method for determination of IMA in rat plasma. The advantages of this method are less sample volume, single step extraction procedure and shorter run time with wide calibration range. Protein precipitation technique was selected because of its advantages such as shorter processing time, lesser organic solvent requirement and efficient plasma sample clean up. The method was validated as per the US-FDA guidelines²¹. This method was used for pharmacokinetic evaluation of IMA liposomes in rat.

Materials and Methods

Materials and Reagents:

Imatinib mesylate was procured from Varda Biotech Lab (Mumbai, Maharashtra, India) and Mizolastine was obtained from Dr. Reddy's laboratory (Hyderabad, Andhra Pradesh, India). Acetonitrile (HPLC grade) and methanol (HPLC grade) were purchased from Qualigen Ltd. (Mumbai, Maharashtra, India). Sodium dihydrogen phosphate and orthophosphoric acid were purchased from Merck (Mumbai, Maharashtra, India). Disodium ethylenediamine tetra-acetic acid (EDTA) vacutainers (BD Franklin Lakes, NJ, USA) were used for collecting blood samples from animals. Milli-Q water purification system supplied by Millipore (Bangalore, Karnataka, India) was used for the preparation of the aqueous mobile phase. Drug free rat plasma samples were obtained from central animal house, MAHE, Manipal and stored at -20°C prior to use.

Equipment:

HPLC analysis was performed using a gradient high pressure liquid chromatograph from Shimadzu (Nakagyo-Ku, Kyoto, Japan) HPLC Class VP series with two LC-10ATVP pumps, SPD-10AVP variable wavelength programmable UV-vis detector, SCL-10AVP system controller and Shimadzu Class VP version 6.12 SP2 data station system. The HPLC column used was Hypersil C₁₈ Phenyl (250×4.6 mm, 5 μ particle size) purchased from Thermo electron corporation (Runcom, UK).

Chromatographic conditions:

The analytes were chromatographically separated using reverse-phase high-performance liquid chromatography (HPLC) with isocratic elution. The mobile phase consisted of acetonitrile – phosphate buffer (pH 3.2; 25.0 mM) (24:76%, v/v) at a flow rate of 1.0 mL min⁻¹. The separation was carried out at room temperature on Hypersil C₁₈ Phenyl (250×4.6 mm, 5 μ particle size) analytical column and eluent was monitored at 268 nm.

Preparation of calibration standard and quality control (QC) samples:

The Main stock solution of IMA and IS were prepared in methanol at free base concentration of 1000.0 $\mu\text{g mL}^{-1}$. Intermediate stock solution was prepared from main stock solutions by dilution with water: methanol (50:50, v/v). This intermediate stock solution was used to prepare the spiking stock solutions for calibration curve and quality control samples. Blank rat plasma was screened prior to spiking to ensure it was free of endogenous interference at retention times of IMA and IS. A nine point standard curve and quality control samples of IMA was prepared by spiking the blank plasma with 5 % v/v of IMA. The calibration curve ranged from 20.0 to 10000.0 ng mL^{-1} and quality control samples were prepared at three concentration levels of 60.0, 3500.0 and 7500.0 ng mL^{-1} .

Sample preparation:

A 50.0 μL aliquot of plasma containing IMA was pipetted into micro centrifuge tube to which 10.0 μL of IS working solution (5.0 $\mu\text{g mL}^{-1}$ Mizolastine) was added and vortexed to mix. 500.0 μL of methanol : acetonitrile (70:30%, v/v) was added and vortexed for two minutes. The mixture was centrifuged at 12000 rpm at 4°C. The supernatant was separated, transferred to polypropylene auto sampler vials and 20.0 μL was injected to HPLC system.

Validation:

This method was validated for selectivity, sensitivity, recovery, linearity, precision and accuracy, stability and dilution integrity.

Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other expected components in the sample. This test was performed by analyzing the blank plasma samples from different sources for interference at the retention time of IMA and IS.

Sensitivity was determined by analyzing blank rat plasma (n=5) spiked with the analyte at lowest level of the calibration curve, 20.0 ng mL^{-1} in replicates.

Linearity of the method was evaluated over the concentration range of 20.0-10000.0 ng mL^{-1} . Calibration standards were freshly prepared on each day of validation. Comparison of the slope, intercept and correlation coefficient was made for three days validation process.

The intra and inter-day accuracy and precision of the assay were tested by analysis of five QC samples at each level and at least five precision and accuracy batches on three separate days. Accuracy and precision of the quality control samples were calculated against the calibration curve. The recovery of the IMA and IS was calculated by comparing the peak area response of extracted analytes with unextracted analytes that represent 100% recovery. Recovery was carried out at three concentrations (low, medium and high quality control sample).

As a part of method validation, stability was evaluated. The stock solution stability was evaluated at room temperature and at 2-8°C by comparing with freshly prepared stock solution. All stability experiments were performed with freshly prepared calibration standards and quality control samples. The in-injector stability was evaluated after keeping processed samples in auto sampler at 10 °C for 24 h. The bench top stability was evaluated for 6 h at room temperature. The freeze-thaw stability was performed by comparing the stability samples that had been frozen and thawed thrice with freshly prepared calibration standards and quality control samples.

The long-term stability was performed by analyzing low and high quality control samples stored at -20°C for 60 days with freshly prepared calibration standards and quality control samples. All stability evaluations were based on back-calculated concentrations.

Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. 2xULOQ concentration was prepared and diluted to 2 and 4 fold with blank plasma. These samples were processed and analyzed. The results were calculated after applying appropriate dilution factor.

Pharmacokinetic evaluation of imatinib liposomes:

Sixty young, male Albino rats (divided into two groups I and II) (body weight 250–300 g) were obtained from the central animal house, MAHE, Manipal, Karnataka, India. The experimental protocol for the animal studies was approved by the Institutional Animal Ethical Committee (No. IAEC/KMC/05/2003-2004). Animals were maintained under controlled temperature (25 ± 2 °C) and humidity ($50 \pm 5\%$ RH) condition in polypropylene cages filled with sterile paddy husk. Animals were fasted 12 h before dosing.

The present method was applied to evaluate the pharmacokinetic behavior of IMA liposome in rats. The effect of liposome on the plasma levels of IMA following intravenous administration was determined. Group I and II received 40 mg/kg body weight of IMA standard drug and IMA liposome formulation respectively. Plasma samples were periodically collected upto 48 h at specified time intervals of 0.083, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0, 24.0, 48.0 h.

Results**Selectivity:**

Chromatographic selectivity of the method was demonstrated by the absence of interfering peaks at the retention times of IMA and IS. Six different lots of EDTA blank plasma were screened. No endogenous interference was observed at the retention time of IMA and IS. Representative chromatogram of extracted blank plasma, extracted plasma samples containing 20.0 ng mL⁻¹ of IMA (LLOQ) and one unknown animal study sample containing IMA is presented in figure 1, 2 and 3 respectively.

Fig. 1. Representative chromatogram of extracted blank plasma

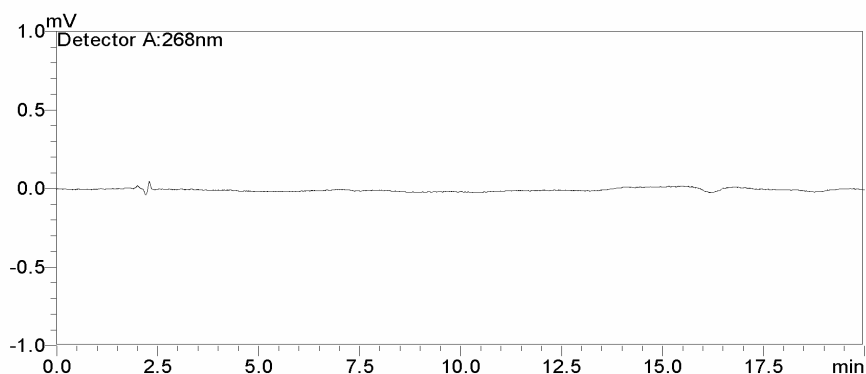


Fig. 2. Representative chromatogram of extracted plasma containing 20.0 (ng/mL⁻¹) of imatinib.

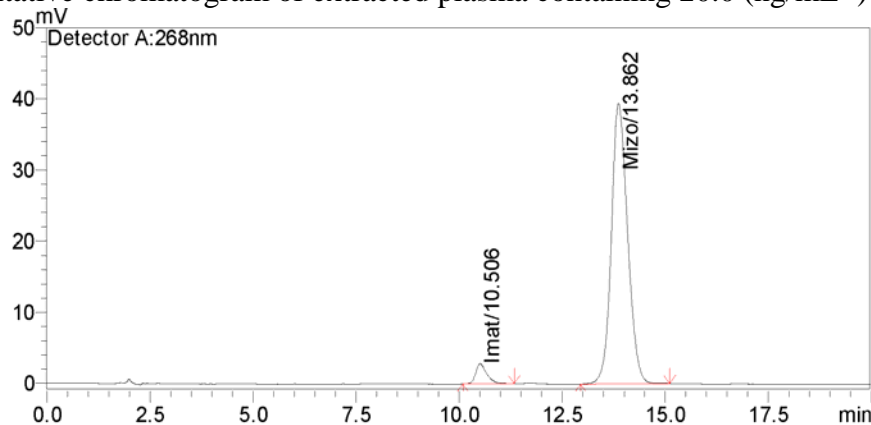
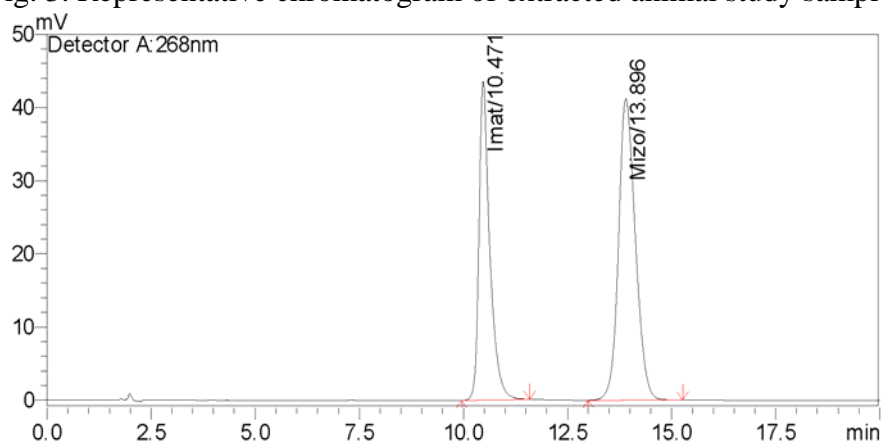


Fig. 3. Representative chromatogram of extracted animal study sample

**Sensitivity (Lower limit of quantification):**

The sensitivity experiment was carried out at LLOQ level. The mean percentage deviation from the nominal concentration was $\leq 4.38\%$ and percentage coefficient of variation was within 5.36% and interference of the plasma endogenous constituents were found to be insignificant.

Recovery:

Peak areas from unextracted analyte with those of extracted analyte determined recovery. The mean absolute recovery of IMA at LQC, MQC and HQC was 80.96% and variability across the QC level was $\leq 5.21\%$. The recovery of internal standard was found to be 90.65% .

Linearity:

The calibration curve data of IMA are listed in table 1. The calibration curves were linear over the range of $20.0-10000.0\text{ ng mL}^{-1}$. The correlation coefficient ($n=5$) was ≥ 0.9986 with acceptable accuracy and precision.

Table No. 1 - Summary of calibration curve parameters for imatinib

Calibration curve	Slope	Intercept	r^2
1	0.10702	0.00360	0.9989
2	0.11671	0.00191	0.9986
3	0.11789	-0.00077	0.9992
4	0.12165	0.00173	0.9996
5	0.11753	-0.00582	0.9989

Precision and accuracy:

Both the intra-day and inter-day accuracy and precision of the method were determined by analysis of the blank rat plasma spiked with IMA at LLOQ, LQC, MQC and HQC levels and calculated against the calibration curve. The accuracy and precision of the method were described as percentage bias of theoretical versus measured concentrations and the percentage of the relative standard deviation, respectively. The intra-day deviation from the nominal concentration was between -3.85 to 3.49% and the intra-day precision was $\leq 6.54\%$ across QC levels. The inter-day deviation from the nominal concentration was between -4.38 to 4.43% and the inter-day precision was $\leq 5.36\%$ across QC level. The results of accuracy and precision are enumerated in Table 2.

Table No. 2 - Intra-day and inter-day accuracy and precision of imatinib in rat plasma

Spiked conc. (ng/mL ⁻¹)	Mean calculated conc. (ng/mL ⁻¹)	%RSD ^a	% Bias
Intra-day (n=5)			
20.000	20.698	4.48	3.49
60.000	58.124	3.25	-3.13
3500.000	3365.254	6.54	-3.85
7500.000	7689.982	2.89	2.53
Inter-day (n=5)			
20.000	19.124	5.36	-4.38
60.000	62.654	1.23	4.42
3500.000	3389.398	0.98	-3.16
7500.000	7256.689	3.09	-3.24

^aRelative standard deviation

Stability:

Stock solution stability experiment was performed at 10000.0 ng mL⁻¹. After storage for 20 days at 2-8°C and at room temperature for 12 h, more than 97.8 % of IMA remained unchanged, based on their peak areas in comparison with freshly prepared solution of IMA (10000.0 ng mL⁻¹). This suggests that the IMA stock solution was stable for at least 20 days when stored at 2-8°C and 12 h at room temperature.

Bench top stability of IMA in plasma and in-injector (4°C) stability after processing of IMA was performed at LQC and HQC levels. IMA in plasma was stable for at least six hours at room temperature and 24 h at auto sampler maintained at 10°C with mean percentage change of ≤ 5.02 % and ≤ 3.22 % respectively. It was confirmed that repeated freeze and thawing (three cycles) of plasma samples spiked with IMA at LQC and HQC level did not affect the stability of IMA with mean percentage change of ≤ 2.55 %. Long-term stability of the IMA in plasma at -20°C was also performed after 60 days at LQC and HQC level with mean percentage change of ≤ 5.88 %. The results of the stability studies are presented in Table 3. The above results indicate that IMA is stable enough to be analyzed using this assay method.

Table No.3 - Stability sample results of imatinib (n=5)

Stability	Specified conc. (ng/mL ⁻¹)	Mean comparison sample conc. (ng/mL ⁻¹)	Mean stability sample conc. (ng/mL ⁻¹)	Mean % change
In-injector ^a	60.000	59.023	57.124	-3.22
	3500.000	3480.679	3580.980	2.88
Bench top ^b	60.000	59.023	61.986	5.02
	3500.000	3480.679	3386.840	-2.70
Freeze and thaw ^c	60.000	59.023	57.519	-2.55
	3500.000	3480.679	3412.325	-1.96
Long-term ^d	60.000	62.348	58.683	-5.88
	3500.000	3598.750	3406.780	-5.33

^a After 24 h in autosampler at 10°C.

^b After 6 h at room temperature.

^c After 3 freeze and thaw cycles at -20°C.

^d At -20°C for 60 days

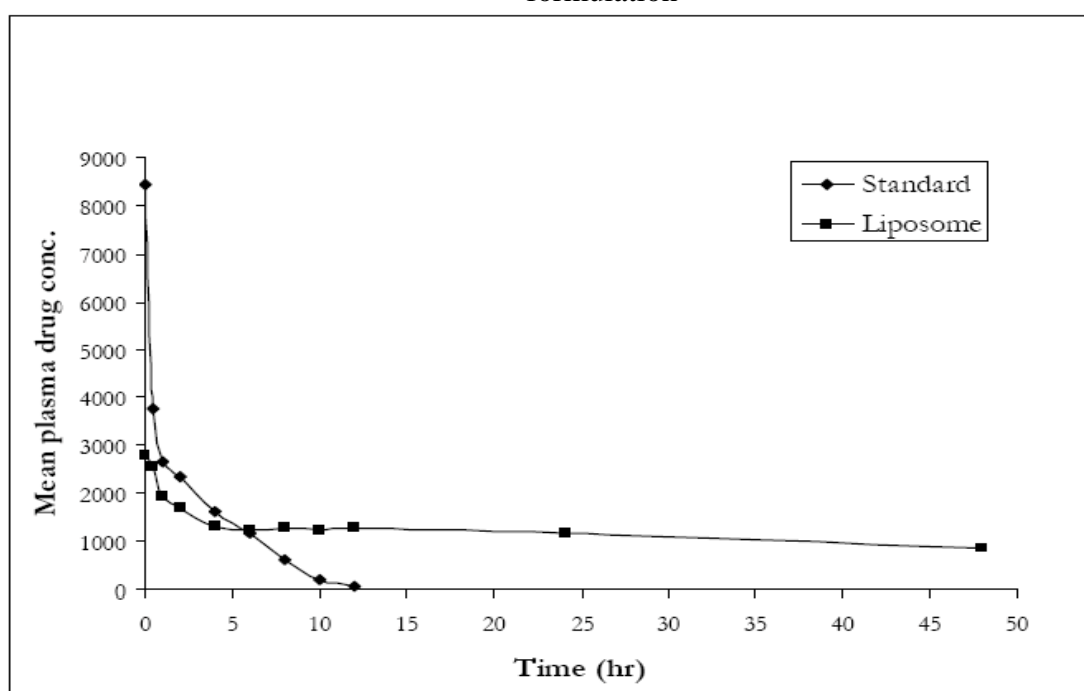
Dilution integrity:

The upper concentration limit of IMA can be extended to 20000.0 ng mL⁻¹ with acceptable precision and accuracy of 15 % by 2-fold or 4-fold dilution with blank rat plasma. The summarized results in table 5 demonstrate a precision of $\leq 3.87\%$ and the mean percentage deviation from the nominal concentration was $\leq 1.58\%$.

Pharmacokinetic evaluation:

The mean maximum plasma concentration obtained for IMA standard and liposome formulation was 8450.32 and 2820.32 ng mL⁻¹ respectively. The AUC measured from 0 h to the last sampling point was higher than 90% of the value of AUC extrapolated from zero time to infinity, which indicates a suitability of the analytical method for pharmacokinetic studies. The mean IMA plasma concentrations versus time profiles are shown in Fig. 4.

Fig. 4. Mean plasma concentrations versus time curve for imatinib standard and liposome formulation

**Discussion**

The objective of this work was to develop and validate a simple and sensitive assay method for the quantification of IMA, suitable for pharmacokinetic evaluation in preclinical studies. To achieve the same, during method development, different options were evaluated to optimize chromatography and sample extraction procedure. A mobile phase containing phosphate buffer (pH 3.2; 25.0 mM) in combination with acetonitrile resulted in improved response and symmetric peaks with excellent resolution as compared to other pH conditions. Use of Hypersil phenyl C₁₈ (250 × 4.6 mm id, 5 μ) column resulted in maximum response with high theoretical plates. The optimized protein precipitation extraction technique enabled the elimination of laborious extraction steps of evaporation and reconstitution involved in generic solid phase and liquid-liquid extraction methods without compromising the sensitivity, which resulted in reduced processing and analysis time.

Conclusion

A selective, sensitive and simple HPLC method for the determination of IMA in rat plasma has been developed and validated, with a lower quantification limit of 20.0 ng mL⁻¹. Validation experiments have shown that the assay has good precision and accuracy over a wide range of 20.0 – 10000.0 ng mL⁻¹. This wide calibration range has an added advantage in pharmacokinetic evaluation of IMA by intravenous administration compared to reported method and no interference by the endogenous compounds was observed. This method was successfully applied for the pharmacokinetic evaluation of IMA liposomes in rat plasma.

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