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Short communication

Development and validation of a simple liquid chromatographic method with ultraviolet detection for the determination of imatinib in biological samples

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Abstract

The aim of this study was to develop a rapid and sensitive HPLC method with UV detection for the estimation of imatinib from the plasma of patients with chronic myeloid leukemia (CML). The robustness of the method was checked by conducting first dose pharmacokinetics on blood samples from four patients who had been administered Gleevec (100 mg) in an oral dose. Samples were prepared in a simple and single step by precipitating the plasma proteins with methanol and injecting 50 μl aliquot from supernatant was subjected for analysis. Assay was conducted using a C8 column (250 mm × 4.6 mm, 5 μm particle size) under isocratic elution with 0.02 M potassium dihydrogen phosphate–acetonitrile (7:3, v/v) at a flow rate of 1 ml/min and detected using photodiode array at 265 nm. Calibration plots in spiked plasma were linear in a concentration range of 0.05–25 μg/ml. The inter and intra-day variation of standard curve was <4% (R.S.D.). This method could be a simple and quick method for the estimation of imatinib from the patient's plasma.

Keywords: Imatinib

1. Introduction

Imatinib is a new anti-cancer agent, which acts by selectively inhibiting the Abl tyrosine kinase. The drug has been approved for oral administration in the treatment of Philadelphia chromosome (Bcr-Abl) positive chronic myeloid leukemia (CML) in blast crisis [1–7]. It has even been found to be effective in those cases of CML-chronic phase where there was no response to interferon-α therapy [8]. Structurally, imatinib is a phenylaminopyrimidine derivative (4-[(4-methyl-3-[[4-(3-pyridinyl)-2-pyrimidinyl] amino]-phenyl]-benzamide methanesulfonic acid salt). It is an optically inactive, creamy-white crystalline powder. The solubility of mesylate salt of imatinib in aqueous medium is pH dependent and is marked at pH 5.8. However, it is slightly soluble to insoluble in organic solvents [9].

In vitro studies have shown that imatinib is mainly metabolized by the enzyme CYP3A4 and in addition, it also competitively inhibits CYP2C9 and 2D6 [9,10]. CYP3A4 has been implicated not only in variable kinetics of many drugs but is also responsible for various drug interactions. In light of these observations, pharmacokinetic–pharmacodynamic correlation studies on CYP3A4 susceptible drugs like imatinib, gain importance. Therefore, the present study was undertaken to develop and validate a simple and rapid HPLC method with UV detection, which could be useful for therapeutic drug monitoring of imatinib in routine setting. The partition coefficient of imatinib mesylate was also evaluated to provide comprehensive data on the physical properties of this newly launched drug.

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2. Materials and methods

2.1. Chemicals

Pure compound of imatinib was the kind gift from Natco Pharmaceuticals (Hyderabad, India). All solvents used were of analytical grade and were purchased from J.T. Baker (Mexico, USA) and E. Merck Ltd. (Mumbai, India). Anhydrous potassium dihydrogen orthophosphate was obtained from Qualigens (Mumbai, India). Water used in the mobile phase was freshly prepared (18.2 MΩ, MilliQ-Academic, Millipore, Bedford, USA).

2.2. Equipment

A Waters solvent delivery pump (510, Waters, Milford, MA, USA) connected to an injector (Rheodyne 77251, California, USA) equipped with a 50 μl loop and Waters 996 photodiode array detector (Milford, USA) was used for all estimations. For the data acquisition and integration, Waters Millennium-32 software operated by Pentium II processor (350 MHz, Intel Inc., USA) was used.

2.3. Chromatography conditions

Imatinib was separated using Kromasil-RP-8 column (250 mm × 4.6 mm i.d.; particle size 5 μm, E. Merck, Worli, Mumbai) maintained at ambient temperature (25 ± 1 °C). The samples were eluted in a mobile phase of 0.02 M potassium dihydrogen phosphate–acetonitrile (7:3, v/v) at a flow rate of 1 ml/min. The mobile phase was filtered through a 0.22 μm filter (Millipore, Bedford, USA) and degassed under vacuum prior to use. The detector wavelength was set at 265 nm and peak purity was determined over 200–400 nm using the custom-made imatinib spectrum-matching library.

2.4. Determination of partition coefficient of imatinib by HPLC method

Partition coefficient was determined at 25 and 37 °C using the method described in OECD guidelines [11]. Briefly, an array of compounds (acetone: 0.791 mg/ml; benzyl alcohol: 1.045 mg/ml; phenol: 0.73 mg/ml; nitrobenzene: 1.204 mg/ml; benzene: 0.879 mg/ml; in methanol), having different log *P* values were mixed with imatinib and chromatographed under above-mentioned conditions. However, these samples were detected at 210 nm in order to have a common absorbance wavelength for all the compounds contained in the sample mixture. An aliquot of 20 μl from this mixture was separated over a run-time of 40 min. This experiment was conducted in quadruplicate at the column temperature of 25 ± 0.1 and 37 ± 0.1 °C. The log *K* values were plotted against log *P* values of known compounds. The line equation of this plot was used for the evaluation of the log *P* value of imatinib mesylate.

2.5. Preparation of standard imatinib mesylate

Stock solution of imatinib mesylate was prepared from accurately weighed (10.1 mg) pure powder dissolved in water (10 ml) and stored at −70 °C. Serial dilutions of the stock, in concentration range of 0.03–31 μg/ml were prepared in methanol: water (1:1, v/v), freshly on each experiment day. Standard curve, intra- and inter-day variation plots were obtained by injecting 20 μl of these dilutions.

2.6. Extraction of imatinib from plasma

Ninety microliters of drug-free plasma was spiked with 10 μl of standard imatinib. The spiked sample was vortexed for 30 s followed by addition of 200 μl of methanol and re-vortexing for 3 min. The sample was centrifuged at 3600 × *g* for 10 min and an aliquot of 50 μl from supernatant was injected into HPLC for quantification.

2.7. Validation parameters

2.7.1. Linearity

Ten microliters of various serial dilutions of imatinib mesylate was added to 90 μl of drug free plasma and vortexed for 2 min to obtain concentrations ranging from 0.025 to 25 μg/ml. For reproducibility, the calibration curves were plotted on each experiment day prior to injecting samples.

2.7.2. Recovery

For the calculation of percentage recovery, the above mention procedure was followed and 90 μl of deionized water was used instead of plasma.

2.7.3. Determination of LOD and LOQ

The limit of detection (LOD) was set four times above the base line noise (signal-to-noise >4). The limit of quantification (LOQ) was calculated using the equation LOQ = 10 (S.D./*S*), where S.D. is the standard deviation and *S* is the slope of the calibration curve.

2.8. First dose plasma levels of imatinib in cancer patients

The first dose pharmacokinetic study of imatinib was conducted by limited sampling on patients of CML undergoing treatment at Institute Rotary Cancer Hospital, All India Institute of Medical Sciences, New Delhi. The patients were orally administered the morning dose (100 mg) of imatinib along with the food and blood samples were withdrawn from antecubital vein in heparinated vial at various time points (0, 1, 2, 4, 6, and 24 h) (Table 1). The plasma was separated and stored at −70 °C and analyzed within a week.

2.9. Results

Under the given conditions imatinib eluted at a retention time of 8.3 min (Fig. 2). The peak was well resolved with λ max at 265 nm. The peak shape was characteristic and well

Table 1
First dose plasma levels of imatinib mesylate estimated by HPLC with UV detection in four CML patients by limited sampling

Sampling (h)	Patient 1 (μg/ml)	Patient 2 (μg/ml)	Patient 3 (μg/ml)	Patient 4 (μg/ml)
1	0.129	0.026	0.468	0.745
2	0.067	0.609	0.734	1.385
4	0.284	0.702	0.766	0.962
6	0.481	0.691	0.726	0.940
24	1.129	0.348	0.319	0.72

The patients were administered 100 mg of imatinib mesylate along with food.

differentiated among 40 drugs library in our PDA match option. The capacity factor (K') for imatinib peak was calculated to be 2.35 with a HETP (using 5σ) of more than 10,000.

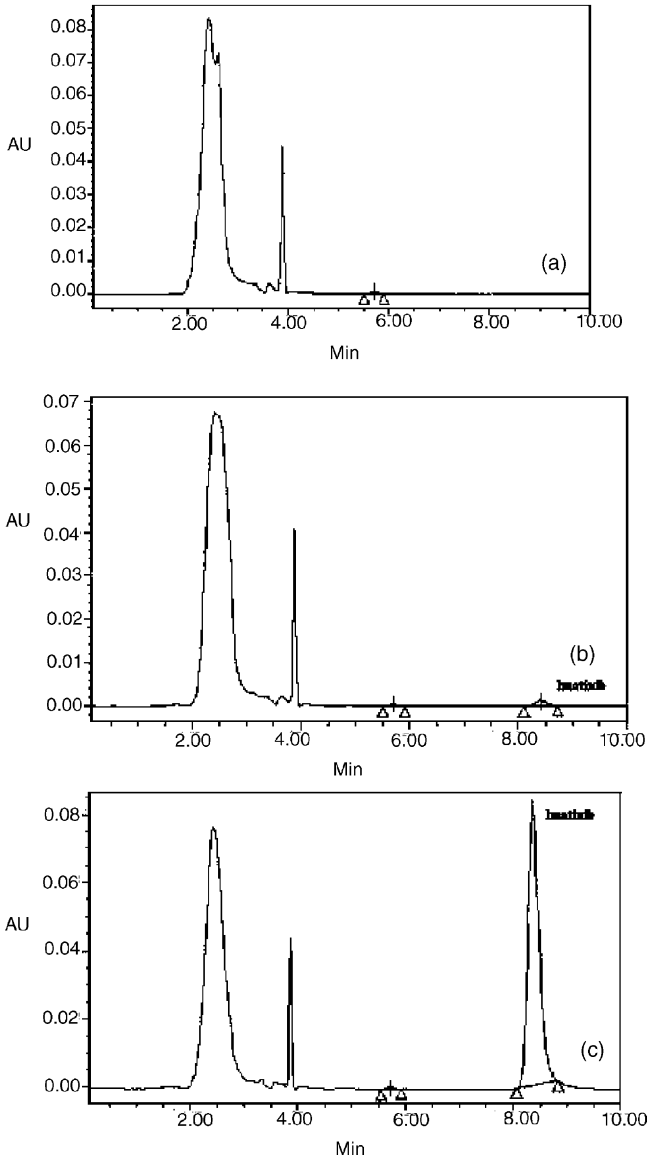

Fig. 1. Representative chromatogram of: (a) blank human plasma, (b) human plasma spiked with 0.19 μg/ml of imatinib, and (c) human plasma spiked with 25 μg/ml of imatinib showing well resolved peaks.

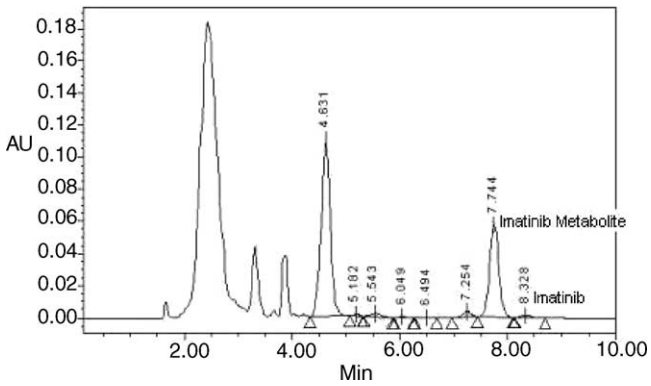

Fig. 2. Chromatogram showing 0.7 μg/ml of imatinib and its metabolite levels in patient's plasma after 100 mg oral administration of capsule formulation of imatinib.

This method is capable of detecting up to 5 ng/ml of imatinib (LOD). The LOQ was calculated as 30 ng/ml and the coefficient of variation was found to be 4.9%.

A perfect linearity was obtained in the concentration versus area curve for standard (0.025–25 μg/ml) from spiked plasma samples. Chromatograms are shown in the Fig. 1a–c. The correlation coefficient (r^2) of the curve was found to be 0.999 and inter- and intra-day variations between standard curves were <4% (R.S.D.). The external calibration curve showed a good linearity in the concentration range of 0.025–25 μg/ml ($r^2 = 0.9992$).

Extraction efficiency, of this direct deproteinization method of plasma, was found to be 105, 94, 97, 98, 99, 104 and 105% at 0.087, 0.168, 0.39, 0.698, 1.39, 2.79 and 5.58 μg/ml, respectively, against water. The accuracy of estimation in the seeded samples of low (0.098 μg/ml), moderate (0.394 μg/ml) and higher (3.156 μg/ml) concentrations of imatinib in the plasma was found to be 92.6, 97.3 and 104%, respectively. The precision (inter-day) of the aforesaid low, moderate and higher concentration seeded plasma expressed as R.S.D. was found to be 0.79, 1.98 and 1.5%, respectively. The partition coefficient ($\log P$) of imatinib mesylate was calculated to be 1.198 and 1.267 at 25 and 37 °C, respectively.

A metabolite of imatinib was also detected at a retention time of 7.7 min (Fig. 2). Its peak was well resolved and easily differentiated from that of the drug. The first dose pharmacokinetic profile of imatinib in CML patients revealed that a peak plasma level (C_{max}) of 0.7–1 μg/ml was achieved within 4 h (T_{max}) after the oral administration of 100 mg of the drug. However, a high degree of variation was observed in the 24 h plasma concentration (0.3–1 μg/ml).

3. Discussion

Imatinib mesylate is an orally acting anti leukemic agent approved by FDA [10,12] for the treatment of myeloid

leukemia in blast crisis, accelerated phase or in chronic phase after the failure of interferon therapy.

Imatinib is metabolized by the enzymes belonging to the CYP3A4 family, which are predominantly housed in the enterocytes of intestinal lumen [9,10]. Many of the CYP3A4 substrates are also reported to be substrates of *P*-glycoprotein (*P*-gp) drug transporter efflux pump present in the enterocytes [13]. Tandem activity of CYP and *P*-gp severely affects the plasma concentration, kinetics and clearance of the susceptible drugs. Such compounds are ideal candidates for therapeutic drug monitoring. The success of therapeutic drug monitoring lies in establishing an accurate, simple, rapid and cheap method for assay. Till date, the only assay method available for imatinib, utilizes LC–MS [14,15]. This technique is quite expensive and as yet inaccessible for many of the third world investigators and cannot be used for routine therapeutic drug monitoring.

Our study addresses this need and provides a rapid and sensitive high-performance liquid chromatographic method with UV detection for the estimation of imatinib. As only 100 μl of plasma suffices for the analysis, small blood samples need to be withdrawn from the patient. In our method, direct deproteinization of plasma gives an extraction efficiency of more than 90% which not only circumvents the need for internal standard but also a multi stage extraction procedure like solid phase extraction [15]. This augments the accuracy of the assay and also helps to cut the time and money required for sample preparation. The inherent property of imatinib of high molar extinction coefficient at 265 nm imparts a high sensitivity to the assay method.

For the evaluation of LOD and LOQ, International Conference of Harmonization Guideline (1996) was followed [16]. According to the guideline signal-to-noise ratio between 3 or 2:1 is generally considered as acceptable but according to our laboratory standard operating procedure the ratio of 4:1 is practiced.

C8 column has been used for the elution of imatinib in the biological samples due to its highly water soluble nature with low partition coefficient value. The low partition coefficient of imatinib denotes that it is unlikely to have high lipid binding. Therefore, plasma level analysis would be a good indicator rather than whole blood analysis, which is appropriate for lipid soluble drugs with preferential accumulations in RBCs. A good degree of separation was achieved when an acidic mobile phase comprising of 0.02 M potassium dihydrogen phosphate was run against the moderately non-polar column like C8. A run time of 10 min is appropriate for eluting samples containing standard drug. However, the run time has to be extended to 12 min for verifying the complete elution of the poly-component clinical samples. However, the first dose imatinib plasma samples did not show any interfering peaks.

Imatinib is reported to quickly reach steady state upon repeated administration [9]. However, its first dose plasma levels in CML patients were low. With limited sampling we could not perform pharmacokinetic estimates but further studies of trough level analysis in patients undergoing treatment is underway for the continuous monitoring of its plasma levels to understand more about its efficacy as well as safety.

To conclude, a simple isocratic HPLC method with UV detection has been developed and validated for the evaluation of imatinib from biological samples.

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