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Automated imatinib immunoassay

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Abstract

Background—Imatinib pharmacokinetic variability and the relationship of trough concentrations with clinical outcomes have been extensively reported. Though physical methods to quantitate imatinib exist, they are not widely available for routine use. An automated homogenous immunoassay for imatinib has been developed, facilitating routine imatinib testing.

Methods—Imatinib-selective monoclonal antibodies, without substantial cross-reactivity to the N-desmethyl metabolite or N-desmethyl conjugates, were produced. The antibodies were conjugated to 200 nm particles to develop immunoassay reagents on the Beckman Coulter AU480TM analyzer. These reagents were analytically validated using Clinical Laboratory Standards Institute protocols. Method comparison to LC-MS/MS was conducted using 77 plasma samples collected from subjects receiving imatinib.

Results—The assay requires 4 μ L of sample without pre-treatment. The non-linear calibration curve ranges from 0 to 3,000 ng/mL. With automated sample dilution, concentrations of up to 9,000 ng/mL can be quantitated. The AU480 produces the first result in 10 minutes, and up to 400 tests per hour. Repeatability ranged from 2.0 to 6.0% coefficient of variation (CV), and within-laboratory reproducibility ranged from 2.9 to 7.4% CV. Standard curve stability was two weeks and on-board reagent stability was 6 weeks. For clinical samples with imatinib concentrations from 438 – 2,691 ng/mL, method comparison with LC-MS/MS gave a slope of 0.995 with a y-intercept of 24.3 and a correlation coefficient of 0.978.

Conclusion—The immunoassay is suitable for quantitating imatinib in human plasma, demonstrating good correlation with a physical method. Testing for optimal imatinib exposure can now be performed on routine clinical analyzers.

Keywords

tyrosine kinase inhibitors; imatinib; oncology; pharmacokinetic-pharmacodynamic

Introduction

Imatinib mesylate (Gleevec®, Glivec®) is a potent inhibitor of BCR-ABL tyrosine kinase and is widely used to treat CML (chronic myelogenous leukemia) and GIST (gastrointestinal stromal tumors).^{1,2} This drug has demonstrated significant clinical efficacy in both CML and GIST, producing durable responses and prolonged survival, becoming the standard of care in the treatment of these diseases. Despite the impressive efficacy, suboptimal responses and treatment failures have been reported.³ Multiple factors contribute to the variation of responses, including biological factors, such as BCR-ABL mutations and other mechanisms of resistance, disease state, and pharmacokinetic (PK) factors that can affect exposure.^{3,4} Additionally, patient adherence to oral medication regimens can be a critical factor influencing outcomes.⁵

A number of studies have demonstrated that imatinib exhibits wide inter-patient pharmacokinetic variability that correlates with lack of efficacy.^{6–20} Imatinib PK parameter values (AUC, $C_{\max}$, and $C_{\min}$) at steady-state are highly correlated to each other.¹⁸ The PK parameter most associated with biological effect is trough concentration ($C_{\min}$). Once the drug has achieved a steady state (after 5 to 7 days of daily dosing), the $C_{\min}$ is determined by taking a sample just before the next dose of the drug.²¹ The inter-patient variability of trough levels can be as high as 16-fold and a CV of greater than 50% has been reported.^{6,7,21} The PK variability of the active N-desmethyl metabolite is similar to that of imatinib, and is present at approximately 20% of the concentration of the parent drug. There are a number of factors that may affect exposure to imatinib. These include: patient adherence, demographic factors, absorption from the gastrointestinal tract, variability in CYP enzyme activity, and drug-drug interactions.^{5,21–26}

Several groups have concluded that Therapeutic Drug Monitoring (TDM) of imatinib would be of value in optimizing exposure. Target trough levels of 1,000 ng/mL for CML and 1,100 ng/mL for GIST have been proposed.^{6,7,10,18,27} This report describes a homogeneous nanoparticle immunoassay developed using an antibody selective for the parent drug. This immunoassay has been validated on the Beckman Coulter AU480 clinical analyzer. The ease of use and low cost of the method will facilitate imatinib testing in clinical laboratories.

Materials and Methods

Immunoassay

The principle of the immunoassay has been described previously and represents a standard approach for a homogeneous nanoparticle immunoassay.^{28,29} Briefly, the assay is a two reagent nanoparticle based system. Reagent one is comprised of a large polymer with repeating units of imatinib derivative. Reagent two is contains 200 nm polystyrene nanoparticles with antibodies covalently attached to the surface. When reagent one and two are mixed the antibody coated particles react with the imatinib polymer to form an agglutination complex that can be monitored spectrophotometrically. The larger the complex the more light is scattered resulting in an absorbance change over time. The relationship of light scattering to the size of the complex is proportional to r^6 where r is the radius of the complex. Imatinib in the sample competes with the drug conjugate for the limited antibody

binding sites. The higher the concentration of the drug the smaller the agglutination complex and the less light is scattered. This results in an inhibition curve with increasing imatinib concentration: no drug in the sample results in maximum absorbance and high drug levels in the sample result in a minimal amount of absorbance. The assay design provides for high sensitivity and liquid stable reagents with shelf lives of 18 to 24 months.

The imatinib monoclonal antibody used in the assay is selective for the parent drug with low cross-reactivity to the active N-desmethyl metabolite and the inactive N-conjugated metabolites (Figure 1). The derivative was prepared by attaching a butyric acid group to the 4-amide position on the imatinib molecule. This acid derivative was activated to an N-hydroxy-succinimidyl ester which was reacted with the carrier protein keyhole limpet hemocyanin (KLH) to form the immunogen. A detailed description of the derivative preparation, immunogen and antibody generation has been described previously.³⁰ This elicited antibodies selective to parts of the molecule that are distal to the point of attachment. Because the antibody requires the N-methyl on the piperazine moiety for recognition, it does not bind to N-desmethyl metabolite conjugates. The parent drug is accurately quantified without interference from metabolites due to the selectivity of the antibody.³⁰

Assay Method

The imatinib reagent kit is manufactured by Saladax Biomedical, Inc. (Bethlehem, PA). The reagent kit consists of two Beckmann AU480 (Brea, CA) compatible bottles: one bottle contains the reaction buffer with a polymeric imatinib carrier (R1), and one bottle contains the monoclonal antibody coated nanoparticle (R2). The kit contains enough reagents to perform 100 tests on the analyzer. The AU480 is an open clinical analyzer, and the assay parameters are applicable to other Beckman Coulter instruments in the AU family. The analyzer pipettes 95 μ L of R1 and 4 μ L of sample into a reaction cuvette. After an incubation time of 3.4 minutes, 95 μ L of R2 is added. Mixing occurs after each step. The agglutination reaction is monitored at 600 nm. The calculated reaction end point is inversely proportional to the concentration of imatinib present in the sample. Additionally these reagents can be run on most open clinical analyzers that perform homogeneous assays. The reagents can be run on the Roche c 501 analyzer with equivalent performance.

Calibrators, Controls, Calibration Curve, and Calibration Interval

The imatinib concentrations of the calibrators were 0, 300, 600, 1,000, 2,000, and 3,000 ng/mL. The low, medium, and high assay control levels (750, 1,500, and 2,500 ng/mL, respectively) were selected to ensure accurate quantitation at medical decision points. Calibrators and controls are formulated in a buffer matrix for ease of use, economy and product stability. Equivalence of the buffer matrix and plasma was demonstrated in recovery studies. Calibration interval was established with three lots of reagent on two analyzers. On day zero, calibrations were performed; thereafter assay controls of each level were run in singlicate at defined intervals (every 3 – 4 days). Within the calibration interval, control results met the recovery specifications of $\pm 20\%$, $\pm 14\%$ and $\pm 12\%$ for low, medium, and high controls, respectively.

Precision

Precision was determined according to CLSI Guideline EP5-A2.³¹ A drug-free plasma pool spiked with imatinib at 4 concentrations (350, 900, 1,600, and 2,700 ng/mL), and the assay controls were tested in duplicate twice daily for 23 days to determine the repeatability and within-laboratory precision of the assay. The twice daily testing was performed at least two hours apart. The repeatability and within-laboratory standard deviation (SD) and CV were calculated.

Linearity

Assay linearity was determined according to CLSI Guideline EP6-A³². Drug-free plasma samples containing imatinib at 251 and 3,316 ng/mL were prepared via spiking. Nine intermediate concentrations were prepared by admixing the two solutions, for a total of 11 linearity samples. For the imatinib immunoassay, the linear range was defined as the range of imatinib concentrations that yielded mean percent recoveries (versus theoretical) ranging from 80 to 120%, and percent deviations of the polynomial fit from the linear that were 15%.

Recovery of Imatinib

To assess recovery, imatinib was spiked into five drug-free plasmas at four concentrations (350, 1,000, 1,600, and 2,700 ng/mL). All samples were analyzed n=5 using three reagent lots on two analyzers. Replicate and average recoveries were calculated from spiked imatinib results compared to assigned values.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were determined according to CLSI Guideline EP17-A.³³ Four and five drug-free human plasmas were spiked with imatinib for LOQ and LOD, respectively. A range of concentrations was chosen to include values above and below the predicted LOD and LOQ of the assay. Samples were analyzed n=3 using three reagent lots on two analyzers. LOD was tested over two days and LOQ over three. Limit of Blank (LOB) was determined in the same experiment with the five drug-free human plasmas over two days. The LOD was the imatinib concentration at which 95% of the results were greater than the assay LOB. The LOQ was the imatinib concentration at which the total analytical error (bias + 2×standard deviation) was 35%.³⁴

Cross-Reactivity

Common medications (prescription and over the counter), including ACE inhibitors, analgesics, anticonvulsants, antifungals, antihistamines, anti-inflammatories, antibiotics, antidepressants, beta blockers, benzodiazepines, chemotherapeutics, diuretics, decongestants, immunosuppressants, protease inhibitors, statins, and steroids were tested in addition to vitamins and herbal supplements. The major imatinib metabolite N-desmethyl imatinib, and the structurally related drug nilotinib (Tasigna®), were also tested for cross-reactivity. Several CYP3A4 inducers and inhibitors, which can alter imatinib exposure were tested to ensure they caused no interference in the assay. These included: mometasone furoate, clarithromycin, clotrimazole, nefazodone, omeprazole, quinidine and valproic acid.

Cross-reactivity was assessed in a drug-free plasma pool, and the same pool spiked with 1,100 ng/mL of imatinib. The samples were evaluated with one lot of reagent on two analyzers. Compounds were dissolved in a suitable solvent and spiked into plasma pools at a minimum concentration of 100,000 ng/mL. The cross-reactivity was calculated from the difference of the mean imatinib values with and without cross-reactant divided by the cross-reactant concentration.

Interference

Interference from lipemia, icterus, total protein, hemolysis, and rheumatoid factor was assessed. Bilirubin, human serum albumin (HSA), and immunoglobulin G (IgG) were spiked into a drug-free plasma pool and a plasma pool spiked at 1,000 ng/mL of imatinib. To prepare lipemic samples, the lipid layer isolated from fresh human plasma was spiked into the plasma pools for interference testing. The concentrations of bilirubin, HSA, IgG, and lipemia were verified using reagent kits from JAS Diagnostics (Miami Lakes, FL, US). Rheumatoid factor was spiked into plasmas from a known stock concentration (Keystone Biologicals, Hatboro, PA, US). Hemolysate was prepared from blood obtained from healthy donors and known hemoglobin concentrations were spiked into plasma samples to simulate hemolysis interference.

Comparative Methods

Imatinib was quantitated using an Agilent 1100 Autosampler and Binary pump (Palo Alto, CA, USA) coupled to a Micromass Quatromicro triple-stage bench-top mass spectrometer (Waters Corporation, Milford, MA, USA). Analytes were separated on a Phenomenex Synergi Polar-RP column (4 μ m, 2 $\times$ 100 mm). The gradient mobile phase system was comprised of solvent A (0.1% formic acid in methanol) and solvent B (0.1% formic acid in water). The initial mobile phase was 55% A and 45% B at a flow rate of 0.3 mL/min. Subsequently, solvent A was increased to 85% over 4 minutes, where it was held until 5 minutes. Finally, A was decreased to 55% at 5.1 minutes and held until 8 minutes with an increased flow rate of 0.4 mL/min, followed by injection of the next sample. Mass spectrometer settings were: capillary voltage 4 kV; cone voltage 50 V; and desolvation temperature 400°C. The MRM m/z ratios monitored were $m/z > 494 > 394$ and $502 > 394$ for imatinib and D₈-imatinib (internal standard), respectively. Aliquots of 100 μ L of plasma were mixed with 10 μ L of internal standard (1 μ g/mL in methanol/water [50/50, v/v]), and were then extracted with 500 μ L methanol. After vortexing and centrifugation, the supernatant was transferred to an auto-sampler vial and 10 μ L of the sample was injected into the LC-MS/MS system. The ion chromatograms were integrated and quantified using Micromass Masslynx Version 4.0 (Waters Corporation). The linearity of this assay was 20–5,000 ng/mL. This assay had an acceptable accuracy (105–109%) and precision (<6.0 CV %), as determined from independent QC samples at 3 levels (N = 6).

Clinical Samples and Method Comparison

Blood samples were collected during an IRB approved clinical trial (ClinicalTrials.gov Identifier: NCT00732784) in heparinized Vacutainers®. Samples were deidentified according to an exempt IRB study approved by the University of Pittsburgh Institutional

Review Board. These samples were analyzed by LC-MS/MS and stored at -80°C for greater than two years. To account for potential sample degradation during storage, 97 samples were reanalyzed by LC-MS/MS before testing with the imatinib immunoassay. To fulfill quality regulations, samples were also tested by a second LC-MS/MS method developed and validated by inVentiv Health Clinical (Princeton, NJ, US), a CRO. Each sample was tested $n=1$ using each method. Results were compared using Deming regression.³⁵ Where samples were outside the total error limit of 15% in the regression analysis between the physical methods ($n=16$), they were excluded from the method comparison. Four samples above the immunoassay test range were of insufficient volume to be diluted and were excluded from the method comparison. Seventy-seven samples were compared between the University of Pittsburgh LC-MS/MS method and the immunoassay.

Results

Calibrators, Controls, Calibration Curve and Calibration Interval

The assay covers the range of expected results using six calibrators (0, 300, 600, 1,000, 2,000, and 3,000 ng/mL) with a four parameter logistic regression curve fit. The calibrator and controls formulated in an aqueous matrix were commutable with imatinib in plasma and were predicted to have a shelf-life of at least two years, based on stability at 37°C and 45°C . The calibration curve is shown in Figure 2. The analyzer was programmed to automatically dilute samples higher than 3,000 ng/mL by 1:3. This gives the assay an effective range up to 9,000 ng/mL. Control values were within specification for at least two weeks; no recalibration of the instrument was required during that time. When controls were outside specifications the instrument was recalibrated and controls were within range.

Precision

The precision study was conducted according to CLSI Guideline EP5-A2.³¹ Repeatability and within laboratory precision were determined for samples prepared from a drug-free plasma pool spiked with imatinib at 4 concentrations (350, 900, 1,600, and 2,700 ng/mL), and the assay controls (750, 1,500, 2,500 ng/mL). The CVs for repeatability were between 2.0 and 6.2% (Table 1). The lowest plasma pool (350 ng/mL) had the highest CV. The within-laboratory CV ranged from 2.9 to 7.4%, with the highest CV occurring at the lowest concentration (Table 1).

Linearity

The imatinib immunoassay was linear from 301 to 3,316 ng/mL. The greatest absolute deviation from linearity was less than 5% and the recovery was between 98 and 120%. Around the medical decision point proposed in the literature (1,000–1,100 ng/mL), the greatest deviation from linearity was less than 3%, and the recovery was within 10%.

Recovery

The replicate and mean recoveries of imatinib spiked at 350 ng/mL ranged from 74–114% and 82–100%, respectively. The replicate recovery of imatinib spiked at 1,000 ng/mL ranged from 83–104% and the mean recovery of imatinib spiked at 1,000 ng/mL ranged from 86–100%. The replicate and mean recoveries of imatinib spiked at 1,600 ng/mL ranged

from 87–104% and 88–100%, respectively. The replicate recovery of imatinib spiked at 2,700 ng/mL ranged from 87–100% and the mean recovery of imatinib spiked at 2,700 ng/mL ranged from 89–96%.

Limit of Detection and Limit of Quantification

The LOQ was defined as the lowest imatinib concentration at which the total analytical error was < 35%. An LOQ of 296 ng/mL was determined. The LOD was defined as the lowest imatinib concentration at which 95% of the results exceeded the assay limit of blank (LOB). An LOD was determined to be 154 ng/mL.

Cross-Reactivity

Cross-reactivity was assessed in a drug-free plasma pool, and the same pool spiked with 1,100 ng/mL of imatinib. Cross-reactants were spiked in the plasma pools at a minimum concentration of 100,000 ng/mL. The cross-reactivity of the major metabolite N-desmethyl imatinib was 0.2%. The cross-reactivity of nilotinib was 0.1%. All of the 100 compounds tested including ACE inhibitors, analgesics, anticonvulsants, antifungals, antihistamines, anti-inflammatories, antibiotics, antidepressants, beta blockers, benzodiazepines, chemotherapeutics, diuretics, decongestants, immunosuppressants, protease inhibitors, statins, steroids, vitamins and others demonstrated cross-reactivities of 0.1% in both normal plasma and plasma spiked with imatinib at 1,100 ng/mL. Medications that could alter imatinib exposure by induction or inhibition of the major metabolizing enzyme, CYP3A4, would not influence the analytical result as they cross-reacted 0.1%.

Interference

Interference from lipemia, icterus, total protein, hemolysis, and rheumatoid factor was within –5 to 2% (Table 2). No significant interference was observed from the following interferents: rheumatoid factor at 500 IU/mL, human serum albumin at 12 g/dL, human IgG at 12 g/dL, icteric samples at 30 mg/dL, lipemic samples at 593 mg/dL and hemolysate samples at 1,000 mg/dL.

Method Comparison

For the 77 samples included in the regression analysis, the range of imatinib concentrations measured by the immunoassay was 438 – 2,691 ng/mL with a mean of 1,492 ng/mL and the range of concentrations measured by the LC-MS/MS method was 399 to 2,643 ng/mL with a mean of 1,475 ng/mL.

The Deming regression of the data set had a slope of 0.995 (95% confidence interval: 0.947–1.043) with a y-intercept of 24.3 ng/mL (95% confidence interval: –51.7 to 100.3), a standard error of the estimate of 124.1 and a correlation coefficient of 0.978 (Figure 3). For all samples, the immunoassay results fell within $\pm 25\%$ of the value predicted by the Deming regression. At 1,000 ng/mL (the target $C_{\min}$ when treating CML), the predicted immunoassay result was 1,019 ng/mL (95% confidence interval: 983 – 1,056 ng/mL). At 1,100 ng/mL (the target $C_{\min}$ when treating GIST), the predicted immunoassay result was 1,119 ng/mL (95% confidence interval: 1,085 – 1,153 ng/mL).

Discussion

A turbidimetric immunoassay was developed with a monoclonal antibody selective for imatinib without interference of the active N-desmethyl metabolite or the inactive metabolites conjugated out of the 4-N position on the piperazine ring.

Studies have used the parent drug to determine relationship between PK and clinical outcomes. Though the major metabolite, N-desmethyl imatinib, is also active, it appears in a consistent ratio (15 to 20%) to the parent drug. Thus, the antibody selectivity is appropriate for a quantitative drug monitoring assay.

The assay demonstrated reliable analytical performance for precision, linearity, and curve stability. The assay was not susceptible to interference from samples with abnormally high lipemia, icterus, rheumatoid factor, or protein samples. Common prescription and over the counter drugs, vitamin and herbal supplements also did not interfere with assay results.

Close to the medical decision points, the repeatability and within-laboratory precision were less than 3% and 4%, respectively. The assay displayed linearity over the entire measuring range – from the LOQ to above the highest calibrator level. Samples with imatinib concentrations > 3,000 ng/mL can be auto diluted three-fold with deionized water to yield acceptable recovery. Given that the majority of samples are below 3,000 ng/mL, sample dilution will occur rarely.⁶

Method comparison between the immunoassay and the physical method demonstrated lack of assay interferences and appropriate selectivity for the parent drug. At the proposed decision points of 1,000 and 1,100 ng/mL, the immunoassay results were within the 95% confidence limits predicted by the Deming analysis.

In clinical use, the dose of imatinib generally ranges from 400 mg to 800 mg per day. At these doses, the median imatinib trough concentrations can range from 1,200 to 2,690 ng/mL. The CV can be > 50%; mean values ($\pm$ SD) of 979 ng/mL ($\pm$ 530 ng/mL), 869 ng/mL ($\pm$ 427.5), and 1452.1 ng/mL ($\pm$ 649.1 ng/mL) have been reported.^{6,10} Given this wide variation and the association of trough concentrations with outcomes, imatinib drug monitoring would benefit patients. An assay readily available in the clinical laboratory would increase accessibility to drug monitoring. Additionally, Gleevec® is off patent, or will soon be off patent in most countries. Newer tyrosine kinase inhibitors have reached the market and investigators have claimed superior clinical performance over imatinib at the 400 mg/day dose. These TKIs are a very expensive class of drugs, and are much more expensive than generic imatinib. Given that imatinib concentrations above 1,000 ng/mL in CML and above 1,100 ng/mL for GIST result in greater clinical benefit, imatinib TDM has the potential to optimize clinical outcomes without the need for newer TKIs. Using generic imatinib with TDM to improve therapeutic response promises substantial savings in health care costs.

Conclusion

The assay evaluated in this report is the first automated immunoassay for the management of imatinib therapy. The availability of this method to clinical laboratories will enable more widespread testing of imatinib and will ensure rapid results. Numerous groups have reported on the extensive PK variability of imatinib in both CML and GIST patients. Statistically significant relationships have been identified between trough concentrations and clinical outcomes. Additionally, patient compliance is a concern as it has a substantial impact on treatment efficacy. With TDM, physicians have a tool to evaluate exposure and adherence, both of which have been shown to profoundly affect response and outcomes. Instead of unnecessarily switching to a more expensive treatment, physicians may find that imatinib remains an effective and appropriate therapy. TDM of imatinib with this assay provides the oncologist with clinically actionable data and a tool to optimize treatment. The availability of an automated assay that can be run on widely used clinical analyzers would provide value and should result in improved patient care.

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Figure 1.
Structure of imatinib and the N-desmethyl metabolite

Figure 2.
Imatinib immunoassay calibration curve generated on the Beckman AU480 analyzer.

Figure 3.
Deming Regression: LC-MS/MS versus imatinib immunoassay: $y = 0.995x + 24.3$

Table 1

Repeatability and within-laboratory precision. N=2 replicates per run, determined twice per day for 23 days

Sample Type	Assigned Value (ng/mL)	Mean (ng/mL)	Repeatability (% CV)	Within-Laboratory Precision (% CV)
Buffer	750	762	3.2	4.2
	1,500	1,468	2.6	3.7
	2,500	2,548	2.0	2.9
Plasma	350	363	6.0	7.4
	900	946	2.8	3.9
	1,600	1,665	2.5	3.5
	2,700	2,649	2.6	3.4

Table 2

Interference Results. N=1 replicate

Interference Type	Interferent Concentration	Interference (%)
Lipemia (Triglycerides)	592.6 mg/dL	-2.0
Icterus (Bilirubin)	30.1 mg/dL	-3.1
Total Protein (HSA)	12.12 g/dL	-2.5
Total Protein (IgG)	12.16 g/dL	-4.8
Hemolysis (Hemoglobin)	1,000 mg/dL	1.8
Rheumatoid Factor	500 IU/mL	0.9