Journal of Chromatography B, 877 (2009) 1894–1900

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

A high-performance liquid chromatography–mass spectrometry assay for quantitation of the tyrosine kinase inhibitor nilotinib in human plasma and serum

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ARTICLE INFO

Article history:
Received 2 April 2009
Accepted 16 May 2009
Available online 21 May 2009

Keywords:
Nilotinib
AMN-107
LC–MS
Mass spectrometry
Chronic myeloid leukemia

ABSTRACT

Nilotinib (AMN-107, Tasigna™) is a small-molecule inhibitor of BCR/ABL, approved for chronic myelogenous leukemia. We developed and validated, according to FDA-guidelines, an LC–MS assay for sensitive, accurate and precise quantitation of nilotinib in 0.2 mL human plasma or serum. After acetonitrile protein precipitation, separation is achieved with a hydro-Synergi column and a 0.1% formic acid in methanol/water-gradient. Detection uses electrospray, positive-mode ionization mass spectrometry. Between 5 (LLOQ) and 5000 ng/mL, accuracy (92.1–109.5%), intra-assay precision (2.5–7.8%), and inter-assay precision (0–5.6%)) were within FDA limits. We demonstrated the suitability of this assay by quantitating plasma concentrations of nilotinib in a healthy volunteer after oral administration of 400 mg nilotinib.

1. Introduction

Although the tyrosine kinase inhibitor imatinib has revolutionized the treatment of chronic myeloid leukemia (CML) and gastrointestinal tumors (GIST) [1–3], some patients experience intolerable side-effects, and the tumors of other patients develop resistance to imatinib [4–6]. As a result, there are active programs to develop newer agents to avoid or overcome these problems [7]. Nilotinib (Tasigna™, formerly known as AMN107,) (Fig. 1) is a second-generation, small-molecule inhibitor of the BCR/ABL tyrosine kinase fusion protein [7]. Nilotinib is approximately 30 times more potent in inhibiting BCR/ABL than is imatinib and also inhibits a number of imatinib-resistant mutant BCR/ABL proteins [8]. Nilotinib has been approved for the treatment of CML and is being evaluated as a single agent and in combination with other antineoplastic agents as a potential treatment for a variety of malignancies [9]. As a result of these studies, there is the need for evaluation of nilotinib pharmacokinetics so that issues such as pharmacokinetic/pharmacodynamic relationships, drug–drug interactions, and patient adherence to daily oral therapy can be evaluated. To date, only one assay for nilotinib has been published [10]. However, that assay is an HPLC-UV method that uses online sample extraction with no internal standard. We sought to develop a facile and sensitive assay for nilotinib that used an internal standard and mass spectrometric detection as well as a smaller sample volume, a simpler sample preparation, and a shorter run time than used in the previously published method. Our assay was validated according to the most recent FDA guidelines [11].

2. Materials and methods

2.1. Chemicals and reagents

Nilotinib hydrochloride monohydrate (M/M+4 > 99.99%) and [C_2^{13}, N_2^{15}]-nilotinib internal standard (M+4/M > 99.9%) were graciously supplied by Novartis Pharmaceuticals (East Hanover, NJ, USA). From here on, nilotinib hydrochloride monohydrate will be referred to as nilotinib. Methanol (Optima grade) and acetonitrile (Optima grade) were purchased from Fisher Scientific Co. (Fair Lawn, NJ, USA). Formic acid was purchased from Sigma Chemical

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doi:10.1016/j.jchromb.2009.05.034

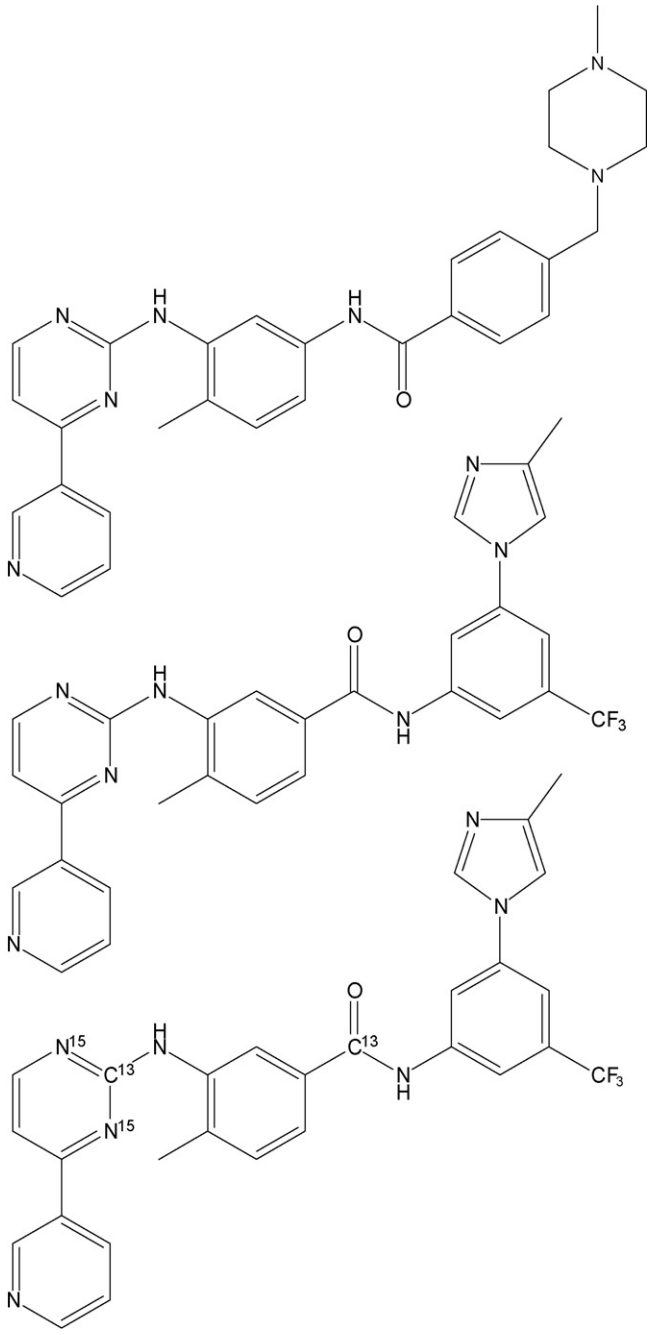

Fig. 1. Chemical structures of imatinib, nilotinib and stable-labeled [C_2^{13}, N_2^{15}]-nilotinib internal standard in their base form.

Co. (St. Louis, MO, USA). Water was purified using a Q-gard® 1 Gradient Milli-Q system (18.2 MΩcm, Millipore, Billerica, MA, USA). Control human plasma was prepared by centrifugation of whole blood (Central Blood Blank, Pittsburgh, PA, USA) at 2000 × *g* at room temperature for 20 min. Control human serum was obtained from Cambrex Bio Science (Walkersville, MD, USA). Nitrogen gas for the mass spectrometer was purified with a Parker Balston Nitrogen Generator (Haverhill, MA, USA), and nitrogen gas for sample evaporation was purchased from Valley National Gasses, Inc. (Pittsburgh, PA, USA).

2.2. Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1100 autosampler and binary pump, a Phenomenex (Torrance, CA, USA) Synergi Hydro-RP (4 μm, 2 mm × 100 mm) column kept at ambient temperature, and a gradient mobile phase. Mobile phase solvent A was 0.1% (v/v) formic acid in methanol, and mobile phase solvent B was 0.1% (v/v) formic acid in water. The initial mobile phase was 60% solvent A and 40% solvent B pumped at a flow rate of 0.2 mL/min. Between 0 and 6 min, the percentage of solvent A was increased linearly to 90%, where it was held for 1 min. At 7.1 min, the flow rate was increased to 0.4 mL/min, which was maintained until 9 min. Between 9 and 10 min, the percentage of solvent A was decreased linearly to 60%, and the flow rate was decreased to 0.2 mL/min. These conditions were maintained until 15 min, at which time the next sample was injected into the LC system.

2.3. Mass spectrometry

Mass detection was carried out using a ThermoFinnigan (San Jose, CA, USA) MSQ single quadrupole mass spectrometer with electrospray ionization in positive-ion mode. The settings of the mass spectrometer were as follows: capillary voltage 4.0 kV; cone voltage 60 V; and probe temperature 400 °C. In single-ion monitoring (SIM) mode, the *m/z* ratios monitored were 530.4 and 534.4 for nilotinib and internal standard, respectively. The dwell time was 0.37 s, and the span was set at 0.2 amu. The LC system and mass spectrometer were controlled by ThermoFinnigan Excalibur software (version 1.4), and data were collected with the same software.

2.4. Preparation of calibration standards and quality control samples

Stock solutions of nilotinib were prepared at 0.5 mg/mL in acetonitrile:water (50:50, v/v) and stored at 4 °C in the dark. On the assay day, this solution was diluted (in steps of 10-fold) with acetonitrile:water (50:50, v/v) to obtain the lower calibration working solutions of 0.1, 0.01, and 0.001 mg/mL. These calibration working solutions were diluted in human plasma or serum to produce the following nilotinib concentrations: 5, 10, 30, 100, 300, 1000, 3000, and 5000 ng/mL. For each calibration series, zero and blank samples were also prepared from 200 μL of control plasma or serum, respectively.

Quality control (QC) stock solutions were prepared independently and from a separate weighing of nilotinib, and stored at 4 °C in the dark. These solutions were diluted in control human plasma or serum to produce the following QC samples: QC low (QCL) 15 ng/mL; QC mid (QCM) 500 ng/mL; and QC high (QCH) 4000 ng/mL. The QC stock solutions were stored at 4 °C in the dark. In addition, from the QC stock solution, plasma and serum samples were prepared at 5 ng/mL, which was the lower limit of quantitation (LLOQ).

2.5. Sample preparation

Two hundred μL of each standard, QC, or sample; 10 μl of a 500 ng/mL solution of [C_2^{13}, N_2^{15}]-nilotinib in acetonitrile:water (50:50, v/v) (internal standard); and 1 mL of acetonitrile were added sequentially to 1.5-mL microcentrifuge tubes that were mixed for 1 min at a setting of 10 on a Vortex Genie-2 (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at 16,000 × *g* at room temperature for 6 min. The resulting supernatants were transferred to 12 mm × 75 mm borosilicate glass tubes and evaporated to dryness under a stream of nitrogen at 37 °C. Dried residues were re-dissolved in 100 μL of methanol:water (50:50, v/v), and 5 μL were injected into the LC–MS system.

2.6. Validation procedures

2.6.1. Calibration curve and lower limit of quantitation (LLQ)

Nilotinib, after addition to human plasma or serum and processing as described in Section 2.5, was injected into the analytical system at decreasing concentrations to achieve a signal-to-noise ratio of at least 5:1. Calibration standards and blanks were prepared as described in Sections 2.4 and 2.5 and analyzed in triplicate to establish the calibration range with acceptable accuracy and precision. The analyte-to-internal standard ratio (response) was calculated for each sample by dividing the area of the nilotinib peak by the area of the internal standard peak. Standard curves of nilotinib were constructed by plotting the analyte-to-internal standard ratio *versus* the nominal concentration of nilotinib in each sample. Standard curves were fit by linear regression with weighting by $1/y^2$, without forcing the line through the origin, followed by the back calculation of concentrations. The deviations of these back-calculated concentrations from the nominal concentrations, expressed as percentage of the nominal concentration, reflected the assay performance over the concentration range.

2.6.2. Accuracy and precision

The accuracy and precision of the assay were determined by analyzing samples with nilotinib at the LLOQ, QCL, QCM, and QCH concentrations in a minimum of five replicates in 3 analytical runs together with an independently prepared, triplicate calibration curve. Accuracy was calculated at each test concentration as:

(mean measured concentration/nominal concentration) × 100%.

Assay precision was calculated by ANOVA as described [12] through SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA). Back-calculated concentrations of calibration and QC samples were entered with the run number as factor. The intra-assay and inter-assay precisions were calculated from the resulting mean squares of the within runs and mean squares of the between runs, respectively.

2.6.3. Selectivity and specificity

To investigate whether endogenous matrix constituents interfered with the assay, six individual batches of control, drug-free human plasma and serum were processed and analyzed according to the described procedures. Responses of nilotinib at the LLOQ concentration were compared with the response in the blank samples.

2.6.4. Extraction recovery and matrix effect

We determined the extraction recovery of nilotinib from human plasma and serum by comparing the absolute response of an extract of control plasma or serum to which nilotinib had been added after extraction with the absolute response of an extract of plasma or serum to which the same amount of nilotinib had been added before extraction. The matrix effect of nilotinib by plasma and serum matrix components was defined as the effect on the signal when comparing the absolute response of an extract of control plasma or serum to which nilotinib had been added after the extraction with the absolute response of reconstitution solvent to which the same amount of nilotinib had been added. Experiments were performed at the QCL and QCH concentrations, in triplicate.

2.6.5. Stability

We investigated the stability of a 0.5 mg/mL nilotinib stock solution after storage at 4 °C in the dark for 10 months. The response was compared with that of a freshly prepared stock solution, and stability was expressed as the percentage recovery of the stored solution relative to the fresh solution. All stability testing in plasma was performed in triplicate at the QCL, QCM, and QCH concentrations. The effect of three freeze/thaw cycles on nilotinib concentrations in plasma and serum was evaluated by assaying samples after freezing and thawing on 3 separate days and comparing them to freshly prepared samples. The stability of a 0.5 mg/mL nilotinib stock solution was assessed by comparing freshly made stock to stock that had been stored on the lab bench at room temperature for 4 h. The stability of nilotinib in plasma and serum during sample preparation was evaluated by assaying samples before and after 4 h of storage at room temperature. To evaluate the stability of nilotinib in reconstituted samples in the autosampler, we re-injected a plasma and serum sample set, respectively, with QCL, QCM, and QCH samples in six replicates after being held in the autosampler for 72 h at 4 °C. This time period corresponds to the time between Friday and Monday, and therefore indicates if a sample set can be re-injected if the run is interrupted during the weekend.

2.6.6. Parallelism

To demonstrate parallelism, the ability to dilute samples from above the upper limit of quantitation to within the validated concentration range, plasma and serum samples ($N = 3$ per matrix) containing 10 μg/mL for 50 μg/mL nilotinib were diluted 10-fold and 20-fold respectively, with control sample and assayed.

2.7. Demonstration of applicability to biological samples

To demonstrate the applicability of the method, we quantitated concentrations of nilotinib in the plasma of a 26-year-old male who received a 400 mg dose of nilotinib orally. Prior to participation in the study, the subject gave written, informed consent as approved by the University of Pittsburgh Institutional Review Board. Blood samples were collected into heparinized tubes before nilotinib dosing and at 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, and 72 h after administration of nilotinib. Each sample was centrifuged at approximately 1000 × *g*, at 4 °C for 10 min, and the resulting plasma was stored at −70 °C until analyzed with the procedure described above.

3. Results

3.1. Method development

Given the similarities between imatinib and nilotinib (see Fig. 1), we tuned our mass spectrometer on the nilotinib and internal standard $[M+H]^+$ molecular ions, and next used our previously described imatinib assay [13,14] as a starting point for the current assay. However, nilotinib was retained much stronger than imatinib. Therefore, we changed the starting mobile phase composition from 30% to 50% organic modifier, with a gradient program to increase the modifier linearly from 50 to 90% between 0 and 6 min. Because this still did not result in a suitable elution time, we changed the column from a Phenomenex Luna C18 (5 μm, 150 mm × 2 mm) to a shorter Synergi Hydro-RP (4 μm, 2 × 100 mm) column. This resulted in adequate retention and assay performance.

3.2. Validation of the assay

3.2.1. Mass spectrometry and chromatography

Nilotinib was diluted in acetonitrile and continuously infused at a rate of 10 μL/min into the HPLC flow, which was an isocratic mobile phase of methanol:water:formic acid (50:50:0.1, v/v/v) pumped at 0.2 mL/min. The tuning parameters of the mass spectrometer were adjusted to maximize the intensity of the $[M+H]^+$ ion at *m/z* 530.4. The procedure was repeated for the internal standard at *m/z* 534.4. Under the chromatographic conditions described, nilotinib and internal standard displayed retention times of approximately 5.4 min (Fig. 2).

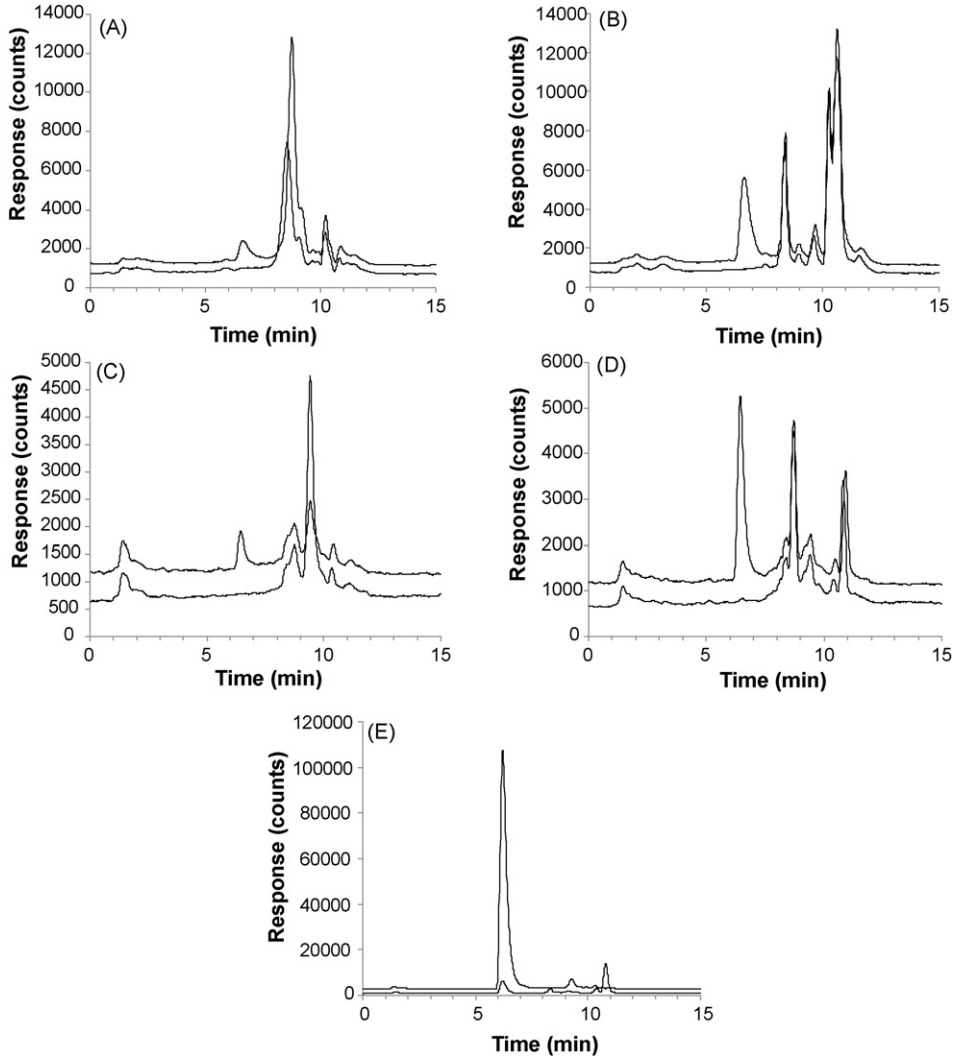

Fig. 2. Representative chromatograms of (A) nilotinib (*m/z* 530.4; 6.5 min) added to control plasma at the LLOQ concentration of 5 ng/mL (top trace with offset of 500 counts) and control human plasma (bottom trace); (B) [C_2^{13}, N_2^{15}]-nilotinib internal standard (*m/z* 534.4; 6.5 min) added to control plasma at a concentration of 25 ng/mL (top trace with offset of 500 counts) and control human plasma (bottom trace); (C) nilotinib (*m/z* 530.4; 6.5 min) added to control serum at the LLOQ concentration of 5 ng/mL (top trace with offset of 500 counts) and control human serum (bottom trace); (D) [C_2^{13}, N_2^{15}]-nilotinib internal standard (*m/z* 534.4; 6.5 min) added to control serum at a concentration of 25 ng/mL (top trace with offset of 500 counts) and control human serum (bottom trace); and (E) nilotinib (top trace with offset of 2000 counts) and internal standard (bottom trace) in the C_{max} sample of a patient 4 h after having been orally administered 400 mg nilotinib.

3.2.2. Calibration curve and lower limit of quantitation (LLOQ)

Triplicate standard curves were performed in plasma and serum on three sequential days. The assay proved to be linear and acceptable, as the regression coefficients were >0.989 for each of the three standard curves in both matrices (see Table 1). The LLOQ was 5 ng/mL, at a signal-to-noise ratio of >10. The individual values for the mean and standard deviations of the back-calculated values at each nominal concentration used in the standard curves are displayed in Table 2, as are the accuracies calculated from those values.

Table 1
Nilotinib assay linearity.

Plasma	Slope	Intercept	R^2
Mean	0.04077	−0.01039	0.9957
CV%	8.1	41.3	0.5
Serum	Slope	Intercept	R^2
Mean	0.04508	−0.03346	0.9910
CV%	15.8	199	0.2

3.2.3. Accuracy and precision

The accuracies for all tested concentrations should be within ±15%, and the precisions should not exceed 15%, except for the LLOQ, in which case these parameters should not exceed 20%. The accuracies and intra- and inter-assay precisions for the tested concentrations (LLOQ, QCL, QCM, QCH) were all within those predefined acceptance criteria (Table 3).

3.2.4. Selectivity and specificity

To test for interference, 6 different sources of plasma and serum were analyzed as blanks and after addition of nilotinib at the LLOQ. The endogenous responses in blank plasma and serum were always less than 2.5 and 7.0%, respectively, of the signal at the LLOQ (mean 1.0%; CV 80% for plasma; mean 3.6%; CV 75% for serum).

3.2.5. Extraction and ion-suppression

Extraction recoveries of nilotinib from plasma and serum were >80%, and matrix effect was less than 15%, see Table 4.

Table 2
Assay performance data of the calibration samples for nilotinib in human plasma and serum.

Plasma concentration (ng/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
5	100.3	4.6	0.5
10	100.6	1.6	5.5
30	99.5	3.7	–*
100	104.5	2.7	6.0
300	101.7	2.5	2.9
1000	106.1	2.4	3.0
3000	97.7	1.6	4.0
5000	94.6	2.7	6.1
Serum concentration (ng/mL)	**Accuracy (%)**	**Intra-assay precision (%)**	**Inter-assay precision (%)**
5	103.1	8.1	–*
10	97.5	7.6	–*
30	97.0	6.4	–*
100	95.9	4.7	–*
300	111.2	13.9	2.7
1000	101.0	12.1	–*
3000	103.9	10.1	–*
5000	109.7	11.0	–*

$N = 9$; triplicate results, each in 3 separate runs, for each concentration.
* The mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variation due to the performance of the assay in different runs [12].

Table 3
Assay performance data for the quantitation of LLOQ, QCL, QCM and QCH nilotinib concentrations in human plasma and serum.

Plasma concentration (ng/mL)	Accuracy (%)	Intra-assay precision (%)	Inter-assay precision (%)
5 (LLOQ)	92.1	3.6	2.2
15 (QCL)	97.4	4.2	2.4
500 (QCM)	101.9	2.6	1.9
4000 (QCH)	94.3	2.5	4.6
Serum concentration (ng/mL)	**Accuracy (%)**	**Intra-assay precision (%)**	**Inter-assay precision (%)**
5 (LLOQ)	101.6	7.8	2.1
15 (QCL)	97.4	5.1	5.6
500 (QCM)	109.5	4.8	0.2
4000 (QCH)	108.3	4.2	–*

$N = 18$; 6 replicate results, each in 3 separate runs, for each concentration.
* The mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variation due to the performance of the assay in different runs [12].

3.2.6. Stability

The stability of a nilotinib stock solution held at 4 °C and in the dark for 10 months was 92.6% (CV 3.9%). The stabilities of a nilotinib stock solution and nilotinib QCL, QCM, and QCH solutions in plasma or serum at room temperature for 4 h, and the stabilities of nilotinib in plasma or serum during freeze–thaw cycling and for 3 months at −80 °C are shown in Table 5. After 72 h in the autosampler at ambient temperature, nilotinib plasma samples showed a stability of 86.0 (CV 11.9%) to 512.0% (CV 39.5%) in absolute counts. However, the ratio of nilotinib to internal standard was stable at 98.9 (CV 2.9%) to 102.6% (CV 2.0%). Similarly, after 72 h in the autosampler at ambient temperature, reconstituted nilotinib serum samples showed a stability of 218.2 (CV 49.8%) to 234.6% (CV 16.7%) in absolute terms. However, the ratio of nilotinib to internal standard was stable at 101.2 (CV 4.4%) to 110.5% (CV 7.2%).

3.2.7. Parallelism

The mean accuracy of diluted plasma samples ($N = 3$) was 94.3%, with a CV of 1.9% at 10 μg/mL, and 100.2%, with a CV of 0.2% at 50 μg/mL. For serum samples, mean accuracy ($N = 3$) was 91.0%, with a CV of 5.4% at 10 μg/mL, and 110.9%, with a CV of 2.2% at 50 μg/mL.

3.3. Applicability to biological samples

We applied the assay to samples obtained from a study subject who received a 400 mg oral dose of nilotinib, which is the lowest standard dose approved by the FDA for treatment of CML. The assay was capable of quantitating nilotinib concentrations in all post-treatment samples from this subject (Fig. 3).

Table 4
Recoveries of nilotinib from human plasma and serum, and ion-suppressions in human plasma and serum extract, with coefficients of variation (CV).

Plasma concentration (ng/mL)	Recovery (%)	CV (%)	Matrix effect (%)	CV (%)
15 (QCL)	87.1	15.1	−12.0	25.1
4000 (QCH)	83.0	8.0	13.3	8.5
Serum concentration (ng/mL)	**Recovery (%)**	**CV (%)**	**Matrix effect (%)**	**CV (%)**
15 (QCL)	107.3	13.5	9.5	17.4
4000 (QCH)	114.3	8.6	−2.5	11.5

$N = 3$, for each concentration.

Table 5
Stability of nilotinib under varying conditions.

Storage condition	Concentration (ng/mL)		Stability (%)	CV (%)	Replicates
Stock solution 4 h					
Ambient temp.	500,000		96.5	4.2	3
Stock solution 10 months					
4 °C	500,000		92.6	3.9	3
Plasma 4 h					
Ambient temp.	QCL	15	94.4	8.9	3
	QCM	500	95.7	6.8	3
	QCH	4000	95.1	5.6	3
Plasma 3 freeze–thaw cycles					
−80 °C	QCL	15	94.9	9.3	3
	QCM	500	100.4	3.4	3
	QCH	4000	99.0	2.7	3
Plasma 3 months					
−80 °C	QCL	15	89.7	3.9	3
	QCM	500	93.4	4.4	3
	QCH	4000	94.2	4.2	3
Serum 4 h					
Ambient temp.	QCL	15	104.5	2.7	3
	QCM	500	103.7	4.9	3
	QCH	4000	107.4	7.9	3
Serum 3 freeze–thaw cycles					
−80 °C	QCL	15	97.5	2.5	3
	QCM	500	101.1	4.2	3
	QCH	4000	105.7	7.5	3
Serum 7 months					
−80 °C	5[a]		106.6	1.1	3
	200[a]		101.8	0.8	3
	5000[a]		100.6	6.1	3

[a] Stability results provided by Novartis.

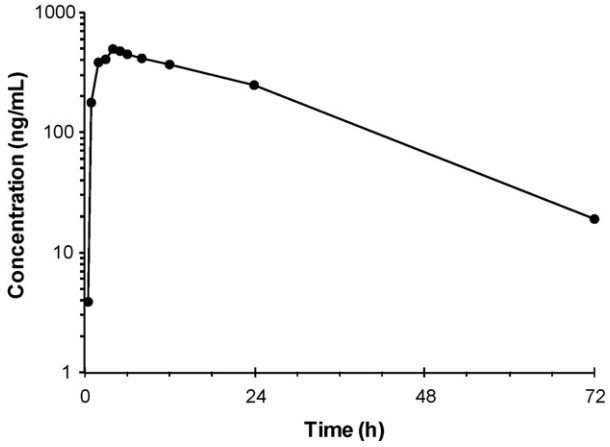

Fig. 3. Time course of nilotinib in plasma of a study subject given 400 mg of nilotinib orally.

4. Discussion

Although an HPLC assay for nilotinib with an identical concentration range in plasma has been published previously [10], its suitability for clinical pharmacology studies of nilotinib is decreased by the use of perchloric acid as the deproteinating agent, the lack of an internal standard, the relatively cumbersome sample preparation, the use of an on-line enrichment step, and the lower selectivity related to its use of absorbance detection. In addition, the published method uses 300 μL of plasma and uses most of the sample for a one-time injection, excluding the possibility of a reinjection. The advantage of the published method over our method is the absence of an evaporation step. The LC–MS method described in the current manuscript is: facile; sufficiently sensitive; applicable to biological matrices; and capable of being implemented in laboratories with standard LC–MS instrumentation. In addition, our assay has been validated according to the most recent FDA guidelines [11].

The incentive for performing additional clinical pharmacology studies of nilotinib is multifactorial. Although nilotinib has striking activity against CML, and is licensed for treatment of that disease, its complete spectrum of activity remains undefined [9]. Numerous studies are actively investigating the utility of nilotinib treatment in a variety of solid and hematologic malignancies. Additional studies are investigating the potential for combining nilotinib with various other antitumor agents as disparate as traditional cytotoxic drugs and monoclonal antibodies. In many of these studies, there is a need to evaluate potential pharmacokinetic/pharmacodynamic relationships and/or potential drug–drug interactions. Of even more importance may be the need to evaluate a number of critical pharmacokinetic issues, such as therapeutic drug monitoring, related to the use of nilotinib in CML for which it has documented activity and is licensed for use. These obvious and overwhelming arguments for generating increased pharmacokinetic data in patients treated with nilotinib are the basis of an equally obvious need for a widely applicable, sensitive and facile assay for nilotinib in biological matrices such as plasma and serum. The LC–MS assay described in this communication fulfills this requirement. The overall applicability is evidenced by the use of this method in a variety of clinical trials at our institution and a large number of collaborative efforts ongoing with other investigators.

Acknowledgements

We thank Mr. Jeremy A. Hedges for excellent secretarial assistance in the preparation of this manuscript. We also thank

Drs. Laurie Letvak, August Salvado, Yanfeng Wang, and Tom Smith for encouragement, logistical support, and funding of our effort to develop this assay. Supported, in part, by a grant from Novartis Pharmaceuticals, grant NCI 2P30 CA47904, and grant NIH/NCRR/GCRC/#5M01RR00056.

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