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LC-MS/MS assay for the quantitation of the tyrosine kinase inhibitor neratinib in human plasma

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Abstract

Neratinib is an orally available tyrosine kinase inhibitor targeting HER2 (ERBB2) and EGFR (ERBB). It is being clinically evaluated for the treatment of breast and other solid tumors types as a single agent or in combination with other chemotherapies. In support of several phase I/II clinical trials investigating neratinib combinations, we developed and validated a novel LC-MS/MS assay for the quantification of neratinib in 100 μ L of human plasma with a stable isotopic internal standard. Analytes were extracted from plasma using protein precipitation and evaporation of the resulting supernatant followed by resuspension. Chromatographic separation was achieved using an Acquity UPLC BEH Shield RP18 column and a gradient methanol-water mobile phase containing 10% ammonium acetate. An ABI 4000 mass spectrometer and electrospray positive mode ionization were used for detection. The assay was linear from 2–1,000 ng/mL and proved to be accurate (98.9–106.5%) and precise (<6.2% CV), and met the FDA guidance for bioanalytical method validation. This LC-MS/MS assay will be an essential tool to further define the pharmacokinetics of neratinib.

Graphical abstract

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Keywords

neratinib; tandem mass spectrometry; assay; validation

1 Introduction

The oncogenes EGFR (ERBB) and HER-2 (ERBB2) are receptor tyrosine kinases (RTK) implicated in multiple cancer pathways [1]. Overexpression has been identified in breast cancer and other solid tumour types [1].

Neratinib is a small molecule tyrosine kinase inhibitor targeting EGFR and HER-2 RTKs [2]. Chemically, it is a Michael acceptor, which allows it to covalently bind to its target, thereby increasing its pharmacological potency [3, 4]. Neratinib is one of several Michael acceptors currently in development [5]. To date, more than 40 clinical trials have been conducted to evaluate neratinib alone or in combination with other agents. Phase I examination of single agent neratinib administration showed a $C_{\max}$ of 5.8-119 ng/mL over a 40 to 400 mg dose range [6]. Pharmacokinetic analysis during dose escalation identified nonlinear dose-AUC and dose- $C_{\max}$ relationship at and above the MTD of 320 mg which is above the recommended phase II dose of 240 mg [6]. This study also identified 4 h as the time of peak concentrations and a $t_{1/2}$ of 14.9 h (± 4.1). Early studies have shown large inter-individual variability and a suggested reliance of response on achieving a minimum efficacious exposure of 431 ng•h/mL as established in a preclinical mouse model, reinforcing the need for further examination of pharmacokinetics [6].

In support of several phase I/II clinical trials investigating neratinib combinations, we developed and validated to FDA guidance a novel LC-MS/MS assay for the quantification of neratinib in human plasma and applied it to clinical samples.

2 Experimental

2.1 Chemicals and reagents

Neratinib maleate (purity > 99.99%) mwt 673.1 g/mol was provided by Excella GmbH (Feucht, Germany). Because the drug substance used clinically, and therefore the reported doses, reflect the free base neratinib, all concentrations hereafter reflect free base neratinib concentrations. The internal standard, [$^2\text{H}_6$]-neratinib (purity > 99.91%), was purchased from Toronto Research Chemicals (North York, Ontario, Canada), see Fig. 1 for location of stable label. Acetonitrile, water, methanol (all HPLC grade) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium formate, ammonium acetate and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Control human plasma was purchased from Valley Biomedical (Winchester, VA, USA) and Lampire (Everett, PA, USA).

2.2 Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1200 SL autosampler and binary pump, a Phenomenex (Torrance, CA) Acquity UPLC BEH Shield RP18 (1.7 μm , 3 mm $\times$ 50 mm) column, a gradient mobile phase. Mobile phase solvent A consisted of methanol:water (90:10, v/v) with 10 mM ammonium acetate buffer and 0.1% formic acid, and mobile phase solvent B consisted of water with 10 mM ammonium acetate buffer and 0.1% formic acid. A 0.3 mL/min flowrate was maintained throughout the duration of analyses. The mobile phase composition was 60% solvent A and 40% solvent B pumped from zero to 0.2 min. Between 0.2 and 1.0 min, solvent A was increased to 100% and maintained until 3.0 min followed by a return to initial conditions between 3.1 and 6 minutes. The total run time was 6 min and the autosampler temperature was 4 °C analysis. The injection volume was 5 μL .

2.3 Mass spectrometry

Mass spectrometric detection was carried out using ABI SCIEX (San Jose, CA, USA) 4000 hybrid linear ion trap tandem mass spectrometer with electrospray ionization in positive-ion, multiple reaction monitoring (MRM) mode. The settings of the mass spectrometer in positive mode scanning parameters were as follows: curtain gas 12, IS voltage 50 V, probe temperature 500°C, GS1 50, GS2 50, declustering potential 50 V, a collision energy of 38 V, and an exit potential of 15 V. The MRM m/z transitions monitored were: 557.0>512.0 for neratinib and 563.0>512.0 for [$^2\text{H}_6$]-neratinib. The LC system and mass spectrometer were controlled by Analyst software (version 1.4.2), and data were collected with the same software.

2.4 Preparation of calibration standards and quality control samples

Stock solutions of analyte neratinib and internal standard [$^2\text{H}_6$]-neratinib were prepared independently at 1 mg/mL in dimethyl sulfoxide and stored at -80 °C. Neratinib stock concentrations were prepared from the maleate salt (673.1 g/mol) in such a manner to reflect 1 mg/mL of the free base (557.0 g/mol). A serial dilution of the 1.0 mg/mL stock solutions was prepared to make 0.1 mg/mL working stock solutions in acetonitrile/water (50/50, v/v). On the day of assay, the internal standard was diluted 500 times and the analyte solution

serially diluted (in steps of 10-fold) in acetonitrile/water (50/50, v/v) to obtain the lower calibration working solutions. These calibration working solutions were diluted in human EDTA plasma to produce the following analyte concentrations: 2, 5, 10, 30, 100, 300, and 1,000 ng/mL. For each calibration series, zero and blank samples were also prepared from control plasma.

Quality control (QC) stock solutions were stored at -80 °C. These solutions were diluted in human plasma to produce the following QC samples of either: QC Lower Limit of Quantification (QCLL) 2 ng/mL, QC Low (QCL) 4 ng/mL; QC Mid (QCM) 50 ng/mL, and QC High (QCH) 800 ng/mL.

2.5 Sample preparation

To each microcentrifuge tube, 100 µL of each sample (standard, QC or sample plasma), 10 µL of 0.2 µg/mL [²H₆]-neratinib and 50 µL of 50 mM ammonium formate were added and vortexed for 1 min on a Vortex Genie-2 set at 8 (Model G-560 Scientific Industries, Bohemia, NY, USA). To each tube, 500 µL of acetonitrile was added followed by vortexing for 2 min. Samples were centrifuged at 13,000 × g at room temperature for 5 min. The resulting supernatants were transferred to borosilicate glass tubes (12 × 75 mm) and dried under a gentle stream of nitrogen using a Multivap Nitrogen Evaporator (Organomation Associates, Berlin, MA). Samples were reconstituted in 100 µL of starting mobile phase (mobile phase A:mobile phase B (60:40,v/v)) and transferred into autosampler vials, followed by injection of 5 µL into the LC-MS/MS system.

2.6 Validation procedures

2.6.1 Calibration curve and lower limit of quantitation (LLQ)—Decreasing concentrations of analytes were injected into the analytical system to determine the minimal concentration with a signal-to-noise ratio of at least 5:1. Calibration standards and blanks were prepared (see paragraph 2.4) 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 analyte peak by the area of the internal standard peak. Standard curves were constructed individually by plotting the analyte-to-internal standard ratio *versus* the known concentrations in each sample. Standard curves were fitted by linear regression with weighting by $1/y^2$, followed by back-calculation of concentrations. The deviations of these back-calculated concentrations from the nominal concentrations were expressed as percentage of the nominal concentration.

2.6.2 Accuracy and precision—The accuracy and precision of the assay were determined by analyzing samples at the QCLL, QCL, QCM, and QCH concentrations in 6 replicates each in 3 analytical runs, together with independently prepared, triplicate calibration curves. Accuracy was calculated at each test concentration as: (mean measured concentration / nominal concentration) × 100%.

Assay precision was calculated by ANOVA as previously described [7], by using SPSS 23.0 for Windows (SPSS Inc., Chicago, IL, USA). Back-calculated concentrations of calibration and QC samples were entered with the run number as factor. From the resulting mean

squares of the within runs and mean squares of the between runs, the intra-assay and inter-assay precisions were calculated.

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 were processed and analyzed according to the described procedure. Responses of analytes at the LLQ concentrations were compared with the response of the blank samples. Cross-talk of neratinib and [$^2\text{H}_6$]-neratinib were characterized by detection in their respective MRM channels by separate injections of neat solutions prepared at 10,000 ng/mL.

2.6.4 Extraction recovery and ion-suppression—We determined the extraction recovery of neratinib from plasma by comparing the absolute response of an extract of control plasma to which this analyte had been added after extraction, with the absolute response of an extract of plasma to which the same amount had been added before extraction. The ion-suppression by plasma matrix components was defined as the decrease of the absolute response of an extract of control plasma to which analyte had been added after the extraction relative to the absolute response of reconstitution solvent to which the same amount of analyte had been added. Experiments were performed at the QC concentrations, in quadruplicate.

2.6.5 Stability—Long-term stability experiments were performed in plasma and in stock solution after storage at $-80\text{ }^{\circ}\text{C}$. Stability in the stock solution was expressed as the percentage recovery of the stored solution (12 months) relative to a fresh solution. Long-term stability of neratinib in plasma was assessed by comparing freshly prepared QCs to those stored at 3 and 12 months. The stability of neratinib in plasma at $-80\text{ }^{\circ}\text{C}$ was determined by assaying samples before and after storage. In addition, the stability of neratinib in stock solution at room temperature for 6 h was determined in triplicate. All stability testing in plasma was performed at the QCLL, QCL, QCM and QCH concentrations. The effect of 3 freeze/thaw cycles analyte concentrations on plasma was evaluated by assaying samples after they had been frozen ($-80\text{ }^{\circ}\text{C}$) and thawed on 3 separate days and comparing the results with those of freshly prepared samples. The stability of neratinib in plasma during sample preparation was evaluated by assaying samples before and after 4 h of storage at room temperature. To evaluate the stability of neratinib in reconstituted samples in the autosampler, we re-injected QC samples and calibration curves approximately 72 h after the first injection and compared the concentrations and absolute responses derived from the second injection with those derived from the first injection. The results of the second run were expressed as a percentage of their respective values in the first run.

2.6.6 Dilutional integrity—To demonstrate dilutional integrity, the ability to dilute samples from above the upper limit of quantitation to within the validated concentration range, plasma samples containing neratinib above the upper limit of quantitation were diluted to within the assay range. Plasma samples (N=3) with analyte concentrations of 10,000 ng/mL were diluted 20-fold (to 500 ng/mL) with control plasma and assayed.

2.6.7 Anti-coagulantia cross validation—To demonstrate the ability of our heparin plasma-based assay to quantitate EDTA plasma samples, we quantitated our heparinized QCLL, QCL, QCM, and QCH samples (N=4) against an EDTA plasma triplicate calibration curve.

2.7 Application of the Assay

To demonstrate the application of the assay, human plasma samples from a clinical trial evaluating neratinib in combination with trastuzumab emtansine in women with HER2-positive breast cancer (Clinicaltrial.gov NCT02236000) were analysed to determine plasma concentrations of neratinib.

3 Results and Discussion

3.1 Assay Validation

3.1.1 Chromatography—The approximate retention time of both neratinib and the [$^2\text{H}_6$]-neratinib internal standard was 2.8 min. At a void time of 1.1 min, neratinib had a retention factor of 1.8. Representative chromatograms of neratinib (at the LLQ), and internal standards in plasma are displayed in Fig. S1.

3.1.2 Calibration curve and LLQ—According to the FDA guidance for bioanalytical method validation [8], the calibration curve adequately describes the concentration *versus* response relationship if the observed deviation and precision are 20% for the LLQ and 15% for all other calibration concentrations. At least 4 of 6 calibration points should meet the above criteria [8].

The selected assay range of 2-1,000 ng/mL fulfilled the FDA criteria for the LLQ concentration and the calibration curve. Accuracies and precisions at the different concentrations were determined from triplicate calibration curves on 3 separate days. Accuracy ranged from 97.8 to 104.2%. Intra-assay precision was below 5% with the exception of the LLQ that was 8.6%. Inter-assay precision yielded similar results. At some concentrations, the mean square of the within runs was greater than the mean square of the between runs, indicating that there was no significant additional variability due to the performance of the assay in different runs [7]. Accuracy and precision for all calibrators are reported in Table S1. A representative calibration curve, residual plot, and corresponding correlation and regression coefficients are shown in Fig. S2.

3.1.3 Accuracy and precision—FDA guidance specifies that the accuracies for all tested concentrations should be within $\pm 15\%$. Moreover, the precisions should not be $> 15\%$ CV except for the LLQ, in which case these parameters should not exceed 20% [8].

The accuracies and intra- and inter-assay precisions for the tested concentrations (QCLL, QCL, QCM, and QCH) were all within the defined acceptance criteria. Accuracy ranged from 98.9 to 106.5%. Intra and inter-assay precision were $< 10\%$ (Table S2).

3.1.4 Selectivity and specificity—According to FDA guidance, the signal at the LLQ must be at least 5 times the signal of any co-eluting peaks [8].

Chromatograms of six individual control plasma samples contained no co-eluting peaks >20% of the analyte areas at the LLQ concentration (interference <19.6%).

Cross-talk calculations were performed at 10,000 ng/mL and revealed that the internal standard cross-talks into the neratinib channel at approximately 0.12% with identical retention times. The internal standard is added at approximately 200 ng/mL, and $0.12\% \cdot 200 = 0.24$ ng/mL is less than 20% at the LLQ of 2 ng/mL. Neratinib cross-talks into the internal standard channel at approximately 0.07% and even at the upper limit of quantitation of 1,000 ng/mL the corresponding 0.7 ng/mL-equivalent would not meaningfully interfere with the approximate 200 ng/mL internal standard concentration.

3.1.5 Extraction recovery and ion-suppression—The FDA-guidance stipulate that extraction recovery be consistent and precise [8]. A recovery of 70% with a variation of 15% is generally accepted [7, 8]. There is no specific requirement for ion-suppression. Ultimately, the assay performance, as expressed in the precision and accuracy, is most relevant; however, a large and/or variable ion-suppression may result in lack of assay robustness.

The recovery of neratinib ranged from 77.7 to 88.7% (CV<15.2%). Ion-suppression ranged from -20.7 to -8.8% (CV<15.6%) meaning ionization was slightly enhanced by the presence of plasma (Table S3).

3.1.6 Stability—Stability in biological samples is acceptable when 85% of the analyte is recovered. The stability of neratinib stock solution at room temperature for 6 h was 90.3% (Table 1). Stability in stock solution for 10 months at -80 °C was 105.4%. The stability of neratinib in plasma at room temperature for 4 h was between 91.4 and 102.2% (CV<12.0%). The stability of the analyte after 3 freeze thaw cycles (-80 °C to RT) was between 91.7 and 108.6% (CV<10.6%). Long-term stability of the analyte in plasma at -80 °C was adequate with recovery between 92.4 and 101.4% (CV<11.0%). The absolute responses of plasma extracts of neratinib at the calibration concentrations, when reconstituted and kept in the autosampler for 72 h, were 71.2 to 77.1% (CV<14.7%) of the initial responses while the response of neratinib relative to the internal standard signal ranged from 100.3 to 104.9% (CV<12.5%). Importantly, the reinjection run passed the requirements of any run set by the FDA [7].

3.1.7 Dilutional integrity—The samples diluted from 10 µg/mL to 500 ng/mL displayed 105.1% accuracy with a CV of 3.3%. This result indicates dilutional integrity for neratinib.

3.1.1 Anti-coagulant cross validation—Accuracy and precision of back-calculated concentrations at the QCLL, QCL, QCM, and QCH levels ranged from -6.4 to 6.3% (CV<8.6%). Results are provided in Table S4.

3.2 Development

3.2.1 Mass Spectrometry—The analytes were scanned in both negative and positive ionization modes and positive ionization was determined to have optimal sensitivity. Analytes were tuned separately using 1 µg/mL neat solutions by both direct injection and

infusion. The $[M+H]^+$ ion was the major m/z identified for analytes resulting in an m/z of 557.0 for neratinib and correspondingly 563.0 for the internal standard. Methanol and acetonitrile were both evaluated as the organic solvent and tuning determined signal intensity was maximized using methanol. Based on a previous description of the use of ammonium acetate buffer (AAB) in the analysis of neratinib [9], we evaluated the introduction of 10 mM ammonium acetate buffer (AAB) in the aqueous phase, which increased signal strength. This component was then added to the methanol based organic phase as well. The addition of 0.1% formic acid to both mobile phases further increased signal intensity. These efforts culminated in the mobile phases composed of methanol and water with each containing 10 mM AAB and 0.1% formic acid.

Evaluation of neratinib fragmentation (m/z 557.0) demonstrated it had two major product ions at m/z 521.4 and 512.0. The internal standard had similar patterns of fragmentation with product ions at m/z 527.1 and 512.0. Parameter optimization of mass spectrometer settings revealed the MRM transitions with the highest signal intensity were 557.0>512.0 for neratinib and 563.0>512.0 for the internal standard at a collision energy of 38 V.

3.2.2 Extraction and sample preparation—A dilute-and-shoot method was initially evaluated to expedite sample preparation but sensitivity was below our desired lower limit of quantitation. Therefore, an extraction using acetonitrile protein precipitation was evaluated. A sample volume of 100 μ L and 500 μ L acetonitrile were used to achieve extraction through protein precipitation. Neratinib was reported as being unstable at a pH of 7 [10]. In an attempt to ensure stability by buffering the pH of plasma, 50 μ L of 50 mM ammonium formate with 0.1% formic acid was added prior to extraction. This method yielded acceptable recoveries.

3.2.3 Chromatography—The following columns were evaluated: Synergi Polar RP 80A (100 $\times$ 2.0 mm, 4 μ m), Synergi Phenyl-hexyl (50 $\times$ 2.0 mm, 4 μ m), Synergi Hydro RP 80A (50 $\times$ 2.0 mm, 4 μ m) and Waters Acquity UPLC C18 BEH (1.7 μ m, 50 $\times$ 2.1 mm). Optimal mobile phases were established during mass spectrometer tuning (see above). Mobile phase A was methanol:water (90:10, v/v) with 10 mM ammonium acetate buffer and 0.1% formic acid and mobile phase B was water with 10 mM ammonium acetate buffer and 0.1% formic acid. Each of the above columns was evaluated using these mobile phases at various gradients and a 0.3 mL/min flow rate.

The Polar RP column demonstrated retention (7.2 min) using isocratic 30% mobile phase B but produced significant peak shouldering as well as slightly differing analyte and IS retention times (0.1 min). Increasing the %B to 80% shifted retention to 3.2 min and produced the same poor peak shape. To shorten retention time and total run time, shorter columns (50 mm) were evaluated moving forward. The phenyl-hexyl column exhibited poor chromatography with multiple peaks eluting. The Hydro RP column exhibited extensive peak tailing. The Acquity UPLC C18 BEH column produced sufficient retention and good peak shape (no shouldering or tailing) and was chosen as the most suitable column. Optimization of gradient methods for this column demonstrated that neratinib interacted strongly with the column and elution occurred only once mobile phase A composition reached 100% (90% methanol). A steep gradient was chosen to allow a total run time of 6

min. The mobile phase composition was 60% solvent A and 40% solvent B pumped from zero to 0.2 min. Between 0.2 and 1.0 min, solvent A was increased to 100% and maintained until 3.0 min followed by a return to initial conditions between 3.1 and 6 minutes.

3.2.4 Chemical Stability—Throughout analyses, it was observed that absolute peak areas decreased throughout a sample sequence. This within-batch decrease in peak areas was observed by continually dropping internal standard area values. The 72 h autosampler stability test confirmed this effect.

Experiments were performed to investigate this effect. A triplicate standard curve and QC sequence of 45 samples was injected and monitored the decrease in internal standard signal directly after being prepared and after sitting in the autosampler for 72 h (Fig. 2A). Analysis of the trendlines produced from this experiment reported a decrease in internal standard response of approximately -3.3%/h. Comparisons between the responses of reinjection of samples 72 h later showed the mean percent decrease was 31.0% ($SD \pm 10.7\%$), as seen in Fig. 2B. The analyte/internal standard response ratio used to calculate the regression curve had a nominal difference of -3.9% ($SD \pm 5.9\%$) during the same time period (Fig. 2B) allowing for appropriate performance of the assay. These experiments demonstrate that while neratinib and the internal standard both experience signal degradation in the autosampler, the effects of degradation are equal between analyte and IS and the total effect is nullified by using the ratio of neratinib to stable-labelled neratinib internal standard.

To determine if the source of degradation could be attributed to thiol bearing plasma components which are known to react with neratinib [11], alternating injections of 1 $\mu\text{g/mL}$ neratinib in either plasma or neat mobile phase were made over the course of 24 h. The decrease in analyte signal in plasma and mobile phase was observed in approximately equal extents and indicated matrix components were not required for decreases in signal strength (Fig. 2C). The degradation rates of neratinib signal in plasma and a neat mobile phase solution were approximately -1.6%/h and -2.1%/h, respectively.

3.3 Application of the Assay

We applied the assay to samples obtained from a patient that received a 120 mg oral dose of neratinib. The patient had signed informed consent on an institutional review board approved protocol (Clinicaltrial.gov NCT02236000). The assay was capable of quantitating neratinib as seen in Fig. 3. The C_{max} was 43.4 ng/mL and is similar to the previously published C_{max} from patients administered the same dose of neratinib (47.6 ± 33.9 ng/mL) [6].

3.4 Incurred sample reanalysis and external validation

Upon completion of the validation, incurred sample reanalysis was performed on samples from 2 ongoing clinical trials being supported with his assay (NCT02236000; NCT01960023). Reanalysis of 27 samples yielded an average difference of -0.7%, an average absolute difference of 5.5%, a range of -10.2% to 14.5%, with no sample exceeding 20% difference [12].

4 Discussion

At 240 mg, the phase II recommended dose of neratinib, a $C_{\max}$ of 68.6 ng/mL ($SD \pm 47$) was reported [13, 14]. Clinical trough neratinib concentrations at steady-state range from 52 to 59 [13]. As also illustrated by our clinical sample analysis, our assay has an appropriate concentration range to support ongoing and future clinical trials.

In clinical trial reports so far, analytical details are either not provided at all [14-17], or a general description is provided without performance characteristics [6, 9]. The latter description details 0.25 mL of plasma sample for an assay over the range of 3-250 ng/mL, a protein precipitation sample preparation, a non-stable isotope internal standard (WAY-178357, HKI-357, PF-5208766, a neratinib analogue where the pyridine is replaced by a 3-fluoro-phenyl moiety), a Genesis C18 column, and a 3-component mobile phase gradient of 50 mM ammonium acetate (pH 4.5) : acetonitrile : methanol (8:1:1 and 1:5:4, v:v:v), followed by detection using a Sciex API 4000 mass spectrometer.

More recently, one publication reports a fluorescence assay for neratinib in buffers with an LLQ of 100 ng/mL [18], and another reports an UPLC-MS/MS assay for bioanalytical purposes [19]. The latter assay utilized double the plasma volume for a 4-fold shorter assay range of 4-500 ng/mL, with a 2-step protein precipitation, had a shorter run-time, and utilized crizotinib as internal standard. Our results showing a decrease in signal over time in the autosampler, potentially related to the reactive nature of neratinib as a Michael acceptor, suggest that the use of a stable isotope internal standard as employed in our assay, is advisable. Our assay is the first to utilize a stable isotope internal standard, which is expected to minimize variability and increase robustness.

In summary, we developed and validate a rapid, facile assay for the quantitation of neratinib in human plasma with a range of 2-1000 which adequately covers the expected clinical concentration range, as illustrated by the analysis of clinical samples and reporting of ISR performance. Advantages of our assay over those previously reported include a lower limit of quantitation, a short sample preparation and analysis time, and determination that the assay is resilient to the observed signal decay. By providing quantitative pharmacokinetic information, our assay will be a valuable tool to support further clinical development of neratinib.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Herbst RS. Review of epidermal growth factor receptor biology. International journal of radiation oncology, biology, physics. 2004; 59(2 Suppl):21-6.

2. Minami Y, Shimamura T, Shah K, LaFramboise T, Glatt KA, Liniker E, Borgman CL, Haringsma HJ, Feng W, Weir BA, Lowell AM, Lee JC, Wolf J, Shapiro GI, Wong KK, Meyerson M, Thomas RK. The major lung cancer-derived mutants of ERBB2 are oncogenic and are associated with sensitivity to the irreversible EGFR/ERBB2 inhibitor HKI-272. *Oncogene*. 2007; 26(34):5023–7. [PubMed: 17311002]
3. Krishnan S, Miller RM, Tian B, Mullins RD, Jacobson MP, Taunton J. Design of reversible, cysteine-targeted Michael acceptors guided by kinetic and computational analysis. *Journal of the American Chemical Society*. 2014; 136(36):12624–30. [PubMed: 25153195]
4. Singh J, Petter RC, Baillie TA, Whitty A. The resurgence of covalent drugs. *Nature reviews, Drug discovery*. 2011; 10(4):307–17. [PubMed: 21455239]
5. Kwak E. The role of irreversible HER family inhibition in the treatment of patients with non-small cell lung cancer. *The oncologist*. 2011; 16(11):1498–507. [PubMed: 22016476]
6. Wong KK, Fracasso PM, Bukowski RM, Lynch TJ, Munster PN, Shapiro GI, Janne PA, Eder JP, Naughton MJ, Ellis MJ, Jones SF, Mekhail T, Zacharchuk C, Vermette J, Abbas R, Quinn S, Powell C, Burris HA. A phase I study with neratinib (HKI-272), an irreversible pan ErbB receptor tyrosine kinase inhibitor, in patients with solid tumors. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009; 15(7):2552–8. [PubMed: 19318484]
7. Rosing H, Man WY, Doyle E, Bult A, Beijnen JH. Bioanalytical Liquid Chromatographic Method Validation. A Review of Current Practices and Procedures. *Journal of Liquid Chromatography & Related Technologies*. 2000; 23(3):329–354.
8. U.S. Department of Health and Human Services Food and Drug Administration, Guidance for Industry-Bioanalytical Method Validation, U.S.Department of Health and Human Services; Food and Drug Administration; Center for Drug Evaluation; and Research Center for Veterinary Medicine, 2001.
9. Abbas R, Hug BA, Leister C, Burns J, Sonnichsen D. Pharmacokinetics of oral neratinib during co-administration of ketoconazole in healthy subjects. *British journal of clinical pharmacology*. 2011; 71(4):522–7. [PubMed: 21395644]
10. Lu Q, Ku MS. Preformulation stability study of the EGFR inhibitor HKI-272 (Neratinib) and mechanism of degradation. *Drug development and industrial pharmacy*. 2012; 38(3):307–13. [PubMed: 21854251]
11. Chandrasekaran A, Shen L, Lockhead S, Oganessian A, Wang J, Scatina J. Reversible covalent binding of neratinib to human serum albumin in vitro. *Drug metabolism letters*. 2010; 4(4):220–7. [PubMed: 20690900]
12. Fast DM, Kelley M, Viswanathan CT, O'Shaughnessy J, King SP, Chaudhary A, Weiner R, DeStefano AJ, Tang D. Workshop report and follow-up--AAPS Workshop on current topics in GLP bioanalysis: Assay reproducibility for incurred samples--implications of Crystal City recommendations. *The AAPS journal*. 2009; 11(2):238–41. [PubMed: 19381839]
13. Burstein HJ, Sun Y, Dirix LY, Jiang Z, Paridaens R, Tan AR, Awada A, Ranade A, Jiao S, Schwartz G, Abbas R, Powell C, Turnbull K, Vermette J, Zacharchuk C, Badwe R. Neratinib, an irreversible ErbB receptor tyrosine kinase inhibitor, in patients with advanced ErbB2-positive breast cancer. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2010; 28(8):1301–7. [PubMed: 20142587]
14. Chow LW, Xu B, Gupta S, Freyman A, Zhao Y, Abbas R, Vo Van ML, Bondarenko I. Combination neratinib (HKI-272) and paclitaxel therapy in patients with HER2-positive metastatic breast cancer. *British journal of cancer*. 2013; 108(10):1985–93. [PubMed: 23632474]
15. Ito Y, Suenaga M, Hatake K, Takahashi S, Yokoyama M, Onozawa Y, Yamazaki K, Hironaka S, Hashigami K, Hasegawa H, Takenaka N, Boku N. Safety, efficacy and pharmacokinetics of neratinib (HKI-272) in Japanese patients with advanced solid tumors: a Phase 1 dose-escalation study. *Japanese journal of clinical oncology*. 2012; 42(4):278–86. [PubMed: 22371427]
16. Awada A, Dirix L, Manso Sanchez L, Xu B, Luu T, Dieras V, Hershman DL, Agrapart V, Ananthakrishnan R, Staroslawska E. Safety and efficacy of neratinib (HKI-272) plus vinorelbine in the treatment of patients with ErbB2-positive metastatic breast cancer pretreated with anti-HER2 therapy. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 2013; 24(1):109–16.

17. Gandhi L, Bahleda R, Tolaney SM, Kwak EL, Cleary JM, Pandya SS, Hollebecque A, Abbas R, Ananthakrishnan R, Berkenblit A, Krygowski M, Liang Y, Turnbull KW, Shapiro GI, Soria JC. Phase I study of neratinib in combination with temsirolimus in patients with human epidermal growth factor receptor 2-dependent and other solid tumors. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology*. 2014; 32(2):68–75. [PubMed: 24323026]
18. Maher HM, Alzoman NZ, Shehata SM. An eco-friendly direct spectrofluorimetric method for the determination of irreversible tyrosine kinase inhibitors, neratinib and pelitinib: application to stability studies. *Luminescence : the journal of biological and chemical luminescence*. 2016
19. Wani TA, Zargar S, Ahmad A. Ultra performance liquid chromatography tandem mass spectrometric method development and validation for determination of neratinib in human plasma. *South African Journal of Chemistry*. 2015; 68

Highlights

Neratinib is an orally available TKI targeting EGFR and HER2 in clinical development

An LC-MS/MS assay from 2-1000 ng/mL in 0.1 mL plasma was developed and validated.

Signal degradation was observed but corrected with isotopic internal standard.

Assay is being utilized to support neratinib combination clinical studies.

Fig. 1.
Structures of neratinib and $[^2\text{H}_6]$ -neratinib internal standard.

Fig. 2.

Signal degradation experiments: A) the internal standard area values of a 45 injection triplicate curve and QC values on the 1st day of analysis (■) followed by a 72 hour reinjection (□). B) The percent change between 72 hours of the internal standard values (△) and to the percent change of neratinib/IS ratio values (▲). C) Neratinib response of identical 1 µg/mL injections over 24 hours in separate matrices of plasma (●) and neat mobile phase (○).

Fig. 3.
Application of the assay. Concentration vs time of a patient orally administered 120 mg of neratinib.

Table 1

Stability of neratinib under varying conditions.

Storage condition	Concentration (ng/mL)	Stability (%)	CV (%)	Replicates
Neratinib				
Stock solution 6 h Ambient temp.	100,000	90.2	7.2	3
Stock solution 12 months -80 °C	100,000	105.4	7.3	3
Plasma 4 h Ambient temp.	QCLL 2	102.2	12.0	6
	QCL 4	92.1	10.3	6
	QCM 50	99.1	4.2	6
	QCH 800	91.4	5.6	6
Plasma 3 freeze-thaw cycles -80 °C	QCLL 2	108.6	7.4	6
	QCL 4	91.7	10.6	6
	QCM 50	102.2	4.5	6
	QCH 800	100.6	5.7	6
Plasma 3 months -80 °C	QCLL 2	92.4	11.0	6
	QCL 4	95.8	8.5	6
	QCM 50	101.4	6.4	6
	QCH 800	93.0	3.1	6
Plasma 12 months -80 °C	QCLL 2	99.3	5.9	4
	QCL 4	102.3	6.5	4
	QCM 50	100.5	5.2	4
	QCH 800	97.1	4.0	4