Development and Validation of Rapid Stability-Indicating RP-HPLC-DAD Method for the Quantification of Lapatinib and Mass Spectrometry Analysis of Degraded Products

Ebrahim Saadat1, Pooya Dehghan Kelishady1, Fatemeh Ravar1, Farzad Kobarfard2 and Farid A. Dorkoosh1*

1Department of Pharmaceutics, Faculty of Pharmacy, Tehran University of Medical Sciences, P.O. Box 14155-645, Tehran, Iran, and 2Department of Medicinal Chemistry, School of Pharmacy, Shahid Beheshtee University of Medical Sciences, Tehran, Iran

Ebrahim Saadat1, Pooya Dehghan Kelishady1, Fatemeh Ravar1, Farzad Kobarfard2 and Farid A. Dorkoosh1*

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A rapid, simple and stability-indicating high-performance liquid chromatography assay method was developed and validated for quantitative analysis of lapatinib (LPT) in bulk pharmaceuticals. The newly developed method was assessed using a C18 MZ-Analytical column (5 μm, 150 × 4.6 mm, OSD-3), which was protected by a (5 μm, 4.0 × 4.6 mm, OSD-3) pre-column with mobile phase that was composed of acetonitrile and water (70/30, v/v) and a detection wave length of 227 nm. The method was validated according to the ICH guidelines with respect to precision, accuracy, linearity, robustness, specificity and system suitability. Forced degradation studies were also performed for LPT to determine the stability-indicating aspect of developed method. The method was found to be specific for LPT in the presence of degradation products. The retention time of LPT was ~4 min. Accuracy of the method was found to be 2.20% bias for all tested samples. The inter- and intra-day precision of the novel method were found to be 2.84 and 2.78%, respectively. The calibration curve was linear over the concentration range of 5–80 μg/mL with a regression coefficient of 0.9990. The limits of detection and quantification were also found to be 1 and 5 μg/mL, respectively. Mass spectrometry analysis was performed in order to better characterize degraded products.

Introduction

Unlimited cell proliferation caused by growth factor receptor-mediated signaling is the main cause of many cancers. Tyrosin kinases are class of receptors that involve in the cell proliferation, differentiation and anti-apoptotic signals produced by growth factors (1). From the clinical point of view, inhibition of these series of receptors would be useful in cancer therapy because this class of drugs showed satisfactory clinical outcomes in the recent trials (2). Tyrosin kinase inhibitors are belonging to a group of compounds that block cell growth signaling by arresting the related pathways, which leads to malignant cell suppression (3).

Lapatinib (GW572016, Tykerb®) (Figure 1) is an orally available selective tyrosin kinase inhibitor, which inhibits both epidermal growth factor receptor and HER-2 receptors. It was indicated in the treatment of HER-2-positive metastatic breast cancer by FDA in 2007 (4). LPT is a highly hydrophobic compound (whose solubility in water is ~0.007 mg/mL), which is synthesized from the quinazoline core found in other tyrosin kinase inhibitors (5). It is designed chemically as N-[3-chloro-4-[(3-flurobanzyl)oxyl]phenyl]-6-[5-[(2-(methylsulfonyl)ethyl[amino]-methyl)-2-fury]-4-quinazolinamine. Liquid chromatography–electrospray–tandem mass (6) and liquid chromatography–mass spectrometry (7) methods were developed for quantitative determination of LPT in biological samples. These methods are not applicable for determination of LPT in pharmaceutical dosage forms directly and need further method development and validation. Recently, an high-performance liquid chromatography (HPLC) method was developed for determination of LPT in pharmaceutical dosage forms (8) but it was not a stability-indicating method. As a quality control point of view, stability is an important issue in pharmaceutical industry; therefore, any analytical method should preferably be stability indicating. International Conference on Harmonization (ICH) guidelines are based on stability-indicating methods that are established from forced degradation studies of drug substances (9). Stability plays an important role in analytical method development and gives a better view of drug quality during a test procedure and also suggests safe storage conditions. The purpose of stability study is to determine how the quality of drug changes under various environmental conditions such as light, humidity and heat. To our knowledge, no validated stability-indicating analytical method has been reported for determination of LPT in pharmaceutical bulk in the literature. Moreover, because LPT is approved recently, no pharmacopoeia monograph is available for assay test that is crucial for quality control department of the pharmaceutical industry. In this report, an isocratic stability-indicating HPLC method is developed for precise and rapid quantitative determination of LPT in pharmaceutical bulk. This study reports forced degradation of LPT under stress conditions such as oxidation, heat, acid and base hydrolysis and ultraviolet light. Moreover, the degraded products were also analyzed using mass spectrometry for better understanding of underlying degradation pathway.

Experimental

Chemicals and reagents

Lapatinib ditosylate (99.9% purity) was purchased from Beijing Mesochem Technology Co. (Beijing, China) and used without further purification. Acetonitrile, sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). All reagents and solutions were from either HPLC or analytical grade and used as received. HPLC-grade water was obtained from distilled water, which was passed through water deionizer system (Hastaran®, Iran) and used freshly for preparation of all solutions.

HPLC instrumentation and conditions

Sample analysis was performed by HPLC system Agilent Technologies model 1260 Infinity, which equipped with autosampler system (Agilent Technologies, USA) fitted with a 25-μL
sample loop and model G1315D diode array detector (Agilent Technologies). Chromatographic data were monitored and analyzed by Chemostation for LC systems software (B.04.02) (Agilent Technologies). Samples were analyzed using a C18 MZ-Analytical column (5 μm, 150 × 4.6 mm, OSD-3), which was protected by a (5 μm, 4.0 × 4.6 mm, OSD-3) pre-column. The mobile phase was composed of acetonitrile–water in a ratio of 70:30 (v/v) with a flow rate of 1.5 mL/min. Samples were monitored at the wavelength of 227 nm. The column temperature was set at 25 ± 8°C and 20 μL samples were injected to the HPLC system every 15 min. The mobile phase was filtered by a polytetrafluoroethylene filter prior to use (0.45 μm).

**Preparation of stock solution**
A stock solution of LPT was prepared in acetonitrile with a concentration of 1 mg/mL. The stock solution was protected from light by wrapping aluminum foil and stored at 4°C. Aliquots of the standard stock solution were transferred into 5-mL A-grade volumetric flasks by means of A-grade bulb pipettes. All solutions were made up to the volume with acetonitrile to yield the final concentrations of 5, 10, 20, 40 and 80 μg/mL.

**Validation procedure**
The newly developed HPLC method was validated according to the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceutical for Human Use (10). This method was validated with respect to linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision, specificity and robustness.

**Forced degradation studies**
Forced degradation studies were performed to assure the selectivity and also stability-indicating characteristics of the newly developed method. In these experiments, pure LPT bulk was tested under stress conditions to perform forced degradation studies. LPT is insoluble in water but soluble in acetonitrile so the acetonitrile was selected as a co-solvent in all forced degradation studies. In all forced degradation experiments, a fixed concentration of LPT (100 μg/mL) was prepared in an equal volume of acetonitrile and one of the following solutions: hydrogen peroxide, sodium hydroxide, hydrochloric acid or distilled water. After degradation experiment, each sample was diluted with mobile phase to the final concentration of 40 μg/mL and analyzed as mentioned above.

**Thermal stress studies**
For thermal stress study, bulk LPT was exposed to the dry heat (150°C) in an oven for 24 h and prepared for analysis as described above.

**Oxidation studies**
In order to perform oxidation studies, solutions of acetonitrile and H2O2 10% (50 : 50, v/v) were prepared and used freshly. Resultant samples were kept for 4 days in these solutions at room temperature and analyzed afterward.

**Acid degradation studies**
Acid degradation studies were performed by the preparation of solutions of methanol and 1 M hydrochloric acid (50/50, v/v). The resultant solutions were analyzed at room temperature after 24 h.

**Alkali degradation studies**
Samples for alkali degradation studies were prepared in acetonitrile and 0.1 M sodium hydroxide (50/50, v/v) and kept for 24 h at room temperature prior to analysis.


**Figure 1.** Chemical structure of lapatinib.
Neutral degradation studies
For neutral degradation studies, solutions were prepared in acetonitrile and water (50/50, v/v) and kept for 24 h at room temperature and analyzed afterward.

Photostability studies
Both LPT solution and powder were introduced into photostability studies to investigate the effect of irradiation on the stability of LPT in solution and solid state. Twenty milligrams of LPT powder were weighed and spread on the glass dish in a layer with maximum 2 mm thickness. A solution of LPT with a concentration of 100 µg/mL was prepared in acetonitrile and HPLC-grade water (50/50, v/v) and radiated at the wavelength range of 300–400 nm at 25°C. Control samples were treated under the same condition except that an aluminum foil was wrapped to protect from light effect. After exposure time, all samples were prepared for analysis as mentioned before.

Mass spectrometric analysis
In order to further evaluate degraded products during the forced test analyses, the LC–Mass method was performed, accordingly. For the purpose of getting significant degraded peaks, LPT was treated with 2 M HCl and 1 M NaOH in room temperature for 24 h, separately. An Agilent 6410 QQQ (triple quad) mass spectrometer (USA) connected to computer software (B.03.01 Agilent Mass Hunter workstation) for data acquisition and processing was used. A mobile phase including methanol, water and formic acid (90 : 10 : 0.1; v/v/v) was delivered to the system at a flow rate of 0.35 mL/min. Samples were introduced into the mass system (20 µL) directly and without any specific column. Mass spectrometric analyses were performed without any collision energy (collision energy = 0), whereas gas temperature was regulated at 300°C. Gas flow and nebulizer pressure were set at 10 L/min and 15 psi, respectively. Analyses were performed in 100–2000 m/z ratio range in a positive mode of electrospray ionization.

Results
HPLC method development and optimization
A C_{18} MZ-Analytical column (5 µm, 150 × 4.6 mm, OSD-3) protected with C_{18} pre-column (5 µm, 4.0 × 4.6 mm, OSD-3) at ambient temperature was used for validation of the LPT method. The composition and flow rate of mobile phase were changed in order to optimize the separation condition by means of stressed samples. The mobile phase was composed of acetonitrile and water (70/30, v/v) and the flow rate of 1.5 mL/min was selected for further studies. Applying the condition described above, all peaks of LPT were defined well and free from tailing.

Validation of the method
Linearity
Linear calibration curves (n = 3) were obtained by plotting peak areas of LPT versus concentration at five levels (5, 10, 20, 40 and 80 µg/mL) each in triplicate. Linearity was determined by least squares linear regression analysis of obtained calibration curves for five points (11, 12). Three correlation coefficients of $R_1 = 0.9982$, $R_2 = 0.9994$ and $R_3 = 0.9995$ were obtained with relative standard deviation (RSD %) values between 0.26 and 2.7%. The regression equation for calibration curve was typically calculated to be $Y = 28.936X - 29.844$ in which $Y$ is the area under the curve and $X$ corresponds to the LPT concentration. RSD% values for slope and intercept were 0.42 and 0.44%, respectively. The standard error of the regression (%) was 0.03%.

Solution stability
The stability of LPT stock solution was investigated by storing it at room temperature for 24 h and analyzing in triplicate at the time points of 6, 12 and 24 h. No significant changes (<1%) were seen in the chromatographic responses for the stock solution in comparison with freshly prepared standards.

LOD and LOQ
The LOQ was determined as a signal-to-noise ratio of ≥10 following triplicate injections of LPT. The LOQ amount for LPT with acceptable precision and accuracy was found to be 5 µg/mL. The % RSD value for these studies was 2.82%. The LOD was determined as a signal-to-noise of ≥3 following injection of LPT. The LOD for LPT was found to be 1 µg/mL.

Precision
The precision or inter- and intra-day variability are summarized in Table I. Repeatability or intra-day precision was evaluated by injection of six independent LPT solutions at three different concentrations. Inter-day precision was carried out by introducing the same three samples over the three consecutive days. Data in Table I show that the method precision has an RSD of maximum 2.78 and 2.84% for intra-day and inter-day assay, respectively.

Accuracy
The accuracy of an analytical procedure reveals the closeness of experimental values to the reference. The accuracy of LPT assay was determined in replicates (n = 6) at three concentration levels (5, 40 and 80 µg/mL). Table II summarizes the accuracy.

<table>
<thead>
<tr>
<th>Table I</th>
<th>Intra- and Inter-Assay Precision Data (n = 6)*</th>
</tr>
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<tbody>
<tr>
<td>Actual concentration (µg/mL)</td>
<td>Measured concentration (µg/mL), RSD (%)</td>
</tr>
<tr>
<td></td>
<td>Intra-day</td>
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<tr>
<td></td>
<td>Mean measured concentration (µg/mL)</td>
</tr>
<tr>
<td>5</td>
<td>5.17</td>
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<td>40.18</td>
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<td>80</td>
<td>80.93</td>
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</table>

*Data expressed as mean for “measured concentration” values.

<table>
<thead>
<tr>
<th>Table II</th>
<th>Accuracy Data of LPT (n = 6)*</th>
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</thead>
<tbody>
<tr>
<td>LPT concentration (µg/mL)</td>
<td>Interpolated concentration (mean ± SD), RSD (%)</td>
</tr>
<tr>
<td>5</td>
<td>5.11 ± 0.14</td>
</tr>
<tr>
<td>40</td>
<td>40.15 ± 0.75</td>
</tr>
<tr>
<td>80</td>
<td>80.74 ± 0.46</td>
</tr>
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</table>

*Data obtained from six replicates at each concentration.
Figure 2. HPLC chromatograms of LPT bulk: (a) untreated, (b) acid hydrolysis-degraded, (c) base hydrolysis-degraded, (d) dry-heated, (e) neutral-hydrolysis-degraded, (f) oxidative-degraded and (g) photo-degraded.
data of the developed method. In each concentration, the percent relative error was calculated and found to be <2.20.

**Specificity**
The reliable analytical method should measure the analyte in the presence of degraded products. In this regard, stress testing studies are used for both determination of the degraded products from original compound and also for establishment of the stability-indicating analytical method. It is important to note that the aim of this study is not to identify degradents of LPT but exploring the peak purity of LPT. Peak purity values for LPT in chromatograms of stress samples and untreated LPT were in the range of 0.999–1, indicating that the peaks were homogenous and so the developed assay method is selective. Typical chromatograms of untreated LPT and stressed LPT solutions under different stress conditions are shown in Figure 2.

**Ruggedness and robustness tests**
Robustness is an important issue in any analytical method with regard to ICH guidelines (13). Robustness of the method was determined by changing the condition of analysis parameters including laboratory temperature, flow rate and mobile phase composition (14). The reproducibility of the result due to the applied small changes indicates the robustness of the method. The recoveries of LPT under newly deliberate changes are tabulated in Table III, which indicates no significant changes under modified important analysis parameters (P < 0.05). The ruggedness of the method is determined by comparison of the results of assay from two different laboratories and two analysts. The % RSD values of inter-day and intra-day assays of LPT in two different laboratories by two analysts were not more than 2.3%, which indicates the ruggedness of the developed method.

**Forced degradation studies**
Whole samples of forced degradation studies were analyzed just after each stress experiment. All samples either in solution or powder state were visually inspected and no evidence of color change was found in comparison with freshly prepared stock solution except for acid and alkaline samples where slight changes were seen (all samples were pale yellow). Following the forced degradation studies, it was found that LPT is completely stable under the dry heat stress test in solid state and also under the neutral stress test. As it is summarized in Table IV, LPT was recovered. Figure 2 shows the chromatogram of LPT under different stress conditions and Table IV represents the percentage recoveries from each series of the stress test. To identify peak purity, diode array detection was performed. These results showed that LPT peaks were homogenous and pure in all stress experiments. The chromatographic response of stock solution relative to freshly prepared solution showed no significant changes (<1%).

**LC mass analysis**
LC mass analyses were performed in order to determine degraded products and proposed destructive pathway of LPT. Acidic and alkaline samples selected for these series of studies due to their lower recoveries (<85% recovery in liquid chromatography analyses) in comparison with other forced tests and also some anomalous peaks in the UV–HPLC analyses. As the main purpose of this study was not to extract and purify the degraded products of LPT from the parent substance (LPT), the mixture of LPT and degraded products in acidic or basic conditions was treated separately using LC-MS.

A positive ion mode LC-MS method showed MH$^+$ at m/z 581, which corresponds to intact LPT (Figure 3a). There are two main degraded products (138 and 301 m/z) under basic conditions (Figure 3b). A peak at 261 m/z is also reported under acidic conditions (Figure 3c). A proposed mechanism and the structure of three degraded compounds are illustrated in Figure 4.

In the basic condition, the acidic hydrogen of the amino group in quinazoline derivatives (compound 1) is captured by a base (NaOH) and led to the formation of an intermediate (2). The intermediate (2) underwent degradation to give the (N-arylaminomethylene)formimidamide intermediate (3). Consequently, loss of hydrogen and formation of a triple bond among C and N atoms afforded to a thermodynamic pathway (Figure 3b). A peak at 261 m/z is also reported under acidic conditions (Figure 3c). A proposed mechanism and the structure of three degraded compounds are illustrated in Figure 4.

Under acid condition, the nitrogen of the amine group in quinazoline derivatives (compound 1) was protonated by H$^+$. Subsequently, migration of the aryl group (Ar1) and removal of NH$_3$ gave the intermediate (compound 7), which tolerated degradation to form compound (8).

**Discussion**
The results of various validation parameters showed that the newly developed HPLC method is suitable for quantitative determination of LPT. Due to many parameters that affect the

| Table III |
| Influence of Changes in Method Parameters on the LPT Recovery (%) |
|----------------|----------------|----------------|
| Parameter | Modification | LPT (% recovery) |
| Flow rate (mL/min) | 1.6 | 90.1 |
| | 1.5 | 102 |
| | 1.4 | 105.6 |
| Acetonitrile composition (%) | 72 | 93.9 |
| | 70 | 101.2 |
| | 68 | 104.3 |
| Lab temperature (°C) | 20 | 99.2 |
| | 25 | 101.3 |
| | 30 | 102.4 |

| Table IV |
| Degradation of LPT Bulk Under Different Stress Tests |
|----------------|----------------|----------------|
| Storage condition | Time | Recovered (%) |
| Acetonitrile and 10% H$_2$O$_2$ (50 : 50, v/v), 25°C | 4 days | 94 |
| Acetonitrile and 1 M HCl (50 : 50, v/v), 25°C | 6 h | 84.7 |
| Acetonitrile and 0.1 M NaOH (50 : 50, v/v), 25°C | 6 h | 82.1 |
| Acetonitrile and water (50 : 50, v/v), 25°C | 24 h | 99.2 |
| Dry heat of 150°C | 24 h | 97.2 |
| UV radiation (300–400 nm) in acetonitrile and water (50 : 50, v/v), 25°C | 24 h | 95 |
optimization and also selectivity of the analysis in gradient mode, the isocratic method was selected. Moreover, application of a gradient method between different kinds of a column and also different laboratories is very difficult (15). In addition, the gradient method is a time-consuming technique compared with the isocratic one. In previous studies, some disturbances were reported regarding the gradient method including baseline noise and eluent mixing, which can potentially affect the

Figure 3. LC mass spectrogram of (a) LPT, (b) alkaline forced test sample and (c) acidic forced test sample.
accuracy and precision of the method (16). The developed method was fully validated and the results were satisfactory in agreement with ICH guidelines. The new analysis method was linear in almost wide concentration range with accepted standard deviation. The precision of method was satisfactory in comparison with other techniques. In the previous studies, which were performed by LC-MC/MC (17, 18) and UPLC-MC/MC (19), the RSD percentages varied from 3.9 to 8.1% while in the present method the maximum RSD was calculated to be 2.84%. Accuracy is another important validation factor, which reported as percent relative error. The accuracy of newly developed method was calculated to be < 2.20%. In the previous published method, the accuracy was expressed as “recovery”, which was reported in the range limit of 100 ± 4.1%, although in this report neither RE% nor % RSD was reported (8). The main goal of the forced test study is evaluating the stability of an analytical sample during the analysis procedure but not long-term stability. The new method is specific for quantitative determination of LPT in the presence of its degradation product. Forced degradation studies demonstrated that LPT is stable under heat and neutral stress tests. These studies showed that LPT was less stable under acid and alkaline stress conditions.

In LC–MS studies, as seen in Figure 3a (LPT without any treatment with acid or base), there is only an intense peak at 581 \( m/z \) which corresponds to the LPT. As seen in Figure 3b, a significant amount of LPT was remained intact under basic conditions. A peak at 581 \( m/z \) corresponds to the intact LPT while other peaks are related to degraded products. However, under acidic conditions (Figure 3c), LPT was completely degraded so there was no peak at 581 \( m/z \) and most probably the other peaks were mainly related to the degraded products. So according to the current results, it seems that LPT is more susceptible in acidic medium than the basic one and it is an important issue in method validation and sample preparation for analysis of this drug. Furthermore, because of LC mass spectrometry analysis, the proposed destructive mechanism of LPT in an alkaline and acidic medium was explored.

**Conclusion**

A simple, rapid, precise and accurate analysis method was developed for analysis of LPT in bulk pharmaceuticals. The newly developed method is selective and stability indicating according to the ICH guidelines. This method is applicable to separate drug (LPT) from its degradation products; therefore, it can be performed for analysis during accelerated stability experiments. Because of the validity and robustness of the developed method, it can be used in the pharmaceutical sector as an accurate and precise analysis procedure.

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