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RESEARCH ARTICLE

Quantitative Determination of Acetic Acid in Gefitinib by Reverse Phase HPLC

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Abstract: A improved, simple and economic reverse phase high performance liquid chromatography (RP-HPLC) method has been developed and validated as per ICH guidelines for the quantification of acetic acid present in Gefitinib as an solvent impurity. Validation studies demonstrated that the proposed RP-HPLC method is simple, specific, rapid, reliable and reproducible. The high recovery and low relative standard deviation confirm the suitability of the proposed method for the routine quality control analysis for the quantification of acetic acid in Gefitinib.

Keywords: Gefitinib; Acetic acid; High Performance Liquid Chromatography; Method Validation

Introduction

Gefitinib[1] chemically known as *N*-(3-chloro-4-fluorophenyl)-7-methoxy-6-[3-(4-morpholinyl) propoxy]-4-quinazolinamide and represented as in Figure 1, is a drug used for certain breast, lung and other cancers. Gefitinib is an EGFR inhibitor[2-5], like erlotinib, which interrupts signaling through the epidermal growth factor receptor (EGFR) in target cells. Therefore, it is only effective in cancers with mutated and overactive EGFR.

Gefitinib is the first selective inhibitor of epidermal growth factor receptor's (EGFR) tyrosine kinase domain. Thus gefitinib is an EGFR inhibitor. The target protein (EGFR) is a family of receptors which includes Her1 (erb-B1), Her2 (erb-B2), and Her 3(erb-B3). EGFR is over expressed in the cells of certain types of human carcinomas - for example in lung and breast cancers. This leads to inappropriate activation of the anti-apoptotic Ras signalling cascade, eventually leading to uncontrolled cell proliferation. Research on gefitinib-sensitive non-small cell lung cancers has shown that a mutation in the EGFR tyrosine kinase domain is responsible for activating anti-apoptotic pathways. These mutations tend to confer increased sensitivity to tyrosine kinase inhibitors such as gefitinib and erlotinib. Of the types of non-small cell lung cancer histologies, adenocarcinoma is the type that most often harbors these mutations. These mutations are more commonly seen in Asians, women and non- smokers

(who also tend to more often have adenocarcinoma). Gefitinib inhibits EGFR tyrosine kinase by binding to the adenosine triphosphate (ATP)-binding site of the enzyme. Thus the function of the EGFR tyrosine kinase in activating the anti-apoptotic Ras signal transduction cascade is inhibited, and malignant cells are inhibited.

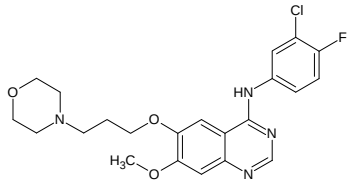

Figure 1. Chemical structure of Gefitinib

Experimental

HPLC grade acetic acid (Assay 99.80%) was obtained from S D Fine Ltd and a pure in-house sample of Gefitinib with assigned purity 99.90% was obtained for method development and validation. Acetonitrile HPLC grade (99.95 % Assay), potassium dihydrogen phosphate A.R. Grade (99.50 % Assay) and ortho phosphoric acid A.R. Grade (85% Assay) were procured from Merck. While highly pure Milli Q Water obtained from Millipore Milli-Q plus purification system was used wherever aqueous preparations were involved. Electronic analytical micro balances XP-26 of Mettler, Micro pipette (Biosystem, 10-100 µl, and 100-1000 µl) were used.

Instrumentation and chromatographic conditions

The chromatographic separation was performed on Waters Alliance 2695 separation module with 2487 dual λ absorbance detector. The data were processed using Empower 2.0 software. The analytical conditions used were, Inertsil ODS-2, 250X4.6 mm, 5 µm column, with a flow rate 1.0 mL/min, a gradient method as depicted in Table 1 with water containing 1.36 g/L potassium dihydrogen phosphate pH adjusted to 3.5 by dilute orthophosphoric acid (Buffer) in pump A and acetonitrile in pump B was performed. The run time was set at 35 min and column temperature was maintained at ambient. The volume of injection was 20 µL. Prior to injection of analyte, the column was equilibrated for 30-40 min with mobile phase. The eluents were monitored at 210 nm. The HPLC chromatograms were integrated for the main peaks and acetic acid while peaks due to blank were not integrated.

Table 1. Gradient program

Time (minutes)	PUMP- A	PUMP-B	Flow
0.00	100	25	1.0
8.00	100	25	1.0
10.00	50	30	1.0
20.00	50	30	1.0
23.00	100	50	1.0
35.00	100	50	1.0

Preparation of mobile phase and diluent

The buffer was prepared by taking 1.36 g of potassium dihydrogen phosphate in 1000 mL of water which was sonicated, filtered through 0.45micron filter and adjusted to pH 3.5 with dilute orthophosphoric acid (Pump A). Acetonitrile of HPLC grade was degassed and used as mobile phase in pump B. Solution of 17.4 mL of concentrate HCl in 1000 mL with Milli-Q water was used as diluent.

Preparation of sample and standard solutions

100 mg of acetic acid was taken in a 100 mL volumetric flask, dissolved in dil. HCl solution (*i.e.* 17.4 mL of conc. HCl in 1000 mL Milli-Q water) and made up to 100 mL. 5 mL of this solution was further diluted to 100 mL using the same dil. HCl solution. 250 mg of Gefitinib was taken into 25 mL volumetric flask and made up to 25 mL using the same dil. HCl solution.

Construction of linearity

The concentrations of analyte were prepared by suitable dilution of stock solution to get concentrations in the linear range of 0.0015 mg/mL to 0.07 mg/mL. Each of these solutions (20 µL) was injected three times into the column, the peak area and retention times were recorded. The calibration curve was constructed by plotting the ratio of the peak area of acetic acid (*Y*) against concentration (*X*) which revealed a good correlation between concentration and peak area as represented in Figure 2.

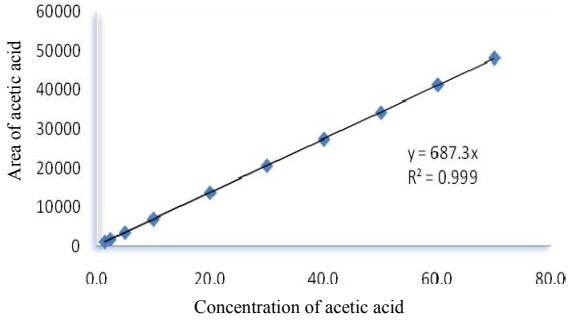

Figure 2. Linearity curve

Results and Discussion

As the routine in-house purification procedure of Gefitinib involved acetic acid as one of the components in the final step it was essential to develop and demonstrate that the manufactured Gefitinib final API was substantially free of acetic acid or more desirably below the ICH limit requirements of 5000 ppm. The main objective thus to develop a quantitative method[6-11] to determine the acetic acid content present in the Gefitinib. In analytical research the time and cost for the method development, validation and the method of quantification is greatly considered hence a HPLC method development was preferred over GC. Thus initially an HPLC method was optimized to provide a good separation of acetic acid and Gefitinib (acceptable theoretical plates and resolution between peaks) with a sufficient sensitivity and suitable peak symmetry (peak tailing factor < 2). For this purpose, the analytical column, solvent selection, mobile phase composition, flow rate and detector wavelength were studied. The use of hydrophobic stationary phases usually provides adequate retention of organic non polar molecules. The chromatographic separation was achieved using an RP C18 column because it was suitable to resolve the acetic acid with adequate resolution and gave symmetrical peak shapes.

Thus a reverse phase high performance liquid chromatographic (RP-HPLC) method was developed and later validated for the quantification of acetic acid in Gefitinib (as discussed in material and method section). In this method the retention times observed for Gefitinib and acetic acid were 13.5 and 7.0 min, respectively. A typical chromatograms of acetic acid spiked in Gefitinib is shown in Figure 3.

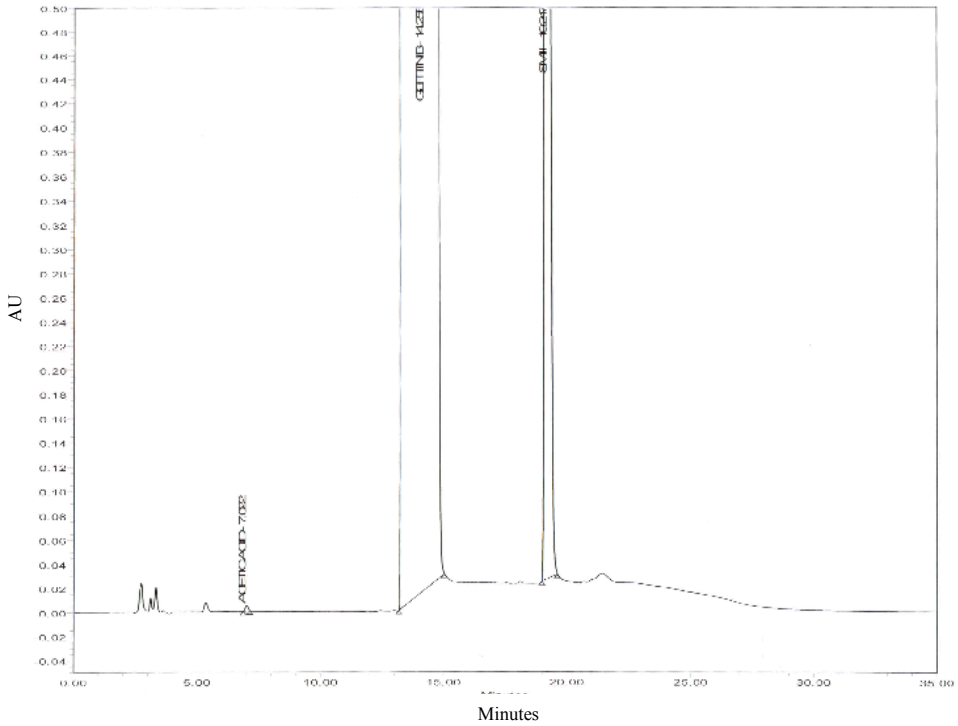

Figure 3. HPLC chromatograph of acetic acid spiked in Gefitinib

A standard plot was constructed by plotting the ratio of the peak area of acetic acid (*Y*) against concentration (*X*). It was found to be linear with a correlation coefficient (r^2) of 0.999, the corresponding linear regression equation being y = 687.3x+00. The results are shown in Table 2 & 3.

Table 2. Linearity of the method

Concentration, mg/mL	Average peak area
0.0015	1205
0.0025	1868
0.005	3628
0.01	7019
0.02	13831
0.03	20628
0.04	27426
0.05	34246
0.06	41288
0.07	48122

Table 3. Results of Linearity

Parameters	Values
Linearity	0.0015-0.07
Regression equation	687.3x+00
Correlation coefficient (r^2)	0.999

The precision of the method was determined by repeatability and intermediate precision studies. Repeatability was evaluated by performing six determinations *(n=6)* at the same concentration, during the same day, under the same experimental conditions. Intermediate precision was evaluated by comparing the results on different days with different analysts. The result revealed the precision with % RSD for intraday and interday 0.66 and 0.82, respectively. The results are shown in Table 4.

Table 4. Intraday and interday precision of the method

Test	Area of acetic acid	
	Day-I	Day-II
1	15456.50	16174.50
2	15489.00	16031.00
3	15404.50	15946.50
4	15308.50	15796.50
5	15213.50	15903.50
6	15410.00	15901.00
Average	15380.33	15958.83
STDEV	102.05	130.10
% RSD	0.66	0.82

To ensure the reliability and accuracy of the method, the recovery studies were carried out by adding a known quantity of acetic acid with Gefitinib and contents were reanalyzed by the proposed method. Accuracy was evaluated at three different concentrations equivalent to 80, 100 and 120% , by adding known amount of acetic acid to the sample of known concentration and calculating the recovery of acetic acid with % RSD and % recovery for each concentration. The mean % recoveries were 97.24 to 97.49 and the values are shown in Table 5. The high recovery of acetic acid indicated that the proposed method for quantification of acetic acid in Gefitinib was highly accurate.

Table 5. % Recovery of the method

Test	80% Level	100% Level	120% Level
1	97.69	97.99	97.44
2	96.90	97.92	97.55
3	97.14	98.16	97.49
Average	97.24	98.02	97.49
STDEV	0.41	0.12	0.06
% RSD	0.42	0.13	0.06

Suitability test was employed to establish the parameters such as tailing factor, theoretical plates, resolution, limit of detection and limit of quantification. The System suitability parameters were established and found to be within acceptable limits and the proposed method indicating that the test method was robust for all variable conditions. The results are shown in Table 6.

The robustness of the method was investigated by making small deliberate changes in the chromatographic conditions at three different levels. The chromatographic conditions selected were flow rate (1, 1.1 and 0.9 mL/min), pH of buffer in the mobile phase (3.5, 3.2 and 3.8 pH) and the column (Inertsil ODS-3V C18, 4.6 mm X 250 mm, 5 µm and Inertsil ODS-2 C18, 4.6 mm x 250 mm, 5µm). The results are shown in Table 7.

Table 6. System suitability parameters

Parameter	Value
Retention time (min)	6.98
Theoretical plates	15005
Tailing factor	1.0
Resolution	5.01
Linearity range (mg/mL)	0.0015-0.07
Limit of detection (mg/mL)	0.0035
Limit of quantification(mg/mL)	0.016

Table 7. Robustness of the method

	Change in Flow		Change in Buffer pH		Column change
	1.1 mL/min	0.9 mL/min	3.2 pH	3.8 pH	Inertsil ODS-2
Area of Acetic acid	12073	14716	13186	14057	12666
% RSD of Area	0.80	0.51	0.39	0.19	0.24
% Acetic acid	0.21	0.22	0.22	0.23	0.20

The limits of detection (LOD) and quantitation (LOQ) were calculated by the method based on the standard deviation (SD) and the slope (*S*) of the calibration plot, using the formulae LOD = 3.3/*S* and LOQ =10/*S*. Specificity of the proposed method demonstrated acetic acid does not interfere in the Gefitinib peak. Furthermore, well shaped peaks indicate the specificity of the method.

Conclusion

A gradient RP-HPLC method for quantification of acetic acid present in Gefitinib was developed also the validation data demonstrates a high precision and accuracy, which proves the reliability of the developed method. In addition to the above the short runtime and cheaper mobile phase involved in the method are advantageous for analyzing routine quality control samples for the quantification of acetic acid in Gefitinib bulk drug.

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