ORIGINAL ARTICLE

Method Development and Validation of a New Stability Indicating Reverse Phase Ultra-fast Liquid Chromatographic Method for the Determination of Gefitinib (Anticancer Agent) in the Presence of an Internal Standard

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Abstract

Introduction: A new stability indicating reverse phase ultra-fast liquid chromatographic (UFLC) method has been developed and validated for the quantification of Gefitinib in pharmaceutical formulations, that is, tablets in presence of an internal standard, Trifluridine. Gefitinib is an anticancer drug and it is used for the treatment of lung cancer, prostate cancer, and breast cancer. **Materials and Methods:** A mixture of tetra butyl ammonium hydrogen sulfate:methanol:Acetic acid (55:45:0.1) (pH 3.4) was used as the mobile phase for the chromatographic study (Flow rate: 0.8 mL/min; ultraviolet detection: 264 nm; Injection volume: 20 µl). Shimadzu Model CBM-20A/20 Alite UFLC system with Agilent C_{18} column and photodiode-array detection detector was used for the present study. **Results and Discussion:** Gefitinib has shown linearity over the concentration range 0.5–100 µg/ml with linear regression equation, y = 0.1027x + 0.0022 (R^2 = 0.9999). The limit of detection and limit of quantitation were found to be 0.1392 µg/ml and 0.4269 µg/ml, respectively. Stress degradation studies were performed by treating Gefitinib with different stress conditions, and the method was validated as per the International Council for Harmonisation guidelines.

Key words: Gefitinib, International Council for Harmonisation guidelines, reverse phase ultra-fast liquid chromatographic, stability indicating, stress degradation studies, Trifluridine (internal standard), validation

INTRODUCTION

Gefitinib is an anticancer agent with chemical name 4-(3'-chloro-4'-Fluoroanilino)-7-methoxy-6-(3-morpholino propoxy) quinazoline [Figure 1] it has a molecular formula $C_{22}H_{24}C_lFN_4O_3$ and molecular weight 446.902 g/mol (pKa 5.4 and 7.2). Gefitinib is a tyrosine kinase inhibitor.[1-3] The internal standard (IS), Trifluridine is an antiviral drug [Figure 2]. Analytical techniques such as high-performance liquid chromatography (HPLC), rapid resolution liquid chromatography (RRLC), and liquid chromatography-mass spectrometry (LC-MS)/MS were established by different authors for the quantification of Gefitinib and its related substances/impurities in biological fluids as well as pharmaceutical formulations.

Sree *et al*.,[4] have proposed a new reversed-phase (RP-HPLC) method with a mobile phase mixture consisting of acetonitrile

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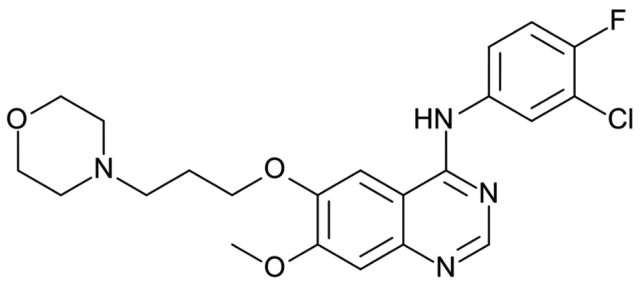

Figure 1: Chemical structure of Gefitinib

and 40 mM ammonium formate buffer pH 2.5 (30:70) for the assay of Gefitinib (Rt 4.476 min) nano formulation using Phenomenex guard column as well as HyperClone Phenomenex C18 column and this method obeys Beer-Lambert's law over the concentration range 0.2–12 mg/ml.

A new RRLC method[5] was developed using Agilent XDB-C_{18} column by Venkataramanna *et al*., with ammonium acetate: acetonitrile (40:60) as mobile phase in which Gefitinib was estimated along two other impurities.

Chandrashekara *et al*.,[6] have proposed a new RP-HPLC method for the assay of Gefitinib (Rt of Gefitinib at 6.777 min) along with its five process related impurities using Inertsil ODS-3V column and mobile phase mixture, 130 mM ammonium acetate: acetonitrile (63:37) (Adjusted pH 5.0 with acetic acid).

A new RP-HPLC technique has been developed by Kumar *et al*.[7] for the determination of Gefitinib using methanol: Dipotassium hydrogen ortho phosphate (90:10) as mobile phase (ultraviolet [UV] detection at 246 nm; Rt 3.667 min), whereas Sankar *et al*.[8] have developed another liquid chromatographic method using the mobile phase, acetonitrile: 0.02M potassium di-hydrogen phosphate (45:55) in which the pH was adjustedto 6.5 with the help of tri ethyl amine and the UV detection was carried at 220 nm.

Mohan and Mangamma[9] have developed another HPLC method for the assay of Gefitinib tablets using Hypersil C_{18} column with mobile phase consisting of di hydrogen potassium phosphate buffer: Methanol (15:85) with UV detection at 247 nm but a low narrow linearity range was reported (0.14–0.52 mg/ml).

Rawat *et al*.,[10] established a reverse phase liquid chromatographic method with Inertsil ODS-2 C18 column on gradient mode for the assay of Gefitinib using phosphate buffer: acetonitrile (pH 3.5) as mobile phase. During the chromatographic study Gefitinib was eluted at 6.98 min (Detection wavelength 210 nm) with linearity 0.0015–0.07 mg/ml.

Raja *et al*.,[11] have established a RP-HPLC method using Agilent column and 0.1% Tri flouro acetic acid: Methanol (35:65) mixture as mobile phase and where Gefitinib was eluted at about 3.4 min and the linearity range was 5–30 mg/ml.

A new LC-MS-MS method was developed by Zheng *et al*., for the estimation of Gefitinib in cerebrospinal fluid and human serum[12] using in the presence of an IS, Icotinib on positive Electrospray ionization (ESI) mode and the linearity was observed as 0.001–1.0 and in human serum and 0.00005–0.050 µg/ml in human serum and cerebrospinal fluid, respectively.

Zheng *et al*., established another LC-MS-MS method for the determination of Gefitinib along with its major metabolites in presence of an IS, dasatinib in tumor-bearing mouse plasma[13] using 0.1% formic acid: Acetonitrile mixture as mobile phase (Gradient mode) and Agilent RRHD SB-C18 column.

Gefitinib was estimated in human plasma, mouse plasma and tissues[14] using LC-MS/MS in presence of an internal standatrd, (d8)-Gefitinib by Zhao *et al*., using a mixture of 0.1% formic acid: Acetonitrile (30:70) andWaters X-Terra C_{18} column and the extraction process was done by protein precipitation and the linearity range was reported as 0.001–1.0, 0.005–1.0 and 0.005–1.0 µg/ml in human plasma, mouse plasma and tissues, respectively.

Pramadvara and Annapurna have proposed LC-ESI-Q-TOF/MS method using Agilent 1200 infinity series HPLC with Inertsil ODS 3V C_{18} column for separation, identification and quantification of 10 process related impurities[15] along with the stress degradants of Gefitinib with mobile phase mixture consisting of acetonitrile: 10 mM ammonium acetate: (37:63) (AdjustedpH to 6.5 with glacial acetic acid).

Kumar *et al*., have proposed a stability indicating liquid chromatographic method for the separation and characterization of related compounds[16] of Gefitinib on gradient mode using Inertsil C_8 column (photodiode-array detection [PDA] detector: 300 nm) withmobile phase consisting ofacetonitrile and ammonium acetate (50 mM) (adjusted to pH 4.7 with tri fluoro acetic acid) with diluent acetonitrile: 0.2% TFA (40:60). The degradants were characterized with the help of LC-ESI-MS/MS- and H nuclear magnetic resonance (^{1}H NMR).

Only two stability indicating RP-HPLC methods were developed for the estimation of Gefitinib by Sreedevi *et al*.,[17] where a mixture ofacetonitrile: phosphate buffer (pH 3.6) (45:55) was used as mobile phase (Hypersil BDS C18 column) and the drug was eluted at about 4.179 min with PDA detection at 248 nm and by Dudekula *et al*.,[18] where a mixture of 0.5% ammonium dihydrogen phosphate buffer: Acetonitrile (70:30) was used as mobile phase (YMCODS-AQ column) and the drug was eluted at about 7.43 min with PDA detection at 205 nm. At present the authors have proposed, a new stability indicating validated

reverse phase liquid chromatographic method for the assay of Gefitinib in presence of an IS Trifluridine.

MATERIALS AND METHODS

Gefitinib API (>99.8 purity) was obtained from Natco Pharma Ltd., India. HPLC grade methanol (Merck, India) and HPLC grade water obtained from Milli-Q Gradient Millipore system were used throughout the study and all other chemicals such as hydrochloric acid, sodium hydroxide, tetra butyl ammonium hydrogen sulfate (TBHS), and hydrogen peroxide (30% w/v) were of AR grade and purchased from Merck (India).

Preparation of TBHS solution (10 mM)

The molecular weight of TBHS ($C_{16}H_{37}NO_4S$) is 339.54 g/mole. 10 mM TBHS was prepared by dissolving about 3.3954 g of TBHS in HPLC grade water in a 1000 ml volumetric flask (pH 3.4), sonicated and was filtered through membrane filter before use.

Preparation of Gefitinib and Trifluridine (IS) stock solutions (1000 µg/mL)

About 25 mg of Gefitinib was weighed accurately and transferred in to a 25 mL volumetric flask and dissolved in HPLC grade methanol (1000 µg/mL). This stock solution was further diluted with the mobile phase to achieve 100 µg/mL (working standard solution) and stored in refrigerator. 25 mg of Trifluridine (IS) was weighed accurately and transferred in to a 25 mL volumetric flask and dissolved in HPLC grade methanol (1000 µg/mL) and 10 µg/mL Trifluridine was used during the entire study.

Optimized chromatographic conditions

A mixture of TBHS:methanol:acetic acid (55:45:0.1) (pH 3.4) was chosen for the chromatographic study (Flow rate: 0.8 mL/min; UV detection: 264 nm; Injection volume: 20 µl). A ultra fast liquid chromatography (UFLC) system, Shimadzu Model CBM-20A/20 Alite withAgilent C_{18} column (PDA detector) was used for the chromatographic study and the run time was 10 min.

Method validation[19]

Linearity

Gefitinib drug solutions (0.5–100 µg/mL) were prepared on dilution from the stock solution along with the IS, that is, Trifluridine (10 µg/mL) and each of these solutions were injected into the UFLC system thrice and the respective chromatograms were recorded. The peak area of Gefitinib as well as the IS were noted the peak area ratio was calculated (Gefitinib/IS) and finally the mean peak area ratio (n = 3) was calculated. The calibration curve was plotted by plotting the concentration of the Gefitinib solutions on the x-axis and the corresponding mean peak area ratio (Gefitinib/IS) on the y-axis. The limit of detection (LOD) and was calculated as 3.3 times of signal to noise ratio (S/N) and that of limit of quantitation (LOQ) as 10 times the S/N.

Precision study

Intra-day and inter-day precision studies of Gefitinib were performed (10, 20 and 40 µg/mL) in presence of the IS (10 µg/mL) within the linearity range on the same day and on 3 different days, that is, Day 1, Day 2 and Day 3, respectively, and the % relative standard deviation (RSD) was calculated.

Accuracy study

The accuracy study was performed by spiking the Gefitinib formulation (20 µg/mL) solution (80, 100, 120%) with a known concentration of Gefitinib (API) in presence of the IS (10 µg/mL) where the final concentrations were found to be 36, 40 and 44 µg/mL. The mean peak area ratio was calculated and the % RSD was finally calculated from the linear regression equation.

Robustness study

Robustness study of Gefitinib (10 µg/mL) was performed in the presence of IS by incorporating small changes in optimized chromatographic conditions such as flow rate (±0.1 mL; 0.7 and 0.9 mL/l), detection wavelength (±2 nm; 262 and 266 nm), mobile phase composition (±2%; 43:57: 0.1 and 47:53:0.1) and pH (±0.1; 3.3 and 3.5), and the percentage RSD was calculated from the mean peak area ratio of the respective chromatograms obtained during the study.

Assay of Gefitinib tablets in presence of IS

Gefitinib is available as tablets (Label claim: 250 mg) with brand names Grexam (SUN Pharmaceutical Industries Ltd.), Gefticip (Cipla Ltd.), Geftinat (Natco Pharma Ltd.), and Iressa (Astra Zeneca). 20 tablets of Gefitinib were accurately weighed and an amount of powder equivalent to 25 mg of Gefitinib was accurately weighed from two different brands and transferred in to two different 25 ml volumetric flasks and Gefitinib was extracted with HPLC grade methanol, sonicated and filtered. This solution was further diluted with the mobile phase (TBHS: Methanol: acetic acid 55:45:0.1, v/v) and 10µg/mL of IS, Trifluridine was added and made up to volume and from this 20 µL of each formulation solution was injected in to the system (n = 3) and the average peak area ratio (Gefitinib/IS) was calculated from the respective chromatograms. Finally the amount of Gefitinib was computed from the calibration curve.

Table 1: Review of literature

Mobile phase (v/v)	λ (nm)	Linearity (µg/ml)	Method	Ref
Ammonium formate buffer (pH 2.5): Acetonitrile (70: 30)	248	0.2–12	HPLC Nanoformulation	4
Acetonitrile: Ammonium acetate: (60:40)	250	–	RRLC (2 Impurities) (Gradient mode)	5
Ammonium acetate: Acetonitrile and (63: 37) (Adjusted pH 5.0 with acetic acid)	260	0.1–2.0 (Impurities) 25–500 (Gefitinib)	HPLC (5 Impurities)	6
Methanol: Dipotassium Hydrogen ortho phosphate (90:10)	246	25–300	HPLC	7
Acetonitrile: 0.02M Potassium di-hydrogen phosphate (45:55) (Adjusted pH 6.5 with tri ethyl amine)	220	10–60	HPLC	8
Dihydrogen potassium phosphate: Methanol (15:85)	247	0.14–0.52	HPLC	9
Acetonitrile: phosphate buffer (pH 3.5)	210	1.5–0.70	HPLC (Gradient mode)	10
Methanol: 0.1% Triflouroacetic acid (65:35)	246	5–30	HPLC	11
Acetonitrile: water (50:50)	–	–	LC/MS/MS (Human serum, Cerebrospinal fluid)	12
Acetonitrile: 0.1% Formic acid	–	–	LC-MS/MS (Gradient mode) (Mouse plasma)	13
0.1% Formic acid: Acetonitrile (30:70)	–	0.001–1.0 0.005–1.0 0.005–1.0	LC/MS/MS (Isocratic mode) (Human plasma, Mouse plasma & Tissues)	14
10 mM Ammonium acetate: Acetonitrile (63: 37%) (pH adjusted to 6.5 with acetic acid)	240	0.2–750	LC-ESI-Q-TOF/MS (Isocratic mode) 10 Impurities Stability indicating	15
Ammonium acetate: Acetonitrile	300	–	LC-MS/MS NMR (Gradient mode)	16
Acetonitrile: Phosphate buffer (55:45) (pH 3.6)	248	25–150	HPLC Stability indicating	17
Acetonitrile: 0.5% Ammonium dihydrogen phosphate buffer (30:70)	205	50–150	HPLC Stability indicating	18
Methanol: Tetra butyl ammonium hydrogen sulphate (10 mM): Acetic acid (45: 55: 0.1) (pH 3.4) (Isocratic mode)	264	0.5–100	RP-UFLC Stability indicating (Internal standard: Trifluridine)	Present method

RP-UFLC: Reverse phase ultra-fast liquid chromatographic, HPLC: High-performance liquid chromatography, LS-MS: Liquid chromatography-mass spectrometry

Stress degradation studies[20]

The stability studies of Gefitinib were performed to determine the specificity of the method. Gefitinib was exposed to the stress conditions such as acidic hydrolysis, basic hydrolysis, oxidation and thermal degradations in presence of IS Trifluridine. The IS was added to the mixture only just before injecting in to the UFLC system so that Trifluridine will not be affected by the stress reagents.

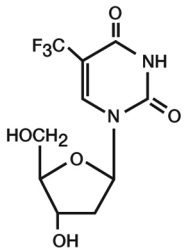

Figure 2: Chemical structure of Trifluridine

Acidic degradation was performed by heating Gefitinib solution with 1 mL of 0.5 N HCl solution on a water bath at 70°C for 1 h. The stressed sample was then cooled, neutralized with 1.0 mL 0.5N sodium hydroxide solution, diluted with mobile phase and then 20 µl of the solution was injected in to the UFLC system after the addition of IS (10 µg/mL).

Alkaline degradation was performed by heating Gefitinib solution with 1.0 mL 0.5N sodium hydroxide solution at 70°C on a water bath for 1 h. The stressed sample was then cooled, neutralized with 1.0 mL of 0.5 N HCl solution, diluted with mobile phase and then 20 µl of the resulting solution was injected in to the UFLC system after the addition of IS (10 µg/mL).

Thermal degradation was performed by heating the Gefitinib solution at 70°C on a water bath for 1 h and then cooled, diluted with mobile phase and 20 µl of the resulting solution was injected in to the UFLC system after the addition of IS (10 µg/mL).

Oxidative degradation was performed by heating Gefitinib solution with 1.0 mL 30% hydrogen peroxide solution at 70°C on a water bath for 1 h. The stressed sample was then cooled, diluted with mobile phase and then 20 µl of the resulting solution was injected in to the UFLC system after the addition of IS (10 µg/mL).

RESULTS AND DISCUSSION

The authors have developed a new validated stability indicating reverse phase UFLC (RP-UFLC) method for the quantification of Gefitinib in bulk (API) and tablets in presence of an IS, Trifluridine. An IS is used to improve the quantitative analysis. A known concentration of the IS is added to the drug solution throughout the study and the ratio of peak area of the drug solution to that of the peak area of the IS is taken for the calibration curve, validation parameters such as precision, accuracy, robustness, etc., and all other studies. Ion pair chromatography is an analytical technique used for the reverse phase chromatography. TBHS is anion pairing reagent. A mixture of methanol: (10 mM) TBHS: acetic acid (45:55:0.1) (pH 3.4) (Isocratic mode) was selected as mobile phase for the present study. Different analytical techniques were found in the literature for the estimation of Gefitinib and these previously published methods were compared with the present proposed method and the details were given in Table 1.

Method optimization

The chromatographic study was performed using an ion pairing agent, TBHS (10 mM). Mobile phase consisting of

Table 2: Method optimization of Gefitinib in presence of IS (Mobile phase: Methanol: TBHS)

Trial	Column	Mobile phase (v/v)	Flow rate (mL/min)	Rt (min)	Theoretical plates	Tailing factor	Observations
1	Sunfire C_{18}	50:50	0.8	4.932	1989	2.324	Theoretical plates < 2000 Peak tailing > 2
2	Agilent C_{18}	50:50	0.8	3.936	3029	2.113	Peak tailing > 2
3	Agilent C_{18}	40:60	0.8	3.422	4129	2.091	Peak tailing > 2
4	Agilent C_{18}	45:55	0.8	3.379	6590	1.371	Method optimized

TBHS: Tetra butyl ammonium hydrogen sulfate, IS: Internal standard

Table 3: Linearity study

Conc. (µg/mL)		*Mean peak area		*Mean peak area ratio (Gefitinib/IS)	% RSD
Gefitinib	IS	Gefitinib	IS		
0	10	0	0	–	–
0.5	10	24413	443841	0.0550	0.31
1	10	47128	443866	0.1062	0.41
2	10	90325	443965	0.2035	0.19
5	10	235149	444026	0.5296	0.35
10	10	462185	443821	1.0414	0.53
20	10	919856	443923	2.0721	0.71
40	10	1815634	443798	4.0911	0.28
60	10	2725143	443991	6.1378	0.48
80	10	3614986	443903	8.1436	0.62
100	10	4592169	444011	10.3425	0.54

*Mean of three replicates, IS: Internal standard, RSD: Relative standard deviation

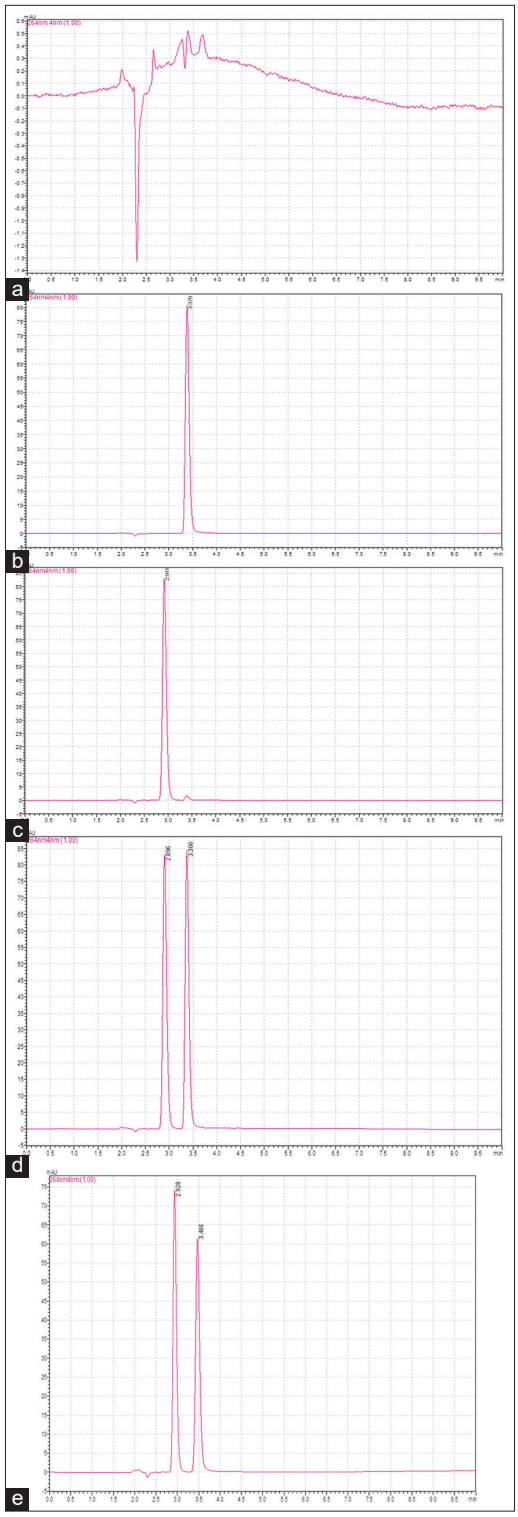

Figure 3: (a) Placebo. (b) Representative chromatogram of Gefitinib API (10 µg/mL) (Rt 3.379 min) theoretical plates: 6589.734; tailing factor: 1.371. (c) Representative chromatogram of Trifluridine (internal standard) (10 µg/mL) (Rt 2.911 min) theoretical plates: 4920.401; tailing factor: 1.355. (d) Representative chromatogram of Gefitinib API (Rt 3.366 min) Trifluridine (internal standard) (Rt 2.896 min) resolution: 2.827. (e) Representative chromatogram of Gefitinib tablet formulation (Rt 3.466 min) and Trifluridine (internal standard) (Rt 2.928 min) Resolution: 2.919

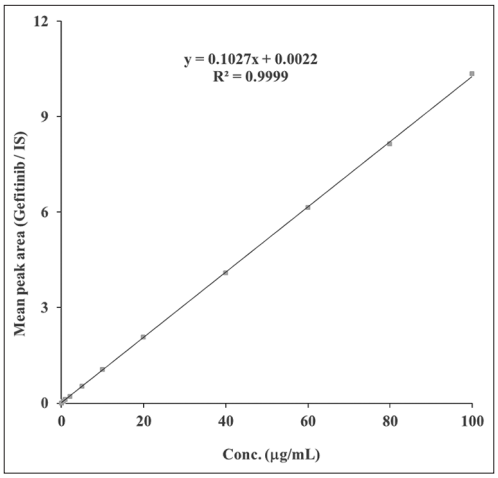

Figure 4: Calibration curve

Table 4: Intraday precision study of Gefitinib in presence of IS

Conc. (µg/ml)		*Mean peak area ratio (Gefitinib/IS)	Statistical Analysis
Gefitinib	IS		*Mean±SD (% RSD)
10	10	1.0414	1.0405±0.000833 (0.0801)
10	10	1.0398	
10	10	1.0402	
20	10	2.0721	2.0708±0.001473 (0.0711)
20	10	2.0692	
40	10	4.0911	4.0904±0.000611 (0.0149)
40	10	4.0899	
40	10	4.0903	

*Mean of three replicates. IS: Internal standard, RSD: Relative standard deviation

10 mM TBHS: Methanol: 0.1% acetic acid was chosen as the mobile phase for the study with UV detection wavelength at 264 nm (Isocratic mode).

A 10 µg/mL Gefitinib was initially injected in to the UFLC system using Sunfire C_{18} column using a mobile composition of methanol:10 mM TBHS 50: 50 with flow rate 0.8 ml/min and Gefitinib was eluted at 4.932 min with theoretical plates 1989 and tailing factor 2.324 (Trial 1). As the theoretical plates are <2000 and the tailing factor was more than 2.0, that is, as the system suitability parameters were not within the acceptable criteria the Sunfire column C_{18} was replaced with Agilent C_{18} keeping the chromatographic conditions same by which the theoretical plates were improved, that is, 3029 (>2000) but the tailing factor was 2.113 (Trial 2) which is not acceptable. The mobile phase composition was modified as 40:60 where the tailing actor was slightly decreased (Trial 3). Finally, the mobile phase composition was slightly modified as 45:55 (Trial 4) with the same flow rate by which Gefitinib was eluted at 3.379 min

where the system suitability parameters were within the acceptable criteria and the method was optimized (Trial 4). After optimizing the chromatographic conditions of Gefitinib then the IS, Trifluridine solution (10 µg/mL) was injected in to the UFLC system. Trifluridine was eluted at 2.911 min with theoretical plates more than 2000 (4920.401) and tailing factor 1.355 which is <1.5. The trial runs observed during the optimization procedure were tabulated in Table 2.

Method validation

Gefitinib obeys Beer-Lambert's law over the concentration range of 0.5–100 µg/mL (% RSD 0.19–0.71) [Table 3] and representative chromatograms of the placebo and Gefitinib API were shown in Figure 3a and b, respectively. Figure 3c represents the typical chromatogram of the IS, Trifluridine and Figure 3d represents the typical chromatogram of Gefitinib API in the presence of the IS with acceptable system suitability parameters such as theoretical plates and tailing factor. The LOD and LOQ were found to be 0.1392 µg/ml and 0.4269 µg/ml, respectively. The linear regression equation was found to be $y = 0.1027x + 0.0022$ ($R^2 = 0.9999$) and the calibration curve was shown in Figure 4. The % RSD was found to be 0.0149–0.0801 (Intraday) [Table 4] and 0.0296–0.2033 (Inter-day) [Table 5] in precision studies which is <2.0 indicating that the method is precise. The % recovery in accuracy studies was found to be 99.47–99.71% [Table 6] and % RSD was (0.29–0.96) <2% indicating that the method is accurate. The % RSD in robustness study was found to be 0.23–1.21 which was <2% indicating that the method is robust [Table 7].

Assay of Gefitinib tablets in presence of IS

Assay of Gefitinib tablets was performed in presence on IS, Trifluridine. The mean peak area ratio of Gefitinib formulations to that of the IS, Trifluridine was calculated from the chromatograms obtained and the percentage of purity of Gefitinib was calculated from the linear regression equation and it was found to be 99.59–99.83 [Table 8]. The typical chromatgram of Gefitinib tablet formulation was shown in Figure 3e and no interference of excipients was found during the assay.

Stress degradation studies of Gefitinib in presence of IS

The pure drug Gefitinib API was eluted at 3.366 min with theoretical plates 6517.250 (>2000) and tailing factor 1.375 (<1.5) and the IS, Trifluridine (IS) was eluted at 2.896 min with theoretical plates 4872.458 (>2000) and tailing factor 1.341 (<1.5) which are within the acceptable criteria.

During the thermal degradation study Gefitinib was eluted at 3.412 min and has undergone <2% degradation (1.57%) whereas the IS was eluted at 2.909 min with resolution 2.865 (>2.0). The theoretical plates for Gefitinib were appears to be

Table 5: Inter day precision study of Gefitinib in presence of IS

Conc. (µg/mL)	*Mean peak area			*Mean±SD (% RSD)
	Day 1	Day 2	Day 3	
10	1.0414	1.0448	1.0453	1.04383±0.0021 (0.2033)
20	2.0721	2.0731	2.0773	2.07416±0.0028 (0.1330)
40	4.0911	4.0929	4.0906	4.0915±0.0012 (0.0296)

*Mean of three replicates, IS: Internal standard, RSD: Relative standard deviation

Table 6: Accuracy study of Gefitinib in presence of IS

Gefitinib Spiked Conc. (µg/mL)	Conc. (IS) (µg/mL)	Gefitinib Formulation (µg/mL)	Total Conc. Gefitinib (µg/mL)	*Gefitinib Conc. obtained (µg/mL)±SD (%RSD)	% Recovery
16 (80%)	10	20	36	35.81±0.1039 (0.29)	99.47
		20	36		
		20	36		
20 (100%)	10	20	40	39.79±0.2905 (0.73)	99.48
		20	40		
		20	40		
24 (120%)	10	20	44	43.87±0.4212 (0.96)	99.71
		20	44		
		20	44		

*Mean of three replicates, IS: Internal standard, RSD: Relative standard deviation

Table 7: Robustness study of Gefitinib (10 µg/mL) in presence of IS

Parameter	Condition	*Mean peak area ratio±SD (% RSD)
Flow rate (±0.1 ml/min)	0.7	1.0316±0.0099 (0.96)
	0.8	
	0.9	
Detection wavelength (±2 nm)	262	1.0429±0.0024 (0.23)
	264	
	266	
Mobile phase composition Methanol: Tetra butyl ammonium hydrogen sulfate: Acetic acid (±2%, v/v)	43:57:0.1	1.0401±0.0061 (0.59)
	45:55:0.1	
	47:53:0.1	
pH	3.5	1.0492±0.0127 (1.21)
	3.4	
	3.3	

*Mean of three replicates, IS: Internal standard, RSD: Relative standard deviation

Table 8: Assay of Gefitinib tablet in presence of IS

S. No.	Brand name	Label claim (mg)	*Observed amount (%w/w)	% Recovery*
1	Brand I	250	248.97	99.59
2	Brand II	250	249.58	99.83

*Mean of three replicates, IS: Internal standard

Table 9: Stress degradation studies of Gefitinib in presence of IS

Stress condition medium/temp/duration	Rt (min)	% Recovery	% Drug degradation	Theoretical plates	Tailing factor	Resolution
Gefitinib	3.366	100	–	6517.250	1.375	2.827
Trifluridine (IS)	2.896	–		4872.458	1.341	–
Thermal degradation 70°C/1 h	3.412	98.43	1.57	5498.132	1.484	2.865
	2.909	–		4836.808	1.345	–
Alkaline degradation 0.5N NaOH/70°C/1 h	3.455	96.91	3.09	5108.165	1.347	2.895
	2.930	–		4794.775	1.446	–
Oxidation 30% H_2O_2/70°C/1 h	3.424	82.45	19.55	5360.562	1.269	2.893
	2.916 2.305	–		5034.480	1.334	3.622
Acidic degradation 0.5N HCl/70°C/1 h	3.374	89.21	10.79	6419.708	1.324	2.891
	2.895	–		5052.963	1.335	–

*Mean of three replicates, IS: Internal standard

5498.132 (>2000) and tailing factor 1.484 (<1.5) which are within the acceptable criteria.

During the alkaline degradation study Gefitinib was eluted at 3.455 min and has undergone 3.09% degradation whereas the IS was eluted at 2.930 min with resolution 2.895 (>2.0). The theoretical plates for Gefitinib were appears to be 5108.165 (>2000) and tailing factor1.347 (<1.5) which are within the acceptable criteria.

During the oxidative degradation study, Gefitinib was eluted at 3.424 min and has undergone 19.55% degradation with an extra degradant peak at 2.305 min whereas the IS was eluted at 2.916 min with resolution 2.893 and 3.622 (>2.0). The theoretical plates for Gefitinib were appears to be5360.562 (> 2000) and tailing factor1.269 (<1.5) which are within the acceptable criteria.

During the acidic degradation study, Gefitinib was eluted at 3.374 min and has undergone 10.79% degradation whereas the IS was eluted at 2.895 min with resolution 2.895 (>2.0). The theoretical plates for Gefitinib were appears to be 6419.708 (>2000) and tailing factor1.324 (<1.5) which are within the acceptable criteria.

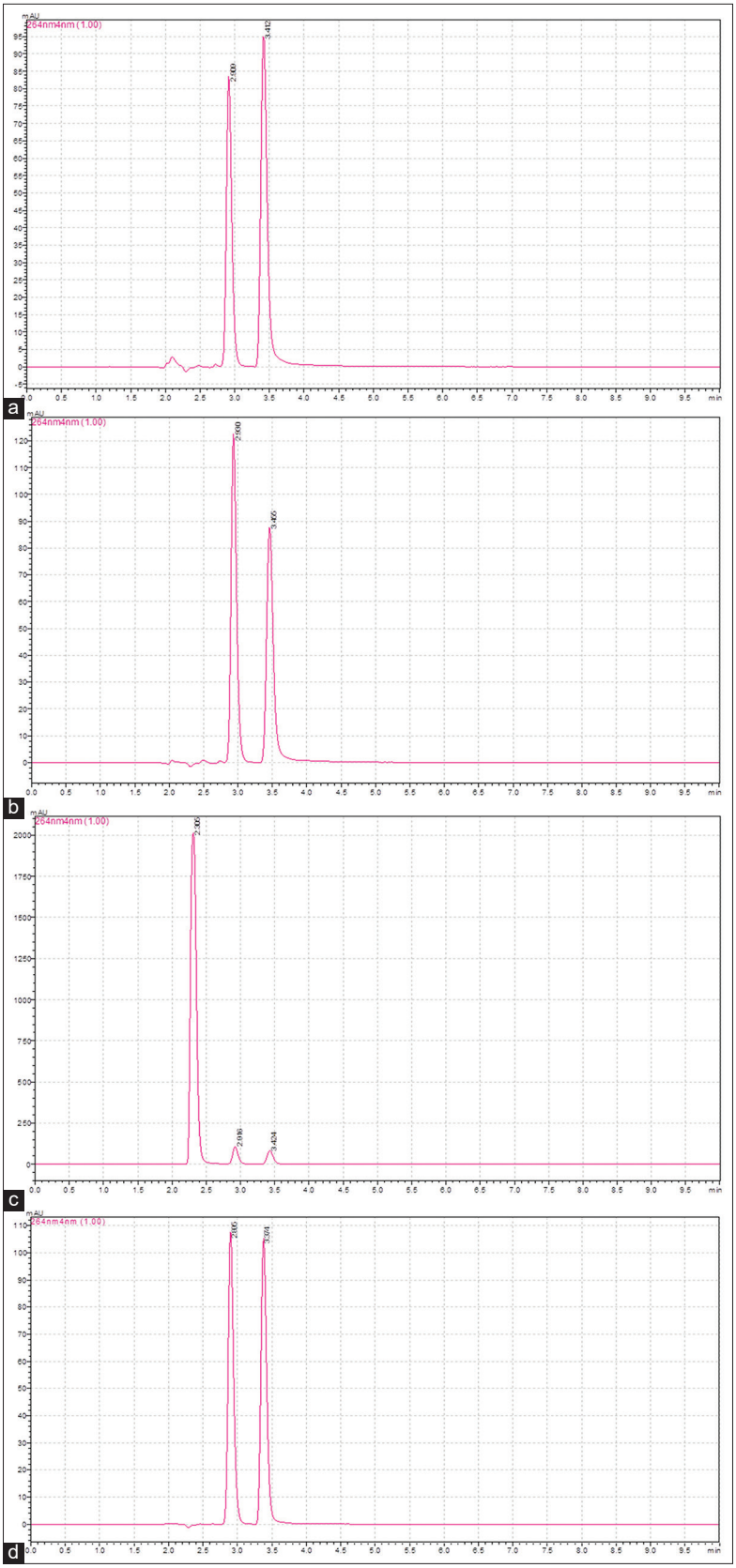

Figure 5: Chromatograms of Gefitinib in presence of internal standard during stress degradation studies (a) Thermal degradation: Gefitinib (Rt 3.412 min) internal standard (Rt 2.909 min) Resolution: 2.865 (b) Alkaline degradation: Gefitinib (Rt 3.455 min) internal standard (Rt 2.930 min) Resolution: 2.895 (c) Oxidative degradation: Gefitinib (Rt 3.424 min) internal standard (Rt 2.916 min) Degradant peak (Rt 2.305 min) Resolution: 2.893 and 3.622 (d) Acidic degradation: Gefitinib (Rt 3.374 min) internal standard (Rt 2.895 min) Resolution: 2.891

The proposed method is highly specific as Gefitinib peak did not interfere with the degradant peaks. The % recovery in all the degradation studies was <20% [Table 9] and the respective chromatograms obtained during the degradation studies were shown in Figure 5.

CONCLUSIONS

A new stability indicating RP-UFLC has been developed for the quantification of Gefitinib in presence of an IS, Trifluridine using an ion pairing agent, TBHS, and the method was validated as per International Council for Harmonisation guidelines. The method is simple, specific, precise, and accurate and there is no interference of degradants with Gefitinib peak and there is no interference of excipients during assay of tablet formulations. Gefitinib is quite stable towards all degradations except oxidation and the percentage of degradation is <20%. The proposed method is simple precise, accurate and robust and is quite suitable for the estimation of Gefitinib in biological fluids as well as for the pharmacokinetic studies of pharmaceutical formulations.

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