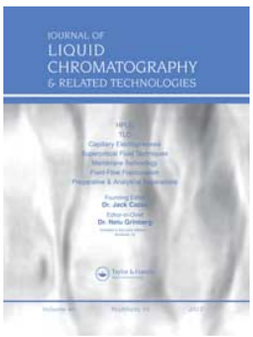

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Quality-by-design-based development and validation of a stability-indicating UPLC method for quantification of teriflunomide in the presence of degradation products and its application to *in-vitro* dissolution

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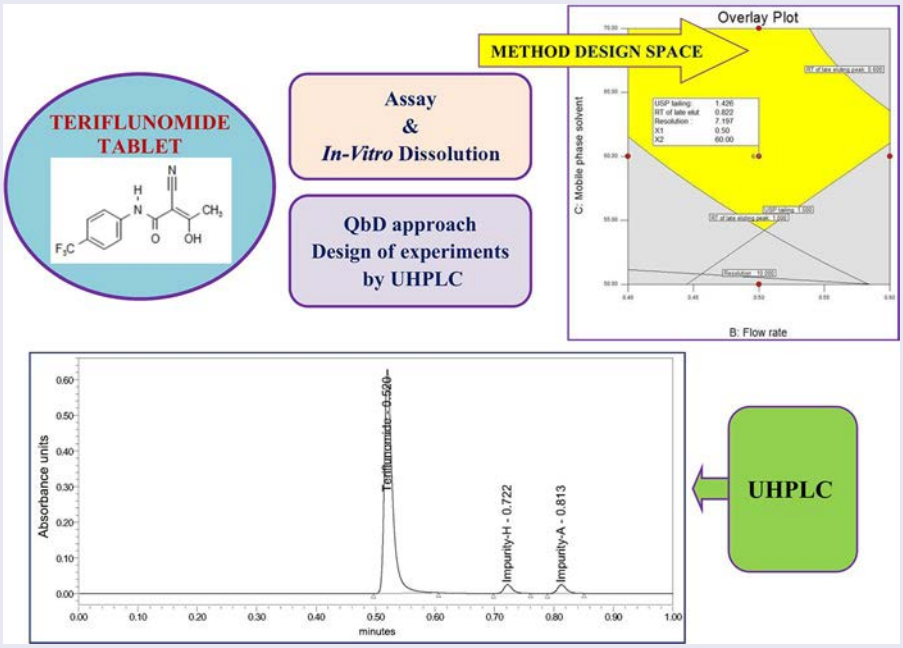
ABSTRACT

A systematic design-of-experiments was performed by applying quality-by-design concepts to determine design space for rapid quantification of teriflunomide by the ultraperformance liquid chromatography (UPLC) method in the presence of degradation products. Response surface and central composite quadratic were used for statistical evaluation of experimental data using a Design-Expert software. The response variables such as resolution, retention time, and peak tailing were analyzed statistically for the screening of suitable chromatographic conditions. During this process, various plots such as perturbation, contour, 3D, and design space were studied. The method was developed through UPLC BEH C18 2.1 × 100 mm, 1.7-µ column, mobile phase comprised of buffer (5 mM K_2HPO_4 containing 0.1% triethylamine, pH 6.8), and acetonitrile (40:60 v/v), the flow rate of 0.5 mL min^{-1} and UV detection at 250 nm. The method was developed with a short run time of 1 min. Forced degradation studies revealed that the method was stability-indicating, suitable for both assay and *in-vitro* dissolution of a drug product. The method was found to be linear in the range of 28–84 µg mL^{-1}, 2.8–22.7 µg mL^{-1} with a correlation coefficient of 0.9999 and 1.000 for assay and dissolution, respectively. The recovery values were found in the range of 100.1–101.7%. The method was validated according to ICH guidelines.

KEYWORDS

Assay and *in-vitro* dissolution; design of experiments; method validation; stability-indicating; teriflunomide; UPLC

GRAPHICAL ABSTRACT



Introduction

Teriflunomide is chemically (Z)-2-cyano-3-hydroxy-but-2-enoic acid-(4-trifluoro methyl phenyl)-amide with molecular formula $C_{12}H_9F_3N_2O_2$ and relative molecular mass of 270.2 g mol^{-1} (Figure 1). Teriflunomide appears as white to almost white, odorless, nonhygroscopic powder. It is a

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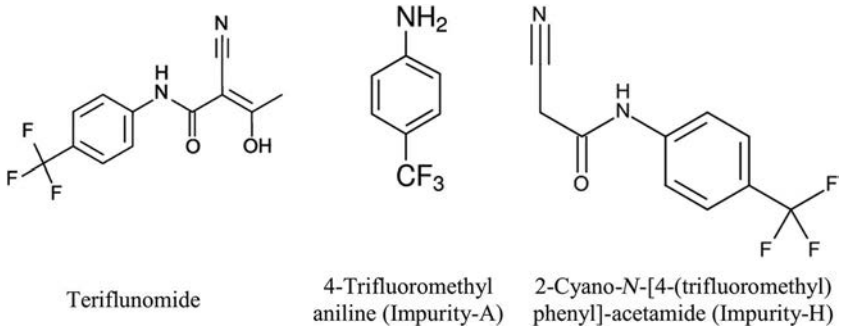

Figure 1. Molecular structure of teriflunomide and its degradation products.

class-2 compound of biopharmaceutical classification system due to its low solubility and high permeability.[1–4] Teriflunomide is weakly acidic with pK_a 3.1 at room temperature and having a pH-dependent solubility. In aqueous buffers at 25°C, the solubility of teriflunomide increases from 0.02 µg mL^{-1} at pH 1.2 to 8 mg mL^{-1} at pH 7.6.[3] Teriflunomide is an immunomodulatory agent with anti-inflammatory properties, inhibits dihydroorotate dehydrogenase, a mitochondrial enzyme involved in *de novo* pyrimidine synthesis.[5–16] Teriflunomide contains two degradation products (Figure 1) namely 4-trifluoromethyl aniline ("leflunomide impurity-A Ph. Eur.") and 2-cyano-*N*-[4-(trifluoromethyl) phenyl]-acetamide ("leflunomide impurity-H Ph. Eur.").[1,17,18]

A stability-indicating method is a quantitative analytical procedure used to detect a decrease in the amount of active pharmaceutical ingredient present due to degradation. According to Food and Drug Administration (FDA) guidelines,[19,20] stability-indicating method is defined as a validated analytical procedure that accurately and precisely measures active ingredients free from potential interferences like degradation products, process impurities, excipients, or other potential degradation products. Quality-by-design (QbD) is a systematic approach to development that begins with predefined objectives and emphasizes product, process understanding, and process control, based on sound science and quality risk management. Key benefits of QbD are as follows.[21–23]

- High level of assurance of analytical method.
  - The method is designed to meet predefined needs and performance requirements.
  - The impact of different reagents and method parameters on analytical method quality is understood.
- Development of robust and cost-effective analytical method.
- Regulatory flexibility.

Teriflunomide is not official in pharmacopoeia such as United States Pharmacopoeia (USP), BP, JP, and Ph. Eur. A wide variety of analytical methods have been reported in the literature for analysis of teriflunomide include estimation of teriflunomide in biological fluids such as human plasma, rabbit plasma, and human blood by high performance liquid chromatography (HPLC) and LC–MS.[24–29] However, there are no methods reported in a study of the effect of stress on pharmaceutical dosage forms, and there is no validated ultra-performance liquid chromatography (UPLC) method on the basis of QbD approach, which enables a stability-indicating UPLC method for quantification of teriflunomide in bulk and pharmaceutical dosage forms. To speed up the analysis, UPLC method gives faster product development for the pharmaceutical industry. In general, an UPLC method provides 3 × higher efficiency and generates a 9 × increase in throughput with no loss in resolution with sub-2-µ particle-size columns than 5-µ particle sizes.[30,31]

The primary objective of the proposed research work is to develop a stability-indicating UPLC method using QbD approach for rapid estimation of assay content and *in-vitro* dissolution release in the presence of degradation products for pharmaceutical tablet dosage forms and to validate the method as per ICH guidelines.[32] Development of shorter chromatography run time reduces the analysis time, cost effectiveness, low solvent consumption altogether increases the pharmaceutical productivity in routine quality control.

Experimental

Materials and reagents

Teriflunomide working standard and teriflunomide film-coated tablets were provided by AET Laboratories Pvt Ltd, Hyderabad, India. Dipotassium hydrogen phosphate (K_2HPO_4), triethylamine, hydrochloric acid, sodium hydroxide (NaOH), phosphoric acid, potassium dihydrogen phosphate (KH_2PO_4), and potassium chloride of Emparta grade were purchased from Merck, India. Acetonitrile, methanol of HPLC grade were procured from Merck, India, and Milli-Q-water was collected from Merck Millipore ELIX 10 system.

Instrumentation

Ultraperformance liquid chromatography system with Empower-3 software, UPLC BEH-C18 100 × 2.1 mm, 1.7-µ column was used for chromatographic analysis. UV spectrophotometer (Lambda 25, Perkin Elmer) was used for the spectroscopic analysis. Analytical balance (XP-205 dual-range model, Metler Toledo), dissolution apparatus (TDT-08 L, Electrolab), Vacuum oven (Thermolab), vacuum filtration unit (Millivac-Maxi 230 V, Millipore), pH meter (Orion-Star-A211, Thermo), Rotary shaker (RS-24BL, REMI), water bath (MSI-8, Meta Lab), photo-stability chamber (NEC103RSPSI, Newtronics), and sonicator (9L250H, PCI) were used.

Chromatographic conditions

Chromatographies conditions were optimized based on design-of-experiments (DoE) studies. The chromatographic

separation was achieved on UPLC BEH-C18 100 × 2.1 mm, 1.7-µ column using mobile phase composed of buffer (5 mM K_2HPO_4 containing 0.1% triethylamine, pH 6.8) and acetonitrile in the ratio of 40:60 v/v. The flow rate was set at 0.5 mL min^{-1}, and UV detection wavelength was performed at 250 nm. Injection volume was 1 µL with a column temperature of 35°C. Total run time of method was 1 min. Fifty mM phosphate buffer (pH 6.8) and acetonitrile in the ratio of 60:40 v/v were used as a diluent.

Standard and sample preparation

Weighed and transferred about 56 mg of teriflunomide working standard into a 100-mL volumetric flask. Added 60 mL of diluent, sonication was done to dissolve and made up to volume with diluent. From this solution, 5 mL was diluted to 50 mL with diluent to obtain a concentration of 56 µg mL^{-1} of teriflunomide. Taken 20 tablets of the test sample into a mortar and pestle, and then crushed to a fine powder. Weighed and transferred powder equivalent to 14 mg of teriflunomide into a 250-mL clean and dry volumetric flask. Then added 150 mL of diluent and sonication was performed for 10 min with intermittent shaking for extraction of the drug. The volume was made up to 250 mL with diluent. The sample solution was filtered through 0.2-µ polyvinylidene difluoride (PVDF) syringe filter and collected the sample with a concentration of 56 µg mL^{-1} of teriflunomide. Teriflunomide was quantified using the following formula, where, "A_x" is the area obtained from sample chromatogram, "A_s" is the average area obtained from standard chromatograms, "W_{std}" is weight of teriflunomide standard in mg, "W_{spl}" is weight of sample in mg, "AW" is the average weight of drug product, "LC" is label claim, and "P" is the percentage assay of teriflunomide standard on as is basis.

$$\text{Assay (\% label claim)} = \frac{A_x}{A_s} \times \frac{W_{std}}{100} \times \frac{5}{50} \times \frac{250}{W_{spl}} \times \frac{AW}{LC} \times P$$

In-vitro *dissolution*

The standard solution was prepared at a concentration of 14 µg mL^{-1} of teriflunomide in phosphate buffer solution of pH 6.8. The *in-vitro* dissolution test was performed using a phosphate buffer solution of pH 6.8 as dissolution media with a medium volume of 1000 mL and media temperature of 37°C. The type of dissolution apparatus used is a paddle with a rotation speed of 50 rpm. The sample was collected after 30 min time point, and samples were filtered through 0.2-µ PVDF syringe filter.

Method validation

The developed UPLC method for quantification of teriflunomide was validated according to a current international conference on harmonization guideline ICH Q2 (R1) validation of analytical procedures.[32] The method was validated for its system suitability, specificity, linearity, accuracy, precision, and robustness. Before analyzing the sample, system suitability criteria were performed to verify whether the analytical system (analytical solutions, UPLC system, column) is suitable or not for giving accurate and consistent precise results. The system suitability of the proposed method was evaluated by calculating parameters such as theoretical plates not less than 2000, tailing factor not more than 2.0, and percentage relative standard deviation (RSD) from five standard injections should not be more than 2.0.[33,34] The specificity of the analytical method was determined by verifying the interference of blank/placebo, impurity peaks at the retention time of teriflunomide. Standard and sample solutions were prepared at a concentration of 56 µg mL^{-1} of teriflunomide. Placebo solution was prepared similarly as sample preparation by taking a placebo and omitting drug substance. Individual preparations of impurity-A and impurity-H solutions were performed at a concentration of 2.8 µg mL^{-1}, i.e., 5% level with respect to sample concentration. Solutions were injected into the chromatography system by giving 200–400-nm wavelength ranges in photodiode array (PDA) system. Recorded the chromatograms, verified for peak purity of teriflunomide as well as interferences of the blank, placebo and impurity peaks.

Forced degradation studies

Performed the forced degradation studies and all the degradation samples were diluted with diluent after completion of the degradation process. Blank and placebo solutions were prepared in the same manner in respective degradation to exclude any contribution from the process. All the degradation sample solutions were injected into UPLC-PDA system and recorded the chromatograms. Peak purity was determined for teriflunomide and verified for any interference of placebo/degradation products at the retention time of teriflunomide. The total percentage of degradation products and a percentage of the assay were calculated. Mass balance was performed by adding together the assay value and total percentage of degradation products to make up to about 100% of the initial assay value of the drug product.

Linearity and range

The linearity of the analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of an analyte in the sample. The linearity of the proposed analytical method was determined by preparing five concentration levels from 28 to 84 µg mL^{-1} of teriflunomide. The correlation coefficient (r), regression coefficient (r^2), y-intercept, and slope of regression line were calculated. The range of the method was proved by performing the linearity, precision, and accuracy at the proposed minimum 50% and maximum 150% concentration levels with respect to sample concentration. Weighed and transferred about 56 mg of teriflunomide into a 100-mL volumetric flask. Dissolved and diluted to volume with diluent to obtain a concentration of 560 µg mL^{-1} of teriflunomide (linearity stock). To prepare 50, 80, 100, 120, and 150% levels, respectively, 5, 8, 10, 12, and 15 mL of linearity stock solutions were diluted to 100 mL.

Accuracy

A known amount of teriflunomide drug substance was spiked at 50, 100, and 150% levels with respect to sample concentration

to the placebo and analyzed by the proposed UPLC method. Percentage recovery was calculated. Weighed and transferred 350 mg of teriflunomide into a 250-mL volumetric flask. Added 150 mL of diluent and sonication was performed to dissolve. The diluent was added up to the mark to get the concentration of 1400 µg mL^{-1} of teriflunomide (accuracy stock solution). Weighed and transferred placebo equivalent to one tablet weight into a 250-mL clean and dry volumetric flask. Added 5, 10, and 15 mL of accuracy stock solution to 150 mL of diluent to obtain 50, 100, and 150% concentration levels, respectively. Solutions were sonicated for 10 min with intermittent shaking and made up to volume with diluent.

Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scattering) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision was performed in repeatability and intermediate precision methods. Repeatability for assay method was demonstrated by preparing six assay sample solutions at a concentration of 56 µg mL^{-1} (100% level of sample concentration) and injected into an UPLC system over a short interval of time as per proposed method and calculated the percentage RSD for assay results. Intermediate precision was demonstrated by preparing six assay sample solutions at a concentration of 56 µg mL^{-1} on a different day by different analysts and then injected into a UPLC system as per proposed method. Percentage RSD of assay results was calculated between two analyst values.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters, and it provides an indication of its reliability during normal usage. Method robustness was established by considering the variations in wavelength (250 ± 2 nm), flow rate (0.5 ± 0.1 mL min^{-1}), column oven temperature (35 ± 5°C), and a mobile phase ratio (40:60 v/v, 38:62 v/v, 42:58 v/v). Solution stability and filter interference were established.

Results and discussion

Method development and optimization

Method development was initiated based on QbD concepts. Quality target analytical profile (QTAP) forms the basis of design for the development of analytical methods. QTAP includes all the target requirements along with justification and objectives of the method and are listed in brief in Table 1.[21,35] Method quality attributes were derived from the QTAP. Critical method quality attributes (CMQA) are the measurable parameters or characteristics of the method that should be within predefined appropriate limits, ranges or acceptance criteria. The CMQA for an analytical method are primarily all the validation parameters of the method including the robustness requirements.[32,35] UV spectrum of teriflunomide was determined in a solution containing 56 µg mL^{-1} of teriflunomide in a diluent containing 50 mM phosphate buffer of pH 6.8 and acetonitrile in the ratio of 60:40 v/v. PDA spectrum was collected from 200 to 400-nm wavelength (Figure 2). Wavelength maxima were observed at 204.8, 248.8, and 295.7 nm. Though the highest absorption is observed at 295 nm, degradation products (impurity-A and impurity H) were observed at 250 nm. Hence, 250 nm was selected as chromatographic detection wavelength for teriflunomide peak. Since all peak responses are at 250 nm, the method is suitable for mass balance studies.

Method optimization

The solubility of teriflunomide was performed in aqueous media with a pH ranging from 1.2 to 6.8. The solubility of teriflunomide in water, hydrochloric acid media of pH 1.2, acetate buffer of pH 4.5 and phosphate buffer of pH 6.8 were insoluble, insoluble, 0.1, and 3.9 mg mL^{-1}, respectively. Based on the solubility of teriflunomide, pH of diluent and the mobile phase buffer was considered in pH above 6. During preliminary method development trials, the chromatographic conditions were used as follows. Mobile phase-A was 10 mM KH_2PO_4 containing 0.5% v/v of triethylamine with pH 6.5. Acetonitrile was used as a mobile phase-B. UPLC BEH C18 2.1 × 50 mm, 1.7 µ column was used with a flow rate of 0.5 mL min^{-1}, the detection wavelength of 250 nm, and an injection volume of 1 µL. Different solvent compositions of mobile phase were studied for teriflunomide peak shape. Poor peak shape was observed in preliminary method development trials and further proceeded with pH scouting studies to evaluate the peak tailing and noninterference of degradation products. pH scouting studies were performed to select the optimum mobile phase pH, to obtain shorter run time with no interference of placebo and impurity peaks at the retention time of teriflunomide. Mobile phases with different pH of buffers were prepared by mixing buffer and acetonitrile in the ratio of 50:50 v/v. The results of pH scouting studies were given in Table 2. pH scouting studies reveal that the retention time of impurity-A and impurity-H was not changing with the change in pH of mobile phase buffer. But, the retention time of teriflunomide peak was moving to lower retention time by increasing pH of mobile phase buffer. Comparatively, the higher USP plate count was observed at buffer pH 2.0, but the impurity peaks were eluted nearby the teriflunomide peak. And also, lower pH was not suggestible due to poor solubility of teriflunomide. Hence, pH of mobile phase buffer between 6.0 and 7.0 was selected for optimization to achieve a lower retention time of teriflunomide without the interference of degradation products and to overcome any solubility issues due to lower pH.

Design-of-experiment studies

The design of experiments was executed to select robust and rugged operational chromatographic conditions within the design space. Ten mM KH_2PO_4 containing 0.5% v/v of triethylamine with pH 6.8 was taken as a mobile phase buffer

Table 1. Quality target analytical profile.

Analytical target profile element	Target/requirement	Justification	Status of current study (yes/no/remarks)
Type of method	Quantification of teriflunomide in the presence of degradation products	To quantify the teriflunomide in the formulation	Yes
Mode of detection and chromatography	UV, isocratic	The molecule is having chromophoric groups and can be detected by UV	Yes, UV detection at 250 nm
Specificity	Blank, placebo, and impurity interference should not be observed	ICH Q_2 (R1) guideline requirements	Yes
Precision of the method, repeatability/ reproducibility	Should have a precision withpercentage RSD below 2.0	ICH Q_2 (R1) guideline requirements	Yes
Accuracy	Percentage recoveryshould be between 98 and 102	The percentage recovery should be good for the drug product and as per requirements of ICH guidelines	Yes
Linearity	Linearity at different concentration levels should be obtained	The correlation coefficient should not be less than 0.99 and as per requirements of ICH guidelines	Yes
Robustness	Assay results should not be affected by small changes in method parameters	Results should not be affected	Yes
Stability indicating nature	The principle peak should be pure even after forced degradation	Purity angle should be less than purity threshold and the mass balance should be close to 100% of initial assay	Yes
Green chromatography	Should use minimum percentage of organic phase as possible	To avoid the use of more organic solvents and to develop an environment-friendly method	Yes, low solvent consumption with a shorter run time of 1 min

for DoE studies. DoE study was executed by considering the mobile phase composition, flow rate, column temperature as control variables. USP tailing, the retention time of late eluting impurity peak and resolution of nearby impurity with teriflunomide were taken as response variables. Design Expert 8.0 software was used for the study. The selected design parameters are response surface as study type, central composite as design type and quadratic as design mode. Proposed ranges of mobile phase solvent composition 50–70%, flow rate 0.4–0.7 mL min^{-1}, and column temperature 25–45°C were selected to design. This data were fed into the design expert software and software given twenty experiments. All the DoE experiments were executed in UPLC and observed results are tabulated in Table 3. The effect of control variables on response variables was graphically evaluated in Figure 3. The observations were derived from perturbation plot and 3D plot which describes the effect of method control variables on the response variables.

Based on the desirability plot (Figure 4), column temperature was set at 35°C. Design space plot (Figure 4) suggests that flow rate of 0.5 mL min^{-1} and mobile phase composition of 40:60 v/v (buffer:acetonitrile) for a robust chromatographic method. Teriflunomide peak was eluted at 0.5 min and run time was set at 1 min. Risk assessment is a valuable scientifically based process used in quality risk management. Risk assessment is helpful in identifying the input material attributes (reagents/chemicals/columns) and method parameters (flow, temperature, solution stability) that are affecting CMQA. The first step of the risk-assessment process involves identification of critical material attributes and method parameters and performing risk assessments of their attributes for the subsequent effect on CMQA. Based on risk assessment, control strategy was established for the selection of diluent pH at 6.8 for solubility of teriflunomide.

Method validation

Specificity

Diluent, placebo, standard, sample, impurity-A, impurity-H, and impurity-spiked sample solutions were injected into

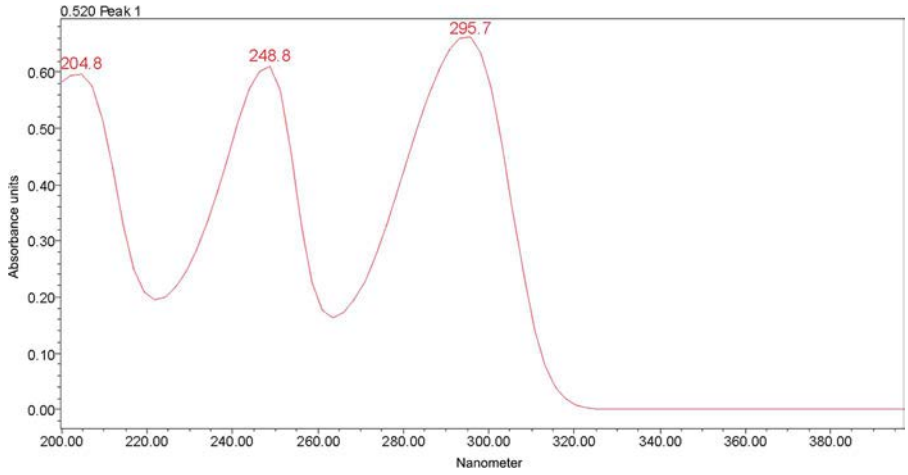

Figure 2. UV spectrum of teriflunomide.

Table 2. Results of pH scouting experiments.

Buffer pH	RT (retention time) of teriflunomide (min)	RT of impurity-A (min)	RT ofimpurity-H (min)	USP tailing	USP plate count
pH 2.0	1.388	1.263	1.130	1.59	9251
pH 3.0	0.808	1.322	1.130	2.02	4694
pH 4.0	0.661	1.329	1.133	1.90	3359
pH 5.0	0.590	1.332	1.134	1.90	3151
pH 6.0	0.587	1.327	1.136	1.90	3157
pH 7.0	0.579	1.333	1.134	1.38	3048

UPLC-PDA system, and chromatograms were extracted. The results are summarized in Table 4. The system suitability test results observed during specificity and precision test are tabulated in Table 5. Acidic, basic, oxidation, thermal, water, humidity, and photodegradation samples were injected into UPLC-PDA system and chromatograms were extracted. No interference was observed with blank, placebo, and impurities at the retention time of teriflunomide. Teriflunomide peak passed the peak purity test for all degradation samples. The results are summarized in Table 4. Mass balance was achieved for all forced degradation samples and the results are summarized in Table 4. The representative chromatogram of specificity is given in Figures 5 and 6.

Linearity and range

Linearity solutions, i.e., 50, 80, 100, 120, and 150% levels were injected into UPLC and chromatograms were recorded. The regression line of analysis shows the linear relationship between concentration and area response of teriflunomide. Results of linearity and range are summarized in Table 5.

Accuracy and precision

Teriflunomide-spiked samples of 50, 100, and 150% levels with respect to sample concentration to the placebo were analyzed by the proposed UPLC method. Recovery of teriflunomide was observed from 100.1 to 101.7% and all the individual results were within the range of 98–102% criteria. The results are summarized in Table 6. The precision of the analytical method was determined by repeatability and intermediate precision. The percentage RSD results for repeatability, intermediate precision, and between two analyst values were 1.03, 1.19, and 1.15, respectively (Table 6). Since the percentage RSD of six assay results is not more than 2.0, the method is repeatable. The percentage RSD of two analyst's assay results is less than 2.0, hence, intermediate precision is acceptable.

Robustness

Method robustness was established by considering the changes in wavelength, flow rate, column oven temperature, and mobile phase ratio. Solution stability and filter interference were studied. Hydrophilic polypropylene (GHP) and Millipore-PVDF syringe filters were evaluated for filter interference and no significant interference was observed. The percentage differences of area response from the unfiltered area for GHP and PVDF were 0.28 and 0.38, respectively. The results of robustness are tabulated in Table 5. Robustness test passed as a variation from initial results is not more than 2.0%.[36–40]

Table 3. Experimental results of DoE study and effect on response variables.

DoE experiments				Results of response variables		
Run no.	Column temperature (°C)	Flow rate (mL min^{-1})	Mobile phase composition (v/v)	USP tailing	Retention time of late eluting impurity (min)	Resolution of nearby impurity with teriflunomide
1	25	0.6	50:50	1.70	0.997	10.35
2	45	0.6	50:50	1.54	0.944	9.76
3	35	0.4	40:60	1.30	1.030	7.63
4	45	0.4	30:70	1.19	0.806	4.42
5	35	0.6	40:60	1.50	0.681	6.87
6	45	0.4	50:50	1.40	1.428	9.61
7	35	0.5	40:60	1.43	0.822	7.17
8	45	0.5	40:60	1.36	0.806	6.84
9	35	0.5	40:60	1.43	0.822	7.17
10	25	0.4	50:50	1.52	1.539	10.51
11	25	0.5	40:60	1.52	0.840	7.33
12	35	0.5	40:60	1.43	0.822	7.17
13	35	0.5	50:50	1.58	1.175	10.7
14	35	0.5	40:60	1.43	0.822	7.17
15	25	0.6	30:70	1.07	0.549	4.12
16	35	0.5	30:70	1.10	0.657	4.27
17	35	0.5	40:60	1.43	0.822	7.17
18	45	0.6	30:70	1.08	0.536	3.88
19	35	0.5	40:60	1.43	0.822	7.17
20	25	0.4	30:70	1.22	0.831	4.62

	Effect on response variables		
Increase of control variable	USP tailing	RT of late eluting peak	Resolution of nearby peak with teriflunomide
Observations from DoE study plots			
A: Column temperature	Decreases	No effect	No effect
B: Flow rate	Increases	Decreases	No effect
C: Mobile phase solvent composition	Decreases	Decreases	Decreases

DoE, design-of-experiments.

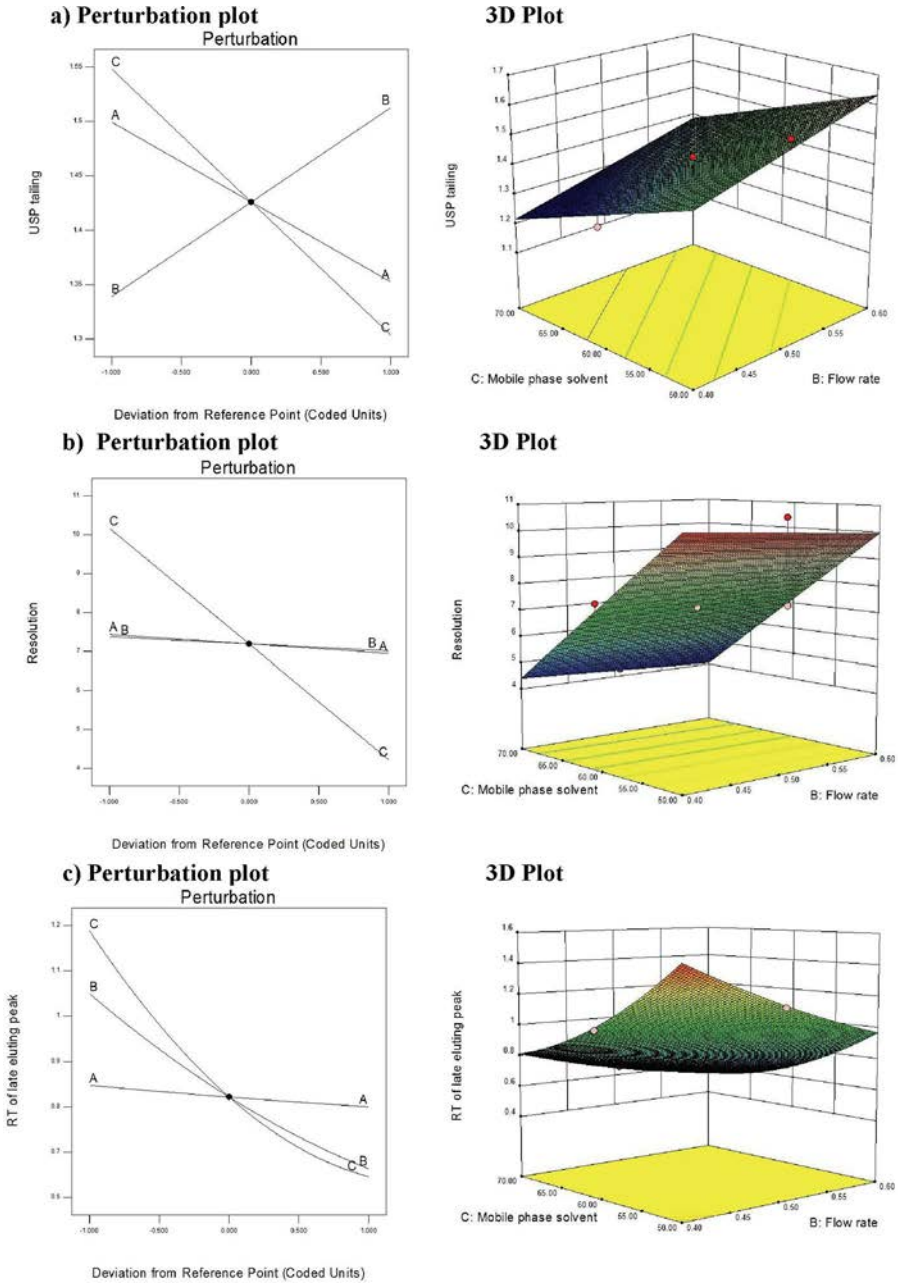

Figure 3. Affect of control variables A (column temperature), B (flow rate), C (mobile phase solvent composition) on response variables a) USP tailing, b) resolution, c) retention time of late eluting impurity.

Comparison with reported methods

Ultraperformance liquid chromatography method was developed for rapid quantification of teriflunomide in the presence of degradation products in the pharmaceutical drug product. The present method uses the shortest run time of 1 min with a retention time of teriflunomide of about 0.5 min compared to earlier reported methods which are having a run time of minimum 2 min with an RT of 1.43 min (Table 7). The method was developed based on QbD approach, and optimum chromatographic conditions were selected based on design space obtained through DoE studies. The developed method is stability indicating as there is no interference in force degradation studies (Table 4). The method was validated as per ICH guidelines and the results of specificity, linearity, accuracy, precision, and robustness were found satisfactory. No interference was observed with blank, placebo, and degradation products at the retention time of teriflunomide. The purity angle was less than the purity threshold indicating that teriflunomide peak was free from interference and passed the peak purity test (Table 4). The reported methods were given for estimation of teriflunomide release rate in biological fluids.[24–28] Present method was developed for rapid quantification of teriflunomide in the presence of potential impurities by QbD in pharmaceutical bulk and finished dosage form. Sample injection volume (1 µL) in the present method is significantly lower, which helps to maintain the good column performance compared to earlier reported methods (minimum of 5 µL). A detailed comparison of selected procedures with the present method is given in Table 7. Teriflunomide contains no asymmetric centers,

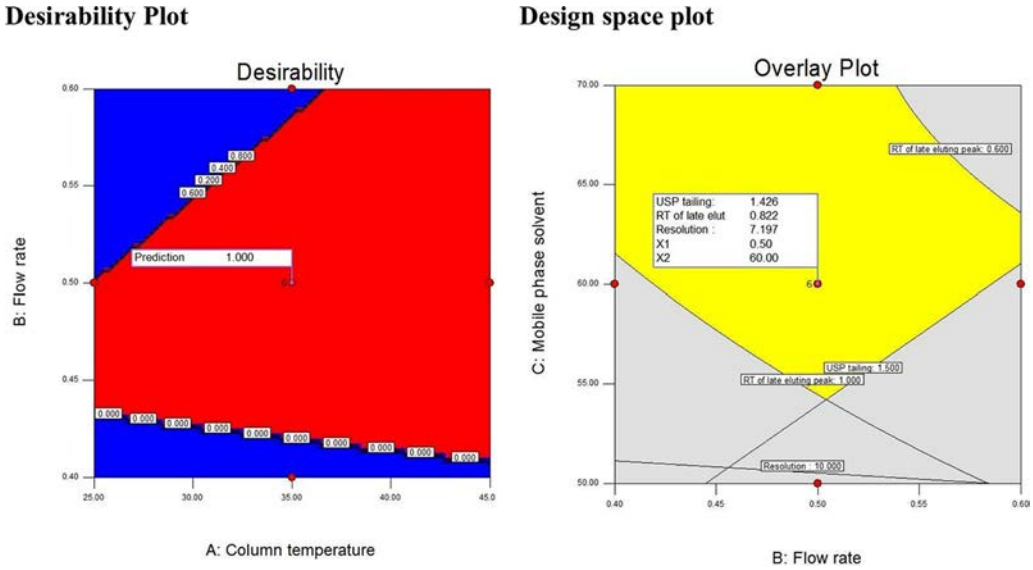

Figure 4. Schematic representations of desirability plot and design space plot.

Table 4. Specificity, forced degradation studies, and mass balance results.

Sample name	Retention time (min)	Purity angle	Purity threshold	Peak purity	Assay (%)	Impurities (%)	Total	Mass balance
Impurity-A	0.813	—	—	—	—	—	—	—
Impurity-H	0.722	—	—	—	—	—	—	—
Teriflunomide standard	0.521	22.675	63.543	Pass	—	—	—	—
Sample	0.521	20.704	73.281	Pass	99.1	Nil	99.1	Yes
Acid-degradation	0.518	23.388	62.978	Pass	99.3	0.9	100.2	Yes
Alkali-degradation	0.524	1.509	72.977	Pass	99.2	Nil	99.2	Yes
Oxidation with $KMnO_4$	0.517	22.161	90.000	Pass	91.6	6.6	98.2	Yes
Photo degradation-UV light	0.518	23.954	71.828	Pass	100.5	Nil	100.5	Yes
Photo degradation-visible light	0.519	21.555	66.176	Pass	100.2	Nil	100.2	Yes
Thermal-degradation	0.518	16.006	46.652	Pass	99.1	Nil	99.1	Yes
Water-degradation	0.519	21.389	59.804	Pass	96.8	2.3	99.1	Yes

Table 5. System suitability evaluation, linearity, and robustness results.

		Results of the test		
Parameter	Acceptance criteria[33]	Specificity	Precision	Remarks
RSD (%) of area/five injections	Not less than 2.0	0.38	0.38	Satisfactory
USP tailing factor	Not more than 2.0	1.66	1.51	Satisfactory
Theoretical plates	Not less than 2000	8134	9938	Satisfactory
Type of test		Assay		*In-vitro* dissolution
Linearity test results				
Test concentration (μg mL^{-1})		28.02–84.06		2.84–22.70
Correlation coefficient (*R*)		0.9999		1.0000
Regression coefficient (R^2)		0.9998		0.9999
Slope		10124.6444		19885449.4184
Intercept		1919.441		1646.58
Parameter		Change done	Results	Remarks
Robustness results				
Solution stability		Initial	100.6	Solutions are stable for 24 hr
		After 24 hr	101.1	
Wavelength (250 ± 2 nm)		250	99.1	No significant variation in results
		248	99.2	
		252	99.3	
Flow rate (0.5 ± 0.1 mL min^{-1})		0.5	99.1	
		0.4	99.8	
		0.6	100.1	
Column oven temperature (35 ± 5°C)		35	99.1	
		30	98.8	
		40	99.2	
Mobile phase ratio (buffer:acetonitrile, v/v)		40:60	99.1	
		38:62	99.2	
		42:58	98.5	

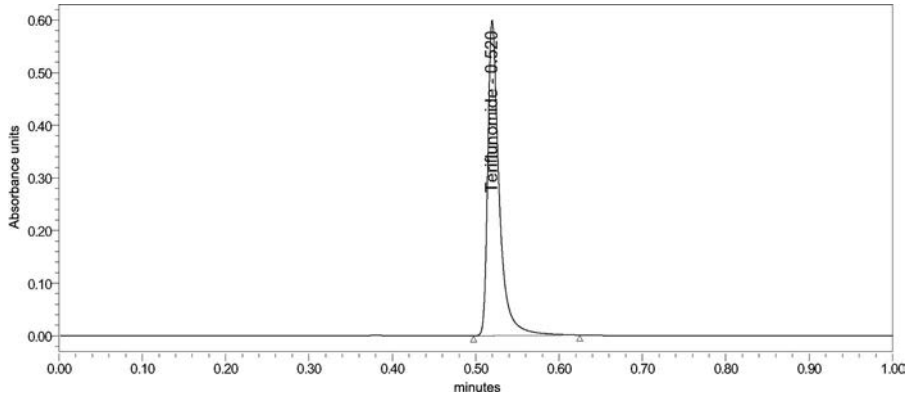

Figure 5. Teriflunomide sample chromatogram.

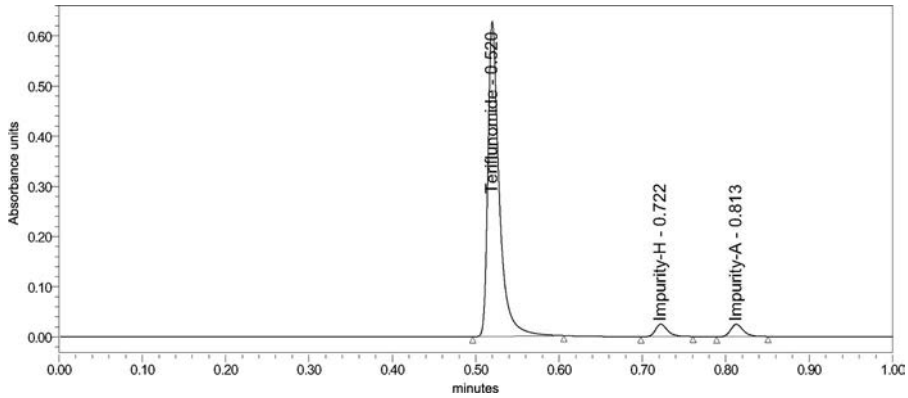

Figure 6. Impurity spiked sample chromatogram.

Table 6. Accuracy and precision test results.

Level	Theoretical concentration (μg mL^{-1}, as teriflunomide)	Experimental concentration (μg mL^{-1}, as teriflunomide)	Recovery (%)
50%-sample-1	27.814	28.101	101.0
50%-sample-2	27.814	28.164	101.3
50%-sample-3	27.814	27.875	100.2
50%-sample-4	27.814	28.278	101.7
50%-sample-5	27.814	28.288	101.7
50%-sample-6	27.814	28.503	101.1
100%-sample-1	55.628	55.999	100.7
100%-sample-2	55.628	55.771	100.3
100%-sample-3	55.628	55.703	100.1
150%-sample-1	83.442	83.538	100.1
150%-sample-2	83.442	83.807	100.4
150%-sample-3	83.442	83.697	100.3
150%-sample-4	83.442	84.207	100.9
150%-sample-5	83.442	84.166	100.9
150%-sample-6	83.442	84.338	101.1
		Mean	100.8
		RSD (%)	0.53

	In-vitro dissolution-precision	Assay-repeatability	Assay-intermediate precision
Preparation	Labelled amount (%)	Label claim (%)	Label claim (%)
Precision results			
1	97	99.1	98.5
2	98	99.8	97.6
3	98	99.5	98.5
4	100	101.7	99.8
5	98	99.2	99.6
6	99	100.8	100.9
Mean	98	100.0	99.2
RSD (%)	1.24	1.03	1.19
	Mean between two analyst values		99.6
	RSD (%) between two analyst values		1.15

Table 7. Comparison of selected analytical methods developed for teriflunomide.

S. no.	Column, elution process, mobile phase, flow rate, injection volume	Sample linear range, detection	Run time (RT of teriflunomide)	Intended use	Refs. no.
1	Phenomenex Luna2 PFP, 2 × 100 mm, 3 µ, gradient, mobile phase-A (0.1% formic acid), mobile phase-B – 0.1% formic acid in methanol:water:acetonitrile (0.5:0.5:9 v/v/v), 300 µL min^{-1}, 100 µL	0.005–200 µg mL^{-1}, LC–MS/MS	4 min (2.18 min)	For estimation of release rate in biological sample	[24]
2	Inertsil-ODS-3 C18 (50 × 4.6 mm, 5 µ); isocratic, 0.02 M CH_3COONH_4 pH 6.5: methanol (25:75 v/v); 0.8 mL min^{-1}, 5 µL	10.1–4000 ng mL^{-1}, LC–ESI-MS/MS	2.0 min (1.43 min)	For estimation of release rate in biological sample	[25]
3	PolySULFOETHYL aspartamide strong cation exchange column; isocratic, 10 mmol L^{-1} KH_2PO_4 and 100 mmol L^{-1} KCl in aqueous 25% acetonitrile, acidified to pH 3 with *o*-phosphoric acid. 0.7 mL min^{-1}, 10 µL	0–200 µg mL^{-1}, HPLC–UV at 280 nm	7 min (2.9 min)	For estimation of release rate in biological sample	[26]
4	XTerra-MS-C18 analytical column (100 × 3.9 mm, 5 µ), gradient, mobile phase-A-0.005 M ammonium formate (pH 9.0) and B-acetonitrile at 1.0 mL min^{-1}, 10 µL	1–4000 ng mL^{-1}, LC–ESI-MS/MS	15 min (6.7 min)	For estimation of release rate in biological sample	[27]
5	Nucleosil 100-5 C18-column, gradient, mobile phase A-0.5 mM ammonium acetate in water–acetonitrile–formic acid (9:5:0.02 v/v/v) and mobile phase-B-0.5 mM ammonium acetate in water–acetonitrile–formic acid (5:95:0.02 v/v/v), 0.5 mL min^{-1}, 60 µL	5–500 µg mL^{-1}, LC–ESI-MS/MS	7 min (3.1 min)	For estimation of release rate in biological sample	[28]
6	Acquity-UPLC BEH-C18 2.1 × 50 mm, 1.7 µ column, isocratic, 5 mM K_2HPO_4 buffer containing 0.1% triethylamine (pH 6.8) and acetonitrile (40:60 v/v), 0.5 mL min^{-1}, 1 µL	28–84.1 µg mL^{-1}, UPLC–UV at 250 nm	1 min (0.5 min)	For quantification of teriflunomide in the presence of potential impurities	Present method

therefore no enantiomers are possible, but can exist in E and Z isomers. E-isomer will not separate in existing developed method. As per literature review, there are no methods available for the separation of these isomers. There is a scope for future research work on separation of E and Z isomers of teriflunomide.

Conclusions

The UPLC method was developed based on QbD concepts for rapid quantification of teriflunomide in pharmaceutical drug products. The developed method was validated as per ICH guidelines. The method was found to be simple, selective, accurate, precise, and robust. The developed method was stability indicating as it was showing no interference of degradation products and placebo at the retention time of teriflunomide. Due to the shorter run time of 1 min, this method provides faster analysis, more work throughput, and reduces the cost of analysis due to the reduction in solvent consumption. The method can be used for *in-vitro* dissolution analysis. Therefore, the developed method can be used for routine assay and *in-vitro* dissolution analysis of quality control samples and stability samples of bulk and finished pharmaceutical dosage form.

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