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An improved validated ultra high pressure liquid chromatography method for separation of tacrolimus impurities and its tautomers

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A selective, specific and sensitive ultra high pressure liquid chromatography (UHPLC) method was developed for determination of tacrolimus degradation products and tautomers in the preparation of pharmaceuticals. The chromatographic separation was performed on Waters ACQUITY UPLC system and BEH C_8 column using gradient elution of mobile phase A (90 : 10 v/v of 0.1% v/v triflouroacetic acid solution and Acetonitrile) and mobile phase B (90 : 10 v/v acetonitrile and water) at a flow rate of 0.6 mL min^{-1}. Ultraviolet detection was performed at 210 nm. Tacrolimus, tautomers and impurities were chromatographed with a total run time of 25 min. Calibration showed that the response of impurity was a linear function of concentration over the range 0.3–6 μg mL^{-1} ($r^2 \geq 0.999$) and the method was validated over this range for precision, intermediate precision, accuracy, linearity and specificity. For precision study, percentage relative standard deviation of each impurity was <15% ($n = 6$). The method was found to be precise, accurate, linear and specific. The proposed method was successfully employed for estimation of tacrolimus impurities in pharmaceutical preparations.

Keywords: UHPLC; tacrolimus; tautomers; impurities; method validation

Introduction

Tacrolimus (TAC) is an active pharmaceutical ingredient with useful immunosuppressive and antimicrobial activity. TAC, also known as FK-506, has the chemical tricyclic structure, as shown in Figure 1.[1] TAC epimerizes to an intermediate tautomer-I (Cis), which is converted into tautomer-II (Trans) to reach equilibrium with three forms, as shown in Figure 1.[2] The immunosuppressive activity was found to be associated with the Cis-Trans conversion through binding to FK506 binding protein.[3] TAC is very sensitive to temperature and will degrade to form impurities with very closely related structures by rearrangement, as shown in Figure 2.[2,4] Separation of TAC, tautomers and its impurities is a major challenge due to structural similarity as shown in Figure 2 and low absorptivity at conventionally used detection wavelengths. TAC exhibits a maximum absorbance at about 205 nm, which poses difficulties in its estimation using ultraviolet spectroscopy.[5,6]

Many analytical approaches, such as liquid chromatography in combination with mass spectrometry,[7–18] fluorescence detection,[19–20] ultraviolet detection[2,21] and HPTLC method[6] are reported for determination of TAC in biological matrices, bulk drugs, and formulations having limit of quantification (LOQ) ranging from μg/mg to pc/mg. Moreover all the above-mentioned methods are orientated to the determination of the active pharmaceutical compound. Now-a-days, the pharmaceutical industry is forced to assess strict control of impurities when manufacturing drug substance and drug products.[22,23] Determination of impurities during the development of separation methods is one of the main and difficult tasks for pharmaceutical analysts, especially if determination of more and more impurities of closely related structures is required. Reports on the determination of TAC-related substances are rather limited. Related substance method (impurities) of TAC capsules appeared in The United States Pharmacopoeia (USP) forum, which is based on normal phase chromatography at 225 nm.[24] The published TAC impurity method demonstrates analysis of two known impurities and one specified unknown impurity with detection wavelength at 225 nm, whereas TAC exhibits maximum absorbance at 205 nm. The published method also demonstrates use of multiple columns for separation of impurities with a total separation time of more than 45 min. By considering all the above factors, a simple and sensitive method should be developed to monitor all impurities of TAC.

In liquid chromatography, the analysis time can be reduced by using small columns packed with sub-2 μm particles. In addition, with sub-2 μm particles, due to the higher efficiency and smaller retention volume, sensitivity is also improved. UHPLC, which uses 1.7 μm particles at a maximum operating pressure of 1000 bar, has proved to be a suitable analytical technique with the advantages of increased linear velocity (speed) and reduced solvent consumption. Although UHPLC has been shown to achieve high resolution in a relatively short time, there are few reports of its application to the analysis of pharmaceutical products and their impurities as an alternative to conventional HPLC analysis.

In order to improve the sensitivity and selectivity of the chromatographic determination of TAC impurities, a simple reversed-phase UHPLC method with UV detection at 210 nm, have been developed, where all four impurities as well as tautomers have been separated in a single analytical column with a run time of 25 min. In our study, Waters ACQUITY UPLC was successfully used

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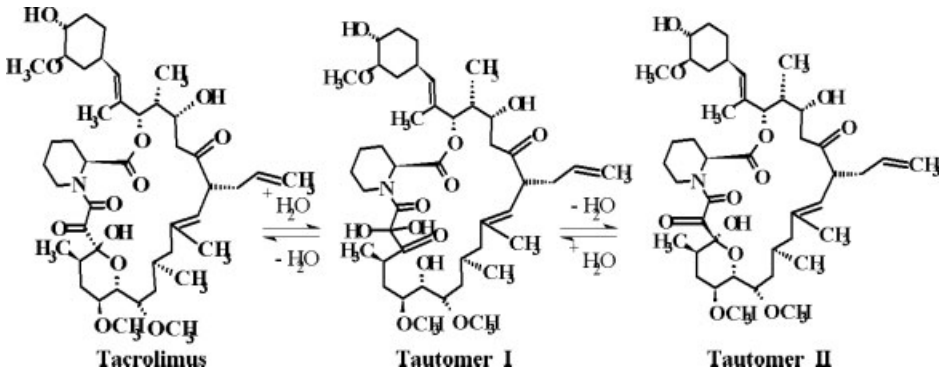

Figure 1. Chemical structure of Tacrolimus & Tautomers.

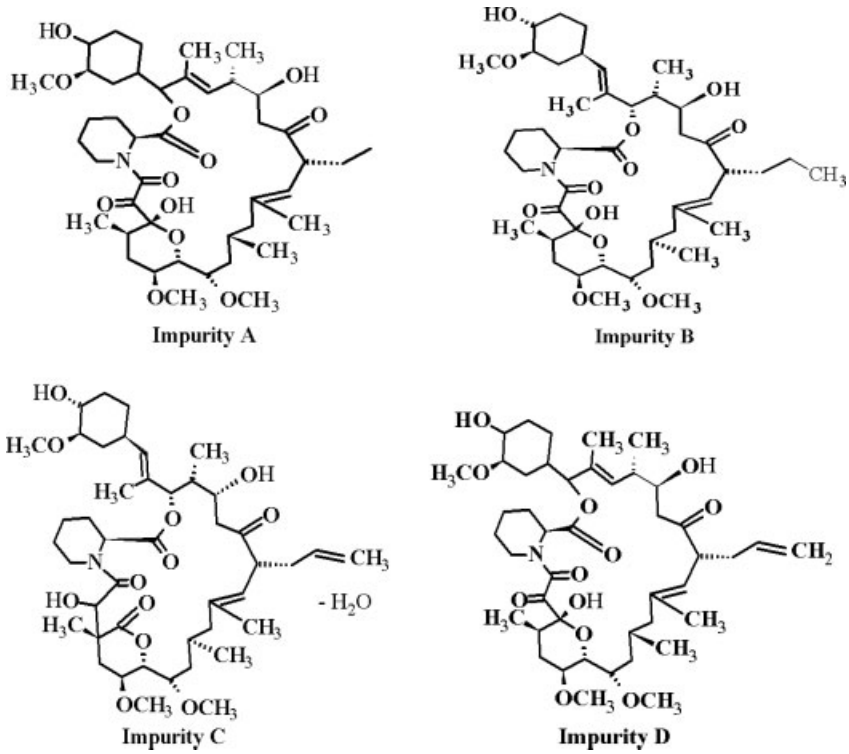

Figure 2. Chemical structure of Tacrolimus Impurities.

for the quantitative estimation of immunomycin (process impurity as Impurity-A), propyl analogue (process impurity as Impurity-B), delta lactone (degradation product as Impurity-C) and regioisomer (thermal degradation product as Impurity-D) by separating from tautomer I and II. A reduction in separation time was achieved, without compromising separation quality compared to other traditional liquid chromatography (LC) methods.

Experimental

Chemicals and reagents

TAC, Impurity-A, Impurity-B and Impurity-C were purchased from Biocon Ltd (Bangalore, India). Impurity-D was isolated at Dr. Reddy's Laboratories Ltd (Hyderabad, India). Acetonitrile (HPLC-grade) was purchased from J.T. Baker (Phillipsburg, New Jersey, USA), and trifluoroacetic acid, sodium hydroxide, hydrochloric acid, hydrogen peroxide were from Merck (Darmstadt, Germany). Water was purified by a Millipore (Bedford, MA, USA) Milli-Q water-purification system and passed through a 0.22 µm membrane filter (Durapore; Millipore, Dublin, Ireland) before use.

Standard and test samples were prepared in HPLC grade acetonitrile as diluent.

Equipment

UHPLC analysis was performed with a Waters (Milford, MA, USA) Acquity UPLC system equipped with a quaternary solvent manager, sample manager, column-heating compartment, and photodiode array detector. This system was controlled by Waters Empower software.

An ACQUITY UPLC™ BEH C_8 column, 100 mm × 2.1 mm, 1.7 µm (Waters (Milford, MA, USA) employed for chromatographic separation. All samples were centrifuged by Thermo Scientific multifuged machine. The specificity study was conducted by

using heating oven, photo stability chamber and heating mantle (Thermo Lab, Thane, India).

Standard and sample preparation

The impurity stock solution was prepared by dissolving an accurately weighed amount of Impurity-A and Impurity-D in acetonitrile, resulting in a concentration of 30 μg mL^{-1} of each impurity.

The identification solution was prepared by dissolving 6 mg of TAC in 5 mL of diluent, mixed with 1 mL of impurity stock solution and diluted to 10 mL in diluent.

The standard stock solution of TAC was prepared by dissolving an accurately weighed amount of TAC working standard in diluent, resulting in a concentration of 0.6 mg/mL. Then the solution was further diluted in diluent to get a final solution of 3 μg mL^{-1}.

The test solution was prepared by dissolving an accurately weighed portion of the powder, equivalent to 6 mg of TAC in 5 mL diluent. After sonicating for around 5 min, the volume was made up to 10 ml. The solution was centrifuged at 3000 rpm for 5 min in order to eliminate insoluble excipients. The supernatant liquid was used for chromatographic analysis.

Chromatography

The analytes were separated on an Acquity UPLC C8 column (100 mm × 2.1 id, 1.7 μm) at oven temperature of 50 °C with a gradient run program at a flow-rate of 0.6 mL min^{-1}. The separation was achieved by gradient elution and the beginning ratio of mobile phase was A–B 55 : 45 (V/V); then the ratio was changed linearly 45 : 55 (V/V); within 20 min. The system came back to initial ratio at 21 min and continued at the same ratio for 4 min. The mobile phase was filtered through a 0.22 μm Millipore filter, before use. UV detection was performed at 210 nm. The sample injection volume was 5 μL in partial-loop mode.

Method validation

The method was validated for specificity, precision, accuracy, sensitivity and linear range as per the International Conference on Harmonization (ICH) guidelines.[26]

Specificity

A study was conducted to demonstrate the effective separation of TAC, tautomers and its impurities. The study was intended to ensure the effective separation of degradation peaks of formulation ingredients at the retention time of TAC, tautomers and its impurities. Separate portions of drug product and ingredients were exposed to the following stress conditions to induce degradation.

The drug product was subjected to base hydrolysis using 1 N sodium hydroxide, acid hydrolysis with 1N hydrochloric acid and neutral hydrolysis with water at 60 °C for a duration of 12h. Oxidation study was performed with 1% hydrogen peroxide solution at 60 °C for 12 h. On photo stability study, drug product was sufficiently spread on petri plates (1 mm thick layer), exposed to sunlight and UV light (1.2 million lux hours) at ambient conditions for 7 days. Humidity study was performed separately by exposing the drug product to humidity at 25 °C, 90% RH for 7 days. Thermal degradation study was performed by heating drug product at 60 °C for 48 h. Similarly, placebo samples were prepared like a drug product by exposing formulation matrices without drug substance.

Stressed samples were injected into the UHPLC system with photo diode array detector by the following test method conditions.

Precision

The precision of test method was evaluated by using six samples of TAC capsules test preparation, spiking with impurities blend solution to get the concentration of 3 μg mL^{-1} of each impurity and analyzed as per test method. Intermediate precision was also studied using different column and performing analysis on a different day.

Accuracy

To confirm the accuracy of the proposed method, recovery studies were carried out by standard addition technique. Samples were prepared in triplicate by spiking impurities in test preparation at the level of LOQ, 50%, 100%, 150% and 200% (a nominal concentration of about 0.3 μg mL^{-1} to 6 μg mL^{-1}) of the standard concentration.

Sensitivity

Sensitivity of the method was established with respect to limit of detection (LOD) and LOQ for TAC impurities (i.e., Impurity-A, Impurity-B, Impurity-C and Impurity-D). A series of concentration of drug solution and its impurities were injected; LOD and LOQ were established by slope method as mentioned below.

$$\text{LOQ} = \frac{3.3 \times \text{standard deviation of y-intercept}}{\text{Slope of the calibration curve}}$$

$$\text{LOQ} = \frac{10 \times \text{standard deviation of y-intercept}}{\text{Slope of the Ccalibration curve}}$$

LOD and LOQ were experimentally verified by injecting six replicate injections of each impurity at the concentration obtained from the above formula.

Linearity of detector response

A series of solutions of TAC impurities in the concentration ranging from LOQ level (0.3 μg mL^{-1}) to 200% (6 μg mL^{-1}) of standard concentration were prepared and injected into the UHPLC system.

Application of developed method

The method suitability was verified by analyzing three different strengths of finished product of both innovator and in-house formulated product. The content of 25 capsules (each containing 5 mg/1mg/0.5 mg of TAC) were emptied and intimately mixed. Quantity equivalent to 6 mg of drug weighed accurately and dissolved in 10 mL of acetonitrile by 5 min of sonication. The solution was centrifuged and injected.

Results and Discussion

A reversed-phase chromatographic technique was developed to quantitate TAC and its impurities at 210 nm. The presence of non-aqueous solvents in the mobile phase, such as methanol and acetonitrile, was studied. Since the sensitivity of the detection system was strongly reduced in the presence of methanol, acetonitrile was chosen as an organic modifier. Satisfactory separation was achieved when the acetonitrile concentration was 10% in mobile phase A and 90% in mobile phase B.

The effect of TFA concentration on analyte retention was studied. TFA is known to improve peak shape and resolution by reducing the analyte interaction with residual silanol groups at the chromatographic surface. TFA is expected to reduce the virtual polarity of the analyte in acidic media. Consequently the retention of the compound increases when the eluent contains TFA. At low pH and high operating column temperature, hydrolysis of the siloxane bond can occur, stripping the bonded phase from the silica support. To avoid this, 10 part of 0.1% v/v TFA solution used in mobile phase A only. Satisfactory resolution was achieved with use of a mixture of water, TFA and acetonitrile as demonstrated in Figure 3C.

C8 and C18 columns were first evaluated as stationary phase for the separation of TAC and its impurities. C8 column was adopted for the analysis because it provided a better separation of the analytes, whereas all analytes were retained so thoroughly that they were not observed 25 min after injection in C18 column. Sensitivity of the method is also improved, compared to conventional HPLC method by reducing the particle size of the stationary phase and detection at 210 nm, where TAC exhibits maximum absorbance. Selectivity, sensitivity, resolution, and speed of chromatographic separation were optimized for the UHPLC method. The optimized UHPLC procedure was compared with previously published HPLC method.[24] Comparing the signal to noise ratio of TAC standard preparation, it is confirmed that proposed method has better sensitivity. Present UHPLC method offers well resolution within 25 min. The retention times of TAC at 15.5, Tautomer-I at 9.3, Tautomer-II at 13.7, Impurity-A at 14.5, Impurity-B at 18.6, Impurity-C at 17.3 and Impurity-D at 12.9 min respectively, under the chromatographic conditions described. Chromatograms obtained from blank, TAC standard, impurity mixture and test spiked with impurities mixture solution are shown in Figures 3A, 3B, 3C and 3D, respectively. The retention times were much more reproducible on a C8 column and a mixture of TFA and acetonitrile mobile phase.

UHPLC system has been proved to be a promising tool for separation of TAC, tautomers and its impurities. Use of small (1.7 μm) particles of stationary phase enabled optimization of UHPLC for both peak selectivity and analysis speed. TAC, its impurities and tautomers were well separated with good peak shape and resolution. No interfering peaks were observed in blank and placebo, indicating that signal suppression or enhancement by the product matrices was negligible. Use of UHPLC resulted in a reduction in run-time to 25 min, without compromising the efficiency, compared with a run-time of approximately 45 min on traditional LC analysis of TAC impurities. LC method will reduce acetonitrile consumption (at least 80%) without compromising productivity and performance.

After satisfactory method development, it was subjected to method validation as per ICH guideline.[26] The method was validated to demonstrate that it is suitable for its intended purpose by standard procedure to evaluate adequate validation characteristics. The result of the system suitability parameter was found to comply with acceptance criteria: relative standard deviation of replicate injection is not more than 5.0% and resolution between Impurity-A and TAC is not less than 2.0 as shown in Table 1. The result of specificity study ascertained the separation of degradation peaks from TAC peak and the spectral purity of all exposed samples were found spectrally pure and data of degradation studies are shown in Table 2. The %RSD of replicate determination was found to be $<5\%$ in both precision and intermediate precision, which indicates

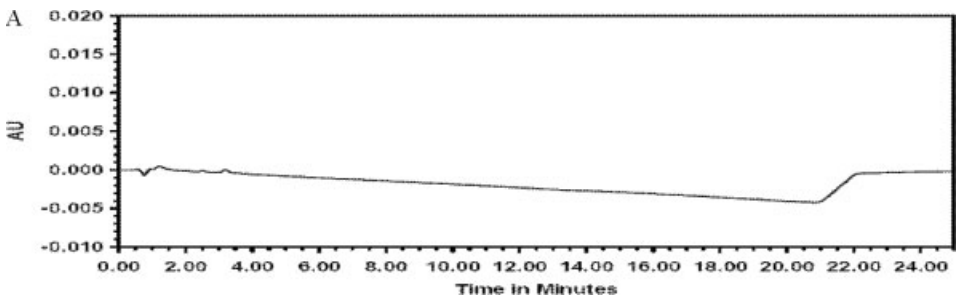

Figure 3a. Typical Chromatogram of Blank.

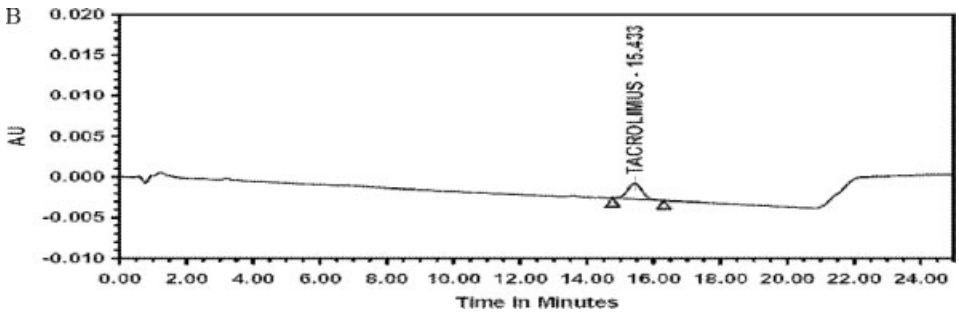

Figure 3b. Typical Chromatogram of Tacrolimus Standard.

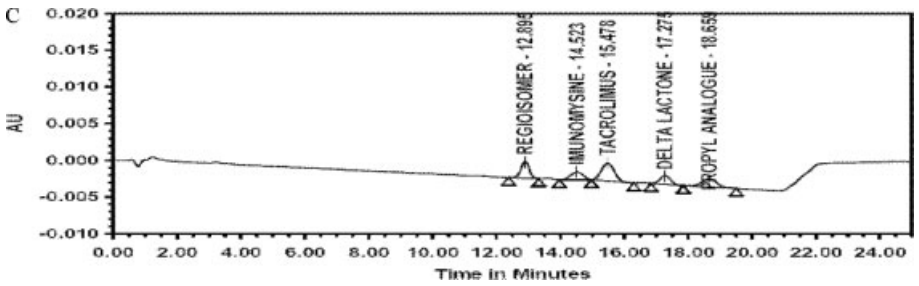

Figure 3c. Typical Chromatogram of Impurities.

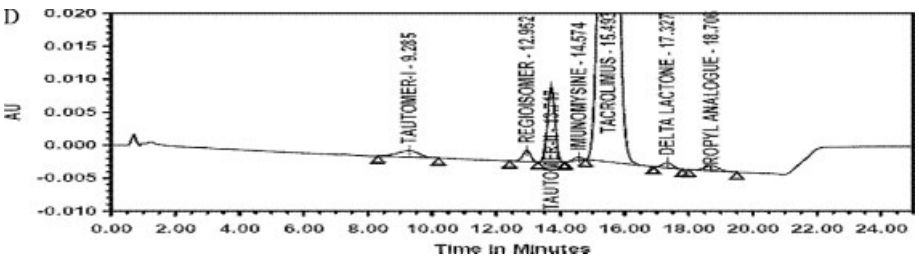

Figure 3d. Typical Chromatogram of Test solution spiked with Impurities.

Table 1. Comparison between HPLC and UHPLC method

Parameters	HPLC	UHPLC
Run time	45 minutes	25 minutes
Resolution between Impurity-A and tacrolimus	1.0	2.2
% RSD of replicate standard injection	2.2	0.6
S/N ratio of Standard preparation	**2.53**	**22.71**

Table 2. Results of specificity studies

Stress conditions	*Total degradation*
Acid hydrolysis	2.44
Base hydrolysis	2.03
Peroxide degradation	0.42
Water hydrolysis	0.87
Humidity degradation	0.36
Sunlight degradation	0.37
Thermal degradation	2.33
UV light degradation	0.28

that the method is precise and the data of precision studies are shown in Table 3. The results obtained from the recovery study were found within the range of 85% to 115% (LOQ to 200%), which indicates that the method is accurate and data for the same are shown in Table 4. Sensitivity of the method was verified and the method was found to be linear, accurate and precise at LOQ and the data of LOD and LOQ studies are given in Tables 4 and 5. The calibration curve of all impurities was obtained by plotting the peak area of individual impurity *versus*

Table 3. Percentage RSD of impurities in precision study

Impurity name	Precision (%RSD) ($n = 6$)	Intermediate precision (%RSD)($n = 6$)
IMPURITY-A	3.9	3.2
IMPURITY-B	2.7	3.0
IMPURITY-C	2.5	2.2
IMPURITY- D	2.1	2.7

Table 4. Percentage recovery of impurities at different level

Nominal concentrations	% Recovery of IMP-A	% Recovery of IMP-B	% Recovery of IMP-C	% Recovery of IMP-D
LOQ level	**112.9**	**104.5**	**87.4**	**93.1**
50% level	107.7	93.2	97.2	99.5
100% level	100.4	100.0	99.4	103.2
200% level	100.6	108.3	113.3	105.5

concentration over the range of about 0.3–6 µg/mL and were found to be linear (r = 0.999). The data of regression analysis of the calibration curves are shown in Table 5. The applicability of the method was verified by the determination of TAC impurities in Prograf Capsules (Astellas pharma) and stability sample of In-house formulation (40 °C/75%RH, 3 month). The impurity content in both formulations was found to be satisfactory and Table 6 summarizes the results obtained.

Conclusion

Although LC is a versatile technique for the analysis of drugs in complex matrices, such as biological or pharmaceuticals, the poor UV absorbency of TAC and its impurities makes the separation and quantification difficult due to the presence of interfering substances. A number of analytical approaches have

Table 5. LOD, LOQ and linearity values of impurities

Parameters (n=7)	IMP-A	IMP-B	IMP-C	IMP-D
LOD μg mL^{-1}	0.1	0.1	0.1	0.1
LOQ μg mL^{-1}	0.34	0.33	0.33	0.31
Calibration range(μg mL^{-1})	0.34–5.984	0.33–6.06	0.34–6.114	0.31–5.992
Calibration Equation	$y = 7348.9x + (152.17)$	$y = 7739.7x + (-618.86)$	$y = 5877.5x + (-549.92)$	$y = 9010.1x + (-205.42)$
Correlation Coefficient	0.999	0.999	0.999	0.999

Table 6. Finished products impurity profile data

Product name	% of IMP-A	% of IMP-B	% of IMP-C	% of IMP-D	% of Total Impurity	% of Tautomer-1	% of Tautomer-1I
Prograf capsules 5.0 mg	0.008	0.02	0.04	0.20	0.37	2.02	2.50
Prograf capsules 1.0 mg	0.02	0.09	0.14	0.20	0.48	1.7	1.8
Prograf capsules 0.5 mg	Not Detected	0.06	0.05	0.19	0.33	1.70	1.30
In-house capsules 5.0 mg	0.03	0.11	0.25	0.33	0.76	3.07	3.09
In-house capsules 1.0 mg	0.03	0.17	0.25	0.35	0.82	2.91	3.09
In-house capsules 0.5 mg	0.03	0.07	0.19	0.37	0.68	2.90	2.89

been previously described to determine TAC in biological materials and pharmaceutical preparation; however, this is the first study reporting a validated reversed phase method for estimation of impurity in TAC formulation. The complexities associated with USP forum reported normal phase chromatographic procedure[25] are: use of multiple columns, lengthy chromatographic run time of more than 45 min, non-optimal separation of impurities, and less sensitivity. These have been addressed by the present UHPLC method. The simple UHPLC method developed in this study makes it suitable for separation and estimation of impurities without interference from excipients and other related substances present in pharmaceutical matrices. The analytical performance and the result obtained from analysis of two different formulations demonstrated that the method is reliable and sufficiently robust. In conclusion, the high sensitivity, good selectivity, accuracy and reproducibility of the UHPLC method developed in this study makes it suitable for quality control analysis of complex pharmaceutical preparation containing TAC and its impurities. The reduction of acetonitrile consumption is one of the best solutions to the current global acetonitrile shortage and will safeguard against future risk.

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