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Original Article

A LIQUID CHROMATOGRAPHIC METHOD FOR THE RELIABLE QUANTIFICATION OF UPADACITINIB AND ITS SPECIFIED IMPURITIES

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ABSTRACT

Objective: In the proposed investigation, a novel RP-UPLC technique for the simultaneous measurement of Upadacitinib and its impurities is developed. The technique's suitability for both tablet and bulk medication dose forms was confirmed.

Methods: A mobile phase consisting of 2.5 g of sodim hexane-1-sulphonic acid in 1 L of water was used to achieve an isocratic elution. The pH was adjusted to 3.0 using diluted formic acid: methanol (60:40) at a flow rate of 1 ml. min^{-1}, using an X-Bridge Phenyl (250 x 4.6 mm, 5 µm) column.

Results: Sharp peaks of Upadacitinib and its impurities were detected at 6.730, 2.823, 3.844, 4.872, 9.991, 10.499, and 10.932 min, respectively, at 256 nm as the peak using the PDA detector. Among other aspects of system suitability, USP resolution is greater than or equal to 2, USP plate count exceeds 4000, and USP tailing is good. Purity Flag "No" means that the peak is uniform (provided by Empower program). If the purity angle is smaller than the purity threshold, the peak is homogeneous. The procedure was verified in accordance with the ICH recommendations. The procedure was validated in accordance with ICH recommendations. The concentration ranges of Upadacitinib (75–450 µg/ml), impurities 1, 2, 4, and 5 (2.50–15.00 µg/ml), and impurities 3 and 6 (1.25–7.50 µg/ml) were all subjected to linear regression for the calibration curve. LOQ (34.41, 0.057, 0.211, 0.181, 0.151, 0.377, and 0.165 µg ml^{-1}) and LOD (11.35, 0.019, 0.070, 0.060, 0.049, 0.124, and 0.055 µg ml^{-1}) for and Upadacitinib and impurities, respectively. Accuracy, precision, and resilience were among the other confirmed characteristics that were within acceptable ranges.

Conclusion: As a result, this method was chosen for shared analysis. Finally, the restrictions of the system-appropriate parameters and validation parameters are acceptable. Stability-indicating experiments under various stress conditions were successfully included to the approach.

Keywords: Upadacitinib, Impurities, UPLC, Empower software and PDA detector

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INTRODUCTION

The regular name of Upadacitinib (UDB) is Rinvoq in the form of tablet dosage. UDB is used in the treatment of psoriatic arthritis, rheumatoid arthritis, ulcerative colitis, atopic dermatitis, axial spondyloarthritis, Crohn's disease, and ankylosing spondylitis [1-3]. UDB was accepted by the states, the European Union, and the United States in 2019 [4-6]. Upadacitinib is an inhibitor of Janus Kinase (JAK) that works by blocking the action of enzymes called (JAK) Janus Kinases. These enzymes play a role in initiating processes that result in inflammation; inhibiting their impact reduces inflammation in the joints [2, 6]. Upper respiratory tract infections (sinuses, common colds), nausea, coughing, and fever are common adverse effects [6].

Upadacitinib quantities in the body are raised by drugs that potently inhibit the liver enzyme CYP3A4, such as clarithromycin, ketoconazole, and itraconazole. Ketoconazole elevated the AUC by 75% in one study. On the other hand, drugs that significantly activate CYP3A4 decrease the levels of Upadacitinib [6, 7]. Upadacitinib has a solubility of about 30 mg/ml in organic solvents such as Dimethyl Sulphoxoide (DMSO) and dimethyl form amide. Upadacitinib has limited solubility in aqueous solutions. Upadacitinib can be dissolved in DMSO first, and then diluted with the preferred aqueous buffer to optimize its solubility in aqueous buffers [8].

Formulation Development for upadacitinib extended-release tablets using *in vitro-in vivo* correlation [9]. UPLC-MS/MS method development for the pharmacokinetics study and quantitative analysis of upadacitinib in beagle dog plasma [10]. Associated substances axitinib, zanubrutinib, and upadacitinib method development and validation using RP-HPLC, and Its degradation products were characterized using LC-MS/MS [11]. Pharmacokinetic application of concurrent UPLC-MS/MS measurement of methotrexate and upadacitinib in rat plasma [12]. The goal of the current work was to develop a new UPLC method with a PDA detector and validate the method according to ICH Guidelines, which was successfully employed to study the pharmacokinetics of Upadacitinib and its related impurities (fig. 1).

MATERIALS AND METHODS

Materials

Merck Chemicals in Mumbai supplied the analytical and HPLC quality solvents. The likely impurity standards for the drug Upadacitinib were submitted by SJS Pharmaceuticals Lab in Hyderabad.

Instruments

Digital pH meters (Elico LI-120) and balances (DENVER brand, SI234 model; Shimadzu AUX-220) were used to weigh and measure the items.

UPLC: An Agilent 1290 Infinity II LC System (Pump: Quaternary; Software: Empower 2.0) with a PDA detector was used.

General procedures

Standard drug (Upadacitinib) preparation

A volumetric (50 ml) flask was filled with 50 mg of Upadacitinib after it had been precisely weighed. Then, it was properly dissolved in acetonitrile, sonicated for about 15 min, and diluted to the appropriate level using a comparable solvent. The stock (1000 µg ml^{-1}) solution was then filtered through a 0.22 µm membrane filter to remove any undissolved particles. This ensured that the final solution was clear and suitable for subsequent analytical procedures) was used to create the obtained solution. To use the solution again, dilute it properly.

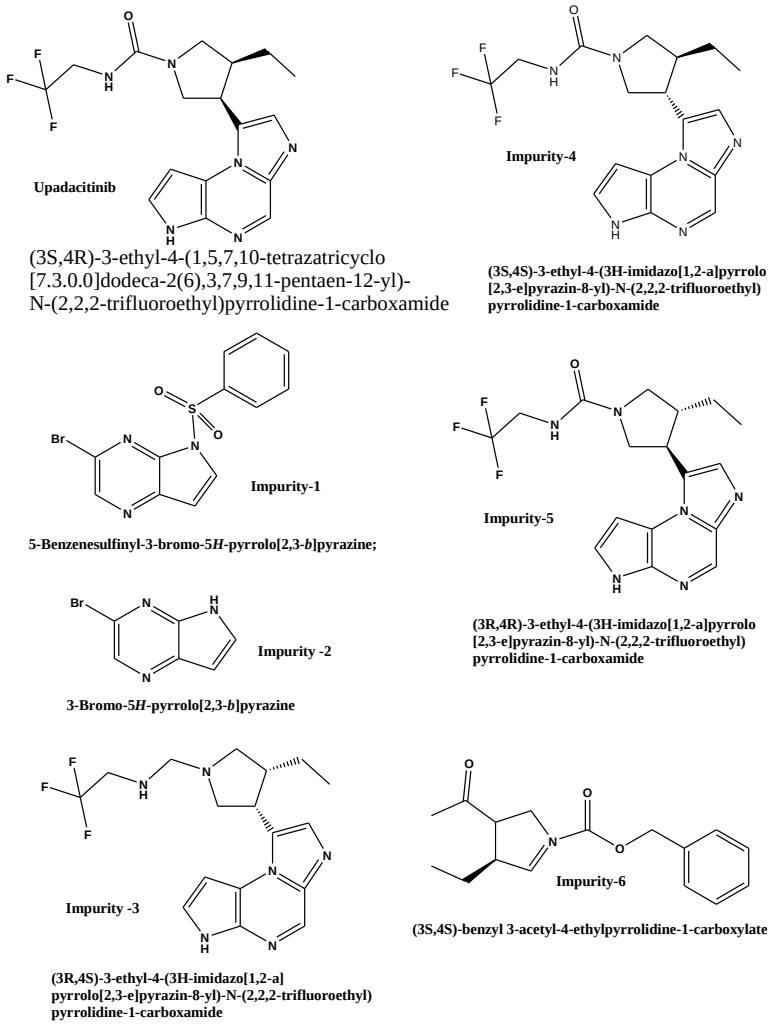

Fig. 1: Upadacitinib and its related impurities

Stock solution-A (from impurities)

A 100 ml volumetric flask was filled with 5 mg of Imp-1, Imp-2, Imp-4, and Imp-5, which had been precisely weighed. After properly dissolving in acetonitrile, sonicate for approximately fifteen minutes.

S**tock solution-B (from impurities)**

5 mg of Imp-3 and Imp-6 were precisely weighed and then added to a 100 ml volumetric flask. After properly dissolving in acetonitrile, sonicate for approximately fifteen minutes.

S**tock solution (Impurity) preparation**

Add 1 ml of each of the impurity stocks A and B to a 50 ml volumetric flask of diluents.

Forced degradation and method validation studies

According to the most recent guidelines, the final optimum conditions are verified [13]. Stress samples were suitably diluted to provide the closing concentration while maintaining the intended technique conditions (50 µg/ml of Upadacitinib), and they were correlated with blank and standard chromatograms.

RESULTS

Analytical method development and optimisation

Selection of diluent

According to USP general requirements, diluent is used to create standards, sample solutions, impurity standards, placebo solutions, and system suitability solutions. These solutions were selected based on solubility experiments carried out for Upadacitinib (table 1).

Table 1: Solubility study (25 °C) of upadacitinib

Solvent	Solubility
Water (H_2O)	Insoluble
Acetone (CH_3COCH_3)	Slightly soluble
Ethyl acetate	Slightly soluble
Methanol	Completely Soluble
Acetonitrile	Completely Soluble

In acetonitrile and methanol, the active ingredient dissolves easily, according to solubility tests. According to solubility studies, compatibility of the mobile phase, and excipient solubility, all of the solutions were prepared using acetonitrile as a diluents.

Determination of detection wavelength

First, methanol and acetonitrile solvents are chosen as mobile phase solvents based on the solubility of Upadacitinib and impurities. Analyte pKa value, polarity, and organic phase concentration were the main factors in the selection of the mobile phase. For simultaneous measurement of Upadacitinib and impurities, the Photodiode Array detector (PDA) wavelength was adjusted at 256 nm (fig. 2) at the iso-absorption point based on the absorption maxima seen for Upadacitinib and impurities.

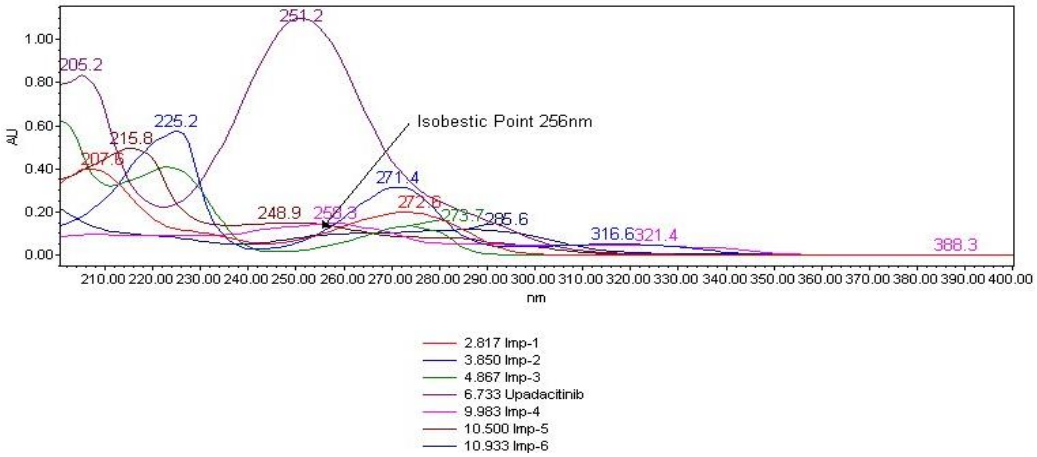

Fig. 2: Spectra of UDB and their impurities (PDA)

Choice of chromatographic situations (Method development)

Using Waters brand HPLC instruments (Alliance model No. e2695; software-Empower 2.0), the current investigation aims to create methodology and validation. Normally (standard) solutions of Upadacitinib and its six contaminants are prepared by diluting the drug with acetonitrile.

The major important solvent of the mobile phase (MP) was chosen to be acetonitrile (LC grade), as it outperforms the other solvents (polar) in the following areas. 1). The lowest transmission density (absorbance) at shorter wavelengths (λmax) results in less sound when UV detection is performed. It advises conducting a more sensitive assessment at shorter UV wavelengths (λmax). 2) For gradient baselines, less ghost cresting is observed. Viscosity and condensed back pressure result in a much larger peak form. (5) The elution strength of acetonitrile (CAN) based solutions is higher than that of methanol (CH_3CH_2OH)-based solutions. 6) Using acetonitrile at modest mixture ratios, one can achieve the same retention duration with less than half the methanol (CH_3CH_2OH) ratio [14]. Upadacitinib is a chemical that has heterocyclic rings (imidazole ring) and one functional group (diamide group). The first is heterocyclic rings, which have a polar character, strong basicity, and are soluble in water [15]. The second is the diamide group, which is nonpolar, less basic, and less soluble in water. Based on the second point, the chemical has a nonpolar nature and is insoluble in water.

The first phase of trials was conducted using a Zorbax SB C18 250x4.6 mm, 5µ column, and mobile phase is 0.01% TFA in water (pH: 3.0): Acetonitrile (30:70, 50:50); this two trials baseline drift and unknown peaks are observed (fig. 3).

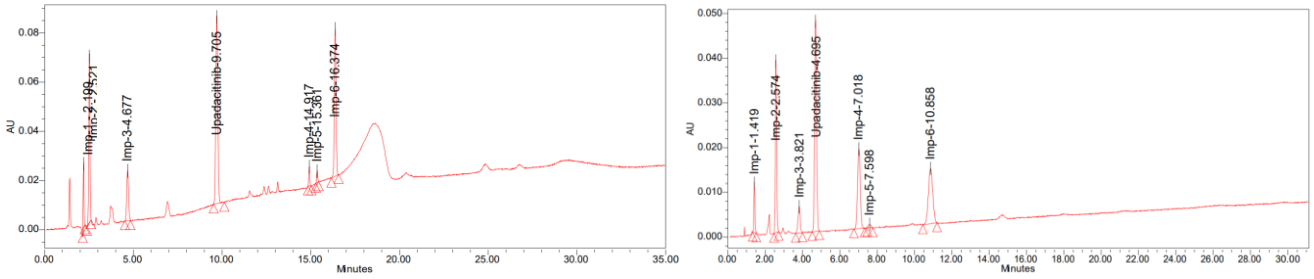

Fig. 3: Chromatograms obtained by TFA (pH: 3.0): Acetonitrile (30:70, 50:50)

The second phase of trials were conducted using Zorbax SB C18 250x4.6 mm, 5µ column and mobile phase 1.36 gm KH_2PO_4 in 1l water (pH: 3.0) adjested with OPA: acetonitrile (50:50), these trial, baseline drift and unknown peaks were observed (fig. 4).

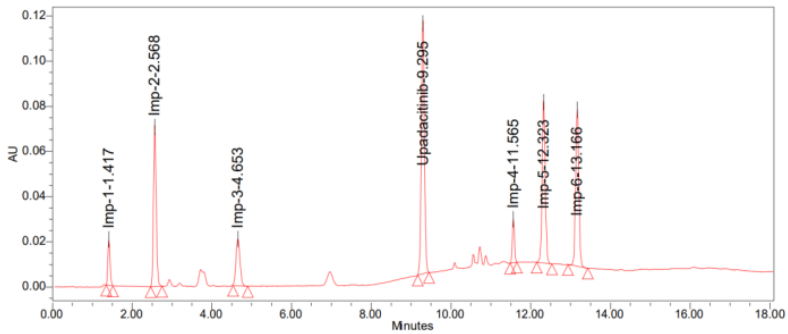

Fig. 4: Chromatograms obtained by KH_2PO_4 (pH: 3.0): Acetonitrile (50:50)

The third phase of trials (table 2) was conducted using a Zorbax SB C18 250x4.6 mm, 5µ column and the mobile phase is 1.36 gm KH_2PO_4 in 1liter water (pH: 3.0) adjusted with OPA: Methanol (50:50), trial, base line drift and unknown peaks are observed (fig. 5).

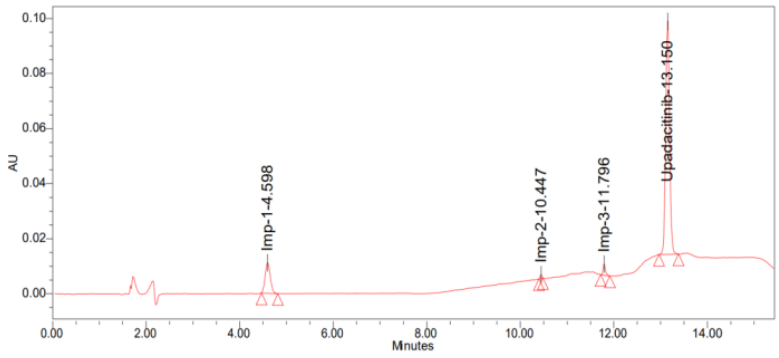

Fig. 5: Chromatograms obtained by KH_2PO_4 (pH: 3.0): Methanol (50:50)

The fourth phase of trials (table 2) was conducted using a Zorbax SB C18 250x4.6 mm, 5µ column and the mobile phase is 1.36 gm KH_2PO_4 in 1l water (pH: 3.0) adjusted with OPA: (Acetonitirile+Methanol (50+50)) (60:40; 40:60), these two trials impurity peaks are not clear, and unknown peaks are observed (fig. 6).

The fifth phase of trials (table 2) was conducted using a Zorbax SB C18 250x4.6 mm, 5µ column and the mobile phase is 770.8 mg of ammonium acetate in 1l water adjust pH-3.0 with Acetic acid: Acetonitirile (50+50; 70:20) these two trials resolutions not within the limit, and unknown peaks are observed (fig. 7).

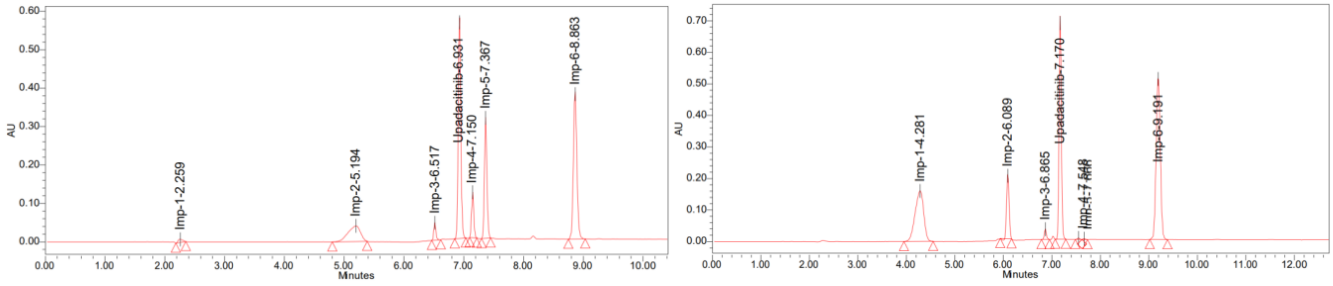

Fig. 6: Chromatograms obtained from KH_2PO_4: ((ACN+CH_3OH) (50+50)) (60:40; 40:60)

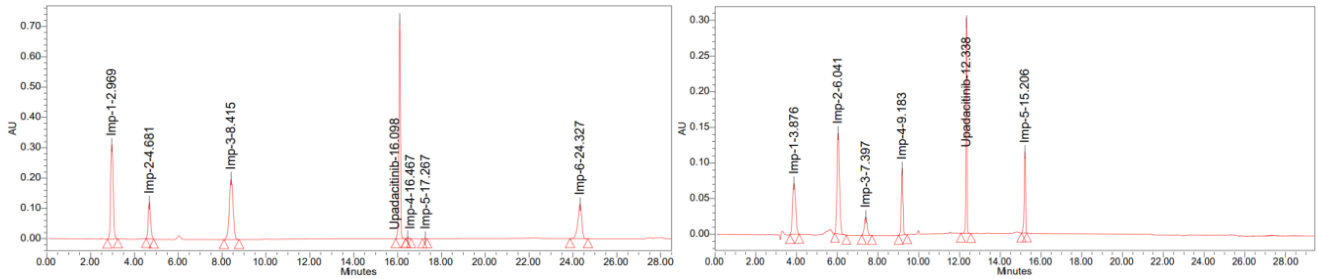

Fig. 7: Chromatograms obtained from KH_2PO_4: Acetonitirile+Methanol (50:50; 70:20)

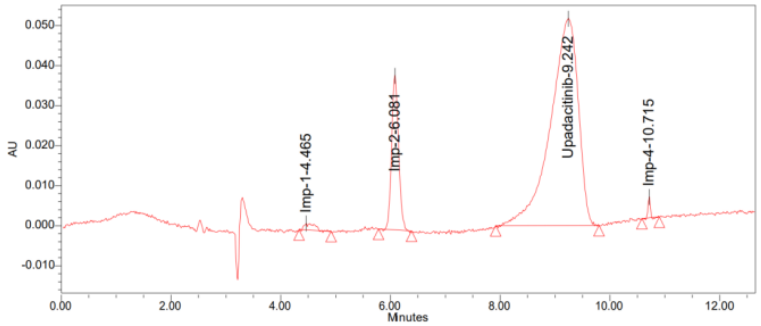

Fig. 8: Chromatograms obtained by ammonium acetate (pH: 3.0): Methanol (50:50)

The sixth phase of trials (table 2) was conducted using Waters X-Bridge Phenyl 250x4.6 mm, 5µ, column and the mobile phase is 770.8 mg of ammonium acetate in 1 L water adjustd pH-3.0 with Acetic acid: Methanol (50+50) these two trials resolutions not within the limit, and unknown peaks are observed (fig. 8).

The seventh phase of trials (table 2) was conducted using Waters X-Bridge Phenyl 250x4.6 mm, 5µ, column and the mobile phase is 2.5 g sodim Hexane-1-sulphonic acid in 1 L water adjust pH-3.0 with Dilted formic acid: Acetonitrile (30:70, 50:50, 40:60) these three trials resolutions not within the limit, and unknown peaks are observed (fig. 9).

The eight phase of trial (table 2) was conducted using Waters X-Bridge Phenyl 250x4.6 mm, 5µ, column and the mobile phase is 2.5 g sodim Hexane-1-sulphonic acid in 1l water adjust pH-3.0 with Diluted formic acid: Methanol (40:60) these three trials resolutions not within the limit, and unknown peaks are observed (fig. 10).

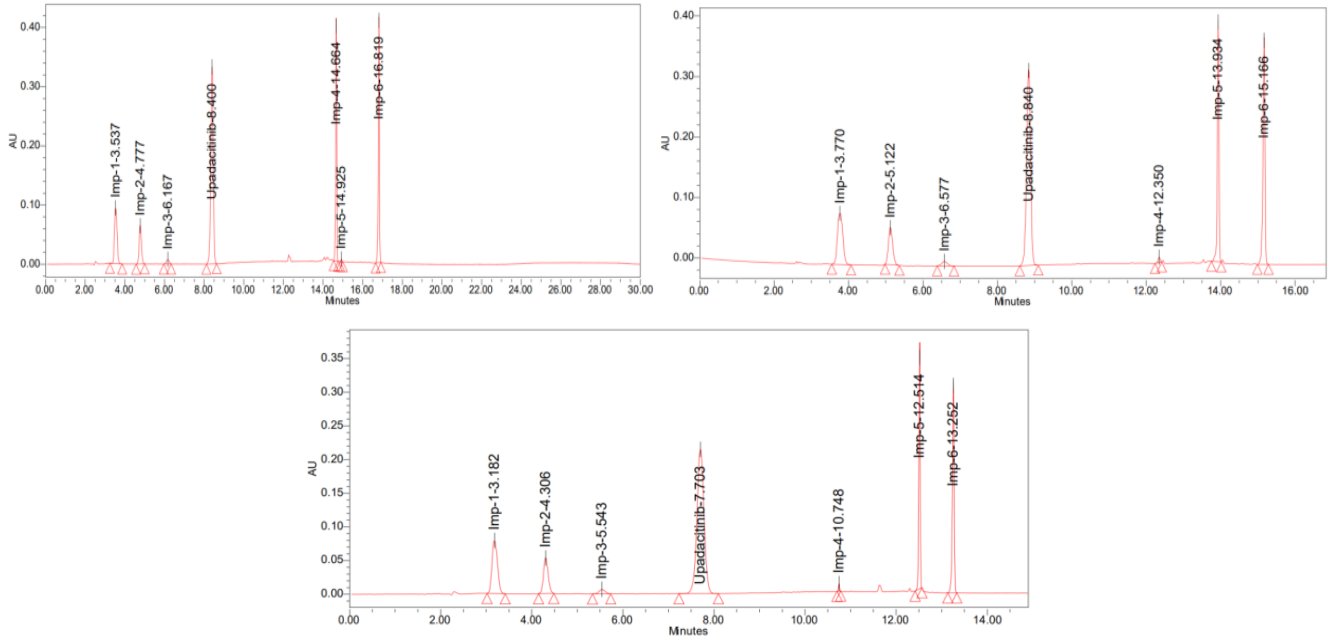

Fig. 9: Chromatograms obtained from sodium hexane-1-sulphonic acid: Acetonitirile (30:70, 50:50, 40:60)

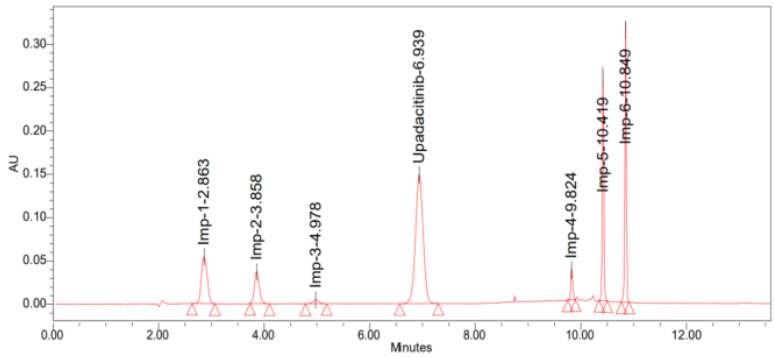

Fig. 10: Chromatograms obtained by sodium hexane-1-sulphonic acid: methanol (40:60)

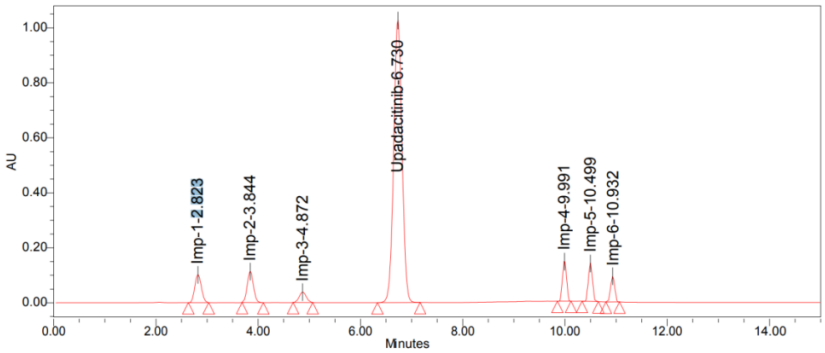

Fig. 11: Optimized chromatograms obtained by sodium hexane-1-sulphonic acid (pH: 3.0): Methanol (60:40, v/v)

The final phase of the trial (table 2) was conducted using a Waters X-Bridge Phenyl 250x4.6 mm, 5µ, column and the mobile phase is 2.5 g sodium hexane-1-sulphonic acid in 1 l water adjusting the pH-3.0 with diluted formic acid: methanol (60:40) this trial was followed by a system suitable for conditions (fig. 11). USP tail factors (acceptable value equal to 2 or less than 2) of drug and its impurities values are 1.05, 1.25, 1.35, 1.58, 1.36,1.24, and 0.69. USP plate count (acceptable value more than 4000) of the drug and its impurities values are 8058, 2225,4768,5748,56921,61902, 68306. USP resolutions (acceptable more than 2) of the drug and its impurities (imp-1, imp-3, imp-4, imp-5, and imp-6) values 6.69, 4.54, 4.87, 13.24, 3.36 and 2.57. Finally, the method is accepted according to ICH rules (table 3).

Table 2: Conducted trails

S. No.	Mobile phase	Column	Observation	Diluent
1	Water pH-3.0 with TFA: Acetonitrile (30:70)	Zorbax SB C18 250x4.6 mm, 5µ	Baseline not sufficient	Acetonitrile
2	Water pH-3.0 with TFA: Acetonitrile (50:50)	Zorbax SB C18 250x4.6 mm, 5µ	Baseline not sufficient	Acetonitrile
3	1.36 g KH2PO4 in 1l water adjust pH-3.0 with OPA: Acetonitrile (50:50)	Zorbax SB C18 250x4.6 mm, 5µ	Unknown peaks observed	Acetonitrile
4	1.36 g KH2PO4 in 1l water adjust pH-3.0 with OPA: Methanol (50:50)	Zorbax SB C18 250x4.6 mm, 5µ	peaks are not separated	Acetonitrile
5	1.36 g KH2PO4 in 1l water adjust pH-3.0 with OPA: Acetonitirile+Methanol (50+50) (60:40)	Zorbax SB C18 250x4.6 mm, 5µ	Imp-2 peak is not clear	Acetonitrile
6	1.36 g KH2PO4 in 1l water adjust pH-3.0 with OPA: acetonitirile+Methanol (50+50) (50:40)	Zorbax SB C18 250x4.6 mm, 5µ	Imp-4and5 resolution not good	Acetonitrile
7	770.8 mg of ammonium acetate in 1l water adjust pH-3.0 with Acetic acid: Acetonitirile (50:50)	Zorbax SB C18 250x4.6 mm, 5µ	Upadacitinb and Imp-4 resolution not good	Acetonitrile
8	770.8 mg of ammonium acetate in 1l water adjust pH-3.0 with Acetic acid: Acetonitirile (70:20)	Zorbax SB C18 250x4.6 mm, 5µ	Imp-6 not eluted	Acetonitrile
9	770.8 mg of ammonium acetate in 1l water adjust pH-3.0 with Acetic acid: Methanol (50:50)	Waters X-Bridge Phenyl 250x4.6 mm, 5µ	Peaks are not good	Methanol
10	2.5 g sodim Hexane-1-sulphonic acid in 1l water adjust pH-3.0 with Dilted formic acid: Acetonitrile (30:70)	Waters X-Bridge Phenyl 250x4.6 mm, 5µ	Imp-4and5 resolution not good	Acetonitrile
11	2.5 g sodim Hexane-1-sulphonic acid in 1l water adjust pH-3.0 with Dilted formic acid: Acetonitrile (50:50)	Waters X-Bridge Phenyl 250x4.6 mm, 5µ	Peaks are separed	Acetonitrile
12	2.5 g sodim Hexane-1-sulphonic acid in 1l water adjust pH-3.0 with Dilted formic acid: Acetonitrile (40:60)	Waters X-Bridge Phenyl 250x4.6 mm, 5µ	Peaks are separed	Acetonitrile
13	2.5 g sodim Hexane-1-sulphonic acid in 1l water adjust pH-3.0 with Dilted formic acid: Methonol (40:60)	Waters X-Bridge Phenyl 250x4.6 mm, 5µ	Peaks are separed; concentration finalize	Methanol
14	2.5 g sodim Hexane-1-sulphonic acid in 1l water adjust pH-3.0 with Dilted formic acid: Methonol (60:40)	Waters X-Bridge Phenyl 250x4.6 mm, 5µ	This method is suitable for validation	Methanol

Table 3: Method conditions

Parameter	Condition
Diluent	Methanol
Detector wavelength	256 nm
Run time	15 min
Column temperature	Ambient
Test temperature	Ambient
Injection volume	10µl*
Flow rate	1 ml min^{-1}
Column	X-Bridge Phenyl (250 mm x 4.6 mm, 5µm)
Mode of separation	Isocratic
Mobile Phase	2.5 g sodim Hexane-1-sulphonic acid in 1l water adjust pH-3.0 with Dilted formic acid: Methanol (60:40)

DISCUSSION

The HPLC process was designed and improved in accordance with ICH (2005) requirements (Q2R1) [13]. Each verified parameter is covered in depth in the section below.

Optimal conditions for the technique System appropriateness parameters are listed in table 4. For Upadacitinib (UDB) and its impurities, the USP tailing factor is 0.69–1.58, indicating that the peaks have precise Gaussian forms and are symmetrical around their axis. By achieving a good resolution between the neighboring peaks, the USP plate count (68306-2225) demonstrates that the selected column is successful in resolving the sample components (fig. 12). Six Upadacitinib (UDB) injections were made in the system precision study, and the percentage RSD was determined to be 0.108 (<1%). This indicates that the amounts produced are accurate. Chromatograms for system appropriateness and system precision are displayed in table 5 and table 6.

A Diluent solution was made using the revised technique and added to the chromatographic apparatus (fig. 13). The retention periods of the active and impurity peaks were not interfering because of the diluents.

To create a placebo solution using the enhanced technique and add it to the chromatographic apparatus (fig. 13). The retention durations of the active and impurity peaks were unaffected by the placebo.

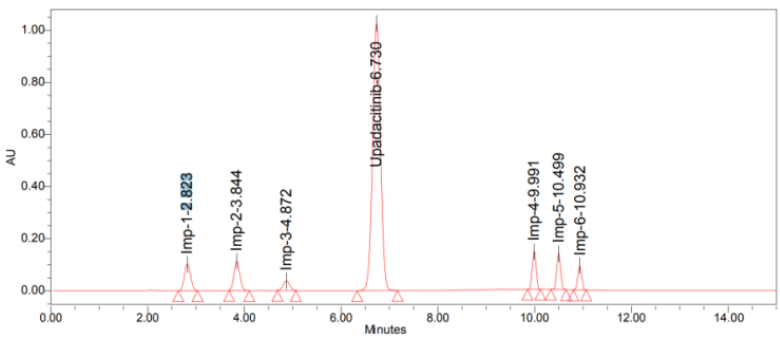

Fig. 12: System suitability chromatogram

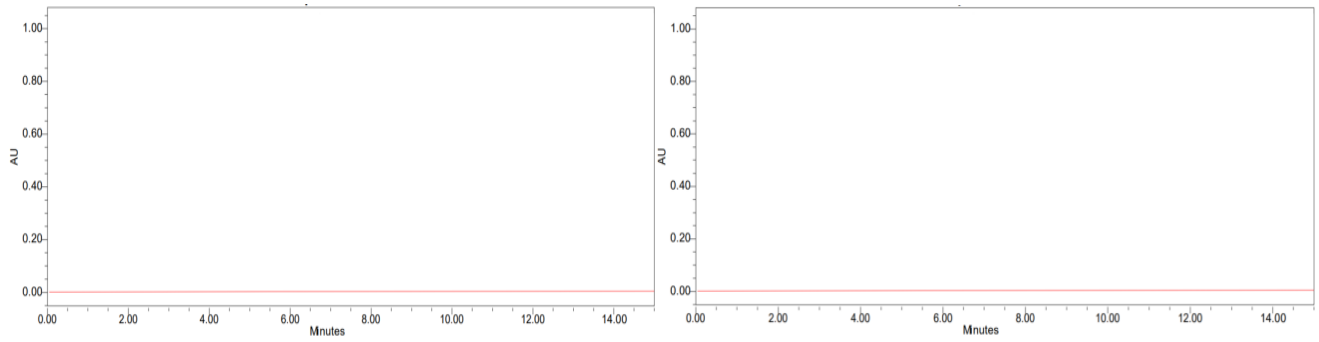

Fig. 13: Chromatogram obtained by blank and placebo

Table 4: Parameters of system suitability

S. No.	Parameter	Parameter values						
		UDB	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6
1	Retention time (min)	6.730	2.823	3.844	4.872	9.991	10.499	10.932
2	Peak area (µV*Sec)	11638823	900850	938896	357027	817722	763990	520317
3	USP tailing	1.05	1.25	1.35	1.58	1.36	1.24	0.69
4	USP plate count	8058	2225	4768	5748	56921	61902	68306
5	USP resolution	6.69	-----	4.54	4.87	13.24	3.36	2.57
6	Purity1 angle	0.028	0.056	0.037	0.567	0.137	1.158	0.115
7	Purity1threshold	5.049	5.027	5.015	5.049	5.019	5.020	5.037
8	Purity1 flag	No	No	No	No	No	No	No

The Purity Flag "No" indicates that the peak is homogeneous (provided by Empower program).
Should the purity angle be below the purity criterion, the peak is homogeneous?

Table 5: System precision

S. No.	UDB	IMP-1	IMP-2	IMP-3	IMP-4	IMP-5	IMP-6
1	11654278	907451	936521	356241	815462	765421	524685
2	11452174	902365	935214	352647	816532	765847	524758
3	11320658	902451	934588	356582	817458	762438	526321
4	11526489	905526	936548	353625	815263	764985	525898
5	11847548	905462	933365	354251	816632	767845	525461
6	11745826	901174	937451	355824	814965	763299	520965
Mean	11591162	904071.5	935614.5	354861.7	816052	764972.5	524681.3
S. D.	178096.9	2213.736	1374.788	1448.86	884.67	1753.17	1760.38
% RSD	1.536	0.245	0.147	0.408	0.108	0.229	0.336

Every peak's retention time can be used to establish whether or not all of the peaks are well-defined in relation to one another. When the resolution between two eluting crests is more than two, it indicates that the crests are well separated.

To find the peak purity, the purity angle and purity threshold are compared. The purity angle provides information about the solvent angle and purity noise angle together. The spectral homogeneity, represented by the purity flag, is stretched when the purity angle and purity threshold are compared [16]. Peak purity assesses a crest's spectral homogeneity.

In the present study, the peak purity of the chromatograms obtained from spiked tasters was estimated using Waters Empower Networking Computer software. Purity angle values and purity threshold values are used to assess both the spectrum homogeneity and peak purity [17]. If the purity threshold is exceeded by the peak purity angle, the analyzed peak is homogeneous (fig. 14-18).

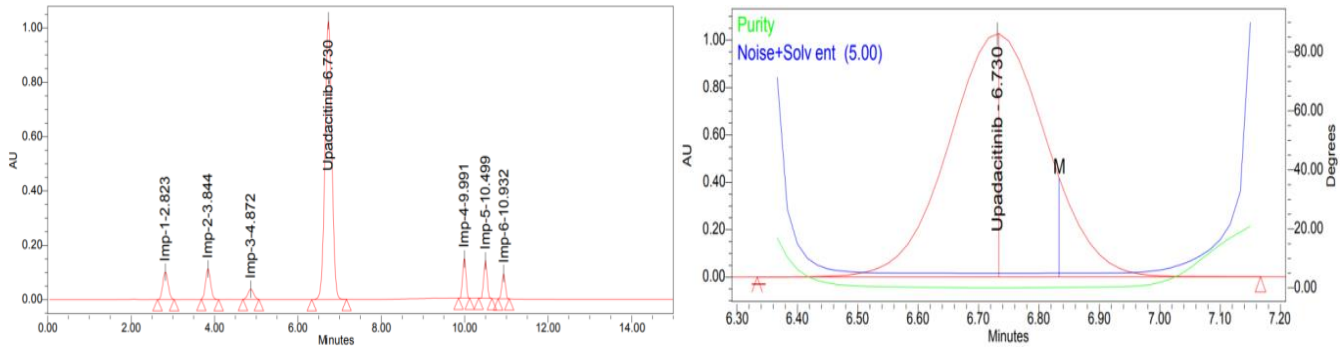

Fig. 14: Typical chromatograms of unspiked sample and purity plot of UDB

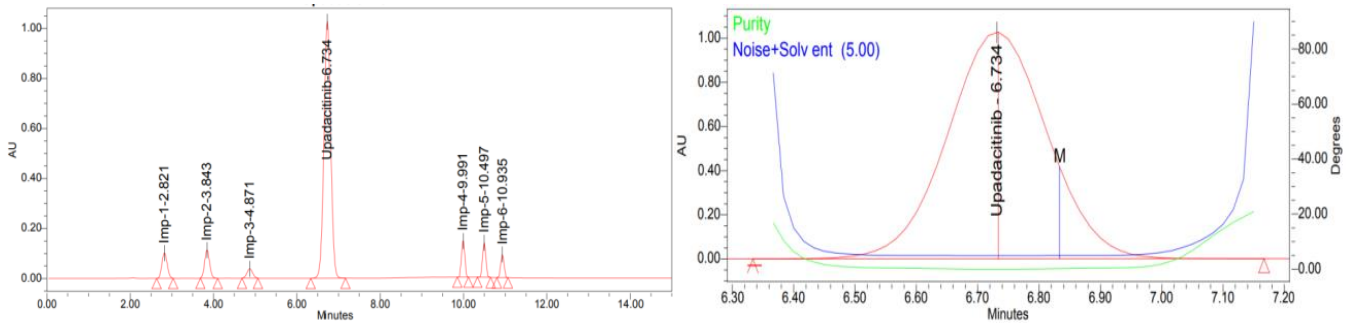

Fig. 15: Chromatograms of sample spiked with impurities purity plot for UDB

Table 6: System precision parameters

UDB						
S. No.	**RT**	**Plate count**	**Resolution**	**Tail factor**	**Peak purity**	**Purity angle**
1	6.734	8067	6.55	1.06	5.027	0.058
2	6.735	8066	6.57	1.24	5.048	0.096
3	6.734	8067	6.63	1.52	5.025	0.048
4	6.733	8078	6.47	1.39	5.087	0.039
5	6.732	8036	6.44	1.25	5.048	0.069
6	6.733	8025	6.14	1.25	5.019	0.037
IMP-1						
1	2.821	2209		1.05	5.028	0.065
2	2.823	2238		1.04	5.015	0.025
3	2.824	2236		1.25	5.057	0.036
4	2.826	2269		1.04	5.027	0.064
5	2.827	2258		1.69	5.021	0.067
6	2.826	2265		1.78	5.014	0.069
IMP-2						
1	3.843	4739	4.33	1.28	5.027	0.025
2	3.842	4735	4.35	1.36	5.048	0.096
3	3.843	4758	4.56	1.52	5.096	0.048
4	3.844	4758	4.85	1.06	5.034	0.089
5	3.843	4768	4.86	1.34	5.048	0.067
6	3.844	4758	4.56	1.69	5.018	0.067
IMP-3						
1	4.871	5792	4.29	1.37	5.015	0.539
2	4.872	5796	4.25	1.41	5.048	0.585
3	4.871	5764	4.20	1.36	5.068	0.578
4	4.872	5725	4.57	1.25	5.018	0.579
5	4.871	5724	4.55	1.14	5.015	0.558
6	4.872	5747	4.85	1.48	5.049	0.537
IMP-4						
1	9.991	56987	13.69	1.41	5.048	0.158
2	9.990	56968	13.65	1.30	5.096	0.157
3	9.992	56924	13.58	1.38	5.041	0.167
4	9.994	56965	13.54	1.67	5.018	0.164
5	9.993	56954	13.58	1.01	5.024	0.149
6	9.995	56698	13.25	1.08	5.019	0.125
IMP-5						
1	10.497	61867	3.05	1.24	5.069	1.121
2	10.496	61856	3.09	1.22	5.037	1.148
3	10.497	61598	3.07	1.20	5.039	1.107
4	10.495	61587	3.09	1.45	5.005	1.131
5	10.494	61527	3.01	1.27	5.063	1.103
6	10. 93	61478	3.69	1.37	5.019	1.137
IMP-6						
1	10.935	68258	2.59	0.96	5.027	0.137
2	10.936	68358	2.57	0.91	5.096	0.148
3	10.934	68364	2.55	1.91	5.076	0.149
4	10.935	68357	2.34	1.69	5.069	0.157
5	10.936	68367	2.37	1.37	5.020	0.134
6	10.934	68304	2.47	1.68	5.019	0.137

Initially, 6h, 12h, 18h, and 24h tests were performed for the degradation parameters (table 7) (acid, base, oxidation, hydrolysis, reduction, thermal, and photolytic (UV)). The base hydrolysis degradation, all conditions are less stable, while in thermal degradation, all conditions are more stable. The approach is exact towards Upadacitinib and impurities, as the purity threshold is higher than the purity angle in all stress parameter, which represents the studied peak, is homogenous (table 8) (fig. 19-25).

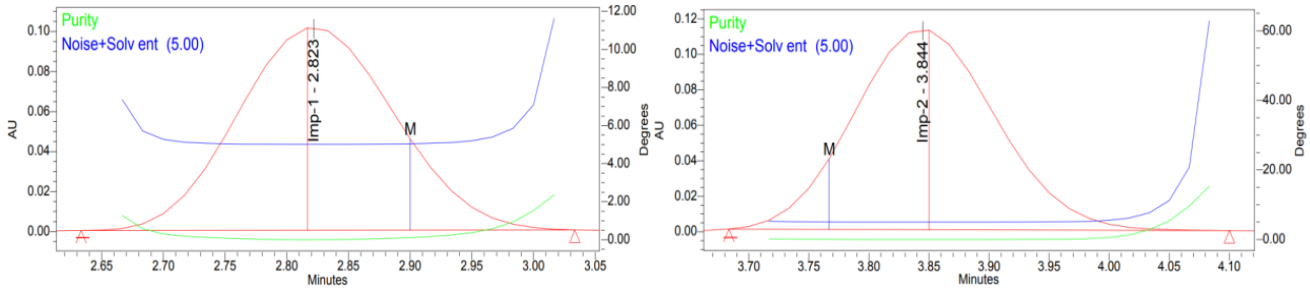

Fig. 16: Purity plot for imp-1 and imp-2 (from impurity spiked sample)

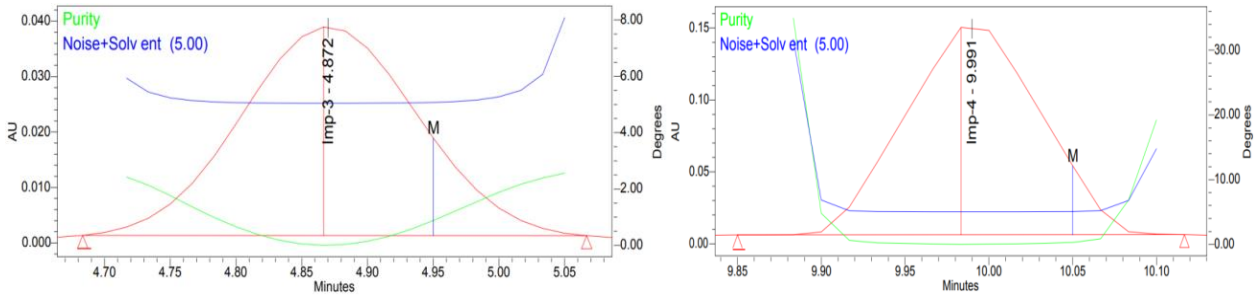

Fig. 17: Purity plot for impurity-3 and impurity-4 (from spiked impurity sample)

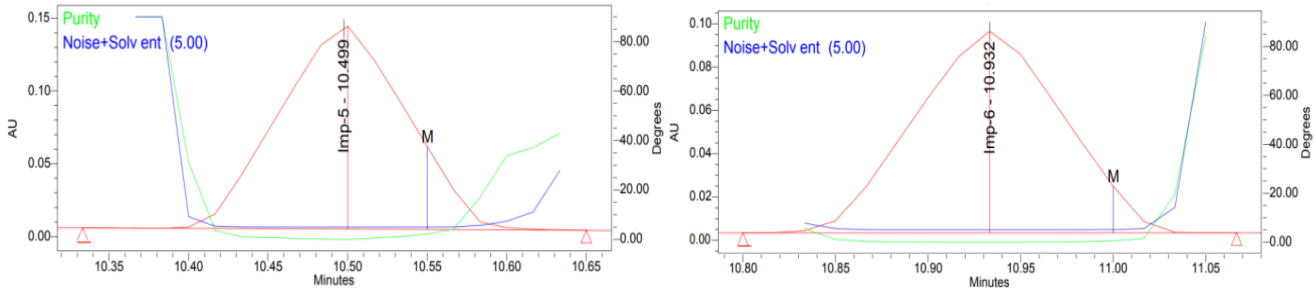

Fig. 18: Purity plot for impurity-5 and impurity-6 (from spiked impurity sample)

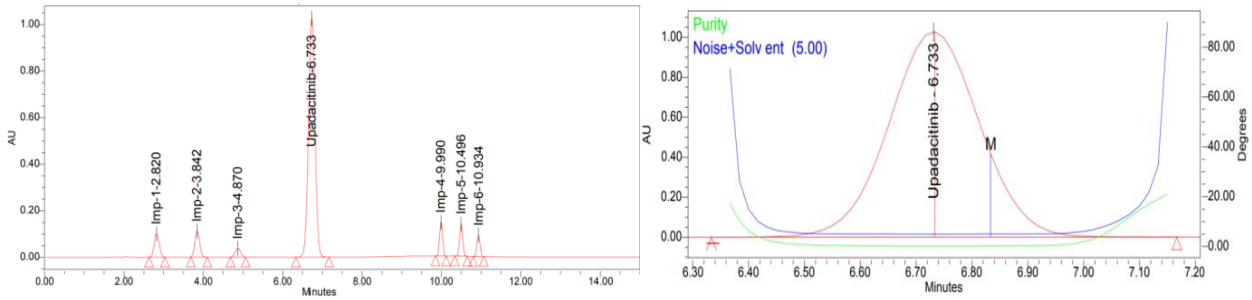

Fig. 19: Chromatograms acid stressed sample and purity plot for UDB

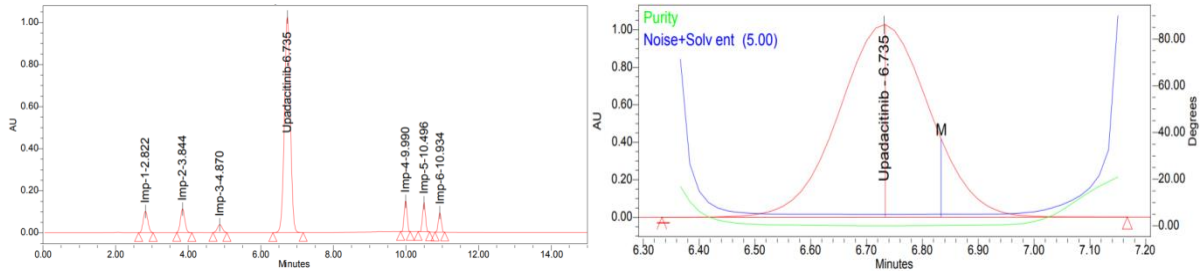

Fig. 20: Chromatograms base stressed sample and purity plot for UDB

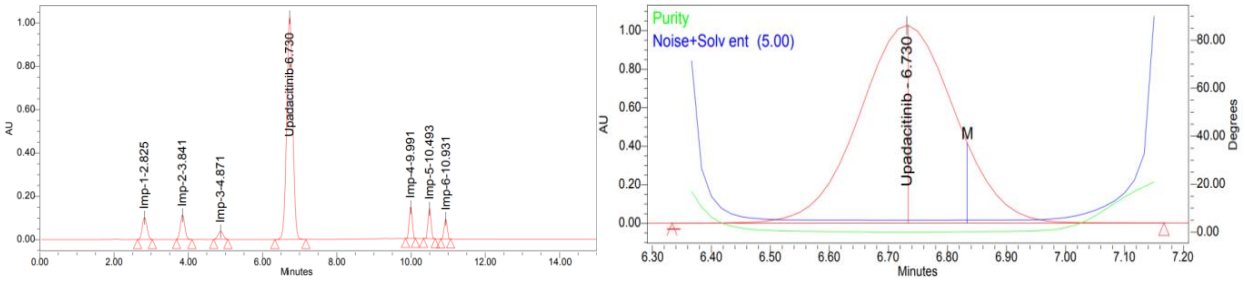

Fig. 21: Chromatograms peroxide stressed sample and purity plot for UDB

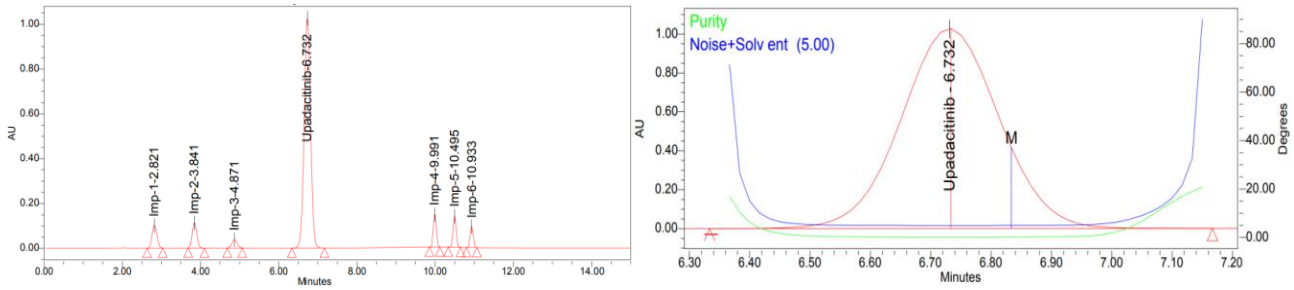

Fig. 22: Chromatograms thermal stressed sample and purity plot for UDB

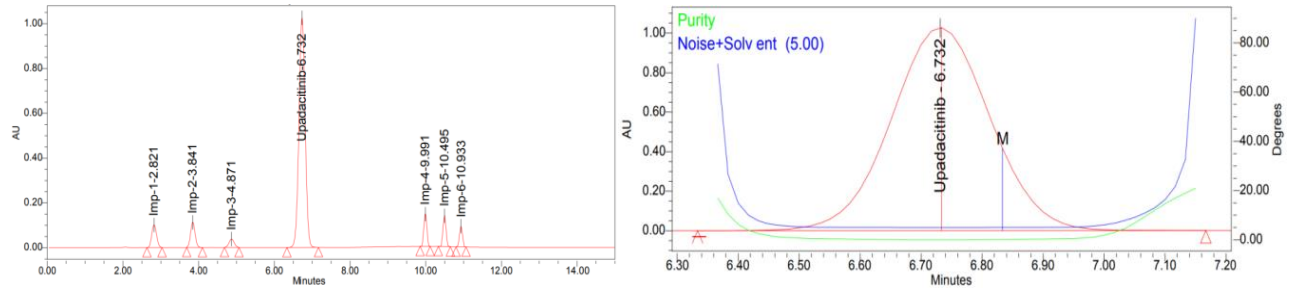

Fig. 23: Chromatograms UV stressed sample and purity plot for UDB

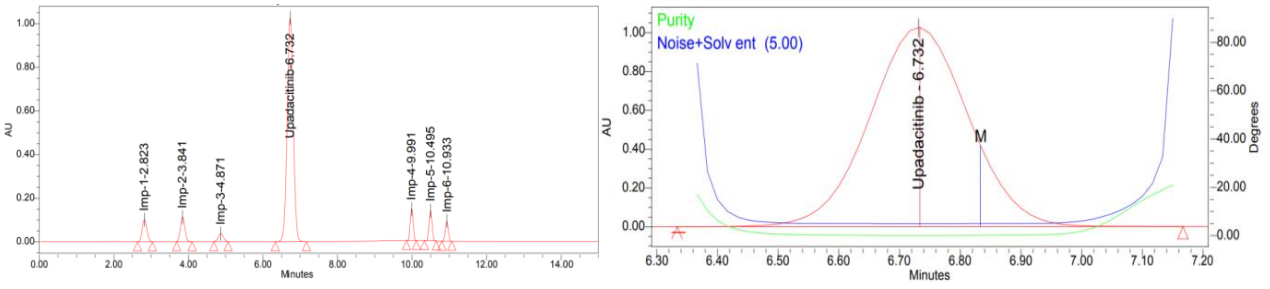

Fig. 24: Chromatograms hydrolysis stressed sample and purity plot for UDB

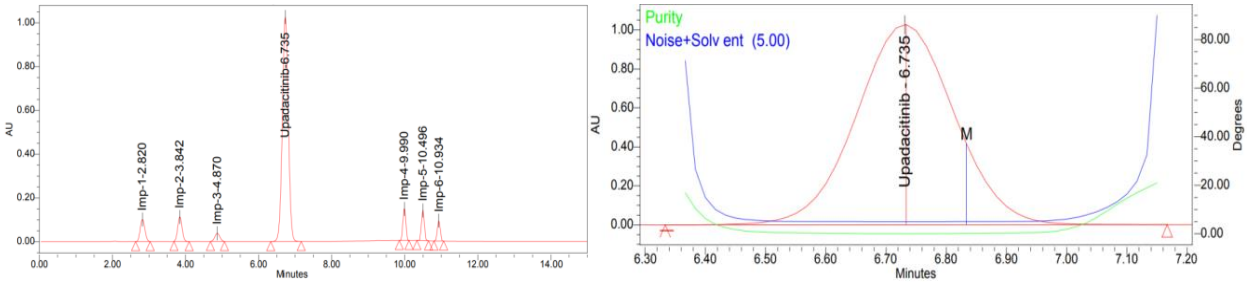

Fig. 25: Chromatograms reduction stressed sample and purity plot for UDB

Table 7: Degradation parameters

Parameter	Initial degradation			6h degradation			12h degradation			18h degradation			24h degradation		
	Amount recovered	% assay	% deg	Amount recovered	% assay	% deg	Amount recovered	% assay	% deg	Amount recovered	% assay	% deg	Amount recovered	% assay	% deg
Control sample (No degradation)	291.3202	97.11	-----	291.3202	97.11	------	291.3202	97.11	------	291.3202	97.11	------	291.3202	97.11	-------
Acid hydrolysis	275.6542	91.88	8.12	267.5515	89.19	10.82	252.5241	84.17	15.83	241.2634	80.42	19.58	220.7904	73.60	26.403
Base hydrolysis	275.0455	91.68	8.32	252.696	84.23	15.77	246.4281	82.14	17.86	235.0564	78.35	21.65	213.9037	71.30	28.698
Oxidation	275.2725	91.76	8.24	277.8712	92.62	7.38	275.2751	91.76	8.24	278.9626	92.99	7.012	284.1078	94.70	5.2973
Hydrolysis	275.0984	91.70	8.30	275.0387	91.68	8.32	275.0127	91.67	8.33	275.7637	91.92	8.08	275.2725	91.76	8.2424
Reduction	275.3656	91.79	8.21	269.3271	89.78	10.22	258.3445	86.11	13.89	249.3793	83.13	16.87	235.1111	78.37	21.629
Thermal	291.7218	97.24	2.76	290.9422	96.98	3.02	290.4225	96.81	3.19	290.1626	96.72	3.28	281.3273	93.78	6.2242
Photolytic (UV)	282.1069	94.03	5.96	281.3273	93.78	6.22	280.9895	93.66	6.34	278.0531	92.68	7.32	277.3774	92.46	7.5408

**B*= (97.11-A)/97.11*100

Table 8: Purity angle and purity threshold values

Parameter	Initial degradation		6h degradation		12h degradation		18h degradation		24h degradation	
	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold	Purity angle	Purity threshold
Control sample (No degradation)	0.028	5.049	0.028	5.049	0.028	5.049	0.028	5.049	0.028	5.049
Acid hydrolysis	0.068	5.065	0.054	5.068	0.065	5.062	0.021	5.035	0.524	5.032
Base hydrolysis	0.065	5.059	0.002	5.096	0.004	5.035	0.005	5.035	0.025	5.069
Oxidation	0.023	5.021	0.054	5.021	0.034	5.023	0.021	5.026	0.041	5.036
Hydrolysis degradation	0.024	5.035	0.046	5.064	0.065	5.056	0.048	5.057	0.048	5.055
Reduction degradation	0.024	5.059	0.035	5.035	0.058	5.035	0.057	5.095	0.095	5.048
Thermal degradation	0.064	5.025	0.064	5.026	0.035	5.026	0.031	5.016	0.031	5.016
Photolytic degradation (UV)	0.059	5.056	0.044	5.097	0.035	5.066	0.032	5.035	0.023	5.021

Concentration (table 9) (75–450 µg ml–1; Upadacitinib, 2.50–15.00 µg ml–1; IMP1, IMP2, IMP5, 1.25–7.50 µg ml–1; IMP3, IMP4) and detector response calibration curves were created. Fig. 26 shows the results of the linear regression technique used to evaluate the linearity. According to table 9, the selected impurities displayed values more than 0.9973, whereas the calibration curves of upadacitinib displayed correlation coefficient (R^2) values greater than 0.9981. The findings show a very good linear relationship between the concentrations and peak areas produced by the proposed method.

Table 9: Linearity concentration and response peak areas

S. No.	Con	(UDB)	Con	IMP-1	IMP-2	IMP-4	IMP-5	Con	IMP-3	IMP-6
1	75	3393309	2.50	251457	250585	198564	192956	1.25	99478	133260
2	150	6013221	5.00	468532	442156	412036	365471	2.50	185469	265124
3	225	9425746	7.50	659837	678546	586325	564216	3.75	282145	402351
4	300	11638823	10.00	900850	938896	817722	763990	5.00	357027	520317
5	375	14865287	12.50	1114574	1185463	993265	946325	6.25	463259	648795
6	450	17842654	15.00	1315646	1366594	1205415	1122354	7.50	555698	786532

Table 10: Regression parameters summary

S. No.	Parameter	Obtained values						
		UDB	Imp-1	Imp-2	Imp-3	Imp-4	Imp-5	Imp-6
1	Residual sum of squares	0.9981	0.9994	0.9973	0.9986	0.999	0.9995	0.9996
2	Slope	38482	85715	92232	72899	80107	75307	103665
3	Y-Intercept	428240	35141	3341.7	4910.8	1287.3	286.07	5862.6

The accuracy of the research approach indicates how close they obtained results are to the correct values. At the description level, a rise in the measured impurity of upadacitinib was used to evaluate the recovery (accuracy) on a known quantity of placebo. In accordance with the suggested methodology, models were set up in three triplicates for the drug and its contaminants at various

concentrations, namely 50%, 100%, and 150% of the target analyte (table 13). For the aforementioned drug and the designated contaminants, separate percentage recovery, standard deviation, percent relative standard deviation, and (n=3) average percent recovery were computed. Upadacitinib recovery ranged from 92.80 to 98.34 (table 11). The percentage RSD ranged from 0.378 to 1.34 (<2 %). The impurity recovery percentage ranged from 103.45 to 93.10, whereas the percentage RSD ranged from 0.116 to 1.43 (table 12). For upadacitinib and its impurities, the percentage RSD values fall well within the acceptable range. These outcomes demonstrate the method's ability to precisely separate the active ingredient and contaminants from the placebo.

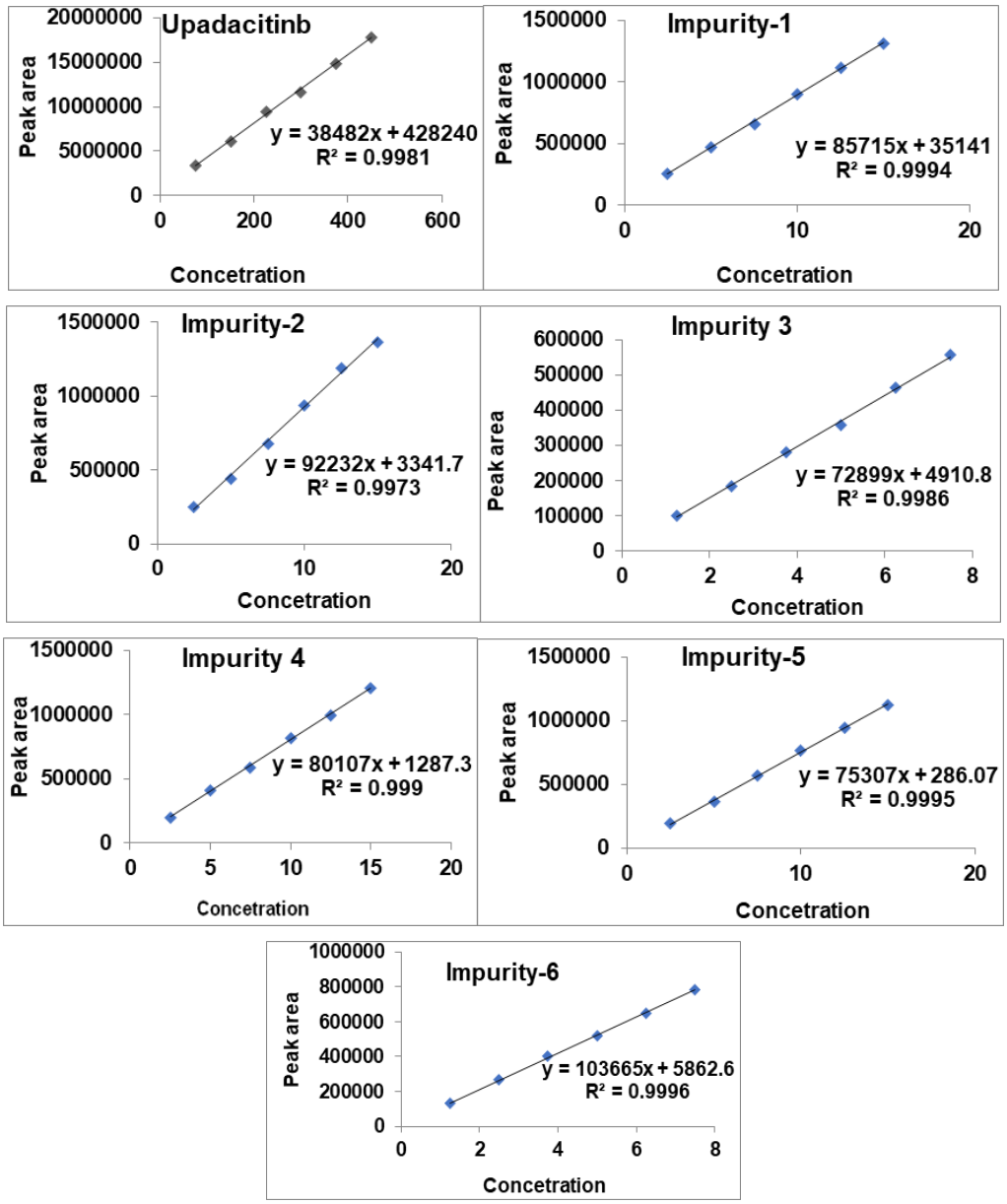

Fig. 26: Calibration curves of upadacitinib and its impurities

Table 11: Results of accuracy for upadacitinib

Level of recovery (%)	Amount added (µg ml⁻¹)	Amount recovered (µg ml⁻¹)	% Recovered	Statistical evaluation	
50	150	139.1927	92.79511	%mean±SD	93.180±0.352
	150	139.8837	93.25583		
	150	140.2313	93.48754	%RSD	0.378269
100	300	286.3189	95.43962	%mean±	96.831±
	300	291.1571	97.05237	SD	1.295
	300	294.0054	98.00178	%RSD	1.337705
150	450	432.0561	96.01247	%mean±SD	97.344±1.202
	450	442.5631	98.34735		
	450	439.528	97.67289	%RSD	1.234418

Value are given in mean±SD; n=3

Table 12: Results of accuracy for impurities

Level of recovery (%)	% Recovered						Statistical evaluation						
	IMP-1	IMP-2	IMP-3	IMP-4	IMP-5	IMP-6		IMP-1	IMP-2	IMP-3	IMP-4	IMP-5	IMP-6
50	98.79	99.57	93.10	102.85	102.85	98.24	%Mean	98.08	99.87±	94.35±	100.65	102.24	97.89
50	97.41	100.51	94.17	101.81	101.81	97.54	±SD	±0.691	0.553	1.35	±0.778	±0.546	±0.347
50	98.03	99.53	95.79	102.04	102.04	97.89	%RSD	0.705	0.554	1.43	0.773	0.534	0.355
100	101.18	101.18	96.39	101.81	101.81	100.63	%Mean	101.17	101.07	96.23±	101.60±	101.53±	100.19
100	101.05	101.28	95.57	101.58	101.58	100.45	±SD	±0.117	±0.282	0.595	0.312	0.305	±0.614
100	101.28	100.75	96.73	101.20	101.20	99.49	%RSD	0.116	0.279	0.619	0.308	0.300	0.612
150	101.15	101.11	96.51	102.16	102.16	98.87	%Mean	102.17	101.56	96.91±	101.08	101.35	99.12
150	101.89	101.57	97.18	100.42	100.42	99.37	±SD	±1.17	±0.449	0.357	±0.879	±0.878	±0.248
150	103.45	102.01	97.04	101.48	101.48	99.13	%RSD	1.15	0.442	0.369	0.870	0.867	0.251

Value are given in mean±SD; n=6

Table 13: Amount recovered and added concentrations

Level of recovery (%)	IMP-1		IMP-2		IMP-3		IMP-4		IMP-5		IMP-6	
	Amount added (µg ml-1)	Amount recovered (µg ml^{-1})	Amount added (µg ml-1)	Amount recovered (µg ml^{-1})	Amount added (µg ml-1)	Amount recovered (µg ml^{-1})	Amount added (µg ml-1)	Amount recovered (µg ml^{-1})	Amount added (µg ml-1)	Amount recovered (µg ml^{-1})	Amount added (µg ml-1)	Amount recovered (µg ml^{-1})
50	5	4.92	5	4.98	2.34	93.10	5	4.10	5	5.14	2.5	2.46
50	5	4.87	5	5.03	2.35	94.17	5	5.03	5	5.09	2.5	2.43
50	5	4.90	5	4.98	2.39	95.79	5	5.07	5	5.10	2.5	2.45
100	10	10.13	10	10.12	4.82	96.39	10	10.19	10	10.18	5	5.03
100	10	10.10	10	10.13	4.79	95.57	10	10.17	10	10.16	5	5.02
100	10	10.13	10	10.07	4.84	96.73	10	10.13	10	10.12	5	4.97
150	15	15.17	15	15.17	7.24	96.51	15	15.16	15	15.32	7.5	7.42
150	15	15.28	15	15.24	7.29	97.18	15	15.30	15	15.06	7.5	7.45
150	15	15.56	15	15.30	7.28	97.04	15	15.03	15	15.22	7.5	7.43

The limit of detection (LOD) is the lowest analyte concentration that can be detected with an S/N ratio of 3:1. The limit of quantitation (LOQ) is the lowest analyte concentration that can be accurately and precisely identified using an S/N ratio of 10:1. An experimental method was used to determine the LOD and LOQ. LOQ (34.41, 0.057, 0.211, 0.181, 0.151, 0.377, and 0.165 µg ml^{-1}) and LOD (11.35, 0.019, 0.070, 0.060, 0.049, 0.124, and 0.055 µg ml^{-1}) for upadacitinib and impurities (IMP 1, IMP 2, IMP 3, IMP 4, IMP 5, IMP 6) were determined using accepted standards [18-26].

The stability of ordinary and sample solutions is examined from the beginning to the end of the 24-hour storage period at room temperature by using the stability methods of Shyamal *et al.* and Kalpana *et al.* [27-29]. They were administered at varying intervals, and the assay's starting and 24-hour percentages differed by less than 2%. The UDB medication is unaffected by storage conditions (table 14).

As per the intended procedure, multiple (six) samples of a comparable batch were evaluated in order to determine the method precision (MP) and intermediate precision (IP). For Upadacitinib, the average assay results in MP and IP are 91.76 and 97.63, respectively, while the impurity range is 94.74 to 102.2. The percentage RSD values of upadacitinib and its impurities vary from 2.263 to 0.057 (table 15), which is much less than the precision acceptability requirements. Furthermore, it was discovered that the system suitability parameters in precision experiments were adequate (table 16). This demonstrates the method's accuracy.

Table 14: Results of stability (sample solutions) (RT and 2-8 °C)

Time intervals	UDB (% assay)		% Difference	
	RT	2-8 °C	RT	2-8 °C
Initial	92.66	94.39	-----	------
6h	85.06	95.54	7.60	1.15
12h	90.34	88.96	2.32	5.43
18h	88.85	88.84	3.82	5.55
24h	88.29	86.02	4.37	8.39

Table 15: Comparison of method precision (MP) and intermediate precision (IP)

S. No.	% Assay													
	Upadacitinib		Impurity 1		Impurity 2		Impurity 3		Impurity 4		Impurity 5		Impurity 6	
	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.	M. P.	I. P.
1	92.66	97.63	101.2	101.2	100.9	100.75	95.02	95.51	101.41	101.4	101.1	100.9	99.39	99.6
2	94.74	91.89	101.2	101.3	101.2	101.18	95.10	94.74	101.14	101.1	102.2	102.1	99.65	99.9
3	93.42	91.89	101.2	101.2	101.4	101.04	95.93	95.77	100.98	100.9	101.4	101.8	99.42	99.7
4	94.08	94.70	101.1	101.3	100.9	101.10	95.07	96.59	100.99	100.9	101.1	101.2	99.43	99.7
5	93.81	91.76	101.3	101.2	101.0	100.50	95.69	96.59	101.00	101.1	101.7	100.9	99.68	99.9
6	96.32	94.18	101.1	101.7	101.0	100.77	95.68	96.59	101.00	100.9	101.5	101.5	100.35	100.5
Mean (n=6)	94.174 ±1.147	93.673 ±	101.2 ±	101.3 ±	101.1 ±	100.88±	95.42±	95.97±	101.09±	101.1± 0.150	101.5± 0.377	101.4± 0.460	99.652±	99.880±
Std. Dev		2.119	0.056	0.184	0.211	0.223	0.362	0.698	0.151				0.330	0.317
%RSD(n=6)	1.2177	2.263	0.057	0.182	0.209	0.221	0.380	0.727	0.149	0.149	0.372	0.454	0.332	0.318

Value are given in mean±SD; n=6

Table 16: Comparison of method precision parameters (MP) and intermediate precision parameters (IP)

System suitability sarameter	Method precision							Intermediate precision						
	UDB	IMP 1	IMP 2	IMP 3	IMP 4	IMP 5	IMP 6	UDB	IMP 1	IMP 2	IMP 3	IMP 4	IMP 5	IMP 6
USP resolution	6.02	-------	4.62	4.52	13.02	3.36	2.14	6.48	-------	4.25	4.69	13.69	3.48	2.28
USP tailing factor	1.68	1.08	1.69	1.48	1.28	1.48	0.38	1.24	1.02	1.36	1.52	1.58	1.39	0.21
USP plate count	8078	2269	4758	5747	56365	61035	61035	8025	2225	4736	5748	56364	61458	68578
Purity1 angle	0.058	0.065	0.025	0.539	0.158	1.121	0.137	0.058	0.065	0.025	0.539	0.158	1.121	0.137
Purity1 threshold	5.027	5.028	5.027	5.015	5.048	5.069	5.027	5.027	5.028	5.027	5.015	5.048	5.069	5.027
Retention time (min)	6.733	2.820	3.842	4.870	9.992	10.496	10.934	6.733	2.822	3.842	4.870	9.990	10.496	10.934
Peak area	113002 90±	9026 44.8±	9353 95±	3526 99.3±	81113 4.7±	764564. 5±	522384. 3±	11242 495±	90358 6±	933737. 2±	354701. 7±	811134. 7±	764099. 2±	52356 9±
SD of area	132399. 2	493.5 408	1947. 27	1319. 9	1208. 84	2839.32	1712.69	24471 0.6	1576.9 7	2057.65	2543.86 6	1208.84	3466.15	1647.0 5
% RSD of area	1.172	0.054 677	0.208	0.374 22	0.149	0.371	0.328	2.177	0.175	0.220	0.717	0.149	0.454	0.3145 8

*from six standard injections

Value are given in mean±SD, n=6

Table 17: Results of robustness/Ruggedness experiment

Altered parameter	Altered cond.	RT (Min)	Tailing factor	Theor plates	USP resolution	Purity threshold	Purity angle	Peak area (mean±SD)	% RSD
UDB									
Control		6.730	1.05	8058	6.69	5.049	0.028		
Flow	0.7	8.364	0.28	14574	7.49	1.069	0.124	11250838.7±182767.89	1.63
(ml/min^{-1})	1.3	5.726	1.99	7545	6.35	1.025	0.057	9545114±21570.14	0.226
Organic	-5	8.325	0.57	27629	9.48	1.018	0.239	9747763.7±19131.3	0.196
solvent (%)	+5	5.563	1.57	69424	5.48	1.035	0.039	11682572±13834.47	0.118
Wavelength	250	8.367	0.95	14681	7.84	1.006	0.180	11371627.7±34693.2	0.305
	262	8.366	0.57	14678	7.96	1.025	0.169	9553714±31114.57	0.326
Impurity-1									
Control		2.823	1.25	2225	----	5.027	0.056		
Flow	0.7	3.508	1.59	2558	----	1.024	0.069	1132391.7± 6080.2	0.537
(ml/min^{-1})	1.3	2.401	1.02	2063	----	1.015	0.052	746928±1440.718	0.193
Organic	-5	3.151	1.06	2305	----	1.036	0.136	914148.7±1567.8	0.172
solvent (%)	+5	2.623	1.35	2135	-----	1.025	0.058	894338.7±1332.41	0.149
Wave length	250	3.509	1.07	2523	-----	1.027	0.082	1124777.7±19244.8	1.711
	262	3.508	1.67	2529	-----	1.058	0.063	742914.7±708.9495	0.095
Impurity-2									
Control		3.844	1.35	4768	4.54	5.015	0.037		
Flow	0.7	4.812	1.68	5757	4.38	1.015	0.069	1159418.3±16955.8	1.462
(ml/min^{-1})	1.3	3.242	1.30	4152	4.01	1.059	0.035	775428.7±1790.04	0.231
Organic	-5	4.281	1.57	5465	4.68	1.030	0.003	912381.7±3354.5	0.368
solvent (%)	+5	3.525	1.69	4359	4.35	1.015	0.036	935092±3069.21	0.328
Wave length	250	4.811	1.06	5780	4.85	1.020	0.064	1146316±27338.12	2.385
	262	4.810	1.59	5737	4.57	1.050	0.096	772525.7±3862.302	0.500
Impurity-3									
Control		4.872	1.58	5748	4.87	5.049	0.567		
Flow	0.7	6.094	1.49	6726	4.35	1.065	0.496	443693.3±3374.9	0.761
(ml/min^{-1})	1.3	4.132	1.69	5254	4.25	1.069	0.396	305863.7±1915.42	0.626
Organic	-5	5.866	1.49	6957	6.25	1.005	0.869	342449±394.9	0.115
solvent (%)	+5	4.220	1.48	5348	3.86	1.068	0.335	332267.7±1761.6	0.530
Wave length	250	6.095	1.05	6780	4.61	1.032	0.424	441035.7±1411.1	0.320
	262	6.094	1.37	6719	4.67	1.065	0.436	304287.3±2052.14	0.674
Impurity-4									
Control		9.991	1.36	56921	13.24	5.019	0.137		
Flow	0.7	10.331	0.67	60284	8.57	1.085	0.165	976808.7±81.13	0.008
(ml/min^{-1})	1.3	9.72 0	1.58	54635	18.65	1.057	0.169	715619.7±2227.72	0.311
Organic	-5	10.203	1.29	59244	10.36	1.007	0.139	832545.7±1984.6	0.238
solvent (%)	+5	9.77 5	0.69	54948	19.02	1.048	0.169	843421.3±3382.96	0.401
Wave length	250	10.332	0.99	60994	8.81	1.028	0.181	976474.7±2708.17	0.277
	262	10.331	0.69	60349	8.57	1.048	0.169	714898±2830.773	0.396
Impurity-5									
Control		10.499	1.24	61902	3.36	5.020	1.158		
Flow	0.7	10.796	1.25	65684	2.58	1.096	1.654	904170±2447.90	0.271
(ml/min^{-1})	1.3	10.252	1.01	58574	3.98	1.024	0.967	665375.3±3099.92	0.466
Organic	-5	10.701	1.18	64369	2.15	1.007	2.439	795548±2393.47	0.301
solvent (%)	+5	10.33 3	1.18	59069	3.35	1.048	1.269	784105.7±2669.24	0.340
Wave length	250	10.799	1.00	65432	2.78	1.025	1.602	906040±3189.2906	0.352
	262	10.798	1.57	65579	2.02	1.048	1.669	664756.3±2847.05	0.428
Impurity-6									
Control		10.932	0.69	68306	2.57	5.037	0.115		
Flow	0.7	11.28 3	1.68	72320	2.68	1.036	0.124	590401±107.5034883	0.018
(ml/min^{-1})	1.3	10.664	1.36	65698	2.68	1.024	0.169	444205.7±3384.241	0.762
Organic	-5	11.364	0.57	71302	3.57	1.039	0.129	514344.3±3488.25	0.678
solvent (%)	+5	10.695	1.27	65040	2.05	1.015	1.296	522490.7±1850.82	0.354
Wave length	250	11.286	1.02	72455	2.89	1.032	0.120	593769±3207.4945	0.540
	262	11.285	1.69	72579	2.39	1.048	0.169	443783±3101.223	0.699

Actual Condition: 2.5 g sodium Hexane-1-sulphonic acid in 1l water adjust pH-3.0 with diluted formic acid: Methanol (60:40); Wave Length: 256; Flow rate: 1 ml/min^{-1}

Value are given in mean±SD; n=3

Research on robustness was done to alter the factors such as wavelength, organic content, and mobile phase flow rate. A ±3% change in flow rate, a ±5% change in organic content, and a ±10% change in flow rate were all examined various chromatographic analyses, including (n=3) %RSD, plate counts, peak regions, SD, retention duration, USP resolution, and USP tail factor (table 17). Upadacitinib's resolution with its impurities is significantly impacted by this information. The other parameters have no effect on how suitable the settings are for the system.

CONCLUSION

Following the establishment of a novel RP-UPLC isocratic technique, Upadacitinib and its designated impurities (impurities 1, 2, 3, 4, 5, and 6) were used for authentication. Among other system suitability parameters, the USP resolution is greater than or equal to 2, the USP plate count exceeds 4000, and the USP tailing is good. The resulting peaks are uniform since there is no purity flag and the purity angle is below the purity threshold. This indicates that the method is robust and reliable for the analysis of Upadacitinib, ensuring accurate quantification of both the active pharmaceutical ingredient and its impurities. Furthermore, these findings support the method's potential for quality control in pharmaceutical applications, contributing to enhanced drug safety and efficacy s technique was validated according to ICH standards, which means that all validating parameters, including robustness, accuracy, and precision, were within acceptable boundaries. As a result, this method was chosen for further studies aimed at assessing the stability of Upadacitinib under various environmental conditions. The comprehensive validation process not only reinforces the method's credibility but also paves the way for its implementation in routine testing to ensure consistent product quality. General analysis. Finally, there are reasonable restrictions on the system-appropriate and validation parameters.

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ABBREVIATIONS

Upadacitinib (UDB), Ultra Pressure Liquid Chromatography-UPLC, Limits of Quantification (LOQ) and Limits of Detection (LOD), Janus kinase (JAK) and Dimethyl Sulphoxide (DMSO) and Photo Diode Array (PDA)

AUTHORS CONTRIBUTIONS

Subhashini Kanthti conceived the study, designed the methodology, performed the experiments and collected the data. Giri Prasad Gorumutchu analyzed the results. Rudraraju Ramesh Raju supervised the project. Subhashini Kanthti wrote the original draft, and all authors contributed to reviewing and editing the final manuscript.

CONFLICTS OF INTERESTS

The authors report no conflicts of interest.

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