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LC and LC-MS/MS Studies for Identification and Characterisation of Related Substances and Degradation Products of Abrocitinib

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Abstract

In the pharmaceutical industry, Related Substances (RCs), impurities or Degradation Products (DPs) are associated with the Active Pharmaceutical Ingredient (API) in the final drug product. These compounds must be within permissible limits for safe therapeutic use for consumers. Hence there is a need to quantify these compounds using an appropriate analytical method. No method is reported in the literature for quantification of these compounds in abrocitinib which is a medical drug prescribed to treat severe atopic dermatitis in adults. This study includes the optimisation of stability indicating the HPLC method for resolution and subsequent quantification of abrocitinib RCs and structural characterisation of stress-induced DPs of abrocitinib. The method was optimised by varying mobile phase solvents, pH, flow rate and wavelength of the detector. The finalised conditions were validated and applied for the resolution and evaluation of stress-induced DPs. The stress was induced in abrocitinib pure drug HCl (0.1M), NaOH (0.1M), hydrogen peroxide (3%), 80°C in an air oven and 254nm in a UV chamber. The generated DPs were structurally characterised with the LCMS experiment. Abrocitinib and DPs along with known RCs were resolved on ACE Ultra Core Super C18 250mm column using 0.9mL/min gradient flow of methanol (Solvent A), acetonitrile and buffer (Solvent B). The resolved compounds were detected through a UV detector at 295nm and a mass detector at NMR positive mode. The method identifies 5.85min, 3.13min, 6.60min and 4.38min respectively for abrocitinib, related compounds 1 to 3 respectively with acceptable system suitability. A very high correlate (< 0.999) linear graph was achieved within 5 to 30 µg/mL concentration level for abrocitinib and 0.05 to 0.3µg/mL for related compounds. Three DPs with molecular mass and formula of $C_{10}H_{13}N_5$ (203.2 g/mol), $C_{11}H_{14}N_4$ (202.2 g/mol) and $C_{13}H_{19}N_5O_2S$ (309.3 g/mol) in acid-induced stress study and two DPs of $C_{12}H_{21}N_5O_2S$ (299.3 g/mol) and $C_{11}H_{19}N_5O_2S$ (285.3 g/mol) in peroxide-induced stress study and one DP of $C_{10}H_{14}N_4$ (190.2 g/mol) in base induced stress study were successfully characterised. The method proposed in this study can effectively resolve unknown degradation products, and known related compounds along with abrocitinib and is hence applicable for quality control analysis of abrocitinib.

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1. Introduction

Pharmaceutical-related substances refer to impurities or Degradation Products (DPs) associated with API in the final drug product. These substances can be present due to various factors, including the manufacturing process, storage conditions or the inherent stability of the drug. Monitoring and controlling the levels of pharmaceutical-related substances are critical for ensuring the safety, efficacy and quality of pharmaceutical products[1].

Forced degradation impurities also referred to as stress degradation impurities emerge deliberately when subjecting a drug substance or drug product to specific stress conditions[2]. The purpose behind stress testing is to expedite the degradation of the pharmaceutical product in a controlled environment, enabling researchers to pinpoint potential degradation pathways and evaluate the drug's stability[3]. Stress degradation studies play a pivotal role in pharmaceutical development, involving the exposure of the drug to diverse stress conditions like acid, base, oxidation, heat and light[4]. The analysis of stress impurities is a critical component in pharmaceutical and chemical development, particularly within the realm of drug manufacturing. The significance of stress impurity analysis lies in its capacity to furnish valuable insights into the stability, quality and safety of a drug substance or product across various conditions[5].

High-Performance Liquid Chromatography (HPLC) plays a crucial role in impurity analysis within the pharmaceutical and chemical industries. It offers high sensitivity, allowing for the detection of impurities even at trace levels[6]. Additionally, its selectivity enables the separation of complex mixtures, making it effective for identifying and quantifying multiple impurities within a sample[7]. The combination of liquid chromatography with mass spectrometry enables the accurate identification of DPs. LC-MS facilitates the structural elucidation of degradation products by providing information on the molecular formula, fragmentation pattern and potential chemical structures. This is crucial for understanding the degradation pathways[8,9].

Abrocitinib (Figure 1) is a Janus Kinase (JAK) inhibitor designed for the treatment of inflammatory conditions with a primary focus on atopic dermatitis[10]. JAK inhibitors target the Janus kinase family of enzymes, which play a crucial role in signalling pathways that regulate the immune response. Abrocitinib is primarily indicated for the treatment of moderate to severe atopic dermatitis in adult patients who are candidates for systemic therapy[11]. JAK inhibitors like abrocitinib work by blocking specific enzymes (Janus Kinases) that are involved in the signalling pathways of certain cytokines and growth factors. By inhibiting these pathways, the drug helps to modulate the immune response, thereby reducing inflammation associated with conditions like atopic dermatitis. It is an oral medication, typically taken in the form of tablets. Common side effects include headache, increased blood pressure, feelings of nausea, sore throat, elevated cholesterol levels and liver enzyme elevations[12]. It has a molecular formula of $C_{14}H_{21}N_5O_2S$ with 323.4138g/mol as molecular mass and *N-{cis-3-[methyl(7H-pyrrolo[2,3-d] pyrimidin-4-yl)amino]cyclobutyl}propane-1-sulfonamide* as systemic name.

The literature reveals the presence of the HPLC method for quantifying abrocitinib in formulations[13-15]. Apart from that method, the LCMS/MS method was published for enantio-separation of abrocitinib in plasma samples[16] and pharmacokinetic study[17-19]. There is no systematic procedure reported for quantification of impurities/RCs of abrocitinib and no work reported for characterisation of stress DPs of abrocitinib. The structural characterisation of DPs is valuable for identifying impurity formation in the synthesis process of the bulk drug. Additionally, this characterisation aids in conducting metabolic studies of

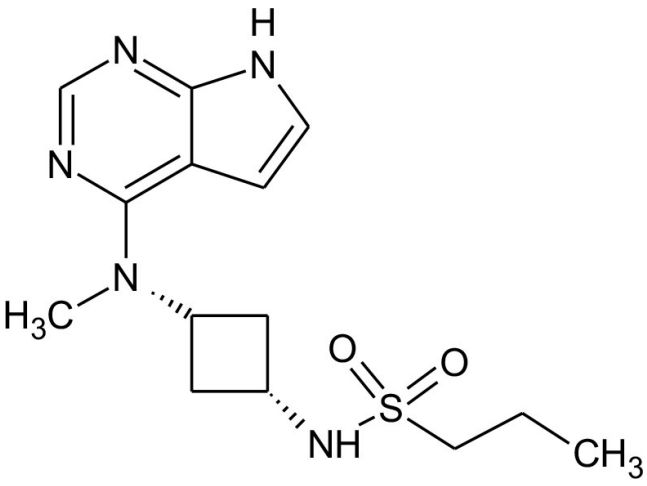
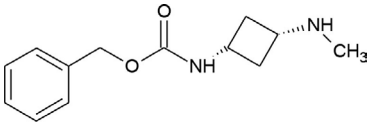
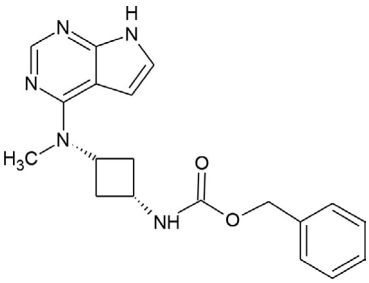
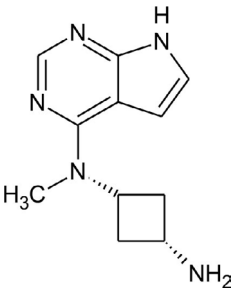


Figure 1. Structure of abrocitinib.

Table 1. Systemic details of abrocitinib and RCs in this study

S No	Structure	Properties
1		**Name**: Related compound 1 (RC 1) **Chemical name**: *benzyl [cis-3-(methylamino)cyclobutyl]carbamate* **Formula**: $C_{13}H_{18}N_2O_2$ **Mass**: 234.29g/mol
2		**Name**: Related compound 2 (RC 2) **Chemical name**: *benzyl {cis-3-[methyl(7H-pyrrolo[2,3-d]pyrimidin-4-yl) amino]cyclobutyl}carbamate* **Formula**: $C_{19}H_{21}N_5O_2$ **Mass**: 351.40g/mol
3		**Name**: Related compound 3 (RC 3) **Chemical name**: *cis-N-methyl-N-(7H-pyrrolo[2,3-d]pyrimidin-4-yl) cyclobutane-1,3-diamine* **Formula**: $C_{11}H_{15}N_5$ **Mass**: 217.27g/mol

abrocitinib. Hence in this research work, we focused on proposing a simple and sensitive HPLC method for the recognition and quantification of Related Compounds (RCs) of abrocitinib and further, DPs were structurally characterised using HPLC coupled with tandem mass spectrometry. Apart from process-related impurities, there are three RCs present for abrocitinib and hence these were selected for this study. Table 1 presents the systematic details of abrocitinib and RCs in the study.

2. Materials and Methods

2.1 Reagents and Drugs

The abrocitinib and RCs in API form were obtained from Metrochem API Private Limited, Hyderabad, Telangana. HPLC grade high pure solvents like methanol, acetonitrile and analytical grade chemicals including ammonium formate, formic acid, hydrochloric acid, hydrogen peroxide, sodium hydroxide and Milli-Q® water purchased from Merck chemicals, Mumbai. Cibinqo® brand abrocitinib formulation tablets with a 50 mg dosage were purchased from the local pharmacy.

2.2 Instruments

LC analysis was performed on 1200 Infinity series (Agilent Technologies, USA) HPLC system equipped with gradient quaternary, auto-sampler, UV detector and Chem Station software. Mass experiments were performed on the Waters LC-MS/MS system (Waters, USA) with a binary solvent delivery pump, autosampler, Triple-Quad mass detector and Mass Lynx software. The mobile phase pH was recorded with a Systronics (India) pH meter.

2.3 Chromatographic Conditions

The chromatographic separation was attained on the ACE UltraCore Super C18 (250mm × 4.6mm; 5µ ID) column (Restek, USA). The methanol (Solvent A), acetonitrile and buffer (Solvent B) were finalised as mobile phase at 0.9mL/min gradient flow with 10µL sample volume. The gradient flow is set as time in min/% composition of solvent B: 0/25, 5/50, 10/75, 25/10 and 28/25. The detector wavelength of 295nm, temperature of a column of 25°C and methanol as diluent were finalised and utilised throughout the analysis.

2.4 Buffer Solution Preparation

Precisely weighed 77.08 grams of ammonium acetate was dissolved in 750mL Milli-Q® water. The solution pH was adjusted to 3.5 and filtered through 0.2µ filters. The final volume was made till mark in a 1 L flask.

2.5 Standard Solution Preparation

An accurately weighed 25 mg of abrocitinib pure drug and RCs were transferred separately into 25mL flasks which were previously filled with approximately 15mL methanol. The flasks were kept in a sonicator to dissolve abrocitinib and RCs in a diluent. The solution was filtered and made till the mark with the same diluent to achieve 1000µg/mL standard solutions separately for abrocitinib and RCs. Then solution was diluted to the desired concentration during analysis by mixing selected concentrations of RCs and standard at fixed volume.

2.6 Assay Sample Solution Preparation

Each formulation tablet containing 50mg of abrocitinib was used for preparing the formulation solution. The tablets were finely powdered and 25mg abrocitinib equivalent powder was transferred to a 25mL calibrated flask having 15mL methanol. The flaks were vortexed for 2 minutes followed by sonication to dissolve analytes in diluent. Then it was centrifuged followed by filtering supernatant through 0.2µ filter into a 25mL fresh flask and the final volume was made till mark in the flask. The resultant formulation solution was diluted to a standard concentration level and utilised for formulation assay analysis.

2.7 Preparation of Stress Degradation Samples

The pure drug solution was mixed individually with HCl (0.1M), NaOH (0.1M) and hydrogen peroxide (3%) and left for 24 hours to induce acid, base and peroxide degradation respectively. The UV light and thermal degradation was induced by exposing pure drug to 254nm in UV chamber and 80°C in an air oven for 24 hours respectively. These stress induced solutions were neutralised whenever necessary and diluted to 100% recovery test level and analysed in the proposed method.

2.8 Method Validation Parameters

The validation of HPLC method adhered to guidelines outlined by ICH[20] and literature that supports this study[21-24]. This encompassed various parameters including suitability of system, range of analysis, repeatability (precision), recovery, robustness, ruggedness, stability, selectivity specificity, Detection Limit (LOD) and Quantification Limit (LOQ).

2.9 System Suitability

The method system suitability was evaluated by repeatedly injecting abrocitinib pure solution having 0.1% of RCs. The column and method efficiency were verified by summarising parameters. The permissible limit set for each parameter as per guidelines that includes tailing factor (<2), resolution (>2), plate count (>3000) and % Relative Standard Deviation (RSD) of area response (<2).

2.10 Linearity

The analytical method is considered linear when a significant relationship exists between the response and the declared working concentration. This relationship extends from the lowest point to the highest point within the tested range, with an R^2 (correlation coefficient) value equal to or greater than 0.999. The linear equation was considered as

$$Y = aX \pm b$$

In which Y represents area response, a and b represents slope and intercept of linear curve whereas X will be claimed analyte concentration.

Six different level concentrations of abrocitinib solution mixed with 0.01% of individual impurity were analysed and area results of resultant chromatographic responses were tabulated for evaluating analytical range of method. Linear curve was plotted individually for abrocitinib and each impurity with concentration against area response.

2.11 Accuracy

Method accuracy was performed in triplicate at 50 to 150 % level spiking to target concentration of abrocitinib and RCs in linearity range. The % recovery of resultant peak area response along with system suitability of each analyte was summarised for evaluating method accuracy. The following formula along with linear equation was utilised for calculating % accuracy of abrocitinib and RCs.

$$\% \text{ Accuracy} = \frac{\text{Actual concentration}}{\text{Theorietical concentration}} \times 100$$

2.12 Precision and Ruggedness

The 100% spiked level solution in calibration range was prepared and analysed in one day (n=6) and in three different days (n=6) to evaluate repeatability and reproducibility of method. Whereas ruggedness was assessed by introducing deliberate and notable variations, encompassing analyst-to-analyst changes by keeping analysis method parameters and conditions constant. The % RSD of individual peak area response in each study for abrocitinib and RCs was calculated in all studies and results of <2 was acceptable.

2.13 Robustness

Robustness was evaluated by implementing carefully planned minor modifications, which involved slight adjustments in parameters such as temperature, mobile phase pH and other relevant factors. The intentionally introduced minor alterations included both positive and negative variations in detector wavelength within a range of ±5nm and adjustments to flow rate within ±0.05mL/min and column oven temperature with ±3°C alteration. The % area response change and system suitability were tabulated for conforming method robustness.

2.14 Sensitivity

LOD and LOQ of RCs in this method was determined for conforming method sensitivity. The analyte minimum concentration in matrix detectable and quantifiable by the instrument was finalised as LOD and LOQ respectively. LOD concentration should remain whereas LOD concentration shouldn't remain consistent across accuracy, precision and linearity ranges upon each injection, without undergoing changes. The determination of LOD and LOQ involved the calculation based on slope (s) and standard error data (σ) derived from calibration linearity as outlined below $\text{LOD} = \frac{3.3\sigma}{s}$ and $\text{LOQ} = \frac{10\sigma}{s}$

2.15 Specificity and Selectivity

For method selectivity confirmation, the solutions such as diluent, buffer, mobile phase, individually reference standard solution of abrocitinib, RCs and combined reference standard solution of abrocitinib and RCs. The stress degradation solutions and tablet formulation solutions were also analysed to evaluate method specificity and selectivity.

2.16 Characterisation of Degradation Products

Initially, stress-degraded solutions were injected into the LC-MS/MS system to facilitate identification, employing the parameters specified in the "Chromatographic conditions" section. The peaks corresponding to abrocitinib, RCs along with DPs were monitored in Electrospray Ionization (ESI) with both positive and negative ionisation modes with a mass range spanning from 10 to 500m/z.

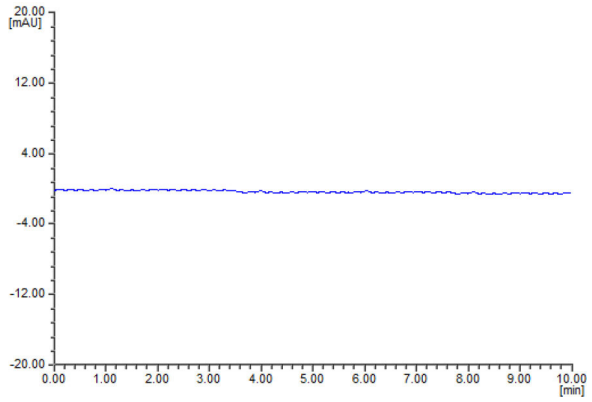
3. Results and Discussion

This study was initiated to propose stability indicating the HPLC method for quantifying RCs 1, 2 and 3 of abrocitinib. Further, the structural characterisation of DPs originated due to stress exposure to abrocitinib with the use of LC-MS/MS. Initially, the HPLC method was optimised with both 250mm and 150mm length ACE Ultra Core Super C18 columns. The efficiency of both columns was evaluated using 0.1% formic acid, ammonium acetate, ammonium formate as pH modifier and methanol acetonitrile as organic modifier. Through experimental trials, it was observed that the utilisation of a longer column resulted in improved separation. Consequently, a column with a length of 250mm was selected for the study. Among the solvents studied as mobile phase, the use of 0.1% formic acid as pH modifier and water as organic modifier doesn't produce acceptable resolution and system suitability of abrocitinib and RCs. Hence these solvents were avoided as mobile phase components. The usage of buffer, acetonitrile and methanol as mobile phase components produces significantly enhanced resolution with acceptable system suitability. Various pH values of buffer were tested, including 3, 4,5, 6 and 7. It was observed that pH 3 and 4 provided superior peak shape and among these pH values, pH 3 exhibited the best outcomes in terms of peak area, resolution and fail factor. Further mobile phase pH was fine-tuned and 3.5 was finalised as suitable for producing the best resolution of analytes. The applicability of gradient elution proved effective in establishing a method for abrocitinib and RCs ensuring sufficient resolution with permissible system suitability.

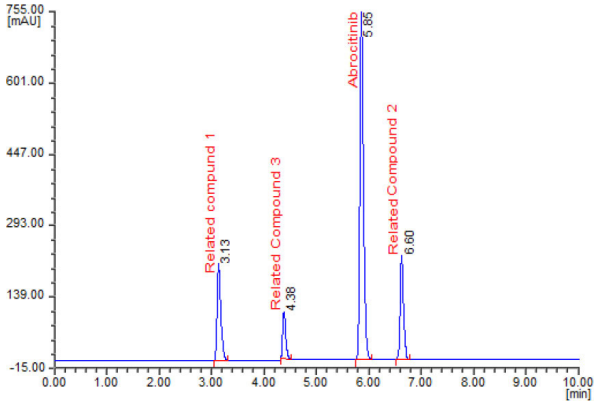
The purity of obtained peaks was verified by computing purity factors through a PDA detector, and they fell within the acceptable noise limit. The total runtime was 10 minutes, with a retention time of 5.85 minutes, 3.13 minutes, 6.60 minutes and 4.38 minutes respectively for abrocitinib, RC 1 to 3 respectively. Figure 2 and Table 2 represent the system suitability chromatogram and results achieved in this proposed method respectively.

The method linearity was assessed across 5 to 30µg/mL for abrocitinib and 0.05 to 0.3 µg/mL for RCs. The method exhibited excellent linearity, as indicated by a high correlation coefficient of more than 0.999 for all analytes. Furthermore, the % RSD of the slope was determined to be 0.01 (less than 2%), indicating minimal deviation around the slope. The results of LOD and LOQ suggest exceptional sensitivity of the method. The sensitivity and linearity results are presented in Table 3.

The 50%, 100% and 150% concentrations to target 10µg/mL of abrocitinib and 0.1µg/mL of RCs were analysed to evaluate method accuracy. The % accuracy of



Chromatogram noticed while injecting mobile phase



Chromatogram noticed while injecting 100 % recovery level standard concentration of abrocitinib and RCs

Figure 2. System suitability chromatogram.

Table 2. System suitability results

S No	Analyte	Results noticed				
		Tailing Factor	Plate Count	Resolution	% RSD	m/z value
1	Abrocitinib	0.99	7905	11.25	0.51	323.41
2	RC 1	1.01	3849	--	0.23	234.29
3	RC 2	0.91	9200	5.83	0.71	351.40
4	RC 3	1.07	5428	8.52	0.55	217.27

Table 3. Linearity and sensitive results

S No	Analyte	Results Noticed			
		Abrocitinib	RC 1	RC 2	RC 3
1	Range (µg/mL)	5-30	LOQ-0.3	LOQ-0.3	0.05-0.3
2	a	51217	416193	217310	593226
3	b	14903	-86.567	868.82	783.63
4	R^2	0.9978	0.9993	0.9985	0.9989
5	LOD µg/mL	0.030	0.015	0.015	0.015
6	LOQ µg/mL	0.099	0.05	0.05	0.05

In the table "a" = slope, "b" = intercept and "R^2" = correlation coefficient

Table 4. Accuracy results in the proposed method

S No	Analyte	Level	Concentration (µg/mL)		% Recovered	% RSD
			Prepared	Actual		
1	abrocitinib	50 %	15	14.771±0.016	98.47±0. 110	0.11
2		100 %	20	19.783±0.091	98.91±0.455	0.46
3		150 %	25	24.730±0.059	98.92±0.235	0.24
4	RC 1	50 %	0.15	0.148±0.001	98.99±0.737	0.74
5		100 %	0.2	0.197±0.001	98.74±0.601	0.61
6		150 %	0.25	0.251±0.001	100.22±0.671	0.67
7	RC 2	50 %	0.15	0.148±0.0002	98.45±0.159	0.16
8		100 %	0.2	0.198±0.002	98.97±0.853	0.86
9		150 %	0.25	0.246±0.0004	98.45±0.191	0.19
10	RC 3	50 %	0.15	0.148±0.001	98.92±0.542	0.55
11		100 %	0.2	0.199±0.001	99.34±.363	0.37
12		150 %	0.25	0.252±0.0008	100.61±0.332	0.33

Values given are the average ± standard deviation of 3 experiments.

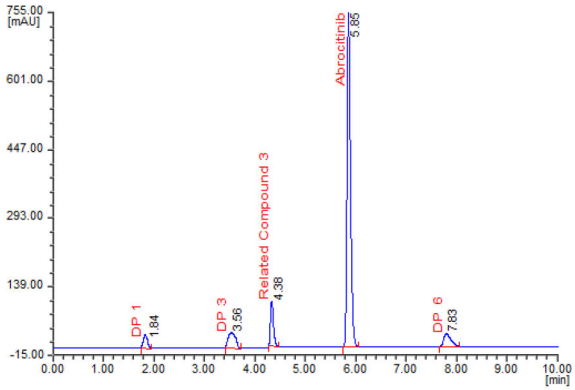
Table 5. Ruggedness results in the proposed method

S No	Altered Condition	Analyte	% Estimated	% Change	Plate Count	Resolution
1	The detector wavelength is 300nm	Abrocitinib	99.02	0.98	7956	11.24
2		RC 1	99.16	0.84	3781	---
3		RC 2	99.50	0.50	9254	5.84
4		RC 3	99.71	0.29	5463	8.52
5	The detector wavelength is 290nm	Abrocitinib	99.38	0.62	8014	11.26
6		RC 1	99.74	0.26	3855	---
7		RC 2	99.74	0.26	9236	5.83
8		RC 3	99.38	0.62	5528	8.53
9	Gradient flow of 0.95mL/minute	Abrocitinib	99.79	0.21	7955	11.53
10		RC 1	99.25	0.75	3864	---
11		RC 2	99.87	0.13	9203	5.51
12		RC 3	99.79	0.21	5563	8.52
13	Gradient flow of 0.85mL/minute	Abrocitinib	99.27	0.73	8055	11.26
14		RC 1	99.27	0.73	3902	---
15		RC 2	99.27	0.73	9256	5.81
16		RC 3	99.27	0.73	5591	8.54
17	Column temperature as 28°C	Abrocitinib	99.11	0.89	8125	11.21
18		RC 1	99.57	0.43	3956	---
19		RC 2	99.14	0.86	9302	5.86
20		RC 3	99.04	0.96	5409	8.55
21	Column temperature is 22°C	Abrocitinib	99.79	0.21	8066	11.22
22		RC 1	99.15	0.85	3800	---
23		RC 2	99.31	0.69	9256	5.85
24		RC 3	99.07	0.93	5567	8.53

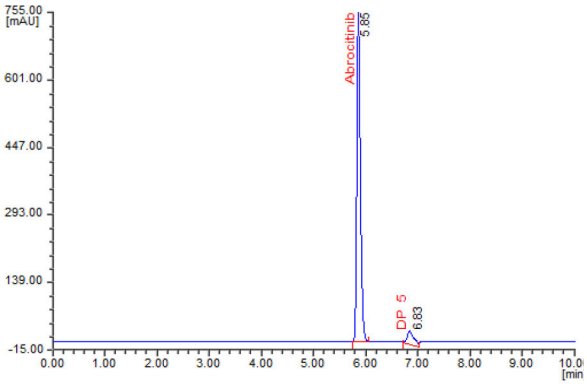
98.47, 98.91 and 98.92 was noticed for abrocitinib in 50%, 100% and 150% levels respectively which falls under the permissible level. The % recovery of studied RCs was also noticed within the permissible level of 98-102%. The % RSD of abrocitinib and RCs in each level was evaluated and an acceptable % RSD of less than 2 (Table 4) was reported suggesting the method's accuracy.

Method robustness was assessed by introducing minor adjustments to the chromatographic conditions. It was observed that there were no notable changes in the measured responses or retention times. The % change in area response of abrocitinib and RCs in the studied adjusted conditions was within a permissible level of less than 2. Insignificant deviations in elution time, plate count and resolution were noticed in all conditions suggesting the method's robustness. Results were tabulated in Table 5.Top of Form

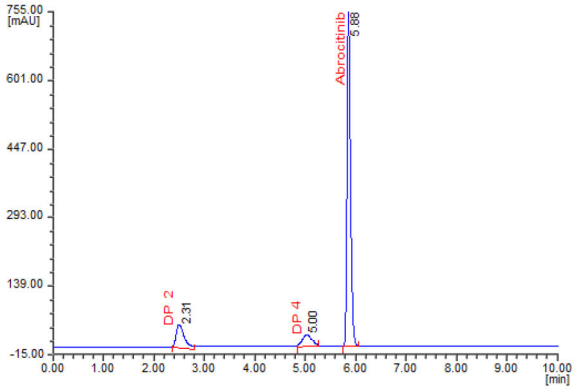
Abrocitinib was degraded at 8.54% in the acid degradation study, due to this, acid DPs emerged in the reaction mixture and were identified at 1.84 minutes, 3.56 minutes and 7.83 minutes and these were designated as DP 1, 3, 6 respectively based on their retention time order. The base degradation induces 6.87% degradation in abrocitinib and as a result of this, one DP was emerged in the reaction mixture. The method resolves and detects these DP at 6.83 minutes and this DP was designated as DP 5 respectively (Table 6). The influence of peroxide induces 5.99% degradation in abrocitinib pure drug and



The acid degradation chromatogram clearly identifies three DPs at 1.84 minutes, 3.56 minutes and 7.83 minutes corresponding to DP 1, 3 and 6 respectively.



The base degradation chromatogram clearly identifies one DP at 6.83 minutes corresponding to DP 5 respectively.



Peroxide degradation chromatogram clearly identifies two DPs at 2.31 minutes and 5.00 minutes corresponding to DP 2 and 4 respectively.

Figure 3. Stress degradation chromatograms of chromatograms of abrocitinib in the proposed method.

Table 6. Stress degradation study results

S No	Degradation Condition	% Degradation	Purity Angle	Mass Balance	No. of DPs Identified
1	Acid	8.54	0.107	91.46	3 (DP 1, 3 and 6)
2	Base	6.87	0.104	93.13	1 (DP 5)
3	Peroxide	5.99	0.106	94.01	2 (DP 2 and 4)
4	Thermal	2.51	0.107	97.49	No DPs noticed
5	UV Light	4.26	0.105	95.74	No DPs noticed

generated one DPs at 2.31 minutes, 5.00 minutes and the retention time was in correlation with DP 2 and 4. The % degradation of 2.51% and 4.26% noticed in thermal and UV light induced degradation studies respectively with no degradation products noticed. The retention time of DPs identified in thermal and UV light were in correlation with DPs noticed in acid and base induced degradation study chromatograms. The purity angle and purity threshold of abrocitinib peak in stress study chromatograms were in the acceptable level suggest purity and homogeneity of abrocitinib peak in stress samples. The mass balance in all stress study chromatograms were more than 99% (Table 6). This study results suggest that the method specificity has the capability to resolve abrocitinib along with known RCs and DPs. The stress study chromatograms of abrocitinib in this proposed method was presented in Figure 3.

The acid-induced stress degradation study chromatogram shows three unknown peaks considered as DPs and based on time of elution, these DPs were marked as DP 1, 3 and 6. In positive ionisation mode, the exact masses of DPs were identified based on protonated molecule ion and nitrogen rule. The molecular formula of $C_{10}H_{13}N_5$, $C_{11}H_{14}N_4$ and $C_{13}H_{19}N_5O_2S$ was confirmed with molecular formulas of 203.2g/mol, 202.2g/mol and 309.3g/mol respectively for DP 1, 3 and 6. DP 1 was characterised by observing fragment ion at 187, 136 and 97 in positive ionisation mode corresponding to $C_{10}H_{10}N_4$ (formed by loss of H_3N from parent ion), $C_6H_7N_4$ (loss of C_4H_3 from 186*m/z*) and $C_4H_6N_3$ (formed by loss of C_2HN from 135*m/z*). The parent ion fragment at 203 (m+1) was noticed in positive ionisation mode suggesting the compound mass of DP 3 as 202.25g/mol. The parent ions noticed at 150 (m+1), 139 (m+1), and 96 (m+1) represent fragments with molecular formula $C_7H_9N_4$ (due to loss of C_4H_5 from parent ion), $C_6H_9N_3$ (due to loss of CN from 149*m/z*), and $C_5H_7N_2$ (due to loss of CH_2N from 138*m/z*). The mass pattern of DP 6 confirms fragment ions at 283 (m+1), 188 (m+1) and 121 (m+1) with the parent ion at 310 (m+1) confirming its molecular mass as 309.38g/mol. The formula of the parent ion confirmed as $C_{13}H_{19}N_5O_2S$ derived from fragment ion formula of $C_{11}H_{16}N_5O_2S$ (generated due to loss of C_2H_3 from parent ion), $C_{10}H_{11}N_4$ (due to loss of CH_5NO_2S from 282*m/z*) and $C_6H_6N_3$ (due to lose of C_4H_5N from 187*m/z*). The possible fragmentation mechanism of DP 1, 3 and 6 was presented in Figure 4. Table 7 presents the accurate mass results and Table 8 presents the structure and other details of DPs identified in this study. The mass spectrum of DPs identified in acid-induced stress was given in supplementary Figures 1, 3 and 5 respectively for DP 1, 3 and 5.

The positive ionisation spectrum of DP 2 confirms 299.3g/mol as molecular mass with the formula of $C_{12}H_{21}N_5O_2S$. The spectrum visualizes fragment ions at 195 (m+1), 178 (m+1), and 97 (m+1). This fragment confirms the fragment formula of $C_9H_{16}N_5$ (origin due to loss of $C_3H_5O_2S$ from parent ion), $C_9H_{13}N_4$ (origin due to loss of H_3N from 194*m/z*), and $C_4H_6N_3$. DP 4 was characterised by the indication of three characteristic fragments at m+1 of 259, 164 and 112 representing the formula of $C_9H_{16}N_5O_2S$, $C_8H_{11}N_4$ and $C_4H_7N_4$. Parent ion noticed at *m/z* of 286 (m+1) with a molecular formula of $C_{11}H_{19}N_5O_2S$. The parent ion at *m/z* of 191 (m+1) was noticed for DP 5 with fragment ions at 150 (m+1), 134 (m+1) and 96 (m+1) corresponding to fragment ion molecular formula of $C_7H_9N_4$ (generated due to loss of C_3H_5 from parent ion), $C_6H_5N_4$ (generated due to loss of CH_4 from 150*m/z*) and $C_5H_7N_2$ (generated due to lose of CN_2 from 134*m/z*). The possible fragmentation mechanism of DP 2, 4 and 5 is presented in Figure 5. Table 7 presents the accurate mass results and Table 8 presents the structure and other details of DPs identified in this study. The mass spectrum of DPs identified in base and peroxide-induced stress was given in supplementary figures.

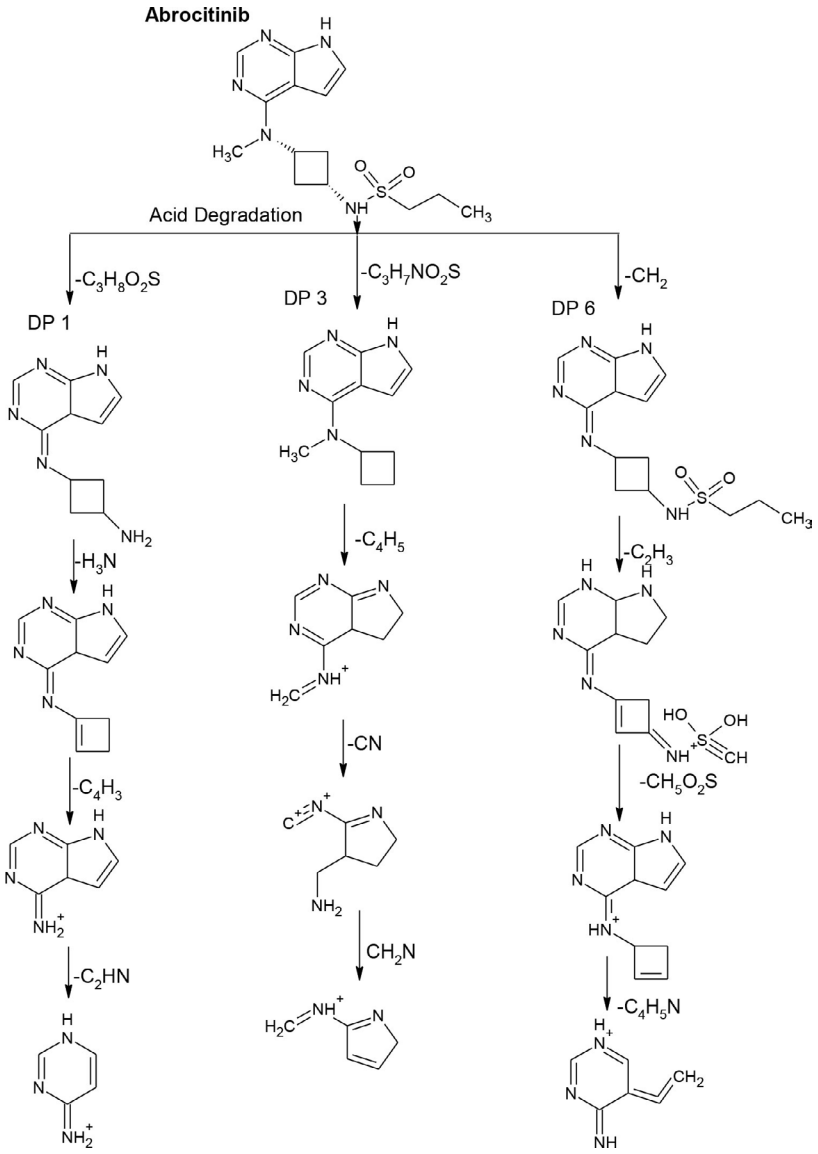


Figure 4. Possible fragmentation mechanism of DP 1, 3 and 6 of abrocitinib.

Table 7. Accurate mass results of abrocitinib, RCs and DPs in ESI positive mode

S No	Compound	Retention Time	Molecular Formula	Calculated Mass *m/z*	Observed Mass *m/z*	Fragment Ion *m/z*	Formula of Fragment Ion
1	Abrocitinib	5.85	$C_{14}H_{21}N_5O_2S$	323.4138	323.1059	119.1433	$C_7H_7N_2$
2	RC 1	3.13	$C_{13}H_{18}N_2O_2.HCl$	234.2942	234.3518	91.1299	C_7H_7
3	RC 2	6.60	$C_{19}H_{21}N_2O_2$	351.4023	351.2066	149.1726	$C_7H_9N_4$
4	RC 3	4.38	$C_{11}H_{15}N_5.2HBr$	217.2703	217.1024	93.1060	$C_5H_5N_2$
5	DP 1	1.84	$C_{10}H_{10}N_5$	203.2437	203.1563	186.2132 135.1460 96.1099	$C_{10}H_{10}N_4$ $C_6H_7N_4$ $C_4H_6N_3$
6	DP 2	2.31	$C_{12}H_{21}N_5O_2S$	299.3924	299.3029	194.2562 177.2257 96.1099	$C_9H_{16}N_5$ $C_9H_{13}N_4$ $C_4H_6N_3$

7	DP 3	3.56	$C_{11}H_{14}N_4$	202.2556	202.3088	149.1726 123.1546 95.1219	$C_7H_9N_4$ $C_6H_9N_3$ $C_5H_7N_2$
8	DP 4	5.00	$C_{11}H_{19}N_5O_2S$	285.3658	285.4167	258.3200 163.1991 111.1246	$C_9H_{16}N_5O_2S$ $C_8H_{11}N_4$ $C_4H_7N_4$
9	DP 5	6.83	$C_{10}H_{14}N_4$	190.2449	190.1692	149.1726 133.1301 95.1219	$C_7H_9N_4$ $C_6H_5N_4$ $C_5H_7N_2$
10	DP 6	7.83	$C_{13}H_{19}N_5O_2S$	309.3872	309.1550	282.3414 187.2205 120.1313	$C_{11}H_{16}N_5O_2S$ $C_{10}H_{11}N_4$ $C_6H_6N_3$

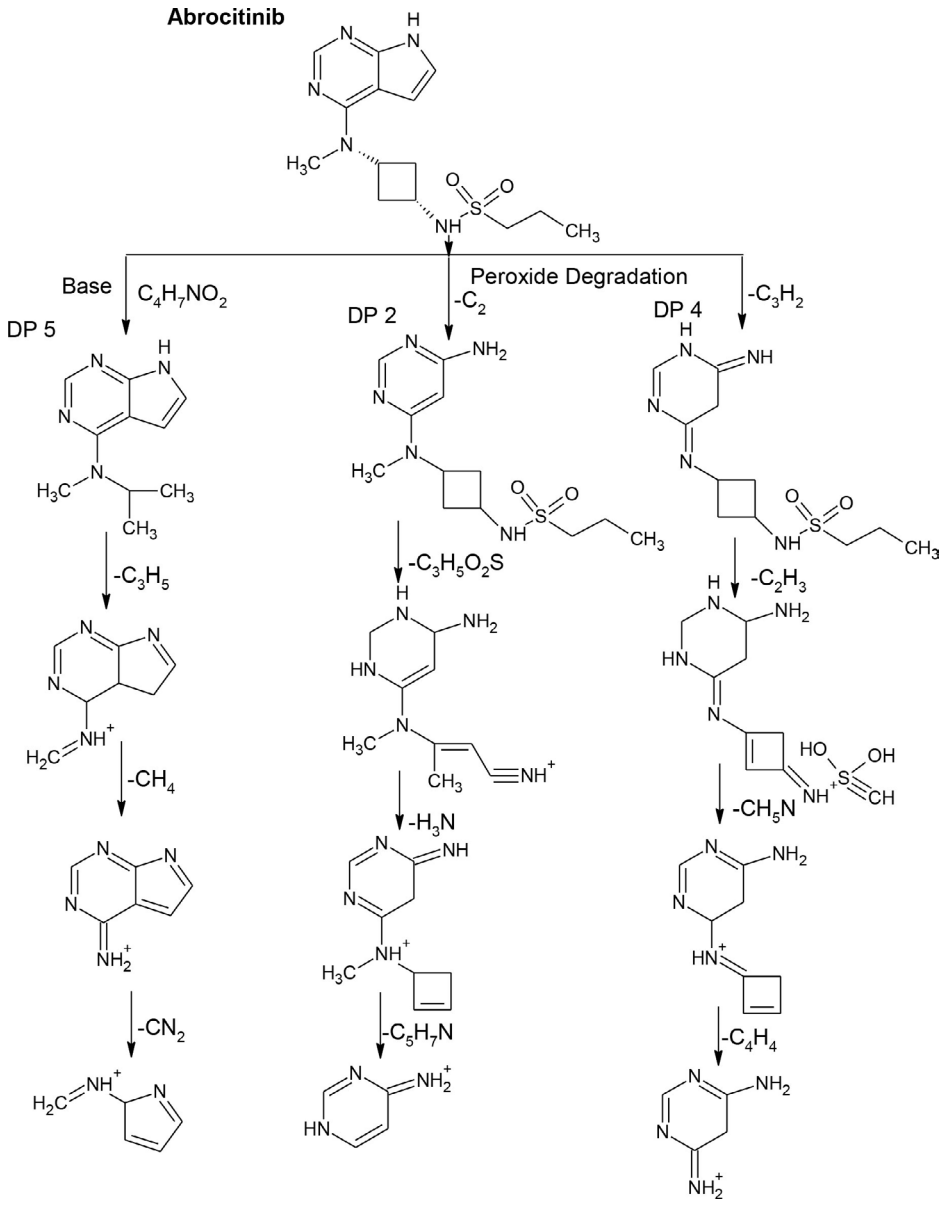
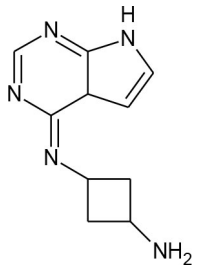
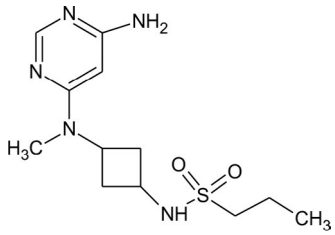
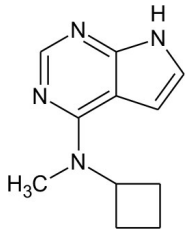
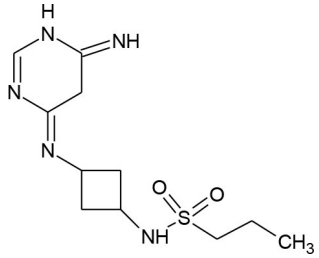
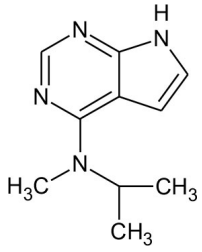
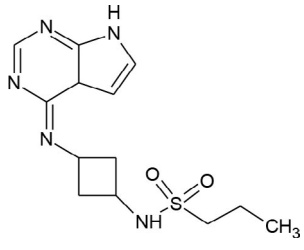


Figure 5. Possible fragmentation mechanism of DP 2, 4 and 5 of abrocitinib.

Table 8. Structure and details of DPs characterised in this study

S No	Retention Time	Structure	Properties
1	1.84 minutes		**Name:** *3-[(E)-4a,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-ylideneamino]cyclobutanamine* **Formula:** $C_{10}H_{10}N_5$ **Mass:** 203.1g/mol
2	2.31 minutes		**Name:** *N-{3-[(6-aminopyrimidin-4-yl)(methyl)amino] cyclobutyl}propane-1-sulfonamide* **Formula:** $C_{12}H_{21}N_5O_2S$ **Mass:** 299.3g/mol
3	3.56 minutes		**Name:** *N-cyclobutyl-N-methyl-7H-pyrrolo[2,3-d]pyrimidin-4-amine* **Formula:** $C_{11}H_{14}N_4$ **Mass:** 202.2g/mol
4	5.00 minutes		**Name:** *N-{3-[(E)-(6-imino-5,6-dihydropyrimidin-4(1H)-ylidene)amino]cyclobutyl}propane-1-sulfonamide* **Formula:** $C_{11}H_{19}N_5O_2S$ **Mass:** 285.3g/mol
5	6.83 minutes		**Name:** *N-methyl-N-(propan-2-yl)-7H-pyrrolo[2,3-d] pyrimidin-4-amine* **Formula:** $C_{10}H_{14}N_4$ **Mass:** 190.2g/mol
6	7.83 minutes		**Name:** *N-{3-[(E)-4a,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-ylideneamino]cyclobutyl}propane-1-sulfonamide* **Formula:** $C_{13}H_{19}N_5O_2S$ **Mass:** 309.3g/mol

The findings derived in this study were correlated with literature and observed that there is no method reported for quantification of abrocitinib RCs as well as no method reported for structural evaluation of DPs of abrocitinib. This study fulfilled the gap noticed in the literature and confirmed that this method was suitable for the evaluation of both RCs and DPs of abrocitinib.

4. Conclusion

A stability-indicating HPLC method, characterised by speed, reliability and precision, was proposed for the first time followed by a structural investigation of forced degradation products of abrocitinib. The study revealed that abrocitinib exhibited high susceptibility to acid and base hydrolysis compared to peroxide while remaining stable under thermal and UV light conditions. The mass approach proved highly effective in characterising and identifying these degradation products. Three DPs namely *3-[(E)-4a,7-dihydro-4H-pyrrolo[2,3-d] pyrimidin-4-ylideneamino]cyclobutanamine* (DP 1), *N-cyclobutyl-N-methyl-7H-pyrrolo[2,3-d]pyrimidin-4 -amine* (DP 3) and *N-{3-[(E)-4a,7-dihydro-4H-pyrrolo[2,3-d]pyrimidin-4-ylideneamino]cyclobutyl} propane-1-sulfonamide* (DP 6) were characterised in acid-induced stress sample, two DPs namely *N-{3-[(6-aminopyrimidin-4-yl)(methyl)amino]cyclobutyl} propane-1-sulfonamide (DP 2)* and *N-{3-[(E)-(6-imino-5,6-dihydropyrimidin-4(1H)-ylidene)amino]cyclobutyl} propane-1-sulfonamide* (DP 4) were characterised in peroxide-induced stress sample whereas one DP namely *N-methyl-N-(propan-2-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine* (DP 5) was characterised in base induced stress sample of abrocitinib. The method underwent validation, including assessments of sensitive levels, linearity, robustness, accuracy and precision. The validated approach was applied to analyse a commercial abrocitinib tablet formulation, showcasing its accuracy, speed, cost-effectiveness and simplicity. The discovery of abrocitinib DPs contributes to expanding research and knowledge on drug stability, offering additional tools for quality control and ensuring safer therapies.

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