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# Method developmentand Validation for the Estimation of Ritlecitinib in rat plasma by LC-MS/MS and its application in Pharmacokinetic studies.

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**Abstract**

A novel LC-MS/MS method was developed and validated for determining the concentration of Ritlecitinib in rat plasma, demonstrating simplicity, precision, accuracy, sensitivity, and reproducibility. The process included liquid-liquid extraction using dichloromethane as the organic solvent, followed by chromatographic separation on an Inertsil ODS-3 column (100 mm x 4.6 mm, 3 µm). The mobile phase, composed of acetonitrile and 0.1% formic acid in a 30:70 v/v ratio, was delivered at a flow rate of 1.0 ml/min, achieving a retention time of 2.724 minutes for Ritlecitinib and 2.727 minutes for Ritlecitinib D6. Detection utilized an Electron Spray Ionization interface with the SCIEX QTRAP 5500 LC-MS/MS system in multiple reaction monitoring mode. This approach enabled simultaneous identification of Ritlecitinib and its deuterated form, showing m/z transitions from 286.159 to 105.7458 and 292.1595 to 111.7468, respectively. The method exhibited linearity across a 5.00–100.00 ng/ml concentration range, with a correlation coefficient ($r^2$) of ≥0.99977. Stability was proven through freeze-thaw cycles and short-term and long-term stability studies. The results show that the proposed strategy can be efficiently and effectively used for routine analysis of Ritlectinib in rat plasma.

**Keywords:** LC-MS/MS, Bioanalytical method, Ritlectinib, rat plasma

## Introduction

The intricate autoimmune disease known as alopecia areata (AA) results in nonscarring hair loss.It can appear at any age and is usually characterized by well-defined, rounded patches of hair loss.Several treatment options are available for the management of AA, such as quercetin, statins, valproic acid, abatacept, interleukin 2, platelet-rich plasma, electroacupuncture, etc.More research into the molecular underpinnings of AA is leading to the recommendation of Janus kinase (JAK) inhibitors and other targeted medicines as the gold standard for treating the disease.According to functional immunological investigations and genome-wide association studies, CD8+NKG2D + T lymphocytes play a significant role in AA pathogenesis by promoting inflammation of hair follicles through the interleukin-15 (IL-15) and interferon-γ (IFN-γ) signaling pathways.The downstream molecular pathway of IFN-γ and IL-15 receptors includes JAK, a signal transducer and transcription activator(Darwin et al., 2018, Yan et al., 2022)).Patients with vitiligo (phase 3), Chron's disease, and other alopecia were the subjects of many Ritlecitinib clinical trials. (National, 2023)

Ritlecitinib, (Brand name-Litfulo) a selective JAK3 inhibitor and a tyrosine kinase inhibitor expressed in the hepatocellular carcinoma (TEC) kinase family,were approved by the US Food and Drug Administration (FDA) in June 2023 and the European Medicines Agency (EMA) in September 2023.It is specifically indicated as a monotherapy for the treatment of severe alopecia areata in adults and adolescents aged 12 years and older.Pfizer developed Ritlecitinib.Ritlecitinib suppressed the phosphorylation of signal transducer and activator of transcription (STAT) generated by cytokines dependent on JAK3, such as interleukin (IL)-2, IL-4, IL-7, IL-15, and IL-21.Suppresses the transmission of signals from immunological receptors such as B cell Receptor (BCR) and T cell Receptor (TCR), which rely on the activity of TEC kinase family members.Chemically Ritlecitinib is chemically known as 1-{(2S,5R)-2-methyl-5-[(7H-pyrrolo[2,3-d] pyrimidin-4-yl) amino]piperidin-1-yl}prop-2-en-1-one.(Figure 1).The molecular formula is $C_{15}H_{19}N_5O$, and the molecular weight is 285.34 g/mol.[3] Ritlecitinib has a basicpKaof 6.6,acidic pKaof 13.59, and log p of 1.47.(National , 2023 and Blair, 2023)

Bioanalysis is the methodology employed to ascertain the concentration of pharmaceutical compounds and their metabolites in biological matrices such as saliva, plasma, serum, cerebrospinal fluid, and urine.Teja and Haque(2022) suggested that bioanalytical techniques are vital forinvestigating bioavailability and bioequivalence. ( Teja et al, 2022)Using bioanalytical methods and validation establishes the compatibility of a quantitative analytical approach with

biochemical processes. (Reddy and Haque, 2022 and Teja et al.,2022)Validation encompasses laboratory examinations to ascertain that the method is appropriate and dependable for its designated applications. ( D'cruz et al., 2017)It is employed in research processes, therapeutic drug monitoring, bioavailability and bioequivalence studies, quantitative evaluation of drugs and metabolites, new drug development, and clinical pharmacokinetics.Bioanalytical techniques are continuously evolving and enduring technologically advanced advancements. ( Tijare et al., 2016)

Quantifying Ritlecitinib in pharmaceutical formulations and biological matrices is vital to understanding pharmacokinetics.There is no published methodology for quantitatively analyzingRitlecitinib in biological matrices using a validated LC-MS/MS technique.Therefore, we recognized the necessity ofcreating an LC-MS/MS technique that encompasses all the criteria for method validation by regulatory guidelines while achieving enhanced sensitivity and utilizing commercially available internal standards.This study presents the creation and verification of a susceptible, specific, and fast LC-MS/MS technique for measuring the amount of Ritlecitinibin rat plasma.The approach was effectively utilized to quantify the levels of Ritlecitinib in a pharmacokinetic investigation conducted on rats.

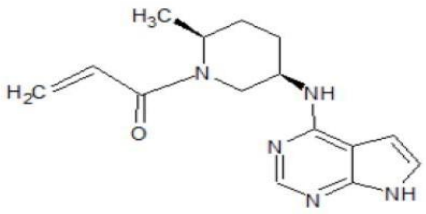


**Figure 1. Chemical Structure of Ritlecitinib**

## Materials and Methods

### Chemicals and reagents

Ritlecitinib (98.0% purity) and Internal standard Ritlecitinib D6 (98.0% purity) (IS Fig. 2) were purchased from Pharma Life Research Lab, India.HPLC grade Methanol, Acetonitrile, Triethyl amine, Water, and Formic acid (LC-MS Grade) were procured from Thermo Fischer Pvt. Ltd., India.

### Ethical considerations

The investigation was conducted strictly following the recommendations and regulations of the Committee for Control and Supervision of Experiments on Animals (CPCSEA).The CPCSEA is a statutory agency established by the Indian government to oversee and regulate animal research activities.The Animal House Facility at Flair Labs in Surat, Gujarat, India, is registered under number 1250/PO/Rc/S/13/CPCSEA.This registration allows for using pharmacokinetic performance methods in experimental investigations on small animals for educational purposes.The rat tests for this research were conducted at Flair Labs Animal House Facility, following protocol No: 1250/PO/Rc/S/13/CPCSEA.Tests are performed without anesthetic while ensuring that all necessary precautions are taken to prevent animals from experiencing unnecessary pain or suffering before, during, or after the experiments.

### Instruments

Waters, alliance e-2695 model HPLC provided with column oven, autosampler, and degasser was operated for analysis.The HPLC system was coupled to a SCIEX QTRAP 5500 mass spectrometer with an electrospray ionization interface.SCIEX software was used tointerpret the data for the chromatogram.

### Mass spectrometer conditions: -

Ritlecitinib was quantified using multiple reaction monitoring modes in positive ion electrospray ionization interface mode.Working parameters utilized wereCollision energy: 14 V, Ion spray voltage: 5500 V, Source temperature: 550$^{o}$C, Drying gas temperature: 120-250°C, Collision gas: nitrogen, Drying gas flow stream: 5 mL/min, Decluttering potential: 40 V, Entrance potential: 10V, Exit Potential: 7 V,Dwell time: 1 sec.

**Mobile Phase:**Acetonitrile: 0.1% Formic acid.0 in a 30:70% v/v ratio. Water is diluent.

**Preparation of phosphate buffer (pH 9.0):** Dissolve 1.74 g of potassium dihydrogen phosphate in 80 ml of distilled water.Adjust the pH with 1 M potassium hydroxide and dilute to 100.0 ml with water.

**Ritlecitinib Stock Solution (200ng/ml)**

Weigh 5mg of Ritlecitinib working standard and transferit into a 100ml volumetric flask diluted to volume with diluent.Then, further dilute 1ml to 10ml with diluent.Take0.4 ml of the above solution into a 10 ml volumetric flask and make it to the mark with a diluent.

**Preparation of Internal Standard Stock Solution (200ng/ml)**

Weigh 5mg of $D_6$-Ritlecitinib working standard and transfer into a 100ml volumetric flask diluted to volume with diluent.Further diluted 1ml to 10ml with diluent.Take0.4 ml of the above solution into a 10 ml volumetric flask and make it to the mark with diluent.

**Preparation of standard working solution of Ritlecitinib and IS (50ng/ml)**

Further dilutions of the standard solution were made with the diluent to obtain two different stock solutions of Ritlecitinib at a concentration of 50ng/ml for calibration and quality control solutions and at a concentration of 50ng/ml for calibration and quality control solutions, respectively. Calibration solutions are prepared by diluting the standard solution of Ritlecitinib (50 ng/ml) with mobile phase to obtain 20, 50, 100, 150, 200, 258,290, and 400 ng/ml.

**Preparation of Calibration standards and Quality control solutions**

Ritlecitinib and Ritlecitinib D6 (IS) are extracted from plasma into the organic phase usinga liquid-liquid extraction technique.Transfer 500µl of the calibration solution into a 2ml centrifuged tube to prepare the calibration standard.To this, add 200µl of plasma, 500µl of internal standard working solution, 600µl of Dichloromethane, and 200µl of buffer.Centrifuge it at 4000 rpmfor 20 min.Separated the organic layer, dried and reconstituted with mobile phase, and transferredit into an HPLC vial.Calibration standards obtained are at concentrations of 5, 12.5, 25, 37.5, 50, 62, 75, and 100 ng/ml.Following the same process, four quality controls, LLOQQC, LQC, MQC, and HQC solutions at 5, 25,50, and 75ng/ml concentrations were prepared.All the samples were freeze-dried.

**Validation of Method**

The optimized method has been validated regarding selectivity, specificity, linearity and range, matrix effect, carry-over, stability, accuracy, and precision per US FDA guidelines.[Guidance, 2001; Kanchanamala et al., 2013; Namburi et al, 2020)

**Selectivity and specificity**

To ensure the accuracy and reliability of the method in detecting the analyte and internal standard, we subjected six unique baseline plasma samples to pre-treatment. To assess the method's specificity regarding endogenous compounds that may co-elute with the analyte and internal standard, six distinct baseline plasma samples underwent a pre-treatment process to identify any possible interferences.This process was conducted to ensure that the results obtained from the analysis were accurate and reliable.The drug and IS elution peaks were identified following their respective retention times and MRM outputs.At the designated retention time, the peak area of Ritlecitinib in blank solution shouldn't surpass 20% of the mean peak area of the lower limit of quantification (LLOQ) for Ritlecitinib.Similarly, the peak area of D6-Ritlecitinib during the designated retention period in a blank solution should be at most 5% of the mean peak area of LLOQ Ritlecitinib.

**Matrix effect**

The matrix factor is calculated by dividing the peak response ratio observed in the presence of the extracted matrix by the peak response ratio observed in neat standards (Aqueous standards).The matrix factor was calculated to measure the matrix effect, which helps anticipate the variance of the matrix effect observed in samples from different subjects.We performed three replications of elution on six sets of blank biological matrices.The elution was done with the pure standard at the low-quality control (LQC) and high-quality control (HQC).Additionally, alternative doses were prepared to compare with the pure standards at the exact dosage.The coefficient of variation (CV %) measures the matrix factor's total precision.The CV % needs to be below 15%.

**Linearity**

A calibration curve was created using a blank plasma sample without Ritlecitinib and IS, along with a zero-sample spiked with sorafenib (IS) and eight concentration levels of calibration standards: 5, 12.5, 25, 17.5, 50, 62.5, 75, 100 ng/ml.Each calibration standard must be analyzed twice.

To establish the concentration-response relationship, the area of peak ratio of Ritlecitinib and D6 Ritlecitinib was plotted against the concentration of Ritlecitinib in ng/ml.The calibration range of Ritlecitinib is defined by the lowest calibration standard of 5ng/ml (LLOQ) and the highest calibration standard of 100ng/ml (ULOQ).

The slope and intercept were calculated, and a regression equation was constructed using a simple regression model.

**Precision and accuracy**

QC samples at four concentration points were analyzed to evaluate accuracy and precision atfour concentration points of 5, 25, 75, and 100ng/ml within and between runs.Each batch of precision and accuracy contains one blank, I zero, Calibration standards, and quality control solutions.Within the run, accuracy, and precision were measured by examining five replicates at each Quality control concentration point in one day.Between runs, accuracy and precision were achieved by analyzing each quality control concentration point for three consecutive days.The mean accuracy was expressed as a percentage, and precision was expressed as the coefficient of variation (%CV).Accuracy and precision should not exceed +15% of nominal concentration, except for the LLOQ, which can deviate within +20%.

**Recovery**

To determine the recovery levels of Ritlecitinib and IS, extracted samples of LQC, MQC, and HQC are compared to spiked LQC, MQC, and HQC levels in a blank solution without a matrix.The recovery levels must be uniform.Six replicates of extracted and post-spiked QC samples are analyzed at the concentration points.The percentage recovery should be at most 15% for each QC sample and across all.

**Stability Studies**

After three freeze-thaw cycles, samples (LQC and HQC; n=6) were collected from the fridge or freezer, following the prescribed clinical procedures.To evaluate stability during freeze-thaw cycles, prepare LQC and HQC samples, each requiring six solutions.Store these samples at -28±5 °C for 6 hours before injecting them into an LCMS device.

To ensure the stability of the Autosampler, Prepare LQC (Low-Quality Control), MQC(Medium Quality Control), and HQC (High-Quality Control) samples.Subsequently, ensure that these samples are pumped into the LCMS (Liquid Chromatography-Mass Spectrometry) network every hour, with a maximum duration of 24 hours.Prepare six solutions for the samples of low-quality control (LQC), medium-quality control (MQC), and high-quality control (HQC).These samples will be used to assess the short-term stability of the LCMS device.After storing the samples at a temperature of 5±3 °C for seven days, introduce them into the LCMS device.For long-term stability, prepare LQC, MQC, and HQC samples, each consisting of six solutions.Store these samples at a temperature of -20±3 °C until they are ready to be injected into the LCMS device.Maintain the LCMS device at a temperature of 5±3 °C.Inject the samples into the device every seven to twenty-eight days.The precision and accuracy of the stability samples should be within 15% and ±15% of their actual concentrations, respectively.

### Pharmacokinetic studies

A method was developed to measure the amount of Ritlecitinib in rat plasma.The study involved administering a dose of ritlecitinib tablet to Male Wistar rats at 7mg/kg.Blood samples were collected at regular intervals of 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, and 3 Hrspost-dose by retroorbital plexus technique into K2EDTA treated vacutainers.The collected blood was centrifuged, and the plasma was stored for future use.These plasma samples were spiked with IS and processed alongside QC samples at four concentration points.We used the WinNonlin (Version 5.2) software package to calculate the pharmacokinetic parameters of Ritlecitinib accurately.This information is essential to understand how the drug behaves in the body and to optimize its effectiveness.The reliability of the study samples was tested rigorously with incurred sample reanalysis (ISR).Two samples were selected from each subject's pharmacokinetic profile, one during the elimination phase and the other near $C_{max}$.The stability of the samples was deemed acceptable if the percentage difference between the two was less than or equal to 20% in rat plasma.

## Results and Discussion

### Method development

Due to its exceptional specificity, accuracy, and repeatability, LC-MS/MS has been widely recognized as one of the most efficient analysis methods.Our investigation aimed to develop and assess a rapid, dependable, and effective method for precisely quantifying Ritlecitinib in plasma samples from rats.Ritlecitinib and D6-Ritlecitinib were isolated from the plasma samples using a Liquid-liquid extraction method.After a series of experiments, the separation conditions for Ritlecitinib and D6-Ritlecitinib were optimized in terms of the composition and properties of the mobile phase to increase signal strength and clarity.The MS-ESI pump optimized the procedure and administered solutions containing Ritlecitinib and D6-Ritlecitinib.By enhancing ionizing and droplet drying processes, additional factors, such as the nebulizer and the thermal gases, were optimized to produce a superior discharge pattern.Our study used two compounds: ionic protonated Ritlecitinib and D6-Ritlecitinib.The ion bands of Rilecitinib (Fig.2) and D6-Ritlecitinib (Fig.3) products generated fragmentation ions of high abundance, with respective m/z values of 286.159105.7458 and 292.1595-111.7468.Separations was carried outInertsil ODS-3 column, 100mm x 4.6mm, 3µmBy supplying the mobile phase with Acetonitrile: 0.1% Formic acid.0 in a 30:70% v/v ratio at a rate of 1.0 ml/min and an injection volume of 10 µl, the samples were effectively separated and eluted with retention time of 2.724 (Fig.4a) minfor Ritlecitinib and 2.727minute for Ritlecitinib D6.(Fig 4b)

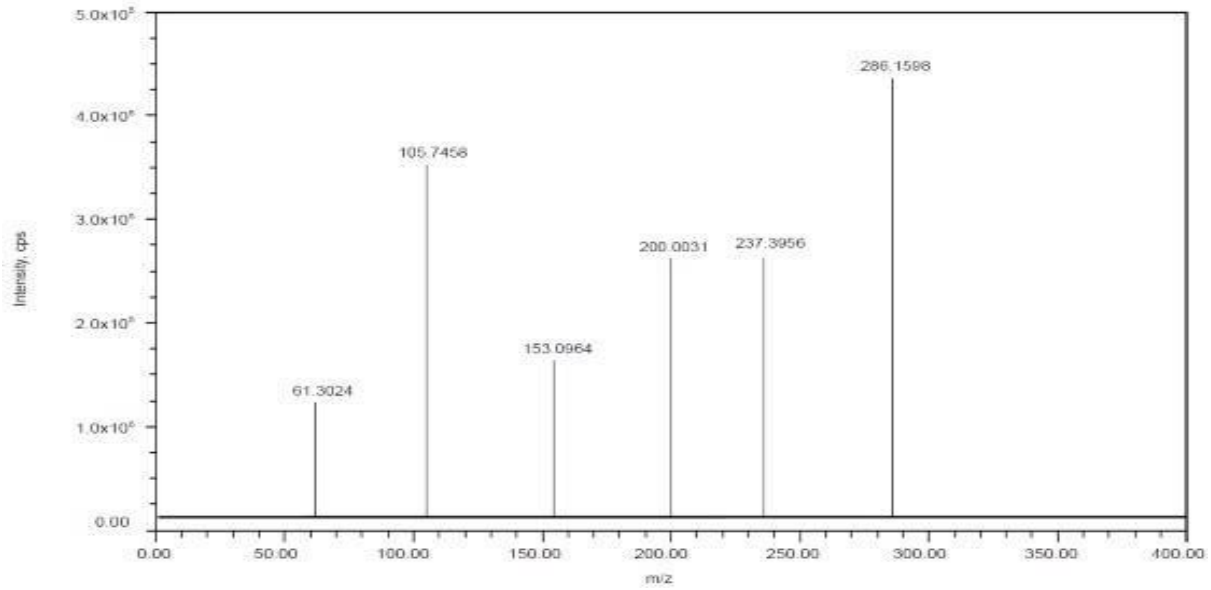


**Figure 2: Multiple Reaction, Monitoring-MRM of the Ritlecitinib, using Positive Polarity**

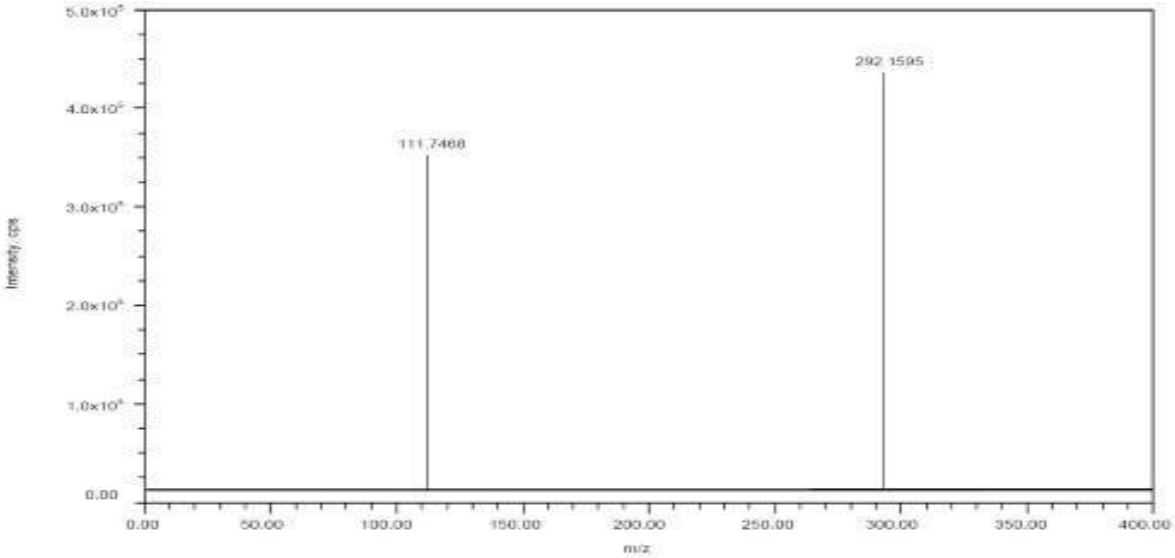


**Figure 3: Multiple Reaction Monitoring-MRM of the D6-Ritlecitinib using Positive Polarity**

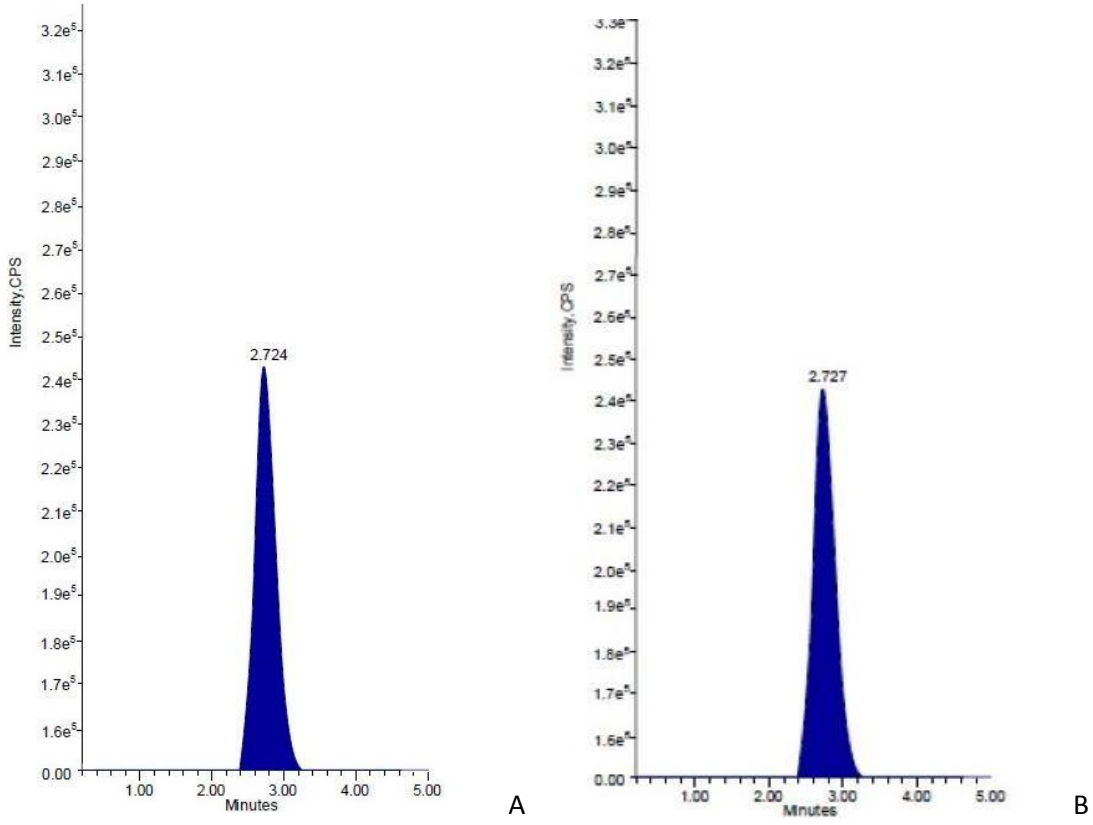


**Figure 4 Chromatogram of Ritlecitinib (A) and Ritlecitinib D6 (B)**

**Method validation**

The proposed approach effectively maintained linearity across a 5.00–100.00 ng/ml concentration range.The validating assessments were conducted per accepted standards.These assessments covered precision, selectivity specificity, linearity, matrix effect, accuracy, stability, and recovery (Guidance, 2001; Kanchanamala et al., 2013; Namburi et al, 2020

**Selectivity and specificity**

Analysis of Ritlecitinib and D6-Ritlecitinib using the MRM technique found no interfering substances in blank rat plasma, proving its selectivity.Specificity tests using six rat plasma batches showed only no interferences at retention times of Ritlecitinib and IS (Fig. 5).Blank-IS chromatograms are shown in Fig. 4.No interfering substances were detected in blank plasma when analyzing Ritlecitinib and D6-Ritlecitinib using the MRM technique.We conducted tests for specificity using six distinct batches of rat plasma.

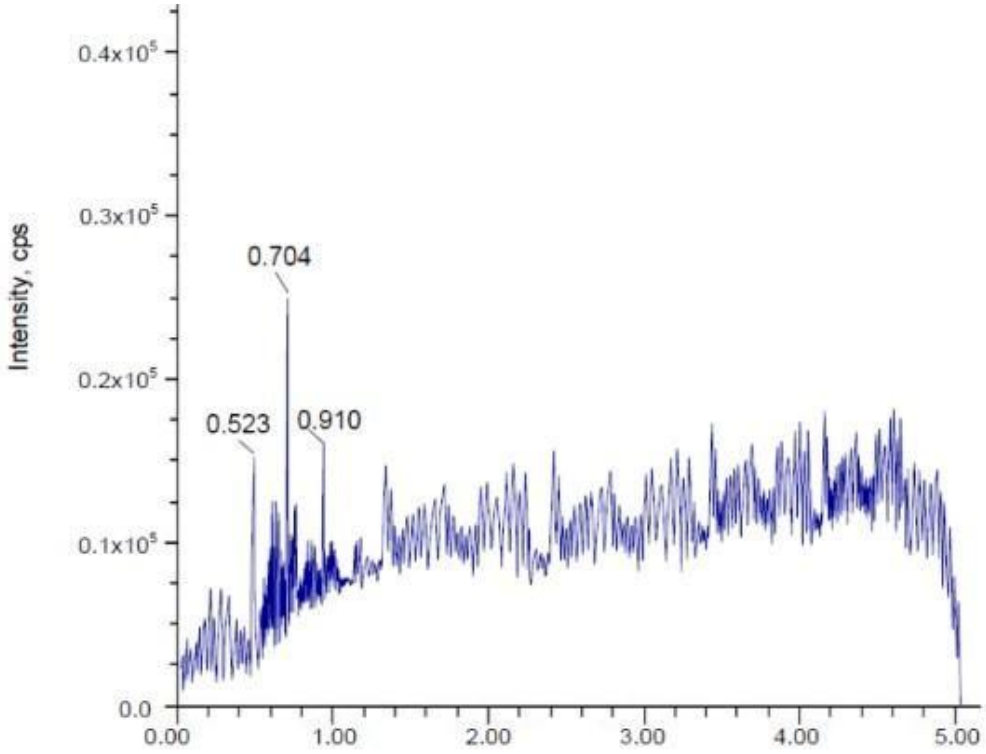


**Figure 5: Chromatogram of Blank rat plasma**

**Matrix effect**

For the Ritlecitinib samples from six lots, the mean percentage accuracy and percentage coefficient of variation were calculated for three replicates of low-quality control (LQC) at 25 ng/mL and high-quality control (HQC) at 100 ng/ml.This analysis was conducted to determine the assay's accuracy and precision. The results are tabulated in Table 1.

**Table 1.Ritlecitinib matrix effect results**

| **Analyte** | **Nominal concentration (ng/ml)** | **% Mean accuracy** | **% Coefficient of Variation** |
|---|---|---|---|
| Ritlecitinib | HQC (75ng/ml) | 99.77% | 043 |
| | LQC (25ng/ml) | 99.79% | 0.58 |

**Linearity**

The linearity between the plasma concentration of Ritlecitinib and the drug/internal standard peak area ratio is unquestionable.The regression equation y = 0.0201x + 0.02541 confidently demonstrates the correlation between the variables. The correlation coefficient value of 0.9999 significantly exceeds the anticipated value of 0.9850, affirming the reliability and strength of the regression model. Moreover, the relative standard deviation was less than 3%, indicating a high precision level.The LLOQ for Ritlecitinib is five ng/ml, which highlights its strong sensitivity, while the ULOQ of 100 ng/ml indicates its upper limit of quantification.The standard curves plotted plasma concentration on the x-axis and drug/internal standard peak area ratio on the y-axis exhibited linearity through the concentration range of 5-100 ng/mL of Ritlecitinib, as demonstrated by the regression equation y = 0.0201x + 0.02541 and the correlation coefficient of 0.9999, which is more significant than 0.9850.The relative standard deviation was less than 3%.(Table. 2 and Fig 7).The limit of quantification (LLOQ) for Ritlecitinib is five ng/ml, while the upper limit of quantification (ULOQ) is 100 ng/ml.

**Table 2: Linearity Results of Ritlecitinib**

| **Calibration standards(ng/ml)** | **Predicted Mean concentration(ng/ml)** | **%RSD** | **Accuracy (%)** |
|---|---|---|---|
| 5.00 | 4.97 | 1.82 | 99.4 |
| 12.50 | 12.67 | 2.01 | 101.36 |
| 25.00 | 23.84 | 3.72 | 95.36 |
| 37.50 | 37.79 | 2.01 | 100.77 |
| 50.00 | 49.48 | 2.33 | 98.96 |
| 62.50 | 62.99 | 0.98 | 100.78 |
| 75.00 | 74.06 | 1.80 | 98.75 |
| 100.00 | 99.92 | 1.06 | 99.92 |

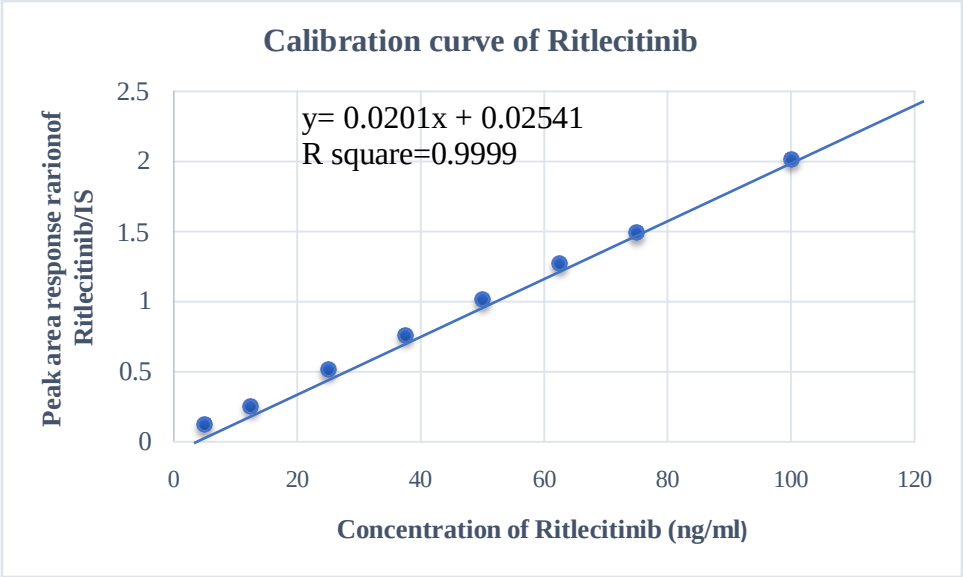


**Fig-6 Calibration plot for concentration v/s Area ratio of Ritlecitinib**

***Accuracy and Precision***

The accuracy and precision of the proposed method were determined through a thorough evaluation process that involved calculating the mean accuracy and precision (% coefficient of variation) within and between the runs of the quality control solution.The solution was tested at different concentrations of Ritlecitinib, including LLOQ, LQC, MQC, and HQC.The data presented in Table 3 indicated that the accuracy and precision ranged from 96.09% to 99.65%, with %CV being less than 2%.The proposed method's accuracy and precision were determined by calculating mean accuracy and precision (%coefficient of variation) within-run and between-runs of quality control solution at concentration points of LLOQ, LQC, MQC, and HQC of Ritlecitinib. Table 3 presents data of accuracy and precision.The accuracy ranged from 97.24% to 104.20%, and %CV was less than 2%.

Table 3.Accuracy and Precision results of Ritlecitinib in rat plasma

| **Nominal concentration (ng/ml)** | **Predicted mean concentration(ng/ml)** | **Accuracy(%)** | **Precision(%C.V)** |
|---|---|---|---|
| **Within run (n=5)** | | | |
| 5 (LLOQ) | 5.09 | 101.80 | 1.54 |
| 25 (LQC) | 24.65 | 98.60 | 0.52 |

| | | | |
|---|---|---|---|
| 50 (MQC) | 49.38 | 98.76 | 0.25 |
| 75 (HQC) | 75.04 | 100.05 | 0.18 |
| **Between runs (n=15)** | | | |
| 5 (LLOQ) | 5.21 | 104.20 | 1.53 |
| 25 (LQC) | 24.31 | 97.24 | 0.75 |
| 50 (MQC) | 49.14 | 98.28 | 0.29 |
| 75 (HQC) | 74.69 | 99.58 | 0.22 |

**Recovery**

The recovery of drug and IS was evaluated at three concentration levels, namely low, medium, and high-quality control.The peak areas of spiked Ritlecitinib and IS in the plasma sample were compared with In the given experiment, the areas of spiked Ritlecitinib and IS in a plasma sample were meticulously compared with those of spiked Ritlecitinib and IS in a blank solution without plasma.This comparison was conducted at three concentration points, namely Low-Quality Control (LQC), Medium Quality Control (MQC), and High-Quality Control (HQC).The percentage recovery was then calculated and found to be within the range of 98.97% to 99.79%.(Table 4)

**Table 4.Percentage recovery of Ritlecitinib in rat plasma**

| **Nominal concentration (ng/ml) (n=6)** | **% Recovery in plasma extracted sample** | **% Recovery in solvent sample** |
|---|---|---|
| 25 (LQC) | 98.97 | 99.63 |
| 50 (MQC) | 99.51 | 99.79 |
| 75 (HQC) | 99.49 | 99.68 |
| IS | 99.34 | 99.71 |

**Stability Studies**

Table 5 displays the stability studies' percentage accuracy and coefficient of variation.The drug's percentage recovery was less than 15% in all cases, indicating that it remained stable throughout the various stages of storage, processing, and analysis of the Ritlecitinib samples.

**Table 5. Stability studies results pf Ritlecitinib in rat plasma**

| **Stability test** | **Plasma** | **Accuracy (%)** | **Precision** |
|---|---|---|---|

| | **concentration (ng/ml)** | | **(%CV)** |
|---|---|---|---|
| Freeze thaw stability | LQC(25ng/ml) | 99.71 | 0.55 |
| | MQC(50ng/ml) | 99.63 | 0.17 |
| | HQC(75ng/ml) | 99.49 | 0.07 |
| Short term stability | LQC(25ng/ml) | 96.58 | 0.31 |
| | MQC(50ng/ml) | 96.75 | 0.29 |
| | HQC(75ng/ml) | 96.50 | 0.11 |
| Long term stability | | | |
| Day 1 | LQC(25ng/ml) | 99.71 | 0.24 |
| | MQC(50ng/ml) | 99.38 | 0.26 |
| | HQC(75ng/ml) | 99.36 | 0.20 |
| Day 7 | LQC(25ng/ml) | 96.25 | 0.57 |
| | MQC(50ng/ml) | 96.34 | 0.25 |
| | HQC(75ng/ml) | 96.83 | 0.15 |
| Day 14 | LQC(25ng/ml) | 89.07 | 0.73 |
| | MQC(50ng/ml) | 90.14 | 0.21 |
| | HQC(75ng/ml) | 90.90 | 0.13 |
| Day 21 | LQC(25ng/ml) | 89.25 | 0.55 |
| | MQC(50ng/ml) | 88.88 | 0.25 |
| | HQC(75ng/ml) | 88.87 | 0.13 |
| Day 28 | LQC(25ng/ml) | 84.48 | 0.40 |
| | MQC(50ng/ml) | 85.30 | 0.30 |
| | HQC(75ng/ml) | 85.58 | 0.14 |
| Autosampler stability | LQC(25ng/ml) | 99.34 | 0.28 |
| | MQC(50ng/ml) | 99.55 | 0.42 |
| | HQC(75ng/ml) | 99.33 | 0.26 |

**Pharmacokinetic studies**

The established method can successfully estimate Ritlecitinib concentrations in rat plasma.The study successfully estimated the concentrations of Ritlecitinib in rat plasma using the established process.The evaluated parameters were maximum plasma drug concentration during the 3-hour

study (Cmax) (as shown in Figure 7), $AUC_{0-10}$, time taken to reach the maximum concentration (Tmax), and terminal half-life.These parameters are summarized in Table 6.The parameters evaluated were maximum plasma drug concentration during 3 hours of study ($C_{max}$) (Fig.7), $AUC_{0-10}$, time taken to obtain maximum concentration ($T_{max}$), and terminal half-life (t1/2), which are summarized in Table 6.

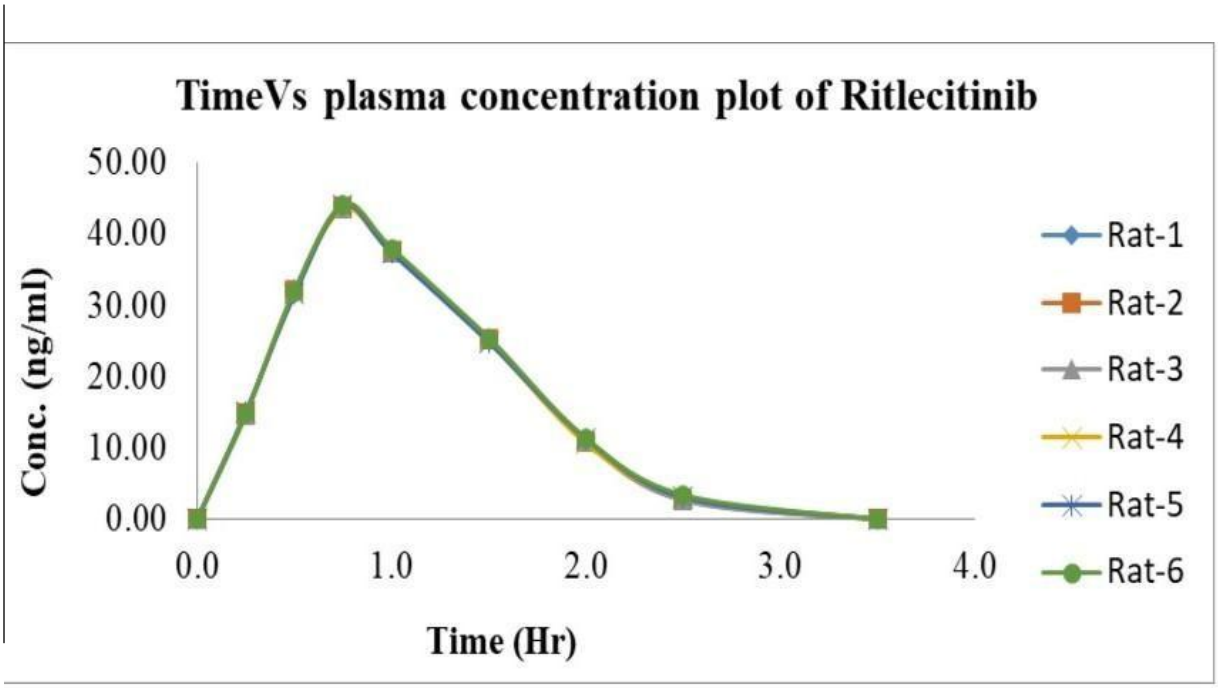


**Figure 7.Time (hrs) vs. plasma Concentration of Ritlecitinib plot**

**Table 6.Pharmacokinetic parameters result of Ritlecitinib.**

| **Pharmacokinetic parameters** | **Ritlecitinib** |
|---|---|
| $AUC_{0-t}$ | 87 ng-hr/ml |
| $C_{max}$ | 43.829 ng/ml |
| $AUC_{0-\infty}$ | 87 ng-hr/ml |
| $t_{max}$ | 0.75 Hrs |
| $T_{1/2}$ | 2.5 Hrs |

## Conclusion

A method of LC-MS was used to achieve chromatographic separation of Ritlecitinib and IS from spiked rat plasma.The technique was accurate and precise during the validation process, with all recoveries falling within the acceptable range of ±20% at the quantification (LLOQ) limit and within ±15% at all quality control (QC) concentrations.The standard deviation and % relative standard deviation also fell within the acceptable range, confirming the method's validity.Frozen plasma samples maintained their expected concentrations even after undergoing freeze-thaw cycles, indicating that the analysis of frozen samples adheres to the guidelines.

This study is the first to introduce and confirm a simple and precise LC-MS/MS method for quantifying Ritlecitinib in rat plasma using a Liquid extraction method with Dichloromethane.The process was susceptible, with a lower limit of quantification (LLOQ) of 5 ng/ml and a concentration range of Ritlecitinib in plasma ranging from 5 to 100 ng/ml.The suggested approach is more pragmatic, efficient, and cost-effective than the current technique and is suitable for the regular analysis of Ritlecitinib in plasma samples with added concentration.This statement highlights the crucial role of obtaining such information to conduct investigations and accurately determine the drug's bioavailability and bioequivalence.

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Professor Galla Rajitha planned the research, while Charumathi Salva collected the required data and conducted the necessary analysis. Authorship of the manuscript was a joint effort that involved all contributors. The final version of the manuscript was approved by all authors.

**Conflict of interest**

The authors who submitted this article have stated that there are no conflicts of interest.

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**Availability of data and materials**

The corresponding author will furnish the datasets utilized or examined in the present work upon a reasonable request.

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