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Development and Validation of Novel Analytical Tecniques for Simultaneous Estimation of Tofacitinib Citrate and Iguratimod in Synthetic Mixture

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ABSTRACT

Background: The Combination of Tofacitinib Citrate (TOFA) and Iguratimod (IGU) is increasing the management of rheumatoid arthritis due to their synergistic suppression of pro-inflammatory cytokines and osteoprotective properties. However, a paucity of simple and cost-effective analytical methods for their simultaneous quantification exists. **Objectives:** This study aimed to develop and validate three distinct analytical methodologies: Vierordt's simultaneous equation (Method I), first-order derivative spectrophotometry (Method II), and a reversed-phase high-performance liquid chromatography (RP-HPLC) (Method III). **Methods:** Spectrophotometric analysis of Tofacitinib Citrate and Iguratimod was performed in methanol, selecting 285 nm and 256 nm for Method I, and zero-crossing points of 262 nm and 345 nm for Method II, respectively. Method III utilized an RP-HPLC system with a mobile phase of Acetonitrile: Methanol: Water (40:30:30, %v/v/v) at a flow rate of 1.0 mL/min and UV detection at 263 nm. **Results:** All methods demonstrated excellent linearity ($r^2 \geq 0.999$) over the concentration range of 1-5 µg/mL for TOFA and 5-25 µg/mL for IGU. The recovery studies yielded results between 98% and 102%, confirming high accuracy. Precision studies showed %RSD values < 2.0 %, indicating robust repeatability and intermediate precision. The RP-HPLC method offered superior sensitivity (LOD: 0.028 µg/mL for TOFA), while the UV methods provided rapid, reagent-efficient alternatives. **Conclusion:** The proposed methods were successfully applied to a laboratory-prepared synthetic mixture with no interference from common excipients. These validated techniques are suitable for routine quality control and pharmaceutical analysis of this drug combination.

1. INTRODUCTION:

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disease characterized by persistent inflammation of the joints, with a prevalence of 0.5–1.0% according to most epidemiological studies. It predominantly affects females, with a male-to-female ratio of 1:2 to 1:3, and can manifest at any age, peaking around 60 years [1-2]. The Co-administration of Tofacitinib citrate and Iguratimod exerts a synergistic pharmacodynamic effect by targeting different signaling pathways in rheumatoid arthritis (RA). The combination of Tofacitinib citrate and Iguratimod represents a superior therapeutic strategy for rheumatoid arthritis (RA) by providing a multi-faceted attack on the disease's inflammatory architecture. By concurrently inhibiting the JAK-

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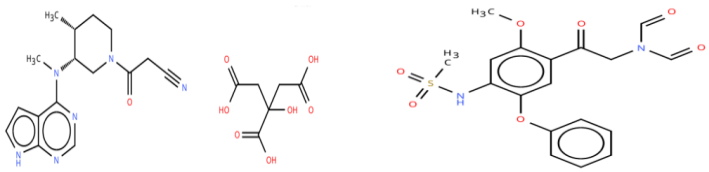
STAT pathway and suppressing mediators like NF-κB and MIF, this dual therapy achieves a more comprehensive blockade of the inflammatory cascade than monotherapy. Beyond merely reducing pro-inflammatory cytokines (IL-6 and IL-1β), the combination offers significant bone-protective benefits and prevents synovial pyroptosis via NLRP3 inflammasome inhibition. Ultimately, this synergistic approach offers a highly effective alternative for patients who are non-responsive to conventional treatments, addressing both systemic inflammation and physical joint preservation [3-5].

Tofacitinib citrate (TOFA) is 3- [(3R, 4R)-4-methyl-3- [methyl (7H-pyrrolo [2, 3-d] pyrimidin-4-yl) amino] piperidin-1-yl]-3-oxopropanenitrile [Figure 1a], disease-modifying rheumatoid drugs (DMARDs). By selectively inhibiting Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, TOFA modulates the inflammatory response, making it a potent therapeutic agent for various autoimmune and inflammatory pathologies[6]. Iguratimod chemically known as N-[7-(methanesulfonamido)-4-oxo-6-phenoxychromen-3-yl] formamide [Figure 1b], conventional synthetic Disease-Modifying Anti-Rheumatic Drug (csDMARD). Iguratimod approved for the treatment of rheumatoid arthritis (RA) in China and Japan. It acts by modulating the immune system and reducing inflammation, with several therapeutic uses within the body. [7]



(a) (b)

Figure 1: (a) Chemical structure of Tofacitinib citrate (b) Chemical structure of Iguratimod

The various analytical techniques have been established for the quantification of TOFA, either as a standalone agent or in combination therapies. These include spectrophotometric approaches [8-9], UV-HPLC [10-11], HPLC [12], RP-HPLC [13-14], Stability indicating RP-HPLC [15], UPLC-MS/MS [16], LC/MS [17] and LC-ESI-MS/MS [18]. Literature survey revealed the estimation methods of Iguratimod or with other drugs by HPLC [19], Stability indicating HPTLC [20-21], UPLC-MS/MS [22] and LC-MS/MS [23]. Given that there is currently no established analytical technique for the concurrent estimation of Tofacitinib citrate and Iguratimod, this study investigates the application of UV-spectrophotometry and RP-HPLC methods to ensure precise, rapid, and reliable simultaneous determination of both compounds in a synthetic mixture as per ICH Q2 (R2) guideline [24]. The utilization of Vierordt's simultaneous equation method, derivative spectrophotometry, and Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) provides a robust, high-resolution analytical framework for quantifying multi-component pharmaceutical mixtures. While spectrophotometric techniques resolve overlapping signals through mathematical transformations without the need for prior separation, RP-HPLC offers superior sensitivity and selectivity by physically separating analytes based on their hydrophobicity.

2. EXPERIMENTAL WORK:

2.1 Chemical and Reagent:

Pharmaceutical-grade Tofacitinib Citrate and Iguratimod (purity > 99%) were generously provided as gift samples by Bueno Salud Care India Pvt. Ltd. and Lewen's Lab Pvt. Ltd., respectively. HPLC-grade Acetonitrile, Methanol, and Water (Finar Chemicals) were employed as solvents, ensuring optimal analyte stability and spectral clarity.

2.2 Apparatus and instrumentation:

Spectral acquisitions were performed using a Shimadzu UV-1900 double-beam spectrophotometer equipped with 1.0 cm matched quartz cuvettes. Chromatographic separation was conducted on an Analytical 3000 RP-HPLC system integrated with a HyperChrom ODS-BP column (250 × 4.6 mm, 5 µm) Data processing was managed via [CXTH 3000] software. Digital Analytical balance- Scale-Tec. Sonicator- Digital Pro+, PS-10A, BROLEO were used in this study.

2.3 Preparation of standard stock solution:

Stock solution for UV spectrophotometry and RP-HPLC was prepared by diluting 10 mg of each drug in sufficient quantity of methanol in separate volumetric flask and volume was made up to 100 ml to get the concentrations of 100 µg/ml for each drug.

3. UV-SPECTROPHOTOMETRIC METHOD AND RP-HPLC METHOD:

Aliquots of the TOFA and IGU stock solutions were appropriately diluted with methanol to yield working standards with final concentrations of 2 µg/ml and 10 µg/ml, respectively. These solutions

were scanned across the ultraviolet region (200-400 nm) to identify the analytical wavelengths (λ).

3.1 Method I: Vierordt's simultaneous equation

Standard stock solutions of Tofacitinib citrate and Iguratimod were made in methanol with concentrations of 100 µg/ml and 100 µg/ml, respectively. The Concentration range of Tofacitinib citrate of 1-5 µg/ml and Iguratimod of 5-25 µg/ml were made in the methanol and absorbance of these solutions was measured at 285 nm and 256 nm. Calibration curves were plotted to confirm the Beer's law and the absorptivity values calculated at the respective wavelengths for both the drugs. Two simultaneous equations as below were formed using these absorptivity values A (1%, 1 cm).

At λ1 A1= ax1bCx+ay1bCy …….....(1)
At λ2A2= ax2bCx+ay2 bCy…… (2)
For measurements in 1 cm cells b=1
Rearrange eq. (2)
Cy= A2- ax2Cx/ ay2
Substituting for Cy in eq (1) and rearranging
Cx = A2ay1-A1 ay2/ax2 ay1-ax1 ay2…. (3)
Cy = A1ax2-A2 ax1/ax2 ay1-ax1 ay2…. (4)

Where Cx and Cy are the concentration of Tofacitinib citrate and Iguratimod, respectively, A1 and A2 are absorbance at 285 nm and 256 nm respectively, ax1 and ax2 are absorptivity of Tofacitinib citrate at 285 nm and 256 nm respectively; ay1 and ay2 are absorptivity of Iguratimod at 256 nm and 285 nm respectively. By solving the two simultaneous equations, the concentrations of Tofacitinib citrate and Iguratimod in sample solutions were obtained.

3.2 Method II: First Order Derivative UV Spectrophotometry:

To prepare for analysis, Tofacitinib citrate and Iguratimod stock solutions were added to a series of 10 ml volumetric flasks and diluted with methanol to achieve concentrations ranging from 1-5 µg/ml of Tofacitinib citrate and 5-25 µg/ ml of Iguratimod. First-order derivative (D^1) spectra were generated using a sampling interval (Δλ) of 2.0 nm and a scaling factor of 4. The Zero-Crossing Points (ZCP) for TOFA and IGU were identified at 345 nm and 262, respectively. Linear regression analysis was subsequently performed by plotting the derivative amplitudes against their corresponding concentrations.

3.3 Method III: RP-HPLC:

Chromatographic separation was achieved by isocratic elution using a mobile phase of Acetonitrile: Methanol: Water (40:30:30 %v/v/v) ratio at a flow rate of 1 ml/min. The effluent was monitored at a detection wavelength of 263 nm. The mobile phase was degassed via ultrasonication for 10 min and filtered through a 0.45 µ m nylon membrane prior to use. A HyperChrom ODS-BP (250 × 4.6 mm, 5 µm) column was used as the stationary phase, and the eluent was monitored by a UV detector from 200 to 400 nm, with chromatograms extracted at 263 nm. Stock solutions of TOFA and IGU were prepared in methanol to contain 100 µg/ml and 100 µg/ml, respectively. Different aliquots of these stock solutions were added to separate 10 ml volumetric flasks and diluted to volume with mobile phase to obtain concentration ranges of 1-5 µg/ml of TOFA and 5-25 µg/ml of IGU, respectively. 2 µg/ml of TOFA and 10 µg/ml of IGU of standard working solution were injected and then analyzed using the optimized chromatographic conditions. The peak areas of TOFA and IGU were measured, and their values were plotted against the relevant concentrations to obtain the calibration curves. The linear regression equations were calculated accordingly.

4. METHOD VALIDATION:

Analytical method validation was performed in accordance with International Council for Harmonisation ICH Q2 (R2) guideline [24]. Parameters evaluated included linearity, range, accuracy (recovery), precision (repeatability and intermediate precision), robustness, and sensitivity (LOD/LOQ).

4.1 Linearity and range:

Appropriate dilutions of working standard solutions for Tofacitinib citrate and Iguratimod were prepared in the concentration range of 1-5 µg/mL and 5-25 µg/ml, respectively and analyzed as per the developed method. Calibration curves were prepared and the linearity was measured by the least square regression method.

4.2 Accuracy (Recovery studies):

Accuracy was assessed via standard addition methodology. Pre-analyzed samples were spiked with known quantities of TOFA and IGU at three distinct levels (50%, 100%, and 150% of the target concentration). Each level was analyzed in triplicate, and the percentage recovery was calculated to determine systematic error.

4.3 Precision:

Precision was checked as intra-day and inter-day variations. Intra-day precision was determined by analyzing Tofacitinib citrate (1, 2, 3 µg/mL) and Iguratimod (5, 10, 15 µg/mL) for three times on the same day. Inter day precision was determined by intermediate precision (Inter-day) was evaluated by analyzing the standard solutions over three consecutive days to ensure method reproducibility across temporal variations.

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4.4 Robustness

Method robustness was investigated by introducing deliberate, minor perturbations to the optimized chromatographic parameters. These included variations in flow rate (±0.2 ml/min) and detection wavelength (±2 nm). System suitability parameters, such as tailing factor and theoretical plates, were monitored for significant deviations. For Methods I and II, samples were analyzed by different analysts to assess the robustness of the methods.

4.5 Limit of Detection (LOD) and Limit of Quantification (LOQ):

The sensitivity of the proposed methods was quantified through the Limit of Detection (LOD) and Limit of Quantification (LOQ), derived from the standard deviation of the response (SD) and the slope of the calibration curve (S) of the developed method was assessed by analyzing ten replicates of standard solutions containing concentrations 2 µg/ml for Tofacitinib citrate and 10 µg/ml for Iguratimod. The LOD and LOQ were calculated as per following equation:

$\text{LOD} = 3.3 * \frac{\sigma}{s}$ and $\text{LOQ} = 10 * \frac{\sigma}{s}$

Where, SD = Standard deviation

Slope = the mean slope of the calibration curve

4.6 Analysis of Synthetic mixture:

The synthetic mixture was prepared to mimic a commercial dosage form, maintaining a TOFA to IGU ratio of 1:5. The synthetic blend incorporated common pharmaceutical excipients, including Microcrystalline Cellulose (MCC), Lactose Monohydrate, and Magnesium Stearate. An accurately weighed quantity of the mixture, equivalent to 10 mg of TOFA and 50 mg of IGU, was quantitatively transferred to a 100 ml volumetric flask. The analytes were extracted using methanol via ultrasonication for 10 minutes, followed by filtration through Whatman No. 41 filter paper. The answers were further serial diluted with methanol to obtain concentrations of 100 µg/ml of Tofacitinib citrate and 500 µg/ml of Iguratimod. The final concentrations were adjusted to get attention within the linearity range and then analyzed using the previously described UV-spectrophotometric and chromatographic conditions. The concentrations of Tofacitinib citrate and Iguratimod were calculated using a regression equation.

5. RESULTS AND DISCUSSION:

5.1 The method I: Vierordt's method:

The zero-order (D^0) absorption spectra of TOFA and IGU (Figure 2a) exhibit significant spectral overlap within the 200–400 nm range. This overlap precludes the use of direct spectrophotometric quantification. Consequently, Vierordt's simultaneous equation method was applied. Analytical wavelengths were selected at 285 nm (λmax of TOFA) and 256 nm (λmax of IGU). The additivity of absorbances at these wavelengths formed the basis for the development of the simultaneous equations.

The regression line equation for Tofacitinib citrate and Iguratimod is as follows,

y = 0.1007x + 0.0799 Tofacitinib citrate at 285 nm (1)

y = 0.029x + 0.021 Tofacitinib citrate at 256 nm (2)

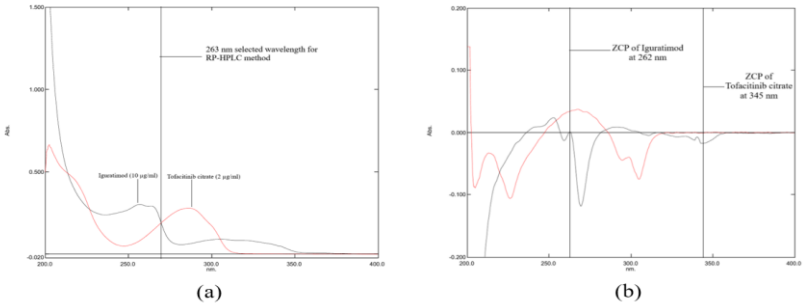
y = 0.0396x – 0.0966 Iguratimod at 256 nm (3)

y= 0.0078x – 0.0152 Iguratimod at 285 nm (4)

The mean specific absorptivity coefficients for TOFA and IGU were calculated at the designated wavelengths. For TOFA, coefficients were determined as ax1= 1376 (285 nm) and ax2 =0.0388 (256 nm). Correspondingly, values for IGU were ay1 =0.0308 (256 nm) and ay2 =0.0060 (285 nm). Calibration curves exhibited excellent linearity ($r^2 \geq 0.999$) across the concentration ranges of 1-5 µg/ml and 5-25 µg/ml for TOFA and IGU, respectively.

5.2 *Method II: First Order derivative spectrophotometry*

First-derivative (D^1) spectrophotometry was employed to resolve the spectral congestion inherent in the zero-order spectra. Instrumental parameters, including scaling factor (4.0) and Δλ (2.0 nm), were systematically optimized to maximize the signal-to-noise ratio at the zero-crossing points (ZCP). As illustrated in (Figure 2b), TOFA was quantified at the ZCP of IGU (262 nm), while IGU was determined at the ZCP of TOFA (345 nm), ensuring mutual non-interference.



(a) (b)

Figure 2: (a) Overlain UV Spectra of Tofacitinib citrate (2 µg/ml) at 285 nm and Iguratimod (10 µg/ml) at 256 nm in Methanol (Zero order) (b) Overlain UV Spectra of Tofacitinib citrate (2 µg/ml) and Iguratimod (10 µg/ml) in Methanol (First order)

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5.3 Method III: HPLC method

A method was developed to simultaneously determine TOFA and IGU using liquid chromatography with UV detection. The primary objective of the RP-HPLC method development was to optimize chromatographic resolution (R_S) and peak symmetry (A_S) while maintaining a brief run time. Among the reversed- phase C8 and C18 columns examined, HyperChrom ODS-BP (250 × 4.6 mm, 5 µm) column gave the best separation with symmetric peaks and shorter retention time. Mobile phase optimization was performed by evaluating various ratios of Acetonitrile, Methanol, and Water. While a (50:40:10 % v/v/v). ratio resulted in peak asymmetry and prolonged elution times for TOFA, a ratio of (40:30:30 % v/v/v) yielded superior chromatographic resolution and sharp peak morphologies. An isocratic flow rate of 1.0 ml/min was maintained, facilitating a total run time is 10 minutes. A typical chromatogram for the separation of the binary mixture is shown in (Figure 3), where Tofacitinib citrate eluted first at 3.5 min, followed by Iguratimod at 6.2 min, with a satisfactory system suitability parameter (Figure 3). Final optimization achieved a high theoretical plate count (N > 2000) and a tailing factor ≤ 1.5

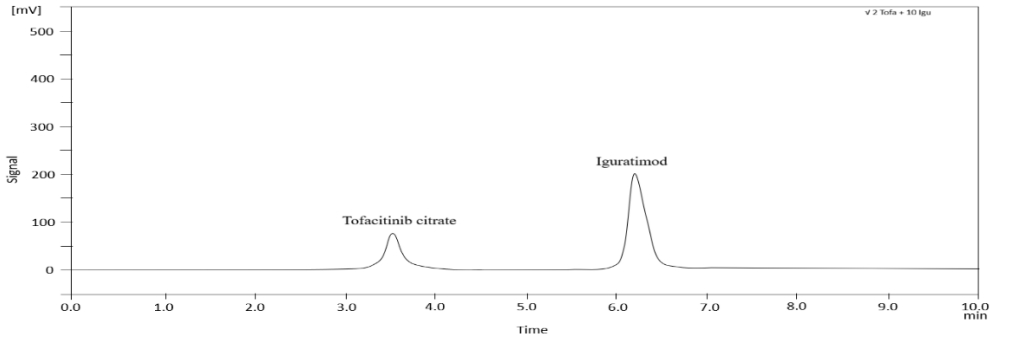

Figure 3: RP-HPLC Chromatogram of Tofacitinib citrate (2 µg/ml) and Iguratimod (10 µg/ml) in Acetonitrile: Methanol: Water (40:30:30 %v/v/v) at 263 nm {Run Time: 10 min, Flow Rate: 1.0 ml/min}

The results presented in Table 1 indicate that the system suitability parameters are satisfactory. The tailing factor for both drugs was less than 1.5, which shows that the peak symmetry is acceptable. The theoretical plate number for both drugs was more significant than 2000, indicating satisfactory column efficiency.

Table 1 System suitability analysis data of proposed RP-HPLC method

Parameters	Retention Time	Tailing Factor	Number of Theoretical plates	Resolution
Tofacitinib citrate	3.5 min	1.2	6822	2.8
Iguratimod	6.2 min	1.0	5485	

5.4 Validation of the proposed methods

5.4.1 Linearity and ranges

The measured responses of Tofacitinib citrate and Iguratimod at the selected wavelengths were treated with least-squares regression (n = 6) to generate linear regression equations. Table 2 summarizes the key statistical parameters for the methods used.

Statistical analysis confirmed a robust correlation between analyte concentration and instrumental response ($r^2 \geq 0.999$). The representative spectra (Figures 4 and 5) and the overlain HPLC chromatograms (Figure 6) illustrate the distinctive peaks utilized for quantification. Comprehensive regression data for all three validated methods are consolidated in Table 2. The calibration curve results are presented in Table 2 for Tofacitinib citrate and Iguratimod using methods I-III.

Table 2: Linearity data for Tofacitinib citrate and Iguratimod by proposed UV Spectrophotometry and RP-HPLC method.

PARAMETER	Vierordt's Method		First order Derivative Method		RP-HPLC Method	
	TOFA at 285 nm	IGU at 256 nm	TOFA at 262 nm (ZCP of Iguratimod)	IGU at 345 nm (ZCP of Tofacitinib citrate)	TOFA	IGU
Linearity Range	1-5	5-25	1-5	5-25	1-5	5-25
Regression Equation	y=0.1007 + 0.0799	y=0.0396x – 0.0966	y=0.0121x + 0.0113	y=0.0021x – 0.0039	y=457392x – 108762	y=238876x + 574750
Regression Coefficient	0.999	0.9991	0.9992	0.9994	0.999	0.9994
LOD	0.081	0.112	0.087	0.125	0.028	0.260
LOQ	0.246	0.341	0.264	0.380	0.084	0.790

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5.4.2 Accuracy:

The accuracy of the developed methods was validated through standard addition and recovery studies. Pre-analyzed sample solutions were spiked with known concentrations of pure drug at three distinct levels (50%, 100%, and 150%). The observed mean percentage recovery values, are presented in Table 3, fall strictly within the ICH-mandated acceptance criteria (98–102%), confirming the absence of significant matrix interference.

5.4.3 Precision:

Method precision was evaluated via intra-day (repeatability) and inter-day (intermediate precision) analyses. Triplicate measurements at three concentration levels across the linearity range were performed. The calculated percentage relative standard deviation (% RSD) was consistently < 2.0 %, demonstrating high reproducibility and method ruggedness. The results of the precision study are shown in Table 4.

5.4.4 LOD and LOQ:

The limits of detection (LOD) and quantification (LOQ) are calculated using the standard deviation responses and slopes obtained from the calibration curves of each drug at their specific wavelengths. Table 2 displays the results of LOD and LOQ.

5.4.5 Robustness:

Method robustness was further validated by assessing the impact of inter-analyst variability. Statistical analysis revealed no significant deviation in the % RSD for Methods I and II. Furthermore, as detailed in Table 5, the RP-HPLC method demonstrated resilience to minor perturbations in flow rate and detection wavelength. These results confirm the reliability of the proposed methods for routine quality control applications.

5.4.4 Analysis of synthetic mixture:

The validated methods were successfully applied to the quantification of TOFA and IGU in a laboratory-simulated dosage form. No spectral or chromatographic interference from excipients was observed, confirming the specificity of the methods. The results (Table 6) indicated no statistically significant difference between the methods at a 95% confidence interval linearity profiles for TOFA (1-5 µg/ml) and IGU (5-25 µg/ml) are depicted in Figures 4(a) and 4(b), respectively.

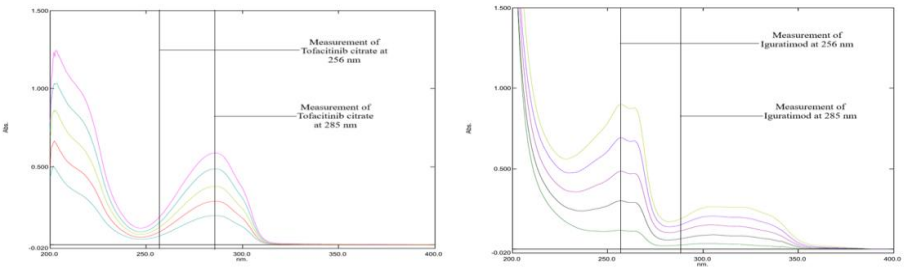

Figure 4: (a) Linearity Spectra of TOFA (1-5 µg/ml) at 285 nm & 256 nm (b) Linearity Spectra of IGU (5-25 µg/ml) at 256 nm & 285 nm

Figure 5 (a) Linearity spectra of TOFA (1-5 µg/ml) at 262 nm (b) Linearity spectra of IGU (5-25 µg/ml) at 345 nm.

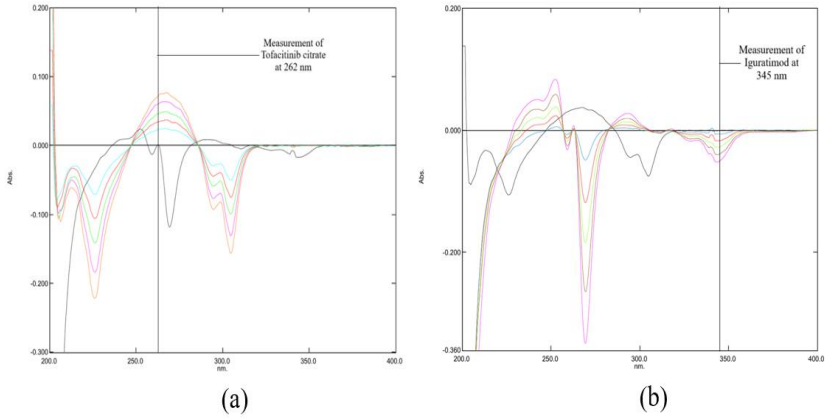

Figure 5: (a) Linearity Spectra of TOFA (1-5 µg/ml) at 262 nm (b) Linearity Spectra of IGU (5-25 µg/ml) at 345nm

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Linearity was established over the concentration ranges of be 1-5 µg/ml for TOFA and 5-25 µg/ml for IGU. Regression analysis confirmed a highly linear response ($r^2 \geq 0.999$) for both analytes across all three validated methodologies.

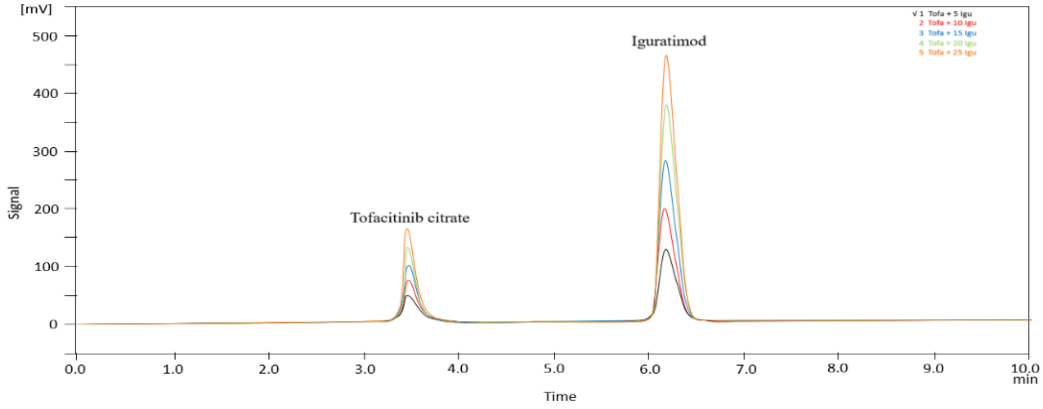

Figure 6: Overlain RP-HPLC Chromatogram of TOFA (1-5 µg/ml) and IGU (5-25 µg/ml) demonstrating stable retention times and baseline resolution under optimized chromatographic condition {Flow Rate: 1.0 ml/min; $\lambda_{det:}$ 263 nm}

Table 3: Accuracy data for TOFA and IGU using the proposed UV-spectrophotometric and RP-HPLC method. (n=3 at each level)

Level	Conc of test µg/ml	Amount of Std added µg/ml	Total Amount µg/ml	Vierordt's Method		First Order Derivative Method		RP-HPLC	
				Total amount of Recovered (µg/ml) Mean±SD	% Recovery	Total amount of Recovered (µg/ml) Mean±SD	% Recovery	Total amount of Recovered (µg/ml) Mean±SD	% Recovery
Tofacitinib citrate									
50	2	1	3	2.995 ± 0.0832	99.84	2.993 ± 0.0152	99.79	2.995 ± 0.0264	99.85
100	2	2	4	3.994 ± 0.0984	99.86	3.992 ± 0.0251	99.81	3.995 ± 0.0611	99.87
150	2	3	5	4.994 ± 0.1193	99.88	4.994 ± 0.0321	99.89	4.996 ± 0.0642	99.92
Iguratimod									
50	10	5	15	14.963 ± 0.0702	99.75	14.975 ± 0.0115	99.83	14.96 ± 0.0115	99.94
100	10	10	20	19.964 ± 0.0763	99.82	19.978 ± 0.0152	99.89	19.96 ± 0.0152	99.96
150	10	15	25	24.976 ± 0.0907	99.90	24.978 ± 0.0208	99.91	24.98 ± 0.0173	99.98

Table 4: Precision data for Tofacitinib citrate and Iguratimod by the proposed UV-spectrophotometric and RP-HPLC method.

Drug	Conc. µg/ml	Vierordt's Method		First Order Derivative Method		RP-HPLC	
		Mean Abs. ±SD	% RSD	Mean Abs. ±SD	% RSD	Mean peak area ±SD	% RSD
Repeatability							
TOFA	2	0.278±0.00333	1.19	0.035±0.00041	1.17	783170±6970.21	0.88
IGU	10	0.296±0.00322	1.08	0.017±0.00016	0.94	2901145±25239.96	0.86
Intra-Day Precision Data of Tofacitinib citrate And Iguratimod							
TOFA	1	0.185±0.00249	1.35	0.023±0.00030	1.30	373564±3885.06	1.03
	2	0.277±0.00351	1.26	0.034±0.00043	1.26	783169±7596.73	0.96
	3	0.374±0.00426	1.13	0.046±0.00052	1.13	1258047±11070.81	0.87
IGU	5	0.110±0.00137	1.24	0.006±0.00007	1.16	1798242 ± 19421.02	1.08
	10	0.295±0.00339	1.14	0.016±0.00018	1.06	2901144 ± 28141.10	0.97
	15	0.482±0.00506	1.04	0.026±0.00025	0.97	4165990 ± 36244.12	0.87
Inter-Day Precision Data of Tofacitinib citrate And Iguratimod							
TOFA	1	0.187±0.00256	1.36	0.025±0.00034	1.36	373566±3922.44	1.04
	2	0.279±0.00359	1.28	0.036±0.00046	1.27	783171±7753.39	0.98
	3	0.376±0.00443	1.17	0.048±0.00056	1.16	1258049±11196.64	0.89
IGU	5	0.112±0.00142	1.26	0.007±0.00010	1.25	1798244 ± 20140.86	1.12
	10	0.297±0.00347	1.16	0.018±0.00020	1.12	2901146 ± 29302.23	1.01
	15	0.484±0.00522	1.07	0.028±0.00028	1.00	4165992 ± 37077.33	0.89

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Table 5: Robustness data of RP-HPLC method

Condition	Variation	Tofacitinib citrate	Iguratimod
		%Assay ± SD (n=3)	%Assay ± SD (n=3)
Flow rate (1 ml ± 0.2 ml/min)	0.8 ml/min	99.25±0.0251	99.78±0.0577
	1.0 ml/min	99.93±0.0115	99.85±0.0702
	1.2 ml/min	99.66±0.0351	99.92±0.0360
Detection wavelength (263 nm ± 2 nm)	261	99.86±0.0152	99.86±0.0351
	263	99.95±0.0404	99.79±0.0519
	265	99.92±0.0305	99.94±0.0208

Table 6: Analysis data of synthetic mixture

DRUG	Tofacitinib citrate			Iguratimod		
	Vierordt's Method	First Order Derivative Method	RP-HPLC	Vierordt's Method	First Order Derivative Method	RP-HPLC
% Recovery ± SD	99.89±0.0346	99.91±0.0351	99.92±0.0577	99.86±0.0081	99.81±0.0449	99.95±0.0152
% RSD	0.0346	0.0351	0.0057	0.0081	0.0450	0.0152

6. CONCLUSION:

In this study, three simple, sensitive, and accurate analytical methods—Vierordt's simultaneous equation, first-order derivative spectrophotometry, and RP-HPLC—were developed and validated for the simultaneous estimation of Tofacitinib Citrate and Iguratimod.

All three methodologies are equally effective in quantifying the analytes without interference from pharmaceutical excipients. While the RP-HPLC method provided the highest level of sensitivity and resolution, the spectrophotometric approaches proved to be rapid, cost-effective, and environmentally friendly alternatives. All validated parameters complied strictly with ICH Q2(R2) guidelines, ensuring the methods' reliability. Consequently, these techniques can be confidently adopted by pharmaceutical industries and research laboratories for the routine quality control of this synergistic drug combination in various dosage forms.

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CONFLICT OF INTEREST:

The authors declare that there is no conflict of interest.

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