

Analytical Method Development And Validation For Tofacitinib Citrate And Baricitinib: A Review Of RGB-Based And Green Chromatographic Strategies

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ABSTRACT

Tofacitinib Citrate and Baricitinib are significant members of the Janus Kinase (JAK) inhibitor class, commonly used in the management of rheumatoid arthritis and several other autoimmune disorders. As the global pharmaceutical industry shifts towards sustainability, there is an urgent need to replace traditional, solvent-intensive analytical methods with environmentally friendly options. This review provides a thorough analysis of both well-established and recently developed analytical methods for identifying these JAK inhibitors in their pure state and in pharmaceutical formulations. The primary focus is on contrasting conventional Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) with modern Green Chromatography methods that utilize biodegradable mobile phases and require less energy. Furthermore, we carry out a critical evaluation of the innovative application of RGB-based digital image processing and colorimetric analysis as cost-effective, rapid, and solvent-free techniques for pharmaceutical quantification. The assessment of these methods is carried out using the Analytical GREENness (AGREE) metric and ICH Q2(R1) validation standards, including sensitivity, precision, and robustness. By integrating existing research, this paper serves as a strategic resource for analytical chemists seeking to implement sustainable and high-throughput quality control methods in contemporary immunosuppressant treatments.

Keywords: Tofacitinib Citrate, Baricitinib, Green Analytical Chemistry (GAC), RGB-based analysis, Janus Kinase inhibitors, Method validation.

INTRODUCTION

The treatment approach for autoimmune conditions, especially rheumatoid arthritis (RA), psoriatic arthritis, and ulcerative colitis, has significantly advanced with the introduction of Janus Kinase (JAK) inhibitors. Tofacitinib Citrate and Baricitinib are the top small-molecule inhibitors within this category. Tofacitinib, a broad-spectrum JAK inhibitor, and Baricitinib, which specifically targets JAK1 and JAK2, work by interfering with the signaling pathways of cytokines that contribute to ongoing inflammation [1]. As these powerful immunosuppressants are increasingly prescribed, there is a growing need for high-throughput, precise, and sustainable analytical techniques to ensure their quality control.

Historically, the pharmaceutical industry has depended on Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) to measure these drugs. Although HPLC provides high accuracy and sensitivity, it is linked to a significant "Environmental Footprint" because of the large use of harmful organic solvents such as Acetonitrile (ACN) and Methanol (MeOH). By 2026, the analytical community is quickly moving towards Green Analytical Chemistry (GAC). GAC emphasizes the "12 Principles," which involve using safer solvents, minimizing waste, and improving energy efficiency [2].

A major development in this transition is the appearance of analytical methods based on RGB technology. Researchers can measure drug quantities by using Digital Image Processing (DIP) and

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smartphone colorimetry, relying on the intensity of the Red, Green, and Blue (RGB) color channels. This method is naturally "Green" since it frequently removes the need for complicated chromatographic devices and large amounts of harmful mobile phases, making it a suitable "Point-of-Care" (PoC) tool for pharmaceutical analysis [3].

Moreover, in cases where chromatography is still necessary, Green Chromatographic Strategies such as employing ethanol-based mobile phases, Micellar Liquid Chromatography (MLC), or reducing run times are being incorporated to meet the Analytical GREENness (AGREE) metric. This review seeks to critically assess the established analytical methods for Tofacitinib and Baricitinib, comparing conventional approaches with these contemporary, environmentally friendly RGB and Green methods, in order to offer a thorough guide for the future of pharmaceutical quality control [4][5].

1. Physicochemical Profiles and Stability Issues

An effective analytical technique, whether chromatographic or based on digital imaging, relies on the fundamental chemical properties of the substances being analyzed. Tofacitinib Citrate and Baricitinib, although part of the same therapeutic class, have different structural characteristics that affect their performance in green solvent systems and their response in colorimetric RGB assays.

2.1 Tofacitinib Citrate

Tofacitinib Citrate: Tofacitinib is given in the form of a citrate salt to improve its solubility in water. Chemically, it is a derivative of pyrrolo[2,3-d]pyrimidine.

Molecular Formula: $C_{22}H_{28}N_6O_8$

Molecular Weight: 504.49 g/mol (as citrate salt)

pKa: 3.1 (Pyrrolopyrimidine nitrogen) and 10.1 (Piperidine nitrogen) The dual-basic characteristic makes the drug's ionization highly responsive to the pH of the mobile phase [6]. **Solubility:** It is highly soluble in water and methanol, but only moderately soluble in ethanol. For "Green" methods, using ethanol/water mixtures necessitates accurate pH control to maintain the drug in a stable and detectable state [7].

2.2 Baricitinib

Baricitinib is a compound derived from azetidine that includes both a pyrazole and a pyrrol pyrimidine ring. It is more compact than Tofacitinib but includes highly electronegative sulfonyl groups.

Molecular Formula: $C_{16}H_{17}N_7O_2S$

Molecular Weight: 371.42 g/mol

pKa: 2.1 (Slightly basic) Compared to Tofacitinib, Baricitinib has a lower pKa, allowing it to stay unionized over a wider acidic range, which is beneficial for specific HPTLC and RGB-based color reactions [8].

Log P: Approximately 1.08. Its moderate lipophilicity enables efficient retention on "Green" stationary phases such as C18 columns when using micellar mobile phases (MLC) [9].

2.3 Stability and Forced Degradation Pathways

- 1) Oxidative Degradation:** Tofacitinib is prone to oxidation at the tertiary amine and the nitrogen in the pyrrolopyrimidine ring. In methods based on RGB, this may result in "color interference" when the degradant interacts with the same chromogenic reagent used by the parent drug [10].
- 2) Hydrolytic Stability:** Baricitinib remains fairly stable in acidic environments, though it may experience nitrile hydrolysis when exposed to highly alkaline conditions. A "Green" approach should be capable of separating the parent Baricitinib peak from its carboxylic acid or amide degradation products using environmentally friendly buffers such as ammonium acetate [11].
- 3) Photostability:** Both JAK inhibitors are prone to breaking down when exposed to ultraviolet light. This is an important factor in RGB-based smartphone analysis, as ambient light during image capture needs to be carefully managed to avoid sample degradation during the test [12].

3. Conventional Chromatographic Methods

The analytical background of Tofacitinib and Baricitinib is based on traditional Reverse-Phase High-Performance Liquid Chromatography (RP-

HPLC). These approaches were mainly created for drug discovery and initial quality checks, where speed and accuracy were more important than environmental sustainability.

3.1 RP-HPLC: The Traditional Workhorse

Most established methods for Tofacitinib Citrate rely on C18 (Octadecylsilane) or C8 columns.

Mobile Phase Composition: Common approaches often use a combination of Acetonitrile (ACN) and Potassium Dihydrogen Phosphate Buffer (pH 3.04.5). ACN is preferred due to its low viscosity and strong eluting capability, although it is classified as a hazardous solvent that produces a significant amount of waste [13].

Detection Parameters: Both JAK inhibitors show significant UV absorption. Tofacitinib is usually monitored at wavelengths of 210 nm or 285 nm, whereas Baricitinib exhibits an absorption peak around 225 nm or 310 nm.

Processing Times: Traditional HPLC techniques for these medications typically take between 10 to 15

minutes. Although efficient, the total amount of solvent waste generated during a 24-hour quality control cycle can surpass one liter per instrument [14].

3.2 UPLC and UHPLC: Effectiveness versus.

Environmental Cost Ultra-Performance Liquid Chromatography (UPLC) has been used for both drugs to cut down the analysis time to less than 3 minutes.

Technology Shift: UPLC offers sharper peaks and increased sensitivity by utilizing columns with a particle size below 2 μm .

Critical Review: Although UPLC decreases the amount of solvent used per injection, it typically necessitates higher levels of organic solvents and produces specialized electronic waste. In a "Green" review, UPLC is considered a temporary advancement rather than the ultimate objective, as it still depends on ACN [15].

3.3 Comparison of Reported Chromatographic Parameters.

Author & Year	Drug	Technique	Column	Mobile Phase	Rt (min)
Kumar et al. (2021)	Tofacitinib	RP-HPLC	C18, 250mm	ACN:Phos Buffer (40:60)	6.2
Reddy et al. (2022)	Baricitinib	UPLC	BEH C18	ACN:0.1% Formic Acid	1.8
Sharma et al. (2023)	Both	RP-HPLC	C18, 150mm	MeOH:Water (70:30)	4.5 / 7.1

3.4 Limitations of Conventional Methods:-

Significant Toxicity: The use of Acetonitrile presents health hazards to laboratory staff and necessitates costly waste management procedures.

Equipment Overhead: HPLC and UPLC systems demand significant electrical power, specialized upkeep, and costly high-purity solvents.

Lack of Portability: These methods are not suitable for "On-site" testing in hospitals or small pharmacies, areas where RGB-based techniques offer a strategic advantage [16].

4. Green Chromatography Strategies

Shifting from traditional to eco-friendly chromatography methods for Tofacitinib and Baricitinib requires a thoughtful re-evaluation of the mobile phase, stationary phase, and total energy usage. Green chromatography aims to substitute harmful, high-waste solvents with environmentally friendly options without compromising the precision and accuracy required by the ICH.

4.1 Ethanol: A Sustainable Alternative to Acetonitrile

Acetonitrile (ACN) is the main solvent used in the analysis of JAK inhibitors, but it is a byproduct of the plastics industry and presents major difficulties when it comes to disposal.

Ethanol as a Modifier: Ethanol (EtOH) is a "Green" solvent derived from renewable plant materials. Although EtOH has a greater viscosity than ACN, which may result in increased backpressure, it is very effective in eluting Tofacitinib and Baricitinib.

Synergy with Column Temperature: To counteract the viscosity of Ethanol, researchers employ higher column temperatures (40-60°C). This decreases the viscosity of the mobile phase, reduces backpressure, and frequently enhances the peak shape for Baricitinib [17].

4.2 Micellar Liquid Chromatography (MLC)

MLC is a specific type of eco-friendly chromatography in which the mobile phase is composed of a surfactant, such as Sodium Dodecyl Sulfate (SDS), used at a concentration higher than its critical micelle concentration (CMC).

Environmental Impact: MLC notably decreases or completely removes the requirement for organic modifiers. The surfactant "micelles" function as a pseudo-stationary phase capable of efficiently separating the basic Tofacitinib from the neutral Baricitinib.

Direct Injection: A significant benefit of MLC when analyzing JAK inhibitors is the capability to inject biological fluids or dissolved tablets directly, without the need for lengthy "clean-up" procedures, thereby saving time and reducing solvent waste [18].

4.3 Assessment of Greenness: The AGREE Metric

- Standard ACN-based HPLC methods used for Tofacitinib typically achieve a score in the range of 0.45 to 0.55.
- Green Strategy Score: Analytical methods based on ethanol or MLC for these drugs target scores above 0.80, reflecting a very sustainable process [19].

4.4 Comparative Analysis of Green vs. Conventional Methods.[20]

Parameter	Conventional (ACN)	Green Strategy (EtOH/MLC)	Improvement Factor
Solvent Toxicity	High (Hazardous)	Low (Biodegradable)	Significant
Waste Generation	15-20 ml per run	< 5 ml per run	times Reduction
Safety (NFPA)	Flammable/Toxic	Low Toxicity	Enhanced Safety
Cost per Analysis	High (Waste disposal)	Low (Renewable)	30% Savings

5. RGB-Based Analytical Methods

Analytical techniques utilizing the Red-Green-Blue (RGB) color model, known as Digital Image Colorimetry (DIC), mark a significant transformation in pharmaceutical analysis. While chromatography physically separates molecules, RGB-based methods determine the amount of drugs by assessing the color intensity resulting from a particular chemical reaction. This method is naturally "Green" since it typically doesn't need organic solvents and makes use of

common devices such as smartphones or flatbed scanners [21].

5.1 The Principle of RGB Colorimetry

In the RGB model, every color is a combination of three primary channels: Red, Green, and Blue, with intensities ranging from 0 to 255.

- **Image Acquisition:** For Tofacitinib and Baricitinib, a color-forming (chromogenic) reagent is added to the drug solution. A digital

image of the resulting color is captured under controlled lighting.

- **Data Extraction:** Using software like **ImageJ** or specialized mobile apps (e.g., PhotoMetrix), the average RGB values are extracted from a defined "Region of Interest" (ROI).
- **Quantification:** According to the Beer-Lambert Law analogy, the "Effective Absorbance" (A_w) can be calculated from the intensity (I) of the most sensitive channel (usually the channel complementary to the color of the solution) [22].

5.2 Chromogenic Reagents for JAK Inhibitors

Since Tofacitinib and Baricitinib lack intense natural colors, a process called "derivatization" is necessary:

Oxidation-Reduction Reactions: Tofacitinib is capable of reducing substances such as Folin-Ciocalteu (FC) in an alkaline environment, leading to the formation of a blue-colored compound. The Red channel, which is complementary to blue, is subsequently used for measurement.

Charge-Transfer Complexes: Baricitinib, functioning as an electron donor, interacts with acceptors such as Chloranilic Acid or TCNQ to generate uniquely colored radicals. The RGB values of these radicals show a linear relationship with the Baricitinib concentration [23].

5.3 Smartphone-Based "Point-of-Care" Testing

The most sophisticated "Green" approach is to utilize a smartphone as the detection device.

Ambient Light Correction: In order to maintain technical accuracy across you need to address "Light Boxes" or "Reference Color Charts," which are used to standardize RGB data in relation to different levels of ambient light in a room.

Analytical Performance: The reported RGB methods for Tofacitinib demonstrate good linearity (r^2) greater than 0.99 and can detect concentrations as low as $2.5 \mu\text{g/ml}$, which is adequate for tablet content uniformity and dissolution testing [24].

5.4 Comparison: RGB vs. Green Chromatography

Feature	Green Chromatography (HPLC/MLC)	RGB-Based Digital Analysis
Cost of Equipment	High (50,000+)	Very Low (Smartphone)
Solvent Use	Low (Ethanol/Water)	Zero to Trace
Speed	3–10 minutes	< 60\$ seconds
User Expertise	Professional Chemist	Minimal Training
Regulatory Status	Widely Accepted (ICH)	Emerging / Supplemental

6. Method Validation and Greenness Evaluation

The creation of "Green" and "RGB-based" analytical methods for Tofacitinib and Baricitinib must follow the ICH Q2(R1) (and the revised Q2(R2)) guidelines in order to be suitable for use in industrial pharmaceutical analysis. This section provides a critical comparison of the validation parameters of traditional HPLC with the new environmentally friendly approaches.

6.1 ICH Validation Parameters: A Comparative Analysis

6.1.1. Linearity and Range

Both Green HPLC and RGB-based techniques demonstrate remarkable linearity. For Tofacitinib Citrate, conventional HPLC typically detects concentrations between $15.90 \mu\text{g/ml}$, achieving a correlation coefficient (r^2) of 0.999 [25]. Interestingly, smartphone colorimetry based on RGB has shown high sensitivity to these drugs, with linear

ranges as narrow as 0.810 µg/ml, making it more effective for testing impurities at low concentrations [26].

6.1.2. Precision (Recovery Experiments)

Precision is generally assessed using "Standard Addition." Green chromatographic methods that use ethanol or methanol as solvents have shown recovery rates for Baricitinib ranging from 98.2% to 100.3%, which falls well within the acceptable regulatory range of 98 to 102% [27]. RGB methods showed comparable accuracy (99.5%–100.1%) when statistical techniques such as the "one-tailed t-test" were used to compare them with HPLC results.

6.1.3. Sensitivity (LOD and LOQ)

Although UPLC-MS/MS is still considered the "Gold Standard" for sensitivity (able to detect nanograms), Green HPTLC and RGB methods are adequate for tablet testing. The detection limit (LOD) for Baricitinib using Green HPTLC is around 50 ng per band, whereas Tofacitinib can achieve an LOD of 0.03 µg/ml with smartphone-based DIC [28].

6.2. Quantitative Greenness Assessment Tools

AGREE Metric: This 12-point pictogram has become the standard in the industry. Standard methods for Baricitinib usually result in an AGREE score ranging from 0.80 to 0.85, while traditional ACN methods have difficulty surpassing 0.50 [29].

Analytical Eco-Scale: Deductions are made based on high energy consumption, harmful chemicals, and waste production. A score greater than 75 is regarded as an "Excellent Green Method."

"The Whiteness Score (GEMAM/BAGI): Introduced in 2026, this is a new approach that evaluates the "Whiteness" of a method by considering a balance between Greenness (Environmental impact), Practicality (Cost and Speed), and Analytical Performance. Your review points out that RGB methods perform well in terms of "Practicality" but might need additional improvements in "Analytical Performance" when compared to HPLC [30].

6.3. Robustness and System Suitability:

In Green HPLC, testing for robustness typically includes making minor adjustments to the pH of the green buffer (e.g., Ammonium Acetate with a tolerance of ± 0.2 units and column temperature with a tolerance of $\pm 5^\circ\text{C}$). For RGB methods, robustness should account for changes in smartphone camera distance, lighting intensity, and image file format (JPEG versus TIFF [31].

CONCLUSION

The systematic review of reported analytical strategies for Tofacitinib Citrate and Baricitinib highlights a significant paradigm shift in pharmaceutical quality control. While conventional RP-HPLC methods remain the cornerstone for routine assays, the integration of Green Analytical Chemistry (GAC) principles has proven that high analytical performance can coexist with environmental sustainability.

1. **Analytical Efficiency:** The comparative study reveals that UPLC and advanced HPLC methods using shorter columns (50, 150\{ mm\}) and sub-2 µm particles provide superior resolution for both Tofacitinib and Baricitinib within a reduced run-time of less than 5 minutes.[31]
2. **Greenness Assessment:** The application of the AGREE metric and the Analytical Eco-Scale confirms that replacing Acetonitrile with greener alternatives like Ethanol or Propylene Carbonate significantly improves the eco-profile of these methods without compromising the ICH-mandated validation parameters (Accuracy, Precision, and Linearity).
3. **The RGB Model:** The introduction of the RGB (Red-Green-Blue) model in this review provides a holistic evaluation of the methods. By balancing Analytical Performance (Red), Environmental Impact (Green), and Productivity/Cost (Blue), this review identifies the most "White" or balanced methods suitable for modern industrial laboratories.[32]
4. **Final Verdict:** For long-term stability studies and impurity profiling of these Janus Kinase (JAK) inhibitors, Stability-Indicating Green HPLC

methods are recommended as they ensure regulatory compliance while adhering to the global sustainability goals of 2026.

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