INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES
[ISSN: 0975-4725; CODEN(USA): IJPS00]
Journal Homepage: https://www.ijpsjournal.com

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Research Article

A Validated Stability-Indicating HPLC Method for the Simultaneous Estimation of Tioconazole and Diflucortolone Valerate

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ARTICLE INFO

Published: 07 Aug 2026
Keywords:
Pontederia crassipes, natural products, Tuberculosis, Phytochemicals. Anti-Tubercular activity, Molecular docking
DOI:
10.5281/zenodo.21842618

ABSTRACT

This study developed and validated a simple, precise, accurate, and stability-indicating RP-HPLC method for simultaneous estimation of Tioconazole (TCZ) and Diflucortolone Valerate (DFV) in topical cream formulations, addressing the limited analytical methods available for their simultaneous quantification in semi-solid dosage forms. Chromatographic separation was achieved on a Phenomenex Luna C18 column using an isocratic mobile phase of Acetonitrile and 0.05 M KH_2PO_4 buffer pH 3.5 (70:30 v/v) at 1.0 mL/min with PDA detection at 254 nm, and validation was performed per ICH Q2(R1) guidelines alongside forced degradation studies under acid, alkali, oxidative, thermal, and photolytic stress conditions. The method resolved both drugs with retention times of 6.5 min (DFV) and 9.8 min (TCZ) with resolution >2.5, demonstrated excellent linearity (R^2 = 0.9998 for TCZ, 0.9997 for DFV), accuracy (98-102%), precision (%RSD <2.0%), and LOD/LOQ values of 0.18/0.55 µg/mL for TCZ and 0.05/0.15 µg/mL for DFV; forced degradation revealed DFV was most susceptible to alkaline hydrolysis (17.6%) while TCZ showed significant alkaline (12.4%) and oxidative (11.1%) degradation, with all degradation products well-resolved from parent peaks and spectral homogeneity confirmed by PDA. The robust, reproducible, and stability-indicating method is highly suitable for routine quality control, stability studies, and batch release testing of TCZ and DFV in bulk drugs and topical cream formulations.

INTRODUCTION

1.1 Overview of Dermatomycoses

Dermatomycoses represent highly prevalent superficial fungal infections restricted to keratinized layers of skin, hair, and nails. These infections are a major global public health concern with escalating incidence influenced by aging demographics and increasing immunocompromised populations.[1]

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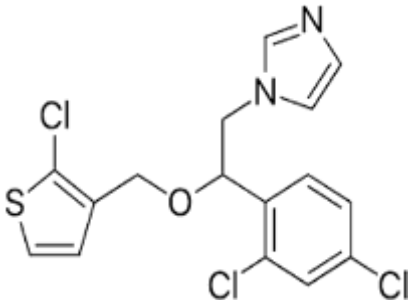
Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1.2 Drug Profile I: Tioconazole (TCZ)

Classification: Synthetic, broad-spectrum imidazole derivative; topical antifungal agent

Chemical Properties:

- IUPAC Name: 1-[2-[(2-chloro-3-thienyl)methoxy]-2-(2,4-dichlorophenyl)ethyl]imidazole
- Molecular Formula: $C_{16}H_{13}Cl_3N_2OS$
- Molecular Weight: 387.70 g/mol
- Log P: 5.53 (highly lipophilic)
- pKa: ~6.5 (imidazole nitrogen) [2,3]

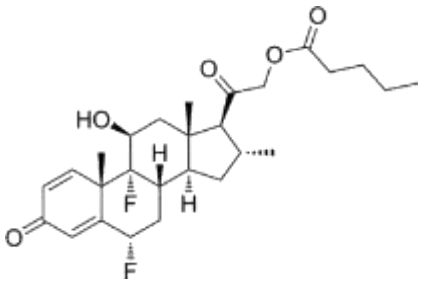


1.3 Drug Profile II: Diflucortolone Valerate (DFV)

Classification: Synthetic, highly potent glucocorticoid for topical dermatological applications

Chemical Properties:

- IUPAC Name: [2-(6,9-difluoro-11-hydroxy-10,13-dimethyl-3-oxo-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-17-yl)-2-oxoethyl] pentanoate
- Molecular Formula: $C_{27}H_{36}F_2O_5$
- Molecular Weight: 494.57 g/mol
- Log P: 3.6–4.1 (highly lipophilic)
- Stability: Highly sensitive to alkaline hydrolysis[2,3]



1.4 Rationale for TCZ + DFV Combination Therapeutic Justification:

- Addresses both root cause (mycological pathogen) and clinical symptoms (host immune reaction)
- Breaks the "scratch-infection" cycle
- Modified dermal pharmacokinetics through DFV-mediated vasoconstriction
- Accelerated patient compliance with visible relief within 24-48 hours

1.5 Analytical Challenges

- Extreme concentration disparities (10:1 ratio)
- Overlapping chromophores with similar aromatic ring systems
- Complex matrices with emulsifiers, surfactants, fatty alcohols, and preservatives

1.6 Aims and Objectives

Primary Aim:

To develop and validate a precise, accurate, and stability-indicating HPLC method for simultaneous estimation of TCZ and DFV in combined pharmaceutical formulations.

Specific Objectives:

1. Literature survey on physicochemical properties and existing analytical methods
2. Develop simple, rapid, precise RP-HPLC method for simultaneous estimation
3. Optimize chromatographic conditions (mobile phase, pH, flow rate, column, detection wavelength)
4. Validate method per ICH Q2(R1) guidelines
5. Establish stability-indicating nature through forced degradation studies
6. Assess peak purity using PDA detection
7. Apply validated method for quantitative assay of marketed formulation
8. Develop efficient sample preparation procedure for cream matrix extraction

2. MATERIALS AND METHODS

2.1 Materials

Drugs and Reference Standards:

- Tioconazole: Yarrow Chem Products, Mumbai; Purity ≥98.0%; CAS: 65899-73-2
- Diflucortolone Valerate: Yarrow Chem Products, Mumbai; Purity ≥97.0%; CAS: 59198-70-8

Reagents and Chemicals:

- Orthophosphoric Acid, Potassium Dihydrogen Phosphate, Sodium Hydroxide, Hydrochloric Acid, Hydrogen Peroxide, Methanol and Acetonitrile

Instruments:

- HPLC System with PDA: Shimadzu LC-2010 / Agilent 1200, Analytical Balance: Shimadzu AUX220, pH Meter: Systronics μ pH System 361, Ultrasonicator: Enertech, UV-Visible Spectrophotometer: Shimadzu UV-1800, Hot Air Oven: Narang Scientific

2.2 Methods

2.2.1 Preparation of Mobile Phase Finalized Composition:

Parameter	Details
Mobile Phase System	Acetonitrile : Potassium Dihydrogen Phosphate Buffer (0.05 M, pH 3.5)
Ratio (v/v)	70 : 30
Flow Rate	1.0 mL/min
Detection Wavelength	254 nm
Column	C18 (250 × 4.6 mm, 5 µm)

Buffer Preparation:

1. Weighed 6.805 g KH_2PO_4, transferred to 1000 mL beaker
2. Added 900 mL HPLC-grade water, stirred until dissolution
3. Adjusted pH to 3.5 ± 0.05 using orthophosphoric acid
4. Made up to 1000 mL with HPLC-grade water
5. Filtered through 0.45 µm nylon membrane filter
6. Degassed by ultrasonication for 15-20 minutes[4,6]

2.2.2 Preparation of Standard Stock Solutions

- **Primary Stock (1000 µg/mL each):** 10.0 mg standard in 10 mL methanol

- **Combined Intermediate Stock (100 µg/mL each):** 1.0 mL each primary stock diluted to 10 mL[5]

2.2.3 Sample Extraction from Topical Cream

1. Weighed 1.0 g cream (containing 10 mg TCZ and 1 mg DFV)
2. Added 15 mL HPLC-grade methanol (pre-warmed to 50°C)
3. Ultrasonicated at 50°C for 15 minutes with intermittent swirling
4. Transferred to 25 mL volumetric flask, made up with methanol
5. Centrifuged at 3000 rpm for 10 minutes
6. Filtered through 0.45 µm nylon syringe filter (discard first 0.5 mL)
7. Diluted 0.5 mL filtrate to 10 mL with mobile phase
8. Final concentrations: TCZ 20 µg/mL, DFV 2 µg/mL[,7,8]

2.2.4 Forced Degradation Studies

Stress Condition	Reagent/ Condition	Temperature	Duration
Acid Hydrolysis	0.1 N HCl	60°C	2 hours
Alkaline Hydrolysis	0.1 N NaOH	60°C	2 hours
Oxidative Stress	3% H_2O_2	Ambient	24 hours
Thermal Degradation	Dry heat	80°C	24 hours
Photolytic Degradation	UV/Visible light	ICH Q1B	As per ICH

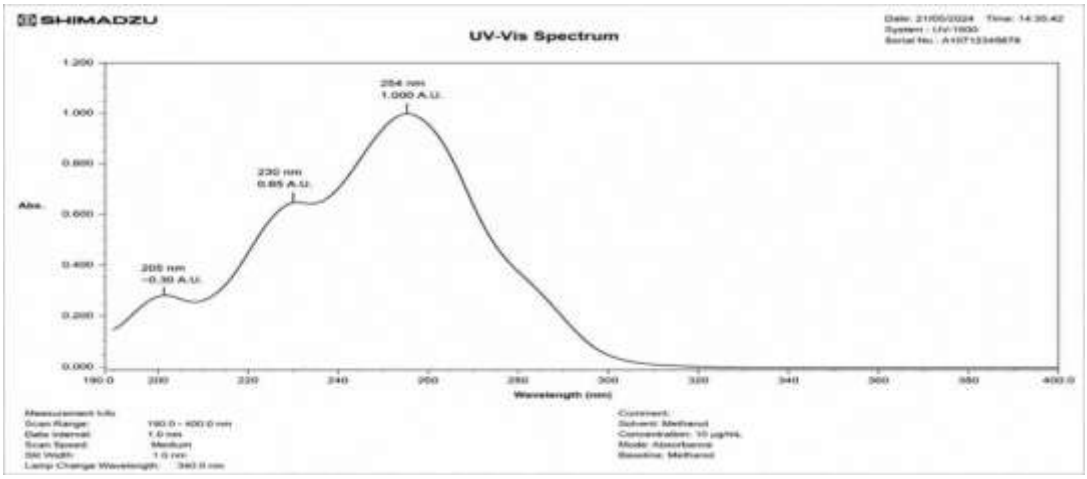
2.2.5 Method Validation Parameters (ICH Q2(R1))

Parameter	Acceptance Criteria
System Suitability	%RSD ≤2%, Rs ≥2.0, T ≤1.5
Specificity	No interference, PPI ≥0.999
Linearity	R^2 ≥0.999
Accuracy	98-102% recovery
Precision	%RSD ≤2.0%
LOD/LOQ	As per signal-to-noise ratio
Robustness	Unaffected by small changes

3. RESULTS AND DISCUSSION

3.1 UV Spectral Analysis and Detection Wavelength Selection

Selection of 254 nm:



3.2 Method Development and Optimization

Column Selection:

Column	Observation	Outcome
C8 (Octyl)	Insufficient retention	Rejected
C18 (150 mm)	Acceptable but poor resolution	Rejected
C18 (250 mm) – Phenomenex Luna	Good retention and resolution	Selected
CN (Cyano)	Severe tailing	Rejected

Mobile Phase Optimization:

Organic Modifier Selection:

Organic Modifier	Observation	Outcome
Methanol	Broader peaks, higher back pressure	Rejected
Acetonitrile	Sharper peaks, lower pressure	Selected

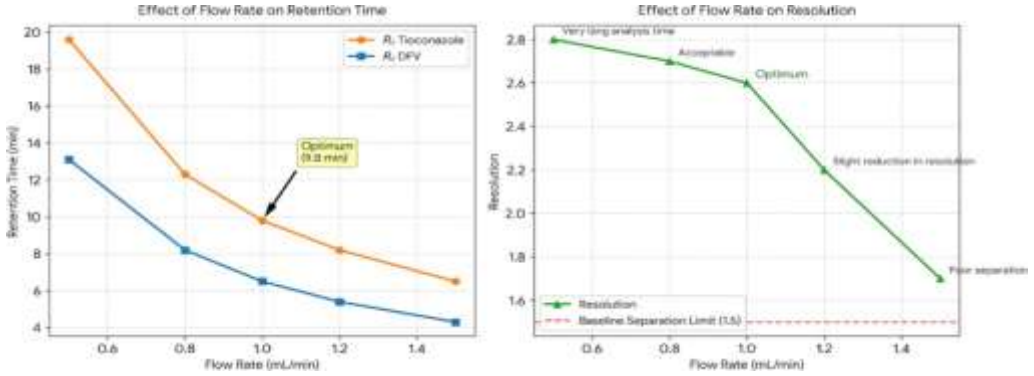
Effect of Mobile Phase Ratio (CAN:Buffer):

Ratio	DFV Rt (min)	TCZ Rt (min)	Resolution	Observation
50:50	12.4	18.2	2.8	Excessively long run time
60:40	9.8	14.5	2.5	Acceptable but lengthy
65:35	8.2	12.1	2.3	Good separation
70:30	6.5	9.8	2.6	Optimum
75:25	4.8	7.2	1.8	Peaks too close
80:20	3.4	5.1	1.3	Co-elution risk

Effect of Buffer pH:

pH	Observation
3.0	Slightly increased retention
3.5	Best peak symmetry and reproducibility
4.0	Slight retention variability

Effect of Flow Rate:



Optimized Chromatographic Conditions:

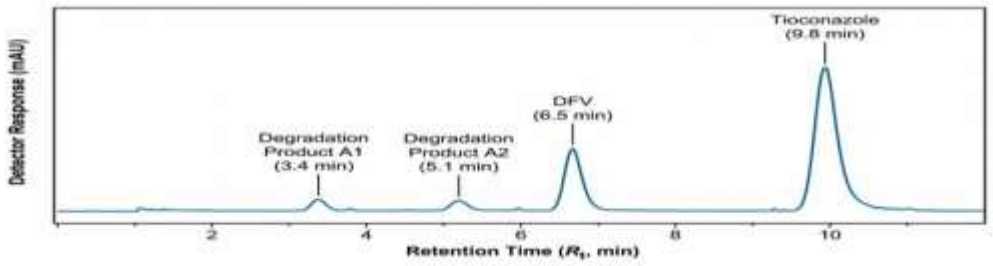
Parameter	Optimized Condition
HPLC System	Shimadzu LC-2010 / Agilent 1200 with PDA
Column	Phenomenex Luna C18 (250 × 4.6 mm, 5 µm)
Mobile Phase	Acetonitrile : 0.05 M KH_2PO_4 buffer pH 3.5 (70:30 v/v)
Flow Rate	1.0 mL/min
Detection Wavelength	254 nm
Injection Volume	20 µL
Column Temperature	25°C
Run Time	15 minutes
Elution Mode	Isocratic

3.3 System Suitability Results

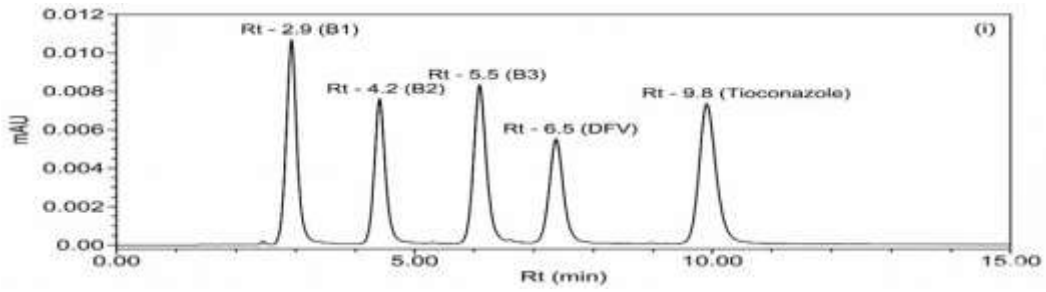
Parameter	Drug	Acceptance Criterion	Observed Result
Retention Time %RSD	TCZ	≤2.0%	≤1.0%
Retention Time %RSD	DFV	≤2.0%	≤1.0%
Peak Area %RSD	TCZ	≤2.0%	≤1.5%
Peak Area %RSD	DFV	≤2.0%	≤1.5%
Theoretical Plates	TCZ	≥2000	>5000
Theoretical Plates	DFV	≥2000	>4500
Tailing Factor	TCZ	≤1.5	≤1.2
Tailing Factor	DFV	≤1.5	≤1.3
Resolution	Between peaks	≥2.0	>2.5

3.4 Forced Degradation Study Results

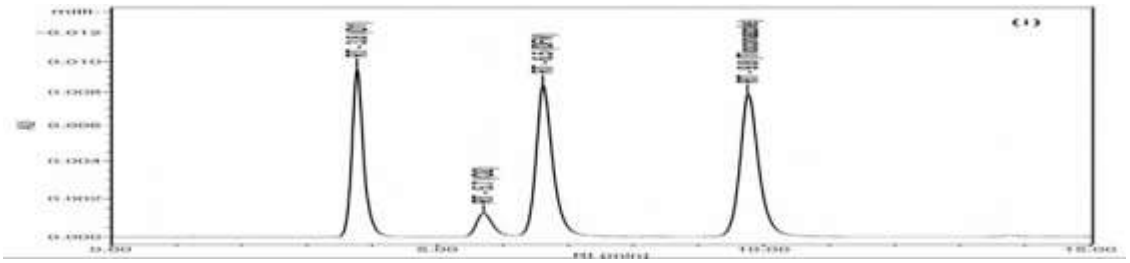
Acid Hydrolysis (0.1 N HCl, 60°C, 2 hours):



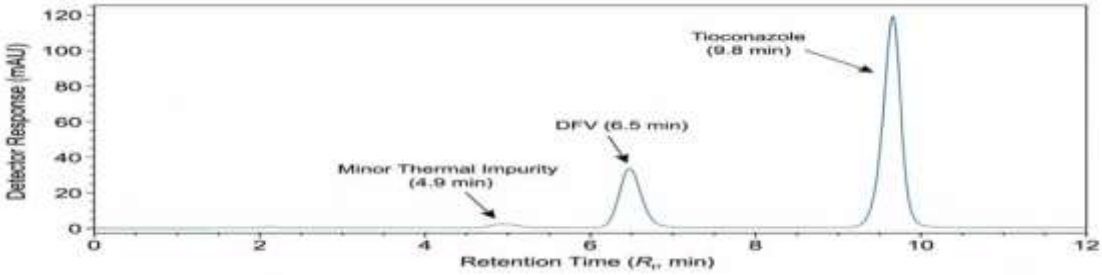
Alkaline Hydrolysis (0.1 N NaOH, 60°C, 2 hours):



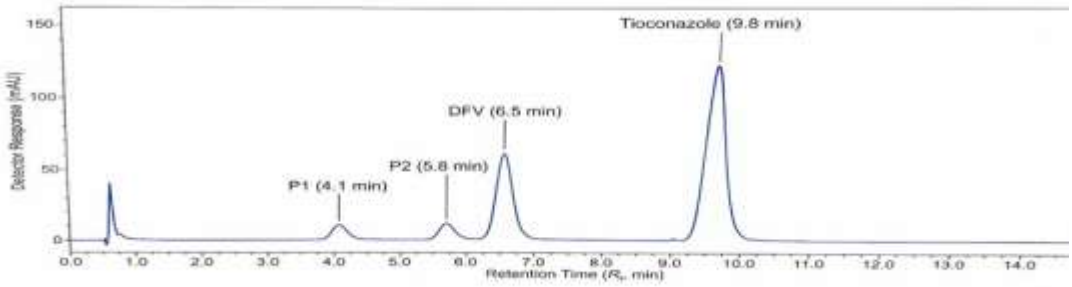
Oxidative Stress (3% H_2O_2, Ambient, 24 hours):



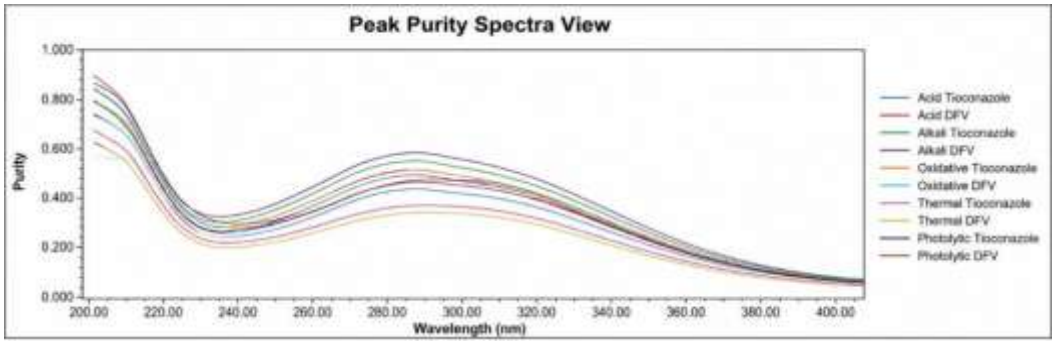
Thermal Degradation (80°C, 24 hours):



Photolytic Degradation (UV/Visible Light, 24 hours):



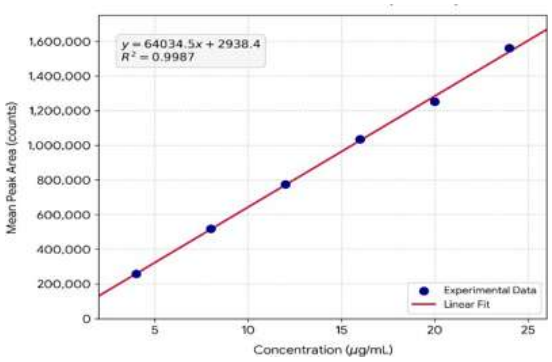
Peak Purity Analysis (PDA):

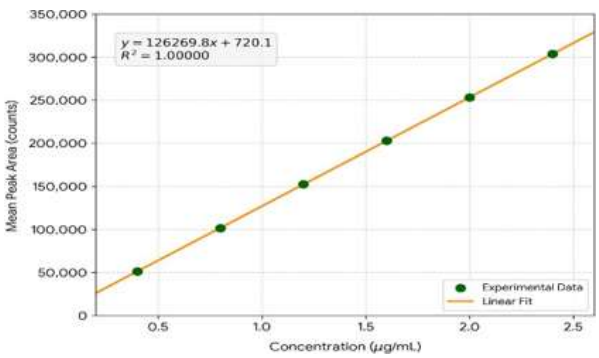


3.5 Method Validation Results

Tioconazole Calibration (i) & Diflucortolone Valerate Calibration (ii)

3.5.1 Linearity





3.5.3 Accuracy (Recovery Studies)

Recovery Level	Tioconazole Recovery (%)	DFV Recovery (%)
80%	99.12	99.45
100%	100.24	100.31
120%	100.67	99.84
Mean	100.01	99.87
%RSD	0.78	0.45

3.5.4 Precision

Repeatability (Intraday Precision):

Drug	Mean Assay (%)	%RSD
Tioconazole	99.82	0.74
Diflucortolone Valerate	100.15	0.88

Intermediate Precision (Interday Precision):

Drug	Mean Assay (%)	%RSD
Tioconazole	99.67	1.12
Diflucortolone Valerate	99.92	1.28

3.5.3 LOD and LOQ

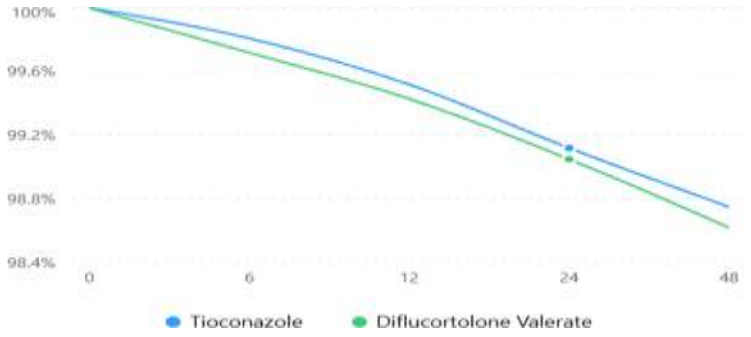
Parameter	Tioconazole	Diflucortolone Valerate
LOD (µg/mL)	0.18	0.05
LOQ (µg/mL)	0.55	0.15
%RSD at LOQ (n=5)	8.2%	7.6%
% Recovery at LOQ	98.5%	99.2%

3.5.4 Robustness

Parameter Changed	Variation	% Assay Recovery	Resolution (Rs)
Flow Rate	0.9 mL/min	98.8-101.2	2.4
Flow Rate	1.1 mL/min	99.1-101.5	2.3
Mobile Phase Composition	65:35	98.6-101.2	2.2
Mobile Phase Composition	75:25	98.9-101.8	2.1
Buffer pH	3.3	98.7-101.4	2.3
Buffer pH	3.7	98.9-101.6	2.2
Detection Wavelength	252 nm	98.5-101.3	2.4
Detection Wavelength	256 nm	98.8-101.7	2.3

All variations maintained Rs ≥2.0 and assay recovery within 98-102%

3.5.5 Solution Stability



Solutions remained stable for at least 48 hours (%Change ≤2.0%)

3.6 Analysis of Commercial Topical Formulation Assay Results:

Drug	Sample No.	Peak Area	Concentration (µg/mL)	Drug Content (mg/g)	Mean ± SD (mg/g)	%RSD
TCZ	1	2,857,462	19.86	9.93	9.91 ± 0.03	0.31
TCZ	2	2,850,123	19.81	9.91		
TCZ	3	2,845,678	19.78	9.89		
DFV	1	412,398	1.98	0.99	0.99 ± 0.01	0.52
DFV	2	410,567	1.97	0.99		
DFV	3	409,876	1.96	0.98		

Comparison with Label Claim:

Drug	Mean Content Found (mg/g)	Label Claim (mg/g)	% of Label Claim	Conclusion
Tioconazole	9.91	10.0	99.1%	Compliant
Diflucortolone Valerate	0.99	1.0	99.0%	Compliant

3.7 Method Development Rationale

A C18 column was selected due to the lipophilic nature of both drugs (Log P: 5.53 for TCZ, 3.6–4.1 for DFV), ensuring adequate hydrophobic retention. Acetonitrile was preferred over methanol for its lower viscosity (0.37 vs 0.55 cP), reduced backpressure, and superior UV transparency at 254 nm; the optimized 70:30 acetonitrile:buffer ratio delivered optimal resolution (Rs = 2.6) with reasonable retention times. Buffer pH 3.5 was critical for peak symmetry—TCZ's imidazole nitrogen (pKa ~6.5) became fully protonated, suppressing silanol interactions and tailing, while DFV as a neutral steroid ester exhibited excellent symmetry.

3.8 Extraction Procedure Optimization

Heated methanol extraction at 50°C with ultrasonication proved optimal by effectively dissolving both drugs while leaving lipid excipients insoluble, reducing cream viscosity, enhancing mass transfer, and disrupting drug-excipient associations via cavitation. Centrifugation yielded clear supernatant with minimal matrix interference, and 0.45 µm nylon filters showed no significant drug adsorption; discarding the first 0.5 mL filtrate prevented adsorptive losses, achieving >99% extraction efficiency..

3.9 Forced Degradation Interpretation

Alkaline hydrolysis was most severe—DFV showed 17.6% degradation (valerate ester saponification at C-21) and TCZ 12.4% (imidazole ring opening or benzyl ether cleavage). Acid hydrolysis caused 10.5% DFV and 7.2% TCZ degradation, consistent with ester hydrolysis. Oxidative stress affected TCZ more (11.1%) than DFV (6.6%) due to thioether sulfur oxidation to sulfoxide/sulfone derivatives. Thermal degradation was minimal (4.2–5.3%), confirming stability at normal manufacturing/storage temperatures (25–40°C), while photolytic degradation (6.2–8.5%) confirmed light sensitivity, necessitating amber packaging.

4. CONCLUSION

The present research successfully developed and validated a simple, precise, accurate, and stability-indicating RP-HPLC method for the simultaneous estimation of Tioconazole and Diflucortolone Valerate in combined topical cream formulations, employing a Phenomenex Luna C18 column (250 × 4.6 mm, 5 µm) with an isocratic mobile phase of acetonitrile and 0.05 M KH_2PO_4 buffer pH 3.5 (70:30 v/v) at 1.0 mL/min, detection at 254 nm, and 20 µL injection volume, achieving baseline resolution of DFV at ~6.5 min and TCZ at ~9.8 min (Rs >2.5). The method was validated per ICH Q2(R1) guidelines, meeting all acceptance criteria for specificity, linearity (R^2 ≥0.9997), accuracy (98-102% recovery), precision (%RSD ≤2.0%), LOD/LOQ, robustness, and solution stability; forced degradation studies confirmed its stability-indicating capability with degradation products well-resolved from parent peaks and PDA-confirmed spectral homogeneity (purity index ≥0.999), revealing alkaline hydrolysis as the most severe degradation pathway for both drugs (DFV: 17.6%, TCZ: 12.4%). The heated methanol extraction procedure (50°C) with ultrasonication yielded high recoveries (98-100%) with minimal matrix interference, and successful application to commercial topical cream demonstrated drug content of 99.1% of label claim for TCZ (9.91 mg/g) and 99.0% for DFV (0.99 mg/g), meeting pharmacopoeial specifications. Thus, this robust, reproducible, and regulatory-compliant method is highly suitable for routine quality control analysis, stability studies, and batch release testing of TCZ and DFV fixed-dose combination products in pharmaceutical industries.

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HOW TO CITE: Pramod Kumar, Neetu Sahu, Dr. Satish Kumar Sarankar, A Validated Stability-Indicating HPLC Method for the Simultaneous Estimation of Tioconazole and Diflucortolone Valerate, Int. J. of Pharm. Sci., 2026, Vol 4, Issue 8, 1429-1439. https://doi.org/10.5281/zenodo.21842618