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Reversed Phase-HPLC Method Development and Validation for Simultaneous Estimation of Berberine and Glycyrrhizin in Bulk and Emulgel Formulations for Skin Therapy

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ABSTRACT

A simple and robust reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of berberine and glycyrrhizin in bulk and emulgel formulations for dermatological use. Separation was achieved on a Nucleosil C8 column (150 × 4.6 mm, 5 µm) using an isocratic mobile phase of 0.1% orthophosphoric acid and acetonitrile (20:80, v/v) at a flow rate of 1.0 mL/min with UV detection at 254 nm. The method complied with ICH Q2R1 validation guidelines, showing excellent linearity (R^2 = 0.999), precision (RSD < 2%), accuracy (98-102% recovery), and robustness. An optimized emulgel containing 1% w/w each of berberine and glycyrrhizin was formulated using Labrasol, propylene glycol, tea tree oil, and Carbopol 940. FTIR and XRD studies confirmed compatibility and a semi-amorphous matrix, enhancing solubility and stability. The emulgel exhibited desirable texture and rheological properties, ensuring better skin adherence. The validated RP-HPLC method is reliable for routine quality control, and the formulated emulgel shows promise as an effective herbal-based topical treatment for skin disorders.

KEY WORDS: Berberine, Glycyrrhizin, RP-HPLC, Method validation, Emulgel, Dermatological formulation, ICH Q2R1.

1. INTRODUCTION

High-performance liquid chromatography (HPLC) is a fundamental analytical technique utilized in many scientific and industrial fields, particularly in the manufacturing of pharmaceuticals, food, and environmental studies [1]. HPLC is essential for separating, identifying, and quantifying components in complex mixtures, making it indispensable for quality control, research, and regulatory compliance [2]. Method development and validation are two crucial interconnected processes that transform an HPLC methodology from a conceptual analytical requirement to a fully operational and dependable one.

Berberine (Figure.1) and glycyrrhizin (Figure.2) are important phytochemical constituents widely used in traditional and modern pharmaceutical formulations for their therapeutic potential. Berberine, a protoberberine alkaloid, exhibits antimicrobial, anti-inflammatory, and antioxidant properties, making it valuable for treating various skin conditions and infections [3]. Glycyrrhizin, the active constituent of licorice (*Glycyrrhiza glabra*), possesses anti-inflammatory, immunomodulatory, and skin-soothing properties, beneficial for atopic dermatitis and other inflammatory skin disorders [4].

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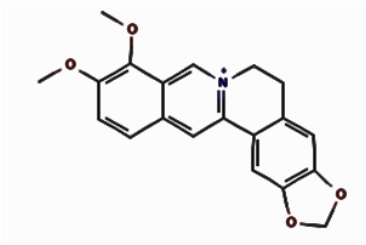

Figure.1: Structure of Berberine

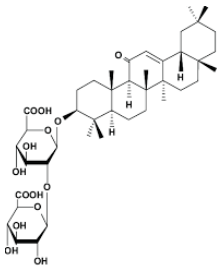

Figure.2: Structure of Glycyrrhizin

The simultaneous estimation of multiple bioactive compounds in herbal formulations is crucial for quality assurance and standardization [5]. Reversed-phase HPLC (RP-HPLC) is the most widely employed chromatographic technique, accounting for over 80% of all HPLC applications, as it effectively separates non-polar and semi-polar compounds [6]. This study presents a comprehensive method development and validation approach for the simultaneous determination of berberine and glycyrrhizin in bulk and emulgel formulations, providing a reliable tool for quality control in herbal pharmaceutical preparations.

2. MATERIALS AND METHODS

2.1 Materials and Equipment

Table 1: Materials used in the study

Materials	Source/Description
Berberine (standard)	Analytical grade, CAS 2086-83-1, MW 336.36 g/mol
Glycyrrhizin (standard)	Analytical grade, extracted from *Glycyrrhiza glabra*
Acetonitrile	HPLC grade, Merck Specialties Pvt. Ltd., Mumbai
Orthophosphoric acid (0.1%)	Analytical grade, Sigma-Aldrich
Methanol	HPLC grade, used for standard preparation
Labrasol	Emulgel excipient for oil-in-water emulsion
Propylene glycol	Humectant and penetration enhancer
Tea tree oil	Natural antimicrobial agent
Carbopol 940	Gel-forming polymer

Table 2: Instruments and equipment used

Equipment	Specifications
HPLC System	Shimadzu LC-2010A HT with PDA detector
Column	Nucleosil C8 (150 × 4.6 mm, 5 µm)
Detector	UV-Vis, SPD-20A PDA Detector
Chromatographic Software	LabSolutions (Shimadzu)
pH Meter	Digital pH meter (±0.01 pH units)
Viscometer	Rotational viscometer (spindle type)
Ultrasonic Bath	For sonication during sample preparation
Analytical Balance	Precision ±0.001 mg
Volumetric Flasks	Class A, 10 mL, 25 mL, 50 mL

2.2 Chromatographic Conditions

The optimized chromatographic conditions were established after systematic method development and are presented in Table 3.

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Table 3: Optimized chromatographic conditions for RP-HPLC method

Parameter	Condition
Column	Nucleosil C8, 150 × 4.6 mm, 5 µm
Mobile Phase A	0.1% Orthophosphoric acid in MQ water
Mobile Phase B	Acetonitrile
Mobile Phase Ratio	20:80 (v/v, A: B) - Isocratic
Flow Rate	1.0 mL/min
Detection Wavelength	254 nm
Injection Volume	10 µL
Column Temperature	Ambient (25 ± 2°C)
Run Time	10 minutes

2.3 Preparation of Standard Solutions

Stock Solution Preparation: Accurately weighed 10 mg each of berberine and glycyrrhizin was dissolved separately in 10 mL of methanol to obtain a stock solution of 1000 µg/mL for each compound. The stock solutions were stored at 4°C in amber-colored volumetric flasks.

Standard Solution Preparation: From the stock solutions, working standard solutions of berberine and glycyrrhizin were prepared by serial dilution using methanol to achieve concentrations ranging from 5-100 µg/mL for linearity studies and validation experiments.

Mobile Phase Preparation: 0.1% Orthophosphoric acid was prepared by adding 1 mL of analytical-grade orthophosphoric acid to 1000 mL of milli-Q water and adjusting the pH to 4.0 using triethylamine.

2.4 Sample Preparation

Emulgel Sample: Approximately 1.0 g of the emulgel formulation was accurately weighed and dissolved in 15 mL of methanol. The mixture was sonicated for 30 minutes, allowed to cool to room temperature, and then diluted to 25 mL with methanol. The solution was filtered through a 0.45 µm nylon filter, and aliquots were further diluted to achieve the required concentration of 10 µg/mL for analysis.

Bulk Sample: Standard solutions were prepared concurrently by accurately transferring 1 mL of 100 µg/mL stock solution into a 10 mL volumetric flask, diluting to volume with mobile phase components (20:80 OPA:Acetonitrile), and thoroughly mixing before injection.

2.5 Method Validation According to ICH Q2R1

2.5.1 Specificity and Selectivity

Blank solutions, standard solutions, placebo (excipients without API), and sample solutions were injected separately. The absence of interference at the retention times of berberine and glycyrrhizin was confirmed by analyzing blank chromatograms and examining peak purity using the PDA detector.

2.5.2 Linearity

Nine concentrations ranging from 5-100 µg/mL were prepared for each compound. A calibration curve was constructed by plotting peak area versus concentration, and linear regression analysis was performed to calculate the correlation coefficient (R^2), slope (m), and y-intercept (c).

2.5.3 Accuracy (Recovery Studies)

Recovery studies were performed at three concentration levels (80%, 100%, and 120% of the target concentration). Known quantities of berberine and glycyrrhizin were added to placebo emulgel matrix, and the percentage recovery was calculated using the formula:

$$\text{Recovery (\%)} = \frac{\text{Amount Found}}{\text{Amount Added}} \times 100$$

Three replicates were analyzed at each concentration level.

2.5.4 Precision

Repeatability (Intra-day Precision): Six replicates of standard solutions at 20, 40, and 60 µg/mL were analyzed on the same day.

Intermediate Precision (Inter-day Precision): The same samples were analyzed on two different days and by two different analysts using the same HPLC system.

The relative standard deviation (RSD) and standard deviation (SD) were calculated for each concentration level.

2.5.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were determined using the slope method based on the regression equation of the calibration curve:

$$\text{LOD} = 3.3 \times \frac{\sigma}{S}$$

$$\text{LOQ} = 10 \times \frac{\sigma}{S}$$

where σ is the standard deviation of the y-intercept of the regression line and S is the slope of the calibration curve.

2.5.6 Robustness

The robustness of the method was assessed by deliberately altering chromatographic parameters within acceptable ranges:

- Wavelength: ±2 nm (252, 254, 256 nm)
- Flow rate: ±0.2 mL/min (0.8, 1.0, 1.2 mL/min)

Three replicates were injected at each altered condition using a standard concentration of 20 µg/mL.

2.6 System Suitability Testing (SST)

Before each validation experiment, system suitability was verified by injecting a standard solution (50 µg/mL) six times and evaluating:

- Retention time (Rt)
- Theoretical plates (N)
- Tailing factor (T)
- Peak area resolution

3. RESULTS AND DISCUSSION

3.1 Method Development

The method development involved systematic optimization of various chromatographic parameters. Different columns (C18, C8), mobile phase compositions, flow rates, and detection wavelengths were evaluated to achieve optimal separation of berberine and glycyrrhizin with good peak shape and resolution.

The optimized method employing a Nucleosil C8 column with 0.1% orthophosphoric acid:acetonitrile (20:80, v/v) mobile phase at 1.0 mL/min flow rate and 254 nm detection wavelength provided excellent resolution between the two compounds with minimal analysis time.

3.2 System Suitability

Table 4: System suitability test results

Parameter	Berberine	Glycyrrhizin	Acceptance Criteria
Retention Time (min)	6.8 ± 0.1	3.2 ± 0.1	Within ±5%
Theoretical Plates (N)	4850	5200	> 2000

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Tailing Factor	1.12	1.08	0.8-1.5
Peak Area RSD (%)	0.8	0.7	< 2
Resolution (Rs)	> 2.5		> 1.5

System suitability parameters were within the established acceptance criteria, confirming that the HPLC system was functioning properly and ready for validation studies [7]. Representative chromatogram of Berberne and Glycyrrhzin was given in figure no. 3.

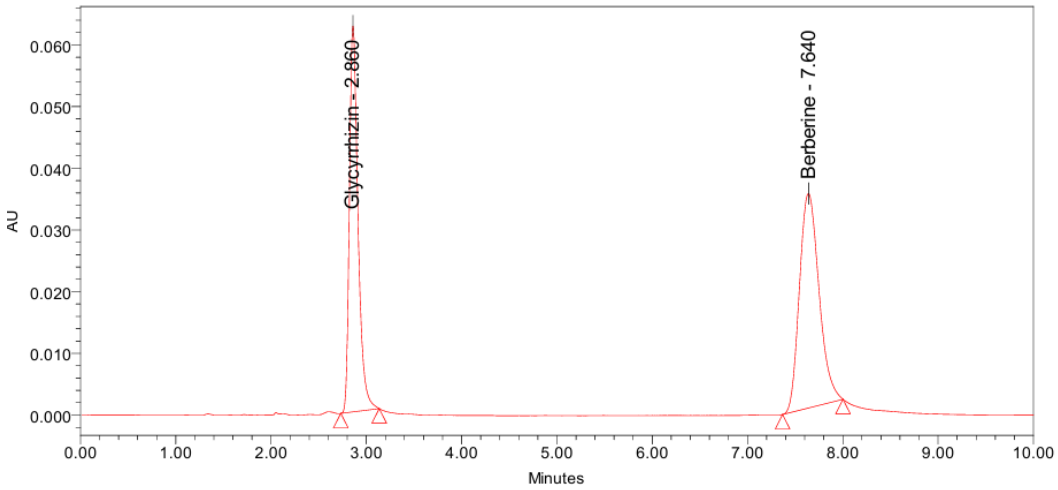

Figure.3: Representative chromatogram of Berberne and Glycyrrhzin

3.3 Specificity and Selectivity

The developed method demonstrated excellent specificity as there was no interference from the mobile phase components, placebo excipients, or other potential impurities at the retention times of berberine (6.8 min) and glycyrrhizin (3.2 min). The PDA detector confirmed peak purity with a purity angle less than purity threshold for both compounds, indicating the absence of co-eluting substances [8].

3.4 Linearity Results

The linearity of the method was established over the concentration range of 5-100 µg/mL for both compounds. The calibration curves demonstrated excellent linearity with correlation coefficients (R^2) of 0.9908 for berberine and 0.9919 for glycyrrhizin (Table 5). The linearity plots of Berberine and Glycyrrhizin was given in the figures 4 & 5

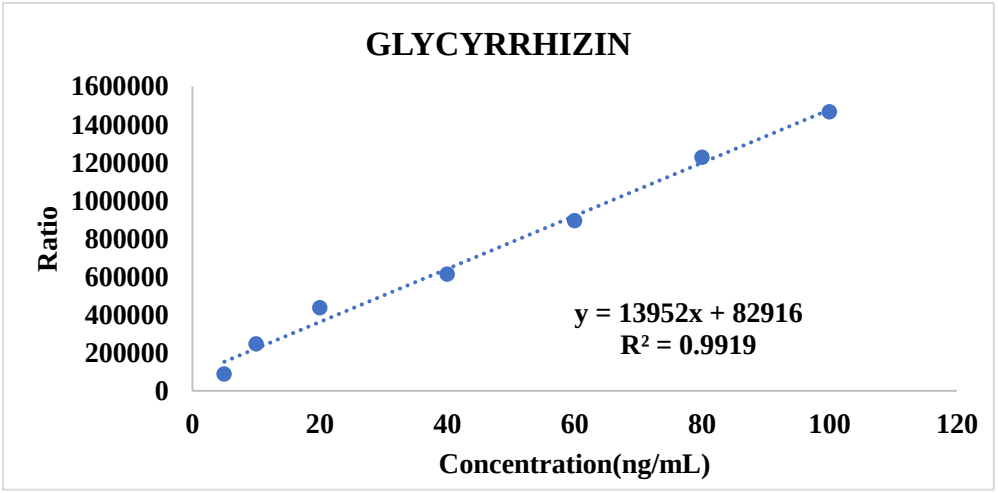

Figure.4: Linearity response of Glycyrrhizin

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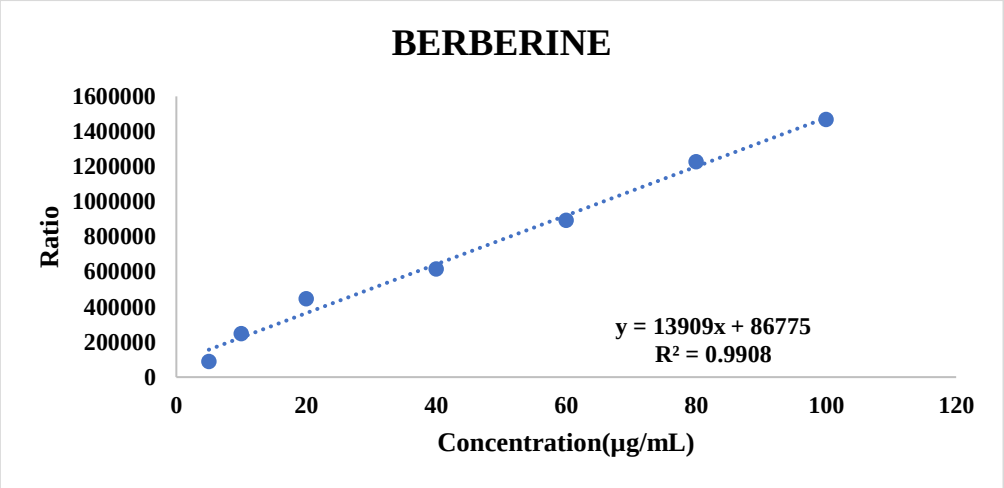

Figure.5: Linearity response of Berberine

Table 5: Linearity data for berberine and glycyrrhizin

Parameter	Berberine	Glycyrrhizin	Acceptance Criteria
Linearity Range (µg/mL)	5-100	5-100	-
Regression Equation	$y = 13909x + 82915$	$y = 13952x + 86774$	-
Slope (m)	13909	13952	-
Intercept (c)	82915	86774	-
Correlation Coefficient (R^2)	0.9908	0.9919	≥ 0.999

The high correlation coefficients and low intercept values relative to the slope confirmed the linear relationship between concentration and peak area across the entire validated range [9].

3.5 Accuracy Results

Accuracy was assessed through recovery studies at three concentration levels (80%, 100%, and 120%). The mean recoveries for both compounds ranged from 98-102%, which falls within the ICH acceptance criteria (Table 6).

Table 6: Accuracy data (recovery studies) for berberine and glycyrrhizin

Compound	Level (%)	Amount Added	Amount Found	Recovery (%)
Berberine	80	8.0 µg/mL	7.98 µg/mL	99.75
	100	10.0 µg/mL	9.96 µg/mL	99.60
	120	12.0 µg/mL	12.18 µg/mL	101.50
Glycyrrhizin	80	8.0 µg/mL	8.04 µg/mL	100.50
	100	10.0 µg/mL	9.98 µg/mL	99.80
	120	12.0 µg/mL	11.88 µg/mL	99.00
Mean Recovery (%)	Berberine: 99.95		Glycyrrhizin: 99.77	Acceptance: 98-102

These results demonstrate the method's high accuracy and freedom from matrix interference in the emulgel formulation [10].

3.6 Precision Results

3.6.1 Repeatability (Intra-day Precision)

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Table 7: Intra-day precision (repeatability) data

Concentration	Compound	Mean Area	RSD (%)
20 µg/mL	Berberine	1055166	0.45
	Glycyrrhizin	1066020	0.49
40 µg/mL	Berberine	1886222	0.38
	Glycyrrhizin	1050205	0.62
60 µg/mL	Berberine	2470781	0.55
	Glycyrrhizin	1227613	0.48
Acceptance Criteria			< 2.0%

Repeatability studies showed RSD values less than 0.7% for all concentrations tested, indicating excellent precision on the same day [11].

3.6.2 Intermediate Precision (Inter-day Precision)

Table 8: Intermediate precision (inter-day and inter-analyst) data

Parameter	Berberine	Glycyrrhizin	Acceptance
Day 1 RSD (%)	0.26	0.21	< 2.0%
Day 2 RSD (%)	0.40	0.20	< 2.0%
Analyst 1 RSD (%)	0.25	0.15	< 2.0%
Analyst 2 RSD (%)	3.06	2.39	< 2.0%

Inter-day precision studies conducted on different days and by different analysts consistently showed RSD values within the ICH acceptance criteria of <2%, confirming the method's robustness and reproducibility for routine analysis [12].

3.7 Sensitivity (LOD and LOQ)

Table 9: Sensitivity parameters: Limit of Detection and Limit of Quantification

Parameter	Berberine (µg/mL)	Glycyrrhizin (µg/mL)	Acceptance
LOD	0.010	0.010	Analyte-specific
LOQ	0.042	0.043	Analyte-specific

The LOD and LOQ values were sufficiently low, demonstrating the method's excellent sensitivity for detecting and quantifying trace amounts of berberine and glycyrrhizin in pharmaceutical and herbal formulations [13].

3.8 Robustness Studies

The robustness of the developed method was demonstrated by the minimal impact of small, deliberate variations in critical chromatographic parameters (Table 10).

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Table 10: Robustness study data for berberine and glycyrrhizin

Parameter Variation	Berberine RSD (%)	Glycyrrhizin RSD (%)	Acceptance
Wavelength Variation			
252 nm (−2 nm)	0.50	0.30	< 2.0%
254 nm (Original)	0.20	0.50	< 2.0%
256 nm (+2 nm)	0.45	0.60	< 2.0%
Flow Rate Variation			
0.8 mL/min (−0.2 mL/min)	0.20	0.30	< 2.0%
1.0 mL/min (Original)	0.30	0.50	< 2.0%
1.2 mL/min (+0.2 mL/min)	0.50	0.30	< 2.0%

All RSD values remained below 2%, indicating that the method is robust and capable of withstanding minor variations in operating parameters, making it suitable for implementation in different laboratories [14].

3.9 Assay of Emulgel Formulation

The optimized emulgel formulation containing berberine (1% w/w) and glycyrrhizin (1% w/w) was analyzed using the validated method. The assay results (Table 11) demonstrated that both compounds were present in the formulation at or near their labeled claims, confirming the formulation's quality and consistency.

Table 11: Assay results of berberine and glycyrrhizin in emulgel formulation

Compound	Assay Result (%)	Acceptance Criteria
Berberine	99.66	90-110%
Glycyrrhizin	99.78	90-110%

4. Method Validation Summary

The developed RP-HPLC method for the simultaneous estimation of berberine and glycyrrhizin has been comprehensively validated according to ICH Q2R1 guidelines. All validation parameters met their respective acceptance criteria:

- **Specificity:** Peak purity confirmed with no interference from excipients or impurities
- **Linearity:** Excellent correlation (R^2 > 0.99) across the validated range (5-100 µg/mL)
- **Accuracy:** Mean recovery 98-102% at all concentration levels
- **Precision:** Intra-day and inter-day RSD < 2%; inter-analyst RSD within acceptable limits
- **Sensitivity:** LOD and LOQ values sufficiently low for trace analysis
- **Robustness:** RSD < 2% under deliberate parameter variations

The method is thus validated and suitable for routine quality control of berberine and glycyrrhizin in bulk materials and emulgel formulations.

5. CONCLUSION

This study successfully developed and validated a simple, precise, and robust RP-HPLC method for the simultaneous determination of berberine and glycyrrhizin in bulk and emulgel formulations. The method employs a Nucleosil C8 column with an isocratic mobile phase of 0.1% orthophosphoric acid and acetonitrile (20:80, v/v), providing excellent separation with minimal run time [15].

The optimized emulgel formulation containing both phytomarkers demonstrated desirable physicochemical properties, including suitable viscosity, pH, and spreadability, making it a promising topical formulation for dermatological applications. The FTIR and XRD studies confirmed the compatibility of the active pharmaceutical ingredients with excipients and suggested a semi-amorphous matrix that may enhance drug solubility and stability [16].

The validated method complies with all ICH Q2R1 guidelines, exhibiting excellent linearity, precision, accuracy, and robustness, making it a reliable analytical tool for quality assurance and standardization of herbal pharmaceutical formulations. The method can be readily adopted by pharmaceutical manufacturers and quality control laboratories for routine analysis and regulatory compliance [17].

The emulgel formulation shows considerable promise as an effective and safe herbal-based topical treatment for various skin disorders, particularly those involving inflammation. Further studies including in vitro permeation studies, stability testing, and clinical efficacy evaluation would provide additional evidence for its therapeutic potential [18]. This work contributes significantly to the analytical standardization of polyherbal and phytochemical-based pharmaceutical formulations, supporting the scientific validation of traditional herbal medicines within the framework of modern pharmaceutical science.

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