

Development and Validation of an HPLC Method for Simultaneous Quantification of Diosgenin and Piperine in Plasma using RP-HPLC

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ABSTRACT

Introduction: The present study aims to develop and validate a robust RP-HPLC method for the simultaneous estimation of Diosgenin and Piperine in human plasma. **Materials and Methods:** Chromatographic separation was achieved using a Phenomenex C18 column (250 mm × 4.6 mm, 5 μm) with an isocratic mobile phase of methanol: water (70:30, v/v) at a 1 mL/min flow rate. Nintedanib was selected as the internal standard based on retention time compatibility. A protein precipitation method was optimized for sample extraction, and the method was validated for selectivity, precision, accuracy, and stability following regulatory guidelines. **Results:** The method demonstrated excellent linearity (0.15-6 μg/mL) with a Correlation Coefficient (R^2) >0.999. The LLOQ was established at 0.15 μg/mL. Recovery studies confirmed efficient extraction with recoveries of 94.3% for Diosgenin and 91.5% for Piperine. Stability assessments, including bench-top and freeze-thaw studies, indicated that both compounds remained stable under different storage conditions. Precision and accuracy values were well within acceptance limits, ensuring the method's reliability. **Conclusion:** The developed RP-HPLC method provides a sensitive, accurate, and reproducible approach for the simultaneous quantification of Diosgenin and Piperine in plasma. This validated method is suitable for pharmacokinetic and bioavailability studies.

Keywords: Chromatography, Diosgenin, HPLC, Limit of quantification, Nintedanib, Piperine.

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INTRODUCTION

Diosgenin and Piperine are naturally occurring phytochemicals with diverse pharmacological properties and growing interest in the field of phytopharmaceuticals. Diosgenin, a steroidal sapogenin derived mainly from *Dioscorea* species, exhibits a wide range of therapeutic effects, including anti-inflammatory, antioxidant, anti-diabetic, and anticancer properties.¹ Piperine, the major alkaloid component of *Piper nigrum* (black pepper), is well known for its bioenhancing potential, improving the bioavailability of co-administered drugs and natural products by inhibiting drug-metabolizing enzymes and enhancing gastrointestinal absorption.² These attributes make diosgenin and piperine an attractive combination in herbal and nutraceutical formulations.

Pharmacokinetic studies of such compounds are essential to understand their absorption, distribution, metabolism, and excretion. In this context, bioanalytical methods play a vital role.³ Bioanalytical methods are defined as techniques used to detect and quantify drugs, metabolites, and biomarkers in biological systems such as plasma, serum, or urine.⁴ The reliability of pharmacokinetic, toxicological, and bioequivalence data depends heavily on the specificity, sensitivity, and reproducibility of the bioanalytical method employed.⁵ A bioanalytical method must undergo rigorous validation to meet international regulatory guidelines covering parameters like accuracy, precision, recovery, linearity, stability, and selectivity.

High-Performance Liquid Chromatography (HPLC) remains one of the most widely used analytical tools for quantitative bioanalysis due to its robustness, sensitivity, and adaptability to complex biological matrices.⁶ Among various chromatographic techniques, Reverse-Phase HPLC (RP-HPLC) is especially prominent.⁷ It utilizes a non-polar stationary phase (e.g., C18 column) and a relatively polar mobile phase, which makes it ideal for separating compounds of intermediate to low polarity, like diosgenin and piperine.⁸ RP-HPLC offers advantages such as shorter run times, better resolution, and compatibility with



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UV and MS detectors, making it a valuable technique in routine bioanalytical laboratories.

While individual methods for the estimation of diosgenin or piperine have been reported.⁹ A validated method for their simultaneous estimation in plasma remains limited. The simultaneous quantification of both compounds is necessary, particularly for pharmacokinetic studies involving combination therapy, as well as for quality control of herbal formulations. Plasma, as a biological matrix, presents unique challenges such as protein binding, endogenous interference, and low analyte concentrations. Therefore, method development must ensure adequate sample preparation, matrix deproteinization, and extraction recovery while retaining chromatographic selectivity.¹⁰

This study aims to develop and validate a rapid, accurate, and reproducible RP-HPLC method for the simultaneous quantification of diosgenin and piperine in human plasma.¹¹ The method will be validated in accordance with ICH and FDA bioanalytical guidelines, making it suitable for application in pharmacokinetic studies, drug interaction assessments, and formulation development involving these compounds.¹²

MATERIALS AND METHODS

Chemicals

Diosgenin (Catlog no: RC02981; Simson Pharma, Mumbai, India) and Piperine (Catlog no: OT746000; Simson Pharma, Mumbai, India), Acetonitrile (Catlog no: ALF-022927-K2; Qualigens Pharma Ltd., Mumbai, India), Formic acid (Catlog no: F/1820/PB17; Qualigens Pharma Ltd., Mumbai, India), Methanol (Catlog no: Q43607; Qualigens Pharma Ltd., Mumbai, India), Ortho-phosphoric acid (Catlog no: Q43626; Qualigens Pharma Ltd., Mumbai, India), Ammonium acetate (Catlog no: Q21335; Qualigens Pharma Ltd., Mumbai, India), Trifluoroacetic acid (Catlog no: Q28462; Qualigens Pharma Ltd., Mumbai, India), and HPLC water (Catlog no: Q43956; Qualigens Pharma Ltd., Mumbai, India). were purchased from. Nintedanib (Catlog no: N600000; Simson Pharma, Mumbai, India). Human plasma was collected from Lokmanya Blood Bank.

Ethical Statement

The human plasma samples used in this study were obtained from a certified Lokmanya blood bank. All procedures involving human samples were conducted in accordance with established ethical standards and guidelines. The study utilized anonymized plasma samples, and no identifiable personal information was collected or used. Therefore, formal ethical approval and informed consent were not required as per institutional and regulatory policies.

Instrumentation

A high-performance liquid chromatography system (Catlog no: 1260 infinity II; Agilent, Santa Clara, USA). equipped with Phenomenex C18 column (250 mm X 4.6 mm, 5 μ M; Catlog no:

00G-4252-E0; Phenomenex, California, USA) with UV-visible detector was employed in isocratic mode.

Mobile phase selection and isocratic optimization

The mobile phase selection and optimization was performed in isocratic mode by varying composition of Mobile phase. The initial trial was conducted with an isocratic method Methanol: Water (70:30). Based on Asymmetry, Theoretical plates, RT and resolution of each drug mobile phase was finalized.

Selection of Internal standard

Telmisartan (Catlog no: T020000; Simson Pharma, Mumbai, India), Azilsartan (Catlog no: A500000; Simson Pharma, Mumbai, India) and Nintedanib drugs were tried as internal standard. Each drug was (10 mg) weighed separately and dissolved in 10 mL of methanol to give 50 μ g/mL solution. Pipette out 1 mL of drug stock solution was pipette out and diluted to 10 mL with mobile phase Methanol: Water (50:50). Based on Asymmetry, Theoretical plates and RT of each drug internal standard was finalized.¹³

Chromatographic conditions

A high-efficiency fine-pore C18 column (250 mm X 4.6 mm, 5 μ M; Catlog no: 00G-4252-E0; Phenomenex, California, USA) was utilized as the stationary phase for chromatographic separation. Methanol: Water (50:50%V/V) was sonicated using ultrasonicator (Catlog no: Z52887; Sigma Aldrich, Mumbai, India), degassed and used as mobile phase with a isocratic mode with run time of 15 min, 40 μ L of standard was injected at a flow rate of 1 mL/min into the HPLC. Chromatographic separations were carried out by keeping column oven temperature at 40°C and detection was done at 219 nm by using UV detector. Nintedanib was used as internal standard during the course of development and validation.¹⁴

Stock solution preparation

In 20 mL volumetric flask Diosgenin (10 mg) and Piperine (10 mg) was dissolved in sufficient quantity of methanol and final volume was adjusted with addition of methanol to get 500 μ g/mL solution. Appropriate dilutions of stock solutions were done using methanol as diluent and used for further experiments.¹⁵

Extraction method

For the extraction of Diosgenin and Piperine from plasma, a precise sample preparation and protein precipitation method was followed.¹⁰ Initially, 475 μ L of plasma was pipetted into a clean microcentrifuge tube, to which 25 μ L of the drug solution containing Diosgenin and Piperine was added. Subsequently, 50 μ L of the internal standard solution (702 μ g/mL Nintedanib) was introduced, followed by the addition of 20 μ L of 2% formic acid to facilitate protein precipitation. To achieve efficient protein removal and extraction, 1 mL of methanol was added to the mixture, and the tube was vortexed thoroughly for 1 min to ensure complete

mixing. The sample was then centrifuged (Catlog no: 75007221; Thermo Fisher Scientific, Mumbai, India) at 12,000 rpm for 10 min at 4°C to separate the supernatant. After centrifugation, 500 µL of the clear supernatant was carefully transferred into a clean HPLC vial without disturbing the precipitated proteins. The extracted supernatant was directly injected into the HPLC system for quantification. A calibration curve was prepared with concentrations ranging from 0.15 to 6 µg/mL to ensure accurate quantification of the analytes.¹⁶

Validation of developed bioanalytical method

Selectivity

The methods selectivity was assessed by comparing interference at Diosgenin's, Piperine's and IS's retention times in blank, hemolyzed, and lipemic plasma. Six different normal blank plasma lot with one haemolyzed and one lipemic plasma lot processed as per method. Spiked blank plasma with the lowest quantifiable concentration of Diosgenin and Piperine (LLOQ level) was injected into the HPLC system.¹⁷

Linearity

The linearity of the developed method was validated using calibration curve. The concentrations of 0.15 µg/mL, 1 µg/mL, 2 µg/mL, 3 µg/mL, 4.5 µg/mL and 6 µg/mL were obtained by diluting stock solution. The chromatograms were recorded after each concentration was injected. The calibration curve was then obtained by plotting an area under the curve vs concentration. The coefficient of correlation (r), equation, slope, and intercept were determined.¹⁸

Sensitivity

A 475 µL of plasma was transferred in 6 different clean dried 15 mL of tars on tubes. Aqueous LLOQ solution (25 µL) was added in each tarson tubet and vortexed for 1 min. Internal standard solution (50 µL) was added and vortexed for 1 min. 20 µL 2% formic acid was added and vortex for 1 min. Added 1 mL of methanol was added and vortexed for 2 min. The solution was centrifuged at 4000 RPM for 3 min and supernatant (0.5 mL) was injected.¹⁹

Carry over

Blank plasma and standard (ULOQ) was prepared and solutions were injected in sequence of Blank-1, Standard (ULOQ), Blank-2 and Blank -3(% interference and area at RT of Diosgenin and Piperine was calculated.¹⁹

Precision and Accuracy

This study was performed by injecting sample solutions of Extracted Blank, extracted blank with IS, Calibration curve standards and QC samples (LLOQ, LQC, MQC and HQC).¹⁹

Recovery

Recovery of Diosgenin and Piperine for QC samples (LQC, MQC, HQC) was calculated by comparing the mean area of extracted QC samples to the mean area of recovery vial QC samples. Recovery vials were prepared by adding specific volumes of LQC, MQC, or HQC solutions to plasma extracts.²⁰

Stability

Various stability tests were conducted including bench top (6 hr), freeze-thaw (3 cycles), autosampler (24 hr), processed sample stability (6 hr), and stock solution stability (24 hr). Percent recovery and accuracy were calculated for LQC and HQC samples based on calibration curves.²⁰

Statistical Analysis

All experimental data were expressed as mean ± Standard Deviation (SD). Statistical evaluation of the method validation parameters, including accuracy, precision, and recovery, was performed using standard statistical tools. The coefficient of variation (%CV) was calculated to assess the precision of the method, while accuracy was determined as the percentage of nominal concentration. Linearity was evaluated using calibration curves, and the Correlation Coefficient (R²) was calculated by linear regression analysis. All calculations were performed using Microsoft Excel software.

RESULTS

Mobile phase selection and isocratic optimization

The first trial conducted with an isocratic method using Methanol: water (70:30) as mobile phase. In this trial Diosgenin was eluted at 3.90 min with very good chromatography while Piperine was eluted at 7.11 min with unacceptable chromatography, hump observed at fronting. This method was rejected as Piperine shows unacceptable chromatography. To address this issue, second trial was performed with Methanol: water (70:30) as mobile phase. Diosgenin was found to be eluted at 5.61 min with acceptable chromatography (asymmetry =1.16 and Theoretical plates= 16982). Similarly, Piperine was eluted at 11.13 min with acceptable chromatography (asymmetry =1.12 and Theoretical plates =16842). Based on these observations mobile phase Methanol: water (50:50) was found to be acceptable. Further to confirm its acceptability, Diosgenin and Piperine in combination was injected at each 100- µg/mL concentration. Both drug showed good chromatography with Diosgenin RT of 5.61 min, Asymmetry of 1.19, and Theoretical plates of 16504. While Piperine showed RT of 11.13 min, Asymmetry of 1.12, with Theoretical plates of 16739.

Selection of Internal standard

Telmisartan, Azilsartan and Nintedanib drugs were tried as internal standard. Telmisartan was eluted at 5.10 min with

good chromatography. (Asymmetry =1.13 and Theoretical plates =11749). But its RT was almost at the same R.T. as that of Diosgenin so Telmisartan was not considered as internal standard.

Azilsartan was eluted at 19.68 min with good chromatography. (Asymmetry =1.09 and Theoretical plates =13429). But considering longer RT Azilsartan was also not considered as internal standard. Nintedanib was eluted at 6.50 min with good chromatography (Asymmetry =1.09 and Theoretical plates =16924) so it was used as internal standard. In order to validate Nintedanib as internal standard, Mixture each of 100 µg/mL of Solution mixture containing 100 µg/mL of Diosgenin, Piperine and Nintedanib as IS was injected to check chromatography in mixture. Each stock solution prepared in methanol and final dilution prepared in mobile phase of trial no.2. All drug showed good chromatography.

Diosgenin (R.T.=5.61 min, Asymmetry =1.16, Theoretical plates =17128). Nintedanib as IS (R.T.=6.38 min, Asymmetry =1.08, Theoretical plates =16408, Resolution =4.16). Piperine (R.T.=11.13 min, Asymmetry =1.10, Theoretical plates =19280, Resolution =18.23). Based on these observations Nintedanib was selected as IS.

Validation of developed bioanalytical method

The validation results for the analytical method used to quantify Diosgenin and Piperine in plasma provide a comprehensive picture of its selectivity, linearity, precision, reliability, and suitability for routine applications.⁸ Below is a detailed breakdown of each parameter:

Selectivity

The method has been shown to be highly selective, with no interference from plasma matrix components like hemolyzed or lipemic samples. This ensures that the measurements of Diosgenin and Piperine are not compromised by common interferences found in plasma, which is crucial in clinical settings where plasma samples may not always be perfectly clean. From selectivity study it was found that there was no blank interference with the Diosgenin, Piperine and IS peak as shown in Figure 2. Diosgenin, Piperine and IS peaks were well separated from each other (Figure 1).

Selectivity LLOQ

Linearity

The linearity of both Diosgenin and Piperine was evaluated by plotting calibration curve between different concentrations and area.¹¹ As shown in Table 1. Excellent linearity was observed between concentration and area between the range of 0.15 µg/mL to 6 µg/mL. The calibration curve for Piperine showed strong linearity (slope=0.6131, $R^2=0.99975$) similar observations were also found in case of Diosgenin (slope=0.32780, $R^2=0.99968$). A high R^2 value ensures the method can produce consistent and accurate results across various concentrations, which is vital for determining plasma levels accurately in pharmacokinetic studies as shown in Figures 2 and 3. This linearity confirms the reliability of the method for quantifying Diosgenin and Piperine, demonstrating its suitability for accurate and reproducible analysis in pharmaceutical applications.¹³

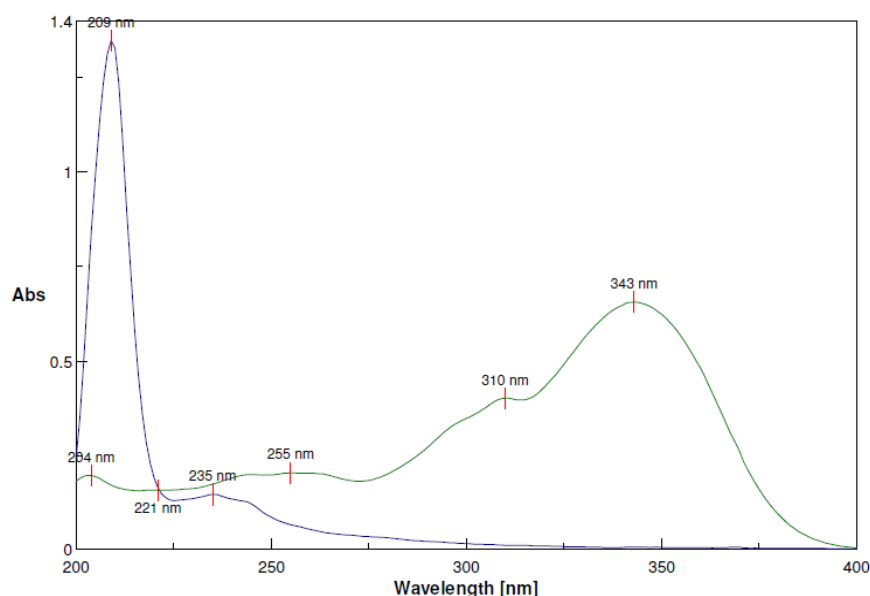


Figure 1: Isobestic Point at 221 nm (Q-Point).

Table 1: Summary of Bioanalytical Method Validation Parameters including Linearity, Accuracy, Precision, Recovery, Selectivity, and Stability for Diosgenin and Piperine in Human Plasma using the Developed RP-HPLC Method.

Validation Parameter	Diosgenin	Piperine	Acceptance Criteria
Linearity Range (µg/mL)	0.15 - 6.0	0.15 - 6.0	Should be linear
Regression Equation	$Y=0.3278X+0.0012$	$Y=0.6131X-0.0069$	-
Correlation Coefficient (R^2)	0.99968	0.99975	≥ 0.99
LLOQ (µg/mL)	0.15	0.15	-
LLOQ Precision (%CV)	1.33%	2.43%	$\leq 20\%$
Accuracy (%Range)	94.67 - 103.30%	98.50 - 106.00%	85-115%
Precision (%CV, QC samples)	2.9-4.2%	2.1-3.6%	$\leq 15\%$
Recovery (%)	94.2%	91.5%	Consistent and reproducible
Recovery (%CV)	1.4%	2.9%	$\leq 15\%$
Selectivity	No interference observed	No interference observed	No significant interference
Carry Over	Not observed	Not observed	Should be negligible
Stability (All conditions)	Stable	Stable	85-115% accuracy, $\leq 15\%$ CV

Sensitivity

The sensitivity of the method was evaluated by analyzing the area values of Piperine and Diosgenin at the LLOQ.¹¹ The mean area values for Piperine and Diosgenin were 324036 and 176381, respectively, with standard deviations of 7865.55 and 2342.67. The calculated coefficient of variation (CV) was 2.43% for Piperine and 1.33% for Diosgenin, both well within the acceptance criteria of NMT 20% as shown in Table 1. Many studies on the quantification of flavonoids like Diosgenin and Piperine report CV values at the LLOQ in the range of 5-10%. The CV values in this study (1.33% for Diosgenin and 2.43% for Piperine) are notably lower, indicating superior precision. This makes the method highly competitive in terms of sensitivity and reproducibility. These low CV values indicate high precision and reproducibility at the LLOQ, demonstrating the method's sensitivity in detecting and quantifying these analytes at minimal concentrations with reliable accuracy.¹² This allows for the detection and quantification of both compounds at low levels, which is particularly important when analyzing drugs or metabolites present in trace amounts, such as in pharmacokinetic studies.

Carry over

The carry-over test confirms that there is no contamination from previous samples. This ensures that the results from one sample do not influence the next, which is essential for maintaining the integrity of the analysis, particularly when high-concentration samples are followed by low-concentration ones as shown in Table 1.

Accuracy and Precision

The accuracy study for Diosgenin and Piperine was assessed over a concentration range of 0.15-6 µg/mL, with results demonstrating

consistent and reliable quantification as shown in Table 1. The % accuracy values for Diosgenin ranged from 94.67% to 103.30%, while Piperine showed values between 98.50% and 106.00%, all within acceptable limits (typically 85-115% for bioanalytical methods). At the lowest concentration (0.15 µg/mL), Diosgenin exhibited 94.67% accuracy, indicating reliable quantification at LLOQ. Similarly, Piperine showed slightly higher accuracy at the same level (106.00%). Across all tested concentrations, both compounds-maintained accuracy close to 100%, with minimal deviations. These results confirm the method's robustness and reliability for precise quantification across a wide concentration range.

Accuracy for Quality Control Samples (QC)

The accuracy of Quality Control (QC) samples for Diosgenin and Piperine was assessed at four concentration levels: Lower Limit of Quantification (LLOQ), Low QC (LQC), Medium QC (MQC), and High QC (HQC). The results demonstrate consistent accuracy across multiple sets, confirming the method's reliability for precise quantification. For Diosgenin, accuracy ranged from 92.00% to 106.03%, with values remaining well within the acceptable range (85-115%). LLOQ accuracy was slightly variable (92.00%-102.00%), while LQC, MQC, and HQC values were consistently close to 100%, ensuring reliable quantification at different concentration levels. Similarly, Piperine accuracy ranged from 92.67% to 106.67%, indicating precise measurement across all QC samples. LLOQ accuracy was slightly higher (97.33%-106.67%), while MQC and HQC values remained within acceptable limits, confirming the robustness of the method. Overall, the results demonstrate the method's accuracy and reproducibility across a wide concentration range, making it suitable for reliable bioanalytical applications as shown in Table 1.

Precision for Quality Control Samples (Qc)

The precision of Diosgenin and Piperine in Quality Control (QC) samples was evaluated at four concentration levels: LLOQ, LQC, MQC, and HQC. The % Coefficient of Variation (CV) for both compounds remained within acceptable limits ($\leq 15\%$ for QC samples and $\leq 20\%$ for LLOQ), indicating high reproducibility. For Diosgenin, the mean accuracy across all QC levels ranged from 97.62% to 101.41%, with %CV values between 2.9% and 4.2%, demonstrating excellent precision. The highest variability was observed at LLOQ (4.28%), but it remained well within regulatory limits, confirming method reliability for low-concentration samples. For Piperine, the mean accuracy ranged from 97.41% to 103.56%, with %CV values between 2.1% and 3.6%, further supporting the method's consistency. The lowest variability was observed at HQC (2.14%), ensuring precise measurement at higher concentrations. % Low CV values at the LLOQ indicate that the method can reliably quantify both compounds even at very low concentrations, which is important for detecting trace amounts in plasma, especially in pharmacodynamics or toxicology studies. Overall, the low % CV values across all QC levels confirm the method's robustness and reproducibility, making it highly reliable for bioanalytical applications involving Diosgenin and Piperine as shown in Table 1.

Recovery

The QC sample recovery study assessed the extraction efficiency of Diosgenin, Piperine, and the Internal Standard across LQC, MQC, and HQC levels. The overall recovery for Diosgenin was 94.2%, with %CV at 1.4%, indicating consistent and efficient extraction. Piperine showed an overall recovery of 91.5%, with a slightly higher %CV of 2.9%, but still within acceptable limits, demonstrating reliable reproducibility. The Internal Standard had the highest recovery at 93.5%, with the lowest %CV (0.7%), confirming its stability and efficiency in the extraction process.

Recovery rates for similar analytical methods in plasma often vary between 80% and 95%. The recovery rates observed in this study (94.2% for Diosgenin and 91.5% for Piperine) are comparable to, or slightly higher than, other studies. This indicates that the method does not suffer significant losses during sample preparation and extraction. The low %CV values across all analytes and QC levels highlight the method's accuracy and reproducibility, ensuring reliable quantification of Diosgenin and Piperine in biological matrices.¹⁵ across various QC levels suggest that the method is reliable for quantitative analysis, providing consistent results even when dealing with biological matrices that have complex compositions as shown in Table 1.

Stability

Stability studies confirm that the compounds remain stable under various conditions, ensuring that the analysis is not compromised by degradation of the compounds during storage or processing. This stability is vital for the method's routine application in clinical settings and supports its robustness for use in long-term studies.² as shown in Table 1.

Bench top stability study

The bench-top stability study for Diosgenin and Piperine demonstrated that both compounds remained stable under the tested conditions, meeting the acceptance criteria for accuracy (85-115%) and precision (CV $<15\%$). The mean accuracy for Diosgenin ranged from 100.37% to 101.92%, with an overall accuracy of 101.15% and % CV values between 2.4% and 4.7%, indicating minimal variability. Similarly, Piperine exhibited a mean accuracy of 99.77% to 102.52%, with an overall accuracy of 101.15% and % CV values between 3.4% and 5.9%, confirming its stability. The low %CV values across both analytes suggest that no significant degradation occurred, ensuring the reliability of the method for sample processing under bench-top conditions as shown in Table 1.

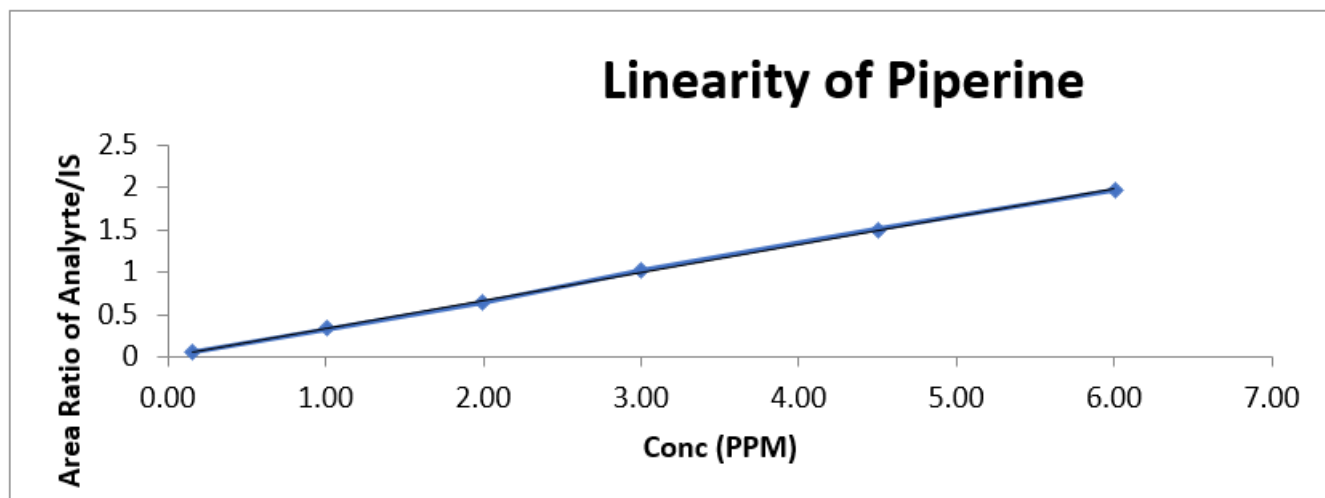


Figure 2: Calibration Curve of Piperine.

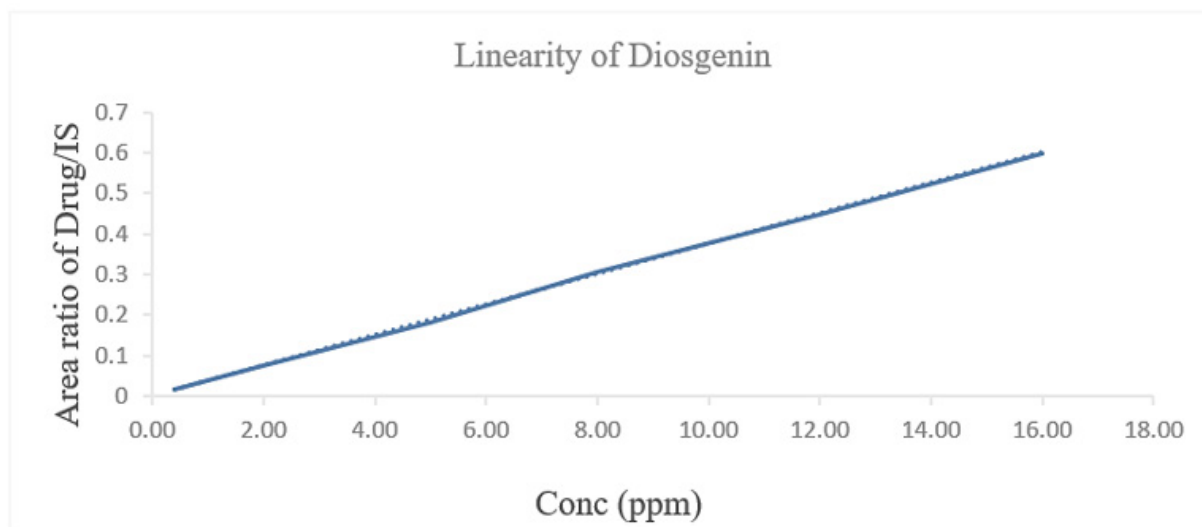


Figure 3: Calibration Curve of Diosgenin.

Freeze thaw Stability

The freeze-thaw stability study for Diosgenin and Piperine confirmed their stability across multiple freeze-thaw cycles, meeting the acceptance criteria for accuracy (85-115%) and precision (CV<15%). For Diosgenin, the mean accuracy ranged from 98.74% to 100.96%, with an overall accuracy of 99.85% and %CV values between 4.1% and 4.9%, demonstrating minimal variability. Piperine exhibited a mean accuracy between 98.44% and 101.15%, with an overall accuracy of 101.15% and %CV values between 1.8% and 4.7%, confirming its stability. The low variability and consistent accuracy values indicate that repeated freezing and thawing do not significantly impact the integrity of either analyte, ensuring method reliability for long-term sample storage as shown in Table 1.

DISCUSSION

The developed RP-HPLC method provides a significant improvement over existing techniques for simultaneous quantification of diosgenin and piperine in plasma. The high linearity, precision, and recovery values demonstrate the method's robustness. Stability and carry-over tests further validate its applicability in pharmacokinetic and bioavailability studies. The absence of matrix effects across plasma types highlights the method's selectivity. Compared to previously reported methods, this protocol offers reduced analysis time and enhanced resolution, enabling its integration into routine bioanalytical workflows. The use of Nintedanib as an internal standard, due to optimal retention and minimal interference, also strengthens the method's reproducibility.

CONCLUSION

The developed RP-HPLC method successfully quantified Diosgenin and Piperine in plasma with high precision, accuracy, and stability while maintaining a balance between analytical

efficiency and environmental considerations. The method demonstrated excellent linearity over the concentration range of 0.15-6 µg/mL, with correlation coefficients (R^2) of 0.99968 for Diosgenin and 0.99975 for Piperine. Accuracy and precision results for calibration standards and QC samples (LLOQ, LQC, MQC, HQC) were within acceptable limits specified by regulatory guidelines (%Accuracy: 85-115%; %CV: ≤15%, ≤20% for LLOQ).

Extraction recovery values were consistent and reproducible, averaging 94.3% for Diosgenin and 91.5% for Piperine, with low %CV across QC levels, confirming efficient analyte extraction from plasma. Matrix effect evaluation, performed using multiple normal plasma lots along with lipemic and hemolyzed samples, showed no significant interference, ensuring selectivity.

The LLOQ was established at 0.15 µg/mL with CV values of 1.33% (Diosgenin) and 2.43% (Piperine), confirming reliable quantification at the lowest concentration. LOD and LOQ were determined based on signal-to-noise and calibration curve statistics, meeting guideline requirements.

Comprehensive stability studies-including bench-top, freeze-thaw, autosampler, processed sample, and stock-solution stability-confirmed that both analytes remain stable under all tested conditions (%Accuracy within 85-115%, CV ≤15%). Recovery studies indicated efficient extraction with overall recoveries of 94.3% for Diosgenin and 91.5% for Piperine. Stability assessments, including bench-top and freeze-thaw studies, confirmed both compounds' stability under different storage conditions. The method exhibited no carryover effect and demonstrated robust performance across multiple validation parameters. The low plasma sample volume and reduced solvent usage enhanced sustainability, while the isocratic mode minimized variability and improved reproducibility. Diosgenin and Piperine pharmacokinetic and bioavailability investigations may be conducted using this validated RP-HPLC technology.

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ABBREVIATIONS

HPLC: High-Performance Liquid Chromatography; **RP-HPLC:** Reverse Phase-High Performance Liquid Chromatography; **IS:** Internal Standard; **LLOQ:** Lower Limit of Quantification; **ULOQ:** Upper Limit of Quantification; **LQC:** Low-Quality Control; **MQC:** Medium Quality Control; **HQC:** High-Quality Control; **RT:** Retention Time; **CV:** Coefficient of Variation.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

Sonal Shaharwale: Prepared the manuscript and performed analytical work, including data acquisition and processing. **Dr. Nirmala Shinde:** Interpreted the results and assessed validation parameters to ensure compliance with regulatory guidelines. All authors have reviewed and approved the final version of the manuscript.

SUMMARY

This study presents the development and validation of a sensitive RP-HPLC method for simultaneous estimation of diosgenin and piperine in plasma using Nintedanib as the internal standard. The method was optimized using an isocratic mobile phase of methanol: water (50:50) and showed excellent linearity (0.15-6 µg/mL), selectivity, and recovery. It exhibited robust performance across various validation parameters including precision, accuracy, carry-over, and stability, making it suitable for pharmacokinetic and bioanalytical applications.

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