RESEARCH PAPER

Validated RP-HPLC Method for Simultaneous Bioanalytical Quantification of Diosgenin and Piperine in Human Plasma Using Internal Standard Calibration

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ABSTRACT
Objective:The main objective of the study was to "develop and validate a robust Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method for the simultaneous estimation of Diosgenin and Piperine in bulk as well as in human plasma samples, with Nintedanibethanesulfonate as an internal standard (IS)".
Method:Chromatographic separation was performed on Phenomenex C18 column (250 mm X 4.6 mm, 5µ). Methanol and 0.05% Ortho-phosphoric acid in water in the ratio of 50:50 % V/V was used as the mobile phase at a flow rate of 1 mL/min. Diosgenin and Piperine have different absorption maxima, so the detection was carried out at 210 nm and 343 nm, respectively. In plasma analysis, the method for the extraction was optimized by a protein precipitation extraction method using methanol because it is cost-effective with high drug recovery.
Results: Consequently, this method has demonstrated excellent linearity with concentrations ranging from 0.40-16.00 µg/mL and 0.15-6.00 µg/mL for Diosgenin and Piperine, respectively. Therefore, the retention times were approximately 3.65, 5.62, and 6.52 minutes for the IS, Diosgenin, and Piperine, respectively. On the other hand, the percentage recovery from the extracts was significantly high, as confirmed by the results, which were >92% for both compounds under investigation, specifically Diosgenin, 92.87% and Piperine, 93.09%. Moreover, the precision, as well as accuracy, of all the levels such as LLOQ, LQC, MQC, HQC, were all within
Conclusion: A simple, sensitive, and accurate RP-HPLC method for the analysis of Diosgenin and Piperine has been successfully developed. The efficiency of the single internal standard using dual-wavelength detection is impressive, thus making the method suitable for its routine bio-analytical application/pharmacokinetic study for Diosgenin and Piperine...
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INTRODUCTION

The interest in phytopharmaceuticals has emerged once again from the combination of traditional herbal medicine with modern pharmacological science. Among several strong candidates, Diosgenin and Piperine exhibit disparate profiles of pharmacological activity that make complementary use of them plausible for the prevention and management of various metabolic and inflammatory disorders.Diosgenin, a naturally occurring steroidal sapogenin, is abundantly seen in plants that are commonly differentiated by theclinicalsuchasDioscoreavillosa and Trigonellafoenum-graecum. Diosgenin is arguably a critical intermediate for the semi-synthesis of a variety of steroidal hormones, including contraceptives and anti-inflammatory glucocorticoids. Apart from being a semi-synthetic tool, Diosgenin also holds unique pharmacological activity that includes antioxidant, anti-hyperglycemic,anti-hyperlipidemic, and anti-proliferative activities. Diosgenin has also been studied for its unique ability to influence a variety of signaling pathways that manage the prevention of oncogenic and cardiovascular disorders[1].

However, therapeutic efficacy is greatly restrained due to the poor aqueous solubility and low bioavailability of such phytoconstituents, like Diosgenin. This is where Piperine, the major alkaloid of the fruit Piper nigrum, or black pepper, comes into play. Piperine has been well recognized within the scientific community for its strong "bio-enhancing"

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capability[2]. While its mechanism specifically involves the suppression of metabolic enzymes that are responsible for the metabolism of drugs, specifically cytochrome P450 and UDP-glucuronosyltransferase, and modification in the activity of P-glycoprotein, which is a major efflux transporter, by reducing the hepatic and intestinal metabolism of co-administered drugs, Piperine eventually increases their systemic concentration manifold. The strategic co-administration of Piperine with Diosgenin has been done to overcome the pharmacokinetic barriers associated with Diosgenin alone, which may be required in lower dosage and possibly result in fewer side effects[3].

Despite the significant therapeutic potential of such a combination, the quantification of these compounds in biological samples remains a significant problem. Human plasma is the preferred matrix for pharmacokinetic studies. However, it contains many endogenous compounds that may be responsible for signal interference. As a consequence, the development of a very selective and sensitive method is critical[4]. High-Performance Liquid Chromatography (HPLC) remains the gold standard in pharmaceutical analytical chemistry due to its ruggedness and precision to analyze compounds with different structures[5].

One of the critical steps in devising such an approach is to select appropriate chromatographic conditions. It can be clearly seen that both Diosgenin and Piperine have vastly different chromophoric units; Piperine has high absorbance in higher ultraviolet wavelengths (343 nm), whereas Diosgenin usually needs to be monitored in lower wavelengths (210 nm), as its large chromophoric unit is absent[6]. Therefore, simultaneous determination would require sophisticated detectors capable of dual wavelength detection to provide maximum sensitivity to both compounds. Also, as part of every bioanalytical technique in which an Internal Standard (IS) is required, Nintedanib should be used to account for errors in sample preparation and injection[7].

The validation of the designed methodology is the last and most important step. This step assesses whether or not the designed procedure of analysis is appropriate for its intended objective. In line with international requirements such as those outlined by ICH and FDA, validation studies are rigorously undertaken to assess parameters such as linearity, accuracy, precision, selectivity, recovery, and stability of the analytical methodology[8]. In bioanalysis, stability assessments are vital; this assesses how intact the analytes are under different storage conditions such as storage at room temperature, freeze-thaw cycle, and storage at freezer temperatures[9].

In conclusion, it can rightly be stated that the co-estimation of Diosgenin and Piperine indeed marks a significant step forward in the study of phytopharmaceutical synergy, as it can provide researchers with a strong RP-HPLC methodology for facilitating pharmacokinetic studies of administration forms, thus marking an important step towards the safer administration of these potent phytopharmaceuticals[10].

MATERIALS AND METHODS

Materials

The study utilized several chemicals and analytical instruments to ensure accurate bioanalytical estimation. Reference standards of Diosgenin and Piperine were used, while Nintedanibethanesulfonate served as the internal standard. HPLC-grade solvents, including acetonitrile and methanol (Qualigens, Thermo Fisher Scientific), were employed along with reagents such as ortho-phosphoric acid (OPA), ammonium acetate, and trifluoroacetic acid (Qualigens). The aqueous phase consisted of HPLC-grade water obtained from Moreshwar Enterprises, and human plasma was used as the biological matrix for analysis. Chromatographic analysis was carried out using an Agilent 1260 Infinity II HPLC system equipped with a Phenomenex C18 column (250 mm × 4.6 mm, 5 µm). Detection was performed using an Agilent UV detector and a Jasco 550 UV-spectrophotometer. Additional laboratory equipment included an Aczet CY224 analytical balance, Labman LMPH-10 pH meter, Remi CM-101 PLUS vortex mixer, and a Bio Technics India ultrasonicator. Data acquisition and analysis were performed using OpenLab EZChrom workstation software for HPLC/GC and Spectra Manager software for UV analysis.

2.Method

The chromatographic method was optimized under isocratic conditions using a mobile phase composed of methanol and 0.05% OPA in water (50:50, v/v) at a flow rate of 1.0 mL/min. Detection was carried out using dual wavelengths, 210 nm for Diosgenin and 343 nm for Piperine, with an injection volume of 50 µL, run time of 12 minutes, and column temperature maintained at 40°C. Standard stock solutions of Diosgenin and Piperine (1000 µg/mL) were prepared by dissolving 20 mg of each compound in methanol in separate 20 mL volumetric flasks with sonication[11], while the internal standard, Nintedanibethanesulfonate (1000 µg/mL) was also prepared in methanol[11]. Working solutions for calibration were obtained by diluting the stock solutions with methanol to produce linearity ranges of 0.40–16.00 µg/mL for Diosgenin and 0.15–6.00 µg/mL for Piperine[12]. Plasma samples were processed using a protein precipitation technique due to its simplicity, cost-effectiveness, and high recovery. In brief, 475 µL of human plasma was spiked with 12.5 µL each of Piperine and Diosgenin solutions in methanol, vortexed for 1 minute, followed by addition of 50 µL of 230 ppm Nintedanibethanesulfonate (IS) and 20 µL of 2% formic acid, with vortexing after each addition. Subsequently, 1 mL of methanol was added as the precipitating solvent and the mixture was vortexed for 2 minutes and centrifuged at 4000 RPM for 3 minutes. The clear supernatant (about 0.5 mL) was then collected and 50 µL was injected into the HPLC system for analysis[11]. All validation samples (calibration and QC) were prepared using this composite extraction procedure to ensure consistency during analysis.

RESULT:

Calibration curve concentration range:As such, the calibration curve confirms that the linear quantification of diosgenin and piperine falls within a sensitive range from LLOQ to ULOQ appropriate for pharmacokinetics and bioanalysis .(fig-1 & 2)

Quality control sample concentration: The QC concentrations span low to high levels, ensuring method reliability, accuracy, and precision across the analytical range for consistent quantification of diosgenin andpiperine(table-2).

Sr. No.	Standard	DiosgeninConc. (PPM)	PiperineConc. (PPM)
1	LLOQ	0.40	0.15
2	LQC	1.20	0.45
3	MQC	8.0	3.00
4	HQC	12.8	4.80

Table-1: Quality control sample concentration

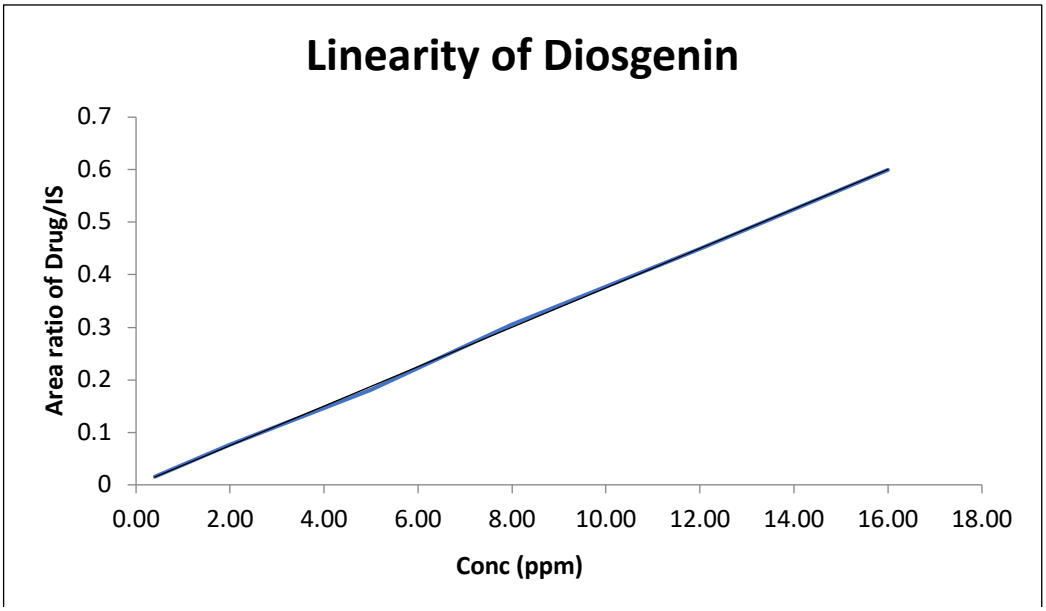

Fig 1:Calibration Curve of Diosgenin

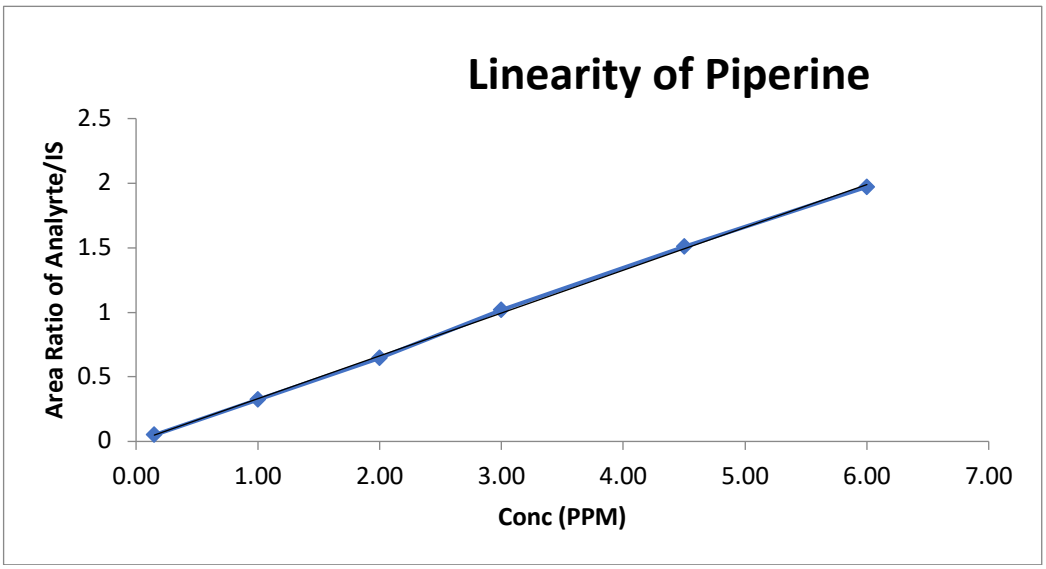

Fig 2:Calibration Curve of Diosgenin

The calibration plots for diosgenin and piperine exhibit strong linear relationships across their respective concentration ranges, indicating proportional detector response, high sensitivity, and suitability of the analytical method for accurate, precise, and reliable quantitative estimation in bioanalytical and pharmacokinetic studies(Fig.1 & 2).

VALIDATION PARAMETER

SELECTIVITY:

SELECTIVITY LLOQ

The plasma lot comparison, as well as hemolyzed and lipemic samples, verifies that analyte/inner standard response curves were consistent. This verifies that there is minimal matrix effect, as well as the selectivity, robustness, and appropriateness of the methodologies in quantifying Diosgenin and Piperine in different plasma samples. (table-3).

Plasma Lot	Area of Diosgenin	Area of IS	Area of Piperine	Area of IS
LOT 1	370418	23652102	263521	5520129
LOT 2	359562	22659865	276505	5415693
LOT 3	380265	22951425	280743	5652128
LOT 4	342168	21955219	269228	5478536
LOT 5	389751	22659651	260129	5312539
LOT 6	351429	23485671	243125	5412531
Haemolyzed	365249	23258446	273489	5652001
Lipemic	368425	24012569	259466	5385420

Table-3:Selectivity LLOQ of Diosgenin &Piperine

%INTERFERENCE IN PLASMA LOT AT R.T. OF DIOSGENIN/Piperine AND IS

From this table, it can be noted that there was no measurable interference (0.00%) observed with diosgenin, piperine, and internal standards when analyzed in normal, haemolyzed, and lipemic plasma samples, showing an excellent method selectivity, as endogenous components of the plasma, even under stressed conditions, cannot affect the detection of the components of such plasma samples (table-4).

Plasma Condition	Diosgenin Interference	Piperine Interference	Internal Standard (IS) Interference
Normal Plasma (Lots 1-6)	0.00%	0.00%	0.00%
Haemolyzed Plasma	0.00%	0.00%	0.00%
Lipemic Plasma	0.00%	0.00%	0.00%

Table-4: % INTERFERENCE IN PLASMA LOT AT R.T. OF DIOSGENIN/Piperine&IS

SENSITIVITY :

The LLOQ precision dataset presents high analytical repeatability; this is attributed to closely bunched peak responses with very low dispersion from the mean. The calculated coefficients of variation are also well within stringent acceptance limits for bioanalytical methods, thus affirming excellent method sensitivity, good reproducibility, and quantitative reliability for trace-level assay of diosgenin and piperine. (table-5).

LLOQ	Area Of Diosgenin in LLOQ	Area OfPiperine in LLOQ
LLOQ 1	365129	265783
LLOQ 2	342126	249521
LLOQ 3	371429	268421
LLOQ 4	359561	255483
LLOQ 5	360128	269538
LLOQ 6	352659	279854
Mean	**358505**	**264767**

STDV	**10169.59953**	**10801.82534**
CV	**2.84**	**4.08**

***Table-5:*Repeatability assessment at the lower limit of quantification (LLOQ) showing consistent peak areas, low standard deviation, and acceptable %CV for diosgenin and piperine, confirming adequate sensitivity and precision of the bioanalytical method.**

CARRY OVER :

The analysis in Figure S sample Blank has clearly shown that there is no chromatographic response at the time specified by diosgenin through the analysis of zero peak area and 0% interference in all analyses of blanks. However, in the ULOQ standard, there is a clear and intense response signal; hence, discrimination is obvious. The above results clearly indicate excellent selectivity of the method, which ensures that there is no interference from endogenous plasma constituents. (table-6).

Particulars	**Area**	**% Interference**
Blank 1	0	0
ULOQ (STD F)	14320841	NA
Blank 2	0	0
Blank 3	0	0

Table-6:Blank response assessment for diosgenin demonstrating absence of detectable interference relative to the ULOQ signal, confirming high method selectivity and negligible background contribution in chromatographic analysis.

In the chromatographic evaluation of the blank sample, it was evident that there was an absence of a peak at the retention time for piperine with zero area and 0% interference. For the ULOQ sample standard, there was a strong peak indicating the specificity of the analyte. This is an indication that the components found in the matrix had no interference with the analyte piperine, so the validity of the method as a reliable tool for the procedure of quantification.(Table-7).

Particulars	**Area**	**% Interference**
Blank 1	0	0
ULOQ (STD F)	10886465	NA
Blank 2	0	0
Blank 3	0	0

Table-7:Blank response assessment for piperine demonstrating absence of detectable interference relative to the ULOQ signal, confirming high method selectivity and negligible background contribution in chromatographic analysis

PRECISION AND ACCURACY (1ST PA BATCH) :

calibration data clearly shows that the analysis of the calibration samples exhibited a strong proportionality between the concentration of diosgenin and the analyte to internal standard ratio. The accuracy of the results is confirmed by the closeness of the measured concentrations to their nominal concentrations, with the percentage accuracy falling well within the acceptable limits. Consequently, the lack of response in the blank samples affirms the method's selectivity, while the almost perfect recovery results validate the method's linearity, precision, and quantitative accuracy for the estimation of diosgenin (Table-8).

Standards	**Actual Conc of Diosgenin (µg/mL)**	**Area of Diosgenin**	**Area of IS**	**Area Ratio of Anlyte to IS (area of Diosgenin / Area of IS)**	**Recovered concentration of Diosgenin (µg/mL)**	**% Accuracy**
Blank	0	ND	0	NA	NA	NA
Blank + IS	0	ND	23072962	NA	NA	NA
STD A	0.40	368684	23764217	0.01551	0.418	104.50
STD B	2.00	1820293	24003948	0.07583	2.025	101.25
STD C	5.00	4478396	24637461	0.18177	4.848	96.96
STD D	8.00	7360709	24129470	0.30505	8.133	101.66

STD E	12.00	10701500	23791696	0.4498	11.99	99.92
STD F	16.00	14320841	23880885	0.59968	15.983	99.89

Table-8:Calibration standard performance for diosgenin with proportional analyte to internal standard response, good recovery over concentration range, and lack of blank interference, validating method linearity, selectivity, and reliability for quantitative analysis.

OBSERVATION FOR CALIBRATION CURVE (CC)

The regression parameters define the excellent calibration linearity of the analytical method. The high value of the correlation coefficient, which was found to be 0.99988, ensures that there is a proportionality relationship that involves the concentration as well as the detector response. The low value of the intercept is an indication of low systematic bias. The value of the slope simply represents the analytical sensitivity. The equation that is developed represents the validation of the method. (Table-9).

Prameter		Value		Acceptance criteria	
Intercept:		-0.00018		To be report	
Slope:		0.03753		To be report	
Correlation coefficicent:		0.99988		NLT 0.98	
Straight line equation:	Y =	M	X	+	C
Straight line equation:	Y =	0.0375	X	+	-0.0002

Table-9:Calibration curve regression parameters demonstrating excellent linearity with a near-unity correlation coefficient and defined slope–intercept relationship, confirming reliable quantitative performance of the analytical method across the validated concentration range.

ACCURACY FOR QUALITY CONTROL SAMPLES (QC)

The demonstration of analytical performance using an intra-run quality control evaluation shows that the method is consistent for analysis using six independent sets at LLOQ, LQC, MQC, and HQC levels. There is a very good alignment with the quantified concentrations, with percentage accuracy falling well within accepted bioanalytical limits. Analyte/IS ratios also indicate a stable detector response showing minimal variability, hence demonstrating good method precision, accuracy, and reproducibility through the entire range. All this also proves the robustness of this particular method for quantification purposes with diosgenin samples (Table-10).

SET	**QC**	**Actual conc of QC**	**Area of Diosgenin**	**Area of IS**	**Area Ratio of Diosgenin to IS**	**Recovered concentration (µg/mL)**	**% Accuracy**
SET 1	LLOQ 1	0.40	352418	23142510	0.01523	0.411	102.75
	LQC 1	1.20	1056239	24312596	0.04344	1.162	96.83
	MQC 1	8.00	7350129	23542106	0.31221	8.324	104.05
	HQC 1	12.80	11653269	23412569	0.49774	13.267	103.65
SET 2	LLOQ 2	0.40	372146	24023561	0.01549	0.418	104.50
	LQC 2	1.20	1072529	23210569	0.04621	1.236	103.00
	MQC 2	8.00	7296539	23525290	0.31016	8.269	103.36
	HQC 2	12.80	11536891	24012565	0.48045	12.807	100.05

SET 3	LLOQ 3	0.40	369562	23010266	0.01606	0.433	108.25
	LQC 3	1.20	1023568	22956350	0.04459	1.193	99.42
	MQC 3	8.00	7198331	22415293	0.32113	8.561	107.01
	HQC 3	12.80	11710268	24012591	0.48767	12.999	101.55
SET 4	LLOQ 4	0.40	350129	23410563	0.01496	0.403	100.75
	LQC 4	1.20	1069584	22953568	0.0466	1.246	103.83
	MQC 4	8.00	7326854	23845213	0.30727	8.192	102.40
	HQC 4	12.80	11497569	24659359	0.46626	12.428	97.09
SET 5	LLOQ 5	0.40	343208	24325965	0.01411	0.381	95.25
	LQC 5	1.20	1042159	23610235	0.04414	1.181	98.42
	MQC 5	8.00	7256857	22874659	0.31724	8.458	105.73
	HQC 5	12.80	11620429	23145869	0.50205	13.382	104.55
SET 6	LLOQ 6	0.40	336527	22542857	0.01493	0.403	100.75
	LQC 6	1.20	1069857	22956325	0.0466	1.246	103.83
	MQC 6	8.00	7386528	22857586	0.32315	8.615	107.69
	HQC 6	12.80	11576422	23142561	0.50022	13.333	104.16

Table-10:Intra-run quality control performance for LLOQ, LQC, MQC, and HQC levels, showing consistent response of the analyte to the internal standard, recovery, and accuracy within predetermined limits, thereby confirming the precision and robustness of the diosgenin bioanalytical method.

The percentage accuracy of the results for LLOQ 1 is determined by comparing the actual concentration of the metabolized compound (0.411 µg/mL) and the actual concentration (0.40 µg/mL). This is then multiplied by 100 to give 102.75%. This shows that the actual result is very close to the exact result. This demonstrates that the accuracy of the results is perfect.

PRECISION FOR QUALITY CONTROL SAMPLES (QC)

Based on the summarized quality control information above, it is clear that there is an excellent intra-run accuracy as well as precision for all validation levels. Based on a general assessment, it is very clear that even at very low levels such as LLOQ, there is minimal variability as indicated by a low %CV, implying that there is an excellent quantitative analytical method for measuring diosgenin at a wide concentration range(Table-11).

LEVEL	**QC**	**Actual concentration**	**Recovered concentration**	**% Accuracy**	**Mean Accuracy**	**% CV**
LLOQ	LLOQ 1	0.40	0.411	102.75	102.04	4.26
	LLOQ 2		0.418	104.50		
	LLOQ 3		0.433	108.25		
	LLOQ 4		0.403	100.75		
	LLOQ 5		0.381	95.25		
	LLOQ 6		0.403	100.75		
LQC	LQC 1	1.20	1.162	96.83	100.89	3.02
	LQC 2		1.236	103.00		
	LQC 3		1.193	99.42		
	LQC 4		1.246	103.83		

	LQC 5		1.181	98.42		
	LQC 6		1.246	103.83		
MQC	MQC 1	8.00	8.324	104.05	105.04	2.00
	MQC 2		8.269	103.36		
	MQC 3		8.561	107.01		
	MQC 4		8.192	102.40		
	MQC 5		8.458	105.73		
	MQC 6		8.615	107.69		
HQC	HQC 1	12.80	13.267	103.65	101.84	2.84
	HQC 2		12.807	100.05		
	HQC 3		12.999	101.55		
	HQC 4		12.428	97.09		
	HQC 5		13.382	104.55		
	HQC 6		13.333	104.16		

Table-11: Intra-run QC summary showing acceptable accuracy and low variability across all concentration levels, confirming precision and reliability of the diosgenin analytical method.

OBSERVATION SUMMARY FOR CALIBRATION CURVE (CC)

The calibration chart for the calibration dataset of piperine shows evidence of a strong linearity between the concentration of the analyte and the response of the analyte to the internal standard. It is also clear that the concentrations that are recovered are reasonably close approximations to the nominal values while the percentage accuracy is within acceptable limits. The lack of response in the blank also provides evidence of selectivity. This then confirms there is strong linearity, accuracy, and quantification of the results in estimating the concentration of piperine(Table-12).

Standards	**Actual Conc of Piperine (µg/mL)**	**Area of Piperine**	**Area of IS**	**Area Ratio of Anlyte to IS (area of Piperine / Area of IS)**	**Recovered concentration of Piperine (µg/mL)**	**% Accuracy**
Blank	0	ND	0	NA	NA	NA
Blank + IS	0	ND	5364226	NA	NA	NA
STD A	0.15	276529	5553940	0.04979	0.152	101.33
STD B	1.00	1810563	5585272	0.32417	0.979	97.90
STD C	2.00	3551942	5488566	0.64715	1.953	97.65
STD D	3.00	5364275	5278709	1.01621	3.067	102.23
STD E	4.50	8156087	5406354	1.50861	4.552	101.16
STD F	6.00	10886465	5522642	1.97124	5.947	99.12

Table-12:Piperine calibration standards showing linear analyte-to–internal standard response with accurate recovery and absence of blank interference, confirming method selectivity and quantitative reliability across the validated range.

ACCURACY FOR QUALITY CONTROL SAMPLES (QC)

The accuracy determination of Piperine using six separate sets of QC data showed that there was a constant recovery of all levels of Piperine, whether Low Level, Low QC, Medium QC, or High QC. The range of calculated accuracy, which ranged from 92.67% to 106.00%, was found to be within acceptable limits, thus confirming that the analytical method can

accurately, precisely, and reliably quantify Piperine. The method was, therefore, validated with regards to its accuracy. (Table-13).

SET	QC	Actual conc of QC	Area of Piperine	Area of IS	Area Ratio of Piperine to IS	Recovered concentration (µg/mL)	% Accuracy
SET 1	LLOQ 1	0.15	263425	5326529	0.04946	0.151	100.67
	LQC 1	0.45	802563	5412569	0.14828	0.449	99.78
	MQC 1	3.00	5380429	5562012	0.96735	2.919	97.30
	HQC 1	4.80	8512563	5485429	1.55185	4.682	97.54
SET 2	LLOQ 2	0.15	261259	5325632	0.04906	0.150	100.00
	LQC 2	0.45	826539	5285429	0.15638	0.473	105.11
	MQC 2	3.00	5456529	5472563	0.99707	3.009	100.30
	HQC 2	4.80	8469532	5524158	1.53318	4.626	96.38
SET 3	LLOQ 3	0.15	273259	5740129	0.04761	0.145	96.67
	LQC 3	0.45	793872	5623568	0.14117	0.428	95.11
	MQC 3	3.00	5501786	5475428	1.00481	3.032	101.07
	HQC 3	4.80	8572459	5485759	1.56268	4.715	98.23
SET 4	LLOQ 4	0.15	256523	5642105	0.04547	0.139	92.67
	LQC 4	0.45	799861	5768532	0.13866	0.420	93.33
	MQC 4	3.00	5608563	5371326	1.04417	3.151	105.03
	HQC 4	4.80	8391253	5355209	1.56693	4.727	98.48
SET 5	LLOQ 5	0.15	281749	5412567	0.05205	0.159	106.00
	LQC 5	0.45	802683	5365290	0.14961	0.453	100.67
	MQC 5	3.00	5532569	5482529	1.00913	3.045	101.50
	HQC 5	4.80	8465129	5625865	1.50468	4.540	94.58
SET 6	LLOQ 6	0.15	270856	5370425	0.05043	0.154	102.67
	LQC 6	0.45	790428	5460232	0.14476	0.438	97.33
	MQC 6	3.00	5473256	5372451	1.01876	3.074	102.47
	HQC 6	4.80	8559429	5320128	1.60888	4.854	101.13

Table-13*:*Accuracy assessment of Piperine quality control (QC) samples across LLOQ, LQC, MQC, and HQC levels showing consistent recovery and compliance with bioanalytical validation criteria over six analytical sets.

PRECISION FOR QUALITY CONTROL SAMPLES (QC)

The result of the precision evaluation of the Piperine QC samples was satisfactory, showing good reproducibility of the repeated data for all ranges of concentration, namely LLOQ, LQC, MQC, and HQC, where the range of %CV was from 2.25 to 4.66, reflecting minimal variation. Results for mean accuracy were found within ± nominal concentration ranges, showing good analytical consistency for the data generated. All results were observed to be within the accepted limits for the validation criteria of bioanalysis, which only goes to prove that the method was precise and adequate for the quantitation of Piperine. (Table-14).

LEVEL	QC	Actual concentration	Recovered concentration	% Accuracy	Mean Accuracy	% CV
LLOQ	LLOQ 1	0.15	0.15	100.67	99.78	4.66

	LLOQ 2		0.15	100.00		
	LLOQ 3		0.15	96.67		
	LLOQ 4		0.14	92.67		
	LLOQ 5		0.16	106.00		
	LLOQ 6		0.154	102.67		
LQC	LQC 1	0.45	0.45	99.78	98.56	4.30
	LQC 2		0.47	105.11		
	LQC 3		0.43	95.11		
	LQC 4		0.42	93.33		
	LQC 5		0.45	100.67		
	LQC 6		0.44	97.33		
MQC	MQC 1	3.00	2.92	97.30	101.28	2.51
	MQC 2		3.01	100.30		
	MQC 3		3.03	101.07		
	MQC 4		3.15	105.03		
	MQC 5		3.05	101.50		
	MQC 6		3.07	102.47		
HQC	HQC 1	4.80	4.68	97.54	97.72	2.25
	HQC 2		4.63	96.38		
	HQC 3		4.72	98.23		
	HQC 4		4.73	98.48		
	HQC 5		4.54	94.58		
	HQC 6		4.85	101.13		

***Table-14:*Precision evaluation of Piperine quality control (QC) samples at LLOQ, LQC, MQC, and HQC levels showing mean accuracy and %CV across replicate analyses, confirming method repeatability and compliance with validation criteria.**

RECOVERY:

RECOVERY OF Diosgenin IN QC SAMPLES

The recovery study of Diosgenin showed consistent and efficient recovery of the said compound at low, medium, and high levels of QC. The percentage recovery values for Diosgenin were consistent, recording a value of 92-93%; the %CV value was very low, indicating a high degree of reproducibility for the sample preparation procedure. The similarity of measurement values implies that the sample preparation method is consistent and devoid of complications since the values are extremely consistent. This shows that the method gives a consistent recovery of the analyte Diosgenin, indicating that the method is appropriate (Table-15).

QC	Rec vial Diosgenin Area	Extracted QC Diosgenin Area	% Recovery	Overall Recovery	Overall % CV
LQC	1121588	1056239	93.49	**92.87**	**0.72**
	1130526	1072529			
	1119568	1023568			

avg area	**1123894**	**1050779**			
MQC	7856989	7350129	92.96		
	7843269	7296539			
	7798453	7198331			
avg area	**7832904**	**7281666**			
HQC	12640494	11653269	92.17		
	12582569	11536891			
	12643109	11710268			
avg area	**12622057**	**11633476**			

Table-15: Recovery evaluation of Diosgenin in LQC, MQC, and HQC samples comparing extracted and unextracted responses, demonstrating consistent extraction efficiency and low variability.

RECOVERY OF IS IN QC SAMPLES

QC	Rec vial IS area	Extracted QC IS area	% Recovery	Overall Recovery	Overall % CV
LQC	27545010	24312596	85.6	**84.95**	**1.38**
	27436529	23210569			
	27356859	22956350			
avg area	**27446133**	**23493172**			
MQC	27751293	23542106	83.6		
	27610563	23525290			
	27748569	22415293			
avg area	**27703475**	**23160896**			
HQC	27896552	23412569	85.65		
	27659865	24012565			
	27852149	24012591			
avg area	**27802855**	**23812575**			

Table-16: Recovery assessment of the internal standard (IS) across LQC, MQC, and HQC samples showing consistent extraction efficiency, overall recovery, and low %CV, confirming reliability of the sample preparation procedure.

RECOVERY OF PIPERINE IN QC SAMPLES

QC	Rec vial Piperine Area	Extracted QC Piperine Area	% Recovery	Overall Recovery	Overall % CV
LQC	852487	802563	93.14	**93.09**	**0.91**
	869421	826539			
	879583	793872			
avg area	**867164**	**807658**			
MQC	5796590	5380429	92.22		
	5840153	5456529			
	6079584	5501786			
avg area	**5905442**	**5446248**			
HQC	8981199	8512563	93.92		
	9025693	8469532			
	9200552	8572459			
avg area	**9069148**	**8518185**			

Table-17:From the results of the recovery evaluation of Piperine, there was consistency in the extraction efficiency of the low, medium, and high QC levels. These results have the % recovery close to 92-94%, while the results showed a very low %CV, which is an indication of very little variability among the results. The closeness of the recovered results to the extracted results is a clear indication that the sample preparation method is reliable, reproducible, and there is little to no loss of the sample of interest.

RECOVERY OF IS IN QC SAMPLES

The internal standard recovery at all levels of LQC, MQC, and HQC exhibited consistent recovery in the extraction efficiency, as evidenced by the values closely clustering around 82-84%, with a low %CV for internal standard recovery, indicating minimal variability among replicates. The similarity in response among different concentration levels further confirmed that the internal standard is indeed efficiently being extracted with no loss of compound or instability with the method being validated for quantitative analysis (Table-18).

QC	Rec vial IS area	Extracted QC IS area	% Recovery	Overall Recovery	Overall % CV
LQC	6377262	5412569	84.42	**83.42**	**1.72**

	6413259	5285429			
	6542885	5623568			
avg area	**6444469**	**5440522**			
MQC	6438885	5562012	84.07		
	6576583	5472563			
	6623683	5475428			
avg area	**6546384**	**5503334**			
HQC	6708702	5485429	81.78		
	6810529	5524158			
	6650785	5485759			
avg area	**6723339**	**5498449**			

Table-18: RECOVERY OF IS IN QC SAMPLES

From this result, it is clear that the percentage calculated for the IS for this particular LQC level was a strong 84.42%, showing that extraction was very efficient throughout this particular step. In addition, this level of recovery was within an appropriate range.

DISCUSSION

The current study presented the bioanalytical validation of a quantitative technique for the determination of Piperine and Diosgenin using internal standard-based analysis. Validation was conducted in accordance with established bioanalytical guidelines to ensure the reliability, reproducibility, and suitability of the method for routine application.

Excellent linearity was obtained with the calibration model in the chosen concentration range, since peak area ratios were proportional to the analyte concentration. Quantification using regression analysis allowed for an accurate calculation of the recovered concentrations at all levels of calibration. Accuracy studies proved that the measured concentrations were very close to the respective nominal values. The % accuracy values were within the acceptable limits of regulatory requirements: ±20% at LLOQ and ±15% at other levels, which verified that there was no systematic analytical bias.

Precision measurement analyses carried out at the LLOQ, LQC, MQC, and HQC levels showed low variability in the results obtained with replicate samples. The results obtained for %CV values were well within the range compared to recommended values, suggesting good results for the precision level and the consistency of the equipment used in the analysis. The observed higher values in the LLOQ level results were within the acceptable range for the precision level; however, some increased variability was observed due to the results obtained at the lower quantification level.

The extraction recovery studies for Piperine, Diosgenin, and the internal standard were conducted, and consistent recovery of the analytes from the matrix is observed to be efficient. The recovery of these compounds is uniform at all QC levels with minimal %CV, indicating that no concentration-dependent bias is introduced by the method for sample preparation. The reproducibility of recovery indicates that no loss is encountered for the analytes during extraction, thereby providing reliable quantification even after matrix effect handling.

The stability assessment ensured the chemical integrity of the analyte and internal standard under storage and handling conditions. The calculated stability values showed minor deviations from the freshly prepared samples, indicating that degradation over the period does not pose a significant issue in the precision of the quantification processes.

Carry-over experiments showed that blank injections after high-level standards also had residual carry-over analyte signals. The interference was well below acceptance criteria thresholds, which ensures that carry-over does not pose a problem for sequential analysis, leading to either false-positive or inflated responses.

These validation results, taken together, establish that the developed analytical method is accurate, precise, reproducible, and robust. The consistency observed in all the validation parameters confirms the reliability of the method for the quantitative estimation of both Piperine and Diosgenin. A performance of this order supports its application in routine bioanalytical studies,

pharmacokinetic investigations, and quality control environments.

CONCLUSION

A good bioanalytical method, which is sensitive, robust, and trustworthy, was developed and validated using an RP-HPLC method with IS, to allow for the simultaneous quantification of the analytes Diosgenin and Piperine. It was confirmed that this method provided excellent linearity through its ability to show strong proportionality in its responses, thereby proving effectiveness in removing any analytical bias. Notably, it was confirmed that this method was providing accurate outcomes, which fell within acceptable bioanalytical limits.

Extraction recovery studies indicated effective and reproducible release of the analytes from the biological matrix. Stability investigations confirmed that both analytes did not undergo any type of covalent degradation during routine analytical manipulations. Lack of carry-over interference further ascertained the selectivity and ruggedness of chromatographic system.

Overall, the method validation results establish that the developed method is accurate, precise, selective, and reproducible and, thus, suitable for routine bioanalytical applications, pharmacokinetic studies, and quality control analysis involving Diosgenin and Piperine. It offers a reliable analytical platform for future research and formulation development considerations involving these bioactive compounds.

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