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Development And Validation Of Hplc Method For The Simultaneous Estimation Of Curcumin And Metformin In Pharmaceutical Dosage Form

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ABSTRACT

Introduction: Curcumin and Metformin are two well-known drugs which have anti-inflammatory and anti- diabetic property respectively but when used in combination they are known to be potent anticancer agents. It was studied that a combination of metformin and curcumin induces the apoptosis without affecting the cell cycle in LNCaP prostate cancer cell line.

Materials and methods: High profile liquid chromatography determination was performed with Agilent Technologies Model 1220 Infinity II LC Software Open Lab, VWD Type Lamp, flow rate was set to 1 ml/min and acetonitrile: water in the ratio 70:30 was used as mobile phase and the effluents were analyzed at 235 nm and 425 nm for metformin and curcumin respectively.

Results and discussion: In the concentration range of 2-10 µg/ml, the HPLC method demonstrated linearity for both curcumin and metformin, with correlation coefficients (r^2) of 0.9953 and 0.9949 at 425 nm and 235 nm, respectively. Data on precision, robustness, and ruggedness revealed %RSD <2%.

Conclusion: the developed HPLC method were found to be simple, precise, specific and sensitive for the simultaneous estimation of curcumin and metformin

Keywords: Curcumin, Metformin, Method development, Validation

1. INTRODUCTION:

Inflammation is a physiological response which is caused by harmful stimuli (like infection) in order to keep the body in a homeostatic condition. It is a series of process which results from oxidative stress or other causes. Inflammation is majorly divided into two types: acute inflammation and chronic inflammation. Acute inflammation exists only for a short period of time and is not that much harmful to the host. If the acute inflammation persists for a long time, then it becomes chronic and can lead to serious chronic diseases. Chronic inflammation is characterised by tissue invasion by inflammatory macrophages. The tissue invasion by inflammatory macrophages.[1]

Curcumin is a well-known anti-inflammatory drug derived from the rhizome of *Curcuma longa L.* (Turmeric) belonging to the family *Zingiberaceae.* It is chemically known as s (1E,6E)-1,7-bis (4- hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione. It is relatively insoluble in water, but dissolves in acetone, dimethylsulph oxide, and ethanol. Chronic inflammation is characterised by tissue invasion by inflammatory macrophages results in expression of inflammatory cytokines and growth factors which is directly associated with the pathophysiology of various diseases.[2] Curcumin acts as an anti-inflammatory agent by inhibiting cyclooxygenase (cox-2) and lipoxygenase (cox) are the enzymes which regulates inflammatory process increases in the activity of these enzymes can lead to inflammation.[3]

Metformin is an anti-diabetic agent which belongs to the class of biguanides. it is chemically know as N, N-Dimethyllimidodicarbonimidicdiamide its administered orally in order to decreases the blood glucose concentration in the patients. its acts by increasing the insulin sensitivity and there by decrease the insulin resistance which has occurred in diabetes mellitus patients. [4] The anti-hyperglycaemic effect of metformin results in decreased absorption glucose from the intestine increased uptake of glucose from blood into tissues decreased production of glucose in the liver. based on clinical studies it has been observed that metformin accumulates in the intestinal wall and decreases glucose absorption by intestine Metformin decreases fatty acid oxidation by 10-20 % which indirectly reduces plasma glucose concentration. metformin also causes an increase in blood lactate concentration id increased due to metformin induced conversion of glucose into lactate by intestinal mucosa.[5] Type-2 diabetes mellitus is a global health problem which is closely linked to obesity, unhealthy, diet and physical activity. insulin resistance and irregular insulin secretion are major defects of type-2 diabetes mellitus. it also leads to several microvascular complications like diabetic retinopathy, nephropathy, neuropathy and macrovascular complications such as cardiovascular problems. The typical characters of type-2 diabetes mellitus include malfunctioning of carbohydrates, lipids, proteins metabolism. disruption of pancreatic β-cells leads to type-2 diabetes mellitus. [6]

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Curcumin and metformin which are two well-known drugs which have anti-inflammatory and anti-diabetic activity but when used in combination they exert synergistic effect as potent anti-cancer drug. The combination of these drugs induces apoptosis without affecting the cell cycle in LNCaP prostate cancer cell line.
curcumin exerts anti-cancer activity by interfering with the pathway of cancer cell proliferation through direct and indirect binding to various targets such as transcription factors, DNA, RNA, and various proteins in cell cycle pathway. Metformin also exerts anti-cancerous activity by preventing the proliferation cancerous cells in breast, prostrate, colon, ovary and glioma etc. [7]
High Performance Liquid Chromatography (HPLC) is a powerful analytical technique used to separate, identify, and quantify components in a mixture. It is a form of liquid chromatography where the sample is dissolved in a solvent and passed through a chromatographic column packed with a stationary phase HPLC works on the principle of chromatography, which involves the partitioning of components between a stationary phase and a mobile phase. [8] In HPLC, the stationary phase is typically a finely packed column made of porous particles, while the mobile phase is a liquid solvent that flows through the column under high pressure. The sample is injected into the mobile phase, and as it travels through the column, the different components of the sample interact differently with the stationary phase, causing them to separate based on their properties. [9]
many pharmaceutical formulations are available in single form but not in combination. survey of literature revels that the HPLC methods for curcumin and metformin have been developed and validated according to ICH guidelines in single forms but as per survey there are no methods developed and validated for their combination.[10] thus, the main aim of the work is to develop a simple, accurate, precise, robust and cost-effective method for simultaneous analytical estimation of curcumin and metformin in both APL as well as in tablet dosage form. [11]
Main objective of this study is to develop analytical methods for curcumin and metformin , to validate the developed analytical methods for curcumin and metformin , to estimate simultaneously curcumin and metformin in pharmaceutical dosage form.

1. MATERIALS AND METHODS

1.1*Reagents and Chemicals*

In our HPLC method, solvent used are Acetonitrile and Water of brand Merck. metformin and curcumin taken as gift sample from elegant drugs pvt.Ltd, chalmatti, hubli. the reagents solution and chemicals were collected from the store of KLE College Of Pharmacy, Hubballi.

1.2 Preparation of tablet

The combination tablet formulation of curcumin (50 mg) and metformin (250 mg) were prepared in the college laboratory by wet granulation method. Formulation was prepared for 20 tablets of 500mg each. granules were obtained by sieving the dough mixture initially through sieve no.18 and 22 and then through sieve no. 60. The granules obtained were dried in hot air oven at 40℃ for 10 minutes. [12] Pre-formulation studies such as angle of repose, carr's index, hausner's ratio were performed and the results obtained were found to be satisfactory. Then, the dried granules were compressed using tablet compression machine in the college laboratory to obtain tablets of 500mg each. [13]

1.3 HPLC method development

HPLC method development involves following steps;

- selection of mobile phase: the initial steps in hplc method development is the selection of mobile phase. through survey of literature, we found out that acetonitrile: water (70:30 v/v) is ideal for simultaneous estimation of curcumin and metformin
- preparation of standard stock solution: stock solutions of curcumin and metformin were prepared by dissolving 10mg of each in 10 ml volumetric flask using acetonitrile: water (70:30 v/v) to give 1000 µg/ml solutions of each.
- preparation of working sample solution: stock solutions (SS-2) of both curcumin and metformin were prepared by dissolving 1ml solution from SS-2 of curcumin into a series of five 10ml volumetric flasks. Pipette out 0.2, 0.4, 0.6, 0.8, 1ml solution from SS-2 of metformin into the same volumetric flasks used in the above step. Make up the volume with acetonitrile: water (70:30 v/v) and we get 2,4,6,8 and 10 µg/ml of curcumin and metformin working solutions respectively.
- determination of HPLC method: Through survey of literature, we found out the reverse phase chromatography was ideal for simultaneous estimation of curcumin and metformin . [14]

1.4 HPLC method validation

The developed method for the simultaneous estimation of curcumin and metformin is validated as per the ICH (International Council on Harmonisation)
Guidelines using the following parameters such as linearity and range, specificity, selectivity, LOD, LOQ, system precision, precision, ruggedness, robustness, assay, accuracy.

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1.4.1 Linearity

linearity is determined by by a series of three to five injections of five or more standards. Plot a graph of peak area (or heights) of the calibration standards are usually plotted in the Y-axis against the nominal standard concentration, and the linearity of the plotted curve is evaluated through the value of the co-relation coefficient (r2). Stock Solution-1 (SS-1): Accurately weighed 10 mg Metformin and 10mg Curcumin are transferred into two individual 10 ml volumetric flasks. Add Acetonitrile: water (70:30 v/v) slowly to dissolve the drugs and then make up the volume to 10 ml mark using the same. We get 1000 µg/ml solution. Stock Solution-2 (SS-2): Pipette out 1 ml solution from SS-1 of both metformin and curcumin and transfer it into two individual 10ml volumetric flasks. Make up the volume to 10 ml using Acetonitrile: water (70:30 v/v). We get 100 µg/ml solution.

Working standard solutions: Pipette out 0.2, 0.4, 0.6, 0.8, 1 ml solution from SS-2 of Curcumin into a series of five 10 ml volumetric flasks. Pipette out 0.2, 0.4, 0.6, 0.8, 1 ml solution from SS-2 of Metformin into the same volumetric flasks used in the above step. Now, makeup the volume in all the five volumetric flasks up to 10 ml using Acetonitrile: water (70:30 v/v). Carry out linearity for both Curcumin and Metformin simultaneously at 425 nm and 235 nm for curcumin and metformin respectively

1.4.2 Specificity

The solvent used in our study i.e., Acetonitrile: water (70:30 v/v) was injected at 425 nm and 235 nm and it showed no interferences at these wavelengths. The chromatograms of solvent are depicted

1.4.3 System precision

System precision was carried out using 6 replicates of the upper concentration of linearity for ZOS method for both Curcumin and Metformin. % RSD was calculated and was found to be within 2% as per ICH guidelines. The system precision data of Metformin and Curcumin by HPLC method is depicted in the Table No. 27 and 28 respectively.

2.4.4 ***precision***

The following 2 ways were carried out to check the precision of the developed method, Intraday precision, Interday precision

a) Intraday precision Here 3 replicates of upper, middle and lower concentrations of linearity for both Curcumin and Metformin were prepared on the same day at 3 different time intervals followed by calculation of mean and % RSD which was found to be within 2%. The intraday precision data of Metformin and Curcumin by HPLC method is depicted in Table No. 29, 30 and 31.

b) Interday precision Here 3 replicates of upper, middle and lower concentrations of linearity for both Curcumin and Metformin were prepared on the 3 different days at 3 different time intervals followed by calculation of mean and % RSD which was found to be within 2%.

The interday precision data of Curcumin and Metformin by HPLC method is depicted in the Table No. 32, 33 and 34

2.4.5 Ruggedness

Ruggedness is performed by changing the analyst and the instrument. Here 3 replicates of upper, middle and lower concentrations of linearity for both Curcumin and Metformin were prepared and analysed followed by calculation of mean and % RSD which was found to be within 2%. The ruggedness data of Curcumin and Metformin by HPLC method is depicted in the Table No. 35 and 36.

2.4.6 Robustness

Robustness is performed by changing the mobile phase ratio and taking 3 replicates of upper, middle and lower concentration of linearity followed by calculation of mean and % RSD which was found to be within 2%. The robustness data of Curcumin and Metformin by HPLC method is depicted in the Table No. 37.

2.4.7 Limit of detection (LOD)

The following equation was used to calculate the limit of detection

$$LOD = 3.3\sigma/s$$

Were, σ – Standard deviation of Y-intercept of calibration curve s–Slope of regression equation.

2.4.8 Limit of quantification (LOQ)

The following equation was used to calculate the limit of quantification

$$LOQ = 10\sigma/s$$

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where, σ – Standard deviation of Y-intercept of calibration curve s – Slope of regression equation

2.4.9 Accuracy

The closeness of a measured value to the true or accepted value is defined as accuracy. In practice, accuracy denotes the difference between the mean value discovered and the genuine value.
Here 3 different concentration levels i.e., 50, 100, 150% of both Curcumin and Metformin were taken in 10 ml volumetric flasks to study the accuracy of the developed method followed by determination of % recovery.

3. Results and discussion

3.1. Development and optimization of the method

A simple, precise and robust HPLC method was established for the simultaneous estimation of Metformin and Curcumin. Initially, a solution containing 10 mcg/ml of both Metformin and Curcumin was prepared using methanol: distilled water (50:50 v/v) as the solvent and was run at 800-200 nm range in UV spectrophotometer. The λ_{max} for Metformin and Curcumin were found to be 235 nm and 429 nm respectively. Both Metformin and Curcumin were analysed by HPLC at 235 nm and 425 nm respectively using VWD detector.
Parameters such as mobile phase selection, their composition, flow rate, detection wavelength were optimized for developing effective chromatographic method. Different mobile phase compositions which include methanol, water, acetonitrile were used for trials to get sharp and well separated peaks of both the drugs. Finally, the mobile phase composition of acetonitrile: water (70:30% v/v) provided good results, well defined peaks without any trailing. The column temperature was set to 25°C which provided acceptable results. Flow rate of 1 ml/min was found to be most reliable and acceptable. The retention time was found to be 3.42 and 2.46 minutes for metformin and curcumin respectively. From fig no. it can be understood that both the drugs display good and acceptable peaks without any trailing.

3.2. Method Validation

The aim of analytical method validation is to ensure that the developed method is acceptable. The developed HPLC method was validated as per ICH guidelines for different characteristics such as linearity, precision, accuracy, LOD, LOQ, ruggedness and robustness.

3.2.1 Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. Linearity of the developed method was tested by analyzing different concentrations ranging from 2-10 mcg/ml solutions of metformin and curcumin at 235 nm and 425 nm respectively. The correlation coefficients(R^2) of the both the drugs were found to be satisfactory which suggests good correlation and linearity for the method.

3.2.2. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.
It consists of two categories:
a) Intraday precision:
Intraday precision was carried out to assess the repeatability of the HPLC method throughout a single day. In this, three replicates of lower, middle and upper concentrations (2, 6 and 10 mcg/ml) of both metformin and curcumin were analyzed three times in three phases of a single day i.e. morning, afternoon and evening. To express the variation in the results, % RSD was calculated in each case. The % RSD was found to be less than 2% each case which indicates good reproducibility of the developed method.
b) Interday precision
Interday precision was carried out to assess the repeatability of the HPLC method across different working days. Three replicates of lower, middle and upper concentrations (2, 6 and 10 mcg/ml) of both metformin and curcumin were analyzed three times in three working days. To express the variation in the results, % RSD was calculated in each case. The % RSD was found to be less than 2% each case which indicates good reproducibility of the developed method.

3.2.3. Ruggedness

Ruggedness was carried out to measure the reproducibility of the method under variations in conditions from analyst to analyst. Three replicates of lower, middle and upper concentrations (2, 6 and 10 mcg/ml) of both metformin and curcumin were analyzed twice by two different analysts in order to assess the reproducibility of the developed method under variations in the analyst. The % RSD obtained for ruggedness of the method was founf to be less than 2% indicating excellent reproducibility.

3.2.4. Robustness

Robustness refers to the adaptabilty of the method to remain unaffected by small variations in the parameters such as mobile phase composition. Three replicates of lower, middle and upper concentrations (2, 6 and 10 mcg/ml) of both metformin and curcumin were analyzed in three different acetonitrile: water (mobile phase) compositions: 69:31 v/v, 70:30 v/v and 71:29 v/v which could potentially affect the results to be obtained. The % RSD obtained in each case was found to be less than 2% indicating excellent adaptability of the method to small variations.

3.2.5. Limit of detection

Limit of detection (LOD) is the smallest amount of analyte that can be detected by a method and not necessarily can quantify the analyte. Limit of detection for Metformin and Curcumin by the developed HPLC method was found to be 0.865 mcg/ml and 0.828 mcg/ml respectively.

3.2.6. Limit of Quantification

Limit of quantification (LOQ) is smallest analyte concentration in a sample that can be detected as well as quantified by the developed method with acceptable precision and accuracy. Limit of Quantification for Metformin and Curcumin by the developed HPLC method was found to be 2.62 mcg/ml and 2.509 mcg/ml respectively.

3.2.7. Accuracy

Here, known amount of combined samples of metformin and curcumin were spiked in three replicates of three concentrations i.e. 50, 100 and 150 % of sample solutions of metformin and curcumin (1 mcg/ml) which were previously analyzed and is further analysed by the developed method. The percentage recovery of was found to be in the range of 98-103 % and 97-102 % for metformin and curcumi respectively.

SUMMARY AND CONCLUSION

In the present work of simultaneous estimation of Curcumin and Metformin, UV Zero Order spectroscopic method. Methanol: Distilled water (50:50 v/v) was used as a solvent in the UV ZOS experiment. In UV zero order spectroscopy, both the drugs were simultaneously analyzed at two different wavelengths i.e., Curcumin was analyzed at 429 nm and Metformin at 235 nm using Shimadzu UV-1900i spectrophotometer.

In the method validation, different parameters like linearity, system precision, ruggedness, robustness, precision, accuracy using different concentrations according to ICH guidelines were studied. Results obtained were up to the mark and the work concludes that the method used is simple and precis

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Table 1: Development method parameter for HPLC

Instrument name:	Agilent 1220 infinity II LC
Flow rate:	1 ml/min
Column:	Pursuit 5 C18 150×4.6mm
Mobile phase:	Acetonitrile: water (70:30 v/v)
Injection volume:	20 µl
Detection wavelength:	235nm and 425nm

Table 2: System suitability parameters for HPLC

Parameters	**Results**
Standard concentration	50 µl/ml
Mobile Phase	Acetonitrile: water (70:30 v/v)
Elution	Isocratic
Wavelength	Curcumin:235nm Metformin:425nm
Column	Pursuit 5 C_{18} 150×4.6mm
Flow rate	1ml/min
Peak area	Curcumin:298.065 Metformin:319.715
Retention time	Curcumin:2.468 Metformin: 3.423
Injected sample volume	20µl

Table 3: LINRARITY

Sl. No.	Concentration (µg/ml)	Area obtained at 235nm	Area obtained at 425nm
1	2	298.065	319.715
2	4	579.689	624.710
3	6	793.319	901.030
4	8	1134.035	1095.18
5	10	1337.04	1392.889
r2		0.9949	0.9953
Slope		131.61	130.84
LOD		0.865	0.828
LOQ		2.62	2.509

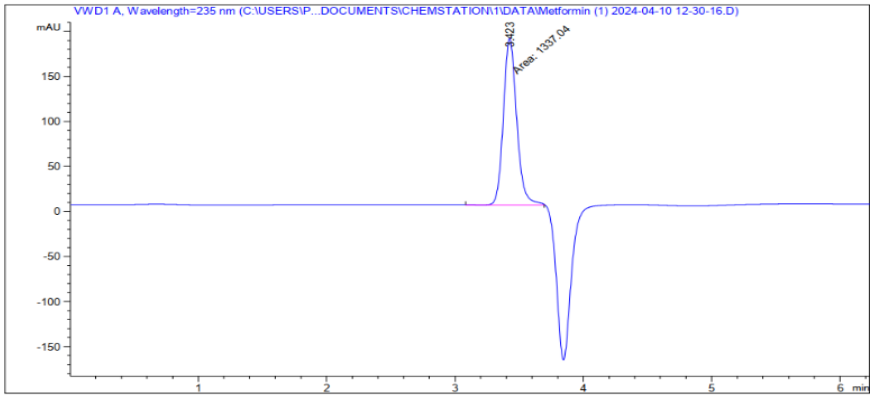

Fig 1: **Linearity- Metformin Chromatogram 2mcg/ml**

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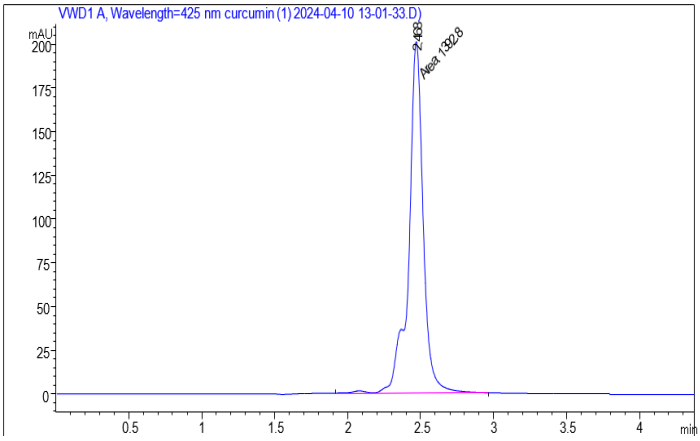

Fig 2: Linearity-Curcumin Chromatogram 2mcg/ml

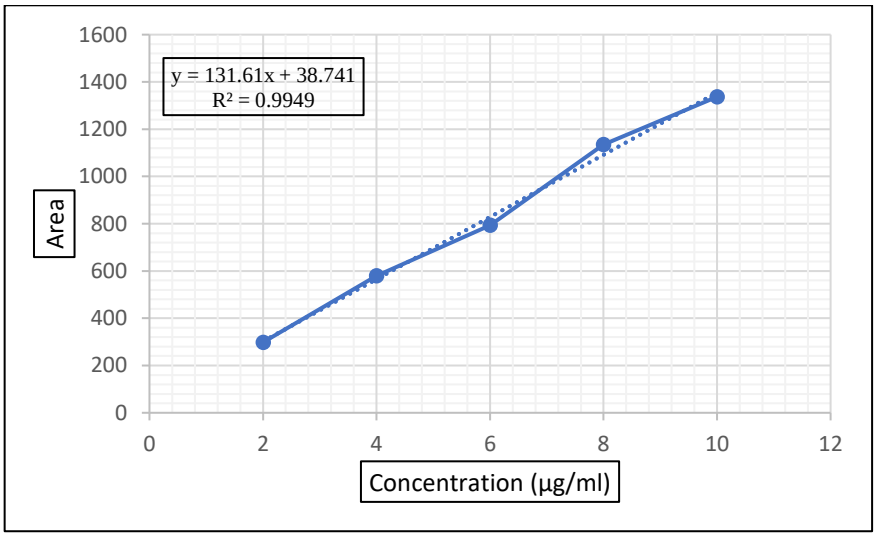

Fig 3: Standard calibration curve of Metformin for HPLC method at 235 nm.

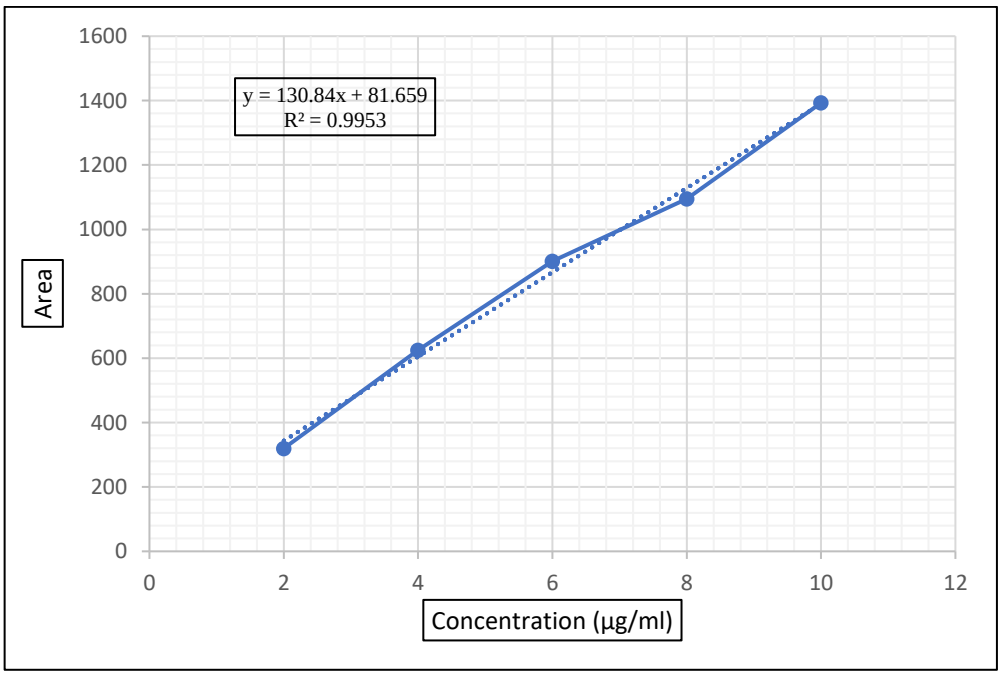

Fig 4: **Standard calibration curve of Curcumin by HPLC method at 425 nm**

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Table 4: System precision of Curcumin

Concentration (µg/ml)	Area obtained (at 425 nm)	Mean	Std Deviation	%RSD
2	325.522	319.399	3.633	1.13
2	317.288			
2	320.102			
2	314.552			
2	319.266			
2	319.665			

Table 5: System precision of Metformin

Concentration (µg/ml)	Area obtained (at 235 nm)	Mean	Std Deviation	%RSD
2	293.884	296.117	2.586	0.873
2	298.118			
2	292.915			
2	296.021			
2	295.889			
2	299.877			

Table 6: Intraday Precision (After 1hr) of Curcumin and Metformin

Concentration (µg/ml)	Area Mean*		Standard Deviation		%RSD	
	235 nm	425 nm	235 nm	425 nm	235 nm	425 nm
2	295.97	320.30	3.9	3.89	1.52	1.21
6	790.355	905.46	6.305	8.31	0.79	0.91
10	1342.26	1396.19	14.36	15.07	1.07	1.07

Table 7: Intraday Precision (After 4hr) of Curcumin and Metformin

Concentration (µg/ml)	Area Mean*		Standard Deviation		%RSD	
	235 nm	425 nm	235 nm	425 nm	235 nm	425 nm
2	295.97	320.30	3.9	3.89	1.52	1.21
6	790.355	905.46	6.305	8.31	0.79	0.91
10	1342.26	1396.19	14.36	15.07	1.07	1.07

Table 8: Intraday Precision (After 8hr) of Curcumin and Metformin

Concentration (µg/ml)	Area Mean*		Standard Deviation		%RSD	
	235 nm	425 nm	235 nm	425 nm	235 nm	425 nm
2	297.11	319.21	5.03	4.96	1.69	1.55
6	791.93	902.91	7.29	8.59	0.91	0.95
10	1342.11	1394.36	11.13	16.79	0.82	1.20

Table 9: Interday Precision of Curcumin and Metformin (2µg/ml)

Concentration (µg/ml)	Day	Area Mean*		Standard Deviation		%RSD	
		235 nm	425 nm	235 nm	425 nm	235 nm	425 nm
2	Day 1	297.17	319.21	5.03	4.96	1.69	1.55
	Day 2	296.27	319.44	4.84	3.6	1.63	1.12
	Day 3	296.36	321.98	4.72	3.12	1.59	0.97

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Table 10: Interday Precision of Curcumin and Metformin (6µg/ml)

Concentration (µg/ml)	Day	Area Mean*		Standard Deviation		%RSD	
		235 nm	425 nm	235 nm	425 nm	235 nm	425 nm
6	Day 1	791.93	902.91	7.29	8.59	0.91	0.95
	Day 2	781.15	902.02	4.93	8.623	0.625	0.956
	Day 3	786.83	899.006	5.93	8.61	0.753	0.958

Table 11: Interday Precision of Curcumin and Metformin (10µg/ml)

Concentration (µg/ml)	Day	Area Mean*		Standard Deviation		%RSD	
		235 nm	425 nm	235 nm	425 nm	235 nm	425 nm
10	Day 1	1342.11	1394.36	11.13	16.79	0.82	1.20
	Day 2	13337.77	1386.47	13.88	15.68	1.03	1.12
	Day 3	1333.10	1382.79	14.42	23.59	1.08	1.69

Table 12: Ruggedness data of Curcumin and Metformin (Analyst 1)

Concentration (µg/ml)	Area Mean*		Standard Deviation		%RSD	
	235 nm	425 nm	235 nm	425 nm	235 nm	425 nm
2	296.27	319.44	4.84	3.6	1.63	1.12
6	789.159	902.02	4.936	8.623	0.62	0.956
10	1337.77	1286.47	13.88	15.68	1.03	1.12

Table 13: Ruggedness data of Curcumin and Metformin (Analyst 2)

Concentration (µg/ml)	Area Mean*		Standard Deviation		%RSD	
	235 nm	425 nm	235 nm	425 nm	235 nm	425 nm
2	296.36	321.98	4.72	3.12	1.59	0.97
6	786.837	899.006	5.93	8.61	0.753	0.95
10	1333.10	1382.79	14.42	23.59	1.08	1.69

Table 14: Robustness data of Metformin

Mobile phase (acetonitrile: water v/v)	Concentration (µg/ml)	Area Mean*	Standard Deviation	% RSD
69:31	2	299.55	3.48	1.16
	6	787.18	7.39	0.93
	10	1331.06	12.49	0.93
70:30	2	296.36	4.72	1.59
	6	786.837	5.93	0.753
	10	1333.10	14.42	1.08
71:29	2	298.40	5.23	1.75
	6	787.73	8.42	1.07
	10	1333.49	10.25	0.77

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Table 15: Robustness data of Curcumin

Mobile phase (acetonitrile: water v/v)	Concentration (µg/ml)	Area Mean*	Standard Deviation	% RSD
69:31	2	320.24	4.42	1.38
	6	896.20	6.95	0.77
	10	1380.51	18.40	1.33
70:30	2	320.98	3.12	0.97
	6	899.006	8.61	0.958
	10	1382.79	23.59	1.69
71:29	2	321.64	6.03	1.87
	6	897.68	9.55	1.06
	10	1391.2	25.95	1.80

Table 16: Accuracy data of Metformin

Total Conc. (µg/ml)	Standard Conc. (µg/ml)	Sample Conc. (µg/ml)	Average Area* (235 nm)		Conc. (µg/ml)	Conc. difference (µg/ml)	% Recovery
			Standard	Sample			
2 (50%)	1	1	298.065	300.667	2.01	1.01	101
4 (100%)	1	3	579.689	580.598	4.00	1.00	100
6 (150%)	1	5	793.319	793.543	5.99	0.99	99

Table 17: Accuracy data of Curcumin

Total Conc. (µg/ml)	Standard Conc. (µg/ml)	Sample Conc. (µg/ml)	Average Area* (425 nm)		Conc. (µg/ml)	Conc. difference (µg/ml)	% Recovery
			Standard	Sample			
2 (50%)	1	1	391.715	391.73	2.00	1.00	100
4 (100%)	1	3	624.710	623.657	3.98	0.98	98
6 (150%)	1	5	901.030	903.039	6.00	1.00	100

*Area mean is the average of 3 replicates of each concentration.

ABBREVIATIONS

ICH - International Conference on Harmonisation
µg/ml - Microgram Per Millilitre
R^2 value - Correlation coefficient
% RSD - Percentage Relative Standard Deviation
λ_{max} - Lambda maximum (Absorption maxima)
SS1 - Stock Solution-1
SS2 - Stock Solution-2
LOD - Limit of Detection
LOQ - Limit of Quantification
Std. - Standard
RP-HPLC - Reverse phase- High Performance Liquid Chromatography
LC - Liquid Chromatography
Conc. - Concentration
pKa - Acid dissociation constant