Development and Validation of RP-HPLC Method for Simultaneous Qualitative and Quantitative Estimation of Curcumin and Quercetin in Bulk Mixture

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ABSTRACT

Objectives: To design a definite, new and explicit reverse phase high performance liquid chromatographic (RP-HPLC) method for simultaneous estimation of quantitative and qualitative curcumin and quercetin in bulk mixture. As per guidelines of International conference on harmonization (ICH) method was validated. **Materials and Methods:** In this study, reverse phase analysis was used to do the analysis Younglin- ACME 9000, C_{18} (250 x 4.6mm i.d) solvent system methanol and 0.05% orthophosphoric acid as mobile phase in proportion of 80:20 v/v was used. The flow rate at constant rate 0.7ml/min. The absorbance of the eluted sample was analyzed by use of UV Visible detector at 254 nm. **Results:** The proposed improved methodology takes 5.25 min to elute curcumin and 8.78 min to elute quercetin. The system suitability parameters were studied for the developed method and were found to be within the acceptable limits. The LOQ and LOD of curcumin was reported to be 0.041 and 0.081g/ml, respectively, while quercetins were 0.0048 and 0.039g/ml. **Conclusion:** The designed methodology validated as per the ICH guidelines for its linearity, precision, specificity, sensitivity, accuracy, raggedness, robustness, LOD, and LOQ. The results of the preceding observations show that the procedure is effective in both qualitative and quantitative analyses of the markers in the mixture.

Key words: RP-HPLC, Curcumin, Quercetin, Validation, Qualitative, Quantitative.

INTRODUCTION

Curcumin is a polyphenolic compound obtained from turmeric (*Curcuma longa*, family Zingiberaceae) and has long been known for its medicinal effects.[1-3] It has attracted medical and scientific attention because it deals with the management of oxidative and inflammatory disorders like cancer, metabolic syndrome and arthritis.[3-7] Its antioxidant and anti-inflammatory qualities are responsible for the majority of these benefits. Curcumin consumption on its own does not result in allied health advantages due to its low blood levels, because of inadequate absorption, rapid metabolism, and rapid excretion. Bioavailability can be boosted by a variety of ingredients. Piperine, for example, is the main active ingredient in black pepper and has been known to improve bioavailability by 2000% when coupled with curcumin. Curcumin, in combination with boosting mediators, has a number of health effects.[8-15]

Quercetin is most significant bioflavonoids existing in about twenty plants and which is recognized due to its antihypertensive, anti-inflammatory, vasodilator properties, anti-obesity and anticholestermic actions.[16-26] The word "quercetin" comes from the Latin word "quercetum," which meaning "oak forest, belongs to the flavanol class of compounds that cannot be synthesized in the human body. It is yellow in color and is poorly in hot water soluble, alcohol soluble in a moderate amount and lipids and insoluble in cold water.[27-31] The chemical name of quercetin is 2-(3,4-dihydroxyphenyl)-3,5,7-
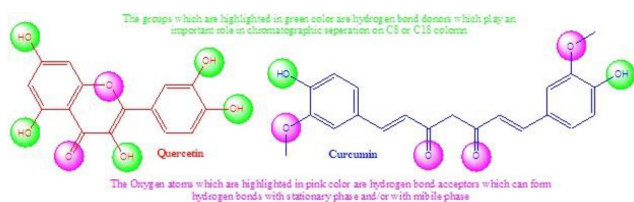
Figure 1: The structures of Curcumin and Quercetin with hydrogen bond donors and acceptors group highlighted in green and pink color respectively.

trihydroxychromen-4-one. The structures of curcumin and quercetin are represented in Figure 1.

Both phyto constituents rarely found in single plant together but numerous nutraceutical formulation contains combination of these compounds. There have been many methods are reported for the analysis of these drugs in bulk majorly on UV Visible spectroscopy.[32-34] HPTLC (high performance thin layer chromatography) and HPLC (high performance liquid chromatography) are two types of chromatography are commonly used methods for quantitative estimation of the curcumin and quercetin.[34-45] There is no any analytical method was reported previously for simultaneous estimation of curcumin and quercetin. In present work, we have developed simple, precise, optimized and validated the RP-HPLC method for simultaneous quantification of curcumin and quercetin in bulk. The approach was validated according to the International Conference on Harmonization's requirements (ICH). This latest validated process will be extended to industry and academics.

MATERIALS AND METHODS

Chemical and Reagents

Curcumin (99%) was obtained as a gift sample from Sun Pure Extract in New Delhi, while quercetin was obtained from Otto Chemicals in Marine Lines, Mumbai, and HPLC grade methanol, water, and orthophoshopric acid were obtained from Avi Chemicals in Mumbai.

Instruments

A HPLC system consisting of Younglin (S.K) Gradient System, Column C_{18} (250 x 4.6mm i.d), and size packing of 5 μm, detector UV 730 D, software-Autochro-3000, and pump SP930 D were used for the method development, data analysis, and interpretation.

Selection of Wavelength

Adequate wavelength for chromatographic separation was calculated by recording UV spectrum in the range of 200-400 nm for curcumin and quercetin. The UV overlain

spectra of these two markers revealed that medications absorbed at 254 nm and 256 nm, respectively, although 254 nm was chosen as the measuring wavelength for the HPLC study since it provided greater resolution. The overlain UV spectra of both the drugs is represented in Figure 2.

Chromatographic Parameter

Reverse phase on a C_{18} column (250 × 4.6mm i.d, particle size 5 μm) was used to develop the process. After running many trials, a solvent system methanol: 0.05% orthophosphoric acid (80:20) with 0.7 ml/min flow rate and 20 μl sample size had provided better chromatographic separation with good resolution. The analysis was performed at 254 nm wavelength and at ambient temperature.

Preparation of Standard Stock Solutions

1 mg of pure medicine was dissolved in 10 ml of methanol to make stock solutions of curcumin (0.1 mg/ml) and quercetin (0.1 mg/ml).

Sample Preparation

Specific aliquots from stock solutions were used to make the sample solutions. To make 10 g/ml of sample solution, 0.1 ml of each stock solution was pipetted out and diluted separately with methanol up to 10 ml.
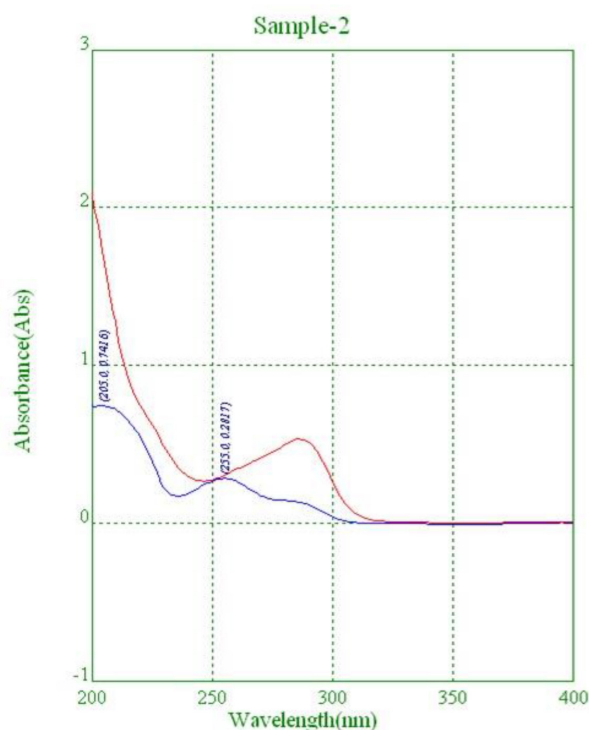
Figure 2: The isobestic point of both the drugs in UV Visible Spectroscopy at 254 nm.

Likewise, the remaining sample solutions were made in the 10 g/ml to 50 g/ml range.

Analytical method validations

The developed RP-HPLC method of curcumin and quercetin was validated as per the ICH guidelines. The method was tested with linearity, precision, specificity, sensitivity, accuracy, raggedness, robustness, limit of quantification (LOQ), and limit of detection (LOD).

Application of developed analytical method

Preparation of plasma samples

To 100 μl of curcumin standard solution and 100 μl of quercetin standard solution, 100 μl of plasma sample, were spiked and added extraction solvent 2 mL of acetonitrile was added and vortexed mixture for 20 min. This sample was ultracentrifuged at 10,000 rpm for 10 min. The supernatant layer was collected and 20 μl was analyzed by HPLC system. The whole procedure was carried out at room temperature.

Estimation of Pharmacokinetic method

Suspension of both compounds in sterile water for injection was administrated to mice (*n*=3) intravenously and blood sample was collected from retro-orbital vein into heparinized bulb at different time intervals 5, 10, 30, 45, 60, 120, 240, 480, 1440 mins to 24 hr. Heparinized blood samples were centrifuge at 5000 rpm for 15 min to obtain plasma same was stored at -80°C till further analysis. Pharmacokinetic parameters were calculated by Kinetica software. The highest plasma drug concentration (C_{max}) and time of highest plasma drug concentration (t_{max}) were determined from plasma concentration vs time curve. Area under the curve (ACU_{0-t}) for curcumin and quercetin plasma concentration v/s time zero to 24 h was also calculated.

RESULTS AND DISCUSSION

The RP-HPLC method was designed with the goal of estimating curcumin and quercetin in bulk, and system suitability criteria such as peak resolution factor, tailing factor, number of theoretical plates, runtime, and cost effectiveness were considered.

Curcumin elution took 5.25 min while quercetin took 8.78 min using the developed improved procedure. The produced chromatogram of curcumin and quercetin is shown in Figure 3. The system suitability characteristics are shown in Table 1. Curcumin and quercetin were discovered to have 3255 and 4266
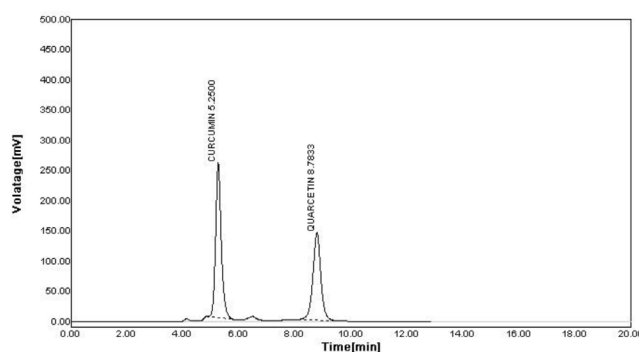

Figure 3: The developed chromatogram of the optimized method for curcumin and quercetin.

Table 1: The system suitability parameters and results.

Parameters	Acceptance limits	Curcumin	Quercetin
Retention time (min)	---	5.25	8.78
Resolution factor	Not less than 2	0.00	6.62
Number of theoretical plate	Not less than 2000	3255	4266
Tailing factor	Not more than 2	1.23	0.95

theoretical plates, respectively. Each trial took 15 min to complete.

Method Validation

Specificity

The developed chromatogram of the optimized method for curcumin and quercetin for standard drug solutions, shown in Figure 3, reveals that the peaks obtained in the standard solutions at working concentrations are only due to the drugs, as the blank has no peak at the retention time of curcumin and quercetin. As a result, it is possible to conclude that the established approach is specific.

Linearity

The detection wavelength was identified by preparing 10ppm solution of curcumin and quercetin in methanol. The study was carried out at 254 nm for both the drugs which had shown better response. The linearity of the curve of peak area versus curcumin and quercetin concentrations (10- 50 g/ml) was determined. The correlation coefficients (R^2) for each drug were greater than 0.99, which passed the technique validation acceptance criteria, indicating that the method is linear. The calibration curves of curcumin and quercetin
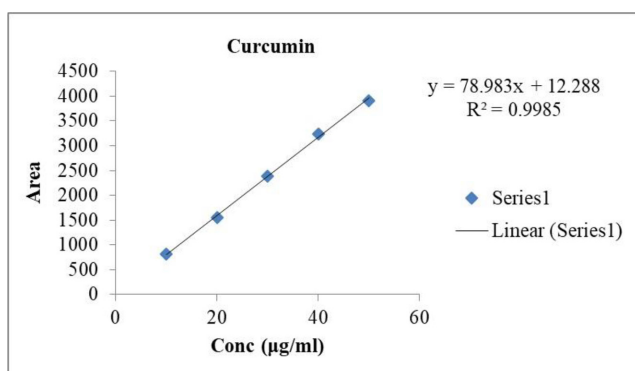
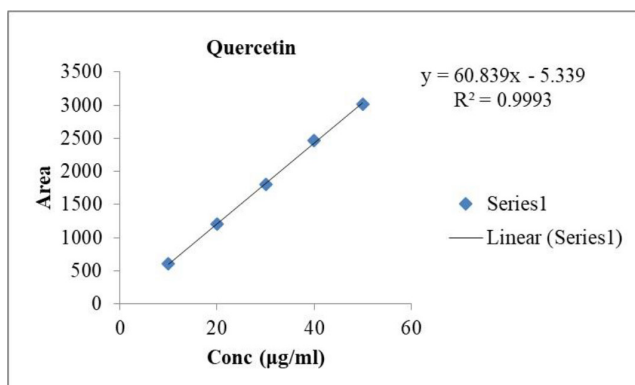**Figure 4: The calibration curve of curcumin at 254 nm (10- 50 µg/ml).**

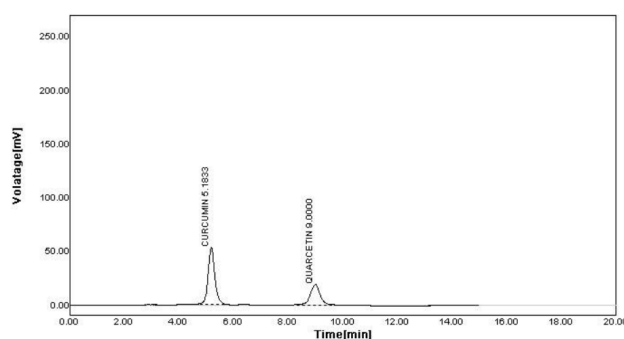**Figure 5: The calibration curve of quercetin at 254 nm (10- 50 µg/ml).**

Figure 6: The chromatogram of curcumin and quercetin in bulk at 10 µg/ml.

precision, the same concentration is injected in three different vials. Intraday precision (10, 30, 50 g/ml) of curcumin and quercetin were tested to see if they were repeatable, as well as interday variation for intermediate precision (10, 30, 50 g/ml) under the same conditions. The relative standard deviation (RSD) and percentage standard deviation (SD) were calculated and found to be within the allowed ranges. The chromatograms obtained as interday precision for 10, 30 and 50 µg/ml
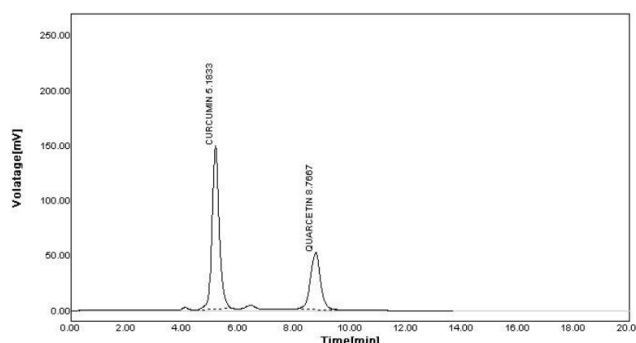

Figure 7: The chromatogram of curcumin and quercetin in bulk at 30 µg/m.
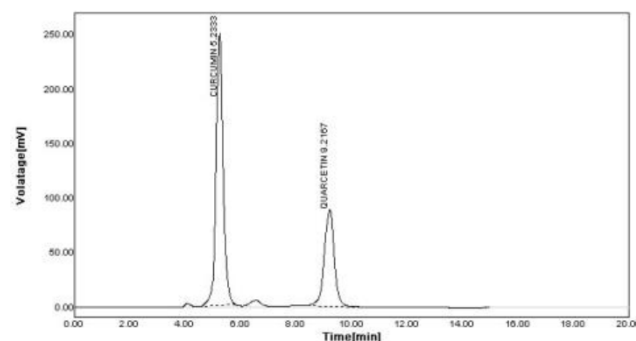

Figure 8: The chromatogram of curcumin and quercetin in bulk at 50 µg/ml.

are represented in Figures 4 and 5 respectively. These Figures also represents the regression equations.

Precision

System precision, method precision, intraday precision, and interday precision were all investigated in the precision research. In system precision, the same concentration is injected six times, while in method

Table 2: The precision results of optimized method.		
Compound	**Repeatability (*n*=6) Mean (µg/ml) ± Standard Deviation**	**RSD (%)**
Curcumin	50.39±0.43	0.86
Quercetin	50.48±0.40	0.78
	Intermediate precision (*n*=6)	
Curcumin	30.14±3.99	0.17
Quercetin	29.99±1.16	0.06
	Reproducibility (*n*=10)	
Curcumin	49.46±1.71	0.04
Quercetin	49.39±1.14	0.04

are represented in Figures 6, 7, and 8 respectively. The precision data of the optimized method is represented in Table 2.

Accuracy

Recovery studies were used to determine accuracy, with the percent mean recovery of each chemical in the bulk measured at three distinct levels (80 percent, 100 percent and 120 percent). As stated in Table 3, the percent mean recovery was determined. The approved mean recovery limits were 98-102 percent, and all of the observed data fell within this range, indicating good recovery values and confirming the accuracy of the established approach.

Sensitivity

It is limit for reliable method to detect minimum level of analyte. Lower level of curcumin and quercetin were evaluated by signal to noise ratio. The smallest concentration of analyte that yields an accurate

response but cannot be quantified is known as the limit of detection (LOD). The smallest quantity of analyte that yields a correct response is known as the limit of quantitation (LOQ). The signal-to-noise ratio was used to calculate LOD and LOQ. The LOD value found for curcumin and quercetin were 0.041 μg/ and 0.0048 μg/ml respectively while LOQ value were found to be 0.082 and 0.039 μg/ml respectively. The LOD, LOQ, and calibration curve parameters are represented in Table 4.

Robustness and ruggedness

These include the ability to overcome adverse instrument, operator, and chromatographic circumstances such as flow rate, pH, column temperature, and wavelength variations. Small purposeful adjustments in the wavelength were used to determine robustness. For analysis, the wavelength was first set to 254 nm, but it was later altered to 221 nm. Second, the flow rate was reduced from 0.7 to 0.5 ml/min. The system's toughness

Table 3: The recovery data of the drugs in bulk.							
Sr. No.	% drug Added	Amount of drug added	Area	Amount Received	% Recovery	Mean	% SD	% RSD
1	80	0.8	150.41	0.79	99.98	99.49	0.72	0.72
			151.22	0.80	100			
2	100	1.0	168.14	0.99	99.81	100.41	0.84	0.84
			169.63	1.1	101.00			
3	120	1.2	184.51	1.18	98.33	99.37	1.47	1.48
			187.36	1.22	100.01			

Table 4: The calibration curve parameters, limit of detection (LOD), limit of quantification (LOQ) for curcumin and quercetin.					
Drug	Calibration curve equation	Correlation coefficient (R^2)	Linear range (μg/ml)	LOD (μg/ml)	LOQ (μg/ml)
Curcumin	y=78.983x+12.288	0.9985	10-60	0.041	0.082
Quercetin	y= 60.839x-5.339	0.9993	10-60	0.0048	0.039

Table 5: The robustness and ruggedness data for curcumin and quercetin.						
Method type	Wavelength (nm)		Conc. of sample (μg/ml)	Mean	% SD	% RSD
	Curcumin	Quercetin				
Developed Method	254	254	2	476.34	5.73	1.20
Changed Method	221	221	2	486.06	6.17	1.27
Flow Rate (ml/min)						
Developed Method	0.7	0.7	2	482.52	3.97	0.82
Changed Method	0.5	0.5	2	429.97	3.80	0.88
Ruggedness						
Operator 1	254	254	2	468.01	10.78	2.3
Operator 2	254	254	2	449.86	4.00	0.89

Table 6: Pharmacokinetic parameters of Curcumin and Quercetin after intravenous administration of suspension in mice.

Pharmacokinetic parameters	Results	
	Curcumin	Quercetin
C_{max} (μg/ml)	7.534	8.899
T_{max} (min)	10	45
AUC_{0-t} (μg. min /ml)	61.434	131.433

was tested by switching the operator. No, after altering the parameters, there were further changes. It denotes that the procedure has been tested. Table 5 depicts the robustness and roughness of curcumin and quercetin.

Application of the developed method

The above method was successfully applied for the pharmacokinetic studies of curcumin and quercetin in plasma of mice.

The Cmax, highest plasma drug concentration and T_{max}, the time of highest plasma drug concentration were found to be 7.534 μg/ml and 10 min for curcumin and The C_{max}, highest plasma drug concentration and T_{max}, the time of highest plasma drug concentration were found to be 8.899μg/ml and 45 min for quercetin. Area under the curve (AUC_{0-t}) found to be 61.434 μg. min /ml for curcumin and 131.433 μg. min /ml for quercetin. (Table 6).

CONCLUSION

For the simultaneous quantification of curcumin and quercetin, the RP-HPLC method reported here is accurate, sensitive, precise, and repeatable. It is cost effective method. It can be utilized for quality control analysis on a regular basis. of curcumin and quercetin in individual or in combination with other Phytophenols as per the regulatory guidelines. With the use of a C_{18} column, the established approach allows for good separation of curcumin and quercetin in bulk mixtures. Using a solvent system of methanol:0.05 percent orthophosphoric acid (80:20) at ambient temperature and a detection wavelength of 254 nm, the flow rate was 0.7 ml/min with a run time of 15 min. Curcumin elution took 5.25 min while quercetin took 8.78 min using the developed improved procedure. The developed method's system suitability parameters were investigated and determined to be within acceptable ranges. Curcumin's LOD and LOQ were reported to be 0.041 and 0.081g/ml, respectively, while quercetin were 0.0048 and 0.039g/ml, respectively. The linearity,

precision, specificity, sensitivity, accuracy, raggedness, robustness, LOD, and LOQ of the devised method were all validated according to ICH recommendations. The results of the preceding observations show that the procedure is effective in both qualitative and quantitative analysis of the in the mixture. The developed method was successfully applied for estimation of pharmacokinetic parameters in swiss albino mice.

ACKNOWLEDGEMENT

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

RP-HPLC: Reverse Phase High Performance Liquid Chromatography; **LOQ:** Limit of Quantitation; **LOD:** Limit of Detection.
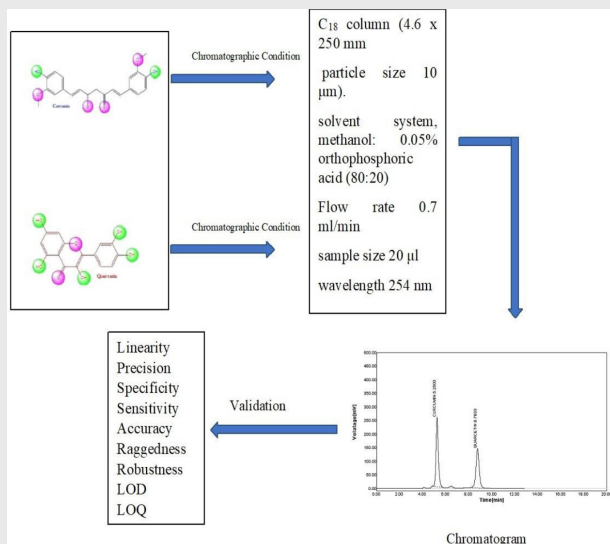
Authors Contributions

Ms. Nayana Patil: Laboratory work and manuscript drafting; **Dr. Hitendra Mahajan:** Concept building, data interpretation and final review.

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PICTORIAL ABSTRACT

Chromatographic Condition

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C$_{18}$ column (4.6 x 250 mm particle size 10 μm).

solvent system, methanol: 0.05% orthophosphoric acid (80:20)

Flow rate 0.7 ml/min

sample size 20 μl

wavelength 254 nm

Validation

Linearity
Precision
Specificity
Sensitivity
Accuracy
Raggedness
Robustness
LOD
LOQ

Chromatogram

SUMMARY

RP-HPLC method for simultaneous estimation of Curcumin and Quercetin was developed. The developed method enables the excellent separation of curcumin and quercetin in bulk mixture. The cleared resolution of two phytochemicals was obtained. The final chromatographic conditions are validated to establish accuracy, precision, LOD and LOQ of the method. Developed analytical method successfully employed for estimation of pharmacokinetic parameters