Research Article

UV Spectrophotometric and HPLC Method Development of Quercetin and Curcumin in Polyherbal Churna and It's Validation

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Article info	Abstract
	A simple, rapid, sensitive, precise, economic and reproducible RP-HPLC method for the quantitative determination of curcumin and quercetin in *Madhujeevan churna(MJC)* was developed and validated. The hydroalcoholic extract of *churna* was obtained by continuous heat extraction method. The extract was considered as sample. Standard curcumin was analyzed using Agilent LC-P-1120 with a HiQ Sil C-18 column by isocratic elution using methanol: acetonitrile: phosphate buffer (pH 5) in the ratio of 42.5 : 42.5: 15 % v/v/v as the mobile phase. The flow rate was 1.2 ml/ min and detection was set at 265 nm as isobestic point.. The method was validated using parameters as linearity, precision, limit of detection, limit of quantification, and recovery as per ICH guidelines. It obeys Beers- Lamberts law in concentration ranges employed for evaluation. The recovery of the method was in the range of 98.50-99.40 % and all the compounds showed good linearity (r = 0.98332) in a relatively wide concentration range. The concentration of quercetin and curcumin present in raw material of madhujeevan churna was found to be 0.1347%w/w and 0.208%w/w respectively in *Madhujeevan churna(MJC)*. The result of analysis has been validated statistically and recovery studies confirmed the accuracy of the proposed method. Hence, the proposed method can be used for the reliable quantification of active marker compound in crude drug and its herbal formulations.

1. INTRODUCTION

The world health organization (WHO) has emphasized the need to ensure the quality of medicinal plant products by using modern controlled technique and applying suitable standards[1]. The collection, identification of crude drug, preparation and evaluation of herbal medicine is challenging era. Drugs made by them were more efficacious, authentic and genuine. In the present age the suppliers make the collection. There are so many drugs, which lost their effectiveness with the passage of time. This causes the lowering the genuine character of the drug and make less efficacious. Until and unless, a method is not being developed to check the adulteration, it is too difficult to achieve the prestigious stage of herbal medicine. The checking of herbal drugs used in the preparation can be checked scientifically through certain well-established norms and standards through the research works**.**[2] For standardization of natural products, crude drugs, single chemical entities, "marker compounds", may be used as potency standards in U.V and HPLC analysis.[3]

Madhujeevan churnam(MJC) consist of seven ingredients as *Curcuma longa, Aegle marmelos, Azardichata indica, Emblica officinalis Salacia reticulate, Syzygium jambulanum and Stevia rebaudiana*. Curcumin is chemically, (1*E*, 6E)-1, 7-bis (4-hydroxy-3-methoxy phenyl) -1, 6-heptadiene-3,5-dione. It is the principal curcuminoid of the popular Indian spice turmeric, which is a member of the ginger family (*Zingiberaceae*).. Curcumin have been reported for many activities. Quercetin is chemically, 2-(3,4-dihydroxy phenyl)-3,5,7-trihydroxy-4*H*-chromen-4-on quercetin , a flavonol, is a plant-derived flavonoid found in fruits, vegetables, leaves and grains. Quercetin has been used to treat many diseases.

Literature survey reveals that several methods as U.V.[4, -6], HPLC[7, 8],

TLC[9, 10], and electrochemical determination of quercetin[11]; have been reported for estimation of curcumin and quercetin separately. Spectrofl1urometric estimation like U.V[13-19] ,   HPLC[14-16,20,21] and HPTLC[17-19, 22-29] have been reported for estimation of curcumin. No simple and accurate RP-HPLC method has been reported for simultaneous estimation and qualitative examination of curcumin and quercetin. Not a single U.V., HPLC or HPTLC method is reported so far for simultaneous analysis of curcumin and quercetin in herbal dosage form.

2. MATERIALS AND METHODS

2.1 Reagents and Materials

Ayurvedic Proprietary –MJC was procured as Gift sample from Dr. Wachasundar, Aniket Clinic, Magalwarpeth, karad. Dist- satara (Maharashtra).

All the chemicals and solvents were used of A. R. grade; standard. Curcumin and Quercetin was procured as gift sample from SDFCL, S.D. fine-chemicals limited, Mumbai.

Instrument used was an UV/Visible double beam spectrophotometer, SHIMADZU model 1800 (Japan) with spectral width of 2 nm, wavelength accuracy of 0.5 nm and a pair of 10 mm matched quartz cell was used to measure absorbance of all the solutions. An electronic analytical balance was used for weighing the sample.

2.2 Preparations of extract of madhujeevan churna (MJC)

The 300g of *Madhujeevan churna (MJC)* was extracted with a mixture of 95% ethanol and water (75:25) at 50-60^{0}C in a Soxhlet apparatus separately[20, 30-37]. The extract obtained was concentrated to dryness in heating mental at a temperature of 35-40^0. The dried of the hydroalcoholic extracts weighed in a required dose and it was dissolved in known volume of distilled water, separately for further treatment.

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2.3 Preparation of standard stock solution of curcumin and quercetin

Further series of dilution were made with ethanol. The stock solution (100μg/ml) of Quercetin and curcumin were prepared by dissolving accurately about 10mg of each drug in sufficient quantity of mobile phase and then volume was adjusted to 100ml with mobile phase, further series of dilution are made with mobile phase.

2.4 Sample preparation

The hydroalcoholic extract was dissolved in 50 % methanol in a volumetric flask.

2.5 Buffer and mobile phase preparation

2.5.1 Buffer preparation

10 mM phosphate buffer was prepared by adding 1.360 gm potassium dihydrogen phosphate in 1000 ml double distilled water. The solution was filtered through 0.45μm filter and sonicated for 15 min.

2.5.2 Preparation of mobile phase

Mobile phase was prepared by mixing methanol: Acetonitrile : phosphate buffer in the ratio of 42.5 : 42.5: 15 % v/v/v. pH of mobile phase was adjusted to 5 with othophosphoric acid.

2.5.3 Degassing of the mobile phase

The prepared mobile phase was degassed by ultrasonication for 20 min, so as to avoid the   disturbances caused by dissolved gases.

2.5.4 Filtration of mobile phase

The degassed mobile phase was filtered through 0.45μ filter to avoid the column clogging due to smaller particles

2.6 Calibration curves

Marker compounds, curcumin and quercetin were accurately weighed and dissolved in 50 % methanol to give serial concentrations within the range of 0.5-50 μg mL-1. The calibration curves were obtained from peak areas of the standard solutions over the concentrations. The content of the markers were determined using a calibration curve established with six dilutions of each standard, at concentrations ranging from 10 to 100 μg/ml. Each concentration was measured in triplicate. The corresponding peak areas were plotted against the concentration of the markers injected. Peak identification was achieved by comparison of both the retention time (Rt) and UV absorption spectrum for standards.

2.7 Chromatographic conditions

Analyses were carried out using an Agilent LC-P-1120 equipped with pump, degasser, photo diode array (PDA) detector and auto-sampler. The generated analytical signals were monitored and integrated using E Z chrome Elite.The chromatographic separation was performed using C_{18} Intersil, 4.6 (i.d.) x 250 mm at a column oven temperatures of 35°C. The method development for simultaneous estimation of curcumin and quercetin was performed using (a) various solvent [i.e. acetonitrile and methanol]; (b) various buffer-solvent ratio [i.e. solvent A (0.1%v/v ortho phosphoric acid aqueous solution) and solvent B (acetonitrile) at the ratio of 40:60, 50:50, 60:40 and 70:30]. 0.1%v/v ortho phosphoric acid aqueous solution was prepared by adding 1mL of ortho phosphoric acid in 1000 mL beaker and about 900 mL of Milli-Q water was added, which was sonicated for 5 minutes and volume was made up to 1000 mL to get 0.1%v/v ortho phosphoric acid and the pH was adjusted to 3.0 by triethylamine; The final mobile phase optimized was methanol: acetonitrile: phosphate buffer (pH 5) in the ratio of 42.5 : 42.5: 15 % v/v/v(c) at various flow rate [i.e. 0.8, 1.0 and 1.2 mLmin-1]; and (e) various elution mode [i.e. isocratic and gradient]. The developed method was validated for system suitability, accuracy, precision, limit of detection (LOD), limit of quntification (LOQ), linearity, range and robustness in accordance with ICH guideline.

Linearity was evaluated at five concentration levels in the range between 10-150 μgmL-1 for curcumin and quercetin The concentration was then plotted against its corresponding peak area and the linearity was determined using least square regression analysis. System suitability parameters were studied to verify the system performance. Six replicate quality control (QC) samples containing curcumin (100μgmL-1) and quercetin (100μgmL-1) were

analysed using the developed method. The accuracy of the developed method was established using recovery studies. Three replicate QC samples containing known amount of curcumin and quercetin at 50, 100 and 150μgmL-1 were spiked into pre-analysed samples (100μgmL-1 of curcumin and 100μgmL-1 of quercetin), which were analyzed using the developed method and the percentage recovery was taken in to consideration for testing accuracy. Precision of the developed method was studied using six replicate QC samples containing curcumin (100μgmL-1) and quercetin (100μgmL-1), which were analyzed using the developed method within a short period of time on the same day and repeatability was taken in to consideration for testing precision. LOD and LOQ of curcumin and quercetin in the developed method were studied using the signal-to-noise ratio; calibtration curve was taken into account. The LOD and LOQ were separately determined which is based on calibration curve. The S.D. of y intercept of regression line may be used as S.D.

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

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

Where, σ = Standard deviation of y intercept of regression lines,
S = Slope of calibration curve.
Robustness was performed with known concentration of curcumin (100μgmL-1) and quercetin (100μgmL-1) using the developed method with negligible but deliberate change in the buffer-solvent ratio, flow rate, column temperature and significant change in the retention time, peak area, and tailing factor were taken in to consideration for testing robustness .
The quantity of Quercetin and Curcumin were determined by following formula,

$$C_{STD} / C_{SAMPLE} = AUC_{STD} / AUC_{SAMPLE}$$

3. RESULTS AND DISCUSSION

3.1 Optimization of separation conditions

Absorption maxima of curcumin and quercetin was observed to be in the range of 200-400 nm on the UV spectra with dimensional chromatograms and a monitoring wavelength for quantitative determination at 265 nm and 256 respectively.It was altered to obtain the baseline separation of marker compound. Isocratic elution was carried out to successfully separate the compounds in hydroalcoholic extract of *Madhujeevan Churna* using methanol: acetonitrile: phosphate buffer (pH 5) in the ratio of 42.5 : 42.5: 15 % v/v/v as the mobile phase. The flow rate was 1.2 mL min-1 and detection was set at 265 nm. There were no interfered peaks within the time frame in which curcumin and quercetin in the extract were detected. It is shown in Figure 1.

3.2 Linearity and range of the developed method

The analytical range was established by the highest and lowest concentrations of analyte where acceptable linearity was obtained. The correlation coefficient (R^2) of curcumin and quercetin were around 0.999, which confirms its linearity in range of 10-150 μgmL-1. The results of linearity are summarized in table 1.

3.3 Regression equation

 Linear regression analysis for curcumin was performed by the external standard method. The calculated results were given in table-1, where a, b and r were the coefficients of the regression equation Y = ax + b, x referred to the concentration of the marker compound (μg mL-1), Y the peak area and r the correlation coefficient of the equation. The marker substance showed good linearity (r = 0.98332) in a relatively wide concentration range. The limits of detection (LOD) for curcumin and quercetin were 18.35 ng mL-1, detected at 265 nm.

3.4 System suitability of the developed method

 Factor such as theoretical plate count, tailing factor, percentage relative standard deviation (%RSD) of peak area and retention time was taken into consideration for testing system suitability. The developed method has produced theoretical plate above 3000 for both curcumin and quercetin with tailing factor less than 1.5 .The %RSD of peak area and retention time of both curcumin and

quercetin were less than 2, which ensures the suitability of the developed method. The results of system suitability study are summarized in table 2 and 3.

3.5 Accuracy of the developed method
The percentage recovery of the spiked curcumin and quercetin were well within 100±2% and %RSD of assay was less than 2%, which ensures the accuracy of the developed method. The results of recovery studies are summarized in table 4.

3.6 Precision of the developed method
The developed method has shown <1.3 %RSD of peak area, tailing factor, retention time and assay, which ensures precision of the developed method. The results of precision study are summarized in table 5.

3.7 Limit of detection and limit of quntification of the developed method
LOD and LOQ of curcumin and quercetin in the developed method were studied using the signal-to-noise ratio of 3 for estimating LOD and 10 for estimating LOQ. The LOD were 0.3 ppm for curcumin and 0.9 ppm for quercetin at a signal-to-noise ratio of 3. LOQ were 0.4 ppm for curcumin and 1.1 ppm for quercetin at a signal-to-noise ratio of 10.

3.8 Robustness of the developed method
The robustness of a method is a measure of its ability to remain unaffected by negligible but deliberate change to the chromatographic condition.. The results of precision study are summarized in table 6.

4. DISCUSSION
The optimum chromatographic condition with adequate resolution for curcumin and quercetin was achieved when the separation was carried using C18 column (Reversed Phase, 150 mm x 4.6 mm with 5 micron particle size, Phenomenex Luna) at a column oven temperature of 35°C with an isocratic elution mode of mobile phase composed of a degassed mixture of isocratic elution using methanol: acetonitrile: phosphate buffer (pH 5) in the ratio of 42.5 : 42.5: 15 % v/v/v as the mobile phase. at 1.0 mLmin-1 flow rate with a total run time of 15 minutes.
The developed HPLC method for quercetin, curcumin and simultaneous estimation of quercetin and curcumin is simple, accurate, precise and robust. This method includes use of C_{18} Intersil, 4.6 (i.d.) x 250 mm columns, mobile phase consist of mixture of methanol, acetonitrile: phosphate buffer (pH 5) in the ratio of 85: 15 % v/v at flow rate 1ml min^{-1}. PH of mobile phase was adjusted with OPA. Retention time was found to be 3.220, 4.287 for quercetin and curcumin respectively. Theoretical plates were found to be 8210, 7955 for quercetin and curcumin respectively. It is must to observe pattern of UV absorbance with prior to HPLC method development for markers. After obtaining the overlaid spectra for markers, 263nm wavelength was selected for further HPLC analysis.
Mobile phase selection[21] is very important for separating two drugs appropriately. While selecting the mobile phase it was observed that mixture of water-methanol gives sharp peak of quercetin with acceptable system suitability parameter but affect the elution of curcumin. By increasing concentration of water in above mixture affect the elution of curcumin and it takes more time for elution. Hence acetonitrile in mixture of water and methanol is suitable for their separation. Keeping this in mind several mobile phases were tried and methanol, acetonitrile: phosphate buffer in the ratio of 85: 15 % v/v was finalized; P[H] of mobile phase was adjusted to 5 with o-phosphoric acid. This mobile phase gives resolved peaks with symmetry within limits and significant retention time. System suitability parameters were studied in order to determine the suitability of chromatographic system for analysis to be done.
The calibration plot quercetin and curcumin follow Beer-Lambert's law. The correlation coefficient, intercept and slope were 0.9992, -0.125, 83644 and 0.994, 0.142, 63501 for quercetin and curcumin respectively. The good correlation coefficient indicates[22] the method is linear over the concentration range. The low values of % R.S.D. (< 2%) for intra and inter day's variation, which suggested an excellent precision of method. Results from recovery studies were within acceptable limits. 98.26-99.35% and 98.32-99.27% for quercetin and curcumin respectively indicating accuracy of method

was good. The limit of detection (LOD) and quntification (LOQ) were near about 0.26 μg/mL, 0.804 μg/mL for quercetin and 0.25 μg/mL, 0.78 μg/mL for curcumin, which indicates adequate sensitivity of method. Low values of SD and % RSD obtained after introducing small deliberate changes in the developed HPLC method indicated the robustness of method. The developed method was validated as per ICH guidelines.[22]
Quantitative estimation of quercetin and curcumin in *MJC* was also determined by standard formula, quercetin was found to be 0.132%w/w of *churna* while curcumin was found to be 0.25 %w/w of *churna* by UV spectroscopy. With the help of HPLC quantity of quercetin and curcumin was found to be 0.1347 %w/w, 0.208% w/w of *churna* . It is invented that the quantity of quercetin and curcumin determined by UV and HPLC is nearby similar hence same analytical techniques are most useful for quantification of target compound. It is concluded that herbal marker compounds can be analyzed by analytical technique as UV, HPLC .Qualitative and quantitative estimation of natural components can be done using this technique, which is part of quality assurance of herbal product.
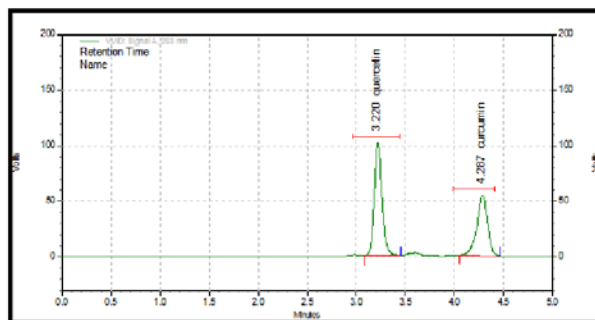
Figure 1: Optimized peaks of Curcumin and Quercetin

Table 1: Linear regression data

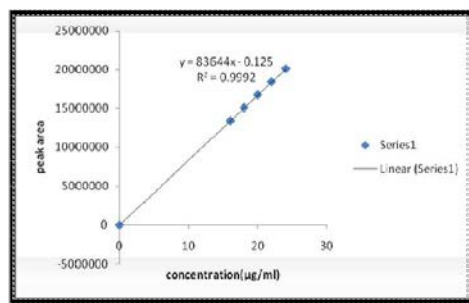| Sr no	Parameter	Quercetin	Curcumin
1	Slope	83644	63501
2	Intercept	-0.125	0.142
3	R^2	0.9992	0.9994

Figure 2: Calibration curve of Quercetin
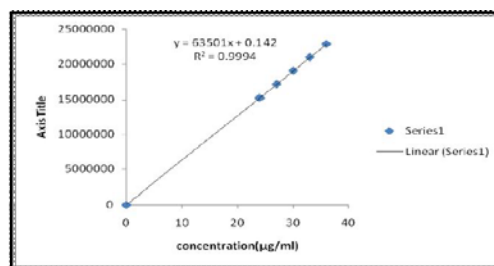

Figure 3: Calibration curve of Curcumin

Table 2: System suitability parameters for mixture

Name	R.T. (min)	Theoretical Plate	Asymmetry	Resolution
Quercetin	3.220	8210	1.15953	0.00000
Curcumin	4.287	7955	0.87525	6.30883

Table 3: System Suitability Test

Sr. no.	Peak area		R.T.(min)		Therotical plate		Asymmetry		Resolution
	QUER	CUR	QUER	CUR	QUER	CUR	QUER	CUR	
1	8364441	6350153	3.22	4.287	8210	7955	1.159	0.875	6.308
2	8364439	6350099	3.213	4.283	8201	7950	1.172	0.878	6.337
3	8364399	6350140	3.2	4.287	8205	7956	1.179	0.876	6.329
4	8364396	6350155	3.207	4.29	8212	7953	1.189	0.867	6.318
5	8364389	6350090	3.21	4.285	8209	7958	1.177	0.88	6.307
Maen	8364412	6350127	3.21	4.2864	8207.4	7954.4	1.1752	0.8752	6.319
SD	25.1037	30.7457	0.0073	0.0026	4.3931	3.0495	0.010	0.0049	0.0131
%RSD	0.0003	0.0004	0.218	0.0607	0.0532	0.0383	0.9329	0.5678	0.2073

Table 4(A): Recovery for Curcumin

Spiked Level (%)	Mean*%Amount Recovery	SD	%RSD
50	98.63	0.291	0.224
100	98.32	0.210	0.213
150	99.27	0.221	0.292

Table 4(B): Recovery for Quercetin

Spiked Level (%)	Mean*%Amount Recovery	SD	%RSD
50	98.26	0.31	0.31
100	99.35	0.314	0.314
150	98.45	0.298	0.298

* Indicates three replicates

Table 5(B): Intra and intraday's precision for Quercetin

Formulation	Intra-day precision (n=3)		Inter-day's precision (n= 3)	
	SD of response	% RSD	SD of response	% RSD
Extract	46370.2	1.78	46499.5	1.43

Table 5(A): Intra and intraday's precision for Curcumin

Formulation	Intra-day precision (n=3)		Inter-day's precision(n=3)	
	SD of response	% RSD	SD of response	% RSD
Extract	29745.9	1.13	40137.94	1.22

Table 6: Robustness evaluation of method of QUER and CUR

Parameters	Quercetin		Curcumin	
	Retention Time	Asymmetry	Retention Time	Asymmetry
A: Flow rate				
0.9 mlmin⁻¹	3.56	1.23	4.74	0.86
1 mlmin⁻¹	3.22	1.19	4.25	0.87
1.1 mlmin⁻¹	2.89	1.19	3.84	0.88
mean±SD(n=3)	3.22±0.33	1.20±0.023	4.27±0.45	0.87±0.01
B: Wavelength				
258 nm	3.33	1.39	4.38	0.95
263 nm	3.22	1.19	4.25	0.87
268 nm	3.30	0.95	4.30	0.97
mean±SD(n=3)	3.28±0.05	1.29±0.10	4.31±0.065	0.93±0.052
C:Mobile phase composition				
83:17	3.19	1.20	4.27	0.88
85:15	3.22	1.19	4.25	0.87
87:13	3.17	1.23	4.20	0.85
mean±SD(n=3)	3.19±0.025	1.20±0.02	4.24±0.036	0.866±0.015

5. CONCLUSION

In present study, a simple, precise, and reproducible HPLC method has been developed for simultaneous estimation of curcumin and quercetin. The developed method was successfully implemented in the estimation of curcumin and quercetin. This is the first report of identification and quantification of curcumin and quercetin in hydroalcoholic extract of *Madhujeevan Churna*. A simple, rapid and accurate assay approach was presented. The experimental results indicated that hydroalcoholic extract of *Madhujeevan churna* contain high concentration of curcumin. Thus developed method can be used for routine analysis of curcumin and quercetin in pharmaceutical dosage forms. Since the phenolic compounds have been of interest of health benefits, the present analytical study

could be a potential application to identify and quantify the phenolic compound in other herbal dosage forms.

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