

HPLC and HPTLC Methods for Simultaneous Estimation of Quercetin and Curcumin in Polyherbal Formulation

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Baghel, *et al.*: Chromatographic Analysis of Quercetin and Curcumin

Fast, sensitive, precise and selective high performance liquid chromatography and high performance thin layer chromatography methods were developed for the simultaneous estimation of curcumin and quercetin in a polyherbal formulation. Validation of these methods were carried out with respect to linearity, range, precision, accuracy and robustness. Both methods were applied to a polyherbal formulation to find the amount of curcumin and quercetin and compared. Curcumin and quercetin were found to be 9.5 mg and 0.613 mg in the polyherbal formulation using the high-performance liquid chromatography method whereas with the high performance thin layer chromatography method, curcumin and quercetin were found to be 9.7 mg and 0.674 mg, respectively. Both methods identified and quantified curcumin and quercetin effectively.

Key words: HPLC, HPTLC, curcumin, quercetin, polyherbal formulation

World Health Organization (WHO) has stressed on the need for scientific validity of herbal drugs and ensuring, devising and implementing sound science^[1]. WHO has published guidelines for quality control of herbal medicines to ensure the identity, purity and content of herbal materials^[2]. A number of techniques can be used for the analysis of herbal medicines such as high performance liquid chromatography (HPLC), gas chromatography-mass spectroscopy (GC-MS), HPLC-nuclear magnetic resonance (HPLC-NMR), ¹³C-NMR, near infrared (NIR). These techniques can be used for the structure elucidation of herbal drugs. HPLC and high performance thin layer chromatography (HPTLC) are the techniques that can be used to analyse most of the herbal drugs^[3]. HPLC and HPTLC have been employed for a number of times in analysing number of herbal drugs^[4-12].

Curcumin (CMN) is yellow in colour and derived from the plant *Curcuma longa* Linn. CMN has a number of activities such as antioxidant, antiinflammatory, antiviral, antibacterial, antifungal and anticancer. CMN has potential in number of diseases such as arthritis, Alzheimer's disease, diabetes and allergies^[13-16]. Quercetin (QCN) is a flavonol, which is a subclass of flavonoids. QCN is found in number of fruits and vegetables such as onion (*Allium cepa* Linn.), apple

(*Malus pumila* Miller), black tea (*Camellia sinensis* (L.) Kuntze) and black currant (*Ribes nigrum* Linn.). QCN has number of activities such as antioxidative, anticarcinogenic, antiinflammatory, antiaggregatory and vasodilating activity^[17-19].

Estimation of QCN has been done by using HPTLC for a number of times individually and also in combination with other compounds^[20-23]. Also a number of validated HPLC methods have been devised for the estimation of QCN both individually and in combination with other compounds^[24-27]. Quantification of CMN has been done by HPTLC technique^[28]. Another stability indicating HPTLC method has been developed to quantify CMN in pharmaceutical formulations^[29]. A HPLC method for simultaneous determination of CMN and silymarin in various dosage forms has also been reported. Analysis of degradation products of CMN has been done using HPTLC^[30]. Other methods are also available for estimation of curcumin^[31,32].

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MATERIALS AND METHODS

HPLC grade water and methanol and analytical grade ethyl acetate, formic acid, glacial acetic acid, acetone, toluene, sodium acetate were purchased from Loba Chemie, Mumbai, India. HPLC grade acetonitrile was purchased from Fisher Scientific, Mumbai, India. Analytical grade ethanol was purchased from Changshu Yangyuan Chemicals, China. Marker compounds, CMN and QCN were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India, and *Divya madhunashini vati* was purchased from Divya Pharmacy, Amritsar. All other reagents employed were of analytical grade.

Shimadzu UV-1700 and Lab India UV-3000 UV spectrophotometers were used for UV/Vis spectrophotometric analysis. UFLC Shimadzu with binary prominence LC-20 AD liquid pump, SPD-20 A prominence UV/Vis detector and a 77251 Rheodyne injector with 20 µl loop was used for HPLC analysis. Inertsil C₁₈ column (250 mm×4.6 mm i.d., 5 µ) with gradient mixer SUS 20 A were used in HPLC analysis. Millipore filter of 0.45 µ pore size was used for filtering mobile phase for HPLC. Camag HPTLC was used for HPTLC analysis. Precoated TLC plates Silica gel 60F254 from Merck, Germany were used with layer thickness 0.2 mm. Samples were applied by means of pressurized nitrogen gas (150 kg/cm²) through Camag automatic TLC sampler 4 fitted with a 25 µl syringe. The bands were visualized in Camag UV cabinet at 254 nm.

The plant materials were collected from local area in Fatehgarh Taprian, Ropar, Punjab, India in the month of October. Then, plants were identified and authenticated as *C. longa* (Specimen number ACL/11/12) and *Syzigium cuminii* (Specimen number ASC/11/12) at NIPER, Mohali, Punjab, India. A herbarium sheet of the plants were submitted in the herbarium of the NIPER, Mohali, Punjab.

Literature survey revealed that several solvents like methanol^[33], ethanol^[34] and acetonitrile^[35] were used for the extraction of CMN and QCN from plant and polyherbal formulation. The efficiency of extraction with methanol was greater than others so methanol was selected for standard and sample preparation. The CMN and QCN standards were also found to be stable in this solvent at room temperature and also during the entire duration of study design.

Preparation of standard solution for HPLC analysis:

Depending on the polarity of both the drugs, solubility

analysis for both the drugs was done in various solvents such as methanol, acetic acid, ethanol, 0.1 N NaOH, 0.1 N HCl. Methanol was selected as the solvent for study. Standard solutions of both CMN and QCN were prepared using HPLC grade methanol giving concentration of 125 µg/ml in each separately and also in combination.

Preparation of sample solution for HPLC analysis:

Twenty tablets of *Divya madhunashini vati* were weighed and powdered. From this, a sample solution containing 10 mg/ml drug was prepared using HPLC grade methanol and filtered through 0.45 µ Millipore nylon filter. Final concentration of CMN was 10 µg/ml and for QCN was 50 µg/ml.

HPLC conditions:

The column chromatography was performed on a HPLC system equipped with binary prominence LC-20 AD liquid pump, SPD-20 A prominence UV/Vis detector and a 77251 Rheodyne injector with 20 µl loop. Inertsil C₁₈ column (250×4.6 mm i.d., 5 µ) with gradient mixer SUS 20 A were used in HPLC analysis. Millipore filter of 0.45 µ pore size was used for filtering mobile phase for HPLC. Wavelength was set at 397 nm. Operation, data acquisition and analysis were performed by using LC solution 1.22 version Single Channel software.

Chromatographic method:

Conditions were optimized for individual CMN and QCN separately. Then, optimization was carried out for simultaneous estimation. Isobestic point was found by using UV spectra at 397 nm. Methanol and water in ratio of 75:25 v/v isocratic elution mode with the flow rate of 1 ml/min were used for HPLC analysis. Each chromatographic run was completed within 10 min. Then, this HPLC method was applied to the sample solution of marketed polyherbal formulation and concentration of CMN and QCN was calculated using the Eqn. 1, (sample area/standard area)×(weight of standard/50)×(100/sample weight×purity of standard/100)×(100–W/100×average weight), where, W is the percent water content in standard drug.

Preparation of standard solution and HPTLC conditions:

A working standard solution of CMN (10 µg/ml) was prepared using methanol. Similarly, for QCN, 50 µg/ml working standard solution was prepared using methanol. The planar chromatography was performed on fresh aluminium plates (10×10 cm) precoated with

silica gel 60 F254 of layer thickness 0.2 mm (Merck, Germany). Before use, the plates were developed in the mobile phase and were activated horizontally for 20 min at $120 \pm 0.5^\circ$ using an oven to remove elutable components. Samples were applied to the plates as bands 6 mm wide, 10 mm from the bottom, by means of a pressurized nitrogen gas (150 kg/cm^2) through Camag automatic TLC sampler 4 fitted with a 25μ syringe. The bands were visualized in Camag UV cabinet at 254 nm. Developing chamber was Camag glass twin through chamber ($10 \times 10 \text{ cm}$). Densitometer consisted of Camag TLC scanner 3 operated by WinCATS software. The scanning speed and data resolution were 20 mm/s and $100 \mu\text{m/step}$, respectively.

HPTLC method for simultaneous estimation of CMN and QCN:

Mobile phase consisting of toluene/ethyl acetate/formic acid (4.5:4:0.1, v/v/v) was used. Sample was applied using automatic TLC sampler. Then, TLC plate was developed over a development distance of 8 cm (migration time of 15 min) in solvent. After this the plates were dried at $60 \pm 0.5^\circ$ and then densitometric analysis was done. Calibration curve was constructed using different volumes of standard solution consisting of mixture of CMN and QCN.

Preparation of sample solution for HPTLC:

Sample solution was prepared using *Divya madhunashini vati* tablets. Twenty tablets were weighed and finely powdered. A quantity of powder equivalent to about 500 mg transferred to a 50 ml volumetric flask, dissolved in methanol, volume made up with methanol and filtered through a $0.45 \mu\text{m}$ Millipore nylon membrane filter under vacuum. Concentration of CMN and QCN was calculated using the Eqn. 1.

Validation of HPLC and HPTLC method:

Linearity and range were established for both the methods. Precision was evaluated by measuring intraday and interday precision. Accuracy was established by performing recovery studies. Robustness was established by making deliberate minor variations in the wavelength and calculating the percent deviation from original method. System suitability testing was performed before starting the experiment. Methanol was used as the extraction solvent as efficiency of the extraction was more with methanol than with other solvents.

RESULTS AND DISCUSSION

Overlay spectra of CMN and QCN in methanol were

recorded on a UV/Vis spectrophotometer and an isosbestic point was chosen at 397 nm. The solvent type, solvent strength, detection wavelength and flow rate were varied to determine the chromatographic conditions giving the best separation. Other conditions such as time required for analysis, flow rate of mobile phase, symmetry of the eluted peaks, assay sensitivity and solvent noise during drug analysis were also considered during optimization. It was determined at 397 nm, both the drugs can be detected simultaneously with good separation, sensitivity, consistent baseline and no mobile phase interference.

Methanol and sodium acetate buffer in the ratio of 70:30 was used in the beginning but it showed tailing. Different ratios of methanol to sodium acetate buffer such as 75:25, 80:20 and 85:15 were tried but they all showed tailing except the ratio 75:25 showed good resolution. However, mobile phase consisting of methanol:water in the ratio of 70:30 offered better resolution but with tailing. Further changing the ratio of methanol:water to 75:25, gave the best resolution and minimum tailing. Any further changes in the ratio of methanol:water resulted in either poor resolution or poor tailing. Finally, methanol:water at the ratio of 75:25 was selected as the mobile phase. Chromatograms obtained using methanol:water mobile phase in the ratio of 75:25. The developed HPLC method was applied to the polyherbal formulation. CMN and QCN were found to be 9.5 and 0.613 mg, respectively in the marketed polyherbal formulation.

Mobile phase optimization for the HPTLC method was started by using a mobile phase consisting of toluene:ethyl acetate:glacial acetic acid:formic acid in the ratio of 4.5:3:1:0.2, but it gave poor resolution. Further modifying the ratio of this mobile phase and increasing the ratio of toluene to 5.5 did not improve results and also gave poor resolution. An improved R_f but poor resolution resulted in further modification of ratio of mobile phase to 5:3:0.5:0.2. Then, ethanol was added to the mobile phase and a ratio of 4.5:3:1:1:0.2 for toluene:ethyl acetate:glacial acetic acid:ethanol:formic acid was used to develop chromatogram, which also resulted in poor resolution. However, by removing ethanol and glacial acetic acid and using toluene:ethyl acetate:formic acid as mobile phase in the ratio of 4.5:4:0.2 gave better resolution. Further modifying this mobile phase ratio to 4.5:4:0.1 resulted in the best resolution and gave R_f value for CMN and QCN as 0.58 and 0.49, respectively. So, toluene:ethyl acetate:formic acid in ratio of 4.5:4:0.1 was finalized for use as

the mobile phase for HPTLC. The optimized TLC plate containing CMN, QCN ran using toluene:ethyl acetate:formic acid in ratio of 4.5:4:0.1 as mobile phase. Typical chromatograms of CMN and QCN are shown in fig. 1A and B, respectively.

Calibration curve was constructed by plotting the peak area ratios of CMN and QCN versus the concentrations of the calibration standards. When the developed method was applied to the sample, HPTLC chromatograms were obtained for CMN and QCN, which were shown in fig. 2A and B, respectively. The CMN and QCN were found to be 9.7 and 0.674 mg, respectively in the marketed polyherbal formulation.

Linearity was established within concentration range 2-12 $\mu\text{g/ml}$ (Table 1). Precision was determined by analysing three concentration and three replicates of each concentration. Data obtained for determining precision was given in Table 2. Accuracy was calculated as the percent recovery by analysing known added amounts of analyte to the sample. A total of nine determinations were made at three concentration levels (three concentrations and three replicates of

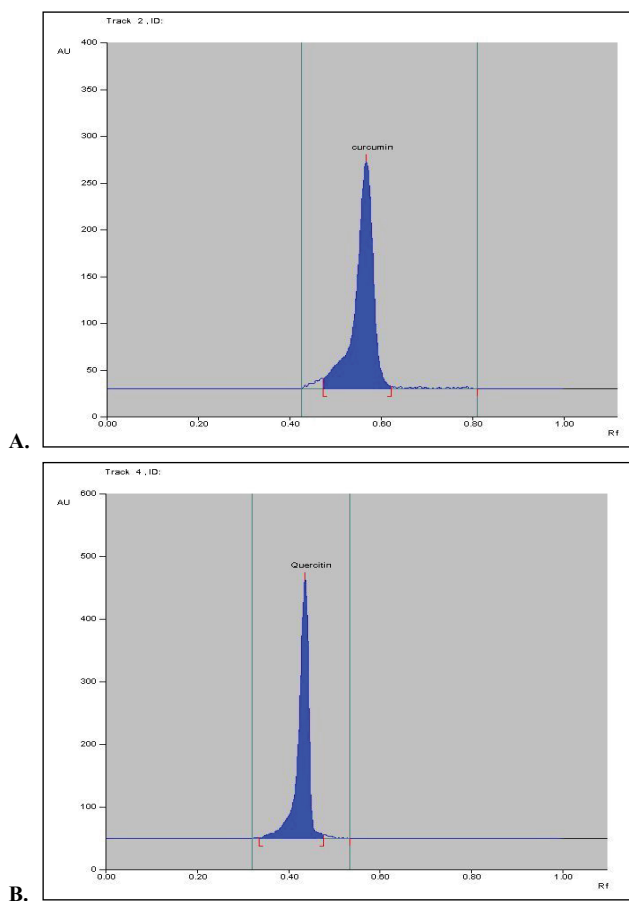


Fig. 1: Typical HPTLC chromatograms of CMN and QCN
A. Typical HPTLC chromatogram of curcumin (CMN) and B. quercetin (QCN)

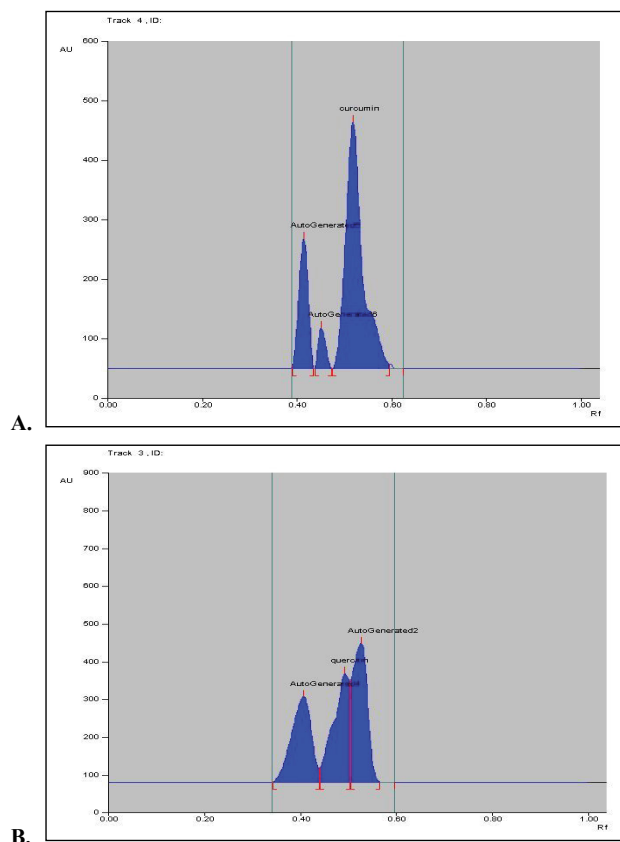


Fig. 2: HPTLC chromatograms of CMN and QCN in samples
A. HPTLC chromatogram of curcumin (CMN) and B. quercetin (QCN) in sample

each concentration), the data of which was presented in Table 3.

Robustness was determined by making small deliberate changes in the wavelength. Analysis was performed thrice, first at the original wavelength of 397 nm, second at 395 nm and third at 399 nm. Data obtained was presented in Table 4. The solution of CMN and QCN mixture was applied on the TLC plate at a concentration ranging from 20 to 1200 ng/band on nine different tracks with 10 mm distance among the tracks from x-axis and y-axis, respectively. Three replications of each standard were performed on different TLC plates. The calibration data was plotted as the peak areas of respective standard (y-axis) against the nominal concentration (x-axis). Linearity data was given in Table 5. For this repeatability studies were performed. Precision and accuracy studies were done by taking 12 determinations (4 concentrations/3 replicates each). Samples at four different concentration levels (20, 40, 60 and 80 ng/band for CMN and 100, 200, 300, 400 ng/band for QCN) were applied in hexuplicate on the same day. Sample concentration was estimated from the calibration curve. The intraday and interday

TABLE 1: SUMMARY OF LINEARITY DATA OF HPLC METHOD

| Compound | Concentration (µg/ml) | Slope (n=2) | | | Intercept (n=2) | | | r ² |
|----------|--------------------------|--------------|-------|------|-----------------|--------|------|----------------|
| | | Mean±SD | RSD | SEM | Mean±SD | RSD | SEM | |
| CMN | 2-12 | 56320.6±3.36 | 0.006 | 1.50 | 25602.6±2.40 | 0.009 | 1.08 | 0.996 |
| QCN | 2-12 | 13969.2±3.63 | 0.026 | 1.63 | 2661.2±1.64 | -0.062 | 0.74 | 0.997 |

SD-standard deviation; RSD-percent relative standard deviation; SEM-standard error of mean; n-number of injection; r²-regression coefficient

TABLE 2: PRECISION DATA OF HPLC METHOD

| Concentration (µg/ ml) | Inter-day precision (n=3) | | | Intra-day precision (n=3) | | |
|---------------------------|---------------------------|--------|--------|---------------------------|--------|--------|
| | Mean area±SD | SEM | RSD | Mean area±SD | SEM | RSD |
| CMN | | | | | | |
| 6 | 385239.1±558.9 | 395.22 | 0.1451 | 385194.8 ± 621.9 | 439.79 | 0.1615 |
| 8 | 471068 ±706.90 | 499.80 | 0.1500 | 470996.4±808.11 | 571.42 | 0.1716 |
| 10 | 594895.8± 913.9 | 646.29 | 0.1536 | 594967.3±1015.13 | 717.81 | 0.1706 |
| QCN | | | | | | |
| 6 | 84729.5± 201.26 | 142.32 | 0.2375 | 84778.4±132.11 | 93.42 | 0.15 |
| 8 | 105354.7± 238.6 | 168.73 | 0.23 | 105403.7±169.31 | 119.72 | 0.16 |
| 10 | 134612.9± 287.0 | 202.95 | 0.2132 | 134678.9±193.67 | 136.94 | 0.14 |

CMN-curcumin; QCN-quercetin; SD-standard deviation; RSD-percent relative standard deviation; SEM-standard error of mean; n-number of aliquots

TABLE 3: SUMMARY OF ACCURACY DATA OF HPLC METHOD

| Amount added (n=3) | Content (mg/ml) | Amount found (mg/ ml)±SD | Recovery (%) | SEM | %RSD |
|-----------------------|-----------------|-----------------------------|--------------|--------|--------|
| CMN | | | | | |
| 80% | 9.0 | 9.003±0.1517 | 100.04 | 0.0876 | 1.6856 |
| 100% | 10.0 | 10.17±0.02 | 113 | 0.0115 | 0.1966 |
| 120% | 11.0 | 11.24 ±0.1167 | 124.92 | 0.067 | 1.0385 |
| QCN | | | | | |
| 80% | 9.0 | 9.023±0.1553 | 100.26 | 0.0897 | 1.7216 |
| 100% | 10.0 | 9.89±0.04 | 109.88 | 0.0230 | 0.4045 |
| 120% | 11.0 | 10.86±0.1686 | 120.70 | 0.0973 | 1.5522 |

CMN-curcumin; QCN-quercetin; n-number of injection; SD-standard deviation; RSD-percent relative standard deviation; SEM-standard error of mean

TABLE 4: SUMMARY OF ROBUSTNESS DATA OF HPLC METHOD

| Method | Wavelength (nm) | | Active drug % | | Percent deviation | |
|-----------------|-----------------|-----|---------------|--------|-------------------|------|
| | CMN | QCN | CMN | QCN | CMN | QCN |
| Original Method | 397 | 397 | 100.83 | 100.15 | - | - |
| Changed Method | 395 | 395 | 100.53 | 99.87 | 0.30 | 0.28 |
| | 399 | 399 | 101.15 | 100.46 | 0.32 | 0.31 |

CMN-curcumin; QCN-quercetin

TABLE 5: SUMMARY OF LINEARITY DATA FOR HPTLC

| Parameter | CMN (n=5) | QCN (n=5) |
|----------------------------------|--------------|----------------|
| Concentration range (mg/band) | 20-100 | 100-1200 |
| r ² | 0.996 | 0.997 |
| Equation of line | y=1139x+6336 | y=699.8x-519.9 |
| %RSD of slope | 0.20 | 0.14 |

CMN-curcumin; QCN-quercetin; n-number of aliquots; RSD-relative standard deviation; r²-regression coefficient

accuracy and precision of the assay were assessed by the average relative percentage deviation (DEV) from the nominal concentration and the relative standard

deviation (RSD) values respectively, based on the reported guidelines^[38,39]. Precision (RSD) and accuracy (DEV) were calculated by using the following Eqns., RSD = standard deviation/average calculated concentration×100; DEV = 1-(average calculated concentration/nominal concentration×100). Precision and accuracy data of HPTLC were given in Table 6.

Novel HPLC and HPTLC methods were developed for the estimation of CMN and QCN in the polyherbal formulation. Both the methods were found to be specific, sensitive, robust, accurate, precise and linear. Both the methods were able to differentiate CMN and

TABLE 6: SUMMARY OF PRECISION AND ACCURACY BY HPTLC

| Nominal amount (ng/ band) | | CMN (n=3) | | | QCN (n=3) | | |
|------------------------------|-----|---------------------------------|------|-------|---------------------------------|------|-------|
| CMN | QCN | Calculated (ng/band) Mean±SD | %RSD | %DEV | Calculated (ng/band) Mean±SD | %RSD | %DEV |
| 20 | 100 | 19.81±0.075 | 0.39 | 0.92 | 102.72±1.86 | 1.81 | -2.27 |
| 40 | 200 | 39.11±0.930 | 2.38 | 2.23 | 197.98±1.55 | 0.78 | 1.008 |
| 60 | 300 | 58.99±1.14 | 1.93 | 1.68 | 296.91±2.40 | 0.81 | 1.03 |
| 80 | 400 | 81.39±2.13 | 2.61 | -1.74 | 397.83±1.59 | 0.39 | 0.54 |

CMN-curcumin; QCN-quercetin; SD-standard deviation; RSD-relative standard deviation; SEM-standard error of mean; n-number of aliquots; %DEV-average relative percentage deviation

QCN simultaneously and quantified CMN and QCN successfully in the polyherbal formulation. It appears that both the methods are suitable for use in quality control.

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Conflict of interest:

The authors declare no conflicts of interest.

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