HPLC method validation for quantification of tetrahydrocurcumin in bulk drug and formulation

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Abstract

Background: Tetrahydrocurcumin (THC), the active metabolite of curcumin, is gaining popularity amongst scientists due to its wide spectrum of pharmacological activities, better stability and colourless nature. The objective of this study was to develop a sensitive, cost-effective RP-HPLC method for the estimation of THC in bulk drug substance and formulation.

Results: Efficient chromatographic separation was achieved on Hypersil BDS, C18 column, 250 mm x 4.6 mm, 5 μm column by isocratic elution with mobile phase comprising of acetonitrile: methanol: water (40:23:37% V/V); adjusted to a pH of 3.0 ± 0.05. The flow rate of the mobile phase was 1.0 ml/min with a column temperature of 25 °C. UV detector was used for the analysis and detection was carried out at 280 nm. The developed method was validated according to ICH guidelines with respect to system suitability, linearity, accuracy, precision and robustness. The theoretical plates were found to be more than 5800. The method showed linearity over the range of 4 to 60 μg/ml with \( R^2 = 0.9998 \). The accuracy of the method in terms of recovery study was 98.23-99.99%. The %RSD for intra-day and inter-day precision were 0.272 and 0.275, respectively. The method was found to be robust with respect to change in wavelength, flow rate and column temperature.

Conclusion: The analytical method was found satisfactory on validation as per ICH guidelines. Hence, it can be routinely used for quantification of THC in bulk drug and formulation.

Keywords: Tetrahydrocurcumin, HPLC-UV, Quantification, Method validation

Background

Curcumin is a principal bioactive component of plant Curcuma longa (Linn). It is a potent antioxidant exhibiting various pharmacological activities [1]. Tetrahydrocurcumin (THC) is an active metabolite of it [2] where both the double bonds are reduced to single bonds (Fig. 1). Chemically, it is 1,7-bis(4-hydroxy-3-methoxyphenyl)-heptane-3,5-dione with empirical formula C\(_{21}\)H\(_{24}\)O\(_6\) and molecular weight 372.4. THC retains a range of therapeutic properties. It has been shown to ameliorate oxidative stress-induced renal injury [3] and was able to mitigate mitochondrial dysfunction in brain vasculature during ischemic stroke [4]. It has shown antidiabetic [5], antiparkinson activity [6] and anticancer activities [7].

Excellent pharmacological effects with added advantage of colourless nature has made scientist to take note of THC. Different dosage forms like cream [8], porous scaffold for cartilage regeneration [9], self-emulsifying floating drug delivery systems [10] have been developed across the globe.

Various analytical methods HPLC, UHPLC, LC-MS/MS etc. have been developed for identification of metabolites of curcumin. THC being one of the metabolite of curcumin has been identified in biological samples like plasma and urine [11–13]. But reference for a simple reliable HPLC method for estimation of THC in the formulation is not available. In the present study, HPLC
based analytical method has been developed and validated for quantitative determination of THC in the gel formulation.

**Methods**

**Chemicals and reagents**

All the solvents were of analytical grade and purchased from Qualigens (Thermo Fisher Scientific). Purified water was purchased from JK labs (Mumbai). The tetrahydrocurcumin (Sabiwhite) was obtained from Sami Labs, Bengaluru, Karnataka, India.

**Method development**

**Preparation of standard solution**

Ten milligrams of tetrahydrocurcumin was accurately weighed and transferred to a 50 ml volumetric flask. Thirty-five milliliters of methanol was added and with the aid of sonication, it was dissolved completely. Volume was made up and solution was mixed well. Ten milliliters of aliquot was withdrawn and diluted to 50 ml with diluent.

**Preparation of sample solution**

Five hundred milligrams of the 1% w/w tetrahydrocurcumin gel was weighed on a butter paper and transferred the gel along with butter paper to a 50 ml beaker containing 25 ml of diluent. The sample was sonicated for 5 min to form clear solution and the contents of the beaker were transferred to a 50 ml volumetric flask. The beaker was rinsed 2 times by 10 ml of methanol and transferred the same to the volumetric flask. The volume was made up to the mark with diluent and mixed well. The solution was filtered through a 0.45 μm Nylon syringe filter discarding 3-5 ml of sample. Ten milliliters aliquot was further diluted with methanol to 25 ml.

**Chromatographic conditions**

The system comprised of a Jasco PU-2089 HPLC Pump equipped with Jasco-2070 UV/Vis detector. The Mobile phase composed of acetonitrile: methanol: water (40:23: 37% V/V); adjusted to a pH of 3.0 ± 0.05 was used for the study. The mobile phase was filtered through a 0.45 μm pore size membrane PVDF filter and degassed ultrasonically after mixing. Twenty microliters of the solution was injected. The flow rate was maintained at 1 ml/min. The column oven temp was maintained at 25 °C. The detection was carried out at wavelength 280 nm. The run time for the analysis was 10 min.

**Validation of method: quantitation of THC in bulk and pharmaceutical dosage form**

The developed method was validated for system suitability, accuracy, precision, linearity and robustness in accordance with ICH guideline for validation of analytical procedures Q2(R1).

**System suitability**

System suitability testing helps to decide whether the developed chromatographic system is suitable for the analysis. %RSD of retention times, asymmetry factors and theoretical plates were the parameters for the study. Six replicate samples containing tetrahydocurcumin were analyzed using the developed method.

Filter study is carried out to ensure that no drug is retained by the filter during sample preparation. The solution was collected after passing through a 0.45 μm PVDF filter and a 0.45 μm Nylon filter, by discarding 5 ml of solution. The results were compared to unfiltered centrifuged sample solution.

**Specificity**

The mobile phase or the excipients in the formulation should not interfere with the analysis. The developed method needs to be specific. The blank, placebo solution, THC solution and gel solution were injected and peak purity was determined.

**Table 1** System suitability study

| Injection | Retention time | Peak area | Asymmetry factor | Theoretical plates |
|-----------|----------------|-----------|------------------|--------------------|
| 1         | 6.62           | 19186889  | 1.16             | 5943               |
| 2         | 6.62           | 19126526  | 1.15             | 5997               |
| 3         | 6.62           | 19234275  | 1.15             | 5985               |
| 4         | 6.62           | 19198526  | 1.15             | 5973               |
| 5         | 6.63           | 19183676  | 1.15             | 5956               |
| 6         | 6.63           | 19196342  | 1.16             | 5883               |
| Mean      | 6.623          | 19187705.67 | 1.153           | 5956.17            |
| SD        | 0.005          | 34986.36  | 0.005            | 40.76              |
| %RSD      | 0.078          | 0.18      | 0.448            | 0.68               |

**Table 2** Filter study

| Sample          | % Absolute difference |
|-----------------|-----------------------|
| 0.45 μm PVDF filter | 0.10                  |
| 0.45 μm Nylon filter  | 0.08                  |
**Linearity**

THC solutions at 5 levels were prepared from 10 to 150% of working concentration. At each level, analysis was carried out in triplicate. The peak areas versus concentrations data was evaluated by linear regression analysis. Three data set corresponding to triplicate analysis were constructed. This data was analyzed by the test one-way ANOVA with post test Tukey using a significant level of $\alpha = 0.05$ (95% of confidence interval).

**Accuracy**

An accuracy study was performed by adding known amounts of THC to the placebo preparation. The actual and measured concentrations were compared and recovery was calculated. Recovery of the method was evaluated at three different concentration levels (corresponding to 50, 100 and 150% of test preparation concentration). For each concentration level, three sets were prepared and measured.

**Precision**

The precision of the assay method was evaluated in terms of repeatability by performing six independent assays (intra-day) of THC. Under the same experimental conditions, intermediate precision of the method was checked by another person performing the same
procedure on a different day (inter-day). %RSD not more than 2 was taken as the limit.

Robustness
The factors chosen for this study were the change in wavelength (+, −3 nm), flow rate (+,−10%) and column temperature (+,−2 °C). The appropriate amounts of THC, bulk drug and formulation were weighed and diluted with methanol. The effect of changed parameters on analysis of THC in bulk drug was evaluated in terms of RT, asymmetry factor, theoretical plates and assay.

Results
System suitability
In system suitability study, the peak showed good symmetry (average 1.15) and sufficient theoretical plates (average 5956). The average retention time was 6.62 min. %RSD of retention times, asymmetry factors and theoretical plates was well below 1% (Table 1). PVDF and nylon both filters were found to be suitable as maximum % absolute difference was 0.10% (Table 2).

Specificity
The method proved to be specific with no interference of mobile phase or excipients. (Fig. 2a-d). The peak purity for the standard solution and sample solution was 0.997 and 0.996 respectively (Table 3).

Linearity
For linearity range, correlation coefficient was 0.9998, $F$ value was less than 0.00002 and $P$ value was 0.99999 (Fig. 3 and Table 4).

Accuracy
The method was accurate at 3 levels with %RSD in the range of 0.02 to 0.194 (Table 5).

Precision
Repeatability and intermediate precision studies showed %RSD, 0.272 and 0.275 respectively (Table 6).

Robustness
Change in wavelength, flow rate and column temperature caused not more than 2% difference in the assay value (Table 7).

Discussion
In HPLC, solvent under pressure moves through the stationary phase packed in the column. The components of a mixture carried by the solvent are separated from each other due to their different degrees of interaction with the stationary particles. This leads to the separation of the components as they flow out of the column and pass over the detector, where they are detected. The right choice of the stationary phase, mobile phase, detection technique, etc. can be done based on guidelines issued by regulatory agencies [14, 15].

Under ideal conditions, chromatographic peak has a Gaussian shape. Asymmetric peak is obtained in case of

| Table 3 Specificity study |
|---------------------------|
| Blank                     | No interference at RT of Tetrahydrocurcumin in blank |
| Standard solution         | Peak purity was 0.997 |
| Placebo                   | No interference at RT of Tetrahydrocurcumin in placebo |
| Sample solution           | Peak purity was 0.996 |

![Linear Regression](image_url)

**Fig. 3** Standard calibration curve of tetrahydrocurcumin
non-uniform migration and non-uniform distribution of drug in the HPLC column. Lesser the deviation, the value of asymmetry factor will be closer to 1. The sharper the peak, the better is the column efficiency. It is measured in terms of the number of theoretical plates per column. Variation in peak area indicates non-suitability of the method. The method showed good system suitability with an average asymmetry factor as 1.15, %RSD for peak area less than 2 and theoretical plates more than 5900 (Table 1). The base-deactivated silica used in the column had blocked –OH groups. Because of reduced silanol activity, tailing was less, and good peak symmetry was observed. THC is insoluble in water and soluble in organic solvents like alcohol and acetone. Hence, methanol was chosen as a diluent for sample preparation. THC was adequately extracted in the methanol, and excipients did not cause interference in the analysis. The mobile phase was composed of acetonitrile: methanol: water (40:23:37% V/V), adjusted to a pH of 3.0 ± 0.05. Presence of methanol in the mobile phase ensured no plug formation after injection of sample solution. The analysis was carried out using a UV detector at a wavelength of 280 nm where THC showed maximum absorbance. High peak purity with no interference from solvent system or excipient indicated results specific to the THC (Fig. 2 and Table 3). With difference less than 2%, the method was found to be accurate with test results close to the true values (Table 5). The method was precise with respect to intra-day and inter-day precision (Table 6). Change in wavelength, flow rate and column temperature did not affect the analysis significantly (Table 7).

**Table 4 Analysis of variance for linearity**

| Source of variation | SS      | df | MS      | F        | P value | F crit | P value |
|---------------------|---------|----|---------|----------|---------|--------|---------|
| Between groups      | 3421080113 | 2  | 1710540056 | 1.38172E−05 | 0.99999 | 3.88529 |
| Within groups       | 148557E+15 | 12 | 1.23798E+14 |          |         |        |         |
| Total               | 148558E+14 | 14 |         |          |         |        |         |

**Table 5 Accuracy study**

| Level | % Recovery | Mean % recovery | %RSD |
|-------|------------|----------------|------|
| 50%   | 98.24      | 98.23          | 0.194|
|       | 98.42      |                |      |
|       | 98.04      |                |      |
| 100%  | 98.31      | 98.32          | 0.016|
|       | 98.32      |                |      |
|       | 98.34      |                |      |
| 150%  | 100.01     | 99.99          | 0.021|
|       | 99.97      |                |      |
|       | 100.00     |                |      |

**Table 6 Precision study-repeatability and intermediate precision**

| Sample | Repeatability Peak area | Intermediate-precision Peak area |
|--------|-------------------------|---------------------------------|
| Sample 1 | 19851008                 | 19863257                        |
| Sample 2 | 19848253                 | 19852637                        |
| Sample 3 | 19752637                 | 19752634                        |
| Sample 4 | 19863415                 | 19752639                        |
| Sample 5 | 19752635                 | 19752633                        |
| Sample 6 | 19863128                 | 19752489                        |
| Mean    | 19821846                 | 19787715                        |
| SD      | 53962.92                 | 54505.19                        |
| %RSD    | 0.2722                   | 0.2754                          |

**Table 7 Robustness study**

| Parameter       | RT | Asymmetry factor | Theoretical plates | % Assay |
|-----------------|----|------------------|--------------------|--------|
| Change in wavelength | +3 nm | 6.61 1.15 | 5671 98.56 |
|                 | −3 nm | 6.62 1.17 | 5661 100.36 |
| Flow rate       | +10% ml/min | 6.64 1.18 | 5680 100.17 |
|                 | −10% ml/min  | 6.66 1.18 | 5652 99.09  |
| Column temperature | +2 °C | 6.56 1.16 | 5627 100.38 |
|                 | −2 °C | 6.56 1.16 | 5641 100.33 |

**Conclusion**

A new HPLC method has been developed for the identification and quantification of THC. The method has satisfactory sensitivity, specificity and repeatability. Method
was successfully validated as per ICH guidelines and can be conveniently employed for routine quality control analysis of THC as bulk drug and in pharmaceutical dosage form.

Abbreviations
THC: Tetrahydrocurcumin; HPLC: High-performance liquid chromatography; UHPLC: Ultra high-performance liquid chromatography; LC-MS: Liquid chromatography-mass spectroscopy; SD: Standard deviation; RSD: Relative standard deviation; SS: Sum of squares; df: Degrees of freedom; MS: Mean sum of squares

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Authors’ contributions
RR carried out the laboratory work, collected and analyzed the data and drafted the manuscript. JS supervised the work and assisted in the data analysis. All authors read and approved the final manuscript.

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