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A Simple, Robust (HPLC–UV) Method for the Quantification of Colchicine in Bulk and Eutectogel Formulation and its Validation

Anil Jindal[1], Anjana kumari[2]

[1]Ph.D, Research Scholar, Department of Pharmacy, Career Point University, Hamirpur, Himachal Pradesh, India.
[2]Associate Professor, Department of Pharmacy, Career Point University, Hamirpur, Himachal Pradesh, India.
Email: aniljindal87@gmail.com

To develop and validate a stability-indicating reversed-phase high-performance liquid chromatography (RP-HPLC) method for determining Colchicine in bulk and eutectoid nano-formulation.The chromatographic conditions were optimized using a stainless steel Hypersil Gold C-18 analytical column with 250 mm x 4.6 mm ID x 5 µm dimensions. The mobile phase consisted of Acetonitrile and 0.1 % O-phosphoric acid in the ratio of 35:65 v/v. The flow rate was set at 1 ml/min, and the detection wavelength was 245 nm. The column was maintained at 300C, and the injection volume was 20 µl. The retention time of pure drug was found to be 3.808. The limit of detection (LOD) and the limit of quantification (LOQ) were 1.279 µg/ml and 3.877 µg/ml, respectively. The procedure was validated for specificity, linearity, accuracy, and precision. A simple, rapid, specific, and stability-indicating HPLC–UV method was successfully developed for determining Colchicine in the pure and eutectoid. The developed method was statistically confirmed to be accurate, precise, and reproducible.
Keywords: Colchicine, RP-HPLC, UV detector, Eutectogel, Anti-gout alkaloid.

1. Introduction

Colchicine (N-[(7S)-1,2,3,10-Tetramethoxy-9-oxo-5,6,7,9-tetrahydrobenzo[a]heptalen-7-yl]acetamide) has a molecular weight of 399.437, and the molecular formula C22H25NO6 is shown in fig 1 [1]. It is a potent alkaloid obtained from the dried corn and seeds of plants of the genus Colchicum, which belongs to the Liliaceae family. The commonly used plants in this family are Colchicum autumnale, “meadow saffron,” and Colchicum autumn crocus [2-

4]. Colchicine has been used to treat acute gout for more than 2000 years. It was also used to treat pseudogout and familial Mediterranean fever for several decades. It is highly effective in treating acute gout, especially when given in the first 12–36 h of the gouty attack [3]. However, colchicine for the treatment of acute gout was only approved by the United States Food and Drug Administration (FDA) in 2009, although colchicine tablets have been

Prescribed in the United States since the 19th century [5].

Eutectogels, which consist of deep eutectic solvent (DES) and polymer networks, have been used for energy storage, ionic conductors, drug delivery, gas absorption, and wearable electronics [6]. Thus, the need for accurate, simple, and sensitive analytical methods to quantify and evaluate the release behaviour of drugs from these formulations is also increasing.

The first few analytical methods developed for determining Colchicine involved colourimetric, fluorometric assay, radioimmunoassay, and thin layer chromatography-densitometry methods [7-11]. Based on the literature review, only a few methods involving high-performance liquid chromatography with UV detection are available for the determination of colchicine in pharmaceutical formulations [12-15]. Other HPLC-UV methods are reported to detect and quantify the drug in biological fluids and samples [16-19]. Several methods using liquid chromatography coupled with mass spectrometry or tandem mass spectrometry and gas chromatography/mass spectrometry were also reported to determine colchicine in different biological fluids [20-26]. Still, these methods are time-consuming, complicated, and expensive compared to a simple HPLC-UV method.

To the best of our knowledge, until now, there is no stability indicating that the HPLC-UV method for determining colchicine has been developed. This study aims to create a simple, rapid, specific, and stability-indicating HPLC-UV method to detect and quantify colchicine in bulk and eutectoid formulation. In this work, the linearity range of the calibration curve was extended to make the method suitable for the assay and the in-vitro release studies of the drug from the prepared eutectogel formulation. The process was validated per the international conference on harmonizing technical requirements for registration of pharmaceuticals for human use (ICH) guidelines [27].

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

Colchicine (purity>98) was purchased from Acros Organics, New Jersey, USA. Acetonitrile (HPLC grade) was purchased from J. T. Baker, Phillipsburg, USA. O-phosphoric acid was obtained from QR.C, Selangor, Malaysia. Carbopol 940. was a kind gift from Lubrizol Corporation, Ohio, USA. Absolute ethanol (purity ~ 99.7) was bought from Fine Chemicals Inc., USA. Elga Purelab's classic UVF water purification system in France prepared ultrapure water. All other organic solvents and chemicals used were either analytical reagents or high-performance liquid chromatography grade.

2.2 Instrumentation

The HPLC system consisted of a Shimadzu (VP series, Kyoto, Japan) pump (LC-20AT VP) with a solvent cabinet, a degasser (DGU-20A3), a column oven (CTO-10S VP), an auto-

injector (SIL20A HTVP), a UV/VIS detector (SPD-20AD VP), and computer software (LabSolutions, Version 5.30 SP1).

2.2.1 Chromatographic conditions

The chromatographic separation was performed using a Hypersil Gold C-18 analytical column with 250 mm x4.6 mm ID x 5 µm dimensions, Fisher Scientific, USA. The flow rate was set at 1 ml/min, and the detection wavelength was 245 nm. The column was maintained at 30^0C, and an injection volume of 20 µl was used. The mobile phase consisted of acetonitrile: 0.1% O-phosphoric acid (35:65 v/v), then it was filtered through a nylon membrane filter 0.45 µm (47 mm) and degassed before use.

2.2.2 Preparation of stock solution, calibration standards, and quality control samples

A primary standard stock solution of colchicine (100µg/ml) was prepared by dissolving 10 mg of colchicine powder in 100 ml of the mobile phase; the solution was then vortexed and sonicated for 5 minutes. Take 3ml of this resulting stock solution and dilute up to 10ml with diluents, concentration found to be 30µg/ml (100%)and filtered through 0.22 µ Millipore membrane filters and injected in HPLC system.

2.2.3 Preparation of the eutectogel nano-formulation

To prepare a Deep Eutectic Solvent Mixture (DESM), choline chloride and selected carboxylic acids (Thio-urea, Citric acid, Urea, Oxalic acid, Benzoic acid, Malonic acid, Cinnamic acid, and Succinic acid) were mixed at different molar ratios. The mixtures were sealed in vials and heated in an oven at 75℃ until homogenous solutions were formed. Colchicine was selected as a model drug for the preparation of eutectogel[28].

2.3 Method validation as per ICH guidelines

2.3.1 System suitability studies

The system suitability test was performed to verify reproducibility and the good performance of the chromatographic system. The chromatographic parameters, such as theoretical plates (N), the height equivalent to the theoretical plate (HETP), and tailing factor (T), were calculated as per the United States pharmacopoeia (USP) and checked at the three QC concentrations in six injections replicates.

2.3.2Linearity and Range

For the present HPLC method to be suitable for the assay, content uniformity, and in vitro release tests, the linearity range was extended to be around 25 to 150% of the target drug concentration (30 µg/ml). Accordingly, the calibration curve was prepared using six solutions of Colchicine at concentrations of 7.5, 15, 22.5, 30, 37.5, and 45µg/ml. Each solution was injected in triplicate. The standard calibration curves for concentration versus peak area were constructed. The homoscedasticity assumption of the linear regression was tested by performing the (F-test), and the linearity was evaluated using a weighted least-squares regression analysis [29].

2.3.3Precision and Accuracy

The intra-day and inter-day variation for determination of Colchicine was carried out six times

on the same day and three consecutive days, and % RSD was calculated. The method was precise due to low values of the %RSD. Three sets of standard solutions were injected over six consecutive days for inter-day precision and accuracy. Precision is defined as the percentage of the relative standard deviation (%RSD) and calculated by dividing the standard deviation of the calculated concentration by the mean value. Accuracy was presented as the relative percentage of error(%RE) and calculated by dividing the difference between the standard solution's calculated and nominal concentrations with the standard solution's nominal concentration.

Analytical recovery experiments were carried out by using the standard addition method at three different percentage levels to check the accuracy of the developed methods and to study the interference of formulation excipients. From the total amount of drug found, the percentage recovery was calculated. A recovery study was carried out using the standard addition method to determine the accuracy of the proposed method. The accuracy was assessed by comparing the theoretical value (100%) with the test recovery values (calculated from the found concentration) at three % levels (low level 80, medium level 100, and high level 120). The percentage recovery of drug amounts was estimated by applying the values obtained to the respective regression line equations. The experiment was repeated three times.

2.3.4Limit of detection (LOD) and limit of quantification (LOQ)

The LOD is the lowest amount of analyte detected in a sample, whereas the LOQ is the lowest amount of the analyte that can be quantified in a sample with acceptable accuracy and precision [29]. Serial dilutions of colchicine stock solutions determined these to obtain a signal-to-noise (S/N) ratio of at least 3.3:1 for LOD and 10:1 for LOQ.

2.3.5Robustness

Robustness is the ability to provide accurate and precise results under various conditions. To measure the extent of method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged and in parallel. The studied parameter was a change in wavelength. 10 mg of the pure compound was taken in a 100 volumetric flask and dissolved in 100 ml with diluents (as mentioned above), and the concentration was found to be 100µg/ml. Take 3ml of this resulting stock solution and dilute up to 10ml with diluents, concentration found to be 30µg/ml (100%). And filtered through 0.22 µ Millipore membrane filters and injected into the HPLC system.

2.3.6Quantification of Colchicine Content in the Eutectogel

Colchicine quantification was done using the method of Ibrahim et al. (2017) with some modifications. Colchicine eutectogel (0.01 g) was dispersed in 20 ml of ultrapure water under continuous mixing with a magnetic stirrer for 60 min. The solution was then sonicated in an ultrasonic bath (Branson 5510, USA) for 10 min. A suitable aliquot was removed and diluted with the mobile phase to get the final nominal concentration of 100 µg/ml and subjected to chromatographic analysis. The regression equation referred to the drug peak area to get the sample concentration [30].

2.3.7Recovery of Colchicine

The blank eutectogel formulation (0.1 g) was dispersed in 20 ml of ultrapure water, and then

10 mg of Colchicine (as this amount is equivalent to the amount of colchicine that should be contained in0.1 g of the Colchicine eutectogel) was added to the solution under continuous mixing with a magnetic stirrer. The solution was then sonicated in an ultrasonic bath for 10 min. Then, 3 ml of this solution was diluted with the mobile phase in a 10 ml volumetric flask and filtered with a 0.45 µm pore size nylon filter to give a final nominal concentration of 30 ng/ml. The sample of 10 µl was injected into the HPLC system. The regression equation referred to the drug peak area to calculate the recovery. The analysis was done in triplicate [30].

2.4 Statistics

All the statistical tests of the developed method, including the weighted linear regression analysis of the calibration curves, the one-way analysis of variance (ANOVA), and a post hoc Tukey's HSD (honestly significant difference), were performed using Minitab software to analyse the results of the robustness and stock solution stability studies. The difference was statistically significant when $p<0.05$ and all values were expressed as mean.

3. Results and Discussion

3.1 Method Development

Developing an efficient chromatographic method depends on optimizing various parameters like the composition of the mobile phase, flow rate, and detection wavelength. Preliminary studies revealed that a mixture of acetonitrile and Orthophosphoric acid (35:65v/v ratio) is the most suitable combination for the efficient elution of colchicine. It was observed that reducing the proportion of acetonitrile in the ratio results in the broadening of the peak. This may be due to the low elution efficiency of orthophosphoric acid from the stationary phase.

Further optimization of chromatographic conditions was carried out to obtain a sharp peak. Parameters like peak shape, peak area, theoretical plate, and tailing factor were utilized to properly select the mobile phase and its flow rate. Chromatograms of standard and commercial colchicine tablets are given in Figure 1.

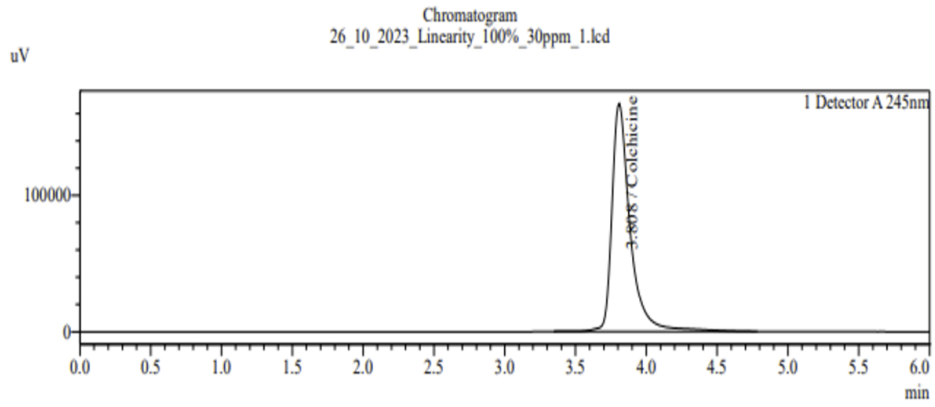

Figure 1. HPLC Chromatogram of Standard Colchicine (30µg/ml)

3.2 Method Validation

ICH guideline (Q2R1) was followed during the validation of the developed method.

3.2.1 System Suitability

System suitability studies ensure the performance of the chromatographic system and its suitability for analysis. The results of the system suitability study are summarized in Table 1. The results obtained for the studied parameters were within the acceptance limits. These results reveal that the chosen chromatographic system was suitable for the analysis of colchicine.

Table 1: Results of System Suitability Study

Parameters	Results	Acceptance limits
Retention Time (tR)	3.808±0.012	-
Tailing Factor (T)	1.253±0.015	≤ 2
No. of Theoretical Plates (N)	4885.65±15.811	> 2000

3.2.2 Linearity

Fig. 2 shows the calibration curve of colchicine. The R2 value of 0.999 indicates that the developed method is linear within the concentration range of 7.5-45 µg/ml. The area of all solutions was taken at their respective wavelengths. The Linearity was constructed by plotting concentration against the area where each reading was taken (Figure 2). The % RSD was calculated, and it was found to be within the acceptable limit (Table 2).

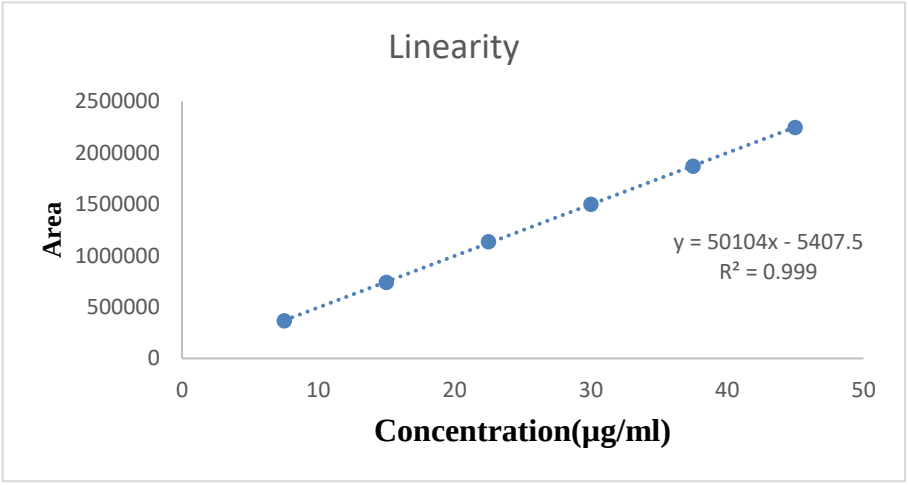

Figure 2: Linearity 1 graph of Colchicine

Table 2: Linearity of Colchicine

Level (%)	Conc.(µg/ml)	Area 1	Area 2	Area 3	Mean	SD	%RSD
25	7.5	365687	394325	360485	373499	18222.43	0.049
50	15	741267	768688	769648	759868	16115.8	0.021
75	22.5	1136066	1089947	1160190	1128734	35690.82	0.032

100	30	1499685	1509484	1542115	1517095	22215.26	0.015
125	37.5	1870068	1889889	1881014	1880324	9928.516	0.005
150	45	2246133	2302249	2403858	2317413	79948.49	0.034

Precision

Table 3 shows the results of the precision study. The study assessed the repeatability of the results using both intra-day and inter-day methods. The % RSD of intra-day and inter-day precision was below 2%, which is within the acceptable limit. Thus, the developed method is precise.

Table 3: Repeatability and Intermediate precision study

Sr.no.	Precision	%Recovery of Colchicine (Mean)	% RSD
1	Repeatability	100.47±0.16	0.012
2	Intraday	101.03±0.21	0.006
3	Interday	101.39±0.15	0.007

Accuracy

The accuracy of the method was studied using the per cent recovery method, and the results are shown in Table 4. The percentage recovery at each level of addition was more than 95%, and the average percentage recovery was 98.774%, which indicates that the method is capable of analyzing the drug accurately.

Table 4: Accuracy Study of Colchicine

Level	Concentration Label Colchicine	% Recovery of Colchicine	% RSD
50%	15(µg/ml)	99.367±0.25	0.003
100%	30(µg/ml)	99.067±0.22	0.012
150%	45(µg/ml)	97.888±0.28	0.009

The results indicated that the recoveries are well within the acceptance range of 97%–100%, indicating a reasonable degree of sensitivity of the method towards detecting analytes in a sample. Therefore, the process was accurate and can be used to estimate drugs.

LOD and LOQ

The LOD and LOQ of the developed method were calculated using the given formulae and found to be 1.279±0.15µg/ml and 3.877±0.24 µg/ml, respectively. The method's Limit of detection and quantification was calculated based on the standard deviation of the response and the slope (s) of the calibration curve at approximate levels of the limit of detection and limit of quantification. The results obtained were within the limit.

Robustness

The robustness was studied by analysing the sample of lower concentration with deliberate variation in the method parameters. The change in the drug responses was noted in terms of %RSD. The method's robustness was studied by a shift in Column temperature, change in flow rate±0.2, or change in wavelenth±5.

Table 5A: Robustness data of Colchicine with deliberate change in wavelength

Conc.(µg/ml)	Wavelength 240nm(% Recovery)	Wavelength 250nm(% Recovery)
30	99.09	96.61
30	99.66	97.53
Mean	99.38±0.18	97.07±0.23
SD	0.4	0.65
%RSD	0.4	0.67

Table 5A: Robustness data of Colchicine with deliberate change in flow rate (ml/min)

Conc.(µg/ml)	Flow rate 0.9ml/min (% Recovery)	Flow rate 1.1ml/min (% Recovery)
30	99.67	97.11
30	99.98	96.00
Mean	99.83±0.11	96.56±0.15
SD	0.22	0.78
%RSD	0.22	0.81

The Percentage RSD should be no more than 2. The %RSD obtained for a flow rate change, and wavelength was below 2, which was within the acceptance criteria. Hence, the method was robust.

Quantification of Colchicine content in the Eutectogel

Colchicine content in the eutectogel formulation was found to be98.75±0.26%.

Recovery of Colchicine

The experiment was conducted to determine the accuracy of the present method for quantifying the colchicine sample. The recovery of colchicine was calculated from the slope and the intercept of the calibration curve drawn in the concentration range of 7.5–45 µg/ml. The percentage recovery of Colchicine ranged between 99.24±0.13% and 99.76±0.17% in the eutectogel formulation sample.

Discussion

In developing an HPLC method, the separation of the analyte should be optimized at a pH where the retention time of the analyte is the least affected by pH changes. Accordingly, the buffer pH should be selected at least±1 pH unit from the pKa of the analyte. This ensures that the analyte is 100% ionised or 100% non-ionized at the chosen pH for reproducible retention time.

Since the pKa of Colchicine is 1.85, in the early stages of method development, the pH value of 3.85 was studied. However, this highly acidic pH may damage specific HPLC columns that cannot tolerate such a low pH value. Therefore, a higher pH value (4.85) was tried, finding no significant difference in the peak area of both tested pH values (i.e., 3.85 and 4.85). Accordingly, the pH value of 4.85 was selected as the optimised pH value of the method.

The solubility of the salt used in mobile phase composition should be considered when

developing an HPLC method, especially if acetonitrile (the commonly used organic solvent) is present in the mobile phase. Compared to other studies, 0.1 % O-phosphoric acid was selected as it is more soluble in acetonitrile than phosphate buffers. Phosphate buffers may precipitate when mixed with acetonitrile, affecting the function of the column and the HPLC device. The oven temperature of 300C was the optimum oven temperature, as good separation, peak shape, and area were obtained.

The LOD (1.279±0.15µg/ml) and LOQ (3.877±0.24µg/ml) obtained in the present study were the lowest values among the previously published methods for the quantification of Colchicine in pharmaceutical formulations. The intra and inter-day accuracy and precision were <1 %, reflecting the high accuracy of the protocol and that it is meeting the United States pharmacopoeia recommendations.

4. Conclusion

A sensitive, straightforward, specific, and stability-indicating HPLC–UV method for determining Colchicine in bulk and eutectogel nano-formulation was successfully developed. The Statistical analysis confirmed that the process was accurate, precise, and reproducible. The method could be used for routine assays, content uniformity studies, and in-vitro release studies of colchicine from the eutectogel nano-formulation.

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