

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF CURCUMIN AND ELLAGIC ACID IN HERBAL FORMULATION

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ABSTRACT

We aimed to ‘‘Development and validation of HPLC method for simultaneous estimation of curcumin and ellagic acid in herbal formulation’’ The developed simultaneous shim-pack HPLC C18 column (250 X 4.6 mm, 5 µm) method is simple, sensitive, specific and rapid for qualitative and quantitative estimation of Curcumin and Ellagic acid either individually or in combination in Dihar Tablet products, herbal antidiabetic medicines as per the regulatory needs. On the other side, estimation in Herbal formulation and impurities estimation are possible by the help of chromatography methods. The presented information is useful for the researchers especially those involved in the Method development and quality control of Ellagic acid and Curcumin.

KEYWORDS: Curcumin, ellagic acid, acetonitrile, 0.02 M potassium dihydrogen orthophosphate, methanol, orthophosphoric acid, shim-pack HPLC.

INTRODUCTION

Analytical method development and validation shows crucial role in the discovery, development and manufacture of pharmaceuticals. These methods used to ensure the identity, purity, potency and performance of drug products. There are many factors to consider when developing methods. The initially accumulate the information concerning the analyte’s physicochemical properties (pKa, logP, solubility) and determining which mode of detection would be convenient for analysis. The many of the analytical development effort goes into validating a stability indicating HPLC method. The pursuit of the HPLC method is to attempt and separate quantify the main active drug, any reaction impurities, all available synthetic interlude and degradants.

Different types of quantitative analytical techniques are there, for eg: HPLC, Gas chromatography, TLC, Ion chromatography and Column chromatography, UV/Visible spectroscopy, FT-IR, LC-MS, GC-MS, MASS and NMR.

Some steps involve in method development which is :

- Physicochemical properties of drug
- Set up HPLC conditions
- Sample preparation
- Method optimization
- Validation of developed method

MATERIAL AND METHOD

Material

Composition of Dihar manufactured by Rajsha Pharmaceuticals, Ahmedabad Antidiabetic herbal formulation (Tablet 100 mg).

Common Name	Botanical Name	Part Used	Family	Composition (%)
Black Berry	<i>Syzygium Cumini</i>	Seed	Myrtaceae	10
Bitter Ground	<i>Momordica Charantia</i>	Fruit	Cucurbitaceae	10
Indian Gooseberry	<i>Embelica Officinalis</i>	Fruit	Euphorbiaceae	20
Rams Horn	<i>Gymnema Sylvestre</i>	Leaves	Asclepiadaceae	10
Nagajivha	<i>Enicostemma littorale</i>	Entire Plant	Gentianaceae	10
Neem	<i>Azadirachta Indica</i>	Leaves	Meliaceae	10
Gulanchara tinospora	<i>Tinospora cordifolia</i>	Root	Menispermaceae	10
Turmeric	<i>Curcuma longa</i>	Rhizome	Zingiberaceae	18

2 Methods

2.1 Selection of wavelength

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of ellagic acid (ELA) and curcumin (CUR) then overlapped. UV overlain spectra of these two markers showed that the drugs absorb appreciably at 366 nm and hence 366nm was taken as a detection wavelength for HPLC analysis (Fig. 1).

2.2 Chromatographic conditions

The method was developed using reverse phase, shim-pack HPLC C18 column (250 X 4.6 mm, 5 μ m). The run time was of 10 min. The mobile phase used was 0.02 M potassium dihydrogen orthophosphate buffer (pH adjusted to 3.5 with orthophosphoric acid) and acetonitrile in the ratio 60:40 at a flow rate of 1.2 ml/min, column temperature maintained at 35 $^{\circ}$ C and a detection wavelength of 255 nm using a UV-visible detector.

2.3 Preparation of 0.02 M phosphate buffer (pH 3.5)

About 2.72 g of potassium dihydrogen orthophosphate was accurately weighed and dissolved in 950 ml of

water. The pH was adjusted to 3.5 with orthophosphoric acid and the volume was made up to 1000 ml in volumetric flask. The solution was then filtered using 0.45 μ membrane filter.

2.4 Preparation of standard solution

100 mg of ellagic acid (ELA) and curcumin (CUR) standard were accurately weighed and transferred into 100 ml volumetric flask respectively. About 70 ml solvent (Methanol) was added, sonicated to dissolve and diluted up to the mark using solvent (1000 ppm). Final concentration of ellagic acid (ELA) and curcumin (CUR) were made to 10 ppm and 7 ppm respectively by suitable dilutions.

2.5 Sample preparation

Accurately about 100 mg of tablet powder was extracted with 100 ml methanol. The sample solution was filtered to obtain a clear solution. The stock solution after suitable dilutions was used for further analysis.

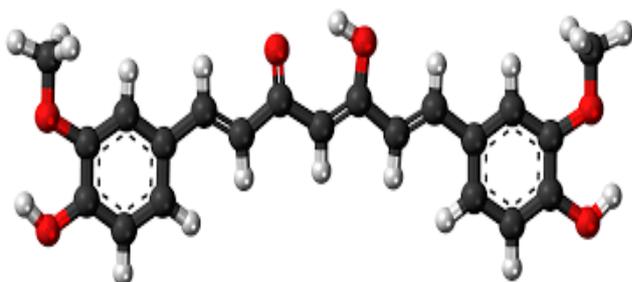


Fig. A. Structure of Curcumin.



Fig. B. Powder form of Curcumin.



Fig. C: Powder form of Ellagic acid.

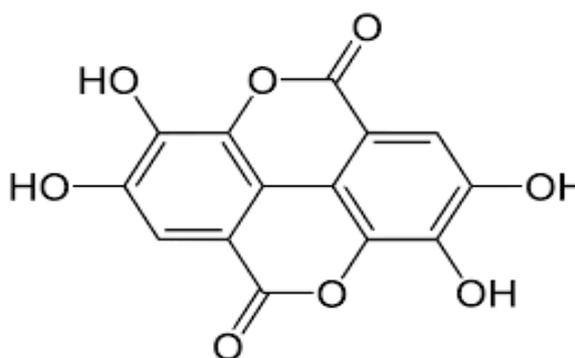


Fig. D: structure of ellagic acid.

2.6 RESULTS

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of ellagic acid (ELA) and curcumin (CUR) then overlapped. UV overlain spectra of these two markers showed that the drugs absorb appreciably at 366 nm and hence 366nm

was taken as a detection wavelength for HPLC analysis (Fig. 1).

Overlapped wavelength 366 nm

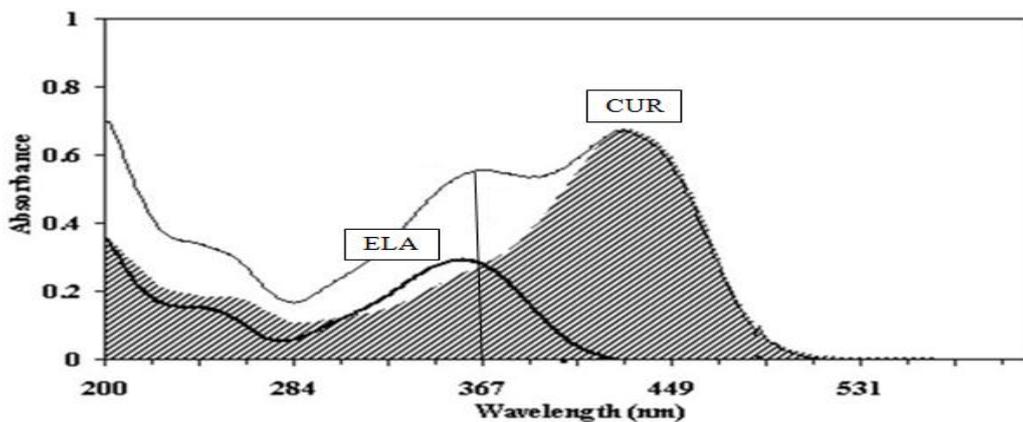


Fig. 1: UV Spectra of ELA and CUR.

A reverse phase HPLC method was developed keeping in mind the system suitability framework i.e. resolution factor between peaks, tailing factor, number of theoretical plates, runtime and the cost effectiveness. The developed optimized method resulted in the elution of

ellagic acid at 1.65 min and curcumin at 2.94 min. Fig. 2 and 3 represent chromatograms of ellagic acid and curcumin standard solution respectively. The total run time was 10 min.

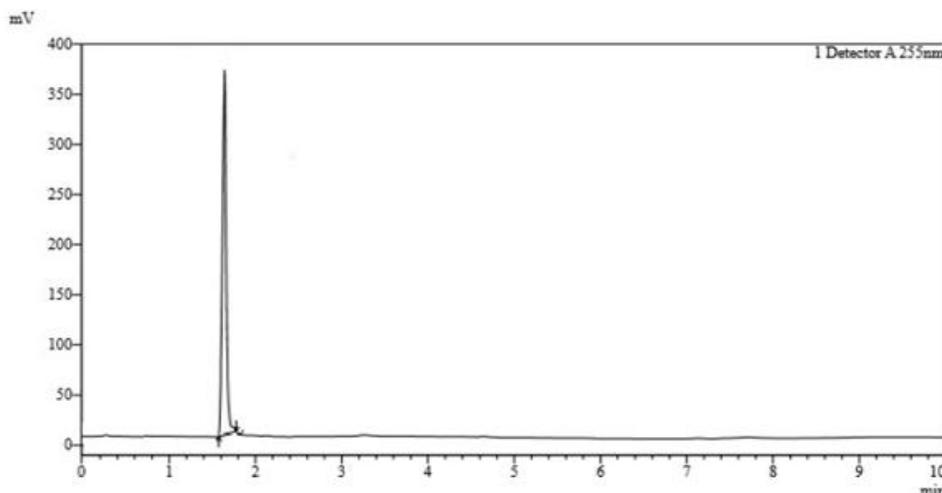


Fig. 2: Chromatograms of ellagic acid standard solution.

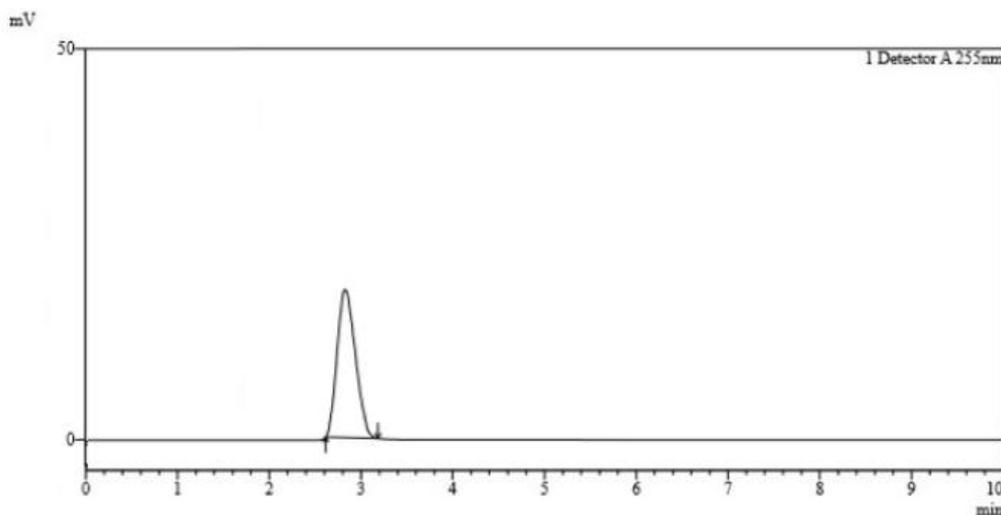


Fig. 3: Chromatograms of curcumin standard solution.

System suitability tests are an integral part of method development and are accustomed to ensure tolerable performance of the chromatographic system. Retention time, number of theoretical plates, peak resolution and

peak tailing circumstance were evaluated replicate injections of the standard working concentration.

The results given in table 1 were within the acceptable limits.

Table 1: System suitability studies results.

S/No.	Parameters	Acceptance Limit	Ellagic acid (ELA)	Curcumin (CUR)
1.	Retention time	-	1.68	2.98
2.	Resolution factor	NLT 2	-	8.14
3.	No. of theoretical plate	NLT 2000	3618	3889
4.	Tailing factor	NMT 2	1.05	1.31

In order to test the applicability of the developed method to an herbal formulation, tablet powder extracts were chromatographed and it is shown in fig. 4.

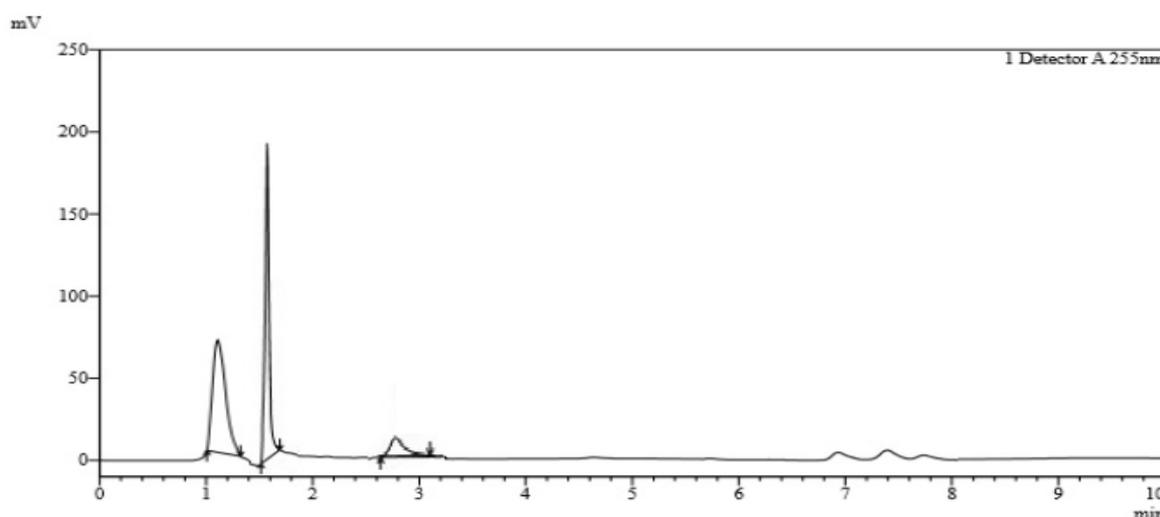


Fig. 4: Chromatograms of herbal formulation.

The sample peaks were identified by comparing the relative retention times with standard markers (fig. 2,3). System suitability parameters were within the acceptance limits, ideal for the chromatographed sample. Integration of separated peak area was done and each marker concentration was determined by using the peak area concentration relationship obtained in the standardization

step. For the analysis of sample, extract of 100 ppm of tablet powder was injected in triplicate and quantified for two active markers using linear regression equation.

The results of tablet powder extract analysis are reported in table 2.

Table 2: Analysis of herbal tablet powder extract.

Formulation	Marker	Amount present (ppm); n=3	Content (%)
Herbal Formulation (Tablet powder extract) 100 pm	Ellagic acid (ELA)	3.92	0.81
	Curcumin (CUR)	2.98	0.63

1. Method validation

Validation of the analytical method is the method that confirmed along laboratory studies in which the performance characteristics of the method meet the stipulations for the intended analytical application. The developed HPLC method was validated according to ICH guidelines for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, method precision, robustness, limit of detection and limit of quantitation.

3.1 Specificity

Fig. 2-4 for standard drug solutions and sample chromatograms reveals that the peaks obtained in the standard solutions and sample solution at working concentrations are only because of the drugs as blank has no peak at the retention time of ellagic acid and curcumin accordingly, it can be concluded that the method developed is said to be specific.

3.2 Precision

3.2.1 System precision

Six replicate injections of the standard solutions at working concentration demonstrated percent relative standard deviation (% RSD) less than 2 relating peak area for each marker, which indicates the tolerable reproducibility and thereby the precision of the system. System precision results are tabulated in table 3.

3.2.2 Method precision

Method precision was determined by performing the analysis of the sample under the test of repeatability at working concentration. Three injections of the sample from the same homogeneous mixture at working concentration showed % RSD less than 2 concerning content of two markers indicate that the method developed is precise by the test of repeatability and

hence can be understood that the method gives consistently reproducible results (table 4).

3.3 Linearity

Standard solutions of ellagic acid and curcumin at different concentrations level were prepared in triplicates. Calibration curves were constructed by plotting the concentration level versus corresponding peak areas for each marker. The results show an excellent correlation between peak areas and concentrations level within the tested concentration range of 6-14 ppm for ellagic acid and as that of 2-10 ppm for curcumin (table 5). The correlation coefficients were greater than 0.99 for each marker, which meet the method validation acceptance criteria and hence the method is said to be linear.

Table 3: Results of system precision.

S/No.	Peak Area	
	ELA	CUR
1	1356600	405178
2	1361151	405276
3	1366110	404024
4	1366288	405671
5	1367917	402517
6	1370101	403210
Mean±SD	1363694.5±5200.94	403927.7±1268.50
%RSD	0.38	0.31

Note: Results are expressed as Mean±SD; n=6; SD: Standard deviation; %RSD: Percent Relative Standard deviation

Table 4: Results of method precision results.

Marker	Intra-day		Inter-day	
	Content (ppm); n=3	%RSD	Content (ppm); n=3	%RSD
ELA	3.90	0.77	3.92	0.81
CUR	2.92	0.59	2.98	0.63

Abbr.: n: number of injections (n=3), %RSD: Percent relative standard deviation

3.4 Accuracy

Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of each compounds in the formulation at three different levels (80%, 100% and 120%). At each level, three determinations were performed. Percent mean recovery was deliberated as demonstrated in table 6. The conventional limits of mean recovery are 98%-102% and fully observed data were within the required range, which indicates good recovery values, okay the accuracy of the method developed.

3.5 Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered, and the system suitability parameter tailing factor and peak area were evaluated. The solution was prepared as per the test method described earlier and injected at different variable conditions like column temperature (33 °C and

37 °C), flow rate (1.1 ml/min and 1.3 ml/min) and detection at specific wavelength. Robustness data clearly shows that the proposed method is robust at small but deliberate change. Robustness data are given in table 7.

3.6 Sensitivity

The sensitivity of measurement of ellagic acid and curcumin by use of the proposed method were estimated in terms of the limit of quantitation (LOQ) and limit of detection (LOD). LOQ and LOD were calculated by the use of the equations $LOD = 3.3\sigma/S$ and $LOQ = 10\sigma/S$ where σ is the standard deviation of intercepts of calibration plots and S is the average of the slopes of the corresponding calibration plot (table 8).

Table 5: Data from linearity studies.

Marker	Conc. range (ppm)	R ²
ELA	6-14	0.997
CUR	2-10	0.991

Table 6: Recovery studies for two markers in herbal formulations.

Compounds	Sample content (ppm)	Standard added (ppm)	Actual amount (ppm)	Total area found (n=3)	Amount recovered (ppm)	% Recovery
ELA	1.87	1.52	3.45	801381	3.39	98.34
		1.91	3.91	826651	3.82	99.50
		2.29	4.22	845421	4.18	99.01
CUR	1.58	1.12	2.54	384992	2.52	99.37
		1.40	2.82	386440	2.83	100.35
		1.68	3.10	388623	3.01	99.10

Table 7: Robustness data for two markers in herbal formulations.

Parameters	ELA (10 ppm)		CUR (10 ppm)	
	Peak area	Tailing factor	Peak area	Tailing factor
Minus temp (33 ^o C)	1356600	1.1	405178	1.28
Plus temp (37 ^o C)	1361151	1.02	405276	1.34
Minus flow rate (1.1 ml/min)	1366110	1.01	404024	1.35
Plus flow rate (1.3 ml/min)	1366288	0.99	405671	1.32
Minus wavelength (365 nm)	1367917	1.1	402517	1.24
Plus wavelength (367 nm)	1370101	1.1	403210	1.28

Table 8: LOD and LOQ for two markers in herbal formulations.

Marker	LOD (ppm)	LOQ (ppm)
ELA	0.63	1.89
CUR	0.15	0.46

RESULTS AND DISCUSSION

Results

Suitable wavelength for the HPLC analysis was determined by recording UV spectrums in the range of 200-400 nm for individual drug solutions of ellagic acid (ELA) and curcumin (CUR) then overlapped. UV overlain spectra of these two markers showed that the drugs absorb appreciably at 366 nm and hence 366nm was taken as a detection wavelength for HPLC analysis (Fig. 1) Overlapped wavelength 366 nm.

A reverse phase HPLC method was developed keeping in mind the system suitability framework i.e. resolution factor between peaks, tailing factor, number of theoretical plates, runtime and the cost effectiveness. The developed optimized method resulted in the elution of ellagic acid at 1.65 min and curcumin at 2.94 min. Fig. 2 and 3 represent chromatograms of ellagic acid and curcumin standard solution respectively. The total run time was 10 min.

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DISCUSSION

Method optimization Simultaneous method development is focused on simple, sensitive and rapid determination of the compounds. Suitable wavelength for the HPLC analysis was resolved by recording UV spectrums in the range of 200-400 nm for individual drug solutions of ellagic acid (ELA) and curcumin (CUR) then overlapped. The optimized chromatographic conditions are tuned by changing the organic phase (0.02 M potassium dihydrogen orthophosphate buffer), pH, flow rate and mobile phase concentration. The optimized chromatographic conditions revealed good separation of curcumin and ellagic acid using a mobile phase 0.02 M potassium dihydrogen orthophosphate buffer (pH adjusted to 3.5 with orthophosphoric acid) and acetonitrile in the ratio 60:40 at a flow rate of 1.2 ml/min, column temperature maintained at 35 °C and a detection wavelength of 255 nm using a UV-visible detector. Curcumin and ellagic acid retention times are found to be 1.68 min and 2.98 min respectively (Table 1). Calibration curves were constructed by plotting the concentration level versus corresponding peak areas for each marker. The results show an excellent correlation between peak areas and concentrations level within the tested concentration range of 6-14 ppm for ellagic acid and as that of 2-10 ppm for curcumin (table 5). The correlation coefficients were greater than 0.99 for each marker, which meet the method validation acceptance criteria and hence the method is said to be linear. Analytical method development was carried out as per ICH guidelines. The developed method was evaluated for validation parameters such as precision, specificity, accuracy, linearity, robustness and sensitivity. Specificity of the method shows drugs as blank has no peak at the retention time of ellagic acid and curcumin accordingly, it can be concluded that the method developed is said to be specific. Precision of the method was carried out in two different days with System precision and Method precision. In System precision Six replicate injections of the standard solutions at working concentration demonstrated percent relative standard deviation (% RSD) less than 2 relating peak area for each marker, which indicates the acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in table 3. And Method precision was determined by performing the analysis of the sample under the test of repeatability at working concentration in table 4. Good linearity and range were achieved with a concentration range of 6-14 ppm for ellagic acid and as that of 2-10 ppm for curcumin (table 5). Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of each compounds in the formulation at three different levels (80%, 100% and 120%). The accepted limits of mean recovery are 98%-102% and fully observed data were within the required range, which indicates good recovery values, okay the accuracy of the method developed Robustness data clearly shows that the proposed method is robust at small but deliberate change. Robustness data are given in table 7. The sensitivity of measurement of ellagic acid

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CONCLUSION

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