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Original Article

Method Development and Validation for Estimation of Curcumin in Fabricated Nano-Sized Formulation: Inter-Laboratory Comparison, Capability and Statistical Analysis

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ABSTRACT

Objective: Current work was designed to develop a simple, rapid, and cost effective spectrophotometric method for the determination of curcumin in fabricated and marketed nano-sized formulation. **Method:** Methanol was optimized as solvent and further spectrophotometric detection was carried at analytical wavelength i.e. 421 nm. Method was further validated as per International conference on Harmonisation (ICH) guidelines for linearity, specificity, accuracy, precision; ruggedness and robustness. **Results:** The concentration of curcumin over range of 1-10 µg/ml obeys Beers law with a correlation coefficient of 0.999. Accuracy was raging from 99.06 to 99.72 %. Percent Recovery estimated was found to be 99.40 ± 0.330. The limit of detection (LOD) and limit of quantification (LOQ) was found to be 0.08 µg/ml and 0.26 µg/ml respectively. Shapiro-Wilk test (P = 0.7323) and Shapiro-Francia test (W' = 0.9815; P= 0.8561) accept the normality of data. Bland-Altman plot revealed an acceptable repeatability coefficient. Youden Plot demonstrated the excellent inter-laboratory reproducibility and it was used to identify Random and total errors. Control charts like Levey-Jennings chart, X- chart and MR chart showed that method is under statistical control. Whereas, CUSUM chart revealed about targetability of the method. Capability analysis demonstrated greater values of Cp (1.18) and Cpk (1.08) than 1, indicating the capability of method to analyze the samples accurately and consistently with minimum variation. **Conclusion:** Validation report demonstrated that common excipients from commercial formulations do not interfere with the proposed method, hence can be applied in regular laboratory analysis. Statistical analysis showed good precision of proposed method.

Keywords: Curcumin, Accuracy, Precision, UV-method development, Validation.

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INTRODUCTION

Although curcumin (Figure 1) is in use since early days, interest of researchers across the world in it is astonishing. Curcumin is the principal component of popular Indian spice turmeric (commonly called as Haldi) and isolated from dried rhizomes of *Curcuma longa* belonging to family *Zingiberaceae*.[1] Curcumin is widely known for its different physico-chemical properties. Curcumin exhibits anticancer, hepatoprotective, Antibacterial, Antiviral and Antifungal, Cardio protective, anti-inflammatory etcetera.[2-6]

Literature survey divulges few analytical methods for the determination of curcumin in bulk and pharmaceutical preparations and biological fluids using High performance thin layer chromatography (HPTLC), High performance liquid chromatography (HPLC), microemulsion electrokinetic chromatography.[7-9] Recently stressed kinetics and pharmacokinetics of curcumin nanoemulsion using validated Ultrahigh-Performance Liquid Chromatography–Synapt Mass Spectrometry has also been studied.[10] Estimation of curcumin in rat plasma by LC-MS/MS has also studied.[11] Spectrophotometry remains to be very popular, due to their simplicity, specificity and low cost amongst the various methods available for the determination of Active pharmaceutical ingredients.[12] Ample work has been going on development of different spectroscopic, HPLC or any other methods for estimation of API in bulk as well as various dosage forms. There is a growing clamour from researchers and academic professional to analyze the data obtained after implementation of analytical techniques statistically. In this study an attempt has been made to analyze the data statistically, to compare inter-laboratory data using novel statistical techniques like Bland-Altman plot, Youden plot and different control charts.

MATERIALS AND METHODS

Materials

Curcumin was obtained from Sami Labs Limited, Bangalore, India as gift sample. Methanol used was of analytical grade and purchased from Merk Chemicals, India. All other chemicals and reagents used were of analytical grade.

Method development

Instrumentation

A Shimadzu UV–visible spectrophotometer (UV mini-1700, Shimadzu Corporation, Kyoto, Japan) was used for all absorbance measurements with matched quartz cells.

Selection and Optimization of Solvent

Different solvents like Chloroform, Acetone and Methanol were screened for solubility of curcumin. Methanol was optimized from all the conditions based on Peak quality and non-interference at the specified wavelength.

Preparation of Standard Stock Solution

Standard stock solution containing 100 µg/ml of curcumin was prepared by initially dissolving accurately weighed 10 mg of curcumin in 50 ml of methanol, followed by sonication for 10 min and the final volume of solution was made up to 100 ml with methanol.

Selection of Wavelength

An ideal wavelength is one that gives good response for the drugs to be detected. The wavelength at which maximum absorption takes place is selected for further analysis. Selection of wavelength was carried out by transferring appropriate volume of 1 ml of standard stock solution into 10 ml volumetric flask, diluted to mark with methanol to give concen-

tration of 10 µg/ml.The resulting Solution was scanned in visible range (400-800 nm). In spectrum curcumin showed absorbance maximum at 421 nm (Figure 2).

Validation of Method

The method was validated in terms of linearity, accuracy, precision and ruggedness as per ICH guidelines.

Linearity Study

Aliquots of 1–10 ml portion of stock solutions were transferred into series of 10 ml volumetric flasks and further volume was made up to mark with methanol, to get concentrations 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/ml, respectively. All the solutions were scanned in the range of 400–800 nm against blank. The absorption maxima were found to be at 421 nm against blank. Further calibration curve was plotted using the data.

Accuracy

To the preanalyzed 4 µg/ml curcumin solutions, a known amount of standard curcumin was added at different levels, i.e. 80% 100% and 120%. Solutions were reanalysed by the proposed method. Three samples were prepared for each recovery level. The accuracy was reported as % recovery.

Precision

Precision of the method was studied as intra-day (Repeatability) and inter-day (Intermediate Precision) variations. Intra-day precision was determined by analyzing 4, 6, and 8 µg/ml of curcumin solution for three times in the same day. Inter-day precision was determined by analysing 4, 6, and 8 µg/ml of curcumin solution for three days.

Sensitivity

The sensitivity of the proposed method was estimated in terms of the limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were calculated using equations,

$$LOD = 3\,Sa/b \quad \text{Eq. 1}$$

$$LOQ = 10\,Sa/b \quad \text{Eq. 2}$$

Where *Sa* is the standard deviation of the response and b is the slope of the corresponding calibration curve.[13] Percent relative standard deviation, standard deviation was reported for each set of data.

Ruggedness

Ruggedness of the proposed method was determined by analyzing 4 µg/ml concentration of curcumin using same instrument by two different analysts under similar operational and environmental conditions.

Robustness

Robustness of the proposed method was also determined by changing the λ_{max} of the analysis by ± 2.0 nm. Percent mean recovery as well as percent relative error was reported.[14]

Statistical analysis of proposed method

Normality of the data and outlier detection

Most of the statistical tests mainly parametric tests rest upon the assumption of normality.

Henceforth it is important to know whether data are normal or non-normal. Normality of data is assayed by normal quantile-quantile plot (Q-Q plot) in which normal score of the observations plotted against expected normal score. Shapiro-Wilk test and Shapiro-Francia test for normal distribution is also implemented.

Shape of the distribution, its central value, its variability and outlier detection is determined by box and whisker plot. Variability in a data set is measured by the interquartile range which is equal to Q3 – Q1, i.e. the difference between the 75th percentile and the 25th percentile.

Coefficient of Repeatability by Bland-Altman plot

The Bland-Altman plot is a graphical technique and generally used to analyzing the agreement between two measurements techniques designed to measure the same parameter.[15,16] It is used to look for any systematic bias and to identify possible outliers.

In this study we have used Bland-Altman plot to compare repeated measurements obtained using one single method on a series of subjects in order to evaluate the repeatability or precision of a method. Therefore, the Coefficient of Repeatability (CR) can be calculated as 2 times the standard deviation of the differences between the two measurements (D2 and D1).[15] Study was performed using sample of known concentration (4µg/ml).

$$CR = 1.96X\frac{\sqrt{\Sigma(D_2 - D_1)^2}}{n} \quad \text{Eq. 3}$$

Reproducibility using The Youden plot

The Youden plots can be used to analyze and compare inter-laboratories data obtained using an analytical method.[17,18] In this work two samples, similar and reasonably close in the magnitude are analyzed in four different laboratories using proposed method. From the data obtained youden plot is constructed. To perform this study two samples of known concentrations (4 µg/ml) was prepared. Youden plot and other analysis are performed using MedCalc Statistical Software version 17.8 (MedCalc Software bvba, Ostend, Belgium)

Statistical control of proposed method

In order to determine measure of precision or ability of the measurement system to reproduces the same result over time and under varying operating conditions quality control of data is studied using control charts. In this study control charts are used to check the ability of the analytical method to produce the accurate results of known concentrated solution. It is useful to determine the deviation from mean value or target value.

Zone Test

Zone test is performed in order to determine whether process in influencing by variables or not. For this study control charts are divided into Zone A, B and C by dividing the area between the average and the upper control limit into three equally spaced areas. Each zone is one standard deviation in width. Region between the average and average plus one standard deviationis denoted as zone C. Region between the average plus one standard deviation and the average plus two standard deviations is denoted as Zone B. Whereas, region between the average plus two standard deviations and the average plus three standard deviations is denoted as Zone A. This is repeated for area between lower control limits and average too.[19]

Capability Analysis of Proposed Method

Capability analysis is used to assess whether a method is statistically able to meet a set of specifications or requirements.[20] It can be use to check whether the process is testing the samples precisely or not. In order to perform capability analysis sample of known concentration is prepared and tested using proposed method. Lower specification limit (LSL), Nominal value and upper specification limit (USL) was set at 3.95, 4.00 and 4.05 respectively.

Process capability (C_p) is calculated by,

$$C_p = \frac{USL - LSL}{6\sigma_{within}} \quad \text{Eq. 4}$$

Process capability index (Cpk) is calculated by,

$$C_{pk} = \text{minimum of}\left(\frac{\text{Mean} - \text{LSL}}{3\sigma}, \frac{\text{USL} - \text{mean}}{3\sigma}\right) \quad \text{Eq. 5}$$

Cp and Cpk should be greater than 1. Capability analysis should be performed only after it has been brought under statistical control.[21] It is performed using SPC for excel.

Application of the Proposed Method for Pharmaceutical Formulation

Twenty tablets of curcumin were weighed and finely powdered. Powder equivalent to 10 mg of curcumin was weighed accurately and transferred into 100 ml volumetric flask. Further 50 ml of methanol was added and resulting solution was sonicated for 15 min to facilitate extraction of the drug from the powder. Later volume was made up to 100 ml and solution was filtered through the Whattman filter paper No. 41. The resulting filtrate was further diluted to get the solution of 10 µg/ml concentration and analyzed for drug content determination against blank using proposed method. The drug content of the preparation was calculated using standard calibration curve.[14,22]

Application of the Proposed Method to Formulation of Curcumin

Amount of curcumin encapsulated in prepared nanoparticles was estimated by measuring the free curcumin in the Nanoformulations. Powdered nanoformulation was extracted using methanol and sonicated for 15 min and volume was made up to 100 ml. The resulting solution was centrifuged at 2500 rpm for 10 min and supernatant was analyzed for drug content.[23]

RESULTS AND DISCUSSION

Method Validation

The proposed method was validated as per the ICH guidelines (Q2 (R1)). In spectrum curcumin showed absorbance maximum at 421 nm (Figure 2)

Linearity Study

Linearity of analytical method within a given range is a plot of signals as a function of analyte concentration or content. The linear regression data for the calibration curves has shown linear relationship over the concentration range of 01- 10 µg/ml (Figure 3). Linear regression equation was found to be Y = 0.1929x + 0.0493 (R^2 = 0.999). Absorbance of the solutions of different concentration is reported in Table 1. Whereas, result of regression analysis has reported in Table 2.

Accuracy

Accuracy of an analytical method is the closeness of test results to true value and studied by recovery experiments.[14] The % recovery for the analysis and all the three concentration levels ranged from 99.06 % to 99.72 % with % RSD from 0.13 to 0.38 showing that any minute change in the drug concentration can be correctly determined with high accuracy (Table 3). Proved accuracy of proposed method using recovery studies indicates its reliability in routine analytical application.

Precision

The precision of an analytical method expresses the degree of scatter between a series of measurements obtained from multiple sampling of the same homogeneous sample under prescribed conditions.[24] Intra-day precision refers to the use of analytical procedure within a laboratory over a short period of time using the same operator with the same equipment whereas Inter-day precision involves estimation of variations in analysis when a method is used within a laboratory on different days, by different analysts. Repeatability (intraday) was assessed by analyzing these three different Concentrations (2.0, 4.0, 6.0 µg/ml), three times a day. Intermediate precision (Interday) was established by analyzing these three different concentrations (2.0, 4.0, 6.0 µg/ml), three times a day for at least three different days. Intraday and Interday precision showed >99% recovery. Detailed result is reported in Table 4 and Table 5.

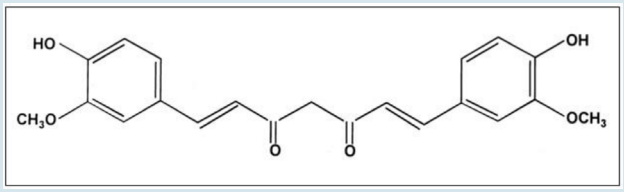

Figure 1: Structure of curcumin.

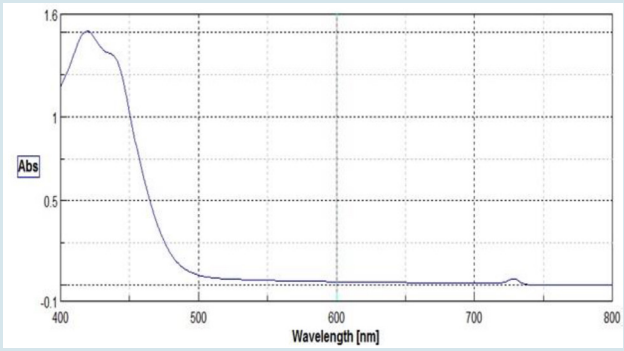

Figure 2: Visible Spectra of curcumin showing λmax at 421 nm.

Table 1: Linearity study of curcumin

Sr. No.	Concentration (µg/ml)	Absorbance
1	1	0.2372
2	2	0.4315
3	3	0.6581
4	4	0.8216
5	5	0.9935
6	6	1.2219
7	7	1.3838
8	8	1.5637
9	9	1.7936
10	10	1.999

Sensitivity

LOD is the lowest analyte concentration that can be detected at a specified level of confidence, so it is greater than limit of blank. Whereas, the LOQ is the limit at which the difference between two distinct values can be reasonably distinguished.[25] The LOD and LOQ for curcumin were found to be 0.08 µg/ml and 0.26 µg/ml.

Table 2: Regression analysis of the data

Least squares regression					
Sample size			10		
Coefficient of determination R^2			0.9990		
Residual standard deviation			0.01988		
Regression Equation					
y = 0.04926 + 0.1929 x					
Parameter	Coefficient	Std. Error	95% CI	t	P
Intercept	0.04926	0.01358	0.01794 to 0.08058	3.6270	0.0067
Slope	0.1929	0.002189	0.1879 to 0.1980	88.1432	<0.0001
Analysis of Variance					
Source	DF		Sum of Squares	Mean Square	
Regression	1		3.0709	3.0709	
Residual	8		0.003162	0.0003953	
F-ratio			7769.2303		
Significance level			P<0.0001		
Residuals					
Shapiro-Wilk test for Normal distribution			W=0.9822 accept Normality (P=0.9758)		

Table 3: Summary of recovery study*

Reanalyzed sample	Level of Recovery (%)	Amount of Drug Added (µg/ml)	Amount of drug detected (µg/ml)	% Recovery	% RSD
4 µg/ml	80	3.2	3.17 ± 0.010	99.06	0.31
	100	4	3.98 ± 0.015	99.42	0.38
	120	4.8	4.78 ± 0.062	99.72	0.13

* Indicates ± SD (n=3)

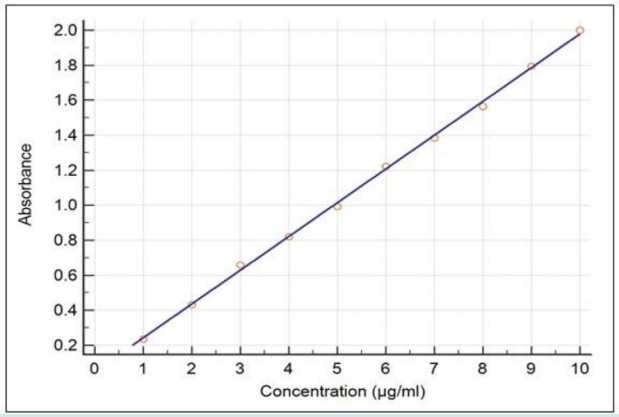

Figure 3: Calibration Curve of curcumin at different concentrations.

Ruggedness

Amount of curcumin recovered by two analysts applying proposed method and working on same instrument are depicted in Table 6. The result showed that the % R.S.D. was less than 2.

Robustness

Robustness of proposed method was studied by monitoring the influence of small variations of wavelength. The results obtained after modification of wavelength were not different compared to the optimum conditions (Table 7). These alterations of wavelength do not affect the assay of curcumin, henceforth the proposed method could be considered robust.

Statistical analysis of proposed method

Normality of the data and outlier detection

The normal Q-Q plot is shown in Figure 4. The data is found to be light tailed with a spike of identical values. Values of Coefficient of Skewness and Coefficient of Kurtosis was found to be -0.1832 (P=0.6811) and -0.2547 (P=0.4722). Shapiro-Wilk test (W' =0.9718; P=0.7323) and Shapiro-Francia test (W'=0.9815; P=0.8561) accept the normality of data.

Box and Whisker plot (Figure 5) showed that the concentration determined is skewed little left. The part of the box to the right of the median is slightly longer than the part to the right of the median. Figure 5 also-shows the descriptive statistics of the data and confirms the left skewness of the data. The median concentration (4.0037) is slightly higher than the mean concentration (4.00). Interquartile range was found to be 0.0125 indicating extremely less variability in the data set. From group of samples whose concentrations were closest to the median, half of them were within 0.0125 µg of each other when they analyzed using proposed method. No outlier was detected which is confirmed by Generalized ESD test (α = 0.05) and Grubbs - left-sided test (α = 0.05).

Repeatability coefficient by Bland-Altman plot

The graph displays a scatter diagram of the differences plotted against the averages of the two measurements. Horizontal lines are drawn at the mean difference, and at the limits of agreement (LOA) which is defined as the mean difference ± 1.96 SD of differences. Coefficient of Repeatability was found to be 83.231. Noteworthy, 95 % confidence intervals of LOA do not exceed the maximum allowed difference between runs, demonstrating the closeness of the results (Figure 6). Henceforth the developed method can be used to perform the routine analysis of the samples repeatedly.[26, 27]

Table 4: Summary of intra -day precision study*

Conc. (µg/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Absorbance (Mean ± SD)	Conc. Found (µg/ml)	% RSD	% Recovery
2	0.398	0.391	0.396	0.395 ± 0.0036	1.998	0.912	99.91
4	0.782	0.776	0.769	0.775 ± 0.0065	3.971	0.838	99.29
6	1.166	1.153	1.151	1.156 ± 0.0081	5.946	0.704	99.114

* Indicates ± SD (n=3)

Table 5: Summary of inter-day precision study

Conc. (µg/ml)	Amount of drug detected (µg/ml)				% RSD	% Recovery
	Day 1	Day 2	Day 3	(Mean ± SD)		
2	1.993	1.988	1.981	1.987 ± 0.0060	0.303	99.372
4	3.966	3.975	3.971	3.970 ± 0.0045	0.113	99.280
6	5.958	5.962	5.946	5.955 ± 0.0083	0.139	99.268

* Indicates ± SD (n=3)

Table 6: Summary of ruggedness studies*

Conc. (µg/ml)	Analyst	Amount detected (µg/ml)	Amount detected (%)	% RSD
4	Analyst 1	3.954 ± 0.0066	98.862	0.862
	Analyst 2	3.964 ± 0.0050	99.121	0.650

* Indicates ± SD (n=3)

Table 7: Summary of robustness studies

Conc. (µg/ml)	Wavelength (nm)	Amount detected (µg/ml)	Amount detected (%)	% RSD
4	419	3.952	98.948	0.720
	423	3.959	98.992	0.880

Table 8: Analysis of the marketed formulation

Conc. (µg/ml)	Conc. Found (µg/ml)	Conc. Found (%)	Conc. Found (Mean ± SD)	% RSD
10	9.892	98.32		
10	9.901	99.01	98.816 ± 0.4336	0.438
10	9.912	99.12		

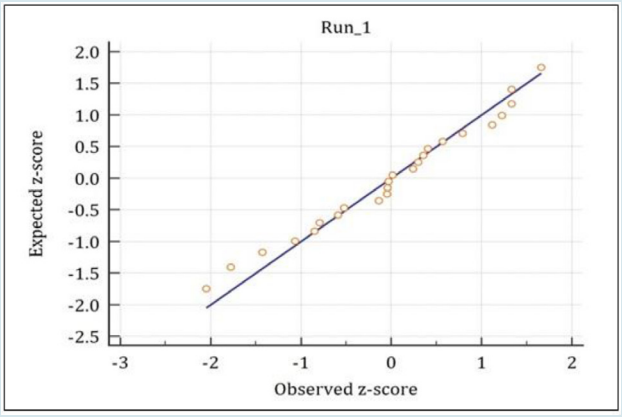

Figure 4: Q-Q Plot showing Goodness of fit.

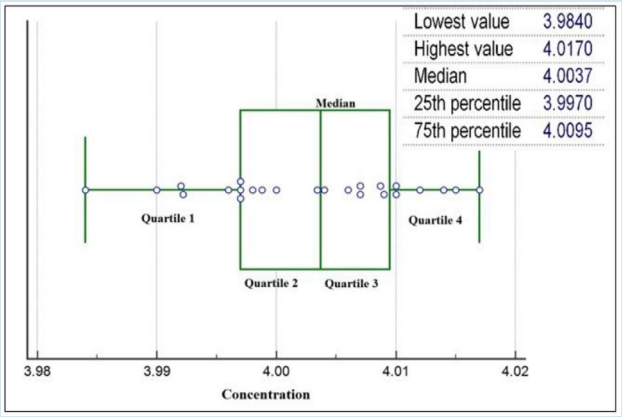

Figure 5: Box and Whisker Plot with descriptive statistics.

Reproducibility using The Youden plot

Youden plot, depicted in Figure 7A and 7B is constructed by plotting response variable 1 on vertical axis: (i.e., run 1) or response variable 2 on horizontal axis: (i.e., run 2). Each point in the graph corresponds to result of run 1 and run 2 of one laboratory. Two median lines parallel to X and Y axis are intersecting at a point called as Manhattan median through which a 45-degree reference line is drawn. Points that lie near the 45-degree reference line but far from the Manhattan median indicate large systematic error. Points that lie far from the 45-degree line indicate large random error.[28]

Statistical control of proposed method

The control charts are the statistical approach which shows picture of a process over time and can be used to study the analytical process for improving its precision. Different quality control chart is depicted in Figure 8.

In Levey-Jennings chart the distance from the mean is measured in standard deviations (SD). Upper control limit (UCL) and lower control limits (LCL) along with target value which is generated in a graph helps to determine the outliers and give a visual indication whether a laboratory method is working well or not. As shown in Figure 8C the

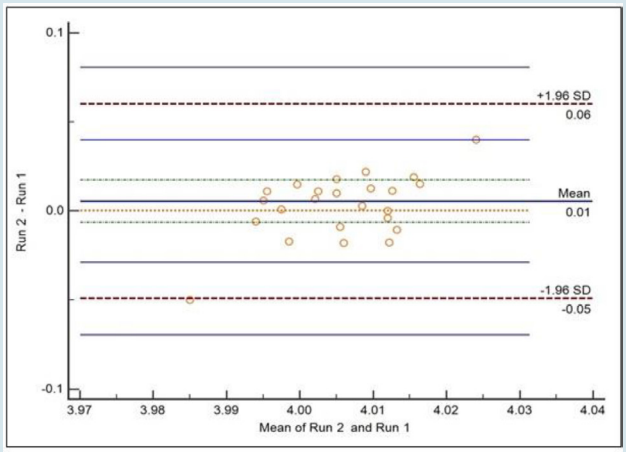

Figure 6: The Bland-Altman plot for repetitive measurements for same method.

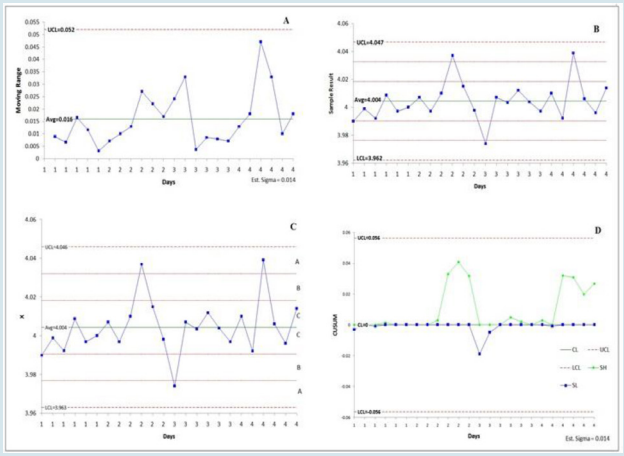

Figure 8: Control charts; A) MR (Moving range) chart, B) X-Individual chart, C) Levey-Jennings chart and, D) CUSUM chart.

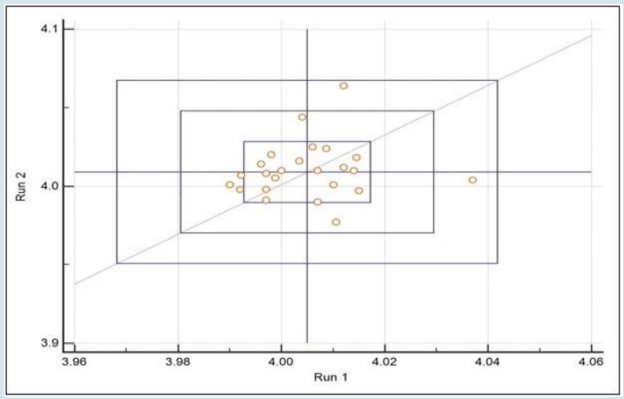

Figure 7A: Youden plot for inter-laboratories data. Rectangles represent 1, 2 and 3 SD.

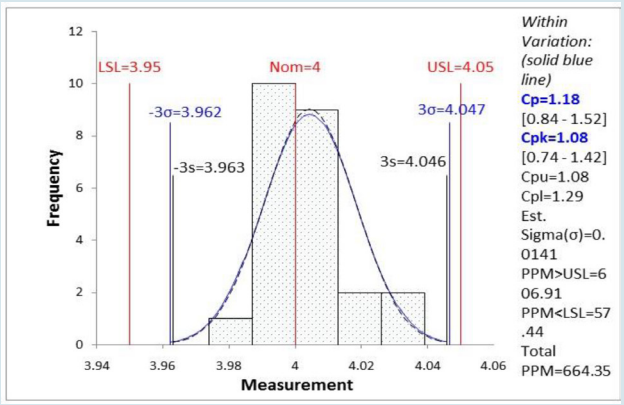

Figure 9: Capability analysis indicating Cp and Cpk for the proposed method.

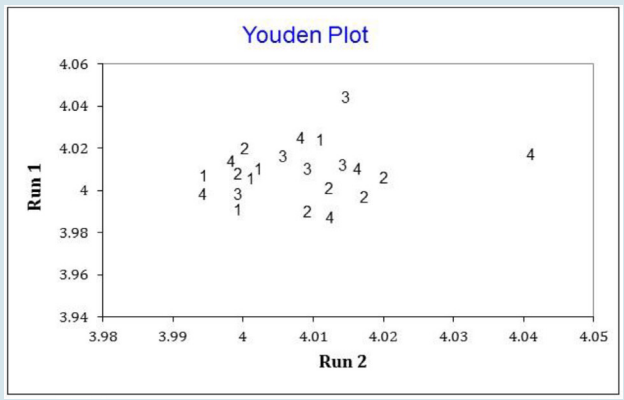

Figure 7B: Youden plot for inter-laboratories data. 1, 2, 3 and 4 indicated different laboratories.

Levey-Jennings charts for the process shows that most points are near the average and no points are beyond the control limits, indicative of absence of special cause variation in the process. Hence the process is under statistical control.[28,29] XmR (individual and moving range) charts also support this, see Figure 8A and 8B.[30]

In the present study CUSUM control chart is used to verify process targetability. Target was fixed by analyzing known concentration sample i.e. 4 µg/ml. The CUSUM chart is plotted with center line indicating zero along with both the cumulative sums on the high side (SH) and lower side (SL). From the Figure 8D it is observed that cumulative sums on the high side instigate to pile up around sample 3rd of 2nd day and continue up to last sample of same day. Same case is observed on day 4 however, process doesn't get beyond UCL or LCL. Conclusively, although the control charts indicated the process in under statistical control, CUSUM chart depicted the drifting of process off-target for few samples.[31]

Zone Test

Each zone is one standard deviation in width. Region between the average and average plus one standard deviationis denoted as zone C. Region between the average plus one standard deviation and the average plus two standard deviations is denoted as Zone B. Whereas, region between the average plus two standard deviations and the average plus three standard deviations is denoted as Zone A.

From the Figure 8C it was observed that two out of three consecutive points does not fall in zone A or beyond, four out five consecutive points does not fall in zone B or beyond and notably seven consecutive points does not fall in zone C or beyond. It means that no special cause variation is present and process in under statistical control.[32,33]

Capability Analysis of Proposed Method

Process capability analysis ensures the performance of a process against its pre-determined specifications.[34] In Figure 9 the dashed black line represents the normal distribution of the data using the overall standard deviation, while the blue solid line represents the normal distribution of the data using the within standard deviation. Process performance (Pp) uses the overall standard deviation and Cp uses the within standard deviation.

From capability analysis it is clear that, 6 sigma less wide than specification width which indicates the capability of process to continuously provides the test results within the specification limits and near to true value. This is confirmed by greater values of Cp (1.18) and CPK (1.08) than 1. Higher CPK value indicates the proposed method meeting the target or true value consistently with minimum variation.[35,36]

Application of the Method to the Fabricated Nano-Formulation

The prepared nano-formulation was analyzed by the proposed method. Notably, the assay value for fabricated formulations was found to be 99.85 %.

Application of the Proposed Method for Pharmaceutical Formulation

Marketed formulation was analyzed by the proposed method. The assay value for marketed formulation was found to be 98.81 % (Table 8).

CONCLUSION

UVspectrophotometric method was successfully developed and validated as per ICH guidelines. Developed method was simple, accurate, precise, reproducible, and sensitive. Quality control analysis and estimation of curcumin from all kind of pharmaceutical formulations can be effortlessly carried out by implementing this method. This is the first report of detail statistical analysis of any analytical method. Statistical analysis showed that process is under statistical control and capable to analyze the samples unceasingly.

ACKNOWLEDGEMENT

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CONFLICT OF INTEREST

The authors declare no conflcit of interest.

ABBREVIATIONS

ICH: International Conference on Harmonisation; **LOQ:** Limit of Quantification; **LOD:** Limit of Detection; **CR:** Coefficient of Repeatability ; **UCL:** Upper Control Limit; **LCL:** Lower Control Limits; **LOA:** Limits of Agreement; **MR:** Moving Range.

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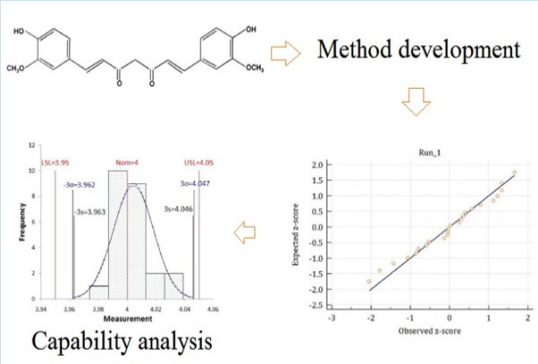
PICTORIAL ABSTRACT



SUMMARY

- Current work was designed to develop a simple, rapid, and cost effective spectrophotometric method for the determination of curcumin in fabricated and marketed nano-sized formulation. Literature survey divulges few analytical methods for the determination of curcumin in bulk and pharmaceutical preparations. In this study an attempt has been made to analyze the data statistically, to compare inter-laboratory data using novel statistical techniques like, Bland-Altman plot, Youden plot and different control charts.

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