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Novel analytical method development and validation for simultaneous estimation of curcumin, ascorbic acid and salicylic acid in bulk and its pharmaceutical formulation by RP-HPLC

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Abstract: A precise and specific reverse phase high-performance liquid chromatographic method has been developed and validated to quantify curcumin, ascorbic acid, and salicylic acid in both bulk and hydrogel. Utilizing a Hypersil BDS C18 column and an isocratic mode, the mobile phase comprised of a mixture of 0.1% orthophosphoric acid and acetonitrile (50:50 v/v). The calibration range spanned concentrations of 100 - 300 µg/mL for curcumin, 50 - 150 µg/mL for ascorbic acid and 50 - 150 µg/mL for salicylic acid. The specificity of the proposed method for estimating these compounds was established through chromatographic peak purity analysis. The limit of detection and the limit of quantification were found to be 18.54 µg/mL and 56.20 µg/mL for curcumin, 10.05 µg/mL and 30.46 µg/mL for ascorbic acid, and 11.39 µg/mL and 34.51 µg/mL for salicylic acid respectively. The accuracy of the method was demonstrated by recovering curcumin, ascorbic acid, and salicylic acid from the hydrogel formulation with a recovery rate exceeding 98%. This indicates the capability of the method to accurately estimate active pharmaceutical ingredients in hydrogel dosage form without interference from excipients. Validation results support the potential applicability of the proposed method for the quantitative estimation of these three drugs in hydrogel.

Keywords: HPLC; hydrogel; method development; curcumin; ascorbic acid; salicylic acid.

1. Introduction

Curcumin, a natural polyphenol derived from turmeric, possesses a unique molecular structure and diverse therapeutic properties [1]. Its traditional use and rich history have led to extensive exploration of its potential health benefits. It has antioxidant, anti-cancer, and anti-inflammatory qualities [2]. It has two aromatic rings containing o-methoxy phenolic group interconnected by seven carbons linkers (α, β-unsaturated β-diketone moiety). Its ability to modulate various cellular pathways and processes underscores its pharmacological effects. However, challenges like poor solubility and low bioavailability hinder its clinical application [3-4]. Researchers are employing innovative approaches to enhance the efficacy of curcumin [5-7].

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Quantification of curcumin, ascorbic acid and salicylic

A water-soluble ascorbic acid, more commonly recognized as vitamin C, is a vital nutrient that plays a crucial role in human well-being [8]. It is found abundantly in various fruits and vegetables, its biochemical significance extends beyond its well-known function as an antioxidant [9]. Its molecular structure includes a six-carbon lactone ring with hydroxyl groups, enabling it to readily donate electrons and scavenge reactive oxygen species. In addition to its role in immune function, ascorbic acid participates in collagen synthesis, neurotransmitter production, and iron absorption.

Salicylic acid, a naturally occurring plant compound, has captured scientific attention for its varied properties and therapeutic applications. It is derived from sources like willow bark and various plants. It is classified as a beta-hydroxy acid due to its distinctive hydroxyl group placement. It is renowned for its role in pain relief and fever reduction, its pharmacological activities extend to anti-inflammatory and antiplatelet effects. In dermatology, it serves as a cornerstone for treating skin conditions such as acne and psoriasis, owing to its exfoliating and keratolytic properties. The interaction between the chemical structure of salicylic acid and its diverse biological effects has positioned it as a vital component in pharmaceutical formulations and medical therapies, rendering it an intriguing subject for comprehensive research.

Simultaneous use of curcumin, ascorbic acid, and salicylic acid represents a dynamic interplay between three bioactive compounds with distinct molecular structures and multifunctional therapeutic properties. Curcumin exhibits potent antioxidant and anti-inflammatory effects, ascorbic acid contributes antioxidant defense, collagen synthesis, and immune support and salicylic acid offers anti-inflammatory and exfoliating benefits [10-12]. This combination presents a unique potential for synergistic effects, enhancing individual benefits of each compound and expanding their collective impact. The intricate interplay between curcumin, ascorbic acid, and salicylic acid presents a promising avenue for innovative therapeutic formulation that capitalize on their complementary mechanisms, potentially yielding enhanced outcomes across a spectrum of health applications.

The development and validation of analytical techniques for the estimation of curcumin, ascorbic acid and salicylic acid has been thoroughly reviewed and the primary focus of the literature review. Curcumin has witnessed extensive method development and validation efforts due to its diverse therapeutic potential. UV- visible spectrophotometric and RP-HPLC estimation of curcumin has been reported in formulation [13-15] and RP-HPLC methods have been commonly employed, with various mobile phase compositions and column types tested to achieve optimal separation and quantification. Validation studies encompassing accuracy, precision, linearity, and robustness have underscored the reliability of these methods. Novel approaches such as spectrofluorimetric and electrochemical techniques have also emerged, enhancing sensitivity and selectivity. Ascorbic acid has been widely studied using various analytical techniques [16,17]. Salicylic acid has been analyzed by HPLC method [18]. HPLC and spectrophotometric methods have been prominent due to their accuracy and ease of use. Parameters such as linearity, precision, and accuracy have been consistently addressed, ensuring the reliability of these methods for quantification [19, 20]. The synthesis of above approaches will help develop the innovative analytical method for simultaneous estimation of curcumin, ascorbic acid, and salicylic acid.

The present research investigation focuses on development of RP-HPLC (Reverse phase High performance liquid chromatography) for the simultaneous estimation of curcumin, ascorbic acid, and salicylic acid in bulk and in the formulated hydrogel. The combination within this hydrogel formulation, consisting of three components (curcumin transferosome, ascorbic acid and salicylic acid) within a single dosage form, is not commercially available [21]. The method developed and validated was specifically tailored for the estimation of the novel hydrogel formulation. This combination presents a unique potential for synergistic effects, enhancing the individual benefits of each compound and expanding their collective impact. [22-24] The intricate interplay between curcumin, ascorbic acid, and salicylic acid presents a promising avenue for innovative therapeutic formulation that capitalize on their complementary mechanisms, potentially yielding enhanced outcomes across a spectrum of health applications. This single method enables the simultaneous estimation of all three components, streamlining the process and offering a comprehensive analysis in a singular procedure. Simultaneous analysis, cost-effective, wider applicability, and enhanced sensitivity are the advantages over the single component analysis.

2. Experimental

2.1. Materials and Methods

Curcumin standard (Product code:RM1449-5G, purity:98.5%) was procured from Himedia Laboratories Pvt. Ltd. Maharashtra, India. Ascorbic acid (Product code: 10220SG100, purity:99.5%) and salicylic acid (Product code: 11443SG500, purity:99.5%) standards were obtained as a gift sample from Finar Ltd, Ahmedabad, Gujarat, India. Orthophosphoric acid (purity 88%) and water (Mili-Q) were procured from Rankem Labs, Vadodara, Gujarat, India. HPLC grade acetonitrile (purity 99.8%) was procured from Merck Life Science Pvt. Ltd, India. All chemicals were of analytical reagent grade and solutions were prepared by using HPLC water.

2.2. Instrumentation and Mobile Phase Preparation for RP- HPLC Analysis

RP- HPLC procedures were executed for analysis by using a Shimadzu prominence-I LC-2030 plus system, which comprised of an auto sampler, a photo diode array detector (PDA),a gradient pump, and a column oven equipped with temperature control. The chromatographic data and relevant parameters were meticulously recorded using Lab solution data acquisition software. The RP-HPLC analysis, conducted using a Hypersil BDS C18, 5µ column, possess dimensions of 250 x 4.6 mm (Thermo Scientific, U.K.). The mobile phase comprised of a buffer solution (prepared by dissolving 1 mL of Orthophosphoric acid and diluting to 1000 mL with HPLC-grade water) and acetonitrile in a 50:50 (v/v) ratio. The mobile phase was filtered using a 0.45µ membrane filter and degassed.

2.3. Preparation of Standard Stock Solution and Working Standard Solutions

Curcumin 40 mg, ascorbic acid 20 mg and salicylic acid 20 mg were accurately weighed into a 20 mL volumetric flask. An appropriate volume of the mobile phase (prepared as per section 2.2) was added to dissolve the weighed compounds in the flask. The solutions were sonicated for 10 minutes and allowed to cool down to room temperature. The solution was diluted up to the mark with mobile phase to get the standard stock solution 500 µg/mL for curcumin, 500 µg/mL for ascorbic acid and 500 µg/mL for salicylic acid. Further dilutions were carried out from this standard stock solution using the same mobile phase so as to get required concentration of working standard solution 100-300 µg/mL of curcumin, 50-150 µg/mL ascorbic acid and 50-150 µg/mL for salicylic acid.

2.4. Preparation of Sample Solution

The formulated hydrogel (sample) 100 mg was accurately weighed and transferred to a 50 mL volumetric flask. Forty mL of mobile phase was added and sonicated for 15 minutes. The volume was made up to the mark with mobile phase and centrifuged for 10 min at 4000 rpm. Accurately measured 10 mL of clear supernatant was further diluted to 20 mL to get the sample concentration 1000 µg/mL.

2.5. Method Development and Calibration Curve Preparation

A 20µgL of working standard solution was injected into RP-HPLC system and the mobile phase was maintained at a constant flow rate of 1.0 mL/min, and the detection wavelength was set at 254.0 nm. The entire process had a run time of 15 minutes. The Chromatogram was recorded. Final concentration of 100, 160, 200, 240, 300 µg/mL for curcumin and 50, 80, 100, 120,150 µg/mL each for ascorbic acid and salicylic acid were prepared by accurately measuring the appropriate aliquots of standard stock solution and diluting it to 10 mL with mobile phase. These solutions (20µgL) were injected into chromatographic system, chromatograms were recorded, and peak area was determined for each concentration of drug solution. Calibration curve of three drugs were constructed by plotting peak area versus applied concentration and regression equation was computed.

2.6. Method Validation

Validation of the proposed method RP-HPLC method was carried out using ICH guideline Q2 (R1). These parameters included linearity and range, repeatability, accuracy, intraday precision, interday precision, limit of detection (LOD), limit of quantification (LOQ), robustness, and system suitability. Detailed results of the validation parameters are tabulated in Table 1 to Table 7. The graphical representation of optimized chromatogram is presented in Figure 1.

2.6.1. Linearity

To assess the linearity of response, solutions ranging over concentrations from 100- 300 µg/mL for curcumin and 50-150 µg/mL for both ascorbic acid and salicylic acid were accurately prepared and subjected to the developed RP-HPLC method. The response data obtained from the calibration curves facilitated the computation of correlation coefficients, slopes, intercepts, and regression line equations. This analytical process robustly determined the linear relationship between analyte concentration and detector response across the specified concentration ranges. The results are shown in Table 2.

2.6.2. Repeatability

The hydrogel formulation was used as the sample matrix in order to assess the repeatability of the suggested approach. Six sample solutions were prepared from the hydrogel formulation and examined using the proposed method in order to evaluate repeatability. The results are shown in Table 3.

2.6.3. Accuracy

Accuracy of the develop method was determined using recovery study wherein, controlled spiking experiments were conducted by adding curcumin API, ascorbic acid API, and salicylic acid API to hydrogel formulation at levels corresponding to 50%, 100%, and 150% of the labelled claim. The resulting concentrations were then analysed, and the percentage recoveries for each compound was calculated across the different spiked levels. The results are shown in Table 4.

2.6.4. Intraday Precision

Intraday precision was determined through the analysis of solutions at three distinct concentrations: 100 µg/mL, 200 µg/mL, and 300 µg/mL for curcumin, as well as 50 µg/mL, 100 µg/mL, and 150 µg/ml for both ascorbic acid and salicylic acid. For each concentration, three replicates were injected into the HPLC system on the same day. The results were analysed to calculate the percentage relative standard deviation (% RSD) as a measure of precision. The results are shown in Table 3.

2.6.5. Interday Precision

Interday precision was assessed under similar conditions as intraday. Solutions with concentrations of 100 µg/mL, 200 µg/mL, and 300 µg/mL for curcumin, as well as 50 µg/mL, 100 µg/mL, and 150 µg/mL for both ascorbic acid and salicylic acid were prepared, and three replicates of each were injected into the HPLC system on three consecutive days. The calculated % RSD from the obtained results offered insight into the precision of the method across different days. The results are shown in table 3.

2.5.6 Limit of Detection (LOD) and Limit of Quantitation (LOD)

Visual method has been used to the find out LOD and LOQ for curcumin, ascorbic acid and salicylic acid. This involved preparing successive dilutions of various concentrations of curcumin, ascorbic acid and salicylic acid, which were subsequently injected into the HPLC system. The lowest concentration at which the relative standard deviation (% RSD) dropped of three replicates below 10.0% was determined to be the LOQ. On the other hand, the LOD was determined as the concentration at which the peaks corresponding to curcumin, ascorbic acid and salicylic acid became visibly detectable in the chromatogram. This meticulous process allowed for the precise determination of the minimum levels of these compounds that the method could reliably detect and quantify.

2.5.7. Robustness

Robustness of the developed method was evaluated through deliberate minor adjustments to chromatographic parameters, including variations in flow rate and mobile phase composition. The altered conditions are detailed in Table 2.

2.5.8. System Suitability Test

Six replicate of a sample solution containing 100 µg/mL for curcumin into injected into the column and the method was run. The peak area, theoretical plates (N), tailing factor (T), and asymmetric factors (As) of each sample were then determined using the appropriate formula and the results are shown in Table 5.

3. Results and discussion

3.1. Chromatographic Separation

The investigation involved the analysis of curcumin, ascorbic acid, and salicylic acid using standard solutions. Their absorption characteristics were individually examined in the 200–800 nm wavelength range to identify their maximum absorption points. This research aims to leverage the Hypersil BDS C18 chromatographic column for the development of an RP-HPLC method to quantify Curcumin, ascorbic acid, and salicylic acid. The method was established using a mobile phase consisting of buffer and acetonitrile in a 50:50 v/v ratio, with the flow rate of at 1.0 mL/min, with detection wavelength set at 254.0 nm. The chromatographic analysis resulted in the retention times of 8.09 min for Curcumin, 2.52 min for ascorbic acid, and 4.10 min for salicylic acid. The formulated hydrogel chromatogram is depicted in Figure 1.

3.2. Method Validation

The method underwent comprehensive validation, including evaluation of linearity and range, repeatability, intraday precision, interday precision, accuracy, LOD, LOQ, robustness, and system suitability. Results for these validation parameters are outlined in tables 1 to 7. The proposed quantification method for curcumin exhibited linearity within the range of 100 to 300 µg/mL, with a high correlation coefficient (r^2) of 0.9999 (Fig. 4). The slope was 33716, and the intercept was 189520, as detailed in table 2. Similarly, the proposed method for ascorbic acid demonstrated linearity within the range of 50 to 150 µg/mL, with r^2 of 0.9996, a slope of 46713, and an intercept of +189590, as shown in table 2 (Fig. 5). The method for salicylic acid was also linear within the 50 to 150 µg/mL range, with r^2 of 0.9996, a slope of 68318, and an intercept of +1235817, as displayed in table 2 (Fig. 6). The interday and intraday precision (table 3) exhibited % RSD values lower than 2, indicating high precision. Intermediate precision results reinforced the method precision for drug analysis. Accuracy was determined via recovery studies using the standard spiking method. Recovery values ± SD exceeding 98.0% affirmed the method's accuracy for drug analysis. The Limit of Detection and Limit of Quantitation for curcumin were 18.54 µg/mL and 56.20 µg/mL, respectively. For ascorbic acid, these values were 10.05 µg/mL (LOD) and 30.46 µg/mL (LOQ), and for salicylic acid, they were 11.39 µg/mL (LOD) and 34.51 µg/mL (LOQ). Robustness testing involved altering certain operating conditions, and the results attested to the method's robust nature. Results are shown in table 6. The system suitability test parameter, as depicted in table 5, also indicated satisfactory outcomes. Applying the liquid chromatographic method to analyze curcumin and ascorbic acid and salicylic acid in hydrogel, the percentage recovery results (tables 4 and 5) implied the process was unaffected by formulation excipients. Therefore, the suggested technique can be used for routine analysis of curcumin, ascorbic acid and salicylic acid in bulk and Hydrogel.

3.3. Uncertainty Assessment

The uncertainty of the proposed method was measured was using the parameters i.e amount of sample mass (u_{Ms}), mass of standard compounds (u_{Sm}), volume (u_v), calibration curve (u_c), stock solution preparation (u_{sprep}), purity of the standards (u_p), and repeatability (u_{rep}). The estimation followed EURACEM CITAC Guides' equations to calculate the overall uncertainty in the method.

$$u_{combined} = \sqrt{u_{Ms}^2 + u_{Sm}^2 + u_v^2 + u_c^2 + u_{sprep}^2 + u_p^2 + u_{rep}^2}$$

$$U = k * u_{combined}$$

Where, U represents the expanded uncertainty, which denotes the range of result and moreover, 'k' signifies the typical coverage factor as 2, in 95 % confidence level. While method validation is a crucial issue to get proper result interpretation and comparison, it's essential to recognize that validation alone may not suffice. The outcomes of individual uncertainty, sourced from literature and the EURACHEM guide, are presented in Table 7 for comprehensive understanding and comparison.

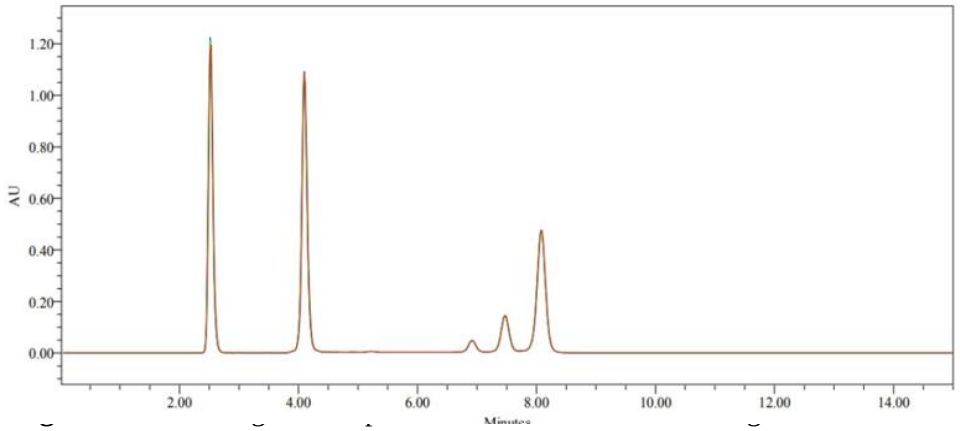

Table 1. Robustness parameters: changed conditions for finding out the Robustness Parameter for validation of the method

Parameter	Normal condition	Changed condition
Flow rate	1.0 mL/min	1.1 mL/min, 0.9 mL/min
Change in mobile phase ratio (0.1% orthophosphoric acid: Acetonitrile)	50:50 (v/v)	48:52 and 52:48 (v/v)

Table 2. Regression analysis data of Curcumin, ascorbic acid and salicylic acid by HPLC method

Parameter	Curcumin	Ascorbic acid	Salicylic acid
λ_{max}	254.0 nm	254.0 nm	254.0 nm
Range	100–300 µg/mL	50-150 µg/mL	50-150 µg/mL
Regression equation (y= mx+ c)	y = 33717x - 189521	y = 61281x + 186673	y= 68319x + 235818
Slope (m)	33717	61281	68318
Intercept (c)	- 189521	+ 186673	+ 235818
Correlation coefficient (r^2)	0.9998	0.9992	0.9996

Table 3. Summary of validation parameters by HPLC method which results indicate the validity of the method

Parameters	Curcumin	Ascorbic acid	Salicylic acid
Linearity	100-300 µg/mL	50-150 µg/mL	50-150 µg/mL
Repeatability	0.21%	0.16%	0.21%
Intraday precision	0.05%	0.26%	0.24%
Interday precision	0.14%	0.09%	0.15%
Accuracy: 50%	99.80±0.81%	100.50±0.80%	99.80±0.15%
100%	101.27±0.28%	98.90±0.34%	99.60±0.19%
150%	99.80±0.80%	99.70±0.56%	99.90±0.10%
Assay	90.25±0.07%	98.76±0.38%	97.28±0.35%
Limit of Detection	18.54 µg/mL	10.05 µg/mL	11.39 µg/mL
Limit of Quantitation	56.20 µg/mL	30.46 µg/mL	34.51 µg/mL
Specificity	Specific	Specific	Specific

Table 4. Recovery study for curcumin, ascorbic acid and salicylic acid

Accuracy level	Conc. of drug in sample (mg)			Nominal amount (mg)			Measured amunt (mg)			Recovery (%)		
	AA	SA	CUR	AA	SA	CUR	AA	SA	CUR	AA	SA	CUR
50%				0.50	0.51	1	0.51	0.50	1.00	100.50	99.80	99.80
100%	1.04	1.00	2.01	1.01	1.01	2	0.99	1.01	2.02	98.90	99.60	101.27
150%				1.51	1.52	3	1.50	1.52	2.99	99.71	99.91	99.82
				Mean						99.70	99.77	100.29
				SD						0.80	0.16	0.84
				%RSD						0.80	0.16	0.85

AA: Ascorbic acid; **SA**: Salicylic acid; **CUR**: Curcumin

Table 5. System suitability parameters for three components in hydrogel

Parameter	Ascorbic acid	Curcumin	Salicylic acid
Retention time	2.52 min	8.09 min	4.10 min
Tailing factor (T)	1.18	1.01	1.06
Theoretical plates (N)	4999	14820	9543
Resolution (R)	-	2.40	10.34

Table 6. Robustness data of the method by normal and changed condition

Parameter	Drug	Mean Assay Value (%)	SD	%RSD
Flow rate 0.9 mL/min	Curcumin	99.59	0.16	0.159
	Ascorbic acid	99.99	0.17	0.169
	Salicylic acid	100.81	0.11	0.110
Flow rate 1.1 mL/min	Curcumin	99.67	0.13	0.129
	Ascorbic acid	99.91	0.12	0.119
	Salicylic acid	100.70	0.07	0.070
Mobile phase composition (0.1% orthophosphoric acid: Acetonitrile) (48:52)	Curcumin	99.78	0.23	0.229
	Ascorbic acid	101.02	0.21	0.212
	Salicylic acid	100.30	0.21	0.210
Mobile phase composition (0.1% orthophosphoric acid: Acetonitrile) (52:48)	Curcumin	100.18	0.45	0.450
	Ascorbic acid	101.84	0.09	0.091
	Salicylic acid	100.80	0.19	0.191

Table 7. Uncertainty assessment for developed RP-HPLC method

Source of Uncertainty	Name of Compound		
	Curcumin	**Ascorbic acid**	**Salicylic acid**
amount of sample mass (u_{Ms})	0.00072	0.00072	0.00072
mass of standard compounds (u_{Sm})	0.00069	0.00069	0.00069
Volume (u_v)	0.01756	0.01756	0.01756
calibration curve ($u_{c)}$	0.00132	0.00342	0.00427
stock solution preparation (u_{Sprep})	0.00357	0.00321	0.00369
purity of the standards (u_p)	0.00342	0.00526	0.0349
repeatability (u_{rep})	0.032	0.043	0.031
$u_{combined}$	0.34	0.38	0.43
$U_{expanded}$	0.69	0.77	0.86

4. Conclusion

An innovative RP-HPLC method was developed to accurately quantify estimation of curcumin, ascorbic acid, and salicylic acid in bulk and hydrogel. This method, developed specifically for hydrogel formulation, demonstrated capability to estimate all the three components simultaneously. Comprehensive validation of the developed method demonstrated encompassed various parameters, including accuracy, precision, specificity, and robustness, of the method and rendering it highly suitable for application in hydrogel formulation without interference from excipients.

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Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

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