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HERBAL STANDARDIZATION OF FORMULATION CONTAINING CURCUMINOIDS, PIPERINE AND BOSWELLIC ACID BY HPLC

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ABSTRACT

The main emphasis of present work is to develop and validate RP-HPLC method for quantification Curcumin C, Piperine and Boswellic acid along with detection and separation of Curcumin B and C from their combined marketed capsule dosage form." The separation of components was carried out by using Hypersil ODS C18 column (250*4.6 mm, 5 µm) and the solvent system contains 65 parts of Acetonitrile, 5 parts of Methanol and 30 parts of Water. The flow rate was maintained at 1ml/min and the chromatograms were observed at 245 nm. Eluted components i.e. Curcumin A, B and C, piperine and Boswellic acid has be detected in chromatogram at 2.52, 3.56, 4.16, 5.21 and 8.59 respectively. The linearity was carried out within the concentration range of 30-190, 1-5 and 15-75 µg/mL and the value of r^2 were found to be 0.995, 0.995 and 0.998 for Curcumin C, Piperine and Boswellic Acid respectively. Recovery of all three components were found between 96.82 ± 95.33 %. The LOD of Curcumin C was found to be 3.17, for Piperine 0.05 and for Boswellic Acid 1.26 µg/mL. The LOQ for Curcumin C, Piperine and Boswellic Acid was found to be 9.60, 0.17 and 3.81 µg/mL respectively. The developed and optimized method was used successfully for assay of market formulations that incorporated with Curcumin, Piperine, and Boswellia serrata extract.

KEYWORDS: RP-HPLC, Herbal standardization, Curcuminoids, Piperine, Boswellic acid, Analytical Method Validation.

1. INTRODUCTION

Turmeric's main active ingredient is Curcumin, which has long been utilised for several health benefits. Curcumin [CUR] is a hydrophobic polyphenolic derivative which is yellow crystalline powder obtained from roots and rhizomes of *Curcuma Longa (family: Zingiberaceae)*. It has been scientifically proven that it helps the body in a variety of ways, including generating anti- inflammatory action, supporting a healthy cardiovascular endurance, and acting as a potent anti-oxidant with neuroprotective properties. CUR is having plethora of uses like anticarcinogen, antimicrobial, anti-Alzheimer and hyperglycaemic. CUR is a mixture of Curcuminoids i.e. Curcumin (77%), Demethoxycurcumin (18%) and bis-demethoxycurcumin (5%).[1-4] Piperine [PIP] is obtained from dried unripen fruits of *Black Pepper (Pipernigrum L.)* Piperine is having plethora of pharmacological properties that are beneficial to the human. health for example antiandrogenic, antiulcer, antidiarrheal, immunomodulatory and anti- inflammatory agent. Piperine is obtained from extract of black pepper that has been clinically investigated to improve absorption nutrients. [1,4-5] The main purpose of using Piperine in this formulation is to enhance the bioavailability of CUR and Boswellic acid [BOS]. The resinous herb *Boswellia Serrata (commonly known as Frankincense)* has been utilised in Ayurvedic system of medicine for ages. In India it is widely known as Indian oli-banum, Sallaki or Salai guggul. Boswellic Acids like alpha and beta boswellic acid are the bioactives of Boswellia that have been scientifically proven to alter effects on enzymes that are involved in tissue repair. Boswellia has been shown to have anti-inflammatory, anti-arthritic, and analgesic properties through various methods.[6]

This combination is available as combined marketed capsule dosage form having 150 mg BOS, 350 mg CUR and 10 mg PIP per serving. Combination is currently marketed as capsule dosage by Healthy hey Nutrition under the category of nutritional supplement.

According to a review of the literature, various methods have been reported for the quantitative analysis of CUR, PIP, and BOS, either alone or in combination with other components[2-15], however at present time, only the HPTLC method.[16] is available to instantaneously quantify CUR, PIP, and BOS from their combined marketed formulation.

2. MATERIALS AND METHODS

2.1. Instrumentation and reagent

Shimadzu HPLC [High Performance Liquid Chromatography] equipment (model: 2010) with manual provision of a 20 µL applicator syringe (Hamilton, Bonaduz, Switzerland). The samples were processed using a UV-VIS detector and a binary gradient operation, the chromatograms being processed and integrated using LCSOLUTION software. For separation, a Hibar ODS [Octa Decyl Silane] C_{18} (5 µm) column with dimensions of 250 mm in length and 4.6 mm in internal diameter was used. Mettler Toledo weighing balance having sensitivity of 0.1 mg was used to weigh exact quantities of samples.

As a gift, all three active ingredients were received. Shaswat Herbals (Vadodara, Gujarat, India) provided analytically pure CUR (99.6% w/w), Pharmanza Herbal Pvt. Ltd. supplied PIP (95% w/w), and BOS (95% w/w) was from Alembic Pharmaceuticals. Smt. S. M. Shah Pharmacy College, Amsaran, provided HPLC grade Methanol, Acetonitrile, and Water (Make: FINAR).

2.2. Method development

To initiate the trials, CUR (10 mg), PIP (10 mg), and BOS (50 mg) were accurately weighed and then taken to 100mL

volumetric flasks. The volume of the flask was raised to the mark with, yielding a solution containing 100 µg mL^{-1} CUR, PIP, and 500 µg mL^{-1} BOS. Working standards of 10 µg mL^{-1} CUR, 10 µg mL^{-1} PIP, and 50 µg mL^{-1} BOS were obtained by diluting 0.1 mL aliquot from the stock solution in 10 mL volumetric flasks with acetonitrile.

2.2.1. Optimization of separation conditions

In order to optimize the separation conditions, Intrinsic Physico-chemical characteristics of active components were thoroughly studied, amongst them solubility and partition co-efficient were having highest priority. Other experimental characteristics, such as flow rate and temperature, were also studied to see if they had an impact on retention time. Because of the large polarity difference between the components, an ODS C_{18} column was used as a stationary phase, allowing nonpolar components to be retained and polar components to be eluted early. Various elution systems were investigated in order to provide enough separation in conjunction with system suitability parameters.

2.2.2. Chromatographic conditions

In order to achieve adequate separation, 65 parts of acetonitrile were used as the organic phase, 5 parts of methyl alcohol and 30 parts of water were used. When above mentioned mobile phase was used under isocratic mode, with Hibar ODS C_{18} column at a flow rate of 1 mL/minute. All active components were well separated along with adequate signal intensity at 245 nm. Finally, to assess the suitability of the conditions, 5 injections at same concentration were injected to get idea regarding the system suitability parameters

2.3. Validation of Optimized method

The new HPLC method was validated for specificity, accuracy, precision, linearity, and range using the International Council for Harmonisation (ICH) criteria Q2(R1).

2.3.1. Linearity

In a 100 ml volumetric flask A, BOS (150 mg) and PIP (10 mg) were combined and their concentrations were set at 100 µg/mL and 1500 µg/mL respectively. A 1000 µg/mL stock solution of CUR (10 mg) was prepared and kept in 10 ml volumetric flask B. Acetonitrile was used to prepare both stock solutions. Following this, separate aliquots were placed into a 10 mL volumetric flask, and a series of diverse concentrations were made with strengths of 30+1+15 µg/mL, 70+2+30 µg/mL, 110+3+45 µg/mL, 150+4+60 µg/mL, and 190+5+75 µg/mL, respectively. Each concentration was injected 5 times. (Figure 1)

2.3.2. Repeatability

Each concentration from range was injected five times and each level was examined for relative standard deviation (RSD) to determine repeatability.

2.3.3. Limit of detection and quantitation

The detection and quantification limits were calculated by utilizing standard deviation of intercept (σ) and mean of slope (S) (From regression analysis of repeatability data)

2.3.4. Accuracy

Accuracy of method was assessed by spiking of Sample (capsule contents) with standard (in mg). The Unspiked concentration used was 100% concentration of formulation, which is 350 mg of CUR, 10 mg of PIP and 150 mg of BOS. The above sample was spiked with standard (in mg) at 50, 100 and 150% of target concentration. Finally, all the

Unspiked and spiked samples were diluted suitably with mobile phase and injected for three times under optimized conditions (For the calculation, area of Unspiked sample was deducted from each spiked level and subjected to regression equation to get recovered concentration). % recovery was analysed at each spiked level.

2.3.5. Robustness

To investigate the impact of minor changes in method parameters on the results, operation parameters such as temperature, pH of the mobile phase, and flow rate were deliberately altered within an acceptable range, and the effect was observed on system suitability parameters such as retention time and % assay, which were also monitored for relative standard deviation. (Table 1)

2.3.6. Ruggedness

This parameter refers to the procedure repeatability, and it was investigated by using two columns with the similar specifications, one of which was the HIBAR ODS C_{18} column and the other was Inertsil ODS C_{18} column, and the difference in the area acquired from the two was monitored using a student t-test.

2.3.7. Method precision

Interday and Intraday precision were used to determine method precision. The entire range (30- 190 µg/ml, 1-3 µg/ml, and 15-75 µg/ml) of a mixture of CUR, PIP, and BOS was evaluated on the same day at different time intervals for Intraday precision and on a different day for Interday precision. (At each level, n = 3 considerations)

2.3.8. Sample analysis (Standardization)

10 capsules were taken and the powder equivalent to 175 mg CUR, 5 mg PIP and 75 mg BOS was taken and transferred to the 100 ml volumetric flask. The volume was made up to the mark using acetonitrile. The mixture was sonicated and filtered from 0.45-micron Whatman filter paper. 1 ml of that filtrate was taken out in 10 ml volumetric flask and the volume was made up to the mark using mobile phase. Resulting solution was chromatographed in triplicates and mean observed area was statistically transformed by linear regression equation to get total % of CUR, PIP and BOS in marketed formulation.

3. RESULT

3.1 Optimization of Chromatographic method

Initially, the trials were conducted with a 65:5:30 v/v mixture of acetonitrile, methyl alcohol, and water. The detection was carried out at 353 nm, although BOS and CUR were not detected in that condition. As, it was not enough to separate and detect all the components so, the detection was carried out at 220 nm in the next trial. However, the identical issue arose, BOS and PIP were not detected. Considering the previously observed problems, for further development and validation 245 nm was selected as an analytical wavelength because at this wavelength, all the components were detected with appropriate resolution. Flow rate was 1ml/minute in all these cases. (Table 2) (Figure 2)

3.1.1 System Suitability

Chromatograms have been observed and examined for a plethora of method suitability factors, including tailing factor and theoretical plates. Five identical working standard solutions were injected to study such parameters, and all the parameters were found to be within the limitations. The parameters of method suitability have been emphasized in (Table 2).

3.2 Validation of Optimized Chromatographic Method

The validation parameters of the proposed optimised RP-HPLC technique were investigated using the ICH Q2R1 recommendations, and each validation parameter was confirmed to follow the criteria. The regression coefficient was shown to be correlating with a standard value when a mixture of CUR, PIP, and BOS in concentration ranges of 30-190, 1-5, and 15-75 µg/mL was chromatographed. (Figure 3) For all components, the accuracy of the optimized HPLC method was established, and recovery tests have been performed at 50-150% levels of targeted concentration (Table 3). The results obtained were found to be within the 95-105% range. The method was confirmed to be robust and rugged, as modest variations in method parameters had no significant effect on results (i.e., retention time and % assay), as shown in Tables 1 and 4. Table 5 provides a summary of all validation parameters.

3.3 Sample analysis (Standardization)

When marketed formulation was analysed by using optimized and validated method, % of CUR, PIP and BOS was found to be 96.55, 99.06 and 98.64 % w/w respectively (Table 6) (Figure 4).

Table 1: Robustness for developed HPLC method.

Para- meters	Level of Changes	Observation					
		Curcumin		Piperine		Boswellic ACID	
		Tr	Peak area ± RSD	Tr	Peak area ± RSD	Tr	Peak area ± RSD
Flow Rate	1.1 mL/min	3.98	3844514±0.71	4.98	184344±2.44	8.79	99322±0.66
	0.9 mL/min	4.20	3893646±0.97	5.34	188571±1.56	8.51	10672±1.07
Composition of mobile phase	60:35:5 (ACN: Water: Methanol)	4.01	3844514±1.29	5.08	187429±2.33	8.62	99179±0.60
	70:25:5 (ACN: Water: Methanol)	4.22	3861670±0.18	5.11	189965±1.92	8.72	99650±0.98

(n=3 determinations)

Table 2: Optimized Chromatographic Condition and System Suitability Prameters for Optimized method.

Optimized Chromatographic Condition			
Stationary phase	Hibar ODS C_{18} (250*4.6 mm, 5µm)		
Mobile phase	Acetonitrile: Methanol: Water (65:5:30 v/v)		
Flow rate	1 ml/min		
Detection wavelength	245 nm		
Injection volume	20 µL		
Run time	15 min		
Retention time	Curcumin A (Rt = 2.52) Curcumin B (Rt = 3.36) Curcumin C (Rt = 4.16) Piperine (Rt = 5.21) Boswellic acid (Rt = 8.59)		
System Suitability Parameters			
Name	**Curcumin C**	**Piperine**	**Boswellic Acid**
Retention time	4.17±0.03	5.18±0.04	8.44±0.14
Peak Area	8880484±616749	36641.2±363.61	23271±2.34
Tailing Factor	1.47±0.01	1.23±0.006	1.59±0.24
Resolution	3.16±0.02	4.22±0.01	4.71±0.50
Number of Theoretical Plates	3836.88±37.21	10756.1±103.001	10659.62±1.14

(n=5 Determination)

Table 3: Accuracy for developed HPLC method.

Drug	Level of Spiking	Quantity of sample (mg)	Amount of drug added (mg)	Amount of drug recovered (mg)	% Mean recovery
CUR	Unspiked	350 mg	-	-	-
	50%	350 mg	175 mg	174.64±0.26	95.6±2.49
	100%	350 mg	350 mg	349.55±0.33	96.82±0.95
	150%	350 mg	525 mg	524.61±0.17	96.45±0.83
PIP	Unspiked	10 mg	-	-	-
	50%	10 mg	5 mg	4.63±0.26	95.33±3.13
	100%	10 mg	10 mg	9.65±0.17	96.66±1.95
	150%	10 mg	15 mg	14.5±0.15	96.66±1.08
BOS	Unspiked	150 mg	-	-	-
	50%	150 mg	75 mg	74.44±0.39	96.58±2.21
	100%	150 mg	150 mg	149.42±0.40	95.71±0.72
	150%	150 mg	225 mg	224.58±0.23	96.40±0.57

(Mean result is average of 3 determinations at each level, SD corresponds to standard deviation.)

Table 4: Ruggedness for developed HPLC method.

Change in Parameter		Result t-test	Inference
Change in column		Curcumin (0.25) Piperine (0.62) Boswellic Acid (0.69644)	No significant difference
Inertsil ODS C18	**Hibar ODS C18**		
Change in instrument			
Agilent	**Shimadzu**	Curcumin (0.25) Piperine (0.62) Boswellic Acid (0.69644)	No significant difference

Table 5: Summary of all validation parameters.

Parameter	Limit	Result			Inference
		Curcumin	Piperine	Boswellic acid	
Linearity and Range	$R^2 > 0.995$	0.995	0.995	0.998	Method was Linear
Repeata-bility	%RSD < 2	1.93-0.71	1.6-0.9	1.4-0.5	Method was repeatable
LOD	-	3.17	0.05	1.26	-
LOQ	-	9.60	0.17	3.81	-
Inter-day precision	%RSD < 2	1.07-0.27	1.03-0.4	0.19-0.8	Method was Precise
Intraday precision	%RSD < 2	1.93-0.83	1.34-0.27	1.03-0.45	Method was Precise
% Recovery	95-105%	95.6-96.82	95.33-96.66	95.71-96.58	Method was Accurate
Robustness	%RSD < 2	0.18-1.29	1.56-2.44	0.60-1.07	Method was Robust
Ruggedness	-	-	-	-	Method was Rugged
Assay	-	96.553	99.06	98.64	-

Table 6: Assay of developed HPLC method.

Drug	Amount Taken µg/mL)	Amount Found µg/mL)	% Assay
CURCUMIN	175	168.97±1.64	96.553±0.97
PIPERINE	5	4.95±0.03	99.06±0.7
BOSWELLICACID	75	73.98±0.68	98.64±0.92

(n=3 determination)

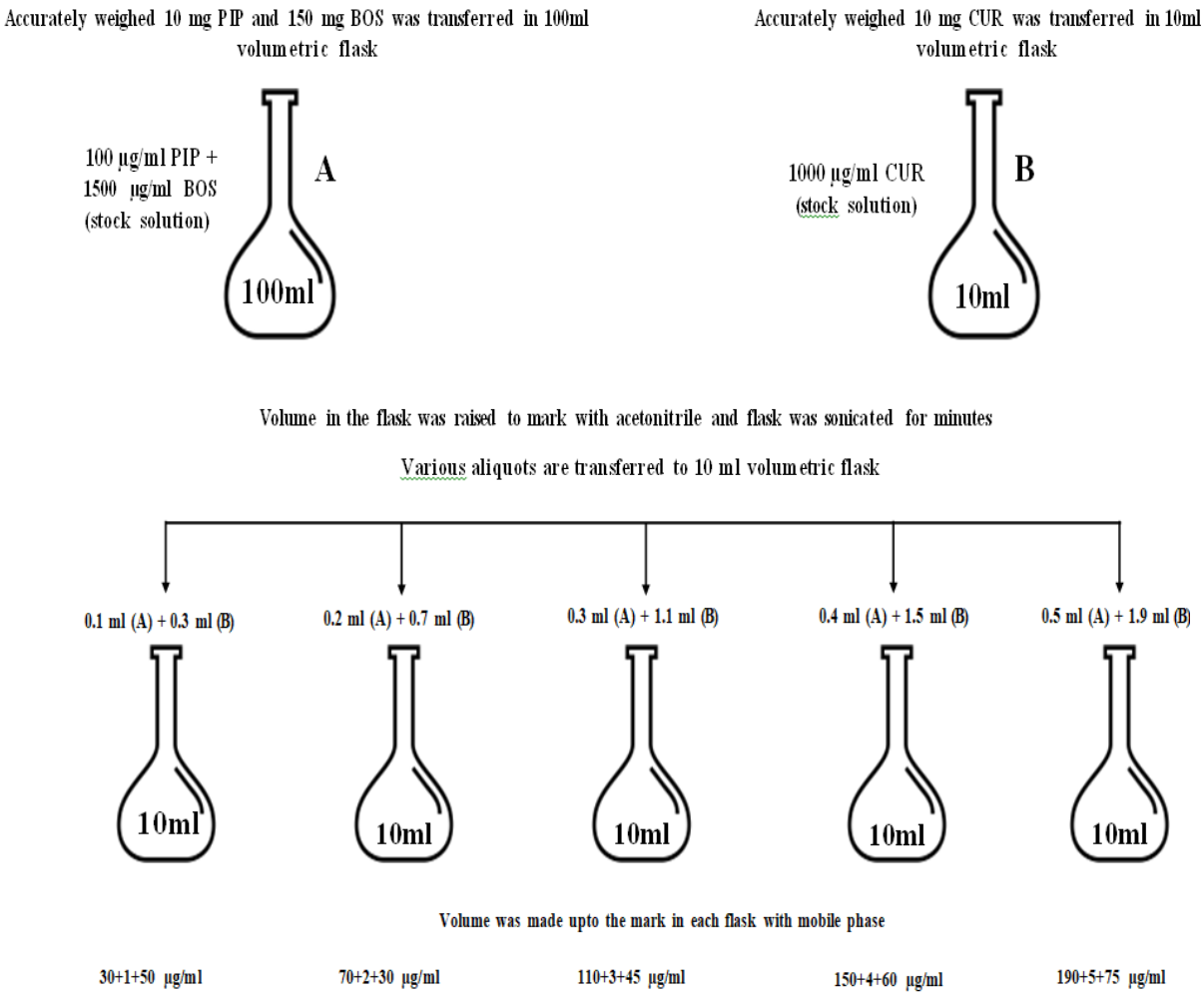

Fig. 1: Procedure for linearity study.

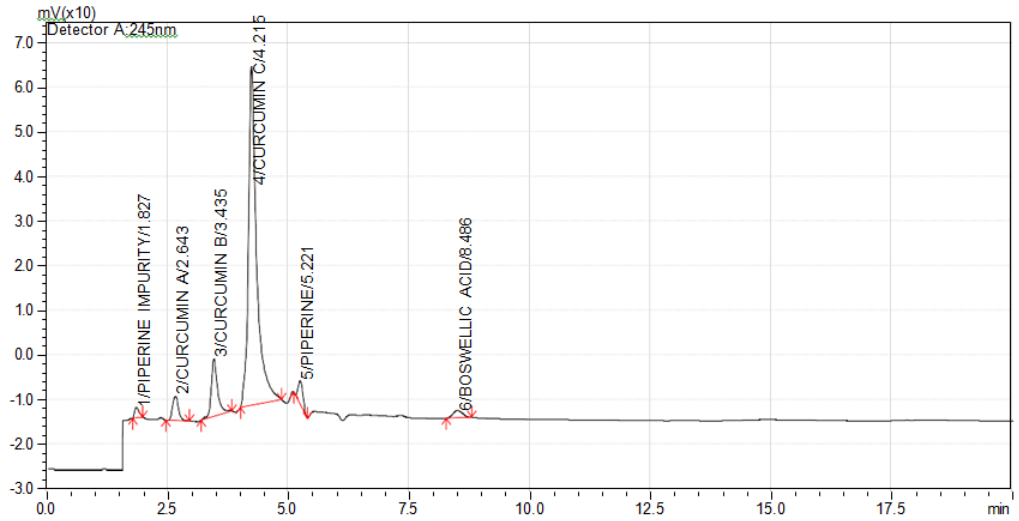

Fig. 2: Chromatogram of mixture of CUR, PIP and BOS under Optimized Chromatographic Condition.

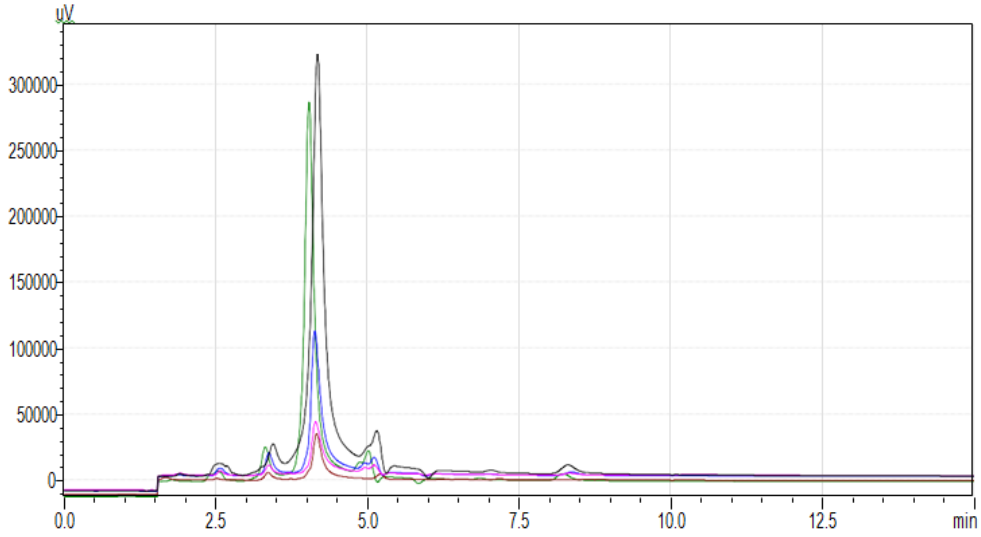

Fig. 3: Overlined Chromatogram of linearity.

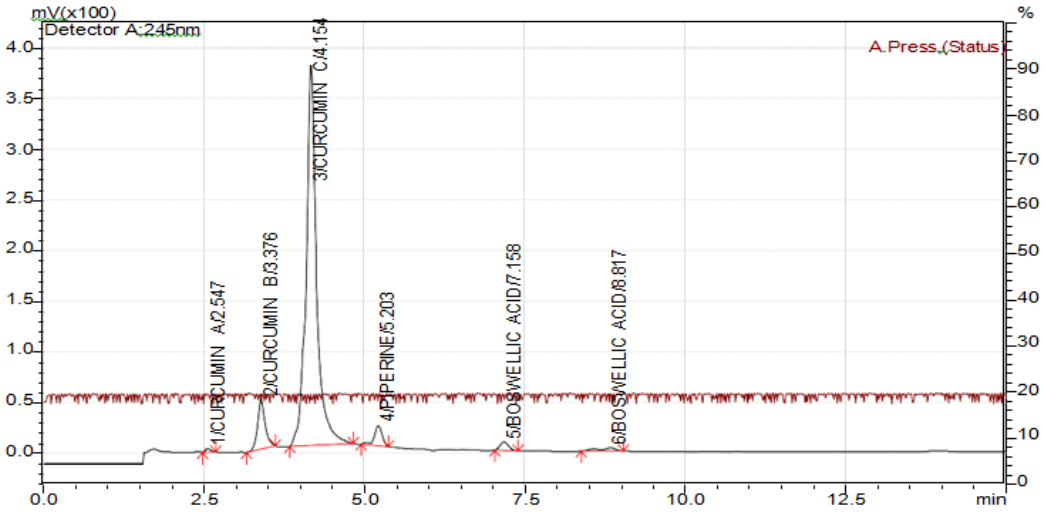

Fig. 4: Assay of marketed Preparation (CUR 175 µg/mL + PIP 5 µg/mL + BOS 75 µg/mL).

4. DISCUSSION

When the system suitability of the optimised method for the simultaneous quantification of CUR, PIP, and BOS was examined, all of the parameters were found to be within the acceptable range. Additionally, the ICH Q2R1 recommendations were used to validate the established RP- HPLC technique; the R^2 values for all three components were within the prescribed criteria. The method can be employed for recovery studies because the percentage of recovery for all three drugs were also within the specified limits. The devised approach proved linear, repeatable, precise, robust, and rugged for simultaneous quantification of CUR, PIP, and BOS, just like all other parameters. The established RP-HPLC method can be used to quantify CUR, PIP, and BOS from commercial capsule formulation simultaneously.

5. CONCLUSION

At present only one HPLC method is available for detection of mention components from food supplements which operates under gradient elution conditions utilizing water, acetonitrile and formic acid with separation on Cyano 150

mm column. Detection of each component were carried out at individual wavelengths. BOS was detected at 206 nm, PIP at 340 nm and CUR at 420 nm. [17] The developed and presented method has relatively more simple conditions for separation and quantification. Even the reported method has the major limitation of extracting components by complex extraction procedure. By looking at above mentioned points, the presented RP-HPLC method suits best for simultaneous quantitative expression of all entitled bioactive components from their combined marketed formulation for this suggested combination of CUR, PIP, and BOS. The suggested RP-HPLC method accurately separates and quantifies the components from their combination. The developed, optimized, and validated method has been found to be far superior then the reported HPTLC and HPLC method in all the aspect specially in terms of reproducibility and accuracy. The method is capable of effectively separating all curcuminoids from all active ingredients. When optimized method was applied to the formulation, it was successfully able to analyze and quantify all the active components.

6. FUNDING

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