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Research Article

METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF ASCORBIC ACID AND FOLIC ACID VITAMINS BY REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY METHOD IN CYANOBACTERIAL METABOLITES AND NUTRACEUTICAL FORMULATION

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ABSTRACT

Objective: It was aimed to estimate ascorbic acid (ASC) and folic acid (FLC) in cyanobacterial metabolite by the reverse-phase high-performance liquid chromatography (RP-HPLC) method, and the work was also extended to nutraceutical formulation.

Methods: RP-HPLC method were developed for simultaneous estimation of two vitamins ASC and FLC in cyanobacterial metabolite and nutraceutical using isosbestic point at wavelength 280 nm. Method was selected after calculating system suitability and validated as per ICH guidelines.

Results: The developed analytical method parameters found within limits prescribed by ICH and USP guidelines. The retention time was found to be 2.334 and 3.892, respectively, for ASC and FLC. Limit of detection and limit of quantification for ASC and FLC were found to be 0.087 and 0.263 µg/ml, 0.052 and 0.159 µg/ml, respectively. Recovery studies show that method is capable of recovering analytes from its formulation. The method is meeting the criteria for validation as per the guidelines.

Conclusion: The method is simple, precise, specific, and accurate. The newly developed method can be used in pharmaceutical industry for routine analysis of ASC and FLC in tablet dosage form.

Keywords: Cyanobacteria, Nutraceutical, Reverse-phase high-performance liquid chromatography, Ascorbic acid, Folic acid, Vitamins, Metabolites.

INTRODUCTION

Cyanobacteria are the Gram-negative photosynthetic prokaryotes found in almost all the ecological habitats. Cyanobacteria are capable of both carbon assimilation and nitrogen fixation, thereby enhancing productivity in a variety of environments. Cyanobacteria inhabit a range of diverse and extreme habitats and have potential to produce an elaborate array of secondary metabolites with unusual structures and potent bioactivity which includes growth promoters, deterrents, herbicides and insecticides, sunscreen compounds, immunosuppressures and antitumor agents, siderophores in the treatment of iron overload.

Vitamins released by cyanobacteria are valuable for the cultivation of plants in terms of the stimulation of root growth in rice and other crops as well as increase in the length of roots and shoots in plants intern increased productivity. Literature survey revealed that there is no quantitative method developed for the simultaneous estimation of vitamins produced by cyanobacteria. However, qualitative methods for estimation have been developed, but there is still a need for the development of a quantitative method for estimation of valuable vitamins. Hence, the aim of this study is to develop fast, simple, inexpensive, sensitive, and validated analytical method for the routine quantitative analysis of vitamins in cyanobacterial metabolites, and the work was also extended for nutraceutical formulation [1-3].

METHODS

Materials

Ascorbic acid (ASC) and folic acid (FLC) standards were procured from S.D. Fine-Chem Limited, Mumbai. Culture filtrate of cyanobacterial metabolites (Fischerella strain) was obtained from the sample provided to the college for analysis. Sample tablets of nutraceutical formulation (Mynerve69-FE) were purchased from local market. All the chemicals and solvents used were of analytical grade/high-performance liquid chromatography (HPLC) grade.

Methods

Selection of suitable analytical wavelength

About 10 mg of reference standard of ASC was dissolved in 100 ml of water to yield stock solution of 100 µg/ml. From this, 1 ml of stock solution was taken and diluted to 10 ml with the same solvent to yield standard dilution of 10 µg/ml. This solution was scanned in spectrum mode over the entire ultraviolet (UV) range between 400 and 200 nm using a UV spectrophotometer. Similarly, the UV spectra of stock solution of FLC (10 µg/ml) was scanned, and then both spectra were overlapped to each other which show a point at higher absorbance of both drugs occurs. The UV overlap spectra so obtained showed the wavelength of isosbestic point is at 280 nm, which was selected as working wavelength for the analysis [4].

Preparation of buffer solution

About 0.02 M orthophosphoric acid was prepared by dissolving 0.115 ml of orthophosphoric acid in 100 ml of HPLC grade water, and this solution was used to adjust the pH (3.0) of the HPLC water to prepare the buffer solution by the digital pH meter and finally filtered through 0.45 µm Whatman filter paper.

Optimization of mobile phase condition

The optimization of mobile phase was done on the basis of trial with standard working solution of 10 µg/ml using Lichrospher, RP-C_{18} column. Mobile phase in the composition of 67:33 buffer:methanol was found to be most suitable for the quantitative analysis of ASC and FLC [5].

Preparation of standard solutions

Standard solution of ASC

The reference standard of ASC (10 mg) was transferred to 100 ml volumetric flask and dissolved in HPLC water. The flask was shaken for

10 minutes, and the volume was made up to the mark with the same solvent to obtain standard stock solution of ASC (100 µg/ml; stock solution). The stock solution was filtered through a 0.45 µ Whatman filter paper. The working standard solutions of ASC were prepared from suitable aliquots of stock solution, and volume was made up to the mark with HPLC water.

Standard solution of FLC

Accurately weighed quantity (10 mg) of reference standard of FLC was transferred to 100 ml volumetric flask. The drug was dissolved in HPLC water with shaking for 10 minutes, and then the volume was made up to the mark with the same solvent to obtain standard stock solution (100 µg/ml). The stock solution was filtered through a 0.45 µ Whatman filter paper. The working standard solutions of FLC were prepared from suitable aliquots of stock solution, and volume was made up to the mark with mobile HPLC water.

Mixed standard solution of ASC-FLC

The reference standard of ASC and FLC were accurately weighed 10 mg and 25 mg, respectively, transferred to 100 ml volumetric flask and dissolved in HPLC water. The flask was vigorously shaken for 10 minutes, and the volume was made up to the mark with the same solvent to obtain standard stock solution of ASC-FLC (100 and 250 µg/ml stock solution). This resultant stock solution was filtered through a 0.45 µ Whatman filter paper. The working mixed standard solutions of ASC-FLC were prepared from suitable aliquots of stock solution, and volume was made up to the mark with water.

Linearity and calibration curve

From mixed standard stock solution, aliquots are made with solvent to obtain concentration of 2-10 µg/ml of ASC, in the same way, FLC dilutions are prepared with solvent to obtain concentration of 5-25 µg/ml. The solution of 20 µl was injected into column with the help of Hamilton syringe. All measurements were repeated three times for each concentration. The calibration curves of the area under curve versus concentration were recorded for these drugs.

Preparation of sample solution of cyanobacterial metabolite

Culture filtrate of cyanobacterial metabolites (Fischerella strain) was taken and concentrated on water bath at temperature below 37°C until approximately 10% of solution remains. Now, this 10% of solution was dissolved in 100 ml of HPLC water (Stock solution). This stock solution was finally filtered through a 0.45 µ Whatman filter paper. The working standard solutions were prepared from suitable aliquots of stock solution, and volume was made up to the mark with the same solvent.

Preparation of sample solution of nutraceutical formulation

About 20 tablets were weighed accurately and powdered using pestle-mortar. The quantity of the powder equivalent to amount labeled for each tablet was weighed, and 73.5 mg of standard FLC was added. Now, this powdered material was dissolved in 100 ml of HPLC water. This stock solution was finally filtered through a 0.45 µ Whatman filter paper. The working standard solutions were prepared from suitable aliquots of stock solution, and the volume was made up to the mark with same solvent [6,7].

RESULTS AND DISCUSSION

UV spectrum showed maximum absorbance for both drugs at 280 nm (Isosbestic Point), which is suitable for HPLC analysis of both drugs simultaneously. Table 1 shows optimized condition for the estimation of ASC and FLC including Column RP-C_{18} with 5 µm particle size using photodiode array detector at 280 nm wavelength and using different ratios of buffer and methanol at pH 3.0 and flow rate 1.0 ml/minutes. Chromatograms Fig. 1 shows retention time of the drug in mix standard.

The developed analytical method parameters shown in Table 2 lie within limits prescribed by ICH and USP guidelines. The retention time was found to be 2.334 and 3.892, respectively, for ASC and FLC. The resolution was found to be 5.04 (it should be more than 2.0 according to ICH). The symmetric factor was found to be 1.61 and 1.55 (it should be <2.0 according to ICH). The tailing factor was found to be 1.61 and 1.55 (it should be <2.0 according to ICH). The capacity factor was found to be 1.333 and 2.891, respectively, for ASC and FLC, which lies in USP limit 1-10.

The recovery studies (relative standard deviation [RSD]) for ASC and FLC were found to be 1.03 and 1.84, respectively, which are under the limit according to ICH guidelines**.** The RSD for ASC and FLC was found to be 1.03 and 1.84, respectively, in cyanobacterial metabolite, which is under the limit according to ICH guidelines as shown in Table 3.

The results of the assay showed in Table 4 for the both drugs ASC and FLC 99.39% and 101.11%, respectively, which are under the limit according to ICH guidelines.

The developed method was validated in terms of accuracy, precision, specificity, robustness, linearity, range, limit of detection (LOD), and

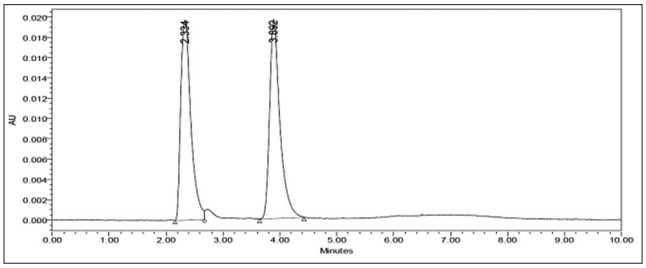

Fig. 1: Chromatogram of mix standard of ascorbic acid and folic acid at final optimized condition

Table 1: Final optimized condition for the estimation of ASC and FLC

S. No.	Parameter	Specification
1	Column	Lichrospher RP-C18
2	Particle size	5 µm
3	Detector	PDA
4	Wavelength	280 nm
5	Mobile phase	Buffer:methanol (67:33)
6	pH	3.0
7	Flow rate	1.0 ml/min
8	Injection volume	20 µl
9	Column temp.	30°C

ASC: Ascorbic acid, FLC: Folic acid, PDA: Photodiode array detection

Table 2: System suitability parameters

System suitability parameters	ASC	FLC	Limits
Retention time	2.334	3.892	-
Resolution	-	5.04	>2
Symmetric factor	1.61	1.55	<2
Tailing factor	1.61	1.55	<2
Capacity factor	1.333	2.891	1-10

ASC: Ascorbic acid, FLC: Folic acid

Table 3: Estimates obtained from analysis of cyanobacterial metabolite

Name of product	ASC	FLC
Found Amount	0.773 mg	0.014 mg
SD	0.008	0.0002
RSD	1.03	1.84

The above value is mean of 3 determinations, ASC: Ascorbic acid, FLC: Folic acid

Table 4: Estimates obtained from analysis of nutraceutical formulation

Name of product	ASC	FLC
Amount claimed	75 mg	1.5 mg
Found amount	74.54 mg	1.51 mg
% purity	99.39%	101.11%
SD	0.381	0.030
RSD	0.005	0.019

The above value is mean of 3 determinations, ASC: Ascorbic acid, FLC: Folic acid, SD: Standard deviation, RSD: Relative standard deviation

Table 5: Summary of validation parameters

S. No.	Parameter	Results	
1.	Specificity	No interference of excipients peaks with analyte peaks.	
2.	Linearity	Linearity between 2-10 µg/ml (ASC) and 5-25 µg/ml (FLC)	
		ASC	FLC
3.	Range		
	Linearity range	2-10 µg/ml	5-25 µg/ml
	Target range	4-8 µg/ml	10-20 µg/ml
	Target concentration	6 µg/ml	15 µg/ml
4.	Accuracy (% recovery)		
	Low	99.56	99.93
	Medium	99.80	99.68
	High	99.84	99.72
5.	Precision (% RSD)		
	Intraday		
	Low	0.629	0.150
	Medium	0.028	1.595
	High	0.058	0.014
	Interday		
	Low	0.261	0.308
	Medium	1.203	0.062
	High	0.048	0.182
	Repeatability	0.271	0.090
6.	LOD	0.087	0.052
7.	LOQ	0.263	0.159

ASC: Ascorbic acid, FLC: Folic acid, RSD: Relative standard deviation, LOD: Limit of detection, LOQ: Limit of quantitation

limit of quantificationof quantitation (LOQ) as per ICH guideline, and it was found that all the parameter lie within limits prescribed by ICH. LOD and LOQ for ASC and FLC were found to be 0.087 and 0.263 µg/ml, 0.052 and 0.159 µg/ml, respectively. Recovery studies show that method is capable of recovering analytes from its formulation. RSD of inter- and intra-day precision is within acceptable limit of 2% proves that method is precise (Table 5).

CONCLUSION

In this work, reverse-phase HPLC method for the estimation of ASC and FLC in cyanobacterial metabolites and Nutraceutical Formulation was developed. The developed method was validated in terms of accuracy, precision, specificity, robustness, linearity, range, LOD, and LOQ as per ICH guideline, and it was found that all the parameter lie within limits prescribed by ICH. It is evident from the study that the method is simple, precise, specific, and accurate. The newly developed method can be used in pharmaceutical industry for routine analysis of ASC and FLC in tablet dosage form.

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