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Stability Indicating RP-HPLC for the Simultaneous Estimation of Proanthocyanidin with Ascorbic

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ABSTRACT

RP-HPLC and UV methods for the simultaneous estimation of Proanthocyanidin and Ascorbic acid in bulk drugs and marketed formulation. The HPLC system used was LC SHIMADZU UFLC-2000 Prominance LC-20AD Binary Gradient System. SPDM 20A detector with Rheodyne injector and Enable C18 G column 250x 4.6mm, 5µm. Injection volume of 20µl was injected and eluted with the mobile phase of Trichloroacetic acid (800 µg/ml): Methanol (pH3.2) in the ratio of 90:10v/v at the flow rate of 1.0ml/min and PDA detection at 277nm. The peaks of Proanthocyanidin and Ascorbic acid were found well separated with retention time 6.10min and 3.70min respectively. The linearity was found to be in the concentration range of 50-150 mcg/ml and 100-300 mcg/ml for Ascorbic acid and Proanthocyanidin. Forced degradation studies were carried out for Ascorbic acid and Proanthocyanidin at different stress conditions like acidic, alkaline, oxidation, reduction, thermal, and photo stability condition it was found that the peaks degraded products did not interfere with the peaks of drug under the study. Hence the stability indicating HPLC method developed and validated can be used routinely for the simultaneous determination of proanthocyanidin and ascorbic acid in bulk drugs and marketed formulation.

INTRODUCTION:

Method development is the setting up of an analytical procedure that will be appropriate for the analysis of a particular sample. It starts with the choice of the technique like UV, HPLC, TLC and GC. It is based upon the technique which provides the best resolution, the shortest analysis times and the highest sensitivity. The next choice will be the phase system that should be used. This will be based on the interactive character of the components of the mixture to be analyzed. The choice will range between predominantly, ionic, polar or dispersive which will indicate an ion exchange stationary phase, a polar stationary phase (hydrophilic) or a dispersive stationary phase (hydrophobic) or a clever blend of two or all three respectively. The liquid chromatography is to be used, then a complementary mobile phase, column length, column diameter must be selected. For a packed column, particle diameter must then be chosen to provide the necessary efficiency to effect the separation in the minimum time. The detector must then be chosen to provide the required sensitivity, the necessary linearity and if needed the desired specificity. These are some of the basic choices but there are many others to be made, an internal or external standard, the method of sampling, the need for gradient elution, or temperature programming, detector sensitivity etc. Efficient method development requires expert knowledge of chromatographic science and extensive practical experience.

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Methodvalidation[5]:

Method validation is the process to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Methods need to be validated or revalidated. Before their introduction into routine use whenever the conditions change for which the method has been validated, e.g., instrument with different characteristics. Whenever the method is changed, and the change is outside the original scope of the method.

Method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and rugged over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use, and is sometime referred to as "the process of providing documented evidence that the method does what it is intended to do." Regulated laboratories must perform method validation in order to be in compliance with FDA regulations (FDA, 2000). In response to this situation, one of the first harmonization projects taken up by the ICH was the development of a guideline on the

"Validation of Analytical Methods: Definitions and Terminology." ICH divided the "validation characteristics" somewhat differently, as outlined in Figure below.

It is not always necessary to evaluate every analytical performance parameter, as different test methods require different validation schemes. The Most common categories of assays for which validation data should be required are as follows:

i) Quantitation of major components or active ingredients.

ii) Determination of impurities or degradation compounds.

iii) Determination of performance characteristics

Category-I: Analytical methods for quantitation of major components of bulk drug substances or active ingredients (including preservatives) in finished pharmaceutical products.

Category-II: Analytical methods for determination of impurities in bulk drug substances or degradation compounds in finished pharmaceutical products. These methods includes quantitative assays and limit tests.

Category-III: Analytical methods for determination of performance characteristics (e.g. dissolution, drug release).

The type of method and its intended use indicates which parameters are required to be investigated. They are illustrated in the following Table.

Data elements required for assay validation

Analytical Performance Parameter	Assay Category-I	Assay category-II		Assay Category-III
		Quantitative	Limit Test	
Accuracy	Yes	Yes	*	*
Precision	Yes	Yes	No	Yes
Specificity	Yes	Yes	Yes	*
LOD	No	No	Yes	*
LOQ	No	Yes	No	*
Linearity & range	Yes	Yes	No	*
Ruggedness	Yes	Yes	Yes	*

*may be required, depending on the nature of specific test

METHODOLOGY & MATERIALS

The mixture of Proanthocyanidin and Ascorbic acid are separated by HPLC method by the principle of partition and then their contents are determined individually.

Instrument	SHIMADZU UFLC-2000 Prominance LC-20AD SPDM 20A Binary Gradient System
Injector	Rheodyne
Column	Enable C-18 G column 250×4.6mm,5µm
Injection volume	20 µL
Wavelength	277nm
Flow rate	1.0mL/min

Reagents and chemicals

1. Aqueous Trichloroacetic acid

2. Methanol HPLC grade

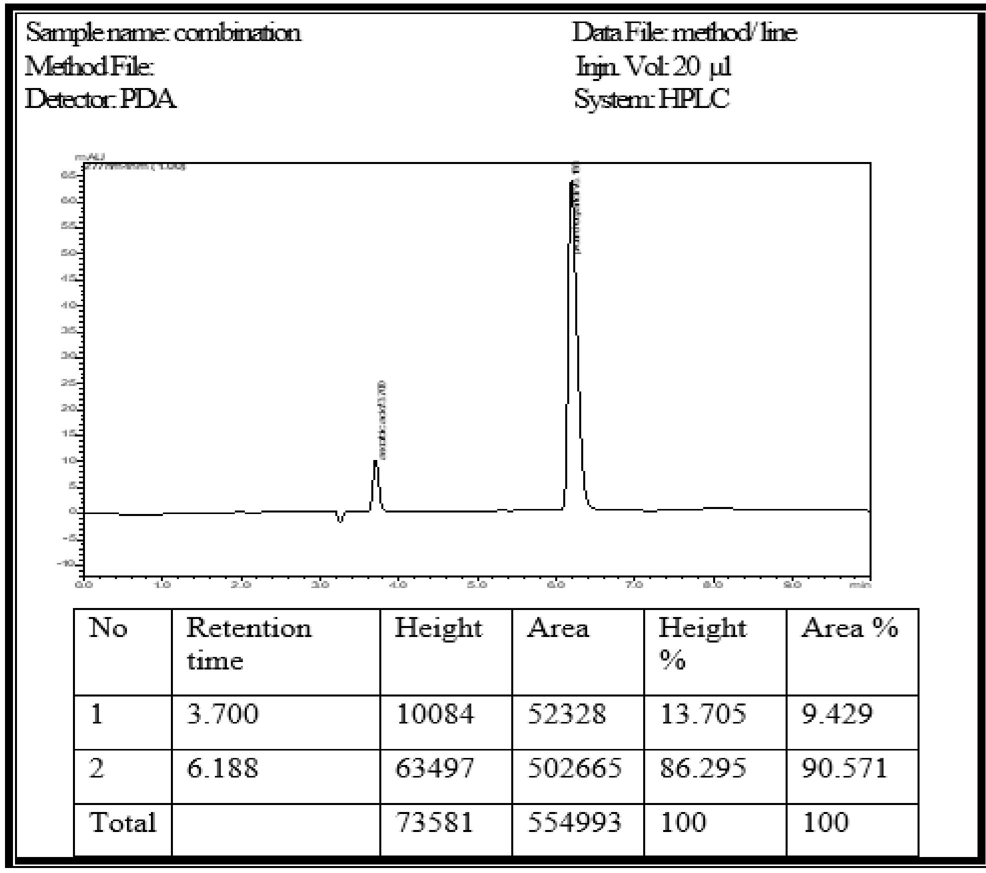
3. Millipore water

4. Proanthocyanidin

5. Ascorbic Acid

Selection and standardization of the mobile phase

Proanthocyanidin and Ascorbic acid are marketed as combined tablet formulation. The proposed method for estimation of Proanthocyanidin and Ascorbic acid required adequate resolution between the peaks of drugs in the chromatogram. Several mobile phase combinations were tried to get good optimum resolution.



No	Retention time	Height	Area	Height %	Area %
1	3.700	10084	52328	13.705	9.429
2	6.188	63497	502665	86.295	90.571
Total		73581	554993	100	100

Fig. No. 1: Chromatogram showing peak separation Peaks of Proanthocyanidin and Ascorbic acid were well resolved with the solvent system of Trichloroacetic acid (800mcg/mL) and methanol in the ratio of 90:10.

Report: Peaks of Proanthocyanidin and Ascorbic acid were well resolved with the solvent system of Trichloroacetic acid (800mcg/mL) and methanol in the ratio of 90:10, Hence the same as selected as mobile phase.

Report: Retention time of Proanthocyanidin and Ascorbic acid were found to be **6.52min** and **3.72min** respectively, when injected as individual solutions.

FORCED DEGRADATION STUDIES:

Forced degradation of each drug substance and combination was carried out under acid, base, oxidation, reduction, photolytic, and thermal stress conditions. After the degradation appropriate dilutions were made to get and injected into HPLC system.

Degradation Studies of Proanthocyanidin and Ascorbic acid in Acidic condition:

Forced degradation studies for Proanthocyanidin and Ascorbic acid were carried out in 0.1 N HCl at room temperature, 1hr and 2hr after heating to 60^0c.

Table No. 1: Degradation data for Ascorbic acid in Acidic condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	51738	47.60	95.21%
2	1 hour	51620	47.52	95%
3	2 hour	51320	47.31	94.6%

Table No. 2: Degradation data for Proanthocyanidin in Acidic condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	232708	104.07	104.07%
2	1 hour	231680	103.94	103.94%
3	2 hour	206012	101.93	101.93%

Report:

The acidic degradation of standard Proanthocyanidin and Ascorbic acid drug was found to be stable in 0.1 N HCl with no degradation peaks.

5.7.2 Degradation Studies of Proanthocyanidin and Ascorbic acid in alkaline condition: Forced degradation studies for Proanthocyanidin and Ascorbic acid were carried out in 0.1 N NaOH at room temperature,1 hr and 2hr after heating to 60^0c.

Table No. 3: Degradation data for Ascorbic acid in alkaline condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	54260	49.35	98.70%
2	1 hour	53619	48.90	97.81%
3	2 hour	52136	47.82	95.76%

Table No. 4: Degradation data for Proanthocyanidin in Alkaline condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	216230	101.95	101.95
2	1 hour	204602	100.47	100.47
3	2 hour	201922	100.12	100.12

Report:

The alkaline degradation of standard Proanthocyanidin and Ascorbic acid drug was found to be stable in 0.1 N NaOH with no degradation peaks.

Degradation Studies of Proanthocyanidin and Ascorbic acid in Oxidation condition: Forced degradation studies for Proanthocyanidin and Ascorbic acid were carried out in 3% hydrogen peroxide.

Table No. 5: Degradation data for Ascorbic acid in Oxidation condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	52420	48.07	96.15%
2	1 hour	51999	47.78	95.5%
3	2 hour	51819	47.66	95.32%

Table No. 6: Degradation data for Proanthocyanidin in Oxidation condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	200696	99.97	99.97%
2	1 hour	199695	99.84	99.84%
3	2 hour	198962	99.75	99.75%

Report:

The Oxidation degradation of standard Proanthocyanidin and Ascorbic acid drug was found to be stable in 3% Hydrogen peroxide with no degradation peaks.

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5.7.4 Degradation Studies of Proanthocyanidin and Ascorbic acid in Reduction condition: Forced degradation studies for Proanthocyanidin and Ascorbic acid were carried out in zinc.

Table No.7: Degradation data for Ascorbic acid in Reduction condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	53260	48.65	97.31%
2	1 hour	53120	48.56	97.12%
3	2 hour	52999	48.47	96.95%

Table No. 8: Degradation data for Proanthocyanidin in Reduction condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	232608	104.06	104.06%
2	1 hour	231002	103.85	103.85%
3	2 hour	229924	103.71	103.71%

Report:

The Reduction degradation of standard Proanthocyanidin and Ascorbic acid drug was found to be stable in Zinc dust with no degradation peaks.

5.7.5 Degradation Studies of Proanthocyanidin and Ascorbic acid in Thermal condition:

Forced degradation studies for Proanthocyanidin and Ascorbic acid were carried out in Hot air oven at 50^0 C for two days.

Table No. 9: Degradation data for Ascorbic acid in Thermal condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	52690	48.20	96.53%
2	24 hour	51899	47.71	95.43%
3	48 hour	51630	47.53	95.06%

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Table No. 10: Degradation data for Proanthocyanidin in Thermal condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	201942	100.13	100.13%
2	24hour	201321	100.05	100.05%
3	48 hour	200931	100.00	100.00%

Report:

The Thermal degradation of standard Proanthocyanidin and Ascorbic acid drug was found to be stable in Hot air oven at 50^0 C for two days with no degradation peaks.

5.7.6 Degradation Studies of Proanthocyanidin and Ascorbic acid in Photostabilty condition: Forced degradation studies for Proanthocyanidin and Ascorbic acid were carried out in for two days.

Table No. 11: Degradation data for Ascorbic acid in Photostability condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	54260	49.35	98.70%
2	24 hour	53986	49.16	98032
3	48 hour	53199	48.61	97.22%

Table No.12: Degradation data for Proanthocyanidin in Photostability condition

S. No	Time interval	Peak area	concentration	Non-degraded %
1	Control	201931	100.13	100.13%
2	24 hour	201269	100.64	100.64
2	48 hour	200240	99.91	99.91%

CONCLUSION:

A HPLC method was developed for the simultaneous estimation of proanthocyanidin and ascorbic acid in bulk drugs and marketed formulation. The HPLC system used was SHIMADZU UFLC-2000 Prominance LC-20AD Binary Gradient System. SPDM 20A detector with Rheodyne injector and Enable C18 G column 250x4.6mm, 5µm. Injection volume of 20µl was injected and eluted with the mobile phase of Trichloroacetic acid (800 µg/ml):Methanol (pH3.2) in the ratio of 90:10v/v at the flow rate of 1.0ml/min The peaks of Proanthocyanidin and Ascorbic acid were found well separated with retention time 6.10min and 3.70min respectively.

The developed methods were validated for various parameters as per ICH guidelines like accuracy, precision, linearity, specificity, ruggedness and robustness. The results obtained were well within the acceptance criteria for all the parameters. The proposed methods were applied for determination of Proanthocyanidin and Ascorbic acid in bulk drug and marketed formulation. The assay results conformed to the label claim of the formulation. Hence the proposed method can be used for the routine analysis of proanthocyanidin and ascorbic acid in their marketed tablet dosage.

Forced degradation studies were carried out for Ascorbic acid and Proanthocyanidin at different stress conditions like acidic medium, alkaline medium, oxidation condition, reduction condition, thermal condition, photo stability condition and it was found that the peaks degraded products did not interfere with the peaks of drug under the study. Hence the stability indicating HPLC method developed and validated can be used routinely for the simultaneous determination of proanthocyanidin and ascorbic acid in bulk drugs and marketed formulation (tablets).

SUMMARY:

A Stability indicating HPLC method for simultaneous determination of Proanthocyanidin and Ascorbic acid was developed and validated using Enable C-18G; 250mm x 4.6mm, 5µm Column with mobile phase of Trichloroacetic acid and methanol in the ratio of 90:10 at flow rate of 1.0 mL/min and PDA detection at 277 nm. The proposed HPLC method was then validated through laboratory studies for various parameters as per ICH Q2 guidelines, viz, accuracy, precision, linearity, LOD, LOQ, specificity, ruggedness and robustness. The results obtained indicated that all the values were well within the acceptance limit. Hence the method is found to be linear, accurate, precise, specific, rugged and robust. The summary of the developed method, all validation parameters and the data obtained are given below.

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Validation Parameters of the HPLC Method

Parameters		Proanthocyanidin	Ascorbic acid	Acceptance Criteria
Specificity		No peak were interrupting the original peaks		No peak was detected
LOD (ng/mL)		33 (ng/mL)	90 (ng/mL)	-
LOQ (ng/mL)		101 (ng/mL)	270 (ng/mL)	-
Linearity range		100 – 300 µg/mL	50– 100 µg/mL	-
Precision	System	0.41 %	1.82 %	NMT 2%
	Method	1.55 %	0.83 %	
	Intra day	1.10 %	1.09%	
	Inter day	0.25%	1.16%	
R0bustness	pH 3	101.16%	93.61%	90-110 %
	pH 4	100.15%	92.80 %	
	0.8 mL/min	90.25 %	91.69%	90-110 %
	1.2 mL/min	100.21 %	91.18 %	
Accuracy (% Recovery)		97–101 %	100–102 %	90-110 %
	No of plates (N)	11218.5	4343.6	>2000
	HETP	0.0022	0.057	-
	Asymmetry	0.77	1	1
	Resolution	1.6		-

Forced Degradation Studies for Proanthocyanidin and Ascorbic acid.

Degradation Condition	Drug Peak Area at		Drug peak area at		Rt of degraded products		% Degradation	
	PAC	AA	PAC	AA	PAC	AA	PAC	AA
Acidic	232708	51738	206012	51320	………	………	2.14%	0.61%
Alkaline 1N NaOH	216230	54260	201922	52136	………	………	1.83%	2.94%
Oxidation	200696	52420	198962	51819	………	…….	0.22%	0.83 %
Reduction condition	232608	53260	229924	52999	………	………	0.35%	0.36%
Thermal Condition	201942	52690	200931	51630	………	………	0.13%	1.47%
Photo	201931	54260	200240	53199	………	………	0.22%	1.48%

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