STABILITY INDICATING RP-HPLC METHOD FOR PROTOCATECHUIC ACID, AN ACTIVE PHYTOCONSTITUENT OF ONION PEELS.

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ABSTRACT

The aim of the present study was to develop and validate a stability indicating reverse phase high performance liquid chromatography (RP-HPLC) method for estimation of protocatechuic acid, which is important phytoconstituent of onion peels.

An isocratic, RP-HPLC method was developed using HiQ Sil C8 (250×4.6mm) column and using 20mM Potassium dihydrogen phosphate buffer (pH 4): Methanol (30:70 v/v) as mobile phase and detection was carried out at 258 nm. The retention time (RT) of drug was 3.05 ± 0.03 min. The method was validated with respect to linearity, precision, accuracy and robustness. The data of linear regression analysis indicated a good linear relationship over the range of 10-50µg/ml concentrations with a correlation coefficient (R^2) of 0.997. Protocatechuic acid was subjected to different stress testing conditions, as per ICH Q1R2 guidelines. The developed method was found to be simple and sensitive for analysis of protocatechuic acid.

KEYWORDS: Protocatechuic acid, HPLC, Stress testing, onion peels, Validation.

INTRODUCTION

Protocatechuic acid (PCA) is a type of widely distributed naturally occurring phenolic acid, commonly found in bran, grain, brown rice, fruits such as plums, gooseberries, grapes and also in onion peels. PCA is chemically 3,4-dihydroxybenzoic acid. PCA has structural similarity with gallic acid, caffeic acid and vanillic acid which are well-known antioxidant compounds. The chemical formula is $C_7H_6O_4$ and molar mass is154.12 g/mol. It is freely soluble in methanol and sparingly soluble in water, insoluble in benzene.[1,2] Protocatechuic acid has many pharmacological activities such as antibacterial, anti-imflamatory, hepatoprotective, anticancer, antidiabetic, antioxidant, antiulcer, antimutagenic, analgesic etc. [3-11]

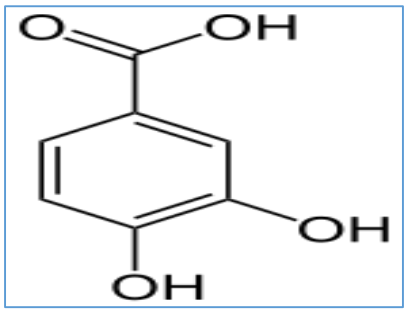

Fig.1 Structure of Protocatechuic acid

As per literature search there is no stability indicating method reported for determination of protocatechic acid by HPLC[12,13], hence based upon these observations we have developed a simple high performance liquid chromatography method for the estimation of protocatechuic acid as pure drug in accordance with International Conference on Harmonisation Guidelines. Development of SIM is based on systematic exposure of protocatechuic acid to various stress conditions. Systematic optimization trials are required to arrive at combination of "concentration of stress reagent and duration of exposure", to obtain degradation preferably in the 10-20% range. Typical degradation conditions involve hydrolysis under different pH conditions, photolysis, oxidation and thermal studies.

MATERIALS AND METHODS

Chemicals and reagents

Protocatechuic acid was procured from SRL. Pvt Ltd, Methanol (HPLC grade), Potassium dihydrogen phosphate buffer, Hydrochloric acid (HCl), 6% v/v Hydrogen peroxide (H_2O_2), Sodium hydroxide (NaOH) were purchased from LOBA CHEMIE PVT. Ltd. Mumbai.

Preparation of Standard Stock Solutions

Standard stock solution of Protocatechuic acid was prepared by dissolving 10 mg of drug in 10 ml methanol to get final concentration of 1000 µg/ml.

Selection of Detection Wavelength

The standard stock solution of concentration 20 µg/ml was scanned over the range of 200-400 nm by using the UV Visible spectrophotometer. It was observed that drug showed considerable absorbance at 258 nm, hence it was selected as the detection wavelength.

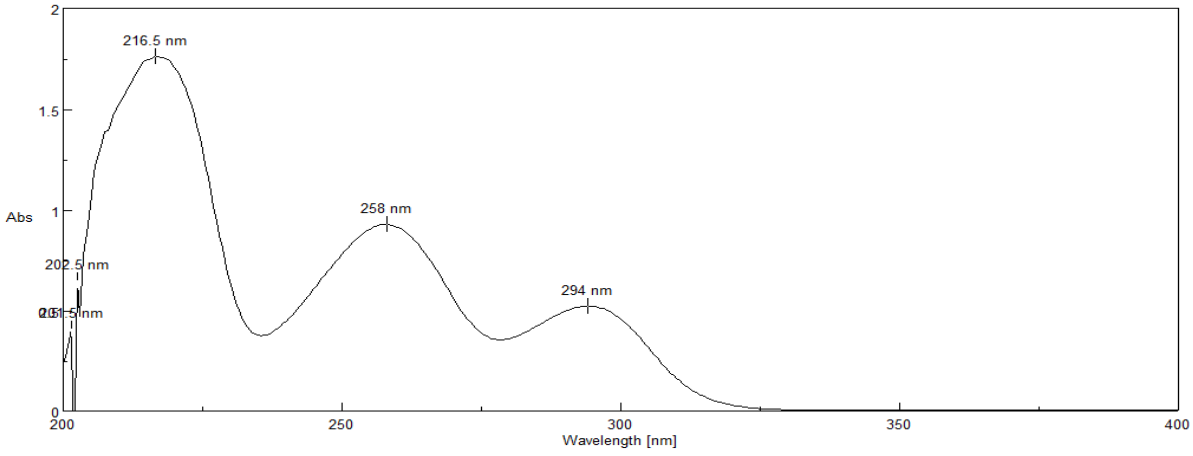

Fig 2. UV absorption spectrum of protocatechuic acid.

Preparation of hydroalcoholic extract of onion peels

Extraction process was carried out by maceration technique for 72 hrs with 70% ethanol and alkaline solution, after completion of extraction macerate was filtered through whatmann filter paper and evaporated on waterbath produced semisolid onion peels extract. 25mg of dried onion peels extract were weighd and dissolved in methanol and madeup the volume 25ml (1000µg/ml) and further diluted to prepare 10µg/ml.

Optimized Chromatographic Conditions

HPLC system used was JASCO system equipped with Model PU 2080 Plus pump, Rheodyne sample injection loop (20µl), MD 2010 PDA detector and Borwin- PDA software (version 1.5). A chromatographic column HiQSil C8 (250×4.6mm) was used for analysis. Separation was carried out at flow rate of 1 ml/min by using 20 mM potassium dihydrogen phosphate buffer pH 4: methanol (30:70 v/v) and detection at 258 nm.

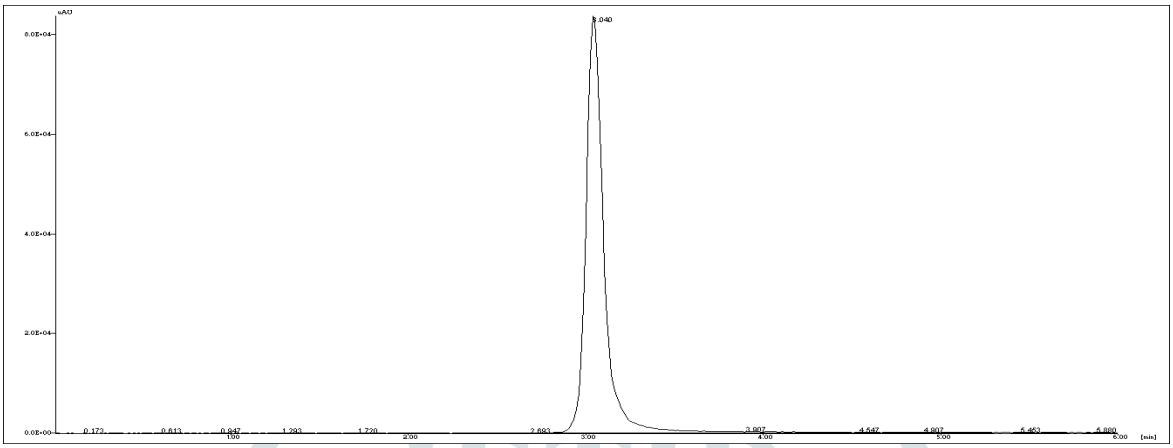

Fig. 2 chromatogram of protocatechuic acid

Preparation of 20 mM potassium dihydrogen phosphate buffer (pH 4)and mobile phase

20 mM potassium dihydrogen phosphate buffer (pH 4) was prepared by dissolving 272 mg of potassium dihydrogen phosphate in 200 ml of HPLC grade water and pH of the solution was checked and pH was adjusted to 4 by ortho-phosporic acid. Mobile phase was prepared by mixing potassium dihydrogen phosphate buffer (pH 4) and methanol in the ratio of 30:70 v/v. It was then filtered through 0.45µm nylon 6, 6 membrane filter and sonicated for 15 min.

STRESS DEGRADATION STUDIES OF BULK DRUG[14,15]

Forced degradation studies were carried out to provide evidence on how quality of drug varies under the influence of a variety of environmental conditions like acidic, alkaline hydrolysis, oxidation, dry heat and photolytic degradation. The hydrolytic studies were carried out by using the solution of 0.01 N NaOH and 0.01 N HCl. The oxidative degradation was carried out by using the 1% H_2O_2 solution. The thermal degradation study was performed by keeping drug sample in oven (80°C) for a period of 3 hours. The photolytic degradation studies were carried out by the exposure of drug sample to UV light upto illumination of NLT 200 watt hr/m2 and to florescent light upto illumination of NLT 1.2×10^6 Lux hr. of florescent light. All studies were carried out at concentration level of of 30µg/ml.

Alkaline hydrolysis

For alkali degradation study, 1 ml of 300µg/ml solutions of Protocatechuic acid was taken into 10ml volumetric flask and 1ml of 0.01N sodium hydroxide was added and diluted with mobile phase to make up the volume 10ml (30µg/ml) and kept it for 2 hrs.. Alkali degradation blank is prepared in the same way without using API. Under alkaline hydrolysis, percent recovery obtained for protocatechuic acid was found to be 80.45% with peak of degradant. The representative chromatogram is shown in Fig. 3.

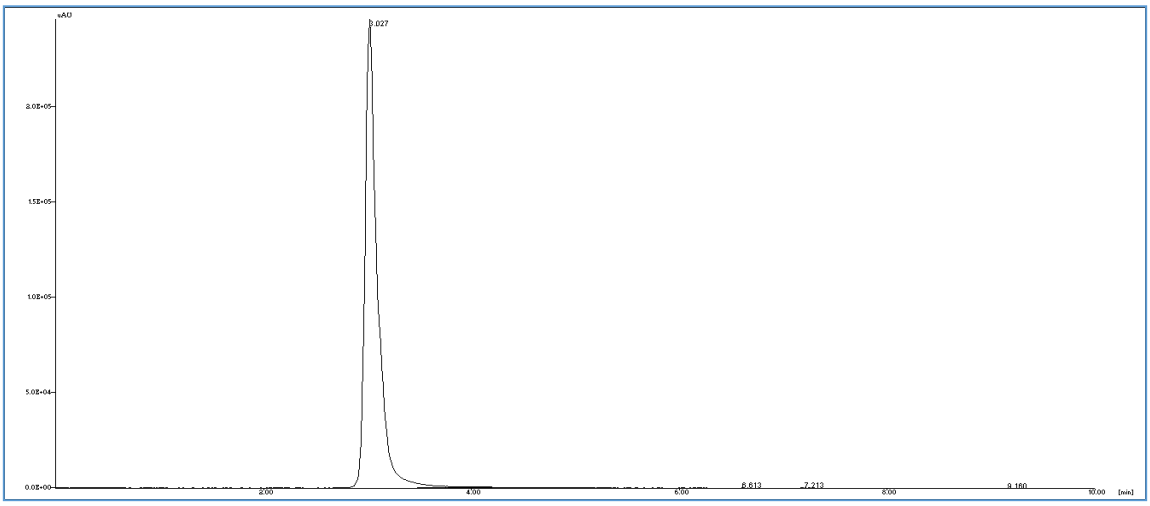

Fig.3 Chromatogram of protocatechuic acid after alkaline degradation.

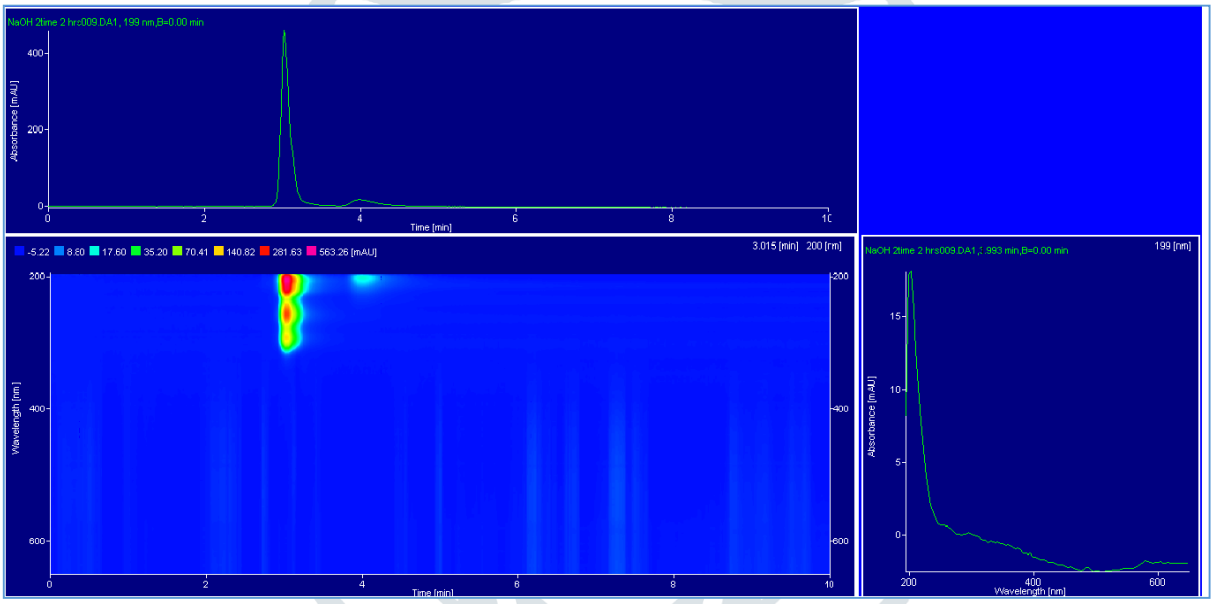

Fig.4: Chromatogram of protocatechuic acid after alkaline degradation.

Acid hydrolysis

For acid degradation study, 1 ml of 0.01 N hydrochloric acid was added and rest procedure was same as alkali hydrolytic condition. Average 89.40% of Protocatechuic acid was recovered with no peak of degradant.

Degradation under oxidative condition

For oxidative degradation study, 1 ml of 1% H_2O_2 was added and kept it for15 min rest procedure was same as alkali hydrolytic condition. Average 88.66% of Protocatechuic acid was recovered with no peak of degradant.

Photo-degradation studies:

The photo degradation of the drug was studied by exposing the drug to UV light providing illumination of NLT 200 watt hr/m^2 and exposure to cool white fluorescence light of NLT 1.2 million Lux-Hr. After exposure accurately weighed 10 mg of drug was transferred to 10 ml of volumetric flask; the volume was made up with methanol. Further dilution made with mobile phase to get 30µg/ml as final concentration and was injected. 88.42 % of Protocatechuic acid was recovered with no peak of degradant after exposure to UV

light and 84.45 % of Protocatechuic acid was recovered with no peak of degradant after exposure to fluorescence light.

Degradation under dry heat

Dry heat study was performed by keeping drug sample in oven (80^0C) for a period of 3 hours. After exposure 10 mg of drug was dissolved in10ml methanol to get solution of 1000 µg/ml and further diluted with mobile phase to get 30 µg/ml as final concentration and was injected. Under dry heat degradation condition, percent recovery obtained for Protocatechuic acid was 80.91% with no peak of degradant.

Degradation under Neutral hydrolytic condition

For neutral degradation study, 1 ml water is added and rest procedure was same as alkali hydrolytic condition. Average 85.32% of Protocatechuic acid was recovered with no peak of degradant.

Table 1: Forced degradation study results

Sr. no.	Parameters	Condition	% Recovery	Rt of PCA
1	Alkali hydrolysis	0.01 N NaOH 2hrs at Room temp.	80.45	3.05
2	Acid hydrolysis	0.01N HCl 2hrs at Room temp	89.40	3.05
3	Neutral hydrolytic	H_2O at Room temp	85.32	3.05
4	Oxidative stress Degradation	1% v/v H_2O_2 15 min at Room temp	88.66	3.05
5	Dry heat degradation	80˚C for 3hrs	80.91	3.05
6	Photolytic Degradation	UV light (200 watt hours/square meter)	88.42	3.05
		cool white fluro light (1.2 million lux hours)	84.45	3.05

VALIDATION OF ANALYTICAL METHOD [16]

Specificity:

The specificity of the method was ascertained by peak purity profile studies. The peak purity values were found to be more than 999, indicating the no interference of any other peak of degradation product, impurity or matrix.

Linearity and Range

From the standard stock solution (1000µg/ml) of Protocatechuic acid, solution was prepared containing 100µg/ml. This solution was further used to prepare range of solution containing different concentrations. The linearity (relationship between peak area and concentration) was determined by analyzing five solutions over the concentration range of 10-50 µg/ml, the equation of calibration curve was found to be y = 55287x + 12784. The peak area of drug was plotted against the corresponding concentrations to obtain the calibration curve as shown in Fig. 6

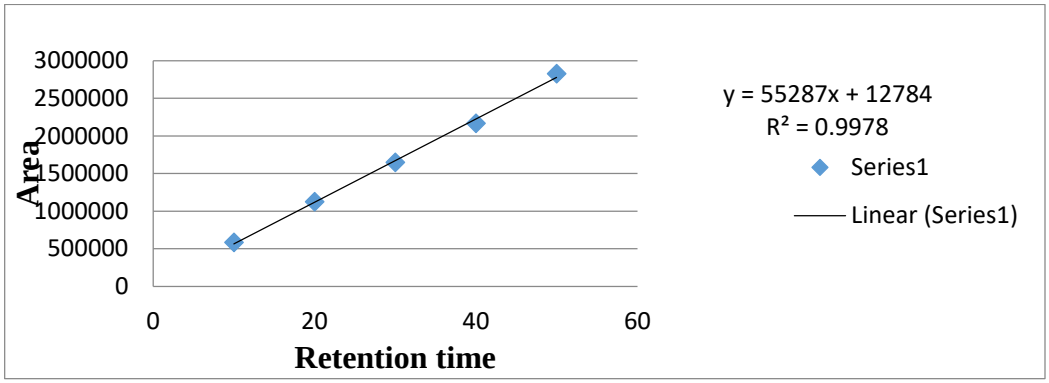

Fig 6: linearity plot for conc. range of 10-50 µg/ml

Precision

The precision of the method was demonstrated by intra-day and inter-day variation studies. In the Intra-day studies, 6 replicates of same concentration were analyzed in a day and percentage RSD was calculated. For the inter day variation studies were analyzed on 3 consecutive days and percentage RSD was calculated. The results obtained for intraday and inter day variations are shown in Table 2.

Table 2: Interday and intraday precision

Concentration (µg/ml)	Intraday			Interday		
	Mean Area	SD	% RSD	Mean Area	SD	% RSD
10	530487.8	5274.381	0.99	542236.74	5963.8072	1.09
10	537818.3	4042.15	0.75	531964.3	5640.02	1.06
10	54702	4578.80	0.84	540451.7	4727.70	0.87

Limit of detection (LOD) and Limit of quantitation (LOQ)

LOD and LOQ were calculated as 3.3 σ/S and 10 σ/S, respectively; where σ is the standard deviation of lowest concentration response and S is the slope of the calibration plot. The LOD and LOQ of protocatechuic acid was found to be 0.35 µg/ml and 1.07µg/ml, respectively.

Accuracy

To check the accuracy of the method, recovery studies were carried out by addition of standard drug solution to pre-analyzed extract solution at three different levels 80 %, 100 % and 120 %. Basic concentration of sample chosen was 18µg/ml of protocatechuic acid from onion peel extract solution. The drug concentrations were calculated from respective linearity equation. The results obtained are shown in Table 3.

Table 3: Recovery Studies of protocatechuic acid

Level (%)	From extract (µg/ml)	From std. addition (µg/ml)	Total Conc. With extract (µg/ml)	Conc. Recovered (µg/ml)	% Recovery

80	18	15	33	33.17	100.51
100	18	18	36	36.42	101.16
120	18	22	40	40.35	100.85

Robustness

Robustness of the method was determined by carrying out the analysis under conditions during which pH and ratio should be varied and the effect on the area of drug were noted. It was observed that there were no marked changes in the chromatograms, which demonstrated that the RP-HPLC method developed is robust.

RESULTS AND DISCUSSION

The developed method was found to be simple and sensitive for analysis of Protocatechuic acid. The results indicated the suitability of the method to study stability of protocatechuic acid under various forced degradation conditions.

Table 4: summary of validation parameter

Sr. No	Validation Parameters	Protocatechuic acid
1	Linearity Equation (r^2) Range	y= 55287x + 12784 R^2 = 0.997 10-50µg/ml
2	Precision	(% RSD)
	Intraday	0.99
		0.75
		0.84
	Interday	1.09
		1.06
		0.87
3	Accuracy	% Recovery
	80%	100.51
	100	101.16
	120%	100.85
4	Limit of Detection	0.35 µg/ml
5	Limit of Quantitation	1.07 µg/ml
6	Specificity	Specific

7	Robustness	Robust

CONCLUSION

The developed method is stability indicating and can be used for assessing the stability of protocatechuic acid in bulk form and in onion peel extract. The developed method is simple and sensitive. The method was developed by using easily available and relatively cheap solvents Methanol hence can be considered as economic.

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