Research Paper

Development of Novel Reverse Phase High Performance Liquid Chromatography Method for Simultaneous Estimation of Gallic Acid, Protocatechuic Acid, Vanillic Acid and Syringic Acid in Sugarcane Roots

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A novel, simple, accurate and precise isocratic high performance liquid chromatographic method was developed for the estimation of four phenolic acids, namely gallic acid, protocatechuic acid, vanillic acid and syringic acid in the sugarcane roots. The selected phenolic acids were analyzed using shim-pack high performance liquid chromatography C_{18} column (250×4.6 mm, 5 µm) by isocratic elution. The mobile phase used was methanol:water containing 0.2 % ortho-phosphoric acid in the ratio of 20:80 at 28°. The flow rate was 1 ml/min and detection wavelength was set to 220 nm. The run time was kept 30 min. The retention time of gallic acid, protocatechuic acid, vanillic acid and syringic acid was found to be 4.864, 8.741, 20.298 and 25.268 min, respectively. Linearity was established for all the selected phenolic acids in the range of 0.25-20 µg/ml. The percentage recoveries of gallic acid, protocatechuic acid, vanillic acid and syringic acid were found to be in the range of 98 %-102 %. The method was found to be robust. Therefore, the method was successfully applied for the determination of the above mentioned phenolic acids in the sugarcane rootss.

Key words: Sugarcane roots, phenolic acids, high performance liquid chromatography, C_{18} column

Sugarcane (*Saccharum officinarum*) is an important crop in tropical and subtropical areas. It is one of the ancient crops known to man[1,2]. India is the highest producer of sugarcane in the world, after Brazil[3]. In India, sugar industry with 400 sugar factories rank as the second major agro-industry in the country[4]. Sugarcane crops are primarily grown for sugar production, after the main use of sugarcane crop, the crop generates large quantities of agriculture waste including green tops, leaves and roots[5,6].

The sugarcane stalk is well studied for its phytochemical composition; the important constituents are phenolic acids, flavonoids, steroids, fatty acids, fatty alcohols[7]. The lesser studied parts of sugarcane are leaves and roots which form a substantial quantity of waste from sugarcane crop. The leaves are reported to contain sterols such as stigmasterol and sitosterol, flavonoids, anthocyanins. Phytochemical nature of roots is still unfamiliar except for the few papers indicating the presence of flavonoids and anthocyanins[8].

The literature survey revealed that roots are unexplored with respect to quantitative determination of phytoconstituents, hence it was necessary to study the roots for the quantitative estimation of polyphenolic compounds. Based on the occurrence of polyphenolics in sugarcane, Gallic Acid (GA), Protocatechuic Acid (PCA), Vanillic Acid (VA) and Syringic Acid (SA) were selected for the present study (fig. 1)[9].

Literature survey also revealed that so far there is no isocratic Reversed Phase High Performance Liquid Chromatographic (RP-HPLC) method reported for simultaneous estimation of these important polyphenolic constituents. Hence it was thought worthwhile to

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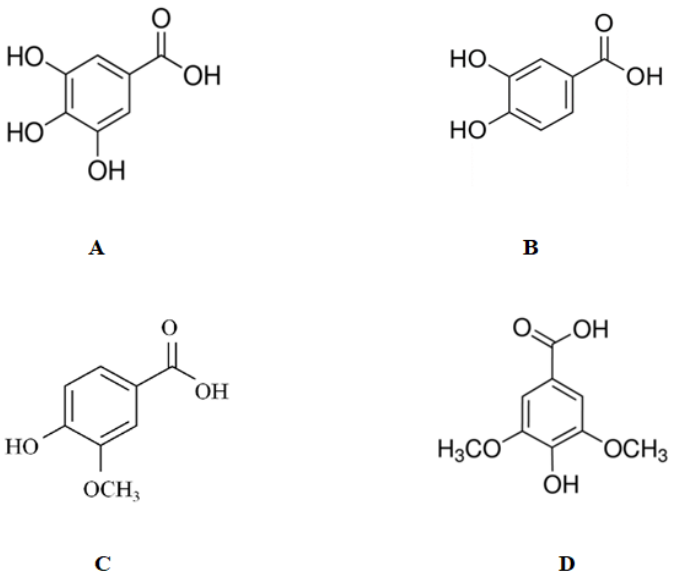

Fig. 1: Structures of (A) GA; (B) PCA; (C) VA and (D) SA

develop a novel, simple isocratic RP-HPLC method for simultaneous estimation of GA, PCA, VA and SA. The developed novel RP-HPLC was applied for the first time for quantification of selected polyphenolics in sugarcane roots.

The selected polyphenolic compounds are well known bioactive constituents. GA is reported to possess strong antioxidant, astringent activity and cytotoxic activity[10]. PCA is reported to act as antibacterial, antifibrotic and anti-ageing[11]. VA is reported to possess anti-inflammatory and neuroprotective effect[12]. While SA is a well-known antioxidant, anti-inflammatory and antimicrobial compound[13].

MATERIALS AND METHODS

GA, PCA, VA and SA (purity 95 %) were procured from Sigma-Aldrich, Mumbai, India. HPLC grade methanol and ortho-phosphoric acid were purchased from Thomas Baker Chemicals Pvt. Ltd. Mumbai, India. High quality pure water obtained from Lab Q Ultra Type-1 was used throughout the analysis.

RP-HPLC system (Shimadzu, LC-2030) comprising of an auto start-up, auto injector, quick batch function and Ultraviolet (UV) detector was used for the analysis. Analytical column used for separation of analytes was shim-pack HPLC C_{18} (250×4.6 mm, 5 µm). The data was noted using "Lab Solutions" software.

Plant materials and sample preparation:

Sugarcane roots were procured from the sugarcane fields in Karad, Maharashtra. The roots were washed with water and subjected to sun drying for 7 d. The roots were then dried in oven at 60° for 2 d. The dried plant material grinded into coarse powder using Hanningfield Uni-Mill M10 (Gansons Pvt Ltd), with screen 37R having size 1 mm grater hole. The resulting powder was subjected to extraction for further HPLC analysis.

Preparation of sample solution:

Accurately 5 g powder of sugarcane roots was weighed and subjected to extraction with 80 ml hydro alcohol for 20 min using reflux assembly. The solution was then centrifuged to get a clear solution. The volume was made up to 100 ml with hydro alcohol and was used for HPLC analysis.

Preparation of standard solutions:

GA, PCA, VA and SA (100 mg) were accurately weighed and transferred into separate 100 ml volumetric flasks and volume was adjusted to 100 ml with HPLC grade methanol. The stock solution was further used for RP-HPLC analysis after suitable dilutions.

Chromatographic conditions:

The method was developed using RP-HPLC, Shimadzu LC Prominance-i 2030 model. The column used for separation of the analytes was shim-pack HPLC C_{18} column (250×4.6 mm, 5 µm). The optimized mobile phase used was methanol: Water containing 0.2 % ortho-phosphoric acid in the ratio of 20:80 at a flow rate of 1 ml/min, column temperature was maintained at 28°. The suitable wavelength for the HPLC analysis was determined by taking separate UV spectrum of

GA, PCA, VA and SA in the range of 200-400 nm. UV overlain spectra of these four standards showed that they absorbed appreciably at 220 nm (fig. 2) and hence, 220 nm was selected as detection wavelength using a UV-visible detector. The run time was kept 30 min. The injection volume was 10 µl.

Method validation:

The developed method was validated for specificity, linearity, accuracy, precision, Limits of Detection (LOD), Limits of Quantification (LOQ) and robustness as per International Council for Harmonisation (ICH) Q2 (R1) guidelines[14].

Specificity: Specificity was confirmed by comparing the retention time of the standards with the retention time of components obtained from the extract.

Linearity: Linearity was evaluated by analyzing the plot area as a function of the concentration of analyte. The standards, GA, PCA, VA and SA showed a linear response in the tested concentration range of 0.25-20 µg/ml. Each standard was run in triplicate. The linearity was constructed by plotting a peak area versus concentration of analyte. The linearity of the detector response for the prepared standards was evaluated by means of linear regression with respect to the amounts of each standard, measured in micrograms (µg) and the area of the corresponding peak from the chromatogram.

Accuracy: Recovery of GA, PCA, VA and SA acid was checked by spiking a known quantity of standards at three concentration levels (i.e., 80 %, 100 % and 120 % of the quantified amount) to the pre-analysed sample in triplicate using HPLC. This way, accuracy was performed and mean recovery was calculated.

Precision: The precision of the method was confirmed by intra-day and inter-day variation studies. Six replicates of mixed standards were injected and the percentage Relative Standard Deviation (% RSD) was calculated.

LOD and LOQ: The LOD of an individual analytical method is the lowest amount of analyte in a sample which can be detected but not essentially quantified as an exact value. The LOD is expressed as LOD=3.3 σ/S, where σ=standard deviation of intercepts of the calibration curve and S is the slope of the calibration curve.

LOQ is a parameter of quantitative assays for low levels of compounds (standards) in extracts. The LOQ is expressed as LOQ=10 σ/S

Robustness: The robustness of the method was determined by making slight changes in the chromatographic conditions (flow rates, wavelength and temperature). Each marker was analyzed in triplicate.

RESULTS AND DISCUSSION

UV overlain spectra of all the four standards showed that drugs absorbed appreciably at 220 nm, so this wavelength was selected as the detection wavelength. Optimization of chromatographic condition was performed to obtain the good peak shape, resolution and peak parameter (tailing factor, theoretical plates). For the selection of mobile phase initially water:methanol and acetonitrile:water has been tried in different proportions but it gave poor peak shape and poor resolution. Finally, methanol:water containing 0.2 % ortho-phosphoric acid in the ratio of 20:80 at the flow rate of 1 ml/min was found to be satisfactory. The optimized mobile phase gave sharp, well-resolved peaks with minimum tailing factor for the selected standards of GA, PCA, VA and SA in mixed standard solution and in sugarcane root extract (fig. 3 and fig. 4).

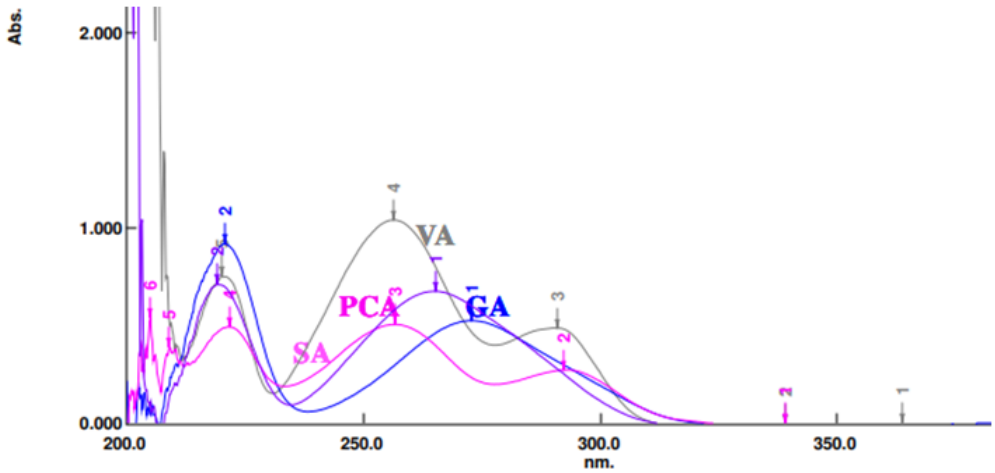

Fig. 2: UV overlain spectra of GA, PCA, VA and SA

The retention time for GA, PCA, VA and SA was found to be 4.86, 8.74, 20.29 and 25.26 min, respectively.

The calibration curve for GA, PCA, VA and SA was found to be linear over the range of 0.25-20 µg/ml. The data obtained for regression analysis of the calibration curves is shown in Table 1.

Accuracy was determined by calculating the percentage (%) recovery. The method was found to be accurate with % recovery between 98.12 %-101.88 % for all the selected standards. The results are tabulated in Table 2. Robustness of the method was confirmed by injecting the standard solution at change in flow rate, wavelength and temperature. No marked changes were noticed in the sample when compared to the standard chromatogram. The results are presented in Table 3 are indicative that the developed HPLC method is robust.

The developed method was found to be specific as there was no interference of any other constituents at the retention time of all four standards, GA, PCA, VA and SA. The % RSD for intra-day and inter-day precision of GA, PCA, VA and SA was found to be 0.43, 0.10, 0.55 and 0.75 respectively, while the % RSD for the intraday precision of GA, PCA, VA and SA was found to be 1.06, 0.48, 0.45 and 1.18 which indicates that the method is precise with % RSD less than 2. The LOD for GA, PCA, VA and SA was found to be 0.062 µg/ml, 0.029 µg/ml, 0.085 µg/ml and 0.116 µg/ml, respectively, while LOQ were 0.188 µg/ml, 0.088 µg/ml, 0.258 µg/ml and 0.353 µg/ml, respectively, which indicates that the

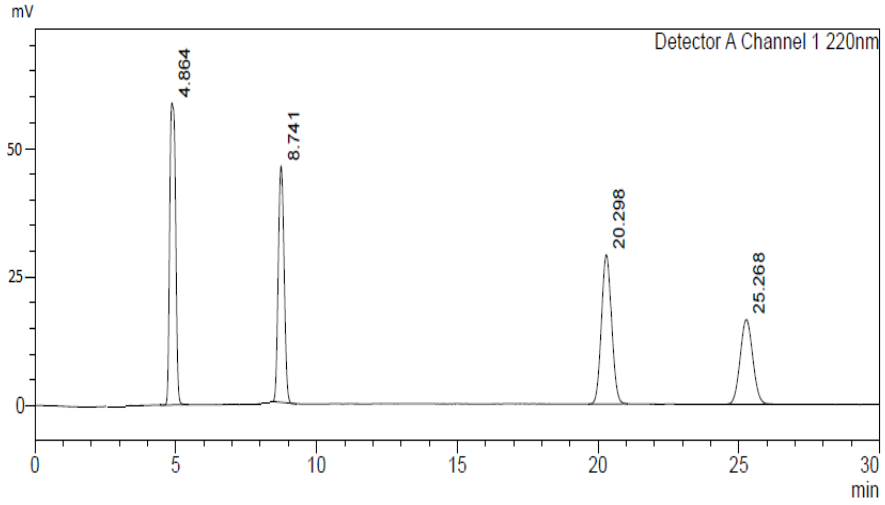

Fig. 3: HPLC chromatogram of mixed standard of GA, PCA, VA and SA at retention time 4.864, 8.741, 20.298 and 25.268 min respectively

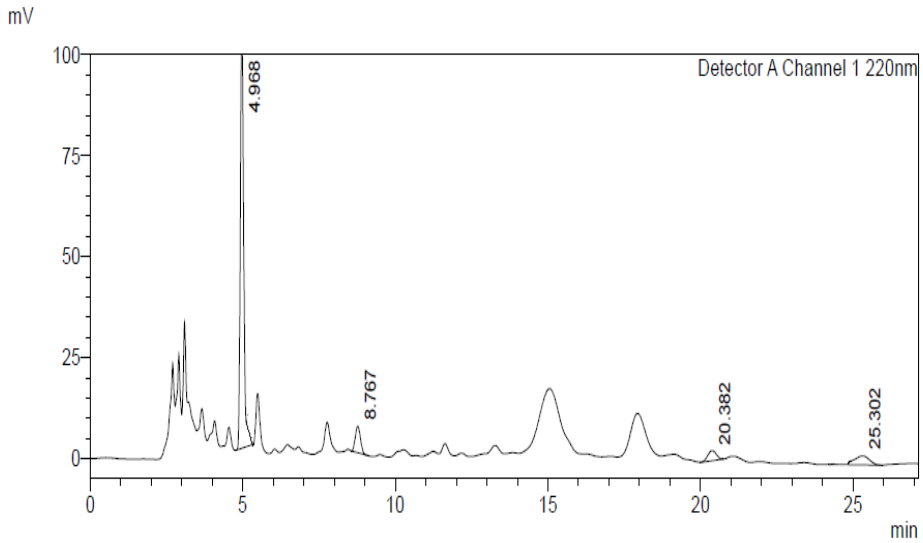

Fig. 4: HPLC chromatogram of sugarcane root sample with peaks of GA, PCA, VA and SA at retention time 4.968, 8.767, 20.382 and 25.302 min respectively

developed method is sensitive. The results for validation and system suitability test parameters are tabulated in Table 4, which shows that the parameters are within the acceptable range as per the ICH guidelines.

In the present study, a novel RP-HPLC method was developed and validated as per the ICH guidelines for the simultaneous estimation of GA, PCA, VA and SA in sugarcane roots. It has been shown that

TABLE 1: LINEAR REGRESSION DATA OF GA, PCA, VA and SA

Parameters (units)	GA	PCA	VA	SA
Linearity range (µg/ml)	0.25-20	0.25-20	0.25-20	0.25-20
Correlation coefficient (R^2)	0.9984	0.9979	0.9985	0.9983
Regression equation	y=203993x-12660	y=115246x+181291	y=195369x+46364	y=102490x+11856

Note: GA-Gallic acid; PCA-Protocatechuic acid; VA-Vanillic acid; SA-Syringic acid

TABLE 2: RECOVERY STUDY OF GA, PCA, VA AND SA

Drugs	Recovery level (%)	Amount of standard (µg/ml)	Standard added (µg/ml)	Total concentration of standard (µg/ml)	Total amount recovered (µg/ml)	% Recovery
	80	3.59	2.87	6.46	6.52	99.07
GA	100	3.59	3.59	7.18	7.26	98.89
	120	3.59	4.30	7.89	7.95	99.24
	80	4.45	3.56	8.01	8.12	98.64
PCA	100	4.45	4.45	8.9	9.07	98.12
	120	4.45	5.34	9.79	9.75	100.41
	80	0.06	0.04	0.108	0.106	101.88
VA	100	0.06	0.06	0.12	0.121	99.17
	120	0.06	0.07	0.132	0.130	101.53
	80	2.65	2.12	4.77	4.80	99.37
SA	100	2.65	2.65	5.3	5.39	98.33
	120	2.65	3.18	5.83	5.85	99.69

TABLE 3: DATA OF ROBUSTNESS STUDIES FOR GA, PCA, VA AND SA

Parameters	% RSD (GA) (n=3)	% RSD (PCA) (n=3)	% RSD (VA) (n=3)	% RSD (SA) (n=3)
Minus flow rate (0.9 ml/min)	1.56	0.92	1.83	1.92
Plus flow rate (1.1 ml/min)	0.69	0.81	0.92	0.76
Minus wavelength (219 nm)	0.01	0.01	0.19	0.51
Plus wavelength (221 nm)	0.23	0.66	0.21	1.56
Minus temperature (27°)	0.36	0.37	0.41	0.68
Plus temperature (29°)	0.29	0.22	0.24	0.54

Note: Robustness parameters were studied by making slight changes in flow rate (±0.1 ml/min), wavelength (±1 nm) and temperature (±1°), for n=3 observations

TABLE 4: SUMMARY OF VALIDATION AND SYSTEM SUITABILITY TEST

Parameters	GA	PCA	VA	SA
Retention time (min)	4.864	8.741	20.298	25.268
Theoretical plates	3011	8050	14970	14709
Tailing factor	1.245	1.087	1.068	1.056
Specificity	Specific, no interference	Specific, no interference	Specific, no interference	Specific, no interference
Precision (% RSD)				
Interday (n=6)	0.43	0.10	0.55	0.75
Intraday (n=6)	1.06	0.48	0.45	1.18
LOD (µg/ml)	0.062	0.029	0.085	0.116
LOQ (µg/ml)	0.188	0.0885	0.258	0.353

the developed method achieved specificity, linearity, accuracy, precision and robustness which prove the reliability of the method. The method was applied for the quantification of selected standards in the sugarcane root for the first time. The results showed that this method can be easily applied for the estimation and isolation of the four above mentioned phenolic acids in sugarcane roots.

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Conflict of interests:

The authors declared no conflict of interest.

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