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Research Article

SEPARATION AND QUANTIFICATION OF PHARMACOLOGICALLY ACTIVE MARKERS P-METHOXYBENZOIC ACID, 3, 4-DIHYDROXYBENZOIC ACID AND GALLIC ACID FROM CAPPARIS SPINOSA L. AND FROM MARKETED FORMULATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Vikas V Vaidya, Manjiri A Shinde* and Pushkar M. Pradhan

Department of Chemistry, Ramnarain Ruia College, Mumbai, Maharashtra, India.

ABSTRACT

A rapid chromatographic method for the determination of phenolic acids; p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid from Capparis spinosa using High Performance Liquid Chromatography was developed. Capparis spinosa Linn. (Fam. Capparidaceae), a thorny shrub distributed in the plains, lower Himalayas, and Western Ghats. In Ayurveda it is used for the treatment of edema, dermatopathies, heart disorders, anaemia, renal disorders, hepatic disorders and inflammatory disorders.

Chromatographic separation and quantification were performed on a reversed-phase Cosmosil C_{18} column with gradient elution using 5mM KH_2PO_4 Buffer (adjusted to pH 2.4) and 0.05% formic acid in Acetonitrile. A flow-rate of 0.6 ml/min, column temperature of 35°C and detection at 273 nm were employed. The method was found to give well separated sharp peaks of p-Methoxy benzoic acid, 3,4-Dihydroxybenzoic acid and Gallic acid at R_t of 14.6min, 5.4min, 3.8min respectively. The quantity of p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid in plant was found to be 0.00655 %, 0.02598 % and 0.0121 %; and 0.01348 %, 0.02434 % and 0.06421 % in formulation respectively. The method was validated in terms of linearity, specificity, precision, stability and recovery. Statistical analysis proved that the proposed method is precise and reproducible. The developed method can be used as a quality control tool for simultaneous quantification of these markers from raw material as well as marketed formulation.

Keywords: Capparis spinosa L., High Performance Liquid Chromatography, Gallic acid.

INTRODUCTION

Capparis spinosa Linn. (syn. C. aculeata Steud, C. microphylla Ledeb) belongs to the family Capparaceae and is known as Himsra in Sanskrit and Caper bush in English. Capparis spinosa Linn. is a thorny shrub growing in dry rocky soils of North-Western India, through Punjab, Rajasthan and Deccan peninsular regions. Widely distributed in the plains, lower Himalayas, and Western Ghats. Capparis spinosa Linn. is widely used in the traditional medicine of several American, African and Asian countries. In Ayurveda it is used for the treatment of edema, dermatopathies, heart disorders, anaemia, renal disorders, hepatic disorders and inflammatory disorders.

Polyphenols are the largest group of phytochemical and many of them have been found in plant-based foods. Phenolic acids are nonflavonoid polyphenolic compounds which can be further divided into two main types, benzoic acid and cinnamic acid derivatives.

p-Methoxybenzoic acid appears to have important hepatoprotective activity. Gallic acid (3, 4, 5-trihydroxybenzoic acid) is one type of

natural phenolic acid compound widely which exists in plants.3, 4-Dihydroxybenzoic acid (protocatechuic acid) and gallic acid are reported to have anti-inflammatory, antioxidant activity.

In this research work, a simple, precise and reproducible HPLC method has been established for simultaneous quantitation of p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid in the plant powder of Capparis spinosa Linn. Further, the proposed method has been validated as per ICH guidelines and applied as a quality control tool for standardization of a commercially available hepatoprotective formulation containing plant extract of Capparis spinosa L. Such a study would not only facilitate standardization of the raw material and commercial products, but also facilitate future pharmacological studies and quality control.

MATERIAL AND METHODS

Chemicals

HPLC grade deionised water, acetonitrile and methanol were procured from Merck Specialities Private Ltd, Mumbai, India.

Analytical grade Formic acid, Hydrochloric acid and Potassium dihydrogen phosphate were procured from Merck Specialities Private Ltd, Mumbai, India.

Reference Standards of p-Methoxybenzoic acid (99 %) and 3, 4-Dihydroxybenzoic acid (97 %) were purchased from Sigma-Aldrich. Reference Standards of Gallic acid (99 %) was purchased from Alpha Aesar.

Plant material

Plant specimen of Capparis spinosa was collected from Tamhini ghat, Pune. Herbarium sample was prepared and authenticated by Blatter Herbarium, St. Xavier's College, Mumbai, India. The whole plant was washed with water to remove dust particles, dried in shade, powdered and then sieved through mesh size 85 and stored in an airtight container.

Polyherbal formulation "Liver Detox capsules" manufactured by Planet Ayurveda, India were procured from Planet Ayurveda manufacturing unit, Punjab.

Preparation of stock solutions

Preparation of stock solution of p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid (1000 ppm)

All the stock solutions of p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid (1000 µg/ 1000 µL) were prepared in methanol. 25 mg of respective standards were accurately weighed and transferred to 25 mL standard volumetric flask. The contents of the flask were initially dissolved in about 5.0 mL of methanol, followed by sonication and then diluted up to the mark with methanol.

From the stock solution 100ppm of standard solution was prepared using appropriate dilution.

Sample Preparation

About 5 gram of dried leaf powder of Capparis spinosa was weighed into a round bottom flask. 100 mL of methanol was added to the flask and the mixture was kept on shaker at 100 rpm speed for 24 hours. The extract was then filtered through Whatmann filter paper no. 41 (E. Merck, Mumbai, India). The filtered solvent was evaporated to reduce volume. The final volume was made to 10ml using methanol.

The formulation Liver detox capsules were opened to get powder material out of them. 1 gram of this powder was treated in the same way as that of plant.

CHROMATOGRAPHIC CONDITIONS

Table 1: HPLC conditions

Parameters	Description
Instrument	Prominence Ultra fast Liquid Chromatographic system (Shimadzu Inc., Japan)
Pump	binary pump (model LC-20AD),
Autosampler	SIL-20AC
Injection volume	10ul
Column	C18 reverse-phase column, (Cosmosil; 4.6 mm I.D * 150mm, 4.5 µm particle size
Column oven temp	oven (model CTO- 20AC), controller (model CBM-20A)
Mobile phase	Solvent A: 5mM KH_2PO_4 buffer, pH=2.4 using HCl Solvent B: 0.05% Formic acid in Acetonitrile
Flow rate	0.6 ml/min
Detector	photodiode array detector (model SPD-M20A)
Detection wavelength	273nm

Table 2: Gradient programme of mobile phase

Time (mins)	5mM KH_2PO_4 buffer, pH=2.4 Solvent A	0.05% formic acid in Acetonitrile Solvent B
0:01	86%	14%
6:00	86%	14%
6:01	35%	65%
15:00	35%	65%
15:01	86%	14%
25:00	86%	14%

VALIDATION OF THE METHOD

ICH harmonized tripartite guidelines were followed for the validation of the developed analytical method. The summary of the validation parameters has been given in Table 3.

System Suitability

System suitability tests for the analysis are used to verify the adequate reproducibility of the equipment. The suitability test was carried out to determine the performance of the operating system. The system suitability experiment was carried out by injecting 10ul of 100.0 µg/mL p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid respectively. The solution was injected six times and % RSD was calculated for each analyte.

Specificity

Specificity is a measure of the degree of interference in the analysis of complex sample mixtures such as an analyte present in matrix containing endogenous substances, and related chemical compounds, etc.

To demonstrate specificity of the analytical method, following determinations were carried out.

Linearity

The method was found to be linear from 1 – 50 ug/ml for p-Methoxybenzoic acid, 20-140 ug/ml for 3, 4-Dihydroxybenzoic acid and 10-70 ug/ml for Gallic acid. The standards were injected in triplicates. The chromatograms were then acquired and the peak areas were recorded for each concentration of standard. RSD of standard peak areas for each linearity level were less than 2%.

Limit of Detection and Limit of Quantification

The limit of detection (LOD) is the lowest concentration of an analyte that can be detected under the operational conditions of the method. The limit of quantification (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy, under the operational conditions of the method.

Recovery

The accuracy of the method was assessed by performing recovery studies at three different levels i.e 80%, 100% and 120 % , spiking p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid in plant matrix and marketed formulation. The percent recovery and the average percent recovery for each component were calculated.

Stability

The stability of the stock solutions of all the three standards was evaluated by storing the stocks in refrigerator at 2-8° C and at room temperature for 72 hours. This was followed by comparing the concentrations of these stocks against freshly prepared stocks for each standard.

RESULT AND DISCUSSION

A HPLC method for separation and simultaneous quantification of p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid in the leaf extract of Capparis spinosa and formulation was developed and validated successfully in the present research work.

Chromatograms

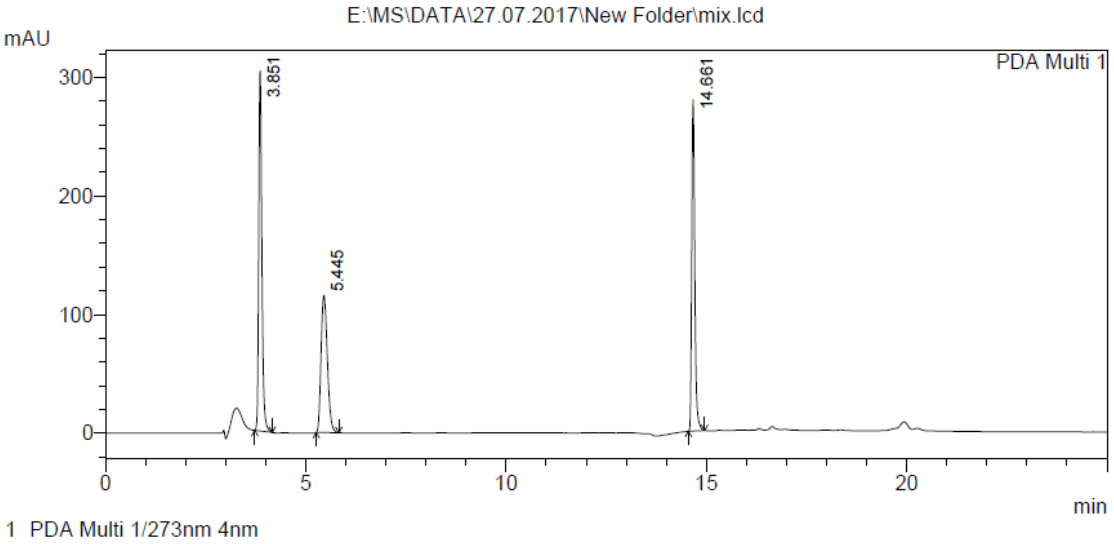

Fig. 1: Chromatogram of mixture of standard
(Peak1: Gallic acid; R_t=3.851min, Peak2:3, 4-dihydroxybenzoic acid; R_t=5.445min, Peak3: p-Methoxybenzoic acid; R_t =14.661min)

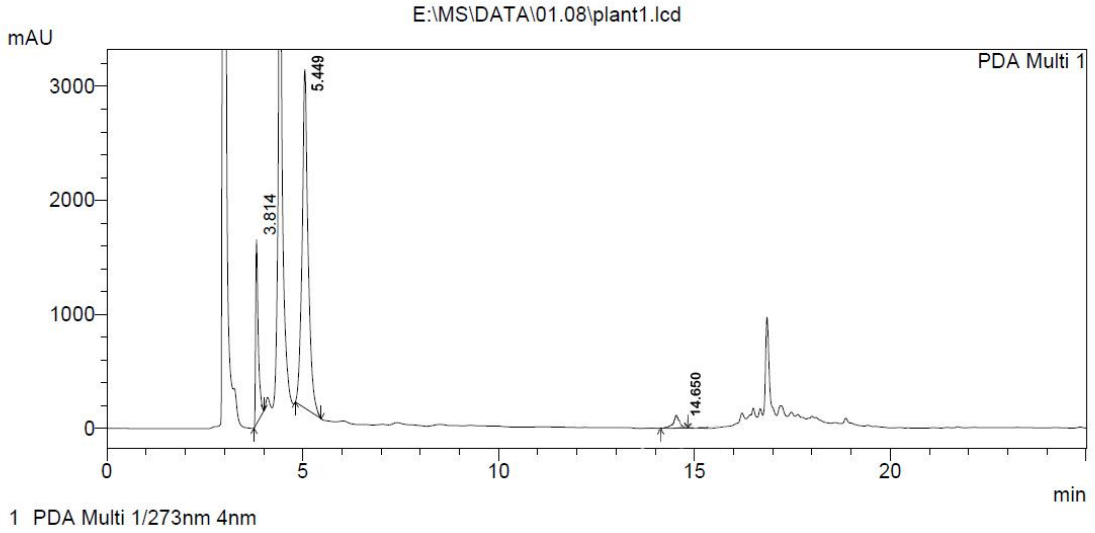

Fig. 2: Chromatogram of methanolic extract of Capparis spinosa

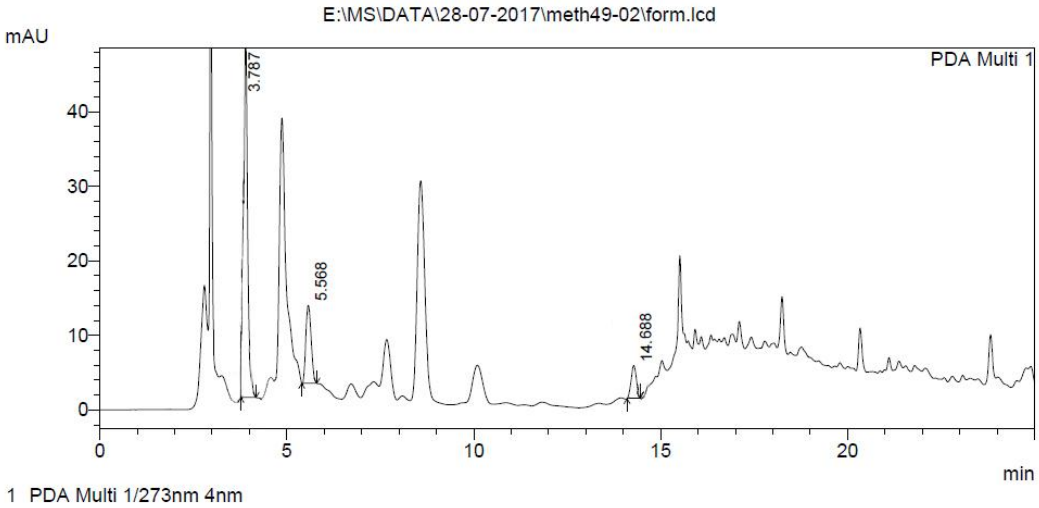

Fig. 3: Chromatogram of formulation

Optimization of Chromatographic conditions

Different mobile phases including methanol, deionized water, acetonitrile, formic acid, and phosphate buffer were tried. Among the different mobile phase investigated, a gradient method including Potassium phosphate buffer of pH adjusted to 2.4 and 0.05% formic acid in acetonitrile was selected with the flow rate of 0.6ml/min. The three peaks were very well separated within 25mins. Since the compound, Gallic acid and 3, 4-Dihydroxybenzoic acid has one OH difference in their structure. So they were eluting at the similar timing, so buffer was included and pH was adjusted according to their pKa values. The above mobile phase composition enabled the peak shape and elution of the component. Also in this separation, the UV detector gave good spectra of the separated components. The detection wavelength was confirmed at 273 nm. The method was found to give well separated sharp bands of p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid at R_t of 14.6 min, 5.4 min, 3.8 min respectively.

Method validation

No interference observed at the retention time of components of interest due to the diluents, Hence the method can considered as specific.

For p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid the % R.S.D values for area and retention time was found to be <2% indicating that the system was suitable to carry out further analysis.

The assay value for samples of *Capparis spinosa* plant powder was found to be 0.006558 %, 0.025987% and 0.0121% for p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic

acid respectively, while for the formulation it was found to be 0.01348 %, 0.02434 % and 0.06421 % for p-Methoxybenzoic acid, 3, 4-Dihydroxybenzoic acid and Gallic acid respectively. The method is specific for the three components because it resolved all standards well in the presence of other phytochemicals in *Capparis spinosa.*

Table 3: Summary of method validation parameters

Parameter	p-Methoxybenzoic acid	3,4-Dihydroxybenzoic acid	Gallic acid
Specificity	Specific	Specific	Specific
Precision (RSD)	< 2%	< 2%	< 2%
LOD (ug/ml)	0.33	6.6	3.33
LOQ (ug/ml)	1	20	10
Linearity (ug/ml)	1-50	20-140	10-70
Assay(Plant)	0.006558%	0.025987%	0.0121%
Assay (Formulation)	0.01348 %	0.02434%	0.06421 %
Stock Solution stability	Until 72 hrs at RT	Until 72 hrs at RT	Until 72 hrs at RT
Recovery (Plant)	95.17 %	103.15 %	99.70 %
Recovery (Formulation)	97.72 %	92.45 %	105.30 %

CONCLUSION

A precise, rapid and reproducible HPLC method is validated for simultaneous quantification of three pharmacologically active markers. This HPLC method serves as a quality control tool for quantification of these markers simultaneously from raw material as well as marketed formulation.

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