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Development and validation of a novel RP-HPLC method for the simultaneous quantification of ascorbic acid, gallic acid, ferulic acid, piperine, and thymol in a polyherbal formulation

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A novel, accurate, precise, and linear RP-HPLC method for the simultaneous estimation of ascorbic acid, gallic acid, ferulic acid, piperine, and thymol in a polyherbal formulation was developed and validated as per ICH guidelines. A good chromatographic separation was achieved using gradient profile with the help of Shim-pack RP-HPLC column (250 mm*4.6 mm, 5 µm) and a mobile phase consisting of acetonitrile and 1% acetic acid was used. The column temperature was maintained at 28 °C throughout the run with a wavelength of 272 nm using UV-Visible detector. The retention time of ascorbic acid, gallic acid, ferulic acid, piperine, and thymol was found to be 3.9, 6.7, 21.5, 44.6, and 45.9 min respectively. The linearity of ascorbic acid, gallic acid, ferulic acid, piperine, and thymol was found to be in a range of 10-100, 5-50, 1-10, 5-50, and 2-20 µg/mL, respectively with correlation coefficient >0.99. The high recovery values (98-102%) indicate satisfactory accuracy. The % RSD values were found to be less than 2% in the precision study which reveals that the method is precise. Hence the developed method can be used for quality control and quantitative analysis of extracts and commercial containing these selected phytoconstituents.

Keywords: ICH, Phytoconstituents, Polyherbal formulation, RP-HPLC, Validation.

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Introduction

Herbal medicines are believed to be effective with lesser side effects throughout the world. However, one of the restraints in the acceptance of these medicines is the lack of standard quality control profiles. The separation of each ingredient in any polyherbal formulation requires optimal separation technique for the quantitative determination of phytoconstituents which must be separated with proper resolution and least interference, making standardization and analysis of phytoconstituents a challenging task[1]. The advances in chromatographic separation techniques have made it possible to quantify the chemical constituents in a mixture with comparatively little cleanup.

A polyherbal formulation used for the treatment of bloating abdomen and constipation was selected for the present study. The selected formulation contains *Trachyspermum ammi* (Umbelliferae), *Piper nigrum* (Piperaceae), *Terminalia chebula* (Combretaceae), *Ferula foetida* (Umbelliferae), *Cuminum cyminum* (Umbelleferae), *Citrus medica* (Rutaceae), sodium bicarbonate, ammonium chloride and black salt. Five phytoconstituents namely thymol, piperine, gallic acid, ascorbic acid, and ferulic acid were selected for quantification. Literature survey reveals that various analytical methods are reported for the estimation of the selected markers alone or in combination with other markers[2-8]. There is no HPLC method reported for the simultaneous separation and quantification of these phytoconstituents and also for this formulation.

In the present research work and investigation, a novel, accurate, precise, and linear validated RP-HPLC method for the quantification and standardization of a polyherbal formulation using the above stated five markers has been developed. The method developed was validated as per the International Conference on Harmonization (ICH) guidelines[9]. This validated novel RP-HPLC method may find wide application for the quality control of herbal formulations containing these markers as ingredients.

Materials and Methods

Chemicals and Reagents

Ascorbic acid, gallic acid, ferulic acid, piperine and thymol from Sigma–Aldrich, St. Louis, USA were used. A marketed sample of a polyherbal formulation

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namely *Gashar Churna* was used for the study. HPLC grade methanol and acetonitrile from Qualigens Thermo Fisher Scientific, India and HPLC grade acetic acid from Sigma–Aldrich, St. Louis, USA was used.

HPLC Instrumentation

Chromatographic analysis was performed on Shimadzu Prominence-i (LC 2030) equipped with Lab solution software. The analytical column used for the separation of analytes was Shim-Pack C18 (250*4.6 mm, 5 µm).

Preparation of sample solution

Accurately 1 g of *Gashar Churna* was weighed and extracted with 50 mL of methanol using reflux assembly. The sample solution was filtered in a 50 mL volumetric flask through Whatman filter paper to obtain a clear solution[10]. The sample solution was injected for HPLC analysis after suitable dilutions.

Preparation of standard solution

Accurately 100 mg of ascorbic acid, gallic acid, ferulic acid, piperine, and thymol was weighed and transferred into 100 mL volumetric flasks separately. About 70 mL of methanol was added and sonicated to dissolve the respective analytes and the final volume was made up to the mark (1000 µg/mL). The final concentration of ascorbic acid, gallic acid, ferulic acid, piperine, and thymol was obtained by suitable dilutions.

Optimized method

Selection of Wavelength

A suitable wavelength at which all the analytes showed maximum absorbance was determined by overlapping the recorded UV spectrums of individual markers. UV overlaying of individual spectra of these five markers showed that they absorb appreciably at 272 nm. Hence 272 nm was selected as the detection wavelength for HPLC analysis of these markers.

Chromatographic conditions

An analytical method was developed using reverse-phase Shim-pack GIST C18 column (250*4.6 mm, 5 µm). The total run time for the analysis was 55 minutes. The mobile phase used was a combination of 1% acetic acid solution and acetonitrile in gradient mode. The column temperature was maintained at 28 °C. Gradient elution program was set for the elution of selected markers at a flow rate of 0.7 mL/min as shown in Table 1.

System suitability

The applicability of the optimized method was tested on the polyherbal formulation. Chromatograms of blank solution (Fig. 1), mixed standard solution (Fig. 2) and the chromatogram of the sample solution (Fig. 3) were taken. The peaks in the sample were identified by comparing the relative retention times

Table 1 — Gradient elution program

Time (min)	Mobile phase A (% v/v)	Mobile phase B (% v/v)
0	90	10
28	60	40
39	40	60
50	10	90

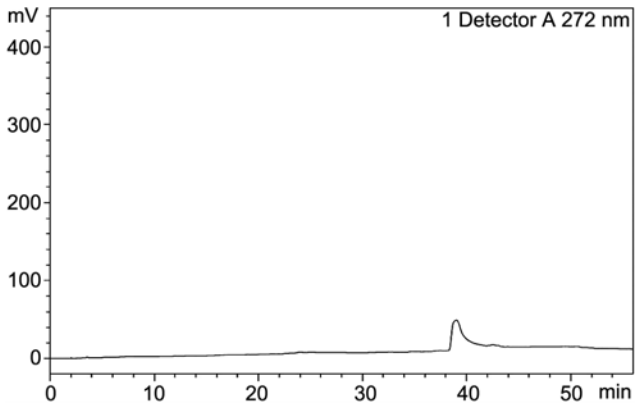

Fig. 1 — Chromatogram of blank solution.

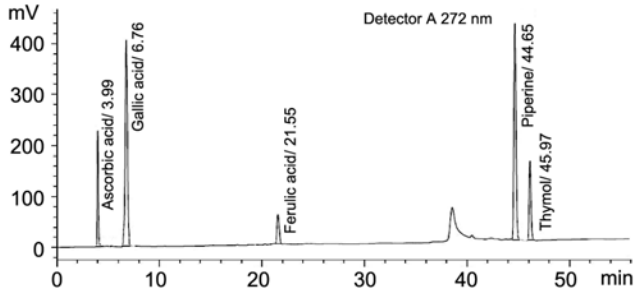

Fig. 2 — Chromatogram of the standard mixture.

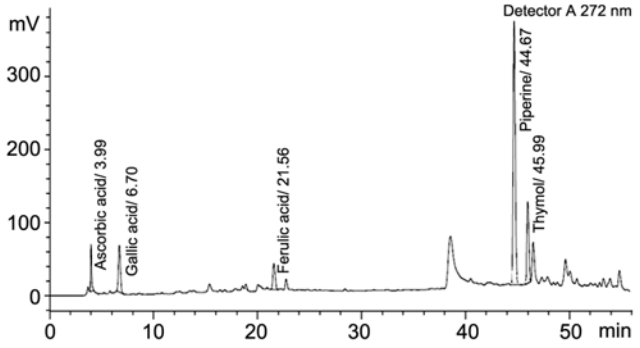

Fig. 3 — Chromatogram of the sample solution.

with the standard chromatogram. The sample was analyzed by injecting it in triplicate and the selected markers were quantified by using a linear regression equation.

Validation of the Method

The performance attributes of the analytical optimized method meet the requirements of the intended analytical application. These acceptance criteria were taken into account with respect to the ICH guidelines and the developed HPLC method was validated according to it. The various validation parameters include specificity, precision, linearity, accuracy, robustness, and sensitivity.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present.

Precision

The precision parameter was assessed as 1) System precision and 2) Method precision. System precision includes six replicate injections of the standard solutions individually or a mixture of the standard solution at working concentrations. Here the % RSD calculated with respect to the individual areas of peaks of each marker should be less than 2% as per ICH guidelines. Method Precision includes six replicate injections of the sample solution at working concentrations to check the consistency of the developed method.

Linearity

Calibration curves of the five different markers were constructed individually by plotting the concentration levels with respect to the corresponding peak areas of each marker as shown in Fig. 4-8.

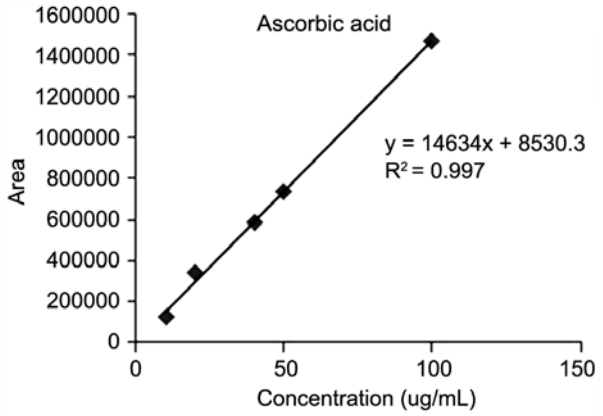

Fig. 4 — Calibration curve of ascorbic acid.

Accuracy

Accuracy was determined by means of recovery experiments in which the % mean recovery of each drug in the formulation at three different levels (50, 100, and 150%) was determined. The analysis was performed in triplicates.

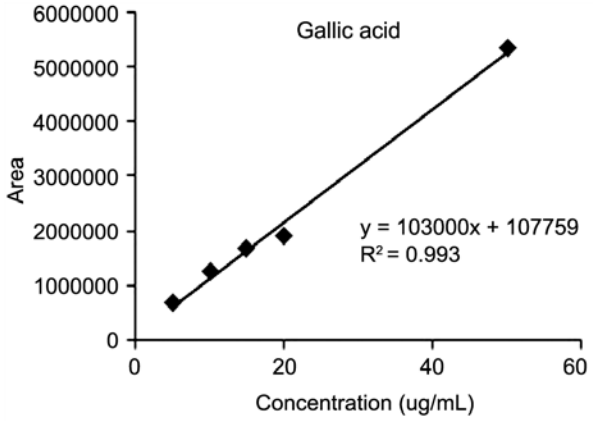

Fig. 5 — Calibration curve of gallic acid.

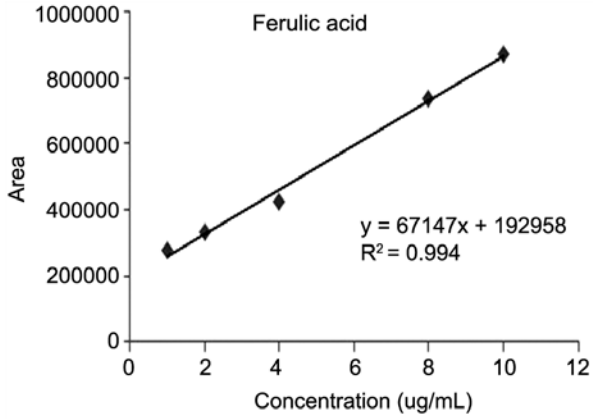

Fig. 6 — Calibration curve of ferulic acid.

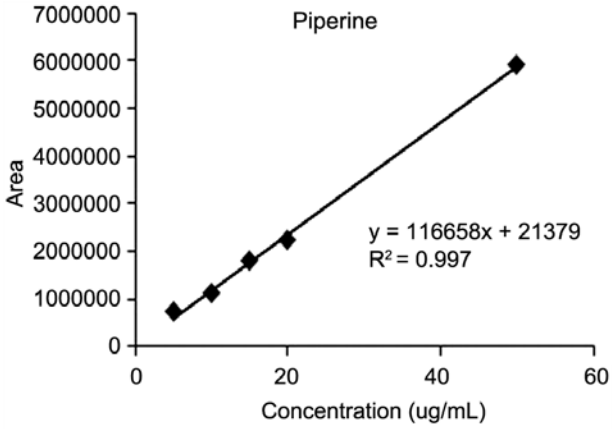

Fig. 7 — Calibration curve of piperine.

Robustness

Robustness corresponds to minute deliberate changes made in the method in which the conditions were altered slightly to check the system suitability factors. The solution to be injected was prepared as per the optimized method mentioned earlier, at different variable conditions such as column temperature (±2 °C), detection wavelength (±1 nm) and flow rate (±0.2 mL/min).

Sensitivity

The sensitivity of ascorbic acid, gallic acid, ferulic acid, piperine, and thymol was estimated in terms of limit of quantitation (LOQ) and limit of detection (LOD). These two parameters were calculated using the equations LOD= 3.3σ/S and LOQ= 10 σ /S where σ is the standard deviation of the intercepts of the calibration plots and S is the average of the individual slopes of the corresponding calibration plot.

Results and Discussion

System suitability

Three parameters were evaluated in system suitability, namelyretention time (min), number of theoretical plates, and tailing factor. The retention time of ascorbic acid, gallic acid, ferulic acid, piperine, and thymol was found to be 3.9,6.7,21.5,44.6, and 45.9 min respectively. Similarly, the number of theoretical plates of these constituents are found to be more than 2000 which is within the acceptable limit at per the ICH guidelines. The tailing factor should not be more than 2 and the results are found to be 1.31 for ascorbic acid, 1.16 for gallic acid, 1.20 for ferulic acid, 1.15 for piperine, and 1.00 for thymol. The results obtained are within the acceptable limits which indicates that the method is suitable for the intended purpose.

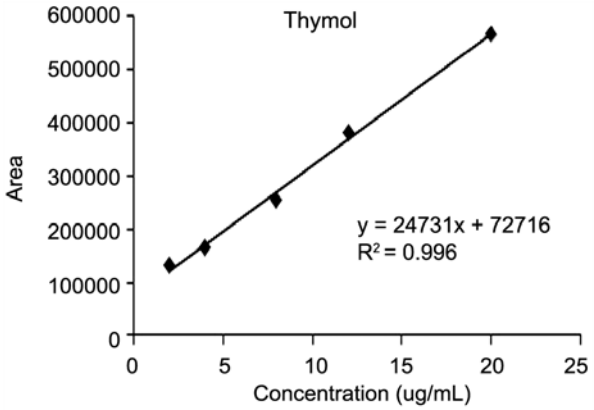

Fig. 8 — Calibration curve of thymol.

After the analysis of the polyherbal formulation extract, the content for ascorbic acid, gallic acid, ferulic acid, piperine, and thymolper 100 g of the formulation was found to be 0.25, 0.03, 0.04, 0.25, and 0.28 respectively.The analytical data of the polyherbal formulation is tabulated in Table 2. The results indicates that the developed method can be successfully applied for the simultaneous quantification of the selected markers.

Method validation

Specificity

Fig. 1, 2 and 3 show the blank, standard mixture and sample chromatograms which reveal that the blank chromatogram does not include the five standard peaks or any of the peaks as seen in the sample. Hence it can be concluded that the developed method is specific for each of the analyte[11,12].

Precision

System precision:% RSD of the six injections of the standard mixture was found to be 0.97, 0.73, 0.78, 1.06, and 0.32 for ascorbic acid, gallic acid, ferulic acid, piperine, and thymol, respectively which is within the acceptable limits.

Method precision: Here the % RSD of the working concentrations of the six replicates were found to be 1.00, 1.07, 0.63, 0.43 and 0.58 for ascorbic acid, gallic acid, ferulic acid, piperine, and thymol, respectively which is within the acceptable limits.

Table 2 — Polyherbal formulation analysis data

Name	Latin name	Content in 100 g of *Gashar churna*	Selected markers for HPLC analysis	Analyzed content of markers in 100 g of *Gashar churna*	Corresponding % of markers in *Gashar churna* content (w/w)
Ajwain	*Trachyspermum ammi*	30 g	Thymol	0.28 g	0.93%
Harar choti	*Terminalia chebula*	20 g	Gallic acid	0.03 g	0.15%
Kali mirch Black pepper	*Piper nigrum*	10 g	Piperine	0.25 g	2.50%
Neembu sat	*Citrus medica*	5 g	Ascorbic acid	0.25 g	5.00%
Hing	*Ferula foetida*	2 g	Ferulic acid	0.04 g	2.00%

This concludes that the developed method is precise by repeatability and hence can give consistent reproducible results.

Linearity

The correlation coefficients were found to be 0.997, 0.993, 0.994, 0.997, and 0.996 for ascorbic acid, gallic acid, ferulic acid, piperine, and thymol, respectively. This concludes that the developed method is linear for each analyte.

Accuracy

The acceptance limits of mean recovery are 98 to 102%. The results of accuracy and % recovery for 50% level were found to be 98.55, 100.57, 98.16, 99.38, and 100.40 for ascorbic acid, gallic acid, ferulic acid, piperine and thymol respectively. For 100% level, the results obtained were 99.11, 101.28, 99.37, 99.54 and 100.22 for ascorbic acid, gallic acid, ferulic acid, piperine and thymol respectively. For 150% level, the results obtained were 98.11, 100.80, 99.40, 99.72, and 101.50 for ascorbic acid, gallic acid, ferulic acid, piperine, and thymol, respectively. It was found that all the observed accuracy data were within the acceptable range affirming the accuracy of the method.

Robustness

The results of robustness studies are expressed in terms of % RSD of area for ascorbic acid, gallic acid, ferulic acid, piperine and thymol respectively which is as follows: Low wavelength (271 nm)- 0.71, 0.72, 0.53, 0.10, and 0.47 ; High wavelength (273 nm)- 0.65, 1.59, 0.58, 0.25, and 0.50 ; Low flow rate (0.6 mL/min)- 0.34, 0.38, 0.48, 0.43, and 0.56 ; High flow rate (0.8 mL/min)- 0.76, 0.80, 0.57, 0.19, and 0.87; Low temperature (26 °C)- 0.41, 0.73, 1.05, 0.24, and 0.67; and High temperature (30 °C)- 0.85, 1.85, 0.94, 0.14, and 1.55. The results indicate that the applied method is robust at small but deliberate change.

Sensitivity

The experimentally determined values of LOD were found to be 1.60, 0.45, 0.30, 0.68, and 0.62 and the LOQ values were found to be 4.86, 1.36, 0.93, 2.06, and 1.89 for ascorbic acid, gallic acid, ferulic acid, piperine, and thymol respectively which indicate that the method is sensitive for the analysis of the polyherbal formulation.

Conclusion

A novel RP HPLC method was developed for the simultaneous estimation of five phytoconstituents namely ascorbic acid, gallic acid, ferulic acid, piperine, and thymol in a polyherbal formulation. The developed method for estimation of selected phytoconstituents is found to be novel, accurate, precise, and linear. The results in this study indicate that the present HPLC method will serve as a promising means for simultaneous estimation of selected phytoconstituents in any polyherbal formulation containing the respective crude drugs. With the growing demand for herbal drugs, the development of HPLC method as a standardization tool will help in maintaining the quality of such polyherbal preparations.

Conflict of interest

Authors declare no conflict of interest.

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