A Validated HPLC Method for Estimation of Cordifolioside A in *Tinospora cordifolia,* Miers and Marketed Formulations

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Abstract

A simple, economic, robust, reproducible, selective, and precise high-performance liquid chromatography (HPLC) method for estimation of cordifolioside A in both 60% methanolic extract of *Tinospora cordifolia* and marketed formulation is developed and validated in the present study. The mobile phase composed of acetonitrile–water (25:75%, v/v) give a sharp and well-defined peak of cordifolioside A at the retention time of 9.52 ± 1.03 min. HPLC estimation of cordifolioside A is carried out at wavelength of 210 nm with flow rate of 1.0 mL/min. The linear regression analysis data for the calibration curve shows good linear relationship with correlation coefficient of 0.997 in the concentration range of 0.5–50 µg/mL. The linear regression equation is $y = 29716x - 4417.4$. The limit of detection and limit of quantification are 0.18 and 0.55 µg/mL, respectively. The developed method is validated for accuracy, precision, reproducibility, and robustness as per ICH guidelines. The proposed method with high degree of precision and accuracy is employed for the estimation of cordifolioside A in methanolic extract and in formulation. Statistical analysis proved that the method is precise, reproducible, selective, and accurate for the estimation of cordifolioside A.

Introduction

Guduchi (*Tinospora cordifolia*) is a large, glabrous, deciduous climbing shrub belonging to the family menispermaceae and is distributed throughout the tropical Indian subcontinent and China (1). The herb is of ancient medicinal repute. In traditional system of medicine, it has been used in treatment of jaundice, rheumatism, urinary disorder, skin diseases, diabetes, anemia, inflammation, and allergic condition (1). The pharmacological activity of guduchi is related to several classes of secondary metabolites like alkaloids (2), glycosides (3), diterpenoid lactones (4), steroids (5), sesquiterpenoids (6), and aliphatic compounds (7). The plant has been reported to have anticomplementary and immunomodulatory activities (8). Different constituents of *T. cordifolia* like in cordioside, cordiofolioside A, and cordial are reported to associated with macrophage activation (7). Cordifolioside A is an important active principle of plant, which has been used for quality control and standardization plant and its formulation as a marker compound.

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A thorough survey of the literature has revealed that no analytical methods have been reported for the estimation of cordifolioside A in the plant, its extract, and in formulations. Therefore, the aim of present study was to develop a simple, economical, selective, precise, reproducible, and robust high-performance liquid chromatographic (HPLC) method for the estimation of cordifolioside A in the natural plant, its extracts, and in marketed tablet formulation using UV detection. The proposed method was validated using International Conference on Harmonization (ICH) guidelines (9).

Experimental

Plant material and chemicals

T. cordifolia stem was collected from three different geographical regions Viz- Bandra (UP), Karera (MP) in March whereas samples from an herbal garden of Jamia Hamdard, New Delhi (ND) were collected in November. These plants were assigned batches like TC/GS/UP, TC/GS/MP, and TC/PA/ND, respectively, in samples obtained from UP, MP, and New Delhi (India). The identity of these batches was confirmed by taxonomist Dr. Gyanesh Shukla (Herbal Drug Research, Ranbaxy Research Laboratories, Gurgaon, India). All the solvents were of chromatographic-grade, and other chemicals used were of analytical reagent-grade obtained from E-Merck (Mumbai, India).

Marketed formulations

Two traditional ayurvedic marketed formulations E and F containing *T. cordifolia* extract was purchased from local market and used for quantification of cordifolioside A.

Preparation of sample solution

About 500 mg each of dried 60% methanolic extracts were weighed and transferred to refluxing flask (501.4, 500.0, 500.3 mg containing UP, MP, and ND samples, respectively), refluxed in 100 mL methanol for 1 h, and filtered through Whatmann filter paper (No. 41). The marc left was again refluxed with methanol 50 mL for 1 h and filtered. Filtrates were combined and concentrated using a rotary vacuum evaporator (Medica Instrument Mfg. Co., Mumbai, India) to a final volume of 25 mL and used as test solution in the HPLC analysis.

Sample preparation for quantification of cordifolioside A in marketed formulation

50 mL syrup was transferred to a separating funnel, and 50 mL of distilled water was added and mixed well. It was then extracted thrice with 70 mL each of extraction media (chloroform–methanol–butanol, 25:2:3, v/v/v). The organic layer was passed over anhydrous sodium sulphate and evaporated to dryness on water bath under vacuum. The residue left was reconstituted in 10 mL of methanol, which was used as test solution in HPLC analysis.

Preparation of standard solution

The cordifolioside A (purity 59.88%), 9.1 mg, was weighed and dissolved in 10 mL; further 1 mL of this solution was diluted to 10 mL, which gives 54.49 µg/mL equivalent of standard cordifolioside A. The 5 mL of previously mentioned solution was diluted to 5.449 mL with fresh solvent using micropipette to prepare standard stock solution of cordifolioside A 50 µg/mL equivalent, which was further diluted to get different concentrations for calibration curve.

Equipment and Conditions

A Shimadzu model HPLC (Kyoto, Japan) equipped with quaternary LC-10A VP pumps, variable wavelength programmable UV/VIS detector SPD-10AVP column oven (Shimadzu), SCL 10AVP system controller (Shimadzu), Rheodyne injector fitted with a 20-µL loop, and Class-VP 5.032 software was used.

The chromatographic column used was a reverse phase C_{18} Zorax RP-HPLC (250 × 4.6 mm, 5 µm). The column and HPLC system were kept at ambient conditions. The mobile phase was acetonitrile–water (25:75, v/v) with a flow rate of 1.0 mL/min. The injection volume was 20 µL, and the elute was analyzed at a wavelength of 210 nm.

Method development

Various solvent systems were tried for the development of a suitable HPLC method for estimation of cordifolioside A in 60% methanolic extract and marketed formulations. Mobile phases tried for these purposes were methanol–water (25:75%), acetonitrile–water (25:75%), methanol–water (50:50%), acetonitrile–water (50:50%), and acetonitrile–water (75:25%). The suitability of the solvent system was decided by cost, sensitivity of the assay, and time required for the analysis.

Calibration curve of cordifolioside A

Different concentrations (0.5–50 µg/mL) were made for the preparation of calibration curve from the prepared stock solution. The mobile phase after filtration through 0.45-µm membrane filter was delivered at 1.0 mL/min for column standardization, and baseline was continuously monitored during the process. The wavelength of detection was selected at 210 nm. The prepared dilutions were injected serially, and areas under the peaks were recorded for each dilution. The stability of drug in solution during analysis was determined by repeated analysis of samples during the course of experimentation on the same day and also after 48 h storage of drug solution at laboratory conditions and in the refrigerator.

Method validation

Linearity

The linearity of cordifolioside A was checked between 0.5–50 µg/m: concentration range. Graph was plotted between concentration and area under peak for linearity.

Accuracy as recovery

Accuracy was determined by standard addition method. The preanalyzed samples of cordifolioside A (10 µg/mL) were spiked with the extra 0, 50, 100, and 150% of the standard cordifolioside A, and the mixtures were reanalyzed by the proposed method. The percent (%) recovery of samples, percentage relative standard deviation (% RSD), and standard error were calculated at each concentration level.

Precision

Precision was determined for repeatability and intermediate precision. Repeatability of sample application was determined as intra-day variation whereas intermediate precision was determined by carrying out inter-day variation for the determination of cordifolioside A at four different concentration levels of 5, 10, 20, and 40 µg/mL in triplicates.

Reproducibility

Reproducibility of the method was checked by obtaining precision on a different instrument, which was analyzed by another person in a different laboratory. Both intra-day and inter-day precision was calculated at four different concentration levels 5, 10, 20, and 40 µg/mL in triplicates.

Limit of detection and limit of quantification

Limit of detection (LOD) and limit of quantitation (LOQ) were determined by standard deviation ($S_{y/x}$) method. For the determination of LOD and LOQ, blank sample was injected in triplicate to the chromatograph, and then peak area of this blank was recorded. The LOD and LOQ were determined using the slope of the calibration curve and $S_{y/x}$ of the blank sample by following formulae: $LOD = 3.3 \times S_{y/x}/S$ and $LOQ = 10 \times S_{y/x}/S$; where $S_{y/x}$ is the standard deviation of the blank response and S is the slope of the calibration curve.

Robustness

Robustness was carried out to evaluate the influence of small but deliberate variation in the chromatographic conditions for the determination of cordifolioside A. Robustness of the method was determined by changing the flow rate (1.1 and 0.9 mL/min) of mobile phase and concentration of acetonitrile in mobile phase (23 and 27%).

Quantification of cordifolioside A in methanolic extract and formulations

The test samples were injected and chromatograms were obtained in the same conditions as that of standard cordifolioside A. The peak area of the peak corresponding to the Rt of standard cordifolioside A was recorded, and content of the same was calculated from the regression equation obtained from calibration curve.

Results and Discussion

Method development

The mobile phase composition was optimized to develop a suitable and accurate HPLC method for quantification of cordifolioside A. Mobile phases tried for this purpose were methanol–water (25:75%), acetonitrile–water (25:75%), methanol–water (50:50%), acetonitrile–water (50:50 %), and acetonitrile–water (75:25%). The chromatogram (Figure 1) obtained with acetonitrile–water (25:75%) solvent system was found to have very good symmetry (1.15) with the lowest R_t (9.53 min) and sharp, well-defined peak. Therefore the mixture of acetonitrile–water (25:75%) was optimized as mobile phase. The drug was stable for a period of 48 h storage at laboratory temperature and under refrigerator temperature in acetonitrile–water (25:75%) mixture.

Calibration Curve

The calibration curve area versus concentration (µg/mL) was found linear in the range of 0.5–50 µg/mL. Statistical calculations were done at 5% level of significance. One-way analysis of variance (ANOVA) test was performed to compare the results. The linear regression data for the calibration curve showed a good linear relationship over the concentration ranges of 0.5–50 µg/mL with respect to peak area as shown in Table I. The correlation coefficient value (R^2) was found to be 0.997, which was highly significant (Table I) ($p < 0.05$). The linear regression equation was $y = 29716x - 4417.40$. No significant differences were observed in the slope of standard curves ($p \geq 0.05$). The retention time and asymmetry factor were found to be 9.525 ± 1.11 min and 1.19 ± 0.12 min, respectively.

Validation of the method

Linearity

The linearity range of cordifolioside A solutions were obtained as 0.5–50 µg/mL. The regression equation was $y = 29716x - 4417.4$ with correlation coefficient of 0.997 (Table I).

Accuracy as recovery

The accuracy of proposed method was calculated by recovery analysis, which afforded recovery of 99.21–99.82% after spiking the additional standard drug solution to the previously analyzed test solution. The % RSD values were found in the range of 1.42–1.97. These results indicated the accuracy of the proposed method.

Precision

Precision was considered at two levels of ICH suggestions (i.e., repeatability and intermediate precision). Repeatability of sample application was determined as intra-day variation whereas intermediate precision was determined by carrying out inter-day variation for the determination of cordifolioside A at four different concentration levels of 5, 10, 20, and 40 µg/mL in triplicates. Results of repeatability and intermediate precision were expressed in terms of % RSD and are shown in Table II. The low values of % RSD indicated the repeatability of the proposed method.

Reproducibility

Reproducibility of the method was checked by obtaining precision of the method in another laboratory using different instruments and analyzed by another person but in the same condition. Both intra-day and inter-day precision was examined in labs. There were no significant differences observed in the % RSD values of intra-day and inter-day precision, which indicates the reproducibility of the method (Table III).

LOD and LOQ

LOD and LOQ of the proposed method were determined by the standard deviation method as described in the Experimental section and were found to be 0.18 and 0.55 µg/mL, respectively, which indicated that the proposed method can be effectively used in a wide range for detection and quantification of cordifolioside A. The low values of LOD and LOQ indicate the sensitivity of the proposed method.

Robustness of the method

There was no significant change in the retention time of cordifolioside A by changing the composition of mobile phase and

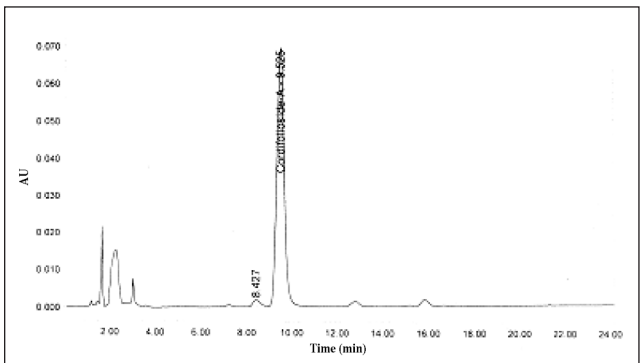

Figure 1. HPLC chromatogram of cordifolioside A in acetonitrile and water (25:75% v/v) showing Rt at 9.52 min.

Table I. Linear Regression Data for the Calibration Curve (n = 3)

Linearity range (µg/mL)	0.5–50
Regression equation	y = 29716x – 4417.40
Correlation coefficient	0.997
Slope ± SD	29716 ± 92.41
Intercept ± SD	4417.40 ± 31.85
Slope without intercept ± SD	29590 ± 90.74
Standard error of slope	53.38
Standard error of intercept	18.38
95% confidence interval of slope	29486.30 – 29945.69
95% confidence interval of intercept	4338.31 – 4496.48
Bias of intercept	–0.0261

Table II. Precision of the Proposed Method

Conc.	Repeatability (Intra-day precision)			Intermediate precision (Inter-day)		
(µg/mL)	Mean area ± SD (n = 3)	SE*	% RSD	Mean area ± SD (n = 3)	SE	% RSD
5	152972.54 ± 467.25	269.77	0.30	152405.38 ± 489.45	282.59	0.32
10	285856.98 ± 2987.51	1724.89	1.04	285104.62 ± 3025.35	1746.73	1.06
20	604272.21 ± 7541.32	4354.11	1.24	602254.11 ± 7845.54	4529.75	1.30
40	1121858.25 ± 2508.65	1448.41	0.22	1121085.14 ± 2921.47	1686.76	0.26

* SE = Standard Error.

Table III. Reproducibility of the Proposed Method

Conc. (µg/mL)	Repeatability (Intra-day precision)			Intermediate precision (Inter-day)		
	Mean area ± SD (n = 3)	SE	% RSD	Mean area ± SD (n = 3)	SE	% RSD
5	145248.44 ± 875.41	505.43	0.60	141254.23 ± 984.42	568.37	0.69
10	265432.11 ± 5214.49	3010.67	1.96	262154.89 ± 5124.33	2958.62	1.95
20	601451.33 ± 8441.22	4873.68	1.40	598954.41 ± 9145.77	5280.46	1.52
40	1116589.55 ± 4907.77	2833.58	0.43	1112154.12 ± 5398.45	3116.88	0.48

Table IV. Robustness of the Method by Changing the Mobile Phase

Conc. (µg/mL)	Mobile phase composition acetonitrile–water			Mean R_t ± SD (min)	% RSD
	Original	Used	Level		
10	25:75	23:77	–2	9.465 ± 1.13	1.74
		25:75	0	9.525 ± 1.11	1.25
		27:73	+2	9.623 ± 1.14	1.82

Table V. Robustness of the Method by Changing Flow Rate

Conc. (µg/mL)	Flow rate (mL/min)			Mean R_t ± SD (min)	% RSD
	Original	Used	Level		
10	1.0	0.9	– 0.10	9.645 ± 1.15	1.66
		1.0	0	9.525 ± 1.11	1.23
		1.1	+ 0.10	9.423 ± 1.16	1.75

flow rate. Low value of the % RSD indicated the robustness of the method as shown in Table IV and V.

Quantification of cordifolioside A in extracts of T. cordifolia

The peaks of cordifolioside A from sample solution were identified by comparing their retention times obtained from the peaks with those of standard. HPLC profile of the 60% methanolic extract of the *T. cordifolia* was developed through the same conditions as estimation of standard cordifolioside A (Rt 9.525 min). It was observed that extract of *T. cordifolia* collected from UP zone showed good content of cordifolioside A followed by samples from MP and ND regions. The cordifolioside A was quantified T. cordifolia extract using the regression equation, and values were found to be 0.70%, 0.20%, and 0.10% w/w in UP, MP, and ND samples, respectively.

Quantification of cordifolioside A in marketed formulations

A single HPLC peak was observed at the same retention time in the samples of formulations. There was no interaction between the cordifolioside A and other excipients present in the marketed formulations. The cordifolioside A content was found to be 0.20% w/w and 0.11% w/w in formulation E and F, respectively. The values of % RSD were also low, which indicated the suitability of this method for the routine analysis of cordifolioside A in marketed formulations.

Applications

The method was used to determine the presence cordifolioside A in *T. cordifolia* from three different parts of the India. The cordifolioside A was present in all the three extract and in marketed formulations, but there was considerable variation in the amounts present. It may be because of genetic variability, the source of plant, as well as external factors like seasonal and environmental variations, drying processes, and storage conditions.

Conclusions

The proposed validated HPLC method is suitable for the quantification of cordifolioside A in both methanolic extracts and marketed formulations. Therefore, this method can be successfully used for the routine analysis of cordifolioside A in both crude drugs and prepared formulations without any interference, which can be explored for standardization and quality control of raw materials and marketed herbal products of traditional system of medicine containing *T. cordifolia* as an ingredient.

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