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Original Article

A Unique Method for Simultaneous HPLC Analysis of Gallic Acid, Glycyrrhizic Acid and E-Guggulsterone in a Polyherbal Formulation

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ABSTRACT

Background: The therapeutic effects of herbal medicines are exerted due to the presence of different phytoconstituents and the effects are further potentiated when compatible herbals are formulated together. High Performance Liquid Chromatography (HPLC) is an important analytical tool towards standardization of polyherbal formulation. Complex nature, co- existence with other constituents trace quantities of active phytoconstituents and their simultaneous estimation are challenges encountered in HPLC analysis of polyherbal formulation. Simultaneous estimation of active biomarkers in polyherbal formulation by HPLC provides a unique method for verifying the quality and efficacy of polyherbal formulation. **Materials and Methods:** Present research work is about inventing an unique, selective and sensitive HPLC (High Performance Liquid Chromatography) method for simultaneous estimation of Gallic acid, Glycyrrhizic acid and E-Guggulsterone from a polyherbal formulation containing thirteen Ethnobotanical herbs, reported to have Cardioprotective effects, marketed as Collasyn tablets by Dr. Palep Research Labs Ltd. in Indian market. Reverse-phase column (C18, 250 mM X4.6 mM i.d., 5µm) was used to analyze the marker compounds Gallic acid, Glycyrrhizic acid and E-Guggulsterone at λ_{max} 224 nm. The mobile phase was composed of acetonitrile and 0.05% OPA (Ortho-phosphoric acid) in water (50:50) with a flow rate of 1 mL/min. **Results:** A unique HPLC method is developed for simultaneous estimation of active markers-Gallic acid, Glycyrrhizic acid and E-Guggulsterone from the polyherbal formulation. Quantitative analysis shows Gallic acid, Glycyrrhizic acid and E-Guggulsterone to be 11.47%, 1.05% and 0.63% respectively in the polyherbal formulation. The developed method is validated in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals guidelines. The method shows good linearity in a relatively wide range of Concentration of 50-150 mcg/mL. The method is also found to be precise, accurate and robust. **Conclusion:** This unique and validated method for simultaneous estimation of cardioprotective biomarkers Gallic acid, Glycyrrhizic acid and E-Guggulsterone from polyherbal formulation, marketed as Collasyn tablets by Dr. Palep Research Labs Ltd. is important for its quality control. It helps in harnessing the complete plant potential for therapeutics. Thus, this approach to standardizing polyherbal formulations will be instrumental in verifying the quality and efficacy of the polyherbal extract. The simultaneous estimation of constituents from plants presents a significant advancement in phytochemical analysis, offering a comprehensive understanding of plant properties and their potential applications. The methodologies employed demonstrate high efficiency and accuracy, allowing for the effective quantification of multiple compounds in a single analytical run. This approach not only streamlines the process of phytochemical profiling but also enhances the ability to explore the therapeutic potentials of various plant species.

Keywords: Cardioprotective, HPLC, Method development, Method validation, Polyherbal formulation.

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INTRODUCTION

The use of Polyherbal formulations in management of various diseases is on rise. Standardization of polyherbal formulations is an essential step in their quality control. High Performance Liquid Chromatography is an important analytical tool towards standardization of polyherbal formulation. However, the same becomes complex as it can involve simultaneous estimation of multiple active biomarkers. The present study aims in developing and validating a unique method for simultaneous estimation of active biomarkers-Gallic acid, Glycyrrhizic acid and E-Guggulsterone from a polyherbal formulation containing thirteen Ethno herbs for cardiovascular disease management, marketed as Collasyn tablets by Dr. Palep Research Labs Ltd.

Literature reveals the cardioprotective role of Gallic acid, Glycyrrhizic acid and E-Guggulsterone in various studies.[1-7] Their presence in the polyherbal formulation used in the current

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study was confirmed by Thin Layer Chromatography and UV spectroscopy. They were chosen as active biomarkers for simultaneous estimation by HPLC. In published literature, there is no reported method for qualitative and quantitative determination of Gallic acid, Glycyrrhizic acid and E-Guggulsterone, simultaneously by HPLC. Therefore, the current study was undertaken to develop a method for simultaneous estimation of Gallic acid, Glycyrrhizic acid and E-Guggulsterone by HPLC in the polyherbal Formulation.

MATERIALS AND METHODS

Selection of analytical wavelength

It is important to find out Q-absorption point of the standards, so that the analytical wavelength for their simultaneous determination by HPLC can be selected.

Preparation of standard stock solutions

Standard 10 mg gallic acid was weighed accurately, transferred into 10 mL volumetric flask and diluted up to the mark with water to obtain 1000 PPM Gallic acid in water. Similarly, 1000 PPM Glycyrrhizic acid and E-Guggulsterone was prepared. 0.4 mL of each solution was further diluted to 20 mL with water to get 20 PPM Concentration for each standard solution. These standard solutions were scanned between 200 nm to 400 nm. Q-absorption point was determined for drugs. It is shown in Figure 1 that 224 nm was found as Q-absorption point. Hence 224 nm wavelength was selected as wavelength for simultaneous determination by HPLC because all drugs showed significant absorption at 224 nm.

Preparation of solution to inject on HPLC for method development

1 mL of 1000 PPM of each standard solution was further diluted to 10 mL with mobile phase of respective trial to get 100 PPM of each standard. This 100 PPM solution of each standard was used to inject HPLC for method development. The chromatographic conditions for HPLC method development were as mentioned in Table 1.

Preparation of standard mixture

Each 1 mL of standard stock solution was pipetted out and transferred to 10 mL volumetric flask and volume was made up to the mark with mobile phase to achieve 100 mcg/mL working concentration of Gallic acid, Glycyrrhizic acid as well as E-Guggulsterone in the standard mixture. As per pharmacopoeial requirement, system suitability was performed to verify the adequateness of the chromatographic system. The tests were performed by collecting data from five replicates injections of standard drug solution and the results were recorded.

Preparation of sample

475 mg of Polyherbal formulation was prepared by using dried aqueous extracts of *Phyllanthus emblica* fruit, *Terminalia arjuna* stem bark, *Glycyrrhiza glabra* root, *Hemidesmus indicus* root, *Terminalia chebula* fruit, *Tinospora cordifolia* root, Shilajit, *Commiphora mukul resin, Sida cordifolia* root, *Trapa bispinosa* fruit, *Withania somnifera* root, *Terminalia bellerica* fruit, *Tribulus terrestrius* fruit. The composition of the polyherbal formulation is same as Collasyn tablets, a cardioprotective marketed by Dr. Palep Research Labs Ltd., in Indian market. Weighed about 100 mg of Polyherbal extract and transferred to clean and dried 100 mL volumetric flask. Added 70 mL of water, sonicated for 30 min with intermittent shaking. After 30 min, allowed the solution to cool at room temperature and made the volume up to the mark with water. Filtered the solution through suitable 0.45 µ syringe filter discarding 3-5 mL of initial filtrate. Further diluted 1 mL of filtered stock solution to 10 mL with mobile phase, injected the resultant solution and chromatograms were recorded.

Method validation

Stability

Stability study was conducted at normal laboratory condition for standard and polyherbal extract (that is test solution). Post initial analysis, both the solutions were analysed after 12 hr and after 24 hr. Standard and Test solution stability study was performed by calculating the difference between results of standard and test solution at each stability time point respectively to that of initial.

Specificity

Blank (plain mobile phase) is injected to prove specificity of the method.

Linearity and Range: Preparation of linearity solution

1000 ppm stock solution of Gallic acid, Glycyrrhizic acid and E-Guggulsterone were prepared. Five levels of Linearity were performed from 50% to 150% of working concentration. Details of levels used for linearity are mentioned in Table 2.

Each level solution was injected in triplicate and mean area of chromatograph peak was calculated. Calibration curve was plotted graphically as a function of analyte concentration in µg/mL on X-axis vs mean area on Y-axis.

Precision (Repeatability)

This was done by repeating analysis six times by the same analyst. Table 3 provides Composition of samples used for Precision (Repeatability) studies.

Intermediate precision

This was done by doing analysis on another day by another analyst to check reproducibility of results. Samples were prepared in same manner as that of Repeatability precision.

Accuracy

Conducted in the range from 50% to 150% of working Concentration. Solution of each accuracy level was prepared in triplicate. Calculated % Recovery for each sample, Mean % recovery for each level and overall recovery. Also calculated % RSD for each level and % RSD for overall recovery.

Robustness

Standard solution was injected under different chromatographic conditions by bringing, a) Change in wavelength (±3 nm), b) Changes in flow rate by ±10%. (±0.1 mL/min) and, c) Change in column oven temperature. (±2°C). Impact of these changes on chromatogram were studied and recorded.

RESULTS

Method development

Chromatographic method development was evaluated as per ICH guidelines, whereby an acceptance criterion for good chromatography is optimum Retention Time, Asymmetry (Tailing factor): 0.8 to 2.0, Theoretical plates: Not Less Than 2000, Resolution between peaks: Not Less Than 2.0

During chromatographic method development, Gallic acid eluted at 3.36 min with good chromatography as Asymmetry was 1.25 and Theoretical plates were found to be 9150. Figure 2 denotes chromatograph of method development for Gallic acid.

For Glycyrrhizic acid retention time was observed to be 4.90 min. Asymmetry was found to be 1.20 and Theoretical plates were 14118. Hence, Glycyrrhizic acid eluted with good chromatography. Figure 3 depicts chromatograph of method development for Glycyrrhizic acid.

E-Guggulsterone eluted at 8.72 min with good chromatography as Asymmetry was 1.05 and Theoretical plates were 17985. Figure 4 reveals chromatograph of method development for E-Guggulsterone.

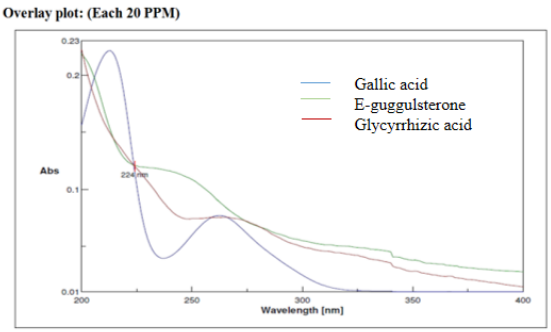
In chromatograph of sample mixture, as observed in below Figure, all peaks eluted with acceptable chromatography. The resolution obtained between Gallic acid and Glycyrrhizic acid was 10.15 Rs and that obtained between Glycyrrhizic acid and E-guggulsterone was 17.61 Rs. Figure 5 depicts chromatogram of mixture of Gallic acid, Glycyrrhizic acid and E-Guggulsterone.

As chromatographic conditions in this trial gave better peak, good retention time and tailing factor, chromatographic conditions in this trial were used for method validation.

System suitability: For the system suitability test, the results are as mentioned in below Table 4.

As per ICH guidelines, for system suitability test acceptance criteria are-RSD (Relative Standard Deviation) should not be more than 2.0% for five replicate injections of standard;2. USP Tailing Factor/Asymmetry Factor not more than 2.0; The column

Determination of Q absorption point for simultaneous estimation of markers by HPLC



Conclusion : overlay of UV spectrum of Gallic acid, E-guggulsterone and Glycyrrhizic acid showed Q absorpotion point to be 224 nm. Hence, this wavelength was chosen for simultaneous estimation

Figure 1: Overlay of UV spectrum of Gallic acid, Glycyrrhizic acid, E-Guggulsterone.

Table 1: Chromatographic conditions for HPLC method development.

Mode	Isocratic
Column	PhenomenexC_{18}
Column Dimension	250 mMX4.6 mM i.d., 5 µm.
Column oven temp	40°C
Detector	U.V. Detector
Wavelength	224 nm
Flow Rate	1.0 mL/min
Mobile phase	Acetonitrile: 0.05% OPA (Ortho-phosphoric acid) in water (50:50).
Injection Volume	20 microliters
Diluent	Stock solution in water Final dilution in Mobile phase.
Run time	17 Min

efficiency as determined for Plate Count should be more than 2000.

It was observed from the data as mentioned in Table 4; the method complies with system suitability parameters. Hence, it can be concluded that the chromatographic method is adequate for intended analysis.

Stability

The results for stability test for Gallic acid, Glycyrrhizic acid and E-Guggulsterone are mentioned in Tables 5-7 respectively.

Acceptance criteria for stability are % Absolute difference of Stability solution should be Not More Than 2.0 with respect to Initial solution. Standard and Polyherbal formulations were found to be stable up to 24 hrs. Hence both solutions can be used up to 24 hrs.

Table 2: Details of levels used for linearity.

Level	Gallic acid stock (mL)	Glycyrrhizic acid stock (mL)	E-Guggul sterone stock (mL)	Diluted to (mL)	Gallic acid Conc (mcg/mL)	Glycyrrhizic acid Conc (mcg/mL)	Guggulsterone Conc (mcg/mL)
50%	1.0	1.0	1.0	20	50.0	50.0	50.0
75%	1.5	1.5	1.5	20	75.0	75.0	75.0
100%	2.0	2.0	2.0	20	100.0	100.0	100.0
125%	2.5	2.5	2.5	20	125.0	125.0	125.0
150%	3.0	3.0	3.0	20	150.0	150.0	150.0

Table 3: Composition of repeatability samples.

Sample	Mg of Gallic acid	mg of Glycyrrhizic acid	mg of E-Guggulsterone	Diluted to (mL)	Volume taken	Diluted to (mL)
Sample 1	10.2	10.1	10.1	10	1.0	10.0
Sample 2	10.1	9.9	10.2	10	1.0	10.0
Sample 3	10.4	10.3	10.3	10	1.0	10.0
Sample 4	9.8	10.5	10.5	10	1.0	10.0
Sample 5	10.1	9.7	9.9	10	1.0	10.0
Sample 6	9.9	10.3	10.2	10	1.0	10.0

Table 4: System suitability test results.

Name of standard	Mean area	Standard Deviation	% Relative Standard Deviation	Asymmetry	Theorotical plates
Gallic acid	143428366	192509.135	0.13	1.23	9039
Glycyrrhizic acid	13683289	42057.830	0.31	1.20	14441
E-Guggulsterone	146030579	672042.42	0.46	1.06	18439

Table 5: Stability of Gallic acid in Standard solution and in Polyherbal formulation (test solution).

Time point	Area	% Absolute difference	Area	% Absolute difference
Initial	143285429	NA	16235986	NA
12 hr	142586943	0.49	16165508	0.43
24 hr	142099704	0.83	16048536	1.15

Table 6: Stability of Glycyrrhizic acid in Standard solution and in Polyherbal formulation (test solution).

Time point	Area	% Absolute difference	Area	% Absolute difference
Initial	13652419	NA	143856	NA
12 hr	13585493	0.49	142562	0.90
24 hr	13486986	1.21	141753	1.46

Table 7: Stability of E-Guggulsterone in Standard solution and in Polyherbal formulation (test solution).

Standard solution			Polyherbal formulation (test solution)	
Time point	Area	% Absolute difference	Area	% Absolute difference
Initial	146285406	NA	916429	NA
12 hr	145922551	0.25	910551	0.64
24 hr	145026485	0.86	902413	1.53

Table 8: Linearity of Gallic acid.

Level	Conc (mcg/mL)	Area	Mean	STD DEV	% RSD
50%	50.00	79350394	78952369	416152.83	0.527
		78520193			
		78986521			
75%	75.00	111488791	111680406	298705.19	0.267
		111527843			
		112024583			
100%	100.00	143509222	143496863	195576.59	0.136
		143685967			
		143295400			
125%	125.00	175360256	175449399	209310.94	0.119
		175299416			
		175688524			
150%	150.00	205842462	205868111	97471.82	0.047
		205975843			
		205786029			

Table 9: Linearity of Glycyrrhizic Acid.

Level	Conc (mcg/mL)	Area	Mean	STD DEV	% RSD
50%	50.00	6818739	6818226	1529.45	0.022
		6816506			
		6819433			
75%	75.00	10151015	10196750	51595.59	0.506
		10252683			
		10186551			
100%	100.00	13632185	13660826	86047.38	0.630
		13757541			
		13592751			
125%	125.00	16882176	16835551	42797.63	0.254
		16826425			
		16798053			
150%	150.00	20423751	20449626	54762.10	0.268
		20512531			
		20412596			

Table 10: Linearity of E-Guggulsterone.

Level	Conc (mcg/mL)	Area	Mean	STD DEV	% RSD
50%	50.00	73123474	73276825	167771.91	0.229
		73250983			
		73456019			
75%	75.00	109957693	110118687	370225.66	0.336
		109856215			
		110542153			
100%	100.00	146152987	145821167	437704.62	0.300
		145325095			
		145985419			
125%	125.00	182778992	182783284	241450.12	0.132
		182544009			
		183026852			
150%	150.00	219175080	219342055	162953.63	0.074
		219350421			
		219500665			

Table 11: Repeatability and inter day precision.

Parameter/Standard	Gallic acid	Glycyrrhizic acid	E-Guggulsterone
Mean % Assay	99.46	99.941	99.518
STD DEV	1.0807	0.7192	0.8736
% RSD	1.087	0.720	0.878

% Assay and % RSD was found well within acceptance limit and hence method is precise (Reproducible).

Specificity

Acceptance criteria for specificity are there should be no Interference at retention time of any standard. No interference was seen due to blank at retention time of Gallic acid, Glycyrrhizic acid and E-Guggulsterone. The standard solution peak was found concordant with that of polyherbal formulation. Hence, the method's specificity was confirmed.

Linearity and Range

The results of linearity of Gallic acid, Glycyrrhizic acid and E-Guggulsterone are as mentioned in Tables 8-10 respectively.

From the calibration curves, it was evident that Gallic acid, Glycyrrhizic acid and E-Guggulsterone show linear response in the range of 50 -150 mcg/mL. The regression value was found well within the limit.

Precision

As per the ICH acceptance criteria, % Assay value for each sample (Individual sample) and mean % assay value for precision (6 samples), mean % assay value of intermediate precision (6 samples) and mean percent assay value for precision plus intermediate precision sample (12 samples) should lie between 98-102%. Percent RSD for precision study samples (6 samples), Intermediate precision study samples (6 samples) and precision plus intermediate precision sample (12 samples) should be not More Than 2.0. The results of repeatability and inter day precision are mentioned in Table 11.

Robustness

It was observed that under each changed chromatographic condition, Asymmetry and Theoretical plates of standard solution pass the acceptance criteria (Asymmetry NMT 2.0 and Theoretical plates NLT 2000). Hence it was concluded that the system suitability test result was found well within the limits and analytical method was robust.

Assay results of sample (Polyherbal formulation): Figure 6 depicts chromatogram of polyherbal formulation.

Gallic Acid

Sample	Area	% Assay	% Mean Assay
Sample 1	16531039	11.50	11.47
Sample 2	16485312	11.44	

Glycyrrhizic Acid

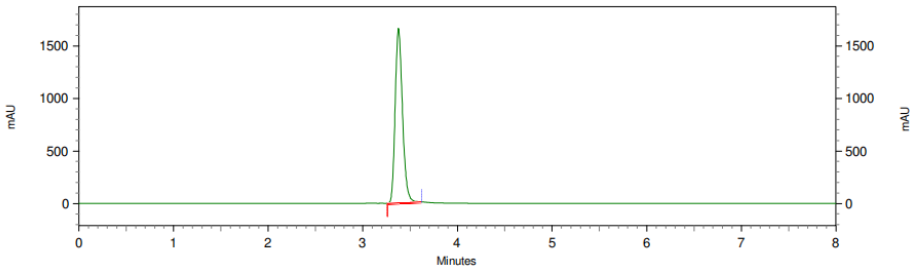
Sample	Area	% Assay	% Mean Assay
Sample 1	144173	1.05	1.05
Sample 2	142851	1.04	

E-Guggulsterone

Sample	Area	% Assay	% Mean Assay
Sample 1	905122	0.62	0.63
Sample 2	934516	0.64	

In the polyherbal formulation, gallic acid, glycyrrhizic acid and E- Guggulsterone were found to be 11.47%, 1.05% and 0.63% respectively.

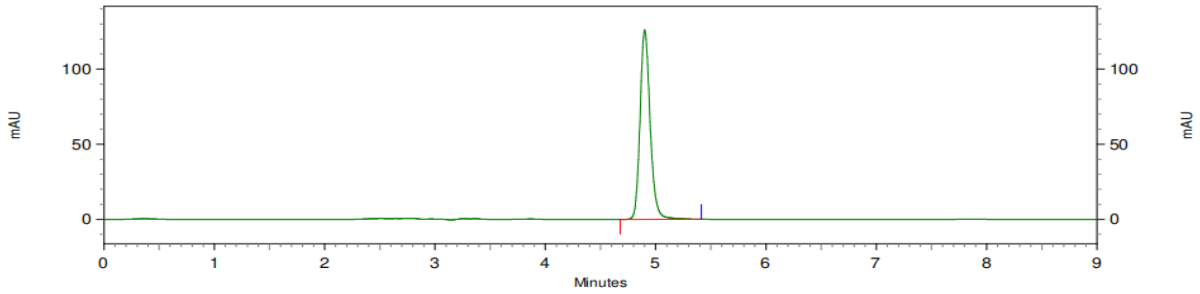
Sample Name: GALLIC ACID 100 PPM



VWD:SignalA, 224nmResults Name	Retention Time	Area	Asymmetry	Theoretical plates(USP)
Gallicacid	3.36	149217346	1.25	9150
Totals		149217346		

Figure 2: Chromatograph of method development for Gallic acid.

Sample Name: GLYCYRRHIZIC ACID 100 PPM

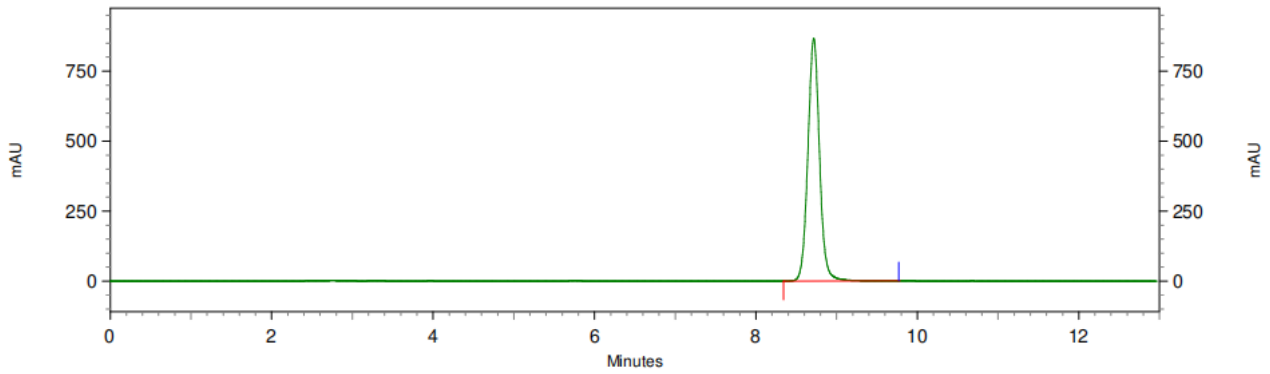


VWD: Signal A, 224 nm Results

Name	Retention Time	Area	Asymmetry	Theoretical plates (USP)
Glycyrrhizic acid	4.90	13482277	1.20	14118
Totals		13482277		

Figure 3: Chromatograph of method development for Glycyrrhizic acid.

Sample Name: E-GUGULSTERONE 100 PPM

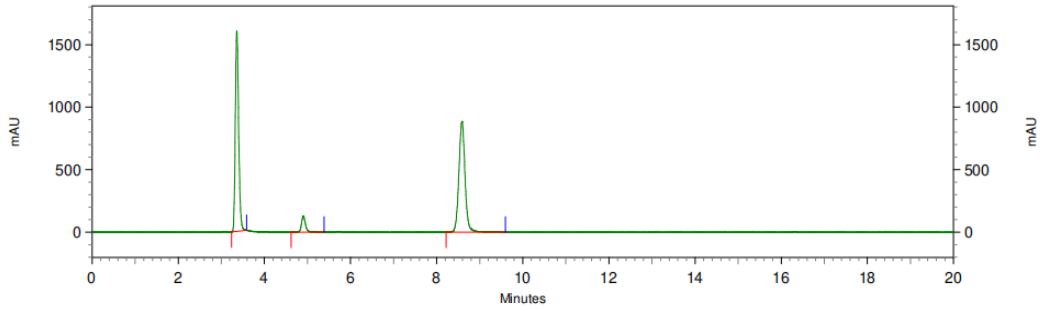


VWD: Signal A, 224 nm Results

Name	Retention Time	Area	Asymmetry	Theoretical plates (USP)
E-Guggulsterone	8.72	145908873	1.05	17985
Totals		145908873		

Figure 4: Chromatograph of method development for E-Guggulsterone.

Sample Name: MIXTURE (EACH 100 PPM)

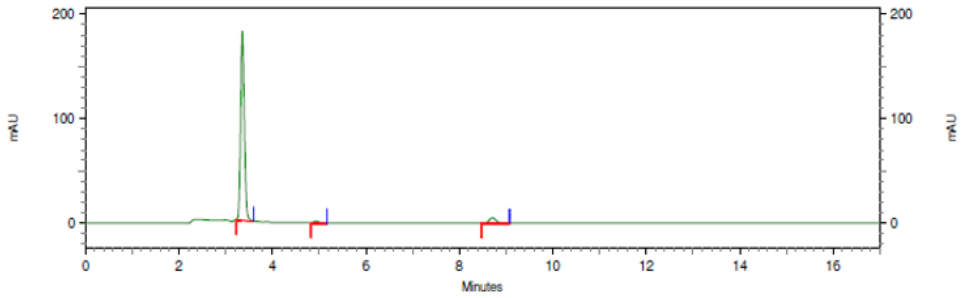


VWD: Signal A, 224 nm Results

Name	Retention Time	Area	Asymmetry	Theoretical plates (USP)	Resolution (USP)
Gallic acid	3.36	143509222	1.23	9085	0.00
Glycyrrhizic acid	4.90	13632185	1.20	14435	10.15
E-Guggulsterone	8.59	146152987	1.06	18115	17.61
Totals		303294394			

Figure 5: Chromartogram of mixture of Gallic acid, Glycyrrhizic acid and E-Guggulsterone.

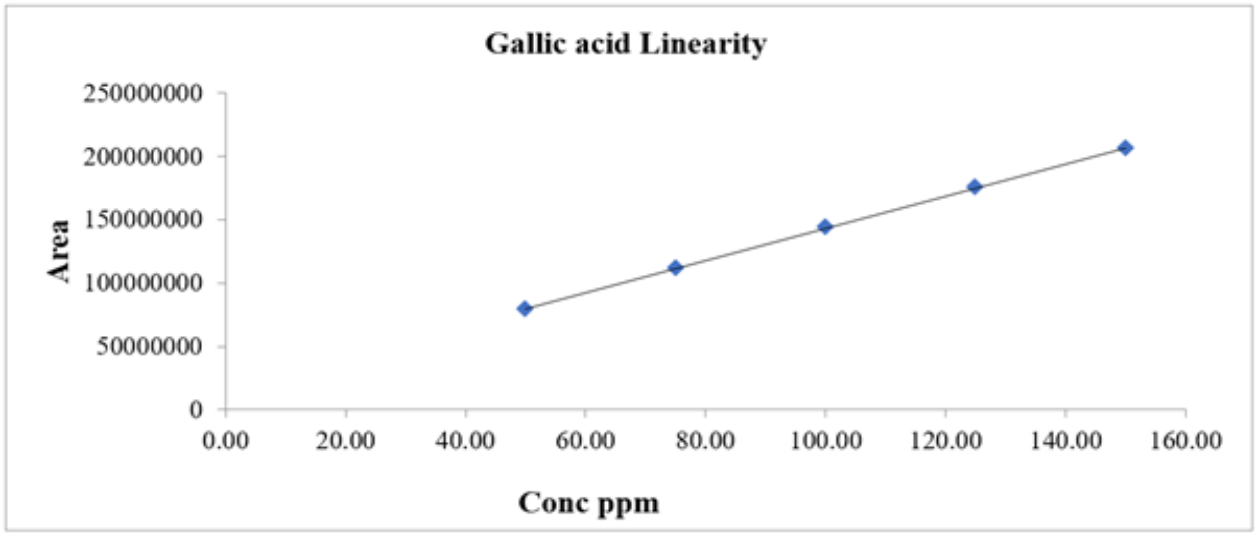
Sample Name: PH EXTRACT SAMPLE_1



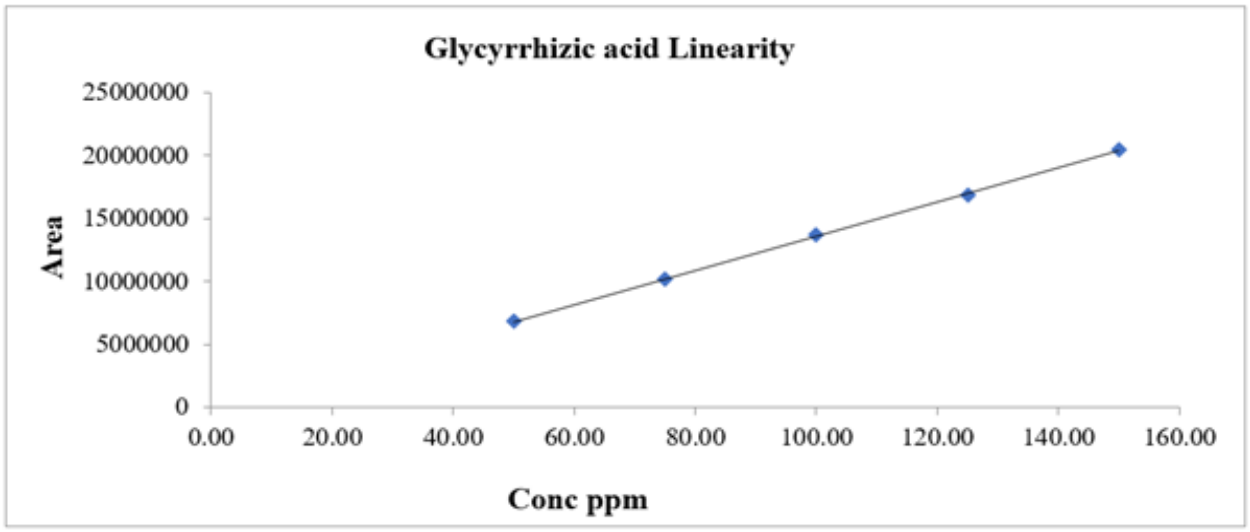
VWD:SignalA, 224 nm Results

Name	Retention Time	Area	Asymmetry	Theoretical plates(USP)
Gallicacid	3.36	16531039	1.13	8847
Glycyrrhizicacid	4.91	144173	1.19	13091
E-Guggulsterone	8.59	905122	1.10	18098
Totals		17580334		

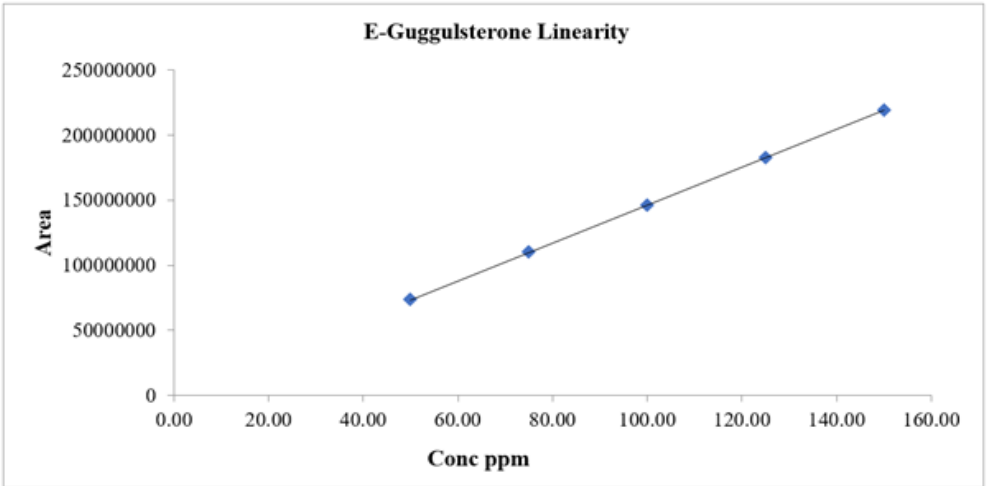
Figure 6: Chromatogram of polyherbal formulation (sample).



Sl. No.	Parameter	Result value
1	Beer's linearity range	50.00-150.00 mcg/mL
2	Correlation coefficient (R^2)	0.99992
3	Intercept	16049238.80
4	Slope	1270401.91



Sl. No.	Parameter	Result value
1	Beer's linearity range	50.00-150.00 µg/mL
2	Correlation coefficient (R^2)	0.99986
3	Intercept	31555.40
4	Slope	135606.40



Sl. No.	Parameter	Result value
1	Beer's linearity range	50.00-150.00 µg/mL
2	Correlation coefficient (R^2)	0.99999
3	Intercept	350380.80
4	Slope	1459180.23

CONCLUSION

It is essential that herbal medicines also get screened through quality control tests to ascertain their quality and efficacy. Standardization of polyherbals by HPLC is challenging due to presence of multiple active phytoconstituents in small Concentration. This research study has identified a novel method for detection and quantification of three cardioprotective biomarkers -Gallic acid, Glycyrrhizic acid and E-Guggulsterone from a polyherbal formulation containing thirteen Ethno herbs, reported to have heart protective effects, marketed as Collasyn tablets by Dr. Palep Research Labs Ltd., in Indian market. The method is also validated as per ICH guidelines and found to be precise, accurate and robust. This research study is a step forward in standardization of polyherbal formulation by HPLC and usage of this method will contribute towards maintaining quality and cardioprotective efficacy of polyherbal formulation marketed as Collasyn tablets by Dr. Palep Research Labs Ltd., in Indian market.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

This study is neither published in any journal so far nor has been submitted to any other journal. There has been no funding for this study.

ABBREVIATIONS

i.d: Internal diameter; **mg:** Milligram; **mcg:** Microgram; **mL:** Milliliter; **UV:** Ultraviolet; **mg/mL:** Milligram/milliliter; **mcg/mL:** Microgram/milliliter; **HPLC:** High Performance Liquid Chromatography; **ppm:** Parts per million; **nm:** Nanometer; **mcg/mL:** Microgram/milliliter; **conc:** Concentration; **RSD:** Relative Standard Deviation; **USP:** United State Pharmacopoeia.

SUMMARY

Standardization of polyherbal formulations is an essential step in their quality control. High Performance Liquid Chromatography is an important analytical tool towards standardization of polyherbal formulation. However, the same becomes complex as it can involve simultaneous estimation of multiple active biomarkers. The present study is on developing and validating an unique method for simultaneous estimation of active biomarkers-Gallic acid, Glycyrrhizic acid and E-Guggulsterone from a polyherbal formulation containing thirteen Ethno herbs for cardiovascular disease management, marketed as Collasyn tablets by Dr. Palep Research Labs Ltd. This research study is a step forward in standardization of polyherbal formulation by HPLC and usage of this method will contribute towards maintaining quality and cardioprotective efficacy of polyherbal formulation

marketed as Collasyn tablets by Dr. Palep Research Labs Ltd. in Indian market.

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