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HPLC Analysis of an Ayurvedic Formulation

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In Ayurveda Intuppukana Churna is commonly prescribed as anti anorectic formulation which is the mixture of *Terminalia chebula* (Combretaceae), *Piper longum* (Piperaceae), *Trachyspermum roxberghianum* (Umbelliferae) and rock salt. Gallic and, ellagic acid and piperine are three markers present in the formulation, which were analyzed simultaneously by HPLC with PDA detector at 280 nm by using waters symmetry C-18 Column (250 × 4.6 mm, particle size 5 µ) at an ambient temperature. The separation was achieved with isocratic elution using a mixture of water: methanol (70:30) at pH 3.0 by addition of orthophosphoric acid at a flow rate of 0.8 mL/min. Calibration curves were linear with correlation coefficients of 0.9941, 0.9968 and 0.9917 for gallic acid, ellagic acid and piperine, respectively over a concentration range of 10-100 µg/mL for each. The validation parameter showed that the method is a simple, economical, specific, reproducible and accurate for the evaluation of these three markers in the Ayurvedic medicines.

Key Words: Gallic acid, Ellagic acid, Piperine, Ayurvedic medicine, HPLC, Method validation.

INTRODUCTION

Ayurvedic medicines are often prescribed by the Indian medical practitioners extensively to prevent and cure diseases from ancient time. These medicines are the complex mixture of the several herbs and / minerals each with multiple component. As a result, analysis of such complex mixture presents a great challenge to the pharmaceutical analysis[1].

Intuppukana Churna is a polyherbal formulation often used to treat gastric disorders and loss of appetite. As per the Ayurvedic Formulary of India, part-I the medicine consist three types of individually powdered herbs: *Terminalia chebula* (Combretaceae), *Piper longum* (Piperaceae), *Trachyspermum roxberghianum* (Umbelliferae) and one mineral-saindhav lavan (rock salt). The formulation contains gallic acid (GA), ellagic acid (EA) and piperine (PN) as markers from *T. chebula* and *P. longum* respectively[2]. *T. chebula* contains high percentage of gallic acid and ellagic acid, which is a strong antioxidant [3]. While *P. longum* is a rich source of piperine alkaloid which is used as bioavailability enhancer[4].

Various analytical methods have been developed for the separation and quantification of the gallic acid, ellagic acid and piperine by different scientists. Weerasak *et al.*[5] developed the method for the simultaneous estimation of the gallic acid, ellagic acid along with other phenolics by using HPLC. Charrier *et al.*[6] also separated the gallic acid and ellagic acid by using different mobile phases in HPLC from the European Oakwood and Eucalyptus. Lei *et al.*[7] separated gallic and ellagic acid by methanolysis and then using HPLC by gradient elution technique

Rai *et al.*[8] described the RP-LC method for the simultaneous determination of piperine, aconitine and solanine in an ayurvedic formulation. Chiang[9] tried to determine the piperine and capsaicin simultaneously by using electrochemical detection and UV detection. Bajad *et al.*[10] analyzed the presence of piperine in rat plasma by HPLC and another method developed for the simultaneous estimation of piperine and ketoconazole in rat plasma[11]

Literature reveals that no analytical work has been reported on the Intuppukana Churna. No method is available for the simultaneous estimation of gallic acid, ellagic acid and piperine. Many of the Ayurvedic formulation contain these three components as a marker (*viz.* avipattikara churna, chyvanprash) but still no work has been reported in these aspects. In the present study attempt has been made to develop and validate the simple, reproducible and economical method for the simultaneous determination of gallic acid, ellagic acid and piperine.

EXPERIMENTAL

The standard marker compound gallic acid, ellagic acid and piperine were purchased from Sigma Aldrich Pvt. Ltd. (USA). Methanol and water were of HPLC grade (Merck, Mumbai, India) and orthophophoric acids (H_3PO_4) of analytical reagent grade were also purchased from the same supplier.

All the other solvents and reagents were of analytical grade and filtered through a 0.2 μm ultipor nylon 66 membrane filter prior to use.

The chromatographic system used was agilent liquid chromatography system series 1200 with quaternary pump, rheodyne injector with 20 μL fixed loop and photodiode array detector. The separation was achieved on waters symmetry C-18 column (250 × 4.6 mm, particle size 5 μ) preceded by an ODS guard column (10 μm, 10 mm × 5 mm ID) at an ambient temperature.

Analytical conditions: The analysis was isocratic with mobile phase methanol:water (70:30) and pH adjusted to 3 ± 0.1. The flow rate was 0.8 mL /min. The mobile phase was prepared freshly every day and filtered through 0.2 μm membrane filter to remove any particulate matter. The mobile phase is mixed and degassed by sonication prior to use. The mobile phase was stable with no precipitation with time or change in temperature. The absorbance of all the three markers (gallic acid, ellagic acid and piperine) was good at 280 nm in this mobile phase. The sensitivity of the detector was set at 0.01 AUFS. Before injecting the solutions, the column was equilibrated for at least 1 h with mobile phase flowing through the system. Each solution was injected in triplicate and the relative standard deviation (RSD) was required to remain below 2 % on peak area basis.

Preparation of standard solutions: The standard solutions were prepared by accurately weighing 5 mg of gallic acid, ellagic acid and piperine standard and dissolved in 5 mL of mobile phase obtaining the stock concentration of 1000 μg/mL of each. Aliquots of each standard were diluted to obtain solutions in the range of 10-100 μg/mL in mobile phase. The stock solutions were refrigerated and were found to be stable for 2 weeks.

Preparation of formulation: The formulation was prepared as per the Ayurvedic Formulary of India part-I. One part of rock salt, two parts fruits of *Trachyspermum roxberghianum* (apiaceae), 4 parts fruits of *piper longum* (piperaceae) and six parts of *Terminalia chebula* (combretaceae) were powdered and mixed uniformly. All the raw materials were purchased from local market. The plant materials were authenticated and voucher specimens were deposited at Agharkar Research Institute, Pune, India. The deposited voucher specimen numbers are *Trachyspermum roxberghianum* (apiaceae) F-147, *Piper longum* (piperaceae) F-145, *Terminalia chebula* (combretaceae) F-146.

Preparation of sample: About 100 mg amount of the Intuppukana Churna and 100 mg of its herbal ingredients were extracted three times with 100 mL methanol by kinetic maceration method. The complete extraction was confirmed by the qualitative tests for the alkaloids and tannins, the major constituents present in the formulation. The extracts were combined and methanol was evaporated under reduced pressure. The residue was dissolved in methanol by sonication and further dilutions were made in mobile phase.

Validation: In order to verify that the proposed method is applicable to formulation analysis, validation was performed as per ICH guidelines Q2B[12]. The following parameters were studied in validation of the method.

Calibration curve: Five different concentrations of piperine, gallic acid and ellagic acid were analyzed and their calibration curve was constructed in the specified concentration range (10-100 μg/mL). The calibration plots were generated by replicate analysis (n = 3) at all concentration levels. The linear relationship was evaluated using the least square method within microsoft excel programme. LOD and LOQ were measured to evaluate the limit of detection and quantitation by using the equations LOD = 3.3s / S and LOQ = 10 s / S (s is the standard deviation of the response and S is the slope of the calibration curve.

Precision

Repeatability: The repeatability was determined by analyzing six consecutive injections of the standards.

Intermediate precision: The intermediate precision was carried out by Intra day and inter day assay.

Reproducibility: Reproducibility was assessed by inter laboratory trials.

Specificity: It is the ability of the analytical method to measure analytical response in the presence of interferences in the sample. It was determined by analyzing the presence of the markers in another proprietary formulation. The resolutions of the intended peaks were determined.

Robustness: Robustness of the method was determined by changing the chromatographic conditions like the brand of the organic solvent used, the brand of the column used.

System suitability: System suitability was performed to evaluate the chromatographic parameters before the validation runs *viz*. theoretical plates, asymmetry of the peaks and the resolution between two consecutive peaks.

Sample analysis and accuracy: Accuracy expressed as recovery of the method was determined by analyzing the percentage recovery of the marker constituents. Known percentages of the markers (80,100,120) were added to the preanalyzed sample solution and chromatograms were obtained.

RESULTS AND DISCUSSION

Method development and optimization of the chromatographic conditions: The mobile phase composition, pH, such factors affects the chromatographic separations significantly. Therefore numbers of trials were conducted by using different combinations of various organic solvents and buffers at various pH values. The pH affected the separation of the piperine significantly. The resolutions of the three peaks were achieved by mobile phase methanol: water (70:30) at pH 3 by isocratic elution. Symmetrical, sharp and well resolved peaks were observed for gallic acid, ellagic acid and piperine having retention time 2.77, 3.74 and 11.13 respectively. The proposed method was validated using following parameters as linearity, precision, detection limit and quantification, specificity, system suitability and recovery.

Validation

Linearity (calibration curve n=5) : The series of the standard mixture solutions of these three compounds were analyzed to determine the linearity between the peak areas and standard mixture concentration. The standard response curve for each of the marker is linear over the concentration range 10-100 μg/mL. The correlation coefficients (r^2) were 0.9941, 0.9968 and 0.9917 for gallic acid, ellagic acid and

piperine respectively. The linearity data obtained is expressed in Table-1.

TABLE-1
LINEAR REGRESSION DATA FOR CALIBRATION CURVE (n = 5)

Parameter	GA	EA	PN
Retention time (min)	2.80 ± 0.05	3.742 ± 0.09	11.04 ± 0.1
Detection wavelength (nm)	280	280	280
Linearity range (µg/mL)	10-100	10-100	10-100
R^2	0.9941	0.9968	0.9917
Regression equation	y = 983041 x – 468789	y = 376651 x – 225295	y = 635683 x – 460436
Limit of detection (µg/mL)	0.319	0.948	0.443
Limit of quantification (µg/mL)	0.969	2.875	1.342

Repeatability (n =6): The injection repeatability shows that the % RSD of the peak areas and retention time for all the three compounds were less than 2 % (Table-2).

TABLE-2
PRECISION: REPEATABILITY DATA (n =6)

	Rt	% RSD Rt	Area	%RSD Area
GA	2.77± 0.035	1.25	5571250 ±54539	0.97
EA	3.693±0.038	1.03	2051616 ±37973	1.87
PN	10.97± 0.06	0.56	3322032± 45604	1.37

Intermediate precision: The intermediate precision result is presented in Table-3. It was seen that the % RSD for interday (for 6 days) and intra day assay (1 h) is low *i.e.* less than 2 % for peak areas and retention time.

Reproducibility and stability: The inter laboratory trials were taken and the method is reproducible. The stability of the crude extract is tested for 24 h at room temperature and the solution was found to be stable. (RSD values of the retention time and peak areas were less than 2%).

Specificity: The results of the proposed method for the determination of the markers in ternary mixtures and formulation containing these three markers were highly specific. No other components from the formulation were interfering in the determination of these three markers. The comparison of the chromatograms of individual Markers and the formulation shows that the retention time of the individual marker and retention time of the markers in the formulation were identical indicating the specificity of the proposed method.

Robustness: Table-4 shows the change in the parameters like brand of solvent and column was not affecting the peak resolution of the three markers indicating that the proposed method is consistent (n = 6). By changing the column brand the retention time of the three markers were shifted but the resolution was not affected.

System suitability: This test is performed to evaluate the chromatographic parameters, number of theoretical plates, capacity factor, asymmetry of the peaks and the resolution between two consecutive peaks (Table-5).

Sample analysis and accuracy: The methanolic extract of the sample was analyzed under proposed condition (Table-6). The content of the each marker were calculated from the respective calibration curve and presented as mean of 5 determinations. The recovery experiments were performed by adding known amount (80,100 and 120 %) of the markers to the preanalyzed sample and results obtained are shown in Table-7.

Conclusion

The presence of two phenolics and one alkaloid in the formulation of Intuppukana Churna makes it difficult to separate them from each other in one mobile phase by previously reported LC methods for alkaloids and for phenolics. Attempt has been made to develop a simple and economical method for the identification and quantification of the gallic acid, ellagic acid and piperine. Addition of orthophosphoric acid in the mobile phase to maintain the pH 3 gives satisfactory chromatographic separation of these three markers within a short run time of 15 min. The method validation data indicates that the present method is reliable, reproducible and accurate for the simultaneous determination of the three markers of the formulation.

Many proprietary Ayurvedic formulation contains these three marker compounds with different combinations as piperine

TABLE-3
INTERMEDIATE PRECISION (n = 6)

Marker	Intra day (n = 6)				Interday (n=6)			
	Retention time		Peak area		Retention time		Peak area	
	Mean ± S.D	% RSD	Mean ± S.D	% RSD	Mean ± S.D	% RSD	Mean ± S.D.	% RSD
GA	2.769 ± 0.050	1.824	5552155 ± 79841	1.438	2.736 ± 0.052	1.918	5561400 ± 6230	0.112
EA	3.713 ± 0.053	1.432	1961117 ± 1.246	1.246	3.704 ± 0.014	0.402	2028487 ± 34180	1.685
PN	11.79 ± 0.078	0.661	3340246 ± 11169	0.334	11.667 ± 0.160	1.373	3344120 ± 13831	0.413

TABLE-4
ROBUSTNESS OF THE METHOD (n = 6)

Chromatographic change factor	GA		EA		PN	
	Mean Rt ± S.D	% RSD	Mean Rt ± S.D	% RSD	Mean Rt ± S.D	% RSD
Methanol brand:						
Qualigens (n=3)	2.72± 0.03	1.29	3.69 ± 0.06	0.17	11.001 ± 0.01	0.16
Merck (n=3)	2.722 ± 0.09	0.350	3.72 ± 0.01	0.402	11.048 ± 0.06	0.55
Column Brand:						
Inertsil (n=3)	2.71 ±0.02	0.90	3.70 ± 0.006	0.17	10.98 ± 0.07	0.64
Waters symmetry (n=3)	3.001±0.041	1.36	4.00 ± 0.025	0.63	11.48 ± 0.04	0.23

TABLE-5
SYSTEM SUITABILITY DATA

Parameter	Gallic acid	Ellagic acid	Piperine
No. of theoretical plates	2346	2392	4881
Asymmetry of the peak	1.857	1.352	1.039
Resolution between two peaks	0.000	3.507	15.690

TABLE-6
SAMPLE ANALYSIS

Marker	Amount found (µg/g)	RSD (%)
Gallic acid	32.96 ± 0.362	1.098
Ellagic acid	23.90 ± 0.255	1.071
Piperine	15.43 ± 0.033	0.216

TABLE-7
RECOVERY STUDY (n= 3)

Marker	Amount added (%)	Mean recovery (%)
Gallic acid	80	99.56
	100	101.62
	120	98.87
Ellagic acid	80	101.89
	100	98.52
	120	99.60
Piperine	80	101.36
	100	98.62
	120	99.95

acts as bioavailability enhancer and gallic and ellagic acid are the potent antioxidants. Therefore the present analytical data could be a potential application in development of standardization parameters of the ayurvedic medicines.

REFERENCES

1. N.P. Yadav and V.K. Dixit, *Int. J. Int. Biol.*, **2**, 195 (2008).
2. Anonymous, The Ayurvedic Formulary of India, Part-I, Government of India, Ministry of Health and Family Welfare, Department of Ayush, edn. 1, p. 87 (1976).
3. A. Saleemm, M. Husheem, P. Harkonen and K. Pihlaja, *J. Ethnopharmacol.*, **81**, 327 (2002).
4. U. Zutshi and J.L Kaul, *Indian Drugs*, **19**, 476, (1982).
5. W. Samee and S. Vorarat, *Thai. Pharm. Health Sci. J.,* **2,** 131, (2007).
6. B. Charrier, M. Marques and J.P. Halik, *Int. J. Biol. Chem. Phys. Technol. Wood*, **46**, 87(1992).
7. Z. Lei, J. Jervis and R.F. Helm, *J. Agric. Food Chem.*, **49**, 1165 (2001).
8. P.D. Rai, A. Pathak and S.J. Rajput, *Chromatographia,* **69**, 1 (2009).
9. G.H. Chiang, *J. Food Sci.*, **51**, 499 (2006).
10. S. Bajad, A.K. Singla and K.L. Bedi, *J. Chromatogr. B,* **776**, 245, (2002).
11. S. Bajad, R.K. Johari, K. Singh, J. Singh and K.L. Bedi, *J. Chromatogr. A*, **949**, 43 (2002).
12. ICH topic Q2B, Validation of Analytical Procedures, Validation of Analytical Procedures: Methodology, Note for Guidance on Validation and Analytical Procedures: Methodology (1996).