RJC

RASĀYAN *J. Chem.*
Vol. 16 | No. 3 |1509-1515| July - September | 2023
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

DETERMINATION OF SHELF LIFE OF AN AYURVEDIC FORMULATION KAISHORA GUGGULU USING RP-HPLC ANALYSIS OF CHEMICAL MARKERS

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ABSTRACT

A new RP-HPLC stability-indicating assay method was developed and stability studies were conducted to determine the shelf life of Kaishora Guggulu. Magnoflorine, Palmatine, and Berberine form the chief chemical markers for the chemical fingerprinting of Kaishora Guggulu. Separation was performed using RP-HPLC equipped with a PDA detector at wavelengths Magnoflorine (231nm), Palmatine (271nm), and Berberine (280nm). The strategy applied for the determination of marker content in the formulation was under prescribed ICH storage conditions of temperature and humidity at specific time points. Stability studies hence were conducted at accelerated conditions at time points (0, 1, 3, and 6 months) and long-term conditions at time points (0, 6, 9, and 12 months). A stability-indicating RP-HPLC method is developed by performing stress studies on markers such as acid and alkaline hydrolysis, photolytic, oxidative, and thermal degradation. Palmatine, Berberine, and Magnoflorine were effectively separated and detected using a photodiode array detector. The stability indicating RP-HPLC assay method was validated and employed for the stability studies. The marker content analysis at the prescribed time period elicited a shelf life of 27.32, 21.37, and 11.29 months considering the markers Magnoflorine, Palmatine, and Berberine. Thus the RP-HPLC method and stability studies data can be utilized for routine commercial stability studies. No pharmacopoeial RP-HPLC method is available so developed this method for laboratory use by local Ayurvedic manufacturers.

Keywords: Stability Studies, Scientific Evidence, Shelf-Life, Magnoflorine, Palmatine, And Berberine**,** Kaishora Guggulu

RASĀYAN *J. Chem.*, Vol. 16, No. 3, 2023

INTRODUCTION

Ayurvedics are complex in nature due to multiple phytoconstituents present. This adds a burden on the insufficiency of evidential data on chemical fingerprinting. Owing to the insufficiency of standard marker content profiles in the Ayurvedic formulations. Various factors play a role in the quality aspect of the medicines which involves the choice of raw material, extraction approaches, operation parameters, container closure design, and most significantly storage conditions.[1] The therapeutic uses of the formulation Kaishora Guggulu include dyspepsia, constipation, gout, diabetes, ulcer, cough, skin disease, edema, anemia, excessive flow of urine, and geriatric disorder. *Tinospora cordifolia* is the major component of the formulation. Alkaloids form the major group of phytoconstituents which includes Palmatine, Berberine, and Magnoflorine. They form the chief chemical markers for the chemical fingerprinting of Kaishora Guggulu.[2-6] On August 12, 2016, AYUSH published a new version of Rule No. 161-B of the 1940 Drugs and Cosmetics Act, which says that the shelf life or expiration date of Ayurvedic formulations must be specified and supported by scientific evidence. Ayurvedic medications shall, in accordance with the amendment, have a shelf life based on scientific evidence as determined by

http://doi.org/10.31788/RJC.2023.1638336

real-time and expedited stability examinations of medications prescribed in Ayurvedic Pharmacopeia of India.[7-8] Therefore an analytical method indicating stability needs to be developed. Thus, markers were subjected to a range of stress conditions, including acidic, alkaline, photolytic, oxidative, and thermal hydrolysis. On the basis of the stability studies, additional shelf life is estimated.[9] The available methods so far, majorly focused on the stability studies concerning an individual secondary metabolite. The outcomes acquired from these stability data do not necessarily reflect the stability of the different phytoconstituents in the formulation. The concomitant compounds present in the polyherbal formulation may play a role in influencing the overall stability of the formulation.[10] Therefore in the present study modern analytical approaches were adopted to determine the chemical stability and shelf life of the Ayurvedic formulation under prescribed storage conditions.

The present study involves the development of an RP-HPLC method for the simultaneous estimation of Palmatine, Berberine, and Magnoflorine that fits the objective of chemical fingerprinting of Kaishora Guggulu. Stability studies were conducted at prescribed temperature and humidity conditions for a period of 12 months. Shelf life was estimated using chemical marker content analysis that assures the efficacy, stability, and safety of Kaishora Guggulu at different storage conditions over time.

EXPERIMENTAL

Material and Methods

Kaishora Guggulu was procured from a local manufacturer. Marker compounds Magnoflorine, Palmatine, and Berberine (purity >97%) were obtained from Merck. Methanol and Acetonitrile HPLC grade from Fine Chemicals.

HPLC Method Development

Separation was performed using Shimadzu HPLC (Make: Kyoto JAPAN) equipped with LC solution software, LC-10 ADVP quaternary pump, SIL-10 ADVP auto-injector, and an SPD M-10A VP photodiode array detector (PDA) detection system. The column used for separation was Phenomenex HyperClone BDS C_{18} (250cm x 4.6mm, 5µ) using a gradient program for elution.

Validation of Analytical Procedure

Validation was performed for the below-mentioned parameters.

Specificity

It is the ability of the method to assess the marker in the presence of interfering constituents in the matrix. These might be inclusive of impurities, degradant, and matrices due to the complex nature of the herbal formulation. A standard solution of 10µg/ml concentration containing a mixture of Magnoflorine, Palmatine, and Berberine was injected into the system. The peak purity index of a system suitability solution of Magnoflorine, Palmatine, and Berberine was determined.

System Suitability

A working standard of 10µg/ml concentration containing a mixture of Magnoflorine, Palmatine, and Berberine was injected. Peak parameters such as retention time, peak area, tailing factor, resolution, and theoretical plates were monitored.

Linearity

A series of standard solutions for linearity containing a mixture of Magnoflorine, Palmatine, and Berberine was prepared in the range from 0.05-3.1 µg/mL.

Limit of Detection (LOD)

The lowest level of marker detected in the sample by an analytical method, which is limited to detection but not quantification.

Limit of Quantitation (LOQ)
The lowest level of marker in the sample that can be quantified with appropriate preciseness and accuracy. This parameter is determined in assays and is also put to use for the resolution of impurities or degraded products.

Accuracy
Accuracy is expressed by the proximity of a standard value and the value found. The standard samples of known concentration were spiked with a predetermined amount of the marker. The marker's recovery was estimated. This is also called a standard spiking method. The concentration levels at which accuracy was evaluated were 0.8µg/ml, 1µg/ml, and 1.2µg/ml for Magnoflorine, Palmatine, and Berberine

Precision
This is usually evaluated at 3 levels: repeatability, intermediate precision, and reproducibility. A series of 6 injections of the standard solution was injected for determining the intraday and interday precision. Retention time and peak area of markers were analyzed. The values were expressed as Mean, SD, and RSD of a series of measurements.

Assay of Marketed Formulation
The average weight of 20 tablets of Kaishora Guggulu was taken and tablets were crushed into a fine powder using a mortar and pestle. 5g of powder was taken in a 50ml volumetric flask and the volume is made up of methanol, sonicated for 45 minutes by frequent stirring for every 5 minutes. 2 ml of the solution was taken and centrifuged for 10 minutes at 1000 rpm. 1ml of the supernatant solution was withdrawn for analysis and % recovery was calculated.

Stress Studies
Stress studies were performed with the standard solution mixture at a concentration of 10µg/ml for Magnoflorine, Palmatine, and Berberine to find out if any degradation products are present. The samples were examined under chromatographic conditions that were tuned using a photo-diode array detector to determine the peak purity. When degradant peaks are present, the peak purity of the marker is calculated to examine any potential degradation products.

Acid Hydrolysis
A mixture of Magnoflorine, Palmatine, and Berberine in an acidic medium was subjected to hydrolysis at heated and room temperature. The initial part of the study was executed with 0.1 M HCl at room temperature. As the degradation obtained was nominal to 10%, studies were executed further at heated temperatures. Accurately weighed 5mg each of Magnoflorine, Palmatine, and Berberine were transferred into a 50 ml round bottom flask, and the reaction was performed with 50 ml of 0.1M HCl. This gives a standard concentration of 100µg/mL of Magnoflorine, Palmatine, and Berberine. The stock solution was further diluted using MeOH. The solution was neutralized by pH adjustment to 7 with 0.1M Sodium hydroxide. A working standard solution was prepared using a diluent. This working standard solution mixture of Magnoflorine, Palmatine, and Berberine (10µg/mL) was used for the determination.

Alkaline Hydrolysis
Initial stock standard solution was prepared in the same manner as described for acid hydrolysis but rather 0.1M Sodium hydroxide was used. The stock solution was further diluted using MeOH. The solution was neutralized by pH adjustment to 7 with 0.1 M HCl. A working standard was prepared with diluent. This working standard solution mixture of Magnoflorine, Palmatine, and Berberine (10µg/mL) was used for the determination.

Oxidative Degradation
A mixture of Magnoflorine, Palmatine, and Berberine was hydrolyzed under different concentrations of peroxide solution (H_2O_2). The initial part of the study was conducted with a 3%v/v solution of H_2O_2 and

5%v/v H_2O_2 at ambient conditions for a duration of 24 hours. As there was no sign of potential degradation for 24 hours, the trials were extended up to 24 hours using 30%v/v hydrogen peroxide solution. The final solution of Magnoflorine, Palmatine, and Berberine (10µg/mL) was used for the determination.

Photolytic Degradation

Accurately weighed 10mg each of Magnoflorine, Palmatine, and Berberine were mounted on Petri plates and were subjected to exposure to direct sun for 24 hours. 5mg of Magnoflorine, Palmatine, and Berberine was weighed and dissolved in MeOH to give a standard stock solution (1000µg/mL). A working standard solution was prepared using a diluent. This working standard solution mixture of Magnoflorine, Palmatine, and Berberine (10µg/mL) was used for the determination.

Thermal Degradation

Accurately weighed 15mg each of Magnoflorine, Palmatine, and Berberine were placed on Petri plates. Further stored at 80°C in a hot air oven for a duration of 24 hours. This working standard solution mixture of Magnoflorine, Palmatine, and Berberine (10µg/mL) was injected into the HPLC system.

Shelf-life Estimation

The developed and validated methods were used in the marker content analyses. The stability study was performed as per ICH. The formulation was stored at accelerated conditions at 40°C ± 2°C/75% RH ± 5% RH for time points of 0, 1, 3, and 6 months. Long term condition involved storage at 30°C ± 2°C/65% RH ± 5% RH for time points 6, 9 and 12 months. The studies were conducted by storing samples in a stability chamber (Thermolab Scientific, India). A USFDA-approved software, Systat SigmaPlot version 15.0 (www.systatsoftware.com) was used in the shelf-life calculation of the formulation.

RESULTS AND DISCUSSION

RP-HPLC Method Development

A gradient elution program was developed for the reverse phase HPLC method. The final chromatographic condition for RP-HPLC is demonstrated below in Table-1. Also, Fig.-1 represents the chromatogram of the RP-HPLC condition.

Table-1: RP-HPLC Parameters

Column	C_{18} Phenomenex HyperClone BDS (250cm×4.6mm, 5µ)
Mobile Phase	A: MeOH: 0.01M pH4.5 Ammonium Acetate buffer (30:70) B: ACN: 0.01M pH4.5 Ammonium Acetate buffer (30:70)
Mode of Flow	Gradient Program: 0.01-8.00 min 100% A, 8.01-35.00 min 100% B, 35.01-40.00 min 100% A, Run time- 40 min
Flow Rate	1 mL/min
Column Temperature (°C)	25
Autosampler Temperature (°C)	4
Injection volume (µL)	20
Detection wavelength (λ_{max})	Magnoflorine (231nm), Palmatine (271nm), Berberine (280nm)
Retention time (min)	Magnoflorine (5.2), Palmatine (18.8), Berberine (20.6)

Analytical Method Validation

Results of method validation show the effectiveness, dependability, and correctness of analytical findings; it is an essential part of a fruitful analytical process. ICH Q2AR1 guidelines were applied during the validation process. The approach was precise because the peak purity indices for magnoflorine, palmatine, and berberine, respectively, were 0.999, 0.997, and 0.998, all of which were considerably below the threshold (>0.99). In blank identification, the active peak and diluents were observed; there was no interference. In the forced degradation studies, the retention time allowed us to monitor active and degradant peaks. At their respective retention time, there was no evidence of interference from other components. The system suitability parameters were determined to assess whether the suggested approach was appropriate, and the results showed that the values met the accepted standards for chromatographic

separation. This shows that the developed method is appropriate for the particular system. Magnoflorine, Palmatine, and Berberine's linearity demonstrated linear responses throughout the range of 0.05–3 µg/mL because their respective correlation constants were found to be 0.999, 0.9996, and 0.9996. After determining the response standard deviation and slope, the LOD and LOQ were determined based on the signal-to-noise ratio. Magnoflorine is 0.001 µg/mL and 0.004 µg/mL while Palmatine and Berberine are 0.005 µg/mL and 0.01 µg/mL and 0.002 µg/mL and 0.006 µg/mL, respectively. The recovery of known quantities of a marker using the common spiking approach served as the basis for accuracy determination. The standard marker was spiked in a known quantity, and the recovery was calculated. It was assessed at three concentrations: 80%, 100%, and 120% of the standard marker. Magnoflorine, Palmatine, and Berberine had concentration values of 0.8 µg/mL, 1 µg/mL, and 1.2 µg/mL, respectively. The method's accuracy was confirmed at each of the three levels, with results falling between 95% and 105%. A series of 6 injections of a standard mixture solution of Magnoflorine, Palmatine, and Berberine was injected into the system. Repeatability or intra- day precision and intermediate or inter-day precision were performed. Mean, SD, and RSD were computed based on the retention time and peak area of the markers. RSD was found within the limits. A summary of the validation report is tabulated below in Table-2.

Table-2: A Summarized Report of RP-HPLC Validation

Parameter	Acceptance criterion	Magnoflorine	Palmatine	Berberine	Inference
Specificity	Peak Purity Index (>0.999)	Active marker peaks are well resolved	Active marker peaks are well resolved	Active marker peaks are well resolved	Passes
Linearity	Correlation Coefficient (r^2>0.99)	0.05-3µg/ml (r^2=0.999)	0.05-3µg/ml (r^2=0.9996)	0.05-3µg/ml (r^2=0.9996)	Passes
Accuracy (95.0% to 105.0%)	80% 100% 120%	96.98 99.24 100.91	98.39 99.77 99.35	98.70 98.64 99.47	Passes
Precision	RSD (<2%) (i)Intra-day (ii)Inter-day	 0.89% 1.24%	 0.90% 1.08%	 0.85% 1.33%	Passes
LOD	-	0.001µg/ml	0.005µg/ml	0.002µg/ml	-
LOQ	-	0.004µg/ml	0.01µg/ml	0.006µg/ml	-
System suitability	RSD (<2%)	0.81%	0.98%		Passes

Assay of Kaishora Guggulu Formulation

The recoveries of Magnoflorine, Palmatine, and Berberine were found to be 96.83%, 79.87%, and 92.85% respectively. This signifies the suitability of the method for the assay of Magnoflorine, Palmatine, and Berberine in Kaishora Guggulu. The mean recovery in Kaishora Guggulu is demonstrated in Table-3. A chromatogram of Magnoflorine, Palmatine, and, Berberine in Kaishora Gugguluis represented in Fig.-2.

Table-3: Mean Recovery in Kaishora Guggulu

S. No.	Magnoflorine	Palmatine	Berberine
1.	96.82%	78.76%	93.17%
2.	96.99%	79.52%	92.73%
3.	96.68%	81.34%	92.64%
Mean	96.83%	79.87%	92.85%
S.D (±)	0.001	0.013	0.002

Stress Studies

During forced degradation studies, there are chances of elution of degradant peaks along with marker peaks at the same retention time as that of the marker. Hence, a photodiode array detector is generally used to demonstrate the purity of the peak. Spectral homogeneity of the marker is indicated at the λ_{max} of Magnoflorine (231nm), Palmatine (271nm), and Berberine (280nm). These results obtained confirmed the absence of other co-eluting peaks, degradants, and impurities at variable stress conditions. No interference was observed with the marker peak. The developed method signifies to be specific for the simultaneous

determination of Magnoflorine, Palmatine, and Berberine in the presence of any degradant. The area normalization method is used for calculating the percentage degradation. The amount of degradation observed for the markers is demonstrated in Table-4.

Table-4: Stress Testing

S. No.	Forced degradation conditions	% Degradation Observed			Peak Purity Index
		Magnoflorine	Palmatine	Berberine	
1.	Acid hydrolysis (0.1M HCL)	20.31%	34.33%	28.01%	Pass
2.	Alkali hydrolysis (0.1M NaOH)	25.14%	26.31%	22.65%	Pass
3.	Oxidative degradation (30% v/v H_2O_2)	15.55%	18.27%	16.96%	Pass
4.	Thermal degradation (80°C in hot air oven)	15.24%	12.95%	13.25%	Pass
5.	Photolytic degradation (exposure to direct sunlight)	21.63%	28.11%	27.37%	Pass

Shelf-life Estimation

Table-5 represents the change in the mean content of markers with respect to time. SD represents the standard deviation of the mean taken for three readings. Shelf life of Kaishora Guggulu was predicted with changes in the marker contents analyzed against time and storage condition. Shelf life was estimated using the marker content analyses and Systat Sigma Plot software. The shelf life for Kaishora Guggulu computed based on Magnoflorine, Palmatine, and Berberine was 27.32, 21.37, and 11.29 months respectively as depicted in Fig 3-4. As the percent content of Palmatine fails to comply with the in-house specification of 90-110%, it does not fit into the curve fitting for shelf-life estimation.

Table-5: Mean Content of Markers with Time

Condition	Time	Magnoflorine		Palmatine		Berberine	
		Mean Content(%)	SD (±)	Mean Content(%)	SD (±)	Mean Content(%)	SD (±)
Initial	0	96.82	0.37	78.76	1.21	93.17	1.01
Accelerated Condition	1	98.21	0.27	77.38	0.78	91.97	0.43
	3	96.41	1.66	76.36	0.95	92.6	1.24
	6	96.35	0.54	77.21	0.69	92.33	0.19
Long-term condition	6	95.22	0.99	76.46	0.75	91.23	1.98
	9	96.01	1.26	75.35	0.72	90.95	1.57
	12	95.97	0.59	75.25	0.31	90.62	0.73

CONCLUSION

The stability of Ayurvedic formulations at their storage and transport condition is very critical in assuring quality. Thus shelf life estimation becomes paramount to quality. However, the complex nature of these phytoconstituents makes it exceedingly challenging to limit characteristic ranges and benchmarks for quality. There is an order released by the Gazette of India on 12th August 2016, by the ministry of AYUSH mandating the AYUSH products to state the shelf-life based on scientific data. The date of expiry of the medicine should be based on the real-time stability studies of medicines following the guidelines prescribed in the Ayurvedic Pharmacopoeia of India. Small-scale industries state the shelf life in the formulations as per the shelf life prescribed by the Ayurvedic Pharmacopoeia of India which is 5 years for Guggulu preparations. The shelf life for Kaishora Guggulu based on Magnoflorine, Palmatine, and Berberine was found to be 27.32, 21.37, and 11.29 months respectively. This data portrays the shelf life mentioned in the formulation is presented without the backup of relevant scientific data. There is a dire need of mentioning the shelf life of these Ayurvedic formulations based on scientific evidence. It becomes an important aspect to set acceptance criteria for shelf-life estimation considering different classes of phytoconstituents present in Ayurvedic formulations. Although the markers used in our study were analytical markers (Magnoflorine, Palmatine, and Berberine). Further studies using active markers (Guggulusterone) can be undertaken. The impact of temperature and humidity on other stability-indicating parameters, container closure systems, and different manufacturing processes must be explored to evaluate the quality and stability of Kaishora Guggulu. The determined expiration dating of the

formulation was limited to the randomly determined batches for research. The shelf life of Kaishora Guggulu estimated in the study was concluded based on a 12-month study which can be extrapolated further for 5 years. A real-time commercial study considering the entire shelf life should be done to validate the shelf life of Kaishora Guggulu.

ACKNOWLEDGMENTS

The authors are grateful to the Department of Pharmaceutical Quality Assurance, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal for providing the necessary facility to conduct the research work.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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[RJC-8336/2023]