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Development and validation of Stability Indicating HPLC method for estimation of Embelin in *Embelia tsjeriam cottam(Vidanga)*

Research Article

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Abstract

Embelin, is an active phytoconstituent obtained from fruits of *Embelia tsjeriam cottam,* commonly known as *Vidanga.* A simple stability indicating HPLC method for the estimation of Embelin has been developed and validated as per ICH Q2A(R1). Sample was eluted using HiQsil C_8 (4.6mm×250mm) column. The mobile phase consisted of Methanol: Acetonitrile: 1% O-phospheric acid in water in the ratio of 70:15:15 v/v/v which was sonicated to degas and delivered at a flow rate of 1ml/min at ambient temperature. The retention time of Embelin was 6.05 ± 0.2 minutes. Studies were performed using an HPLC system equipped with a UV detector; the response was monitored at 291 nm. A good linear relationship over the range of 2–10 µg/ml concentrations with correlation coefficient value of 0.999 was obtained. The accuracy of the method is indicated by good recovery in the range of 98.9-101.2 % and precision less than 2% RSD. The limit of detection and limit of quantification were found to be 0.47 and 1.44µg/ml respectively. Embelin was subjected to stress conditions as per ICH Q2A(R2) and a significant degradation was found to occur by acid hydrolysis, oxidation and thermal stress. Stress degradation studies on embelin provide an insight into its stability.

Keywords: Embelia tsjeriam cottam, Embelin, Stability indicating HPLC, Validation, stress degradation

Introduction

Herbal medicines are getting popularized in developing and developed countries owing to its natural origin and lesser side effects. Chemical fingerprints obtained by chromatographic techniques are strongly recommended for the purpose of quality control of herbal medicines, since they represent appropriately the "chemical integrities" of the herbal medicines and therefore be used for authentication and identification of the herbal products.

Embelin (2, 5-dihydroxy-3-undecyl-p-benzoquinone), is an active principle of *Embelia tsjeriam cottam* commonly known as *vidanga*. *Vindanga* is official in Indian pharmacopia 2014(1) and Ayurvedic Pharmacopia (2). *Embelia tsjeriam cottam* belonging to the family Myrsinaceae, is a climber found in the Western Ghats of Lonavala and also seen in the southern states of Maharashtra, Karnataka, Kerala, Tamil Nadu and Andhra Pradesh upto an altitude of 1600m. The fruits of this plant contain (2.5-3.1%) embelin on dry weight basis. Embelin shows diverse pharmacological activities including chemo prevention in hepato□carcinogenesis observed in Wistar rats(4), antifertility effects(5), wound healing(6), antibacterial (3,7), free radical scavenging(8) and in vitro cytotoxic activity (9)

Literature survey reveals UV spectrophotometric method reported for the estimation of Embelin in plant and in pharmaceutical dosage form(10-11),HPLC(12-14,16) and HPTLC(15).In the present study, HPLC method have been developed as chemical fingerprints for *Vidanga* extract and *Vidanga* churna formulation using Embelin as an active chemical marker and to observe the effect of various stress conditions on Embelin.

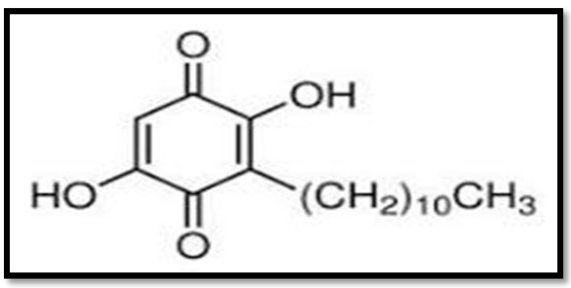

Fig.1:Chemical structure of Embelin

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Materials and Methods

Chemical and Reagents:

Vidanga berries and Embelin was purchased from Yucca enterprises, Mumbai and was used as such, without any further purification. Methanol (HPLC), Acetonitrile (HPLC grade), o-phosphoric acid, were purchased from S. D. fine chemical Laboratories, Mumbai. Hydrochloric acid (HCl), hydrogen peroxide (H_2O_2, 3% v/v) and sodium hydroxide (NaOH) were purchased from LOBA CHEMIE PVT. LTD., Mumbai.

Equipment:

Quantitative HPLC was performed using isocratic high performance liquid chromatography (Jasco HPLC system) with a LC-PU 2080 Plus pump, manual injector with loop volume of 20µL (Rheodyne), programmable MD 2010 UV detector and HiQsil C8 Column (250 mm x 4.6 mm, 5µm particle size). The HPLC system was equipped with "Borwin- UV software (version1.5) software. An electronic balance (Shimadzu AY-120), UV-Visible (Jasco model V-550) spectrophotometer, Elga Labwater (PURELAB UHQ-II) water purification system were used in this study.

Optimized Chromatographic conditions:

Embelin was analyzed using various mobile phases like water and methanol, Acetonitrile and water in varying proportion. After several trials, Methanol: Acetonitrile: 1% o-phospheric acid in water in the ratio of 70:15:15 v/v/v was selected as the mobile phase. Analysis was performed on HiQsil C_8 column (250 x 4.6 mm i.d, 5 µm) which gave good resolution and acceptable system suitability parameters. The flow rate of mobile phase was maintained at 1 ml/min and the response was monitored at 291 nm with a run time of 10 min.

Preparation of Standard stock solution:

Standard stock solution was prepared by dissolving 10 mg of Embelin in 10 ml of methanol to get concentration of 1000 µg/ml. From the standard stock solution, working standard solution was prepared containing 100 µg/ml of Embelin.

Preparation of Extract solution:

The 50g coarse sized dried berries powder was weighed and used for the extraction by using maceration technique and separately using the soxhlet apparatus for 5 cycles. Solvent used was Ethyl alcohol The extract was concentrated on water bath at 50^0C. % yield obtained was 9%.The assay was carried out by weighing 0.25g of extract and dispersed in 10 ml of methanol which was diluted further to get concentration of 2.5ug/ ml solution.

Preparation of sample solution (Formulation Analysis):

Formulation analysis is carried out as per label claim of the marketed formulation (*Vidanga Ghana*) which is claimed to contain 250mg of aqueous extract of *vidanga.* The assay was carried out by weighing tablet equivalent to 0.25g of extract in 10 ml of methanol which was diluted further to get a concentration of 2.5ug/ml extract solution.

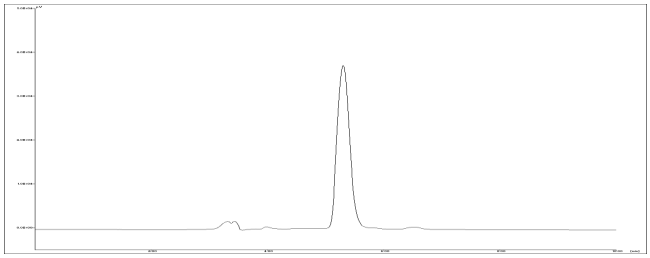

Fig.2: Chromatogram of Methanol: ACN: 1% o-phosphoric acid in water (70:15:15v/v/v)

The system suitability parameter is given in Table.1 below

Table.1: System suitability parameter

Name	RT (min)	Conc. (µg/ml)	Area (µV. Sec)	Plates	Asymmetry
Embelin	6.05 ±0.3	4	383203	2204	1.01

Stress degradation studies

Stress degradation studies were carried under condition of acid/ base/ neutral hydrolysis, oxidation, dry heat and photolysis. For each study, samples were prepared as per ICH Q1A(R2)(17) using High-Performance liquid chromatography (HPLC) analysis. The endeavor was to quantify degradation of embelin under various stress conditions.

Acidic degradation

Acid induced degradation was performed by adding 1 ml of 0.01 N Hydrochloric acid (HCl) to volumetric flask containing 1ml of Embelin standard solution (100µg/ml). The volume was made up to 10 ml with methanol & kept for 24 hrs in dark place. The solution was neutralized with Sod. Hydroxide solution. Final solution (10µg/ml) was injected into the HPLC system.

Alkaline degradation

Base induced degradation was performed by adding 1 ml of 0.1 N Sodium hydroxide (NaOH) to volumetric flask containing 1ml of Embelin standard solution (100µg/ml).The volume was made up to 10 ml with methanol & kept for 24hrs in dark place. The solutions were processed in the same way as acid degradation and neutralized with hydrochloric acid before injection.

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Neutral hydrolysis

Neutral hydrolysis was performed by adding 1ml of Embelin standard solution (100µg/ml) was mixed with 1ml of water in 10ml of volumetric flask and the volume was made upto the mark with methanol. Solution was kept for 24hrs, 1 ml of neutral stressed solution (100µg/ml) diluted with mobile phase and volume made up to 10 ml. 20 µl aliquots was injected into HPLC system.

Oxidative degradation

Oxidative degradation was performed by adding 1ml of Hydrogen peroxide (H_2O_2, 3% v/v) to volumetric flask containing 1ml of Embelin standard solution (100µg/ml). The volume was made up to 10 ml with methanol & kept for 6 hrs in dark place.

Degradation under dry heat (thermal degradation)

Dry heat degradation was performed by adding 1 ml of the standard Embelin to 1ml of dry heat exposed Embelin solution (100ug/ml) which was placed in an oven at 80°C for 8 hrs. The solution were mixed in 10 ml volumetric flask and volume was made with mobile phase, 20 µl was injected into HPLC system.

Photo-stability studies (18)

For photo stability study, the standard drug was exposed to UV light up to 200 watt hours/square meter and subsequently to cool fluorescent light to achieve an illumination of 1.2 million Lux.Hr. Sample was weighed, dissolved and diluted, 1 ml of photo exposed drug solution (100 µg/ml) and 1 ml of standard Embelin were mixed in 10 ml volumetric flask and volume was made with mobile phase. Then analyzed under the optimized chromatographic conditions.

Validation of Analytical method

The method was validated as per ICH Q2 (R1) guidelines (19)

Specificity

The specificity was carried out to determine whether there are any interference of any impurities (presence of components may be expected to be present). The specificity of the method was ascertained by peak purity profiling studies.

Linearity and Range

It is the ability to attain test results that are directly proportional to the concentration of analyte in the sample. From the standard stock solution (1000µg/ml), the linearity of the method was established by a spotting a series of sample of Embelin, the solutions of five different concentration levels 2-10µg/ml was injected. Calibration curves for the standard Embelin were constructed by plotting the peak area against their respective concentrations. Linear regression was applied and slope, intercept, and correlation coefficient-R^2 were determined five replicates per concentration were injected. The linearity (relationship between peak area and concentration) was determined

Accuracy

Accuracy is determined in terms of percentage recovery. The accuracy study was performed at 80%, 100% and 120 % for Embelin. Standard and sample solutions were applied to TLC plate in triplicate and percentage recoveries of Embelin were calculated. The area of every level was used for calculation of % recovery.

Assay

The raw material and marketed formulation of *Vidanga* were selected for the assay method.

Assay of Embelin from Formulation

The assay was carried out by weighing tablet equivalent to 0.025g of extract in 10 ml of methanol from which 5ml of solution was diluted to get a concentration of extract solution Analysis was repeated for three times. Sample solution was injected and area was recorded. Concentration and % assay was calculated.

Formulation Name	Manufacturer	Mfg. Date	Expiry Date
Vidanga Ghana	Chaitanya Pharmaceuticals, Pvt,ltd	September 12 ,2014	September 15, 2015

Assay of Embelin from Extract

Assay of extract was done by weighing 0.25g of extract in 10ml of methanol. From which 0.1ml was further diluted to 10ml and further to get a concentration of 2.5ug/ml**.** Analysis was repeated for three times. Sample solution was injected and area was recorded. % assay was determined from linearity equation.

Precision

Express the closeness of agreement between the series of measurement obtained from multiple sampling of same homogeneous sample under the prescribed conditions. System precision was determined in terms of repeatability.

Intraday precision:

Precision of the system was evaluated by analyzing six independent standard solutions of 2ug/ml and the peak area was determined and expressed as a mean and %RSD calculated from the data obtained.

Interday precision:

Precision of the system was evaluated by analyzing three independent standard preparations on

three different days and %RSD calculated from the data obtained.

Limit of Detection (LOD) and Limit of Quantitation (LOQ)

LOD and LOQ were calculated by using the formula:
LOD = 3.3(SD)/S and LOQ= 10 (SD)/S,
Where SD = the standard deviation of response of Embelin area for the lowest conc. in the range and S = the slope of the calibration curve.

Robustness:

Robustness of the method was determined by carrying out the analysis under conditions during which wavelength, flow rate were altered and the effects on the area were noted.

RESULTS AND DISCUSSION

Stress degradation studies:

The results of stress degradation studies are summarized below;

Acid Hydrolysis

Degradant was observed at a Rt of 6.7 with percent recovery of 76.94%.

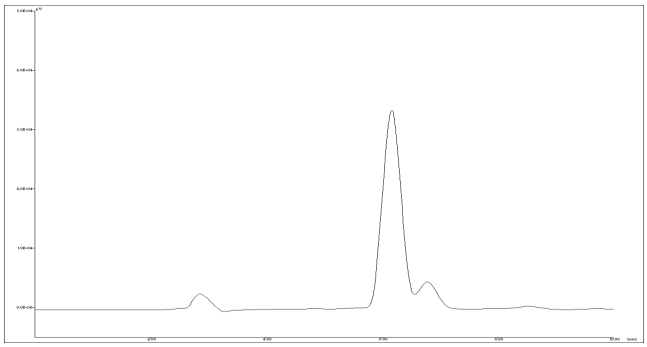

Fig.3: Chromatogram of Acid treated Embelin (10µg/ml).

Oxidative Degradation

Degradant was observed at a Rt of 3.4 with percent recovery of 75.29%.

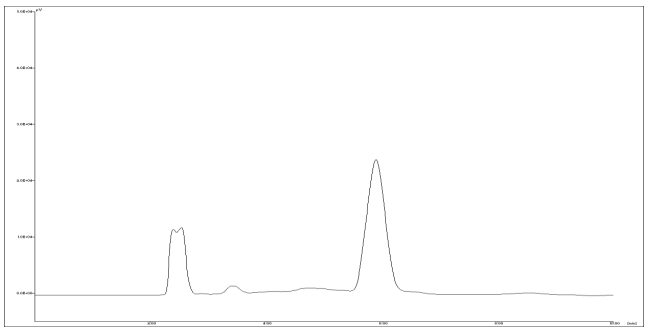

Fig.4: Chromatogram of H_2O_2 treated Embelin (10µg/ml).

Dry heat Degradation

Degradant was observed at Rt of 4.2 with percent recovery of 79.22%

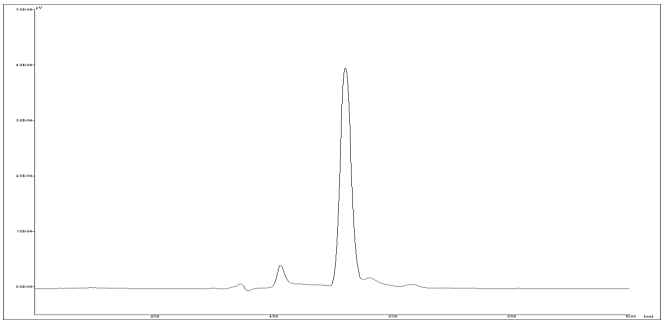

Fig.5: Chromatogram of Dry heat treated Embelin (10µg/ml).

Alkali Degradation

Average 83.50% of Embelin was recovered with no peak of degradation in alkaline condition.

Photolytic Degradation

Average 82.63% of Embelin was recovered with no peak of degradation after photolytic degradation.

Neutral Degradation

Average 87.63 % of Embelin was recovered with no peak of degradation in neutral condition.

Table.2: Summary of Stress degradation studies

Stress Degradation Condition	Peak Area	% Recovery	Rt of degradation product
Base (0.1 N NaOH, kept for 24 hrs)	838662	83.50	-
Acid (0.1 N HCl, kept for 24 hrs)	775116	76.94	6.7
Neutral (kept for 24hr)	882513	87.63	-
H_2O_2 3% (kept for 6 hrs)	758524	75.29	3.4
Heat dry (60^0C, 8 hrs)	798023	79.22	4.2
Photo stability (UV, 200 watt hrs/square meter and Florescence 1.2 million Lux. Hrs)	832256	82.63	-

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The results of stress degradation studies of Embelin obtained by us fairly match the ones reported by Ferreira G and Laddha KS[16].

The results indicate that Embelin is prone to oxidative, thermal and acid hydrolytic degradation. A well resolved product of degradation was obtained under these conditions. This method can distinguish between standard and degraded Embelin.

Validation of analytical method

Specificity:

The peak purity values were found to be more than 980, indicating the non interference of any other peak of degradation product, impurity or matrix.

Linearity and Range:

In the linearity parameter calibration curve was constructed by plotting peak area against respective concentration of Embelin. The plots were found to be linear in the range of 2-10µg/ml with coefficient of correlation (r2) 0.999 for Embelin as shown in Table 3 below.

Fig.6: Chromatogram of Linearity of Embelin

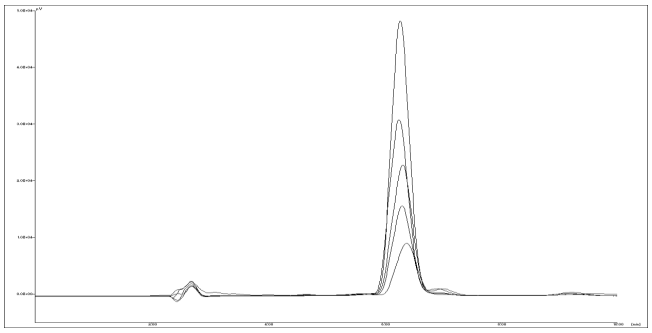
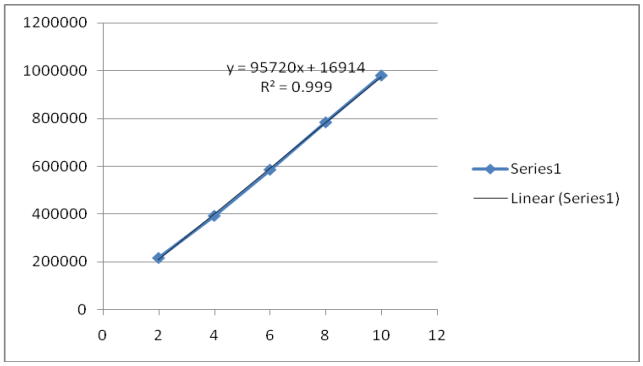


Table.3: Linearity study of Embelin

Sr.no.	Conc (µg/ml)	Peak Area
1	2	216714
2	4	392519
3	6	585241
4	8	783059
5	10	978647

Fig.7: Calibration curve of Embelin



Accuracy

To check the accuracy of the method, recovery studies were carried out by addition of standard marker solution to pre-analyzed sample solution at three different levels 80 %, 100 % and 120 %. The percentage recovery was found to be in a range of 98.9-101.2%.

Assay

The raw material and marketed formulation of Vidanga were selected for the assay method.

Assay of Formulation

Table.4: Assay for Embelin from Vidanga Ghana

Injected Sample (µg/ml)	Area	% Assay
2.5	228531	0.88
2.5	226538	0.87
2.5	228918	0.88

Assay of Raw material

Table.5: Assay for Embelin from Vidanga extract

Injected Sample (µg/ml)	Area	% assay
2.5	273231	1.07
2.5	270282	1.05
2.5	272699	1.06

Precision

The Intraday precision was found to be 0.66. Interday precision was calculated at three levels 2,4,6ug/ml which was found to be 0.56%,0.78%,1.68%.Thus the Intraday and Interday precision were found to be within the limits.

Limit Of Detection(LOD) and Limit Of Quantitation (LOQ)

The LOD and LOQ values were found to be 0.47and 1.44µg/ml respectively.

Robustness:

Robustness of the method was determined by carrying out the analysis under conditions during which wavelength, flow rate were altered and the effects on the area were noted. The % RSD was found to be within the limits and the method was found to be robust.

Table.6: Summary of validation parameters

Sr.no.	Validation parameter	Embelin
1.	Linearity Equation (r2) Range	Y=95720x-16914 r^2=0.999
2.	Precision (% RSD)	
	Intraday	0.66%
	Interday	1.17%
3.	Accuracy	
	80	101.2%
	100	99.7%
	120	98.90.%
4.	Limit of Detection	0.47ug/ml
5.	Limit of Quantification	1.44ug/ml
6.	Specificity	Specific
7.	Robustness	Robust

Conclusion

The developed method was found to have all parameters within limits of ICH guidelines. When exposed to stress conditions Embelin was found to be very much susceptible to Acidic, Oxidative and Dry heat condition. Since the stress degradation products of Embelin are well resolved, this method has stability indicating property. It may be used in phytopharmaceuticals formulation industry to monitor the stability of active marker compound, to analyse the plants for Embelin content before taking up the production. Since the reported method involves a higher percentage of costly solvent Acetonitrile, our developed method is cost effective Hence our validated HPLC method can serve as a Quality control parameter for herbal raw materials containing Embelin and can be readily adapted for routine analysis of Embelin.

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