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High Performance Liquid Chromatography Method Validation and Forced Degradation Studies of Chrysin

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ABSTRACT: Chrysin is belonging to flavone class. It is found in *Passiflora incarnata, Oroxylum indicum*, mashroom, honey and propolis. An isocratic LC method was developed, optimized and validated for Chrysin. The method was validated accord¬ing the Q2 (R1) guidelines of ICH with respect to linearity, range, system suitability, LOD, LOQ, accuracy, precision. Chromatographic separation were performed on Agilent EC-C-18 column (100 x 4.6 mm, 2.7 um). The mobile phase composed of 0.1% acetic acid in Water: Methanol (25:75 v/v). The flow rate was 0.8 mL/min. The developed method was applied to the different extracts of *Passiflora incarnata* for estimation of Chrysin using advanced extraction techniques. Chrysin was also estimated in marketed formulation. The retention time for Chrysin was 2.57 min. the calibration curve of Chrysin was linear (R2-0.999) in the concentration range of 2-20 ng/ml. The inter-day accuracy ranged -0.52 to 4.77 % and intra-day accuracy ranged from -0.45 to 4.88%. The interday precision was from 0.368 to 1.68 % and intraday precision was ranges from 0.0121 to 1.35 %. The limits of detection and quantification were 0.004748 and 0.014244 respectively. Chrysin showed significant degradation when exposed to water, acid, base, oxidizing agent, and UV light. Chrysin was estimated in Passiflora incarnata leaves extracted by different extraction techniques viz. SAE, UAE, ASE and in Marketed formulation. An accurate, precise and novel HPLC method was developed and validated. The method was successfully used to estimate the Chrysin in *Passiflora incarnata* extracts and its marketed formulation.

KEYWORDS: Chrysin; HPLC method development; Validation; Forced degradation studies.

1. INTRODUCTION

Chrysin (5, 7-di hydroxyl-2-phenyl-4H-chromen-4-one) (Fig.1) is chemical compound found in *Passiflora incarnata*, Oroxylum indicum, mashroom, honey and propolis belongs to flavone class. Chrysin is use worldwide in many herbal medicines for various diseased conditions. In recent years, chrysin is reported as bioactive principle. It is used as an anti-aging component [1]. Chrysin also showed modification in urinary concentrations of testosterone in human male. Chrysin has shown to be an inhibitor of aromatase enzyme activity. Chrysin also exhibit anti-inflammatory activity. Previous studies also reveal that Chrysin is rapidly excreted from the body upon oral administration and therefore its bioavailability is very poor [2].
Several analytical methods were developed for Chrysin like UV spectroscopy, IR spectroscopy, HPTLC, HPLC and RP-HPLC [3-14].

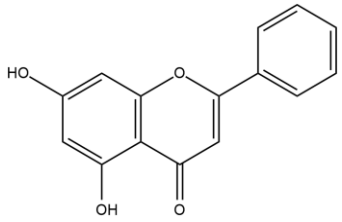

Figure 1. Chemical Structure of Chrysin

In this study, HPLC method development, validation and force degradation studies were carried out for Chrysin. Amount of Chrysin was determined and quantified in *Passiflora incarnata* extracts and its marketed

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formulation by HPLC method. Till today, there is no any report on estimation of Chrysin from *Passiflora incarnata* leaves by HPLC method.

2. RESULTS AND DISCUSSION

2.1 Optimization of RP-HPLC Method

Under the prescribed experimental conditions as shown in table 1, peak of Chrysin was well defines and free from tailing with short retention time i.e. 2.57 min shown in figure 2.

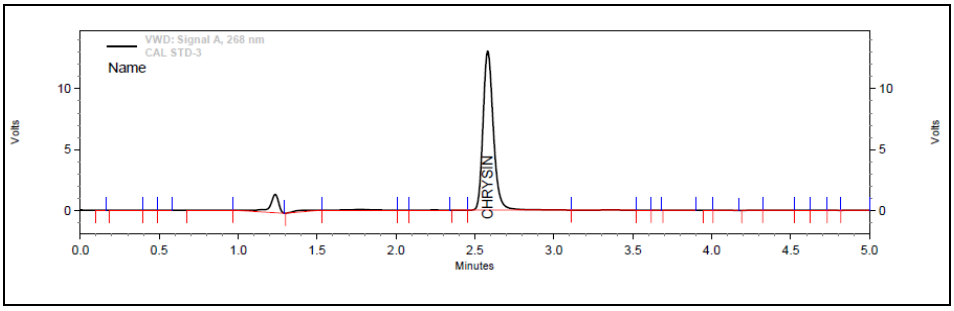

Figure 2. HPLC chromatogram of Chrysin standard

Table 1. The optimized chromatographic conditions

Separation variable	Optimized conditions
Chromatography	Agilent 1260 Series
Column	EC-C-18, 100 x 4.6 mm, 2.7 um, Agilent
Mobile phase	0.1% acetic acid in Water: Methanol (25:75 v/v)
Flow rate	0.8 mL/min
Total Run Time	5 min
Pressure	146-147 bar
Temperature	40 degree Celsius
Detection wavelength	268 nm
Retention time of Chrysin	2.57 min

2.2 System suitability

System suitability parameters were used to ensure that the method can generates the results of acceptable accuracy and precision. The system suitability parameters, such as retention time, peak area, and theoretical plates were calculated and compared with the standard values. The results are shown in Table 2.

Table 2. System suitability parameters for Chrysin

Parameter	Acceptance criteria	Results	
		Chrysin	%RSD
Retention Time	%RSD ≤ 2%	2.582	0.115
Peak Area	%RSD ≤ 2%	417718	1.222
Theoretical plates	≥ 2000	8501	0.421

2.3 Method validation

2.3.1 Linearity and Range

Least squares linear regression analysis of the calibration curve was established for determination of linearity. The calibration curves were linear over the concentration range of 2-20 ng/ml. Peak areas of Chrysin was plotted against respective concentration (Fig. 3). Correlation coefficient was found to be 0.999. The regression equation was

$$y = 5.89581x + 0.0885205 \ (R = 0.9999)$$

The peak area against concentration is shown in table 3.

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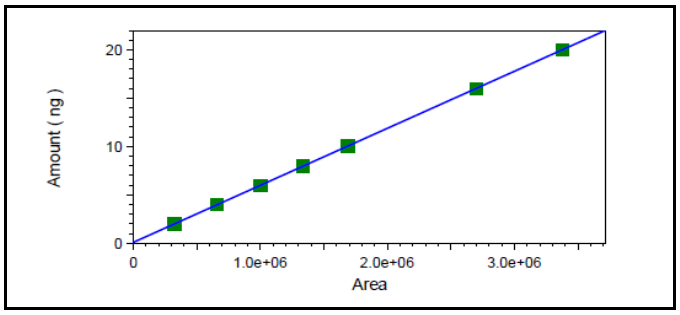

Figure 3. Calibration curve for Chrysin

Table 3. Linearity of Chrysin

Conc. (ng/mL)	Peak Area
2	328256
4	659797
6	1002005
8	1337557
10	1685545
16	2699366
20	3376758
Slope	5.8958
y-intercept	0.0885
R^2	0.9999

2.3.2 Accuracy

The accuracy of developed method was determined by recovery experiments which were carried out by spiking aliquots of Chrysin with different concentration range at three levels. The % relative standard deviation and percent recovery were calculated as depicted in table 4.

Table 4. Intra-day & Inter-day accuracy data for Chrysin

Sample	Nominal Conc. (µg/ml)	Accuracy (Diff. %)					
		Intra-run (n=9)			Inter-run (n=9)		
		Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
LQC	3	4.12	4.88	3.91	4.18	4.56	4.77
MQC	9	2.22	4.10	3.04	2.93	2.54	3.62
HQC	19	-1.34	-0.84	-0.45	-0.98	-0.74	-0.52

2.3.3 Precision

The precision of method was studied at 2 levels intraday (repeatability) and interday precision (intermediate precision). The repeatability was performed by testing three different solution of Chrysin solution at 3, 9 and 19 µg / mL on the same day and three sample solution also evaluated at three different days. Results were reported in terms of RSD (stable 5).

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Table 5. Intra-day & Inter-day precision data for Chrysin

Sample	Nominal Conc. (µg/ml)	Precision (% CV)					
		Intra-run (n=9)			Inter-run (n=9)		
		Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
LQC	3	1.35	0.9247	0.8211	1.13	1.54	1.29
MQC	9	1.32	0.7076	0.9474	1.68	1.59	1.73
HQC	19	0.9312	0.6927	0.0121	1.32	1.06	0.368

2.3.4 Robustness

Evaluation of robustness is depending on the assay method, type of procedure conditions etc. It was tested by minor changes in chromatographic conditions as depicted in table 6.

Table 6. Robustness study for Chrysin

Parameter	Setting	Chrysin			
		RT	% RSD	Amount (ng)	% RSD
Column temperature (°C)	38	2.23	0.0633	8.95	0.5658
	40	2.67	0.0162	9.08	0.8553
	42	2.23	0.1199	8.39	1.3868
Mobile phase flow rate (ml/min)	0.7	2.89	0.0854	8.87	1.7123
	0.8	2.6	0.0267	9.01	0.5433
	0.9	2.41	0.0675	8.95	0.8545
Mobile phase composition (%, v/v)	24:76	2.79	0.0946	8.87	0.9341
	25:75	2.58	0.0214	9.01	0.2279
	26:74	2.45	0.0843	8.88	0.9905

2.3.5 Force degradation studies

Forced degradation is essential study for the development of stability-indicating analytical method. This study helps in estimation of shelf life of drug. The forced degradation study of Chrysin was performed. Significant degradation were observed when exposed to water, acid (0.1N HCl), base (0.1N NaOH), oxidizing agent (10% H_2O_2), and UV light. Percent degradation under different conditions is summarized in table 7 and Chromatograms are shown in Figure 4.

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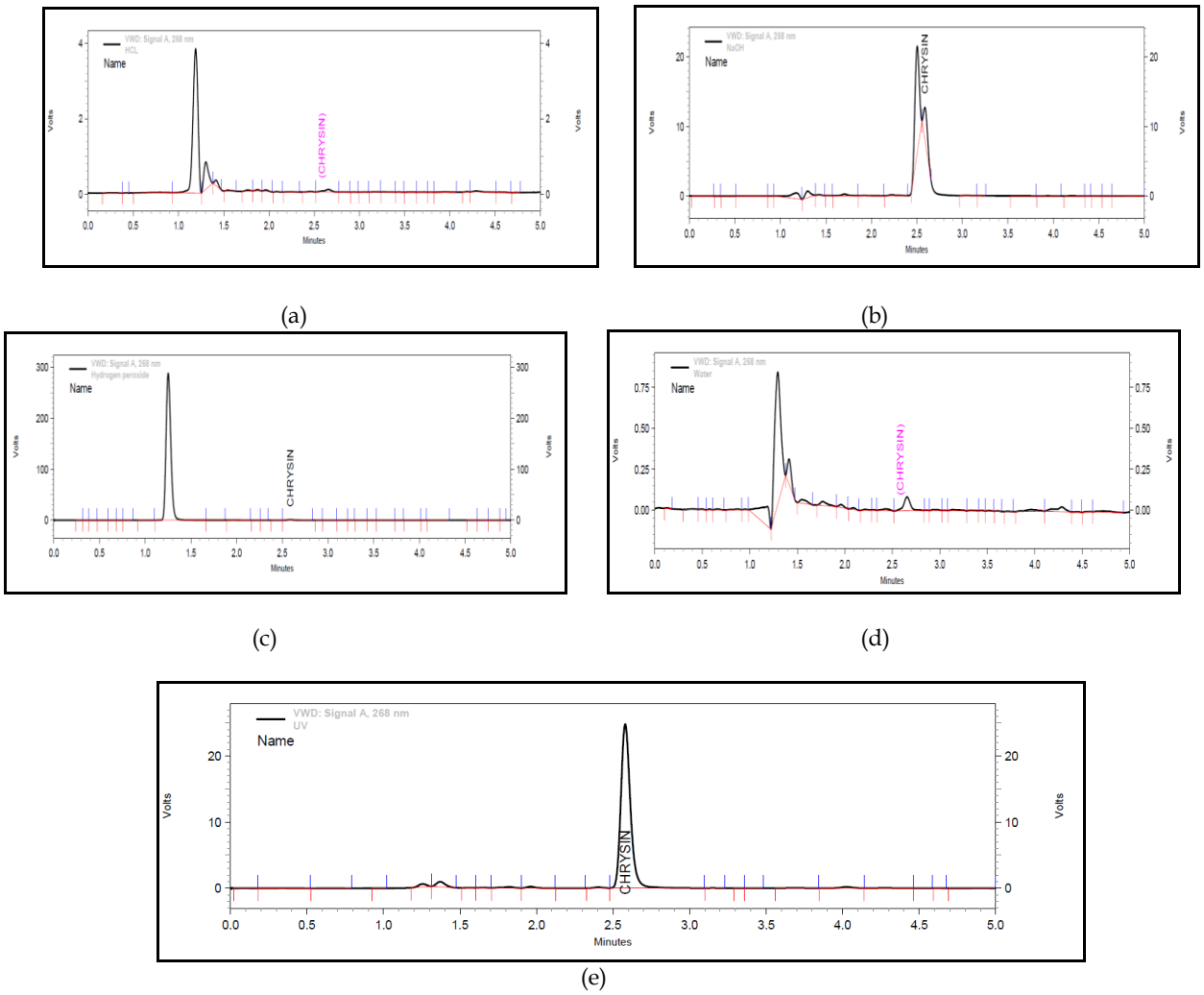

Figure 4. Forced degradation study representing Chrysin behavior in 0.1N HCl (a), 0.1 N NaOH (b), 10% H_2O_2 (c), Hydrolytic degradation (d), Exposed to UV light (e)

Table 7. Summary of stressed degradation studies

Drug	**% of degradation**				
Chrysin	**Acid**	**Base**	**Oxidation**	**Water**	**UV**
	100	87.52	66.80	100	14.64

2.4 Application of proposed HPLC method

Chrysin content was estimated using developed HPLC method in *Passiflora incarnata* leaves extracted by SAE, UAE, ASE and its marketed formulation. % Chrysin content is depicted in table 8. Extraction using SAE showed highest amount of Chrysin.

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Table 8. % Chrysin content in different extracts and in marketed formulation

Extraction Method	% Chrysin content
SAE	0.15 ± 0.04
UAE	0.10 ± 0.03
ASE	0.11 ± 0.06
Marketed formulation	0.09 ± 0.02

3. CONCLUSION

A simple and efficient HPLC method was developed and validated for Chrysin. Force degradation studies were performed under acid, base, oxidation, neutral hydrolysis and UV light. The method was found to be precise, linear, specific, accurate and robust. Validation results were found acceptable limits as per the guidelines. Proposed HPLC method successfully used for estimation of Chrysin in in *Passiflora incarnata* extracts and its marketed formulation.

4. MATERIALS AND METHODS

4.1 Chemicals and Reagent

Chrysin was purchased from TCI chemicals (India) Pvt. Ltd. HPLC grade Methanol and acetic acid was procured from Rankem. The high-purity HPLC grade water was used for the study.

4.2 Instrumentation

An agilent HPLC with a G1329B auto sampler, a G1311C pump, G1314F UV detectors and EZchrome software was used. Chromatographic separation was performed using EC-C-18, 100 x 4.6 mm, 2.7 um, Agilent column. Mobile phase was degassed by using Ultrasonicator (PCi Analyticals). Vibra HT (Essae) analytical balance was used for weighing of chemicals, Lab link "Extra pure" water purification system was used to obtained HPLC grade water.

4.3 Chromatographic conditions

Mobile phase consisted of 0.1% acetic acid in Water: Methanol (25:75 v/v) used for HPLC analysis. The mobile phase was degassed prior to use by applying ultrasonication for 10 min. It was filtered through 0.22µm filter by applying vacuum. Flow rate was set at 0.8 ml/min. Column oven temperature was set at 40°C. The detection was carried out using UV detector at 268 nm.

4.4 Preparation of standard stock solution

Stock solutions-I of 1 mg/mL was prepared by solubilizing 1 mg of Chrysin in 1 ml of HPLC grade methanol. The prepared stock solution was then filtered through 0.45µm nylon membrane syringe filter.

4.5 Method Validation

Method validation studies were performed based on protocol given in ICH guidelines and USFDA. The method was validated for accuracy, precision, robustness, linearity, specificity, LOD and LOQ [15-22].

4.5.1 System Suitability

System suitability is performed to determine and verifies resolution and reproducibility. It is generally used to ensure that the complete system is suitable for intended analysis including instruments, reagents, column analyst etc. The standard concentration of 1 ng/mL of Chrysin was repeatedly analyzed. The RSD of area and retention time was determined as per the ICH guidelines.

4.5.2 Linearity & Range

The linearity of Chrysin was obtained by preparing the calibration standard curve in HPLC grade methanol. The calibration standard was prepared using seven replicates (n = 7). The different concentrations of Chrysin were injected to HPLC covering range of 2 to 20 ng/mL (2, 4, 6, 8, 10, 16, 20 ng/mL). The peak areas

obtained from each calibration standard were plotted against concentration. The linearity was determined by linear regression analysis.

4.5.3 Accuracy (% Recovery)
Accuracy of developed method was evaluated as per ICH guidelines by injecting the Chrysin at three different levels of concentration (80%, 100% and 120%) which produces the final concentration 3, 9 and 19 µg/ mL. Percent recovery was calculated from the obtained peak area.

4.5.4 Precision
The precision of the developed analytical method was estimated by performing Intra-day and Inter-day precision studies. It was evaluated by repeatability of three concentrations three times a day (i.e. morning, afternoon an evening). Intra-day and inter-day precision results were expressed in terms of % RSD.

4.5.5 Robustness
Robustness of analytical method is measure by applying minor changes in method parameters. Robustness was studied by assessing the effect of deliberate variation in chromatographic conditions. These changes were made to determine its effect on the method. Robustness was evaluated by calculating % RSD and percent of recovery.

4.5.6 Limit of detection (LOD) and Limit of quantification (LOQ)
The limit of detection (LOD) is the lowest amount of sample that can be detected, but not necessarily quantitated, while the limit of quantification (LOQ) is the lowest amount of sample that can be quantitatively determined with suitable precision [23, 24]. LOD and LOQ were determined by injecting the low concentration of Chrysin using developed HPLC method.

4.5.7 Force degradation studies
Forced degradation commonly known as stress testing carried out to develop and validate a stability indicating method. Forced degradation is the degradation of drug and drug substances more severe than accelerated conditions. ICH guidelines illustrate several degradation conditions like heat, light, oxidation, acidic, basic etc [25, 26]. A force degradation study is very essential step for validation of stability indicating method [27-31].

A. Acid and alkali hydrolysis
Solution of 1 mg/mL was prepared by solubilizing 1 mg of Chrysin in 1 ml of HPLC grade methanol. From stock, 125 µl solution of Chrysin was added to 5 ml 0.1N HCL and 0.1 N NaOH and the mixture was refluxed in water bath (80°C) for 2 hours. Sample was then cooled, neutralized with water and analyzed using the HPLC method.

B. Oxidation
125 µl aliquot of a 1 mg/ml solution of Chrysin was added to 5 ml of 10% hydrogen peroxide solution and refluxed at a temperature of 80°C for 2 hrs. Sample was neutralized with water and analyzed using the HPLC method.

C. Thermal conditions
125 µl solution of Chrysin was added to 5 ml HPLC grade pure water and mixture was refluxed in water bath at 80°C for 2 hrs. The resulting solution was then neutralized and analyzed using the HPLC method.

D. Photolytic conditions
1 mg of Chrysin powder was exposed to UV light (254 nm) for 2 hrs. Then it was dissolved in 1 ml water and analyzed using the HPLC method.

4.6 Application of proposed HPLC method
The proposed HPLC method was applied for the estimation of Chrysin in different extracts of *Passiflora incarnata* leaves by using various extraction techniques. The resultant extracts were analyzed by developed HPLC method. The fresh leaves of *Passiflora incarnata* were collected from local area of Aurangabad, Maharashtra. Leaves were washed and dried in micro tray dryer (S. B. Panchal & Company). Dried leaves

were subjected to mixer grinder (Devika mixer grinder) for size reduction and sieved from 120 mesh sieve to get uniform particle size. The final powder was then used for extraction.

4.6.1 Soxhlet assisted Extraction (SAE)

Twenty grams of *Passiflora incarnata* leaves were placed in thimble (Borosil, Mumbai, MH, India) inserted into a Soxhlet apparatus and extracted with 90% ethanol for 8 hr. After the extraction, the resulting sample was collected, filtered and analyzed by developed HPLC method for estimation of Chrysin. The SAE of *Passiflora incarnata* leaves were performed in triplicates.

4.6.2 Ultrasound assisted Extraction (UAE)

Dry powder of *Passiflora incarnata* leaves (20 gms) were extracted in 500 ml glass beaker, immersed in ultrasonic bath (Labman scientific instruments Ltd.) for definite period of time (5, 10 and 15 min). The irradiated powder was transfer to a beaker containing a volume of extraction solvent (mass: solvent ratio 1:5) and sonicated for 5 min at 30°C±5°C. The resulting sample was collected, filtered and analyzed for Chrysin content by HPLC method.

4.6.3 Accelerated Solvent extraction (ASE)

Accelerated solvent extractor (Buchi, Speed extractor E-914) was used for extraction. Powdered drug (5 gms) were homogenized with specific proportion of diatomaceous earth and placed in stainless steel cells. Extraction was performed in different conditions of temperature, pressure, no. of cycles and hold time as shown in table 9.

The sample from all batches were collected, filtered and analyzed by developed HPLC method. Amongst three batches, one optimized batch was selected based on results of HPLC analysis.

Table 9. Extraction batches for ASE of *Passiflora incarnata*

Extraction Batches	Temperature	Pressure	No. of cycles	Hold Time
Batch 1	50°C	50 bar	2	5 min
Batch 2	70°C	70 bar	4	10 min
Batch 3	90°C	90 bar	6	15 min

4.6.4 Marketed formulation

The Chrysin content was estimated in marketed formulation (Solaray dietary supplement) containing *Passiflora incarnata* extract. The marketed formulation was purchased from local market (Aurangabad). 20 capsules were taken, shells were removed and powder was transferred to conical flask. An amount equivalent to the labeled claim (350 mg of *Passiflora incarnata* extract) was accurately weighed and taken in an extraction flask followed by 10 mL of ethanol. The mixer was subjected to sonication (Labman scientific instruments Ltd.) for 30 min and centrifuge at 10,000 rpm for 10 min (Thermo scientific Sorvall Legand Micro 21 R Centrifuge). The supernatant was filtered across 0.22 µm syringe filters. Resulting sample was analyzed for Chrysin content using developed HPLC method.

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