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SHORT COMMUNICATION

Development and Validation of a High-Performance Liquid Chromatography Method for Hesperidin in Pharmaceutical Formulations

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Abstract

Background: Hesperidin, a bioflavonoid with diverse pharmaceutical applications, necessitates a reliable analytical method for its quantification in formulations. This study developed a straightforward, sensitive, and economical High Performance Liquid Chromatography (HPLC) method to address this need. **Aim:** To develop an easy, rapid, accurate, precise and reliable analytical method for the hesperidin. **Methods:** The separation was performed using a C-18 column HSS (250 x 4.0 mm, 5µm particles). The mobile phase used was a mixture of Methanol:Water in ratio of 50:50 v/v isocratic at 40° C, with a flow rate of 0.9 mL/min and an injection volume of 10 µL. Detection was carried out at 280 nm using a PDA detector. **Results:** The new RP-HPLC technique yielded a correlation value of 0.99 and was demonstrated to be linear in the 10-30 µg/ml range. Hesperidin was shown to have retention period of 4.888 minute. The technique was validated using criteria such as specificity, accuracy, LOD, LOQ, precision, repeatability, robustness. **Conclusion:** The validated HPLC method shows excellent reliability and reproducibility, making it suitable for routine quantitative analysis of hesperidin in pharmaceutical formulations. This technique is effective for quality control, offering a cost-efficient and rapid approach for the determination of hesperidin. It met validation criteria for specificity, accuracy, and precision, proving to be a reliable and cost-effective tool for routine analysis in pharmaceutical formulations.

Major Findings: The newly developed RP-HPLC method for quantifying hesperidin exhibited high linearity (correlation coefficient of 0.9907) within the 10-30 µg/mL range, with a retention time of 4.892 minutes. It met validation criteria proving to be a reliable and cost-effective tool for routine analysis in pharmaceutical formulations.

Keywords: Hesperidin, High Performance Liquid Chromatography, Method Development, Validation

1. Introduction

Hesperidin, a flavonoid, is a flavanone glycoside ($C_{28}H_{34}O_{15}$) that is plentiful in citrus fruits. Hesperidin is the aglycone form of it. Hesperidin, a plentiful and cost-effective bioflavonoid found in (*Citrus aurantium*) peel, has been shown to have various pharmacological benefits such as antioxidant, anti-inflammatory, hypolipidemic, radioprotective, vaso-protective, anticarcinogenic, and cholesterol lowering effects. Hesperidin acts as an enzyme inhibitor, blocking phospholipase A2, lipoxygenase, HMG-CoA reductase, and cyclo-oxygenase-8. Hesperidin enhances capillary health by decreasing capillary permeability. Citrus fruits, berries, onions, parsley, and green tea are some sources of hesperidin[1-5]. As a naturally occurring flavanone glycoside, hesperidin consists of a hesperetin molecule (Figure 1) attached to a rutinose moiety, making it of significant interest in pharmaceutical and nutraceutical industries[6]. Given its therapeutic potential and increasing demand in quality control for formulations, the accurate and reliable quantification of hesperidin is essential.

High-Performance Liquid Chromatography (HPLC) has become the method of choice for the analysis of flavonoids, including hesperidin, due to its sensitivity, precision, and ability to handle complex

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matrices[7]. HPLC methods allow for the separation and quantification of bioactive compounds in plant extracts, pharmaceutical formulations, and biological samples, making them indispensable tools for quality control and pharmacokinetic studies[8]. However, developing and optimizing an HPLC method for hesperidin requires careful selection of chromatographic conditions such as mobile phase composition, detection wavelength, column type, and flow rate to ensure robustness, accuracy, and reproducibility. Multiple HPLC techniques were developed to measure hesperidin, either by itself or in combination with other flavonoids, in plant juices and pharmaceutical products. Certain RP-HPLC methods were cost-inefficient due to the mobile phase composition, column dimensions, and run times[9].

A search in the literature revealed that many analytical methods for quantification of hesperidin from nutraceutical preparations or citrus fruit juices, were simultaneous estimations from a mixture of non-citrus phytoconstituents and were complex[10-13]. Despite the widespread use of this citrus bioflavonoid, a cost-effective, simple, and rapid method with high sensitivity, accuracy, and reproducibility for its quantification remains a critical need. Addressing this gap, the present study aims to develop an easy-to-perform RP-HPLC method for the estimation of hesperidin in pharmaceutical formulations. Utilizing a C18 column, this method is designed to be time-efficient while ensuring excellent precision, accuracy, linearity, and reproducibility.

2. Materials and Methods

2.1 Chemicals and Reagents

Hesperidin, with a purity of > 98%, was purchased from Yarrow Chem Products (Mumbai). HPLC-grade methanol was obtained from Rankem Chemicals (Avantor brand), and all other reagents were of analytical grade. Additionally, HPLC-grade water was sourced from a Milli-Q EQ 7008 lab water purification system (Merck Millipore).

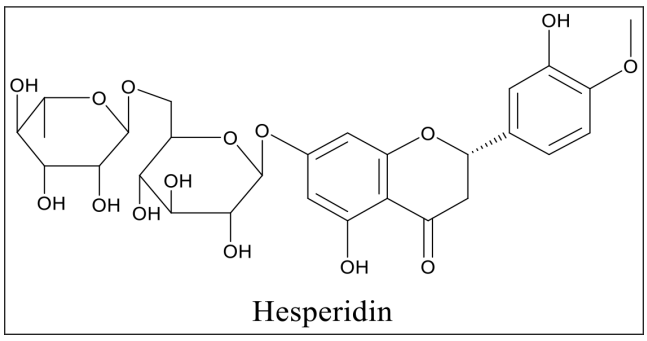


Figure 1. Chemical structure of Hesperidin - $C_{28}H_{34}O_{15}$.

2.2 Instrumentation

HPLC analysis was conducted using a Shimadzu i-Series LC-2050C 3D System (Shimadzu, Japan), equipped with a Photodiode Array (PDA) detector and an autosampler. Chromatographic data analysis was performed using LabSolutions software version 5.106. Additional instruments utilized in the study included an electronic weighing balance (Mettler Toledo ME204), a sonicator bath machine (Analytical Technologies Limited), and a vacuum pump, facilitating precise measurements and sample preparation.

2.3 Selection of Wavelength of Detection

The wavelength for detection of hesperidin was selected on the basis of preliminary studies of hesperidin using UV spectrophotometer. The absorbance maximum was scanned and was compared with reported values.

2.4 Chromatographic Conditions

C18 column containing high strength silica, or HSS, (250 x 4.0 mm, 5µm particles) was heated to 40° C in a column oven. The mobile phase used was a mixture of Methanol:Water in ratio of 50:50 v/v isocratic with an injection volume of 10 µL and a flow rate of 0.9 ml/min. After degassing, each solution was passed through a 0.45µm pore size filter. The UV detector was set at 280 nm, and the column was maintained at 40° C for the duration of the analysis. The total run time was 10 minutes. Peak area was used for quantitative evaluations.

2.5 Preparation of Standard Solution

To create a 1000 µg/ml stock solution, 10 mg of hesperidin was dissolved in 10 ml of methanol. To obtain a stock solution of 100 µg/ml, 1 ml of the aforesaid solution was taken out and the volume was made up to 10 ml with HPLC-grade methanol. To produce10 µg/ml, 15 µg/ml, 20 µg/ml, 25 µg/ml, and 30 µg/ml hesperidin solutions, the 100 µg/ml stock solution was serially diluted. HPLC-grade methanol was used for all of the dilutions.

2.6 Preparation of Sample Solution

The hesperidin formulation was sonicated with HPLC-grade methanol for 5 minutes at 40° C to create a methanolic extract as hesperidin gets solubilized in the methanol. A micro-separation filter (0.45µm, 25mm) that was connected to a syringe was then used to filter the extract to remove the particulate matter from the extract. To get a solution equal to 20 µg/ml hesperidin, the extract is further diluted with methanol. After being moved into a 1 ml HPLC vial, all standard and sample solutions were placed into the HPLC rack.

2.7 Method Validation

The International Council on Harmonization's (ICH) requirements were followed in the validation of the approach[14,15]. According to ICH guideline Q2(R1), the validation metrics were specificity, linearity, accuracy, precision, LOD and LOQ, and robustness. The methods specificity was examined by looking for any indications of interference from the formulation excipients. The blank formulation methanolic extract was injected into the column to check for interference from the excipients. Linearity in a method refers to its capability to produce test results that are directly proportional to the concentration of the sample within a specified range. In HPLC methods, this is assessed by evaluating the correlation between the detector response (measured as peak area/height) and the sample concentration. Linearity was determined by the serial injection of different concentration of drug and recording their chromatograms. The calibration curve, which was created by graphing the solution concentration versus peak area in the range of 10-30 µg/ml. Accuracy studies are typically conducted by measuring the recovery of an analyte spiked into the sample matrix (placebo). Recovery studies were conducted to determine the methods accuracy. In order to assess the recovery and retention time for measuring precision, samples were injected three times in a single day and three times on separate days. For both intra-day and inter-day changes, the peak locations and area were noted and the percentage RSD was computed. The standard deviation of the slope and intercept of the regression equation (S) was obtained by performing the linearity of the standard in triplicate in order to determine the LOD and LOQ. The Limit of Detection (LOD) of an analytical procedure is the minimum amount of analyte in a sample that can be detected but not necessarily quantified with precision. The quantitation limit of any analytical procedure (LOQ) is the lowest amount of analyte in the sample which can be quantitatively determined with suitable precision and accuracy. These determinations are based on the standard deviation of the response and the slope of the calibration curve. The following formula was used to calculate LOD and LOQ: $LOD = 3.3 \times SD/S$; $LOQ = 10 \times SD/S$. The robustness of an analytical procedure refers to its ability to withstand small, intentional variations in method parameters without being significantly affected. It serves as an indicator of the methods reliability under normal operating conditions. Changing the flow rate allowed for the analysis of robustness.

3. Results and Discussion

The present study deliberated the HPLC method development and validation for estimation of hesperidin in pharmaceutical formulations.

3.1 Assay Specificity

Specificity is a critical criterion for the validation of the HPLC analytical procedure, ensuring that the method can accurately identify and quantify hesperidin in the presence of other components. In alignment with the optimized test protocol, several formulations were systematically produced, including blank samples, placebo formulations, standard hesperidin solutions, and drug-loaded formulations containing hesperidin. Each formulation was injected into the HPLC system under controlled conditions. During the analysis, the retention time for hesperidin was carefully monitored, and the resulting chromatograms were examined for any potential interferences from co-eluting substances. As shown in Figure 2, the chromatograms exhibited no significant interference at the retention time of hesperidin, confirming that the method effectively discriminates hesperidin from other constituents. Additionally, the peak purity analysis demonstrated that the methods specificity[13]. These results collectively affirm that the developed HPLC technique is robust and specific for determining hesperidin concentrations in pharmaceutical formulations, making it suitable for quality control and assurance purposes.

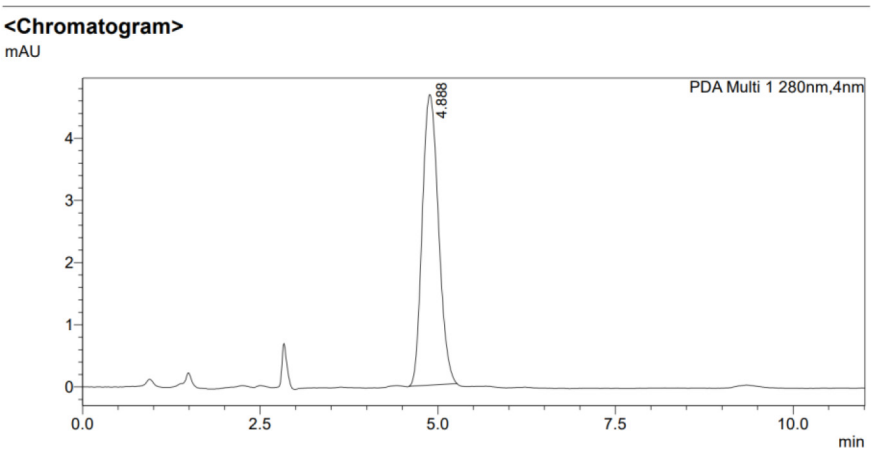


Figure 2. Chromatogram of hesperidin.

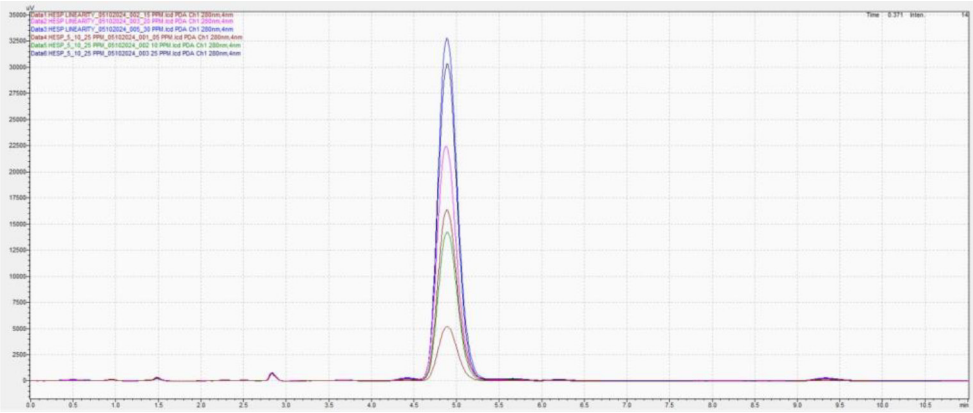


Figure 3. Overlay chromatograms of hesperidin.

3.2 Linearity

Concentration versus area was used to create calibration curves for the standard solution. For the range of standard solutions investigated, the correlation coefficient was computed and found to be 0.9907. All sample concentrations between 10 and 30 µg/ml are collected in the overlay (Figure 3), peak area of hesperidin were plotted against concentration on the calibration graph (Figure 4).

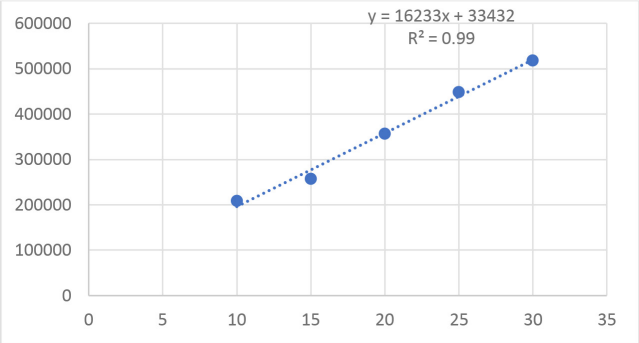


Figure 4. Calibration plot of Hesperidin-concentration range of 10 to 30 µg/ml.

3.3 Accuracy

The proposed method for the analysis of placebo in known quantities of hesperidin solutions at concentrations of 12 µg/ml, 15 µg/ml, and 18 µg/ml of hesperidin was used to evaluate the accuracy. The chosen concentration is equivalent to 80%, 100%, and 120% nominal values and spans the methods linear range. The suggested HPLC method yielded recovery results ranging from hesperidin, demonstrating the validity of the current approach. The accuracy results meet the requirements for acceptance[13] (Table 1).

3.4 Precision

Intra-day and inter-day precision were used to evaluate the method's accuracy. In the morning (T1), afternoon (T2), and evening (T3) of the same day, the intra-day precision was confirmed. For concentrations of 10 µg/ml, 15 µg/ml, and 20 µg/ml, the intra-day precision and percentage RSD were determined to be 0.28%, 0.43%, and 0.65%, respectively. On different days, the inter-day precision was carried out. The observed inter-day percentage RSD was 0.20%, 0.38%, and 0.57%, in that order. This indicates that the current approach complies with ICH recommendations Q2 (R1) and is consistent with good precision (Table 2 and 3)[14].

3.5 LOD and LOQ

The LOD and LOQ of the developed method was calculated to 3.014 µg/ml and 9.13 µg/ml respectively. The developed method's LOD and LOQ can permit successful quantitation and detection of the analyte in the formulations (Table 4).

3.6 Robustness

The ability of an HPLC process to withstand changes in parameters, such as flow rate, is referred to as robustness. The flow rate was adjusted by 10% (0.8, 0.9, and 1 mL/min) to see if the peak area changed. The retention time increased with decreasing flow rate. The devised approach was resilient, as evidenced by the % RSD of 1.73%, which fits within the acceptance standards (Table 5)[14].

The validation parameters are summarized in Table 6, which show the successful development of a RP-HPLC method for estimation of Hesperidin. One of the widely cited and used method for hesperidin quantitation by HPLC used Methanol:Water (0.1% orthophosphoric acid (50:50) as the mobile phase and reported an LOD and LOQ of 12.49 and 37.86 µg/ml[11].

Table 1. Observations for accuracy measurement

Level	Std. Conc. (µg/ml)	Conc. Found (µg/ml)	% Recovery	Mean ± SD (n=3)	% RSD
80%	12	11.87	101.09		
	12	11.77	101.95		
	12	11.85	101.26	101.43 ± 0.45	0.449
100%	15	14.76	101.62		
	15	14.91	100.60		
	15	14.83	101.14	101.12 ± 0.51	0.505
120%	18	17.66	101.92		
	18	17.93	100.39		
	18	17.84	100.89	101.067 ± 0.78	0.772

Table 2. Observations for intra-day repeatability

Concentration (µg/ml)	Time	Area	Mean ± SD(n=3)	% RSD
10	T1	208913	209171.7 ± 598.5	0.29
	T2	209856		
	T3	208746		
15	T1	256969	257471.7 ± 1123.7	0.44
	T2	258759		
	T3	256687		
20	T1	358865	357953 ± 2333.7	0.65
	T2	359693		
	T3	355301		

Table 3. Observations for inter-day repeatability

Concentration (µg/ml)	Day	Area	Mean ± SD (n=3)	% RSD
10	Day-1	209586	209100.7 ± 431.1	0.21
	Day-2	208954		
	Day-3	208762		
15	Day-1	255987	256990 ± 987.9	0.38
	Day-2	257962		
	Day-3	257021		
20	Day-1	325462	324595.3 ± 1858.3	0.57
	Day-2	325862		
	Day-3	322462		

Table 4. Observations for LOD and LOQ

Parameter	Observation
LOD (µg/ml)	3.014
LOQ (µg/ml)	9.13

Table 5. Observations for robustness

Parameter	Variable	Condition	Retention Time (min)	Area	Mean ± SD (n=3)	% RSD
Flow rate	0.1%	0.8mL/min	5.379	239954		
		0.9mL/min	4.888	246969		
		1mL/min	4.321	239586	239954 ± 4160.4	1.73

Table 6. Summary of validation parameters data of Hesperidin

Parameter		Results
Linearity range (µg/ml)		10 - 30
Retention time (min)		4.888
Run time (min)		10
Correlation coefficient (R^2)		0.9907
Theoretical plate		2159.16 ± 58.30
Tailing factor		1.1496 ± 0.032
Peak purity index		1.0000
LOD (µg/ml)		3.014
LOQ (µg/ml)		9.13
Accuracy (% RSD)		0.44 - 0.77
Precision (%RSD)	Inter-day	0.20 - 0.57
	Intra-day	0.28 - 0.65
Robustness (0.9 ± 0.1 mL/min) (% RSD)		1.73

The developed method herein, reports a lower LOD, LOQ and pH independent method of chromatographic analysis of hesperidin.

4. Conclusion

In order to quantify hesperidin in pharmaceutical formulations with accuracy, precision, and resilience, the HPLC method used in this study was optimised. Throughout the intended concentration range, the method showed outstanding linearity, sensitivity, and selectivity. The resulting technique offered effective separation and a quick analysis time through methodical optimisation of important parameters. The method's validation validated its dependability in terms of precision, accuracy, robustness, LOD, and LOQ, which makes it appropriate for routing analysis in application areas such as environmental monitoring and pharmaceutical quality control. All things considered, the established HPLC method offers a reliable and repeatable instrument for the analysis of hesperidin, making it a significant contribution to pharmaceutical analysis.

5. Author Contribution

NC – Investigation, Writing - Original Draft; JP - Formal analysis, Data Curation; ML – Conceptualization, Writing - Review and Editing. All authors have read and agreed to the published version of the manuscript.

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