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Research Article

### DEVELOPMENT AND VALIDATION OF RP-HPLC-PDA METHOD FOR THE QUANTIFICATION OF HESPERIDIN IN BULK AND ITS PHARMACEUTICAL DOSAGE FORM

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**Abstract:**

*Flavonoids do have a lot of pharmacologic effects and health benefits. And are a group of polyphenolic compounds. For the effective delivery of flavonoids, to improve the bioavailability they are being formulated in many ways and for this an accurate analysis in a formulation, a suitable analytical method is needed. For the analysis of flavonoids, High-Performance Liquid Chromatography offers an accurate, sensitive technique. Hence in the present work, an attempt is made to develop and validate an economic, rapid and sensitive method for quantitative determination of Hesperidin in solid lipid nanoparticles based on isocratic reversed phase high-performance liquid chromatography. Chromatography was performed on a C-8 reverse-phase analytical column and photo diode array detector, the following conditions were chosen as optimal: mobile phase – Mobile phase A (methanol : acetonitrile – 60:40) 15% v/v; Mobile phase B (purified water acidified with 0.20 % ortho-Phosphoric acid-pH 2.5) 85% v/v, flow rate – 1.0 mL/min and ambient temperature. Linearity was observed in the concentration range 1-25 µg /mL with a correlation coefficient of 0.999 and the limit of detection (LOD) is 0.25µg/mL and limit of quantification (LOQ) is 0.81µg/mL. The proposed method allowed direct determination of Hesperidin with a high degree of accuracy, precision, robustness, specificity in solid lipid nanoparticles in the presence of formulation excipients.*

**Keywords:** Hesperidin, Solid Lipid Nanoparticle Formulation, Quantitative Analysis, Isocratic Reverse Phase High-Performance Liquid Chromatography

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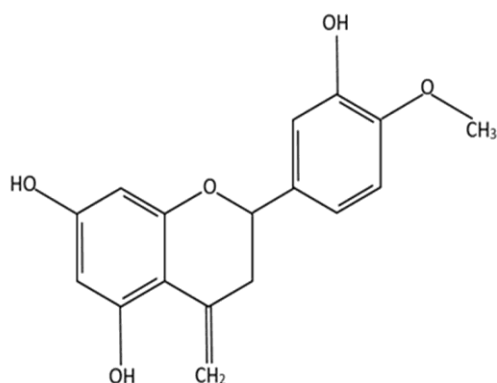
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**INTRODUCTION:**

Dissolution rate of Hesperidin in aqueous media and biological fluids was limited by its poor aqueous solubility (Md Khalid Anwer, 2014). For the efficient delivery of lipophilic molecules, nanotechnology is an excellent tool which increases the bioavailability as the physicochemical characteristics of the nanoparticles influence the pharmacokinetics of drugs which affect their bioavailability and bio-distribution. Hesperidin is a flavonoid synthesized by phenyl-propanoid pathway and belong to group of polyphenolic compounds having a benzo- $\gamma$ -pyrone structure that is present in plants. Hesperidin is a hydroxylated phenolic substance that is synthesised by plants in response to microbial infections and have protective effects against many infections (bacterial and viral diseases), cancers and degenerative diseases like cardiovascular and other age-related diseases (Pandey, 2013).



**Figure 1 Structure of Hesperidin**

Hesperidin was reported to have many biological activities such as antioxidant, anti-microbial, anti-spasmodic, anxiolytic and anti-inflammatory activities. Hesperidin has been shown to induce an anti-inflammatory effect (Gacche, 2016). Hesperidin is most well known for being a testosterone boosting plant compound, although this seems to be a misleading claim. But the therapeutic potential of Hesperidin is severely limited by its poor oral bioavailability, which is because of its poor aqueous solubility (4.93  $\mu\text{g/mL}$ ) and poor oral bioavailability, due to extensive pre-systemic metabolism in the intestine and the first-pass metabolism in the liver (Md Khalid Anwer, 2014).

Poorly aqueous soluble drugs often exhibit low bioavailability as their absorption can be kinetically-limited by low rates of dissolution and capacity-

limited by poor solubility. Lipid-based formulations can reduce the inherent limitations of slow and incomplete dissolution of poorly water soluble drugs, and facilitate the formation of solubilised phases from which absorption may occur (Milan Stuchlík, 2002). Solid lipid nanoparticles (SLNs) have been proposed as an alternative drug carrier system to other novel delivery approaches, such as microemulsions, microspheres, liposomes and polymeric nanoparticles, due to various advantages, including feasibility of incorporation of lipophilic and hydrophilic drugs, improve physical stability, low cost, ease of scale-up and manufacturing (Mohammad Javed Ansari, 2016). Nanoemulsions are reported to be less physically stable (Kareem Rahn-Chique, 2012) while polymeric nanoparticles are proved to be more toxic than SLN (Ramaraio, 2013). Further SLNs are easier to prepare and offers more stability than other delivery systems and thus improve bioavailability.

Hence in the present work, it was proposed to formulate Hesperidin loaded solid lipid nanoparticles to improve its bioavailability and develop an accurate, precise, robust, specific and suitable analytical method to quantify Hesperidin in the formulation to determine entrapment efficiency, total drug content with high specificity and prevent interference of excipients. The analysis of flavonoids by high-performance liquid chromatography offers an accurate, sensitive technique. Ward and Pelter in 1974 published the first application of HPLC to flavonoid analysis (Kim KS, 2002).

**MATERIALS and METHODS:****Chemicals and Reagents**

Hesperidin, stearic acid, soya lecithin and poloxamer-188 were acquired from Himedia (Mumbai, India), HPLC grade Methanol, Acetonitrile and ortho-phosphoric acid were acquired from MERCK Life Sciences (Mumbai, India), Triple distilled water is from Merk Millipore distillation unit.

**Preparation of Hesperidin SLNs**

Modified hot high shear homogenization ultrasonication method was used to prepare Hesperidin loaded SLNs. High shear homogenization and ultrasound liquid processing technology are techniques which were primarily utilised for the preparation of solid lipid nanoparticles. Both techniques are popular and comprise ease of handling. Briefly, solid lipid was melted, co-surfactant was dispersed in the lipid melt and dissolved in organic solvent and the drug was added to the lipid solution. Organic solvent evaporated on a

water bath. Further, an aqueous phase containing the surfactants prepared and lipid phase was dispersed in the hot aqueous phase by utilising high shear homogenizer 4000 rpm to 12000 rpm for 5 min. The resulting suspension was sonicated using a probe sonicator by adjusting frequency and amplitude (Garg A, 2017). Processing parameters needed to optimise depend on various lipids used in the preparation. The final product obtained is kept at room temperature to obtain stable solid lipid nanoparticles.

In preliminary studies, Hesp-SLNs were prepared by using different lipid matrix such as stearic acid tristearin, glyceryl monostearate, and compritol ATO 888. Hesp-SLN prepared using GMS as lipid matrix stabilised with 1 % v/v Tween 80 and 1% w/v poloxamer-188 was found to be aggregated within 30 minutes and 24h of preparation, respectively. Therefore, poloxamer-188 (1% w/v) was selected as a surfactant to prepare the further batches of Hesp-SLN. Hesp-SLNs prepared using tristearin and compritol as lipid matrix showed visual aggregation after 24 h and hence stable up to 24h. Hesp-SLN prepared with stearic acid as lipid was found to be stable even after 24 hours as no visible aggregation was observed. Hence, stearic acid was selected as solid lipid matrix for preparing additional batches of Hesp-SLN to achieve the desired characteristic including particle size less below 500 nm and higher entrapment efficiency (more than 90%).

#### Preparation of samples

Standard stock solutions of Hesperidin were prepared with HPLC grade DMSO and dilutions were prepared

with methanol to obtain 1 to 25 µg/mL (1, 5, 10, 15, 20 and 25µg/mL) solutions, Hesp-SLN samples were prepared with HPLC grade methanol and to ensure extraction of Hesperidin the samples were sonicated for 10 mins, further diluted with HPLC-methanol and were duly filtered with 0.2µ pore size PVDF Whatman syringe filter prior to injection.

#### High-Performance Liquid Chromatography Instrumentation

The liquid chromatographic system consisted of a Waters 515 HPLC binary pumps, degasser, Waters 2998 photo diode array (PDA) detector and waters column oven. Samples were separated on Sun Fire C-8 column (4.6 mm x 150 mm, 5 µM) at an ambient temperature. All the parameters of HPLC were controlled and the results were integrated with Empower-2 software.

#### Chromatographic conditions

Mobile phase – Mobile phase A (methanol : acetonitrile – 60:40) 15% v/v; Mobile phase B (purified water acidified with 0.20 % ortho-Phosphoric acid) 85% v/v. Mobile phase-A consisting of methanol, acetonitrile and mobile phase-B consisting of acidified triple distilled water were duly filtered through 0.2µ PTFE and Cellulose Nitrate filters respectively and degassed by sonicating for 10 min before use. The flow rate of mobile phase and the injection volume was 1.0 mL/min and 20 µL respectively. The column effluent was monitored at 280 nm.

**Table 1 Chromatographic Conditions**

Column	Waters Sunfire C-8 reverse phase column (150 × 4.6 mm, particle size 5 µm)
Flow rate	1 mL/Min
Retention time	2.67 ± 0.08 Min
Detector	PDA detector (Waters 2998)
Detection wavelength	280 nm
Injection volume	20 µL
Temperature	Ambient temperature
Elution type	Isocratic
Run time	6 minutes

**METHOD VALIDATION:**

Validation of proposed RP-HPLC method was done according to the International Conference on Harmonization guidelines (ICH Q2(R1)). All measurements were performed in triplicate.

Linearity was evaluated in the range 1–25 µg/mL. Peak area versus concentration was subjected to linear regression analysis and the slope, intercept and correlation coefficient for the calibration was determined. Correlation coefficient is a statistical tool used to measure the degree or strength of this type of relationship. Limits of detection and quantification were determined from the calibration curve using the following expressions:

$$\text{LOD} = 3.3\sigma/S \quad \text{Eq. 1}$$

$$\text{LOQ} = 10\sigma/S \quad \text{Eq. 2}$$

Where  $\sigma$  is the standard deviation of intercepts and  $S$  is the slope of the calibration curve.

Precision of the method was evaluated by repeatability (intra-day) and intermediate precision (inter-day). Analysis of different Hesperidin standards three times each on the same day was carried out to evaluate the repeatability or intra-day precision. The intermediate precision was determined by analysing the three standard solutions on three different days. The precision results were reported as the standard deviation (SD) and the relative standard deviation (RSD).

Accuracy was determined by recovery studies via standard addition method. Pre-analysed samples of the formulation of known quantity are spiked by 50%, 100%, 150% standard Hesperidin solution and reanalyzed to determine any change in the linearity of the plot. This is to prove that the matrix of the formulation does not have any effect on the concentration of drug and accuracy of the method. Recovery studies can also be considered as method applicability for formulated solid lipid nanoparticles.

Robustness of the method was evaluated by deliberately introducing very small changes in the analytical methodology at a single concentration level. Robustness of the proposed method was determined in different ways, i.e., by making deliberate changes in the mobile phase ratio, flow rate, and detection wavelength of analysis. The percentage of relative standard deviation (%RSD) of the experiment was calculated to assess the robustness of the method.

The specificity of the method was ascertained by analysing the standard drug and sample. The peak for Hesperidin in SLN samples was confirmed by comparing the  $R_t$  values with that of the standard. The peak purity of Hesperidin was assessed by comparing the spectra at three different levels, that is, peak start, peak apex, and peak end positions of the spectrum.

Factors such as the tailing factor and the capacity factor were taken into consideration for testing the system suitability and system performance.

**RESULTS:****Method Development**

Initial tests were conducted using a mobile phase mixture of methanol, acetonitrile and acidified water in different ratios. However, the method has not proved repeatable or accurate. Consequently, we used different ratios of the mobile phase, methanol, acetonitrile and acidified water and then symmetrical peaks were observed. The best peak with respect to width and symmetry was observed with a mobile phase of methanol, acetonitrile and acidified water (pH-2.5) in the ratio of 9:6:85 (v/v) and a flow rate of 1.0 mL/min. The peak was detected at 2.65 min (Fig.2).

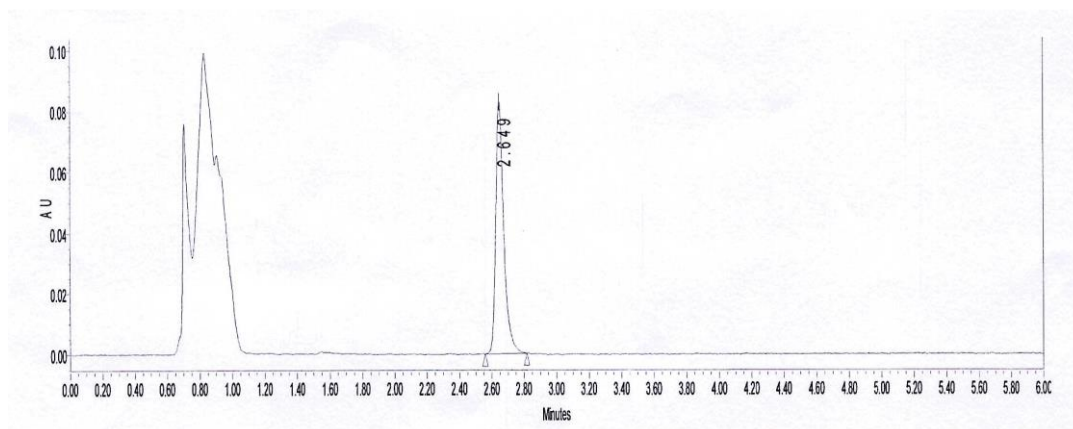


Figure 2 Chromatogram of Standard Hesperidin at 2.65 minutes

### Method Validation

#### Calibration curve

Chromatogram of Hesperidin in the developed HPLC method is shown (Fig.2). A retention time of 2.65 mins can be observed from the HPLC chromatogram in Figure. Calibration curve of Hesperidin in the range between 1–25 µg/mL by the developed HPLC method was shown (Fig.3).

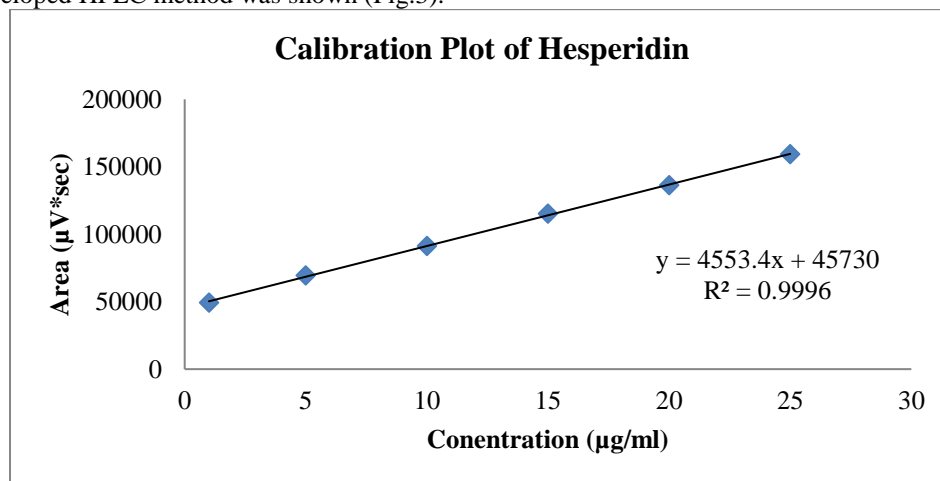


Figure 3 Calibration plot of Hesperidin

#### Linearity and Range

The linear regression data for the calibration curve with equation  $y = 4553.4x + 45730$  (Where Y is the peak area (µV\*sec) and C is the concentration (µg/mL) of the standard solution in µg/ml) demonstrated a good linear relationship over the concentration range of 1 to 25 µg/mL. A good linearity was established by a correlation coefficient ( $R^2$ ) value of  $0.9996 \pm 0.0003$  (Table 2). A high correlation coefficient value (a value very close to 1.0) indicates a high level of linear relationship between the concentration of Hesperidin and peak area.

Table 2 Linear regression data for the calibration curve (n = 3)

Parameter	Mean $\pm$ SD	%RSD
Linearity range (µg/mL)	1 to 25	-
Correlation coefficient ( $R^2$ )	$0.9996 \pm 0.0003$	0.03
Slope	$4553.4 \pm 41.48$	0.91
Intercept	$45730 \pm 805.31$	1.76



### Specificity

The specificity is considered the first analytical validation step, it must be ensured that the components of the formulation such as excipients, does not interfere in the quantification of the drug. The specificity of the method was evaluated by comparing the chromatograms of Hesperidin from nanoparticles with Hesperidin standard solution. The representative chromatogram of the Hesperidin sample showed the peak at approximately 2.65 min, which was in agreement with that obtained for the Hesperidin standard (Fig.2). No peaks at this retention time were observed in the chromatogram of the blank nanoparticles (Fig.4), which indicates that there was no interference in the quantitative determination of Hesperidin from the formulation components.

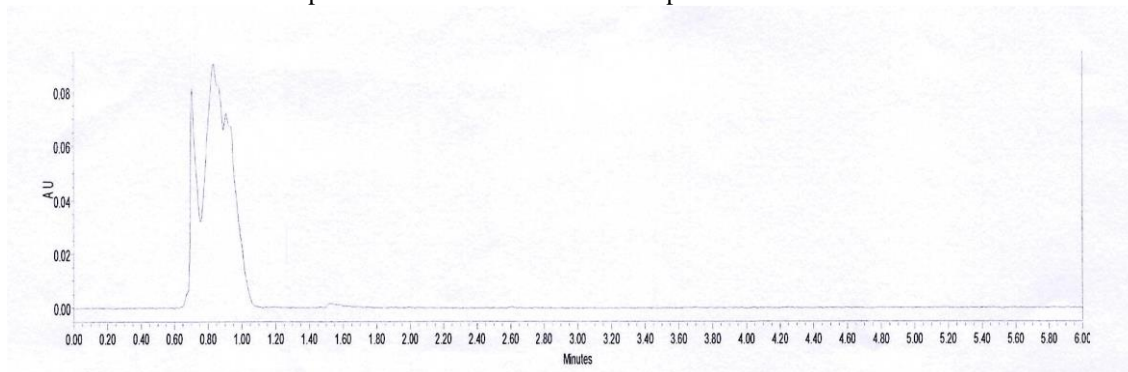


Figure 4 Chromatogram of Placebo SLNs

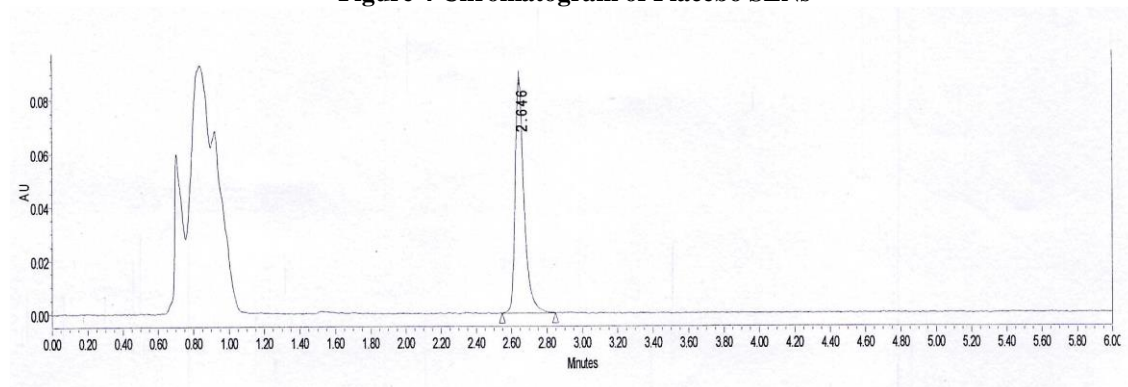


Figure 5 Chromatogram of Hesp SLNs sample

### Precision

Precision is a measure of the relative error for the method and is expressed as the RSD of the repeatability and intermediate precision. Three concentrations of Hesperidin (1, 15 and 25 µg/mL) were prepared in triplicate and analysed to evaluate the intra- and inter-day variations, respectively. The RSD of the responses were calculated for each case and are shown in Table 3, presenting a maximal RSD of 1.45%, which indicates the method precision.

Table 3 Repeatability and intermediate precision of developed method

Concentration (µg/mL)	Repeatability (n = 3)		Intermediate precision (n = 3)	
	Mean peak area ± SD (µv*sec)	%RSD	Mean peak area ± SD (µv*sec)	%RSD
1	50478 ± 389.6	0.77	49834 ± 456	0.91
15	115840 ± 556.3	0.48	114805 ± 667.36	0.58
25	161792 ± 1213.8	0.75	150341 ± 1525.5	1.01

**Accuracy**

The accuracy was assessed by calculating the percent recovery and the RSD of the mean concentration of the analyte. The detailed results are presented in Table 4. The mean percent recovery of Hesperidin from the samples was 100.89% (RSD = 1.49%, n=9), which indicates agreement between the experimental and theoretical values.

**Table 4 Recovery data for the accuracy of the HPLC method (n = 3)**

Excess of Hesperidin spiked (%)	Concentration of sample (µg/mL)	Theoretical concentration of spiked sample (µg/mL)	Concentration of spiked sample ± SD (µg/mL) (n=3)	Recovery ± SD (%)	%RSD
50	10	15	15.11 ± 0.04	100.73 ± 0.26	0.26
100	10	20	19.98 ± 0.11	99.9 ± 1.84	1.84
150	10	25	25.20 ± 0.13	100.8 ± 1.75	1.75

**Robustness**

A robustness is the parameter used to verify the influence of small changes in the analytical procedures/parameters on the response. The evaluation of robustness was based on the comparison of % RSD values obtained by using optimised method and by making deliberate changes to the chromatographic parameters like mobile phase composition, flow rate and detection wavelength. The method was found to be robust despite of the deliberate alterations in chromatographic parameters (Table 5).

**Table 5 Robustness data of the HPLC method (n=3)**

Parameter	Study Condition			Mean Area ± SD (µv*sec)	%RSD of Area	Rt ± SD (mins)	% RSD of Rt
	Original	Used	Level				
Mobile phase ratio (A : B)	15 : 85	83:17	-1	116182 ± 565.66	0.48	2.67 ± 0.18	6.7
		85:15	0				
		87:13	+1				
Flow rate (mL/min)	1.0	0.9	-1	115852 ± 472.78	0.41	2.67 ± 0.21	7.8
		1.0	0				
		1.1	+1				
Detection wavelength (nm)	280	278	-1	114888 ± 84.66	0.07	2.67 ± 0.03	1.1
		280	0				
		282	+1				

**LOD and LOQ**

The LOD and LOQ were determined as per the ICH Guidelines Q2(R1) (2005) and were found to be 0.25 and 0.81 µg/mL, respectively.

**System Suitability**

System suitability was defined as the proof that system is working perfectly. System suitability testing has to be done before sample analysis. Capacity factor (K') and Tailing factor (T) are the commonly checked system suitability parameters in HPLC. The results are mentioned in table 6.

**Table 6 System suitability of the HPLC method**

Chromatographic Parameter	Result (n=3)
Capacity factor (K')	2.05 ± 0.14
Tailing factor (T)	1.49 ± 0.31

**DISCUSSION:**

Based on the observed results it is proved that an economic, rapid and sensitive method was developed for the estimation of Hesperidin. According to this method, there was no need to employ any salts, and therefore the risk of saturation, breakdown or overpressure in the column was reduced.

**CONCLUSIONS:**

The ISOCRATIC RP-HPLC-PDA method for the quantification of Hesperidin in solid lipid nanoparticles was successfully developed and validated with a run time of 6 minutes. The method was validated in terms of linearity and range, accuracy, precision, specificity, robustness, detection limit, and quantitation limit according to the guidelines of the International Conference on Harmonization (ICH) and fulfilled all of the requirements to be considered a reliable method. This method can be applied for other assays such as free drug content, % drug loading, Entrapment efficiency, *in-vitro* release profile and compatibility studies etc.

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