Technical paper

Direct Spectrophotometric Determination of Hesperidin in Pharmaceutical Preparations

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Abstract

A simple, rapid and reliable direct spectrophotometric method for the determination of hesperidin is proposed and validated. The influence of wavelength, solvent, the ionic strength, pH and temperature on hesperidin determination were investigated. Under the optimum conditions, $\lambda = 283$ nm, 60% methanol as the solvent, ionic strength, $I = 2.5 \times 10^{-5}$ mol L^{-1}, pH = 6.4 and T = 37.0 °C, the Beer's law is obeyed in the concentration range 1.83–24.5 μg mL^{-1}. The molar absorptivity and Sandells sensitivity were found to be 1.8×10^4 L mol^{-1} cm^{-1} and 0.03 μg cm^{-2}, respectively. The sensitivity of the proposed method was 0.9 μg mL^{-1} (as limit of detection) and 3.2 μg mL^{-1} (as limit of quantification). Applicability of the proposed method to the direct determination of total flavonoids as hesperidin equivalents in pharmaceutical formulation (Vitamin C with citrus bioflavonoids & Rose Hips) was demonstrated. Although the presence of ascorbic acid may cause problem in identification and measurements, hesperidin has been determined successfully.

Keywords: Direct spectrophotometric method, flavonoids, hesperidin, drug analysis

1. Introduction

Hesperidin (Fig. 1.) ((S)-7-[[6-0-(6-deoxy-alpha-L-manno-pyranosyl)-beta-D-glucopyranosyl]oxy]-2,3-dihydro-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-4*H*-1-benzopyran-4-one) (hereinafter referred as Hesp), belongs to the flavonoids (or bioflavonoids) of flavanone type, a ubiquitous group of polyphenolic substances which are present in most plants, concentrated in seeds, fruit skin or peel, bark, and flowers.[1] Hesp is the predominant flavonoid in *citrus* species, occurring mainly in the peel and membranous parts of the lemons and oranges. Hesp is thought to reduce capillary permeability and to have anti-inflammatory action, hence it is used to shrink hemorrhoids, reduce varicose veins and battle viral infections.[2–5] A variety of flavonoid products are either being actively developed or currently sold worldwide as dietary supplements and/or herbal remedies.

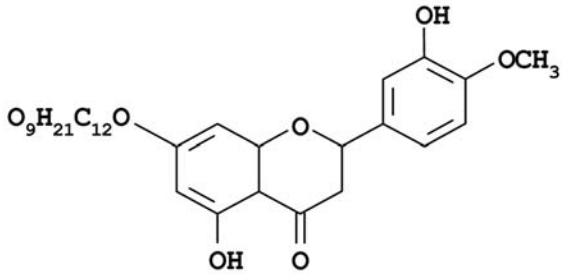

Fig. 1. Chemical structure of hesperidin

Until now, a number of analytical techniques have been described for Hesp quantification. Liquid chromatography is currently the most commonly applied method for routine determination of all flavonoids, including Hesp. The reversed phase RP-HPLC approaches established in the late '80s aim to separate,[6] identify and quantify Hesp in crude plant materials/extracts and plant-based beverages.[7–9] The radioimmunoassay,[10] spectrofluorimetry,[11] spectrophotometry,[12] as well as kinetic analytical method[13] are also described for Hesp quantification.

Knowing that Hesp easily reacts with different metal ions forming chelates which are usually coloured, as well as that absorption spectra of these *Hesp-metal* chelates is typically bathochromic shifted related to absorption spectra of Hesp, we optimise and utilize different chelating reactions for Hesp determination by indirect spectrophotometric methods.[14–18] The intensity and the hue of the color of the new formed complexes *Hesp-metal ion* depend strongly on the metal ion properties, as well as the

reaction conditions, what was assembled in the review article.[18]

In this paper, we developed and validated a simple and inexpensive direct spectrophotometeric method for Hesp determination by simply selecting optimum parameters such as wavelength, solvent, pH, the ionic strength and temperature. In order to assess the analytical potential of the proposed method which would be suitable for the analysis of total flavonoids as Hesp equvalent in pharmaceutical formulation, the effects of possible interfering substances, predominantly ascorbic acid (AA) were also studied (Hesp is present in nutritional supplements such as *vitamin C with bioflavonoids*).

Some other methods exhibit very high sensitivity for Hesp determination,[8–11] rather than spectrophotometric method. Nevertheless, the principal advantage of this method over the other techniques is that it may be applied directly to the analysis of pharmaceutical formulation without the need for separation or complex sample preparation. Therefore, the method is fast, and cost-effective, requiring less expensive equipment and chemicals and may be considered as a suitable alternative to the existing analytical techniques.

2. Experimental

2. 1. Apparatus

For all absorbance measurements UV-VIS spectrophotometer Beckman DU-650 (Fullerton, CA, USA) with 1-cm optical path length quartz cuvette was used. All of spectra were registered with a scan rate of 600 nm min^{-1}. Spectral scans were continuously collected from 260 to 400 nm during each run. For pH measurements, a pH-meter (pHM-82 Radiometer Copenhagen), accuracy of ± 0.001 pH, equipped with the combined electrode (No. CW. 733 Series No.35162, Russel) was used. The temperature was controlled within ± 0.2 °C by circulating water thermostat (Series U, MLW Freital, Germany). Ultrasonic bath (L.U.5.7 Fungilab, S.A, Spain) was used for dissolving the samples.

2. 2. Chemicals

Only analytically graded reagents without further purification and de-ionized water with the specific resistance of 18 MΩ cm (Milli-Q, Millipore, USA) for solutions preparing were used. Hesp, quercetin, rutin, morin, naringin and naringenin from Fluka (Buchs, Switzerland), ascorbic acid, methanol, $NaNO_3$, HNO_3 and NaOH from Merck (Darmstadt, Germany) as well as glutaminic acid, citric acid, glucose, sucrose and starch from Sigma-Aldrich (St. Louis, United States) were used. A *Vitamin C with citrus bioflavonoids & Rose Hips* capsules was obtained from Optimum Nutrition, Coral Springs, USA.

The standard stock solution of Hesp ([Hesp] = 1×10^{-4} mol L^{-1}) was prepared by dissolving 61.06 mg of Hesp in 1000 mL absolute methanol. To prepare standard curves, appropriate volumes of stock solution were transferred and diluted to final concentrations of (1.83, 3.05, 4.27, 5.49, 6.10, 9.77, 18.31 and 24.60) μg mL^{-1}. All standard solutions were prepared in a 20-mL volumetric flasks, by mixing appropriate volumes of the standard stock solution of Hesp (V_1), absolute methanol (V_2), water (V_3) and $NaNO_3$ (V_4) following this order. To obtain final solutions of the required molar concentrations in 60% (v/v) methanol, it is necessary that $V_1 + V_2 = 12$ mL and $V_3 + V_4 = 8$ mL. Since volumes are not strictly additive, volumetric flasks were filed to the mark with 60% methanol. A blank solution without Hesp was prepared in a 20-mL volumetric flask, by mixing appropriate volumes of absolute methanol, water and $NaNO_3$.

All solutions were stored in refrigerator, protected from daylight and appeared to be stable during the period of study.

The pH was adjusted by adding of 2.0 mol L^{-1} HNO_3 and/or 2.0 mol L^{-1} NaOH, and the ionic strength of the final solutions was kept constant by addition of 1.0 mol L^{-1} $NaNO_3$.

For interference study, appropriate amounts of possible interfering substances such as flavonoids, ascorbic acid (AA), sugars, starch, citric acid anhydrous and glutaminic acid were dissolved in 60% methanol. After further dilution with the same solvent, the final concentrations for interference study were obtained.

2. 3. Sample Solutions

To determine Hesp in the pharmaceutical dosage forms, the mass content of twenty capsules were weighed and the average mass of one capsule was evaluated. The equivalent of two capsules was accurately weighed and transferred to a 1000-mL volumetric flask. About 50 mL of 60% methanol was added to dissolve the drug. After sonicating and shaking the mixture for 30 min, it was completed to volume with the same solvent. Solution was filtered through the Whatman No. 1. filter paper. The sample solution was obtained by transferring 4 mL of this solution into 100-mL volumetric flask and adding 60% of methanol to the volume. The procedure was repeated five times. Here, we underline that according to the factory declaration, two capsules contain 751 mg of *Citrus* flavonoids and 1000 mg of vitamin C.

3. Results and Discussion

3. 1. Optimization of Experimental Variables: Wavelength, Solvent, pH, Ionic Strength and Temperature

Preliminary experiments were run to find out the best operative conditions to obtain the calibration curve,

as well as for the measurements of real sample. The parameters optimized included wavelength, solvent, the ionic strength, pH and temperature.

Typically, two major absorption maxima (Fig. 2) are observed in the UV-VIS spectrum of Hesp: in the range around 280 nm (referred to as band II in flavonoids) and the one around 330 nm (band I). Absorbance measurements are carried out at 283 nm because this band is less sensitive to matrix influence, since absorption band II may be considered as to have originated from electron transitions in the A-ring benzoyl system of Hesp. [1]

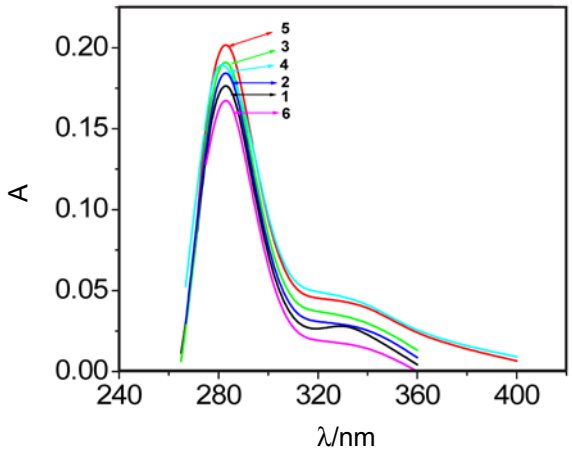

Fig. 2. Absorption spectra of hesperidin in 60% methanol at different pH values: 2.6 (curve 1), 3.7 (curve 2), 4.1 (curve 3), 4.9 (curve 4), 6.4 (curve 5) and 8.8 (curve 6); [Hesp] = 6.1 μg mL^{-1}.

The optimum medium for direct Hesp determination related to 60% methanol and pH = 6.4 (Fig. 2, curve 5). Although higher percentage of water favourites Hesp dissociation and pH decreasing which consequently affect absorbance, it was found that change of pH caused a negligible absorbance blue shift of the bend II, because of the lack of conjugative effect in Hesp molecule (the shift is evident only at pH = 4.9, curve 4). Solvent with this percentage of methanol enables the best compromise among Hesp solubility (Hesp is very sparingly soluble at room temperature in aqueous media, and relativity soluble in boiling water), and sufficient solubility of AA, and related substances.[19]

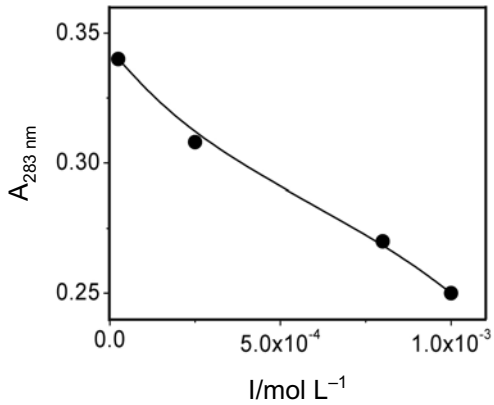

Fig. 3. The influence of ionic strength on absorbance of the hesperidin solution; [Hesp] = 10.4 μg mL^{-1}, pH = 6.4 and T = 37 °C.

The influence of the ionic strengths on absorption spectra of Hesp, at the constant value of both, pH and temperature (pH = 6.4 and T = 37.0 °C) were investigated (Fig. 3). From the Fig. 3, it is obvious that the best conditions (the higher absorbance) relate to the value of ionic strength I = 2.5 × 10^{-5} mol L^{-1}.

The influence of temperature on the absorption of Hesp solution at the selected ionic strength is also investigated. The greatest absorbance was found for the temperature T = 37.0 °C.

3. 2. Influence of Ascorbic Acid

Worldwide, Hesp is present in nutritional supplements such as vitamin C with bioflavonoids. Therefore, the effect of AA on values of absorbance of Hesp was also investigated. After fixing all investigated parameters, the effect of AA was studied with mixture containing 10.4 μg mL^{-1} of Hesp and different amounts of AA (110.0 μg mL^{-1} and 305.0 μg mL^{-1}), Fig. 4. The first data points shown in the Fig. 4., were taken at 5 min after mixing Hesp and AA (recording time is 0 min) while the end data points were taken at 42 min after that (recording time is 37 min).

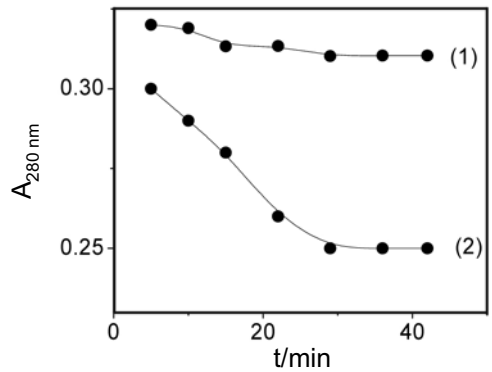

Fig. 4. The influence of ascorbic acid on absorbance of hesperidin under optimum conditions. (1) [Hesp] = 10.4 μg mL^{-1} and [ascorbic acid] = 110.0 μg mL^{-1} (2) [Hesp] = 10.4 μg mL^{-1} and [ascorbic acid] = 305.0 μg mL^{-1}

From the Fig. 4. it is obvious that not more than 0.7% difference was produced by up to 10-fold excess of AA (curve 1) as well as not more than 3.7% difference was produced by up 30-fold excess of AA (curve 2), in the first 5 minutes of recording. Significantly decreased amount of Hesp in time is obvious only after 10 minutes of recording, i.e. 15 minutes after Hesp and AA mixing, in the case of 30-fold excess of AA. The evident change of absorbance of Hesp in time may be the result of their synergy with AA, due to the process where each antioxidant improves the oxidant activity of the other.[20, 21] Namely,

antioxidant activity of flavonoid has been related to their protection against AA oxidation; the inhibitory effect of flavonoids on AA oxidation is considered to be due to free-radical trapping by flavonoids. It appears that addition of AA in concentration higher than 110.0 μg mL^{-1} causes a complex kinetic interplay of competing as well as parallel reactions of flavonoid aroxyl radicals and ascorbyl radicals.[22] For that reason, all measurement sequences (mixing Hesp and AA, filling the test cuvette with this mixture as well as absorbance readings at optimal experimental conditions) have to be performed in the time period less than 10 minutes.

3. 3. Analytical Features

Based on previously described examination, the best conditions for direct spectrophotometric Hesp determination correspond to 60% methanol as solvent, λ = 283 nm, pH = 6.4, the value of ionic strength I = 2.5 × 10^{-5} mol L^{-1}, T = 37.0 °C, and the assay time less than 10 minutes. Thus, under optimum experimental conditions, the absorbance of the standard solutions was measured at 283 nm against the reagent blank. The concentration of Hesp content was established by reference to the calibration graph.

Under the above described experimental conditions, the calibration curve (graphs of the absorbance versus the concentration of Hesp) is obeyed in the concentration range from 1.83–24.5 μg mL^{-1}. Regression analysis of Beers law plots revealed a good correlation (r = 0.998). The Beer-Lambert law limit, molar absorptivity, Sandells sensitivity, parameters of the regression equation (intercept, slope, RSD values of the slope and intercept) as well as limit of detection (LOD), and limit of quantification (LOQ) are all given in Table 1. Both detection and quantification limits were calculated from the standard deviation of the absorbance measurements obtained from a series of 10 blank solutions. The LOD and LOQ were established according to the IUPAC definitions.[23]

In order to prove the applicability of the proposed method and the reproducibility of the results obtained, five replicate experiments at different concentration of Hesp were carried out. The precision of the method was expressed by the relative standard deviation ($RSD^{*} = t\,RSD/\sqrt{n}$ with theoretical t-value at 95% confidence limit for seven degrees of freedom). It can be seen from Table 2 that% RSD (intra-day and inter-day precisions) were in the range 0.8%–2.4%. These results for RSD show that the described method is precise. Also, the lowest RSD average value (1.0%) is for 10.0 μg mL^{-1}, so in further work this concentration was used for sample analysis. Ten runs using this concentration of sample were performed.

Table 1. Features of the calibration plot and analytical figures of merit for the determination of hesperidin

The parameters of the regression line	
Beers law limit (μg mL^{-1})	1.83 – 24.5
Molar absorptivity (10^4 L mol^{-1} cm^{-1})	1.8
Sandells sensitivity (μg cm^{-2})	0.03
Intercept	0.019
Slope (μg^{-1} mL)	0.0299
Intercept (RSD %)	0.005
Slope (RSD %)	0.0005
LOD[a] (μg mL^{-1})	0.9
LOQ[b] (μg mL^{-1})	3.2
Regression coefficient	0.998

[a] Limit of detection defined as dynamic concentration of Hesp that produce signal-to-noise ratio = 3.
[b] Limit of quantification is assess at a minimum signal-to-noise ratio of 10.

Table 3. Recovery data for hesperidin spiked to pharmaceutical

Sample	Added (μg mL^{-1})	Found (μg mL^{-1})	Recovery (%)
Pharmaceutical formulation	0.0	7.21	–
	1.0	7.83	95.4
	5.0	12.04	98.6
	10.0	17.06	99.1
	12.0	19.74	102.8
			$\bar{x}$ = 99.0
			RSD = 2.8%

Hesp recovery from pharmaceutical formulation after spiking with 1.0, 5.0, 10.0 and 12.0 μg mL^{-1} of additional standard was 99.0% with mean RSD = 2.8% (Table 3) confirming the accuracy of the method. Accuracy was measured as recovery value (RCV) *i.e.* as percentage error as (concentration found / known concentration) × 100. Taking into account that the recoveries of Hesp added to capsule were close to 100% (Table 3), it may be assumed that no interfering substances were encountered.

Table 2. Precision of the assay for three different concentrations of hesperidin. All values derived from n = 5 independent measurements

	Repeatability(intra-day variation)				Intermediate precision (inter-day variation)		
Compound	Taken (μg mL^{-1})	Found ±SD (μg mL^{-1})	Recovery (%)	RSD (%)	Found ±SD (μg mL^{-1})	Recovery (%)	RSD (%)
Hesperidin	2.0	2.06 ± 0.04	103.0	1.9	2.09 ± 0.05	104.5	2.4
	10.0	9.89 ± 0.08	96.9	0.8	10.20 ± 0.12	102.0	1.2
	20.0	20.15 ± 0.35	101.0	1.7	20.26 ± 0.19	100.5	0.9

3. 4. Interferences

In order to assess the analytical potential of the proposed method, the effects of possible interfering substances were studied. The experiment was performed under the optimum experimental conditions, with standard solution containing Hesp and different amounts of potential interferents commonly present with Hesp in the dosage forms such as AA, glucose, sucrose, starch, citric acid and glutaminic acid. Since Hesp is available as citrus bioflavonoids, some other flavonoids (morin, rutin, naringin, narirutin, quercetin) which might be present in citrus complex were also investigated. Their influence on the determination of 10.4 μg mL^{-1} of Hesp was systematically studied with the aim of obtaining the efficiency and selectivity of the proposed method to pharmaceutical formulations. The criterion of interference was an error of not more than ± 3% in the absorbance of Hesp. The species examined should not interfere below tolerable ratios (defined as the concentration of interferent and concentration of analyte ratio).

Experiments showed that there was no interference from excipiens (glucose, sucrose, starch) for the proposed method up to 100-fold excess. Also, not more than 1% of error was produced by up to 10-fold excess of AA (Fig. 4). Since a typical dose of vitamin C in products is not more than about six times higher than Hesp, the developed method can be taken for the routine analysis of Hesp in the presence of vitamin C. For Hesp determination, the following species, when present in amounts for which tolerable ratio is shown in brackets, do not influence on absorbance: morin [60], rutin [40], quercetin [40], glutaminic acid [30], citric acid [20], narirutin [0.7] and naringin [0.5]. The strong interference of structurally related compounds such as narirutin and naringin, which all have the identical absorbtion maximum, is evident. However, there are numerous reports of HPLC analysis of the composition of citrus fruits and its derived products which all indicate that Hesp predominates among the citrus flavonoids, with naringin and narirutin present in considerably smaller amounts.[24–27] These mentioned relevant facts as well as our results all indicate that the proposed method is convenient for quantitative determination of total flavonoids as Hesp equivalent in citrus complex samples, i.e. in samples in which Hesp is a principal flavonoid.

3. 5. Analytical Application

Validity of the proposed method was tested for pharmaceutical preparation by assaying *Vitamin C with citrus bioflavonoids & Rose Hips* capsules. It was found that each capsule contained 260.75 ± 2.25 mg of Hesp (RSD = 0.9%), whereas the total citrus flavonoid content, according to the producer's declaration, was 751 mg per two capsules.

The applicability of the proposed method was assessed by calculating t- and F-values[28] compared with the kinetic analytical method.[13] The calculated t and F, for both five degrees of freedom and 95% confidence level, are 1.96 and 5.87, respectively (theoretical value for both, t and F are 2.571 (P = 0.05) and 6.26 (P = 0.05), respectively). Since the calculated value of t is less than 2.571, the null hypothesis is not rejected: the proposed methods do not give significantly different results for Hesp concentration.

4. Conclusion

The developed spectrophotometric method is convenient and efficient for determination of Hesp in bulk drug as well as in pharmaceutical preparation. It operates without any derivatization reaction and shows good analytical features. There was no interference of excipients and ascorbic acid in the examined products, thus no additional extraction or separation procedures were required. The main limitation of the proposed method is a strong interference with some structurally related compounds having concentration larger than 7.3 μg mL^{-1} (narirutin) and 5.2 μg mL^{-1} (naringin) Therefore, it may be applicable for Hesp determination in pharmaceuticals that contain sufficiently low concentrations of these substances, as it is in our case. In the cases when these compounds are present in large excess, unequivocal identification is not possible using UV absorption for detection, because some other flavonoids have very similar UV absorbances/spectra. Therefore, a Hesp determination in complex matrices (orange juice, for example) can not be achieved by the proposed method. Nevertheless, it is demonstrated that the proposed method is very suitable for routine analysis of pharmaceuticals with Hesp as principal flavonoid, without any pretreatment of the samples apart from its dissolution.

5. Acknowledgments

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Povzetek

Razvili in validirali smo enostavno, hitro in zanesljivo spektrofotometrično metodo za določanje hesperidina. Raziskovali smo vpliv valovne dolžine, topila, ionske moči, pH in temperature na določanje hesperidina. Pri optimiziranih pogojih (λ = 283 nm, 60 % metanol kot topilo, ionska moč I = 2.5 × 10^{-5} mol L^{-1}, pH = 6.4 in T = 37.0 C) velja Beerov zakon v koncentracijskem območju 1.83–24.5 μg mL^{-1}. Določili smo molarno absorptivnost (1.8 × 10^4 L mol^{-1} cm^{-1}), Sandellovo občutljivost (0.03 μg cm^{-2}), mejo zaznave (0.9 μg mL^{-1}) in mejo določanja (3.2 μg mL^{-1}). Metoda je uporabna za direktno določanje celokupnih flavonoidov kot ekvivalentov hesperidina v farmacevtski formulaciji.