Simultaneous Determination of Diosmin and Hesperidin in *Cissus quadrangularis* Capsules by Ultraviolet-Visible Spectroscopy

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Abstract

The aim of this study was to develop and validate the analytical method for simultaneous determination of diosmin and hesperidin in *Cissus quadrangularis* capsules, using UV-visible spectroscopy based on simultaneous equation method. Standard and sample solutions were prepared in 0.2 N NaOH and measured the absorbances at 267.5 and 284.0 nm, as the maximum absorption wavelength of diosmin and hesperidin, respectively. Validation results of showed acceptable linearity of diosmin and hesperidin over the concentration range of 1 – 5 and 4.8 – 24 µg/mL, respectively. Unfortunately, the percentage recoveries and %RSD for hesperidin did not meet the acceptance criteria. The developed method was applied to quantify the content of diosmin and hesperidin in the capsules, the assay result revealed that each 250-mg capsule (125 mg of *C. quadrangularis* powder) contained 29.88 ± 0.08 and 3.176 ± 0.45 mg of diosmin and hesperidin, respectively. However, more specific and precise method, such as high-performance liquid chromatography (HPLC) must be further developed for the estimation of diosmin and hesperidin in multicomponent formulations.

Keywords : Diosmin; hesperidin; Cissus quadrangularis capsules; ultraviolet-visible spectroscopy

1. Introduction

Cissus quadrangularis L. (Vitaceae) or commonly known in Thailand as "Phet Sang Khat" is a medicinal plant that has been used in Ayurveda for very long time. *C. quadrangularis* is a climbing herb with tendrils grown throughout Asia and Africa. Stem and root portions of this plant provide anthelmintic, antibacterial, dyspeptic, digestive, and analgesic effects, including general tonic, especially for the patient with bone fractured. Moreover, many scientific studies reported various potential benefits by using of *C. quadrangularis* stem powders or extracts for the treatment of irregular menstruation, bone fractures, back pain, and hemorrhoids (Brahmkshatriya *et al.*, 2015, pp. 169-173; Stohs & Ray, 2013, pp. 1107-1114). In Thailand, dried powder of *C. quadrangularis* in combined capsules has been widely used as an alternative medicine to relieve hemorrhoid symptoms. The clinical efficacy of 500-mg *C. quadrangularis* powder capsules and micronized purified flavonoid fraction (MPFF) did not show statistically significant difference compared with placebo for the treatment of acute hemorrhoids (Panpimanmas *et al.*, 2010, pp. 1360-1367). Interestingly, several groups of phytochemical constituents are identified from *C. quadrangularis* powders and extracts, such as triterpenoids, carotenoids, phytosterols, tannins, ascorbic acid, calcium oxalate, and flavonoids (Nawghare *et al.*, 2017, pp. 443-445).

The bioflavonoids (i.e., quercetin, diosmin, and hesperidin) are found to be major components in *C. quadrangularis* stem powders. The active constituents that play a crucial role for the treatment and prevention of hemorrhoids are supposed to be diosmin and hesperidin, which are generally found in 9:1 ratio particularly in citrus species. These 2 flavonoid glycosides possess antioxidative, vasoprotective, and venotonic properties by

promoting vascular sustainability and elasticity (Cospite, 1994, pp. 566-573; Ivanova *et al.*, 2018, pp. 61-70). Thus, the amount of diosmin and hesperidin contained in various formulations should be determined or standardized for effective treatment outcomes.

Previous studies have introduced different analytical methods, such as high-performance liquid chromatography (HPLC) (Kanaze *et al.*, 2003, pp. 243-249), and UV-visible spectroscopy (Bennani *et al.*, 2020, pp. 100-107; Ivanova *et al.*, 2018, pp. 61-70; Srilatha *et al.* 2013, pp. 1-4) for concomitant analysis of diosmin and hesperidin in beverages, food supplements, and pharmaceutical dosage forms, but no reports for *C. quadrangularis* powders or extracts. For UV-visible spectroscopy, this technique was found to be a simple, rapid, reliable, economical, and suitable for simultaneous determination of samples in binary mixture. Therefore, the rationale of this study was to determine the quantity of diosmin and hesperidin in *C. quadrangularis* capsules by developed and validated UV-visible spectrophotometry using simultaneous equation method.

2. Objectives

To develop and validate an analytical method for determination of diosmin and hesperidin in *C. quadrangularis* capsules, using UV-visible spectroscopy based on simultaneous equations (Vierodt's method).

3. Materials and methods

3.1 Materials

Diosmin (>85.0% purity) was purchased from Tokyo Chemical Industry (Tokyo, Japan) and Hesperidin (≥80% purity) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Sodium hydroxide pellets (NaOH, AR grade) was purchased from Ajax Finechem (New South Wales, Australia) for preparation of 0.2 N NaOH in deionized water. Marketed available 250-mg *C. quadrangularis* capsules (Mho Iang Brand, Kongka Herb, Nakhon Pathom, Thailand), labeled to contain 50 g of *C. quadrangularis* in 100 g of powder, was purchased from a local drugstore in Bangkok, Thailand.

UV spectra and absorbances were recorded using JASCO double-beam UV-visible spectrophotometer model V-630 (Tokyo, Japan) with a pair of matched 1-cm quartz cells. All spectral data were analyzed using Spectra Manager Software.

3.2 Methods

3.2.1 Method development

Preparation of stock standard solutions

Diosmin and hesperidin were weighed accurately about 5 and 12 mg, respectively, into 50-mL volumetric flask, separately. Dissolved and adjusted to the volume with 0.2 N NaOH to obtain diosmin and hesperidin stock standard solution at concentration of 100 and 240 µg/mL, respectively.

Preparation of standard solutions

Diosmin and hesperidin standard solutions were prepared by diluting the stock standard solutions with 0.2 N NaOH to get series of diosmin standard solution at concentrations of 1, 2, 3, 4, and 5 µg/mL. Similarly, a series of hesperidin standard solution at concentrations of 4.8, 9.6, 14.4, 19.2, and 24 µg/mL was also prepared. These solutions were scanned to determine the wavelength of maximum absorbance (λ_{max}) and measured the absorbances at corresponding wavelengths.

Preparation of sample solution

The capsule powder was weighed accurately equivalent to 125 mg of *C. quadrangularis* into a 50-mL volumetric flask. The powder was dissolved in 20 mL of 0.2 N NaOH, shook for 5 min, adjusted to the volume with the same solvent, and mixed. The mixture was filtered through a 0.45-µm filter paper and discarded the first 5 mL of filtrate. Pipetted 2.0 mL of the filtrate into a 50-mL volumetric flask and adjusted to the volume with 0.2 N NaOH to obtain 0.1 mg/mL of *C. quadrangularis* powder.

3.2.2 Method validation

The developed method was validated for specificity, linearity, accuracy and precision according to the ICH and AOAC guideline.

Specificity

Diosmin and hesperidin standard solutions were scanned in the UV range 200 – 400 nm to examine the λ_{max}. Specificity of the method was determined by comparing the pattern of UV spectra obtained from individual and combined standard solution to the spectrum of sample solution.

Linearity and range

Linearity was established by the linear regression analysis of calibration curves. Standard solutions in the concentration range of 1 – 5 µg/mL for diosmin and 4.8 – 24 µg/mL for hesperidin were prepared. The absorbances obtained simultaneously from corresponding wavelengths were plotted against the concentrations to determine the regression equation and correlation coefficient (r). The linearity was accepted when $r > 0.995$.

Accuracy and precision

Accuracy was evaluated by calculating mean recoveries of diosmin and hesperidin using standard addition method (at the 100% level). The samples were prepared in 6 replicates by adding each 5.0 mL of 90 µg/mL diosmin and 10 µg/mL hesperidin standard solution into 25-mL volumetric flasks, containing 5.0 mL of sample solution. Adjusted to the volume with 0.2 N NaOH and mixed. The absorbances of standard addition mixture were measured at the corresponding wavelengths compared with un-spiked sample solution. The accuracy was accepted when percentage recoveries (%recovery) were in range of 97 – 103.

Precision was consequently determined by calculating percentage of relative standard deviation (%RSD) from average %recovery. The precision was accepted when %RSD values were not more than 2.7.

3.2.3 Assay of *C. quadrangularis* capsules

Pooled powder from 20 capsules were accurately weighed accurately and prepared by a stepwise procedure for sample solution (3.2.1), then diluted with 0.2 N NaOH to obtain 0.02 mg/mL of *C. quadrangularis* powder. The absorbances of the resulting solution were measured at the corresponding wavelengths.

3.2.4 Simultaneous equation method

The absorbances of sample mixtures were measured at corresponding λ_{max} of diosmin and hesperidin. The concentration of diosmin (x) and hesperidin (y) in the mixtures were calculated by simultaneous equation using the following formula:

$$C_x = \frac{A_2 ay_1 - A_1 ay_2}{ax_2 ay_1 - ax_1 ay_2} \qquad C_y = \frac{A_1 ax_2 - A_2 ax_1}{ax_2 ay_1 - ax_1 ay_2}$$

Where C_x and C_y are the concentration of diosmin and hesperidin, respectively; A_1 and A_2 are the absorbance of sample solution at λ_{max} of hesperidin (284.0 nm) and diosmin (267.5 nm), respectively; ax_1 and ax_2 are absorptivity of diosmin at 284.0 and 267.5 nm; and ay_1 and ay_2 are absorptivity of hesperidin at 284.0 and 267.5 nm, respectively. The

absorptivity (a) values of diosmin and hesperidin were obtained from the slope of their calibration curves.

4. Results

The developed method was applied to determine the quantity of diosmin and hesperidin in *C. quadrangularis* capsules without the separation of each compound prior to analysis by UV-visible spectroscopy based on simultaneous equation method. In this study, the analytical method was validated in terms of specificity, linearity, accuracy and precision.

4.1 Method validation

Specificity

Absorption spectra of diosmin and hesperidin showed the λ_{max} of 267.5 and 284.0 nm, respectively. Comparisons between combined standard solution and sample solution, the result illustrated that overall spectral pattern were different (Figure 1). Absorption peak of the sample at 267.5 nm was comparable to the standard mixture, suggested that the method might be more specific to diosmin than hesperidin.

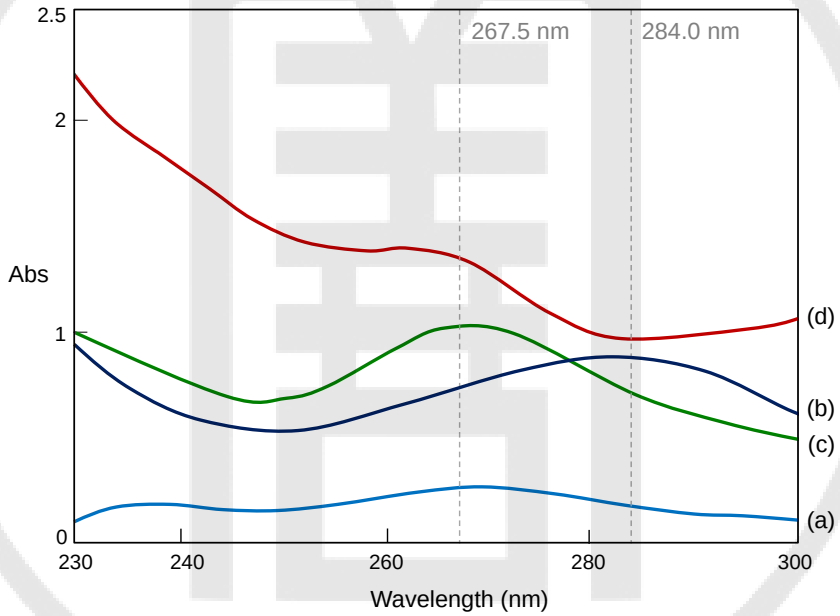

Figure 1 UV overlay spectra of diosmin at 5 µg/mL (a); hesperidin at 24 µg/mL (b); combined standard solution contained 18 and 2 µg/mL of diosmin and hesperidin, respectively (c); and *C. quadrangularis* sample solution (d).

Linearity and range

The calibration curve of diosmin was linear over the concentration range of 1 – 5 µg/mL with the regression equation and correlation coefficient (r) of y = 0.0498x + 0.003 (r = 0.9997), and y = 0.034x + 0.0027 (r = 0.9997) at 267.5 and 284.0 nm, respectively. In similar fashion, hesperidin also showed good linearity over the concentration range of 4.8 – 24 µg/mL with the regression equation and r-value of y = 0.0194x - 0.0063 (r = 0.9999), and y = 0.0304x - 0.0078 (r = 0.9999) at 267.5 and 284.0 nm, respectively.

Accuracy and precision

Using standard addition method for evaluation of analytical method accuracy and precision, 6 replicates of spiked samples were analyzed and calculated the amount of diosmin and hesperidin recovered at the 100% level. The data were displayed in Table 1. For accuracy, %recoveries varied from 97.20 – 97.69% (average 97.47%) for diosmin and 96.88

– 107.8% (average 103.9%) for hesperidin. For precision, %RSD values calculated from mean percentage recoveries were 0.16% for diosmin and 4.73% for hesperidin. These findings suggested that the developed method was accurate and precise for the quantification of diosmin more than hesperidin.

Table 1 Accuracy and precision results for diosmin and hesperidin (n = 6)

No.	Diosmin			Hesperidin		
	Amount added (µg/mL)	Amount found (µg/mL)	%Recovery	Amount added (µg/mL)	Amount found (µg/mL)	%Recovery
1	20.94	20.41	97.45	0.64	0.69	107.8
2	20.94	20.36	97.20	0.64	0.63	98.44
3	20.94	20.46	97.69	0.64	0.69	107.8
4	20.94	20.41	97.44	0.64	0.68	106.3
5	20.94	20.41	97.45	0.64	0.68	106.3
6	20.94	20.43	97.55	0.64	0.62	96.88
Average			97.47	-	-	103.9
SD			0.16	-	-	4.92
%RSD			0.16	-	-	4.73

4.2 Assay of *C. quadrangularis* capsules

Sample solution containing 0.02 mg/mL of *C. quadrangularis* powder was prepared and measured the absorbances in triplicate at 267.5 and 284.0 nm. The amount of diosmin and hesperidin calculated by simultaneous equation (mean ± SD) were found to be 29.88 ± 0.08 and 3.176 ± 0.45 mg/capsule (125 mg of *C. quadrangularis* powder), respectively.

5. Discussion

Determination of diosmin and hesperidin by UV-visible spectroscopy using simultaneous equation method was found to be simple, precise, and accurate. Srilatha, *et al.* (2013) had introduced the success of this analytical method to quantify the amount of diosmin and hesperidin in tablet dosage form (Daflon® 500 mg, each tablet contains diosmin 450 mg and hesperidin 50 mg), using 0.2 N NaOH as a solvent. The results showed satisfied validation parameters in terms of linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and ruggedness with a simple procedure for sample preparation. Thus, the main concept of previous research was adapted to our study for simultaneous determination of diosmin and hesperidin in *C. quadrangularis* capsules. For method validation, the results of diosmin met the criteria in terms of linearity, accuracy, and precision. On the other hand, the validation results of hesperidin seemed to be unsatisfied especially for specificity and precision. This may due to a very low proportion of hesperidin which usually occurred in combination with diosmin in 1:9 ratio, and the effect of other compounds in the matrix that possibly interfered the measured absorbances. Therefore, an appropriate extraction process must be applied for sample preparation to primarily separate the interferences, resulting to an increase of method specificity. Moreover, most of commercially available *C. quadrangularis* capsules are in combined traditional medicines which the exact quantity of diosmin and hesperidin is unknown. For this reason, the amount of these compounds in *C. quadrangularis* powder was estimated by calculating from total flavonoid content found, which 100 g of *C. quadrangularis* stem powder contained 7.86 g of total flavonoids (Nawghare *et al.*, 2017, pp. 443-445). However, this tentatively estimation was useful to provide a starting information for the preparation of sample solution to contain

appropriate concentrations of diosmin and hesperidin for further analysis, including the setting of accuracy and precision acceptance criteria.

Our findings showed the concept of simultaneous analysis to determine the quantity of diosmin and hesperidin in *C. quadrangularis* capsules without the separation of each compound. Although this analytical method was found to be simple and easy to operate, the presence of other components in the analyte still affected the specificity as mentioned above. Thus, using of more efficient method to quantify and isolate the interested compounds in samples was expected to overcome this problem. Kanaze, *et al.* (2003) had previously reported a reversed-phase high-performance liquid chromatographic method (RP-HPLC) for simultaneous determination of diosmin and hesperidin in Daflon® tablets, including marketed and fresh citrus fruit juices. Compared with the UV-visible spectrophotometric method (Srilatha *et al.* 2013, pp. 1-4), LOQ of hesperidin obtained from HPLC method was noticed to be lower (0.1 vs. 0.42 μg/mL), whereas the other validation parameters were resemble and met the criteria.

6. Conclusion

The analytical method in this study was considered to be simple, accurate and precise, especially for the quantification of diosmin. However, the application of the developed method for simultaneously determination of diosmin and hesperidin in *C. quadrangularis* capsules still had some limitations. Thus, separation technique with qualitative and quantitative analysis, such as high-performance liquid chromatography (HPLC) could be a specific, accurate, and precise method to estimate the content of diosmin and hesperidin in multicomponent formulations for further study.

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8. References

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