Bulletin of Faculty of Pharmacy, Cairo University (2013) **51**, 81–88

Cairo University

Faculty of Pharmacy

Cairo University

Bulletin of Faculty of Pharmacy, Cairo University

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ORIGINAL ARTICLE

Fluorimetric determination of diosmin and hesperidin in combined dosage forms and in plasma through complex formation with terbium

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Received 10 November 2012; accepted 29 December 2012
Available online 28 January 2013

KEYWORDS

Fluorimetric determination;
Ternary complex;
Terbium;
Dosage forms;
Plasma

Abstract A sensitive and simple fluorimetric method was developed for the determination of diosmin and hesperidin. The proposed method involves the formation of ternary complex with Tb^{3+} in the presence of Tris buffer. The fluorescence quenching of Tb^{3+} at 549 and 494 nm (λ_{ex} at 275 and 248 nm) due to the complex formation was quantitatively measured for diosmin and hesperidin, respectively. The reaction conditions and the fluorescence spectral properties of the complexes have been investigated. Under the described conditions, the proposed method was applicable over the concentration range (4.93×10^{-6}–1.81×10^{-5} mol) and (3.28×10^{-6}–1.64×10^{-5} mol) with mean percentage recoveries 100.22 ± 0.89 and 99.13 ± 0.72 for diosmin and hesperidin, respectively. The proposed method was applied successfully for the determination of studied drugs in bulk powder, dosage forms and plasma samples. The results obtained by applying the described method were statistically analyzed and compared with those obtained by applying a reported method. The method was validated according to ICH recommendations.

1. Introduction

Flavonoids are important polyphenolic secondary metabolites that are widely distributed in medicinal plants and foods of plant origin providing much of the flavor and color to fruits and vegetables.[1] Among these flavonoids; the flavone glycoside diosmin (3′,5,7-trihydroxy- 4′-methoxyflavone 7-rutinoside) and its flavanone analog hesperidin (3′,5,7-trihydroxy-4′-methoxyflavanone 7-rhamnoglycoside). The two flavonoids are common constituents in many citrus species.[1,2] Diosmin and hesperidin possess antioxidant, blood lipid lowering, anti-carcinogenic activities.[3,4] In addition, both drugs improve venous tone, enhance microcirculation, assist healing of venous ulcers and they are used for the treatment of chronic venous insufficiency, hemorrhoids and the prevention of post-operative thromboembolism.[1] In view of the increasing interest in these bioflavonoids, especially for the treatment of chronic venous insufficiency, chronic hemorrhoids and as antioxidants, several methods have been reported for the determination of

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Peer review under responsibility of Faculty of Pharmacy, Cairo University.

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http://dx.doi.org/10.1016/j.bfopcu.2012.12.001

diosmin and hesperidin in plant extracts, biological fluids and dosage forms, the majority of which are chromatographic in nature.[1–3,5–11] In addition, capillary electrophoretic techniques[12–14] as well as electrochemical methods have been also used for the determination of both drugs.[15–18] However, few spectrophotometric methods for analyzing diosmin[19–21] and hesperidin[20,22,23] have been found in the literature. Although fluorimetric methods of analysis offer great sensitivity and selectivity for the determination of a large number of analytes, however, to the best of our knowledge, no spectrofluorimetric method is available in the literature for the determination of diosmin. In addition two methods were developed for the determination of hesperidin based on the formation of fluorescent metal chelates either with magnesium[24] or aluminum[25], however, they lack application to the determination of the drug in dosage forms or in plasma. Where, the latter method was applied for the determination of hesperidin in orange peel and orange juice.

Luminescence spectrometry offers possibilities of sensitive and selective detection, by the use of lanthanide sensitized luminescence. The specific strong fluorescence of lanthanide ions, which usually occurs as a result of intramolecular energy transfer through the excited triplet state of the ligand to the emitting level of the lanthanide ion, is characterized by large Stokes shifts, narrow emission bands and long fluorescence lifetimes.[26] As a result of spectral and temporal discrimination of the analytical signal, background fluorescent emission interferences from the complex matrices are eliminated, selectivity and detection limits are improved. In some instances, however, when the organic compound has a triplet state level below the excited state level of the lanthanide ion, the organic compound can quench the background luminescence of the ion.[27,28] The sensitizing or quenching effect is more important with chloride than the nitrate salts of the lanthanide, because the probability of collision leading to energy transfer is larger for the chloride salt.[27,28] Based on energy level considerations and luminescence quantum yields, Tb^{3+} and Eu^{3+} are the best lanthanide ions to be applied to the determination of organic compounds. The terbium metal ion (Tb^{3+}) has been the lanthanide of choice in most applications of ternary complex formation and was used as fluorescent probes for the determination of several compounds.[29–35]

The lack of spectrofluorimetric methods for the analysis of both diosmin and hesperidin in combined dosage forms and in plasma has motivated us to develop a specific and sensitive spectrofluorimetric method for their determination. This method is based on the quenching of fluorescence produced by these drugs on Tb^{3+} – Tris system. The fluorescence properties were investigated using different buffer systems and different solvents. Factors affecting complexation such as the concentration of Tb^{3+} and the ratio of Tb^{3+} to the studied drugs were carefully investigated. The method was subsequently used to determine the concentration of the drugs in dosage forms and in spiked plasma samples. Our experimental results were validated by a well-established reported method.

2. Materials and Methods

2.1. Instrumentation

The spectrofluorimetric measurements were performed using a Jasco FP-6200 Spectrofluorometer using quartz cell, band width (ex): 5 nm, band width (em): 5 nm, response: medium, sensitivity: medium, scanning speed: 250 nm/min. For buffer preparation a digital pH meter, HANNA instruments HI2211 pH/ORP Meter was used.

2.2. Chemicals and reagents

Diosmin and hesperidin were obtained as gifts from Sedico Pharmaceutical Co., 6 October City, Egypt. Their purities were certified and analyzed by a reported method[3] and were found to be 99.44% and 98.54%, respectively. They were used as provided. Terbium chloride 99.99% was obtained from Sigma–Aldrich, St. Louis, USA. It was prepared as 2×10^{-3} M in methanol. Tris(hydroxyl methyl)amino methane was purchased from Fluka-Biochemica, St. Louis, USA. Tris buffer was prepared by dissolving 2 g of Tris and 2.4 g of sodium chloride in 100 ml distilled water, pH was adjusted with 1 M HCl and then the volume was diluted to 200 ml with distilled water. Methanol and sodium hydroxide were obtained from Sigma–Aldrich, St. Louis, USA. Hydrochloric acid was purchased from Riedel-de Haen, Seelze, Germany. Acetonitrile was obtained from Merck, Darmstadt, Germany. Sodium chloride, boric acid and dipotassium hydrogen orthophosphate were purchased from ADWIC; El Nasr Pharmaceutical Chemicals, Egypt.

Dosage forms of Daflon 500 mg Tablets: Diosmin 450 mg, Hesperidin 50 mg B.N. 17162 (Servier Egypt Industries Limited, 6th October City, Giza, Egypt), Dioven 500 Tablets: Diosmin 500 mg B.N. 614710[A] (Amriya Pharm Ind., Alexandria, Egypt), Diosed C 500 mg Tablets: Diosmin 450 mg, Hesperidin 50 mg B.N. 1209195 (Sedico Pharmaceutical Co., 6 October City, Egypt), Veinatonic Tablets: Diosmin 450 mg, Hesperidin 50 mg B.N. 12227 (Sigma Pharmaceutical Industries, Egypt, S.A.E.) were purchased from local market. Human Plasma: B.N. 1005250102 was obtained from Vacsera, Giza, Egypt.

2.3. Standard solutions

Diosmin (1.64×10^{-4} mol), was prepared by dissolving the appropriate amount in a 100 ml volumetric flask in 5 ml of 0.1 N NaOH and the volume was completed to the mark with methanol, dissolution was aided by sonication. Hesperidin (1.64×10^{-4} mol) in methanol, dissolution was aided by sonication. The stock solutions of both drugs were protected from daylight and stored in a refrigerator.

2.4. Procedures

2.4.1. Construction of calibration curve

2.0 ml of terbium solution (2×10^{-3} mol) was transferred into a series of 10 ml volumetric flasks. Aliquots equivalent to 4.93×10^{-5}–1.81×10^{-4} mol for diosmin and 3.28×10^{-5}–1.64×10^{-4} mol for hesperidin were transferred from the corresponding stock solutions, followed by 2 ml of Tris buffer of pH 8 and pH 8.5 for diosmin and hesperidin, respectively. The volume was completed with distilled water. The solution was left for 15 min then the fluorescence quenching was measured at 549 and 494 nm using 275 and 248 nm as excitation wavelengths for diosmin and hesperidin, respectively. The concentration versus the decrease in fluorescence intensity was

plotted to obtain the standard calibration graphs and the linear regression equations were computed.

2.4.2. Procedure for dosage forms

2.4.2.1. Dioven 500 tablets. Five tablets were pulverized well; an accurate amount of the powdered tablets was weighed and transferred to a 100 ml volumetric flask. The drug was dissolved in 5 ml of 0.1 N NaOH and the volume was completed to the mark with methanol to obtain 100 ml of 1.64×10^{-4} mol diosmin. The resulting solution was sonicated for 30 min and filtered then the procedure described under calibration curve was carried out.

2.4.2.2. Daflon 500, Diosed C and veinatonic tablets.

For diosmin determination: Five tablets were pulverized well; an accurate amount of the powdered tablets was weighed and transferred to a 150 ml beaker. Then 25 ml of methanol was added and the resulting mixture was sonicated for 30 min and filtered. The filtrate (containing hesperidin) was rejected. The residue (containing diosmin) was collected in the beaker and dissolved in 5 ml 0.1 N NaOH then transferred quantitatively to a 100 ml volumetric flask with the aid of methanol. The volume was completed to the mark with methanol to obtain 100 ml of 1.64×10^{-4} mol diosmin. The resulting solution was sonicated for 30 min and filtered. Finally the procedure was completed as described under calibration curve.

For hesperidin determination: Five tablets were pulverized well; an accurate amount of the powdered tablets was weighed and transferred to a 100 ml volumetric flask. The drug was dissolved in methanol and the volume was completed to the mark with methanol to obtain 100 ml of 1.64×10^{-4} mol hesperidin. The resulting solution was sonicated for 30 min and filtered then the procedure described under calibration curve was carried out.

2.4.3. Procedure for spiked human plasma

Plasma samples were stored at −80 °C then thawed at room temperature on the day of analysis. One ml aliquots of human plasma were transferred into a series of centrifugation tubes, spiked with increasing quantities of diosmin and hesperidin to give a final concentration range of 4.93×10^{-6}–1.81×10^{-5} mol and 3.28×10^{-6}–1.64×10^{-5} mol, respectively. Then 2.5 ml of acetonitrile was added and centrifugation at 6000 rpm for 45 min was performed. Aliquots from the clear supernatant were transferred quantitatively to 10 ml volumetric flasks and evaporated to dryness. The residue was reconstituted in the dissolving solvents and the steps described under calibration curve were carried out.

3. Results and discussion

3.1. Spectral characteristics

The fluorescence spectra of diosmin and hesperidin in their dissolving solvents were investigated, it was observed that both drugs showed no notable fluorescence. The spectral characteristics of Tb^{3+} in methanol are shown in Fig. 1. A very weak fluorescence could be detected which was not surprising. It is well known that generally lanthanide ions emit weak fluorescence because of the weak absorption bands, and because they can be non-radiatively deactivated by the solvent molecules.[36]

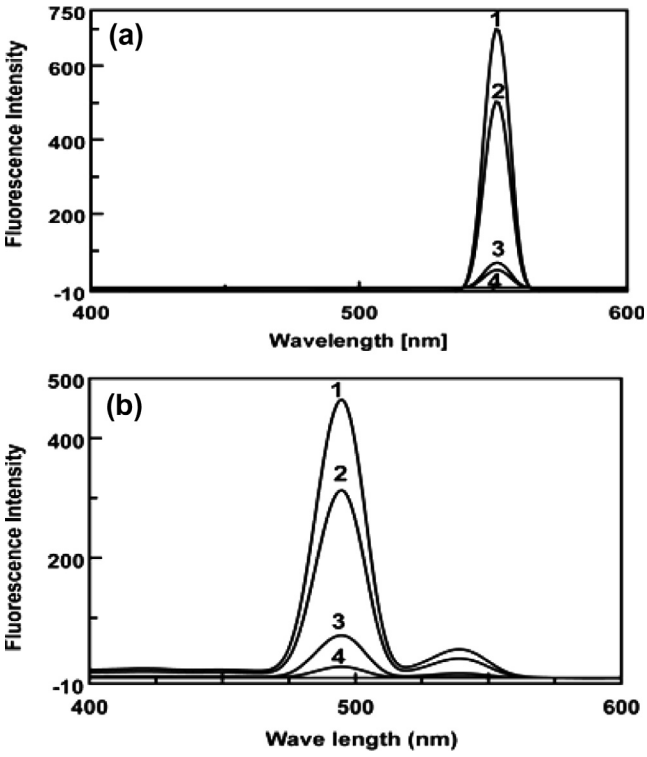

Figure 1 (a) Emission spectra at 549 nm; 1. Tb^{3+} – Tris (pH 8.0); 2. Tb^{3+} – Tris – Diosmin (1.48×10^{-5} mol); 3. Tb^{3+}; 4. Diosmin (1.48×10^{-5} mol). (b) Emission spectra at 494 nm; 1. Tb^{3+} – Tris (pH 8.5); 2. Tb^{3+} – Tris – Hesperidin (1.15×10^{-5} mol); 3. Tb^{3+}; 4. Hesperidin (1.15×10^{-5} mol).

However, addition of Tris buffer to Tb^{3+} solution resulted in an intense emission at 549 and 494 nm after excitation at λ_{ex} 275 and 248 nm. Clearly, the intensity of the emission line was greatly enhanced in the presence of Tris buffer as compared to Tb^{3+} alone. On adding the studied drugs immediate fluorescence quenching was observed. This phenomenon could be attributed to the level of the triplet state of the organic compounds which is below the excited state level of the lanthanide ion, so, the organic compounds can quench the background luminescence of the ion. The relative emission spectra are shown in Fig. 1. The decrease in fluorescence intensity was proportional to the concentration of the added drugs at λ_{em} 549 and 494 nm (λ_{ex} 275 and 248 nm) for diosmin and hesperidin, respectively.

3.2. Method optimization

Having established that diosmin and hesperidin caused fluorescence quenching of the Tb^{3+} – Tris complex; we embarked on studying the optimum reaction conditions.

3.2.1. Effect of pH

The maximum fluorescence quenching of the drug solutions was studied by varying the pH of Tris buffer in the range pH 5.5–10.0 while keeping other experimental factors constant. Fig. 2 shows that on increasing the pH, fluorescence quenching increases and reaches maximum at pH 8.0 and 8.5 for diosmin and hesperidin, respectively. However, when the pH was

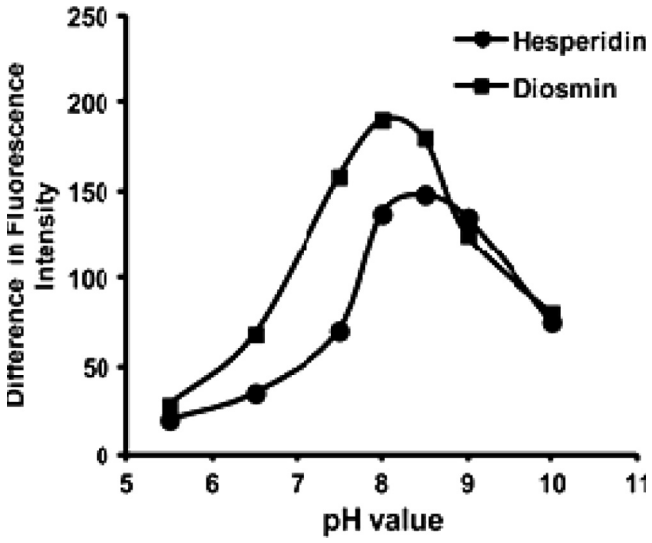

Figure 2 Effect of different pH values on the reaction between Tb^{3+} and Tris buffer with diosmin (1.48×10^{-5} mol) and hesperidin (1.15×10^{-5} mol).

further increased, the intensity of fluorescence quenching decreased sharply. At pH higher than 10.0, non-reproducible quenching was observed which is attributed to the competitive hydrolysis of Tb^{3+} with the deposition of $Tb(OH)_3$. So, the choice of the suitable pH was critical for obtaining maximum fluorescence quenching by the drugs.

3.2.2. *Effect of buffer*

The experiments indicated that the kind of buffer has a great influence on both the enhancement of the fluorescence intensity of Tb^{3+} ions as well as the quenching of fluorescence of the Tb^{3+}—Tris complex by the studied drugs. So, different buffers including Tris, borate and phosphate buffers at the chosen pH were tested (Fig. 3). The results indicated that Tris buffer showed the sharpest increase in the fluorescence intensity of

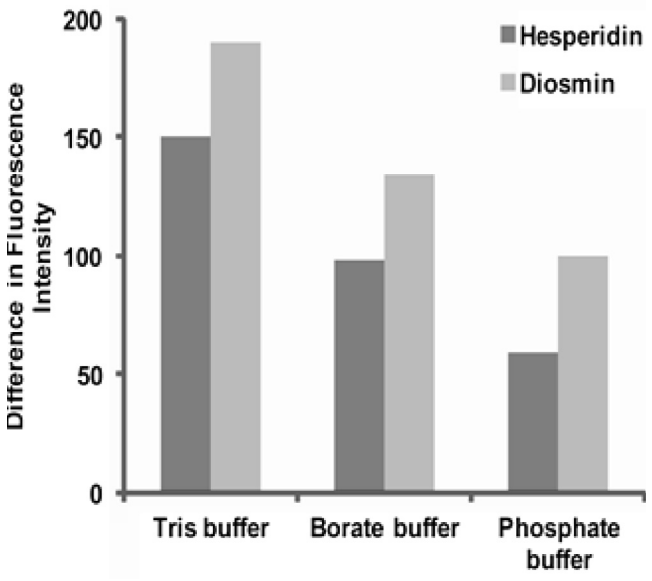

Figure 3 Effect of different buffer types on the reaction of Tb^{3+} with diosmin (1.48×10^{-5} mol) and hesperidin (1.15×10^{-5} mol).

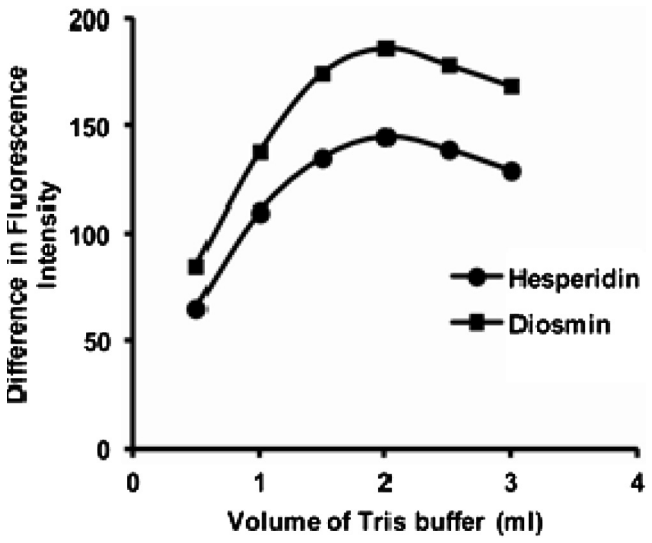

Figure 4 Effect of Tris buffer volume on the reaction of Tb^{3+} with diosmin (1.48×10^{-5} mol) and hesperidin (1.15×10^{-5} mol).

Tb^{3+} ions. Similar behavior was previously observed by several authors, where it was reported that Tris buffer may chelate with Tb^{3+} to give an intense fluorescence.[27,28,31] In addition, Tris buffer also resulted in the best quenching of fluorescence upon addition of diosmin and hesperidin. It is worthy to note that, several cephalosporin antibiotics[27] as well as some sulfur containing drugs[28] were reported to cause quenching of the fluorescence of Tb^{3+}—Tris complex. Also the effect of volume of Tris buffer was studied and it was found that 2 ml was adequate for complete reaction (Fig. 4).

3.2.3. *Effect of Tb^{3+} ion concentration*

The effect of Tb^{3+} concentration on fluorescence intensity was studied by using different volumes of terbium chloride (2×10^{-3} mol). It was found that 2 ml was appropriate for producing maximum intensity of the emission line of terbium ions,

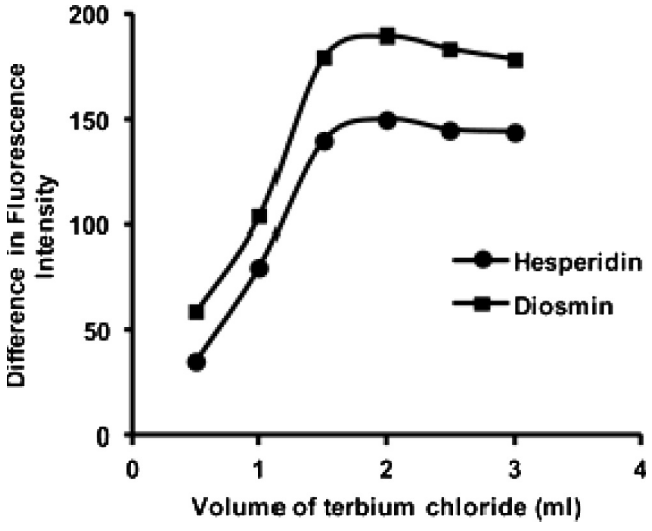

Figure 5 Effect of Tb^{3+} volume (2×10^{-3} M) on the reaction of Tb^{3+} with diosmin (1.48×10^{-5} mol) and hesperidin (1.15×10^{-5} mol).

as well as, best fluorescence quenching by the studied drugs, (Fig. 5). In addition, the stoichiometry of the reaction was studied by the molar ratio and it was found to be 1 Tb: 3 drug.

3.2.4. Effect of diluting solvents

It is a well known phenomenon that the type of solvent used influences the luminescence intensity of the molecule. Therefore an attempt was made to study the effect of different solvents (water, methanol, ethanol and acetonitrile) on the luminescence intensity of the Tb^{3+}—Tris—drug complex. It was found that optimum conditions are obtained when using water as a solvent. This conclusion is in agreement with the literature stating that water molecules strongly quench terbium sensitized luminescence.[32] The low sensitization in water may be due to the O–H oscillators of water molecules that are known to quench the excited state of the lanthanide ions by interaction with high-energy vibrations associated with O–H. Other solvent molecules such as methanol or ethanol can usually replace coordinated water and avoid high frequency O–H bond vibration, thus greatly decreasing energy loss resulting through the coordination of water molecules, and increasing the fluorescence intensity of the emitting lanthanide ion.[29] This suggests that the chemical environment plays an important role in determining the fluorescence intensity of the complexes; hence the choice of water was ideal in studying the luminescence of the Tb^{3+}—Tris—drug complex.

3.2.5. Stability test

The experiments indicated that the fluorescence intensity of the system reached a maximum in 15 min after all reagents had been added and remained stable for at least 1 h.

3.3. Method validation

3.3.1. Linearity and range

The calibration graphs for the determination of diosmin and hesperidin by the proposed method were constructed under the optimum experimental conditions. The graphs were found to be rectilinear in the concentration ranges of 4.93×10^{-6}–1.81×10^{-5} mol and 3.28×10^{-6}–1.64×10^{-5} mol for diosmin and hesperidin, respectively, and with the regression equations cited in Table 1.

Table 1 Performance data for diosmin and hesperidin by the proposed fluorimetric method.

Parameter	Diosmin	Hesperidin
Linearity range	4.93×10^{-6}–1.81×10^{-5} mol	3.28×10^{-6}–1.64×10^{-5} mol
Regression equation	$\Delta FI = 1.27 \times 10^{7} C + 1.66$	$\Delta FI = 1.23 \times 10^{7} C + 8.16$
Slope (b)	1.27×10^{7}	1.23×10^{7}
Intercept (a)	1.66	8.16
Correlation coefficient (r)	0.9999	1
SE of slope	1.07×10^{-5}	5.05×10^{-4}
SE of intercept	1.33	0.55
$S_{y/x}$	1.12	0.52
LOD	2.91×10^{-7}	1.40×10^{-7}
LOQ	8.82×10^{-7}	4.23×10^{-7}

$\Delta FI = a + bc$, ΔFI = relative fluorescence intensity, a = intercept, b = slope, C = concentration in mol, $S_{y/x}$ = standard deviation of residuals, LOD = limit of detection, LOQ = limit of quantitation.

3.3.2. Accuracy

The accuracy of the proposed method was evaluated by analyzing six levels of standard solutions of the studied drugs, each three times. The mean percentage recoveries obtained by the proposed method were found to be 100.22 ± 0.89 and 99.13 ± 0.72 for diosmin and hesperidin, respectively. These results were favorably compared with those of a reported one.[3] Statistical analysis[37] obtained by the proposed and reported methods using Student's t-test and variance ratio F-test, showed no significant difference between the performance of the two methods as indicated by the smaller calculated values compared to the corresponding theoretical values of t and F (Table 2).

3.3.3. Precision

The intraday precision was evaluated through replicate analysis of the standard solutions of the drugs. Whereas the interday precision was performed through replicate analysis of the standard solutions of the drugs on three successive days. The percentage recoveries as well as the percentage relative standard deviations were calculated as abridged in Table 3.

3.3.4. Selectivity

The selectivity of the method was investigated by observing any interference encountered from the common tablet excipients such as talc, lactose, starch and magnesium stearate. It was found that these compounds did not interfere with the assay of the proposed method.

3.3.5. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to ICH recommendations.[38] The LOD was found to be 2.91×10^{-7} and 1.4×10^{-7} while the

Table 2 Statistical comparison for the results obtained by the proposed fluorimetric method and a reported one[3] for the analysis of diosmin and hesperidin in pure form.

Item	Diosmin		Hesperidin	
	Proposed	Reported[3**]	Proposed	Reported[3**]
Mean*	100.22	99.44	99.13	98.54
±SD	0.89	0.79	0.72	0.66
% RSD	0.89	0.80	0.73	0.67
% REr	0.36	0.40	0.30	0.34
Variance	0.79	0.63	0.52	0.44
t-Test	1.41. . . ..**(2.31)**		1.30. . . ..**(2.31)**	
F-test	1.25. . . ..**(9.013)**		1.19. . . ..**(9.013)**	

S.D. = standard deviation, % RSD = percent relative standard deviation, % REr = percent relative standard error, the values in parenthesis are the corresponding theoretical values of t and F at $P = 0.05$, where $n = 6$ for the proposed method and $n = 4$ for the reported method.

* Average of three determinations.

** The reported method is an HPLC method using LiChrosorb RP-18 column, a mixture of methanol–water (60:40, v/v) as a mobile phase in an isocratic mode at a flow rate of 1.5 ml/min and UV detection at 345 nm.

Table 3 Intraday and interday statistical data for the studied drugs in pure form by the proposed fluorimetric method.

Parameter	Concentration of diosmin in mol			Concentration of hesperidin in mol		
	4.93×10^{-6}	1.15×10^{-5}	1.81×10^{-5}	3.28×10^{-6}	9.83×10^{-6}	1.64×10^{-5}
Intraday						
Mean* (X)	100.72	99.33	100.51	99.93	98.04	99.35
±S.D	0.48	0.86	0.86	0.59	0.95	0.78
% R.S.D	0.48	0.86	0.85	0.59	0.97	0.79
% REr	0.27	0.50	0.49	0.34	0.56	0.45
Variance	0.23	0.73	0.73	0.35	0.90	0.61
Interday						
Mean* (X^-)	101.05	99.80	100.48	98.98	100.70	99.31
±S.D	0.72	1.06	0.45	1.03	0.77	0.70
% R.S.D	0.71	1.06	0.45	1.04	0.76	0.71
% REr	0.41	0.61	0.26	0.60	0.44	0.41
Variance	0.51	1.13	0.21	1.06	0.59	0.49

* Average of three determinations.

LOQ was 8.82×10^{-7} and 4.23×10^{-7} for diosmin and hesperidin, respectively as cited in Table 1.

3.3.6. Robustness of the method

The robustness of the method adopted was demonstrated by the consistency of the fluorescence values with the deliberate minor changes in the experimental parameters. So, when changing the pH value of Tris buffer by ±0.2 units or varying the volume of Tb^{3+} ion or Tris buffer by ±0.5 ml, the fluorescence intensity was not greatly affected.

3.4. Application of the proposed method

The proposed method was successfully applied to the determination of diosmin and hesperidin in tablets of different brands. The concentrations of the drugs were calculated referring to

Table 4 Statistical comparison for the results obtained by the proposed fluorimetric method and the reported one[3] for the analysis of diosmin in its dosage forms.

Item	Diosed C 500 mg tablets		Veinatonic tablets		Daflon 500 mg tablets		Dioven 500 tablets	
	Proposed	Reported[3]	Proposed	Reported[3]	Proposed	Reported[3]	Proposed	Reported[3]
Mean*	97.82	97.18	99.02	98.23	98.57	97.91	98.29	97.21
±SD	0.77	0.62	0.50	0.76	0.85	0.77	0.97	0.60
% RSD	0.79	0.64	0.51	0.78	0.86	0.78	0.99	0.62
% REr	0.32	0.32	0.21	0.39	0.35	0.39	0.40	0.31
Variance	0.59	0.39	0.25	0.58	0.72	0.59	0.95	0.36
t-Test	1.39. . . . . **(2.31)**		1.99. . . . . **(2.31)**		1.24 . . . . . **(2.31)**		1.95. . . . . **(2.31)**	
F-test	1.52. . . . . **(9.013)**		2.31. . . . . **(5.409)**		1.23. . . . . **(9.013)**		2.62. . . . . **(9.013)**	

The values in parenthesis are the corresponding theoretical values of t and F at $P = 0.05$, where $n = 6$ for the proposed method and $n = 4$ for the reported method.
* Average of three determinations.

Table 5 Statistical comparison for the results obtained by the proposed fluorimetric method and the reported one[3] for the analysis of hesperidin in its dosage forms.

Item	Diosed C 500 mg tablets		Veinatonic tablets	
	Proposed	Reported[3]	Proposed	Reported[3]
Mean*	97.36	97.93	98.90	98.56
±SD	0.69	0.66	0.69	0.56
% RSD	0.71	0.67	0.69	0.57
% REr	0.29	0.34	0.28	0.28
Variance	0.48	0.43	0.47	0.32
t-Test	1.30. . . . . **(2.31)**		0.83. . . . . **(2.31)**	
F-test	1.11. . . . . **(9.013)**		1.49. . . . . **(9.013)**	

The values in parenthesis are the corresponding theoretical values of t and F at $P = 0.05$, where $n = 6$ for the proposed method and $n = 4$ for the reported method.
* Average of three determinations.

Table 6 Determination of diosmin and hesperidin in their dosage forms by the proposed method with the application of the standard addition technique.

Item	Taken (mol)	Added (mol)	Diosmin recovery%*			
			Diosed C 500 mg tablets	Veinatonic tablets	Daflon 500 mg tablets	Dioven 500 tablets
	4.93×10^{-6}	4.93×10^{-6}	97.28	99.58	99.28	97.88
		8.27×10^{-6}	98.74	99.28	99.14	98.74
		1.15×10^{-5}	98.05	98.38	98.45	98.15
Mean			98.02	99.08	98.96	98.26
±SD			0.73	0.62	0.44	0.44
% RSD			0.75	0.63	0.45	0.45
% REr			0.43	0.36	0.26	0.26
Variance			0.53	0.39	0.20	0.19
			Hesperidin recovery%*			
	4.91×10^{-6}	3.28×10^{-6}	98.05	98.59		
		4.91×10^{-6}	97.04	99.69		
		9.83×10^{-6}	98.34	98.72		
Mean			97.81	99.00		
±SD			0.68	0.60		
% RSD			0.70	0.61		
% REr			0.40	0.35		
Variance			0.47	0.36		

* Average of three determinations.

the corresponding regression equation. Commonly used tablet excipients did not interfere in the analysis as indicated by the percentages found. The results obtained are abridged in Tables 4 and 5 and were in accordance with those obtained from the reported method.[3] Statistical analysis[37] obtained by the proposed and reported methods using Student's *t*-test and variance ratio *F*-test, revealed no significant difference between the performance of the two methods regarding the accuracy and precision, respectively. The results of the standard addition technique are cited in Table 6.

The method was further applied for the assay of the two drugs in spiked plasma samples. It has been reported that after oral administration; diosmin[5] and hesperidin[39] are rapidly hydrolyzed by enzymes of the intestinal microflora into their flavone aglycone, diosmetin and hesperitin, respectively, which are subsequently absorbed into the systemic circulation. However, the metabolites still carry the target chemical groups that undergo the fluorigenic reaction of concern thus; analysis of the cited drugs in plasma could be applied. The plasma samples were spiked with different concentrations of the standards of both drugs and deproteinization was carried out by the addition of acetonitrile which is the most effective solvent for precipitation of proteins. However, the fluorescence intensity of the final experimental solution was greatly decreased by the presence of acetonitrile, hence, the acetonitrile layer was evaporated to dryness and the residues containing the drugs were reconstituted in the corresponding dissolving solvents. After optimizing the extraction procedure, the proposed method proved to be suitable for the determination of diosmin and hesperidin in spiked plasma samples with mean recoveries 95.74% and 96.32% and percentage relative standard deviations of 1.99 and 1.58, respectively, as shown in Table 7.

Table 7 Statistical data of the proposed fluorimetric method for the analysis of diosmin and hesperidin in spiked plasma samples.

Parameter	Diosmin	Hesperidin
X^{-} ± SD	95.74 ± 1.90	96.32 ± 1.52
% RSD	1.99	1.58
% REr	0.81	0.64
Variance	3.62	2.31

4. Conclusion

In this study a fluorimetric method was successfully developed for the determination of diosmin and hesperidin. This method is based on the quenching of fluorescence produced by these drugs on Tb^{3+}—Tris system. The proposed method is characterized by being simple, rapid, cheap and needs neither expensive solvents nor sophisticated apparatus. The method has proved to be successful for the determination of the studied drugs in bulk powder, combined dosage forms and spiked plasma samples. The simplicity and sensitivity of this method allow its use in the quality control of the cited drugs and can be extended for routine analysis of the drugs in their dosage forms.

5. Conflict of interest

None.

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