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A new improved Stability-Indicating RP-HPLC method for Determination of Diosmin and hesperidin in combination

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Abstract

A rapid, simple, precise and cost effective stability-indicating RP-HPLC method has been developed and validated for determination of diosmin and hesperidin in combination. The mobile phase consisted of mixture of methanol: water (45:55 v/v). UV detection was performed at 346 nm. The method was linear over the concentration ranges 4.8-7.2 µg mL^{-1}, and 20-40 µg mL^{-1} with correlation coefficient 0.999 both for diosmin and hesperidin. The developed method was validated as per ICH guidelines. The recovery of diosmin and hesperidin were in the range of 100.3 to 102.2 %. The limit of detection and limit of quantitation were 0.0102 and 0.0311µg mL^{-1}for diosmin and 0.0558 and 0.1693 µg mL^{-1}for hesperidin respectively. The proposed method is reproducible, accurate, robust and suitable for the simultaneous quantitative analysis of the studied drugs in bulk and dosage formulation.

Keywords: Diosmin, hesperidin, HPLC, simultaneous determination.

Introduction

Diosmin and hesperidin are important flavonoids that are widely distributed in medicinal plants and foods of plant origin. Disomin is chemically flavones glycoside (3′,5,7-trihydroxy- 4′-methoxyflavone 7-rutinoside) while hesperidin is its flavanone analog (3′,5,7-trihydroxy-4′- methoxyflavanone 7-rhamnoglycoside). Diosmin and hesperidin are the common constituents that is found in various citrus plants.Diosmin differs molecularly from hesperidin by the presence of a double bond between two carbon atoms in diosmin's central carbon ring[1].Diosmin can be manufactured by extracting hesperidin from citrus rinds, followed by conversion of hesperidin to diosmin[2,3]. Diosmin and hesperidin has a wide spectrum of biological and pharmacological activities, including antioxidative, blood lipid lowering and anticarcinogenic activities[4-7]. In adition, these falvoids also used to treat chronic venous insufficiency (CVI), hemorrhoids, lymphedema, and varicose veins. Biological effects of diosmin and hesperidin have also been reported to possess anti-inflammatory, anti-allergenic, antihypertensive, antimicrobial, and vasodilatory properties and to decrease bone density loss[8-10].

In recent years pharmaceutical preparations containing both these flavanoids have been available commercially. A thorough literature search reveals that fewHPLC methods are available for the simultaneous determination of diosmin and hesperidin[11-13].Because use of this pharmaceutical preparation is increasing rapidly, however, it is essential to develop a suitable analytical method for simultaneous estimation of diosmin and hesperidin in pharmaceutical dosage form. HPLC methods have been widely used for routine quality-control assessment of drugs, becauseof their sensitivity, repeatability, and specificity. We havedeveloped a RP-HPLC for the simultaneous determination of diosminand hesperidin in pharmaceutical preparations. However, our method provides easy, fast and cost effective method for the analyses.

In the present study, an attempt has been made to develop a method for the simultaneous estimation of the two flavanoids, diosmin and hesperidin. It can also be applied for routine analysis of either one or any combinations of these drugs in dosage forms. The method was validated in terms of stability, linearity, specificity, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ).

Material and Methods

Instrumentation: Waters HPLC system, equipped with Waters 600 controller pump, auto-sampler (Waters 717 plus) fitted with a 20 µl loop and Waters 486 tunable absorbance detector was used. The output signal was monitored and processed using a Breeze Software. The chromatographic column used was a 150 mm x 4.6 mm, Symetry R RP-C18 with 5µm particles.

Reagents and chemicals: Diosmin and hesperidin were purchased from Sigma-Aldrich, (St. Louis, MO, USA). All the solvents were of HPLC grade, and other chemicals used were of analytical reagent (AR) grade.

Chromatographic conditions: The isocratic mobile phase consisted of mixture of methanol: water (45:55 v/v) was

circulated through a stainless steel analytical column at flow rate of 0.6 mL min^{-1}. The variables UV-VIS detector was set at 346 nm. All the analyses were performed at ambient temperature, and the volume of solution injected onto the column was 5µL.

Preparation of stock solution: Stock solution of diosmin was prepared separately at concentration of 1 mg /ml, using 10% dimethylsulfoxide in methanol. The standard stock solution containing diosmin and hesperidin in same sample were prepared at concentration of 1mg /ml hesperidinand 0.2 mg/ml disomin by adding 2 ml of above stock solution into 10 ml of volumetric flask volume was adjusted with 10% dimethylsulfoxide in methanol.

Calibration graph for simultaneous determination of diosmin and hesperidin: Standard solution of diosmin (at concentration of 2, 4, 6, 8, 10 µg mL^{-1}), and hesperidin (at concentration of 10, 20, 30, 40, 50 µg mL^{-1}) were prepared in same sample using the previously prepared stock solution. A 5µl volume of each standard solution was injected in triplicates onto the HPLC column.

Method Validation: The proposed HPLC method was validated according to the guidelines of international conference on harmonization (ICH)[14].

Specificity: Specificity is the ability of the method to measure the analyte response in the presence of all potential impurities. The specificity was checked by stressing the mixture of diosmin and hespiridine, under extreme conditions, such as 0.1 MHCl and 0.1 MNaOH, at 70°C for 6 h. The stability of the each drug in mixture to oxidation was studied by stirring a solution containing 1% (w/v) of the drugs and 3% H_2O_2 for 2h.

Linearity: Linearity was studied by preparing standard solution at five concentration levels from 80 to 120% of the target analyte concentrations i.e. Concentrations ranging from (4.8-7.2 µg mL^{-1}, and 20-40 µg mL^{-1}) for diosmine and hespiridine respectively. These analyses were performed in triplicate.

Precision: The precision of the method for the assay was studied by repeatability and intermediate precision. Repeatability is an intra-day variation in assay obtained at three concentrations, with nine analyses in one laboratory, on the same day. It expressed in terms of %RSD calculated for each day.

Accuracy: The accuracy of the method was determined by standard addition method by spiking the pre analyzed samples with extra 80, 100 and 120 % of the standard4.8-7.2, 20-40µg $mL^{-1}$for diosmin and hesperid in respectively in triplicate.

The Limit of detection (LOD) and limit of quantitation (LOQ): The Limit of detection (LOD) and limit of quantitation (LOQ) represent the concentration of analyte that would yield signal-to-noise ratios of 3 for LOD and 10 for LOQ respectively. LOD and LOQ were determined by standard deviation of y-intercepts of regression lines and slope of calibration curve for samples containing an analyte in the range of DL.

Robustness: The robustness of the method was determined by small changes in the variables such as flow rate (0.6± 0.25 mL min^{-1}) of mobile phase and concentration of methanol in mobile phase (45:55 ± 2%). The reliability of the method during normal usage was checked by robustness.

Preparation of test sample (tablets) for the assay: Ten tablets of single batch were accurately weighed and powdered in a mortar. A quantity of the powder equivalent to 15 mg diosminewas weighed accurately, transferred to a 50 ml volumetric flask and suspended using 10% DMSO in methanol with sonication upto 20 min, finally the volume was made up with the same solvent. Extract was filtered later the 200µl of solution was transferred to a 10 ml volumetric flask and volume was brought up to mark with methanol. A suitable volume (usually 5µl) of this solution was injected onto HPLC column, concentration of diosmin and hesperidin were obtained from the preconstructed calibration graph.

Results and Discussion

Optimization of chromatographic conditions: In this paper we described an HPLC method for simultaneous analysis of diosmin and hesperidin in tablets. Among different isocratic and gradient mobile phases investigated, the use of an isocratic mobile phase of 45:55 (*v*/*v*) methanol:water was selected on the basis of simplicity, better column regeneration, and appropriate system suitability (theoretical plate number, tailing factor, resolution, capacity factor, *k*, and repeatability) for the analyte peak which complied with limits stipulated (table 1).

Method validation: The developed method was validated according to ICH guideline with respect to specificity, linearity, precision, accuracy, limit of detection, limit of quantitation and robustness.

Specificity: Drug recovery of degradation product is given in table 2. The chromatogram of acid degraded sample showed an additional peak at 5.15 min. The presence of additional degradation peaks at mild conditions (0.1N HCl) reconfirmed the vulnerability of diosmin to degradation in acidic condition and hespeidin was found to be more stable (figure 1). The chromatogram of degradation ofdiosmin and hesperidin in basic medium showed no additional peaks. Heating on water bath did not result in any increase in area of base degraded product indicating that diosmin and hesperidinare stable under basic conditions (0.1 N NaOH) (figure 2).

The chromatogram of 3% H_2O_2 degraded sample showed an additional peaks at 4.94 min.

Linearity: The data were subjected to statistical analysis using a linear- regression model; regression equation and correlation coefficient are given in table 3. The results have indicated good having a broader range compare to previous method. The typical simultaneous chromatogram resulted in a sharp, symmetrical, and well resolved peak at RT value of 2.859 min and 3.736 min for hesperidin and diosmin respectively (figure 4).

Table-1
System suitability parameter

Components (n = 3)	Theoretical plates	Asymmetry	Resolution	Capacity factor
Diosmin	2662.07	1.41	5.10	2.2
Hesperidin	4019.48	1.61	5.10	3.2

Table-2
Results of forced degradation studies of diosmin and hesperidin

Exposure	Diosmin		Hesperidin	
	% Remaining	% RSD	% Remaining	% RSD
Acidic degradation (0.1 N HCl)	40.8	0.26	80.6	0.93
Basic degradation (0.1 N NaOH)	84.6	0.26	93.1	1.11
Oxidation (3% H2O2)	71.2	0.27	82.2	0.95

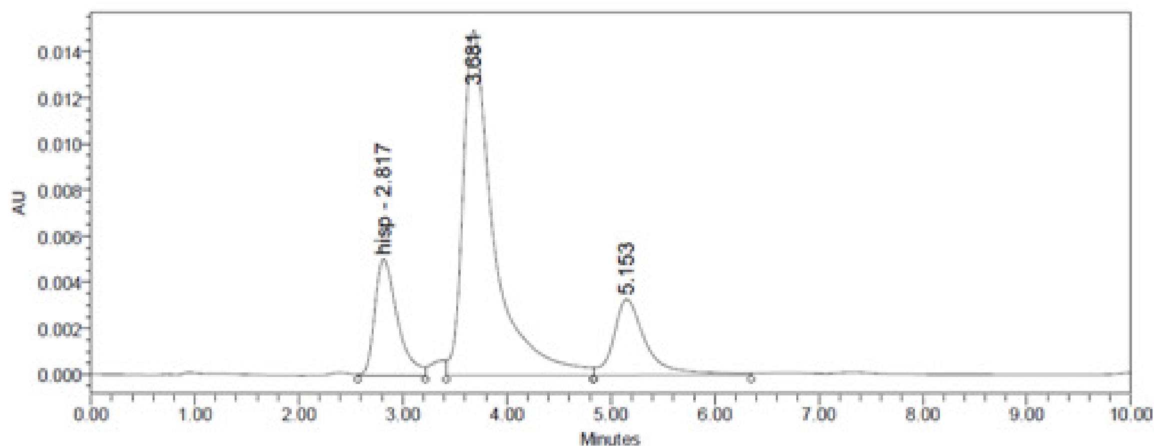

Figure-1
Chromatogram of acid (0.1 N HCl) treated diosmin and hesperidin

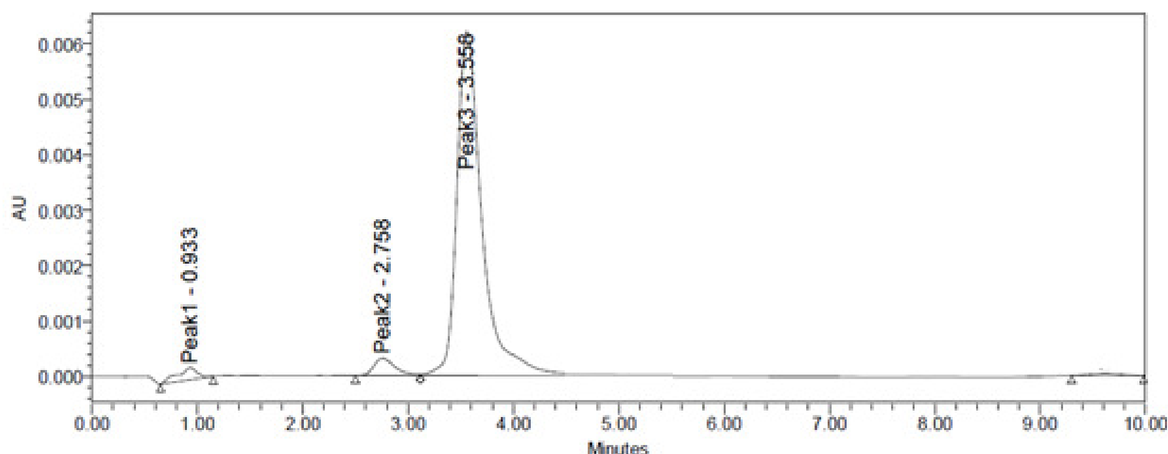

Figure-2
Chromatogram of basic (0.1 N NaOH) treated diosmin and hesperidin

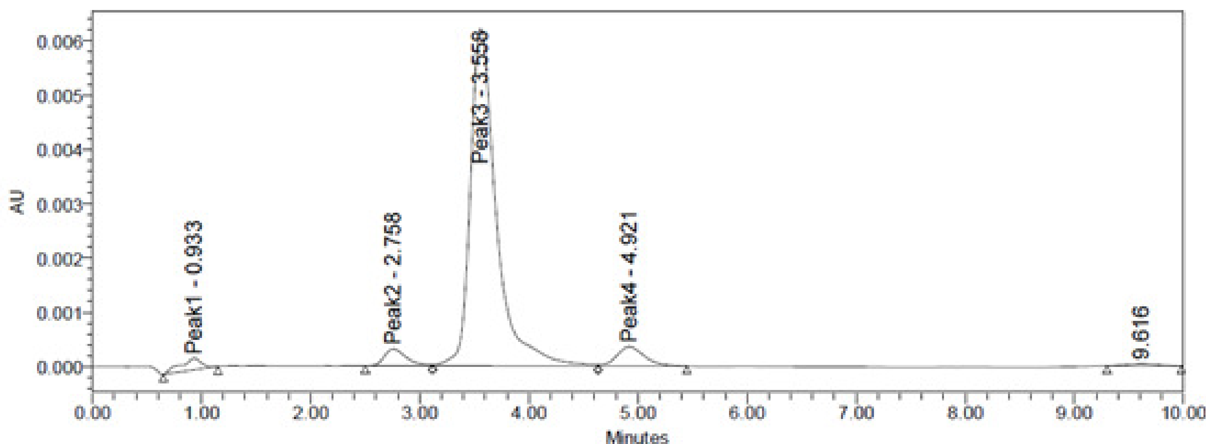

Figure-3
Chromatogram of 3% H2O2 treated diosmin and hesperidin

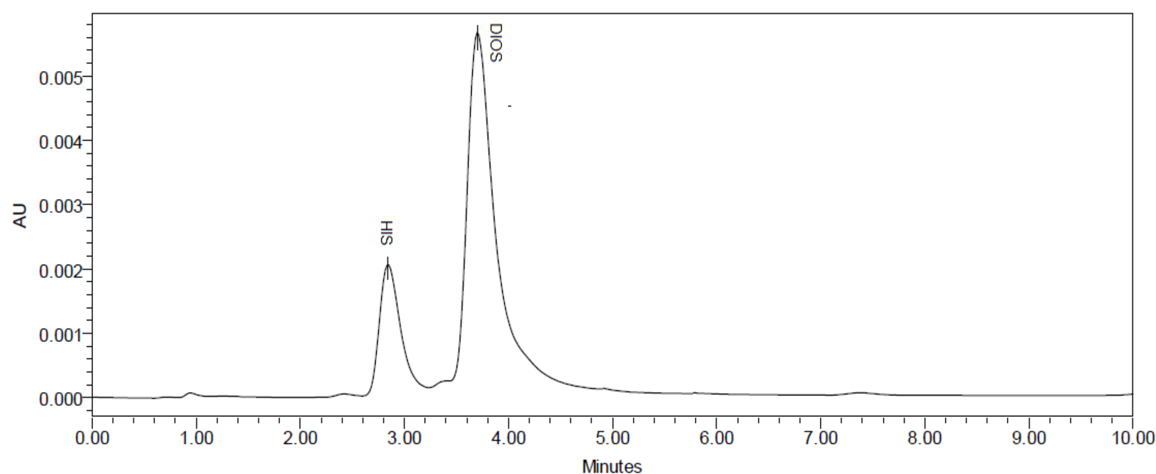

Figure-4
A typical simultaneous chromatogram ofdiosmin and hesperidin

Table-3
Regression statistics

	Diosmin	Hesperidin
Linearity range (µg mL^{-1})	4.8-7.2	20-40
Regression equation	y = 21231x + 81492	y = 1541.x - 113.3
Mean correlation coefficient	0.999	0.999
Mean slope ±SD[a]	21311 ± 75.49	1547 ± 30.55
Mean intercept ±SD[a]	81565 ± 66.29	141.33± 26.2
Standard error of slope	43.58	17.63
Standard error of intercept	38.27	15.12

n=3, average of three determination, SD (±): standard deviation.

Precision: The intra-day variations were studied for three successive days at three concentration level of diosmin and hesperidin (4.8-6-7.2, and 24-30-36µg mL-1respectively). The RSD values were found to be below 1.5 % for each day indicating a good repeatability (table 4). Intermediate precision is the inter day variation at the same sample in the same way on successive days. The inter day variations calculated for three concentration level from the above data expressed in terms of % RSD values. The RSD values were found to be below and equal to 2 %, indicating a good intermediate precision. It is acceptable according to acceptance limit of these parameters. It shows variability of analysis. In all cases, as it could be expected, hesperidin the compound with higher concentration in the sample, presented low variability, while Diosminwith low concentration, presented higher variability.

Accuracy: The percentage recovery of diosmin and hesperidin were found to be in the range of 100.3 to 102.5 and % RSD range from 0.49 to 1.5 respectively. The results of accuracy are shown in table 5.

Table-4
Intraday and inter-day precision data

Amount of drug taken (µg mL^{-1})	Mean conc. found	± SD[a]	RSD (%)[a]	SEM[a]
Diosmin (DIO)				
Intra-day (1st day) (n=3)				
4.8	4.85	0.03891	0.8022	0.02246
6	5.97	0.06802	1.1393	0.03927
7.2	7.27	0.07377	1.0175	0.04259
Inter-day (2nd day) (n=9)				
4.8	4.89	0.09145	1.8435	0.03048
6	6.02	0.1225	2.0348	0.04084
7.2	7.25	0.09066	1.2470	0.03022
Hesperidin (HES)				
Intra-day (1st day) (n=3)				
24	23.74	0.3027	1.2750	0.1748
30	30.11	0.2163	0.7183	0.1249
36	35.99	0.3137	0.8716	0.1811
Inter-day (2nd day) (n=9)				
24	24.37	0.5656	2.3208	0.1885
30	30.44	0.6382	2.0693	0.2127
36	36.37	0.7451	2.0263	0.2484

n=3: average of three injections, SD: standard deviation, RSD: relative standard deviation.

Table-5
Accuracy and Assay data

Components(n=3)	Theoretical recovery level [a], %	Amount added (µg mL^{-1})	Amount recovered (µg mL^{-1})±SD[b]	Mean %Recovery	% RSD[c]	SEM[d]
Diosmin	80	10.8	10.9± 0.125	100.9	1.03	0.072
	100	12.0	12.2± 0.080	101.6	0.49	0.046
	120	13.2	13.5± 0.194	102.2	0.97	0.112
Assay	0	6	6.1[e]± 0.121	101.6	0.21	-
Hesperidin	80	54	54.2± 0.105	100.3	1.5	0.075
	100	60	60.5± 0.060	100.8	0.96	0.56
	120	66	66.9± 0.075	101.3	0.99	0.121
Assay	0	30	30.2[e]± 0.098	100.6	0.33	-

n=3: average of three determinations. a. Recovery procedure: Spiking the pre analyzed samples with extra 80, 100 and 120 % of the standard at three concentration level; and analyzed by the proposed method, b. SD: standard deviation, c. RSD: relative standard deviation, d. SEM: standard error of mean, e. assay concentration in mg.

The Limit of detection (LOD) and limit of quantitation (LOQ): The Limit of detection (LOD) and limit of quantitation (LOQ) of the method were found to be 0.010 and 0.0311; 0.05588 and 0.1693; µg mL^{-1} for diosmin and hesperidin respectively, which indicated that the proposed method is sensitive for detection and quantification of analytes in a mixture, even in very low concentration as compared to reported method.

Robustness: It showed that no significant change in the retention time of analytes of diosmin and hespiridine by changing the composition of mobile phase (45:55 ± 2%) and flow rate (0.6± 0.25 mL min^{-1}). The % RSD values of retention time ($t_{R,}$min) ranged from 0.159 to 0.276 for both variables.

Assay of diosmin and hesperidin: The developed and validated method was applied for the assay of diosmin and hesperidinin tablet dosage form. Results were found out as mean % recovery 98.4% and 99.3%fordiosmin in and 98.6% and 97.4% for hesperidin in daflon and dafrex tablets respectively. The results are shown in table 6. It indicates the successful application of developed method for the simultaneous quantification of active contents.

Table-6
Analysis of tablets for estimation of diosmin and hesperidin

Tablets	Diosmin			Hesperidin		
	Leblled(mg/tab)	Found (mg/tab)	% Recovered	Lebelled (mg/tab)	Found (mg/tab)	% Recovered
Daflon	450	443	98.4	50	49.3	98.6
Dafrex	450	447	99.3	50	48.7	97.4

Conclusion

This HPLC method is stability-indicating and, as revealed by the validation data, enables specific, accurate, robust, and precise simultaneous analysis of diosmin and hesperidin in pharmaceutical preparations. The method is sensitive enough for quantitative detection of the analytes in pharmaceutical preparations and can thus be used for routine analysis, quality control, and for studies of the stability of pharmaceutical preparations containing these drugs.

Conflict of Interest Statement: We declare that we have no conflict of interest.

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