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Rapid Analytical Method Development and Validation of RP-HPLC Method for Simultaneous Estimation of Diosmin and Hesperidin

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ABSTRACT: *Developing a single analytical method for estimation of individual drug from a multidrug composition is a very challenging task. A simple, rapid, precise, and reliable reverse phase HPLC method was developed for the separation and estimation of Diosmin and Hesperidin in bulk drug mix and pharmaceutical dosage forms. The estimation was carried out using Symmetry C18 (4.6×150mm, 5µ) column; mobile phase consisting of Methanol: Water (65:35v/v); the flow rate of 1mL/min and ultraviolet detection at 250 nm. All the drugs were properly resolved having run time of 8 minutes for Diosmin, and Hesperidin, respectively. The method was validated as a final verification of method development with respect to precision, linearity, accuracy, ruggedness, and robustness. The validated method was successfully applied to the commercially available pharmaceutical dosage form, yielding very good and reproducible result.*

KEYWORDS: *Reverse phase HPLC method, Diosmin, Hesperidin, Symmetry C18 (4.6×150mm, 5µ) column and mobile phase consisting of Methanol: Water (65:35v/v)*

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I. INTRODUCTION:

The most potent analytical method that contemporary chemists have at their disposal is most likely chromatography. Its ability to quantitatively identify several distinct components present in a mixture using a single analytical process is what gives it its strength. [1, 2]. In analytical chemistry and biochemistry, high-performance liquid chromatography (HPLC) is a chromatographic method that can separate a mixture of substances and is used to identify, measure, and purify the mixture's constituent parts[3]. In the field of biological separation and purification, reversed phase chromatography has been used for both analytical and preparative purposes. Proteins, peptides, and nucleic acids are examples of molecules with some degree of hydrophobicity that may be separated using reversed phase chromatography with good recovery and resolution[4]. The hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, or stationary phase, is what drives the separation mechanism in reversed phase chromatography. Although there is much disagreement on the precise nature of the hydrophobic binding interaction, it is generally accepted that a beneficial entropy[5] effect is the cause of the binding interaction. Ideally, the sample should dissolve in the first mobile phase. If stability or solubility issues prevent this, the sample can be made more soluble by adding salt, acetic acid, or formic acid. In order to produce a consistent and repeatable solution that can be injected into the column, sample preparation is a crucial step in HPLC analysis. The goal of sample preparation is to create an aliquot of the sample that:

- Is comparatively interference-free;
- Won't harm the column; and
- The sample solvent will dissolve in the mobile phase without compromising sample retention or resolution, making it compatible with the specified HPLC procedure[6]

The main aim of the study is to develop and validate a rapid and reliable RP-HPLC (Reverse Phase High Performance Liquid Chromatography) method for the simultaneous estimation of Diosmin and Hesperidin in pharmaceutical formulations.

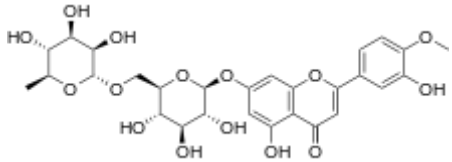
Objectives:

- To optimize the RP-HPLC method parameters such as mobile phase composition, pH, and column type for achieving good resolution and peak shape of Diosmin and Hesperidin.

- To develop a robust chromatographic method that provides accurate and reproducible quantification of Diosmin and Hesperidin within a short analysis time.
- To validate the developed RP-HPLC method as per ICH (International Council for Harmonisation) guidelines with respect to parameters including specificity, linearity, precision, accuracy, robustness, and system suitability.
- To apply the validated RP-HPLC method for the simultaneous estimation of Diosmin and Hesperidin in marketed pharmaceutical formulations.
- To compare the developed method with existing methods in terms of speed, efficiency, and sensitivity for the analysis of Diosmin and Hesperidin.
- To demonstrate the applicability and reliability of the developed RP-HPLC method by analyzing real pharmaceutical samples and comparing the results with label claim.

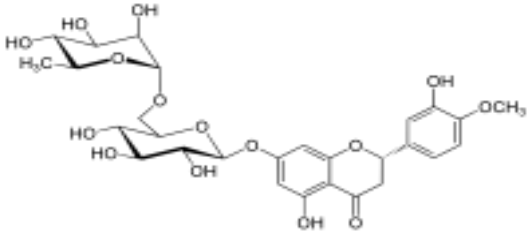
Diosmin (3',5,7-Trihydroxy-4'-methoxyflavone-7-rutinoside), helps to maintain circulatory system structure and function, particularly vein strength and competence[7]. Oral diosmin affects how varicose veins in vitro metabolise noradrenaline, which may improve vascular health and lessen haemorrhoid symptoms.

Structure:



Hesperidin, 2-(3,5-dihydroxy-4-methoxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxychromen-4-one, is a flavonoid glycoside that exhibits antioxidant and anti-inflammatory properties. Along with lowering oxidative stress, it may help enhance vascular health. For its possible health advantages, including as cardiovascular support and antioxidant properties, it is used as a dietary supplement. looked into for possible advantages in lowering inflammation and boosting immunity.

Structure:



II. EXPERIMENTAL METHODS

Table 1: Instruments used

S.No	Instruments And Glass wares	Model
1	HPLC	WATERS Alliance 2695 separation module, software:
2	pH meter	LabIndia
3	Weighing machine	Sartorius
4	Volumetric flasks	Borosil
5	Pipettes and Burettes	Borosil
6	Beakers	Borosil
7	Digital ultra sonicator	Labman

Table 2: Chemicals used

S.No	Chemical	Brand names
1	Diosmin	Procured from Sun pharma, provided by Sura
2	Hesperidin	Procured from Sun pharma, provided by Sura
3	Water and Methanol for HPLC	LICHROSOLV (MERCK)

4	Acetonitrile for HPLC	Merck

HPLC METHOD DEVELOPMENT:

Preparation of standard solution:

Accurately weigh and transfer 10 mg of Diosmin and Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7ml of Methanol and sonicate to dissolve and removal of air completely and make volume up to the mark with the same Methanol.

Further pipette 0.15ml of the above Diosmin and 0.1.35ml of Hesperidin stock solutions into a 10ml volumetric flask and dilute up to the mark with Methanol.

Procedure:

Inject the samples by changing the chromatographic conditions and record the chromatograms, note the conditions of proper peak elution for performing validation parameters as per ICH guidelines.

Mobile Phase Optimization:

Initially the mobile phase tried was Methanol: Water and Water: Acetonitrile and Methanol: TEA Buffer: ACN with varying proportions. Finally, the mobile phase was optimized to Methanol: Water in proportion 65:35 v/v respectively.

Optimization of Column:

The method was performed with various columns like C18 column, X-terra and Zodiac column. Symmetry C18 (4.6×150mm, 5µ) was found to be ideal as it gave good peak shape and resolution at 1ml/min flow.

OPTIMIZED CHROMATOGRAPHIC CONDITIONS:

Instrument used	:	Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature	:	35^0 C
Column	:	Symmetry C18 (4.6×150mm, 5µ)
Mobile phase	:	Methanol: Water (65:35v/v)
Flow rate	:	1ml/min
Wavelength	:	250 nm
Injection volume	:	10 µl
Run time	:	8 min

VALIDATION

PREPARATION OF MOBILE PHASE:

Preparation of mobile phase:

Accurately measured 650 ml of Methanol (65%) and 350 ml (35%) of Water were mixed and degassed in digital ultrasonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Diluent Preparation:

The Mobile phase was used as the diluent.

VALIDATION PARAMETERS

SYSTEM SUITABILITY

Accurately weigh and transfer 10 mg of Diosmin and 10mg of Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Diosmin and 1.35ml of Hesperidin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

SPECIFICITY STUDY OF DRUG:

Preparation of Standard Solution:

Accurately weigh and transfer 10 mg of Diosmin and 10mg of Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Diosmin and 1.35ml of Hesperidin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution:

Take average weight of Tablet and crush in a mortar by using pestle and weight 10 mg equivalent weight of Diosmin and Hesperidin sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent.

Further pipette 1.35ml of Sample stock solution into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

PREPARATION OF DRUG SOLUTIONS FOR LINEARITY:

Accurately weigh and transfer 10 mg of Diosmin and 10mg of Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (5ppm of Diosmin & 45ppm of Hesperidin):

Pipette out 0.05ml of Diosmin and 0.45ml of Hesperidin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (10 ppm of Diosmin&90ppm of Hesperidin):

Pipette out 0.1ml of Diosmin and 0.9ml of Hesperidin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (15ppm of Diosmin&135ppm of Hesperidin):

Pipette out 0.15ml of Diosmin and 1.35ml of Hesperidin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (20 ppm of Diosmin&180ppm of Hesperidin):

Pipette out 0.2ml of Diosmin and 1.8ml of Hesperidin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (25 ppm of Diosmin&225ppm of Hesperidin):

Pipette out 0.25ml of Diosmin and 2.25ml of Hesperidin stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Procedure:

Inject each level into the chromatographic system and measure the peak area.

Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient.

PRECISION

REPEATABILITY

Preparation of Diosmin and Hesperidin Product Solution for Precision:

Accurately weigh and transfer 10 mg of Diosmin and 10mg of Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Diosmin and 1.35ml of Hesperidin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits.

INTERMEDIATE PRECISION:

To evaluate the intermediate precision (also known as Ruggedness) of the method, Precision was performed on different days by maintaining same conditions.

Procedure:

DAY 1:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

DAY 2:

The standard solution was injected for six times and measured the area for all six injections in HPLC. The %RSD for the area of six replicate injections was found to be within the specified limits.

Accuracy:

For preparation of 50% Standard stock solution:

Accurately weigh and transfer 10 mg of Diosmin and 10mg of Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.075ml of the above Diosmin and 0.67ml of Hesperidin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution:

Accurately weigh and transfer 10 mg of Diosmin and 10mg of Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Diosmin and 1.35ml of Hesperidin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution:

Accurately weigh and transfer 10 mg of Diosmin and 10mg of Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.22ml of the above Diosmin and 2.02ml of Hesperidin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:

Inject the Three replicate injections of individual concentrations (50%, 100%, 150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Diosmin and Hesperidin and calculate the individual recovery and mean recovery values.

ROBUSTNESS:

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results.

For preparation of Standard solution:

Accurately weigh and transfer 10 mg of Diosmin and 10mg of Hesperidin working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Further pipette 0.15ml of the above Diosmin and 1.35ml of Hesperidin stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Effect of Variation of flow conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of mobile phase organic composition:

The sample was analysed

III. RESULTS AND DISCUSSION

Trails for Method Development

Trail 1:

Column	: Hypersil C18 (4.6mm×250mm) 5µ particle size
Column temperature	: 30ºC
Wavelength	: 252nm
Mobile phase ratio	: Acetonitrile: Water (60:40% v/v)
Flow rate	: 1.0ml/min
Injection volume	: 10.00µl
Run time	: 10 Minutes

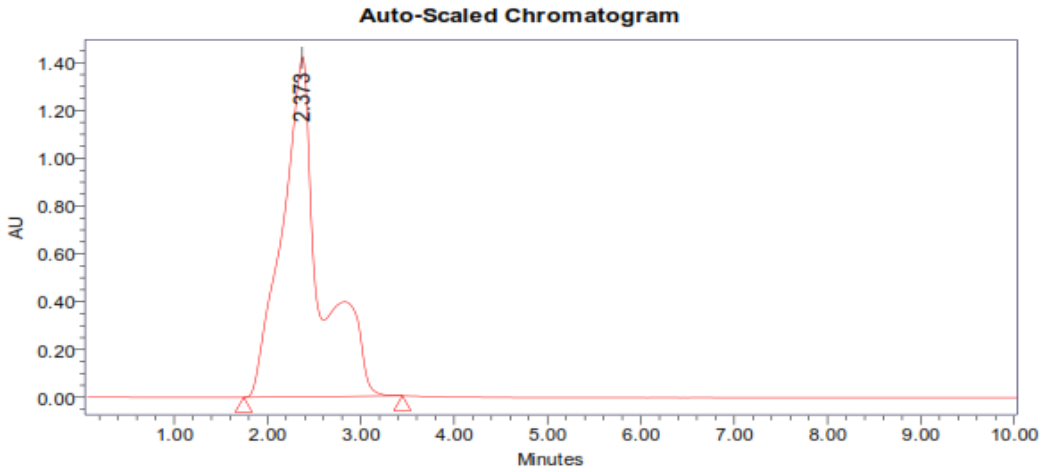


Fig.No.1: Chromatogram for Trail 1

Table No-3: Peak Results for Trail 1

S.No.	Peak Name	R_t	Area	Height	USP Tailing	USP Plate count
1	Diosmin	2.373	5263562	165955	1.08	1365

Observation:
In this trial it shows less plate count and improper separation of two peaks in the chromatogram. So it's required more trials to obtain good peaks.

Trail 2:

Column : Zorbax ODS C18 (4.6mm×250mm) 5µm
Column temperature : 35ºC
Wavelength : 252nm
Mobile phase ratio : Methanol: Acetonitrile (70:30% v/v)
Flow rate : 1.0ml/min
Injection volume : 20.00µl
Run time : 9.5Minutes

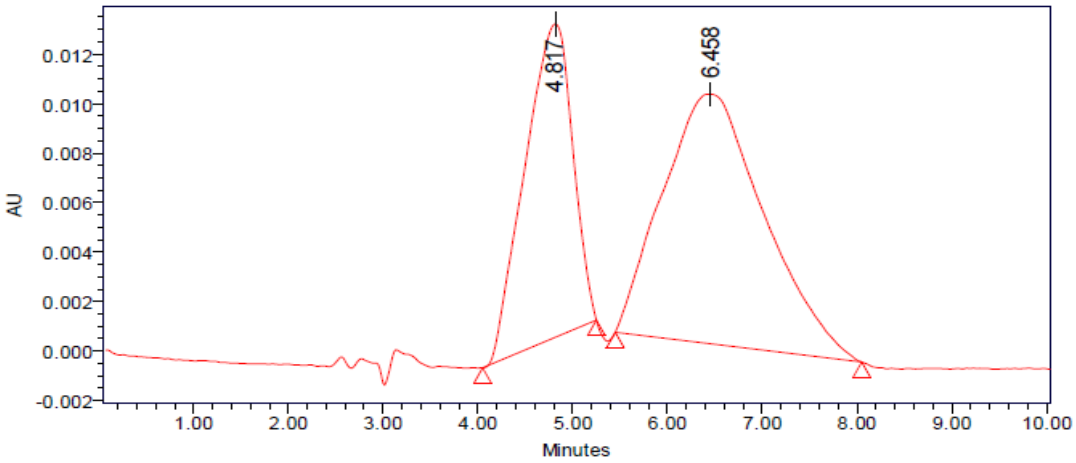


Fig.No.2: Chromatogram for Trail 2

Table No-4: Peak Results for Trail 2

S.No	Peak Name	R_t	Area	Height	USP Tailing	USP plate count
1	Diosmin	4.817	3865852	41565	0.96	3562
2	Hesperidin	6.458	69589451	5668	0.99	4758

Observation: In this above trail it shows one sample peak and less platecount, improper baseline in the chromatogram. So its required more trails to get optimized chromatogram.

Trail 3:

Column	: Hypersil C18 (4.6mm×250mm) 5µm
Column temperature	: 40ºC
Wavelength	: 252nm
Mobile phase ratio	: Methanol: Phosphate Buffer (35:65% V/V)
Flow rate	: 0.8ml/min
Injection volume	: 20µl
Run time	: 9.5minutes

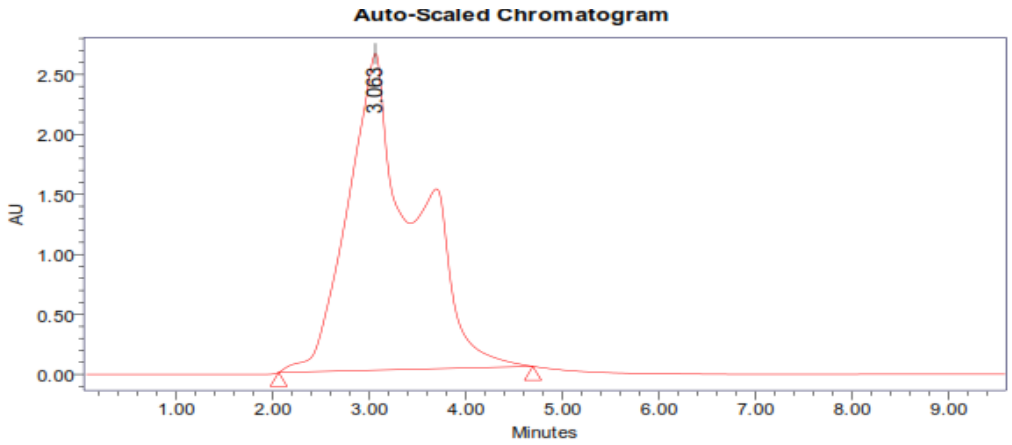


Fig.No.3: Chromatogram for Trail 3

Table No-5: Peak Results for Trail 3

S.No	Peak Name	R_t	Area	Height	USP Tailing	USP Plate Count
1	Diosmin	3.063	4365525	91568	1.14	5651

Observation:
In this trail only one sample peak is eluted. And it shows less platecount and improper baseline in the chromatogram. More trails required to get perfect peak.

Trail 4:

Column	: Symmetry (C18) (150mm x 4.6mm, 5µm) Column
Column temperature	: 38ºC
Wavelength	: 252nm
Mobile phase ratio	: Methanol: Phosphate Buffer (pH-5.8) (40:60v/v)
Flow rate	: 1.0ml/min
Injection volume	: 20µl
Run time	: 7.5minutes

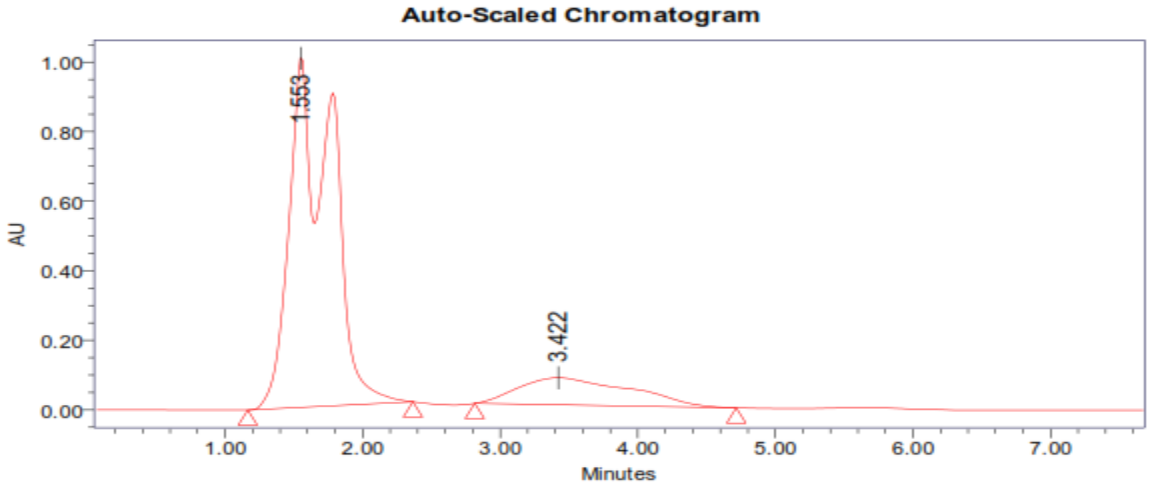


Fig.No.4: Chromatogram for Trail 4

Table No-6: Peak Results for Trail 4

S. No.	Peak Name	R_t	Area	Height	USP Tailing	USP Plate Count
1	Diosmin	1.553	189856952	6985454	2.68	5628
2	Hesperidin	3.422	6452154	52465	1.07	6454

Observation:
In this above trail more tailing shows and improper peak shape in the chromatogram. More trails required to get optimized peaks.

Optimized Chromatogram (Standard)

Temperature : 35^0 C
Column : Symmetry C18 (4.6×150mm, 5µ)
Mobile phase : Methanol: Water (65:35v/v)
Flow rate : 1ml/min
Wavelength : 250 nm
Injection volume : 10 µl
Run time : 8minutes

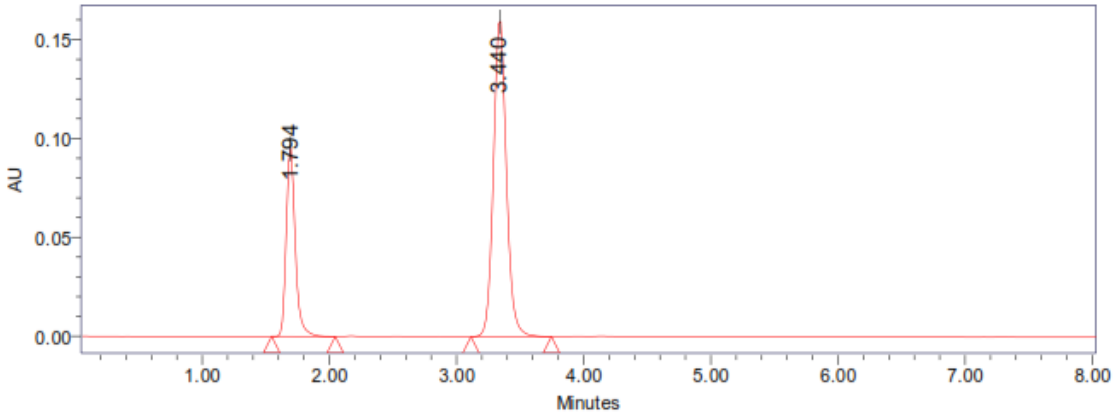


Fig.No.5: Optimized Chromatogram (Standard)

Table No.7: Optimized Chromatogram (Standard)

S.No.	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Diosmin	1.794	545265	7462	1.09	7564
2	Hesperidin	3.440	7768545	43652	1.12	8695

Optimized Chromatogram (Sample)

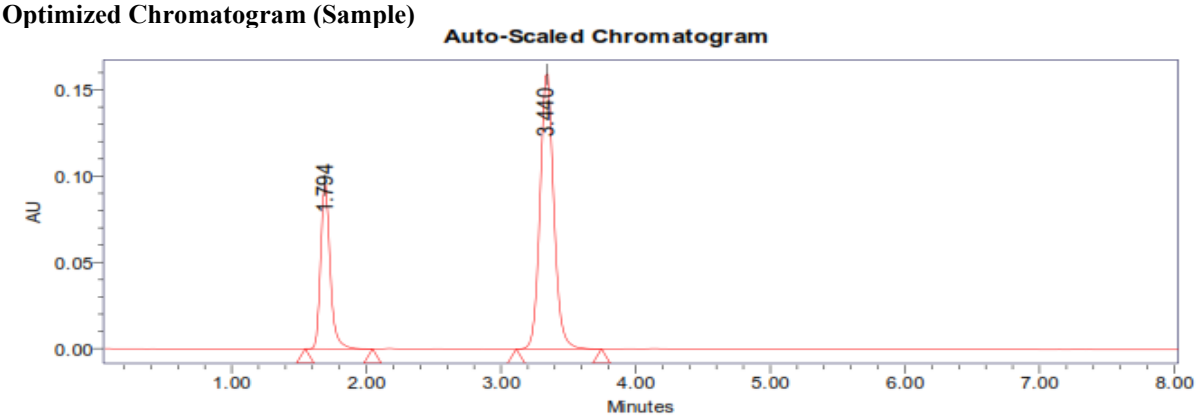


Fig.No.6: Optimized Chromatogram (Sample)

Table No. 8: Optimized Chromatogram (Sample)

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Diosmin	1.794	558659	7584	1.10	7659
2	Hesperidin	3.440	7856985	44658	1.13	8743

Acceptance Criteria:

- Theoretical plates must be not less than 2000.
- Tailing factor must be not less than 2.
- It was found from above data that all the system suitability parameters for developed method were within the limit.

METHOD VALIDATION

Blank:

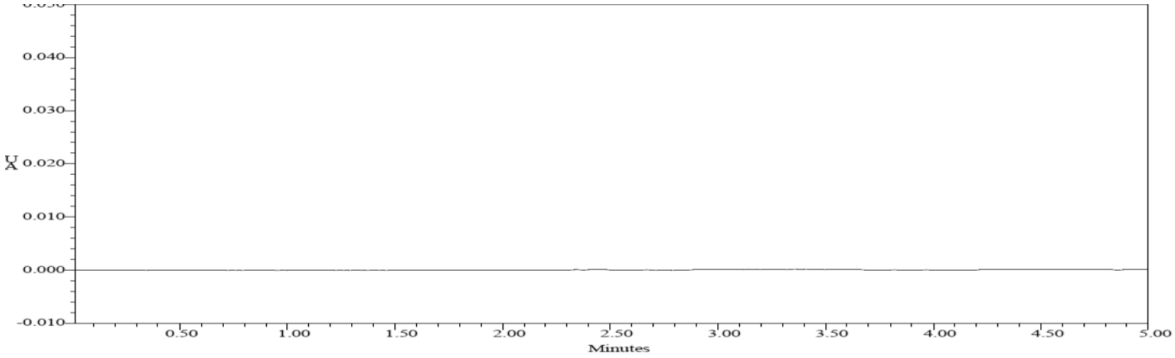


Fig.No.7: Chromatogram showing blank (mobile phase preparation)

SPECIFICITY

The ICH documents define specificity as the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components.

Analytical method was tested for specificity to measure accurately quantities Diosmin and Hesperidin in drug product.

Assay (Standard):

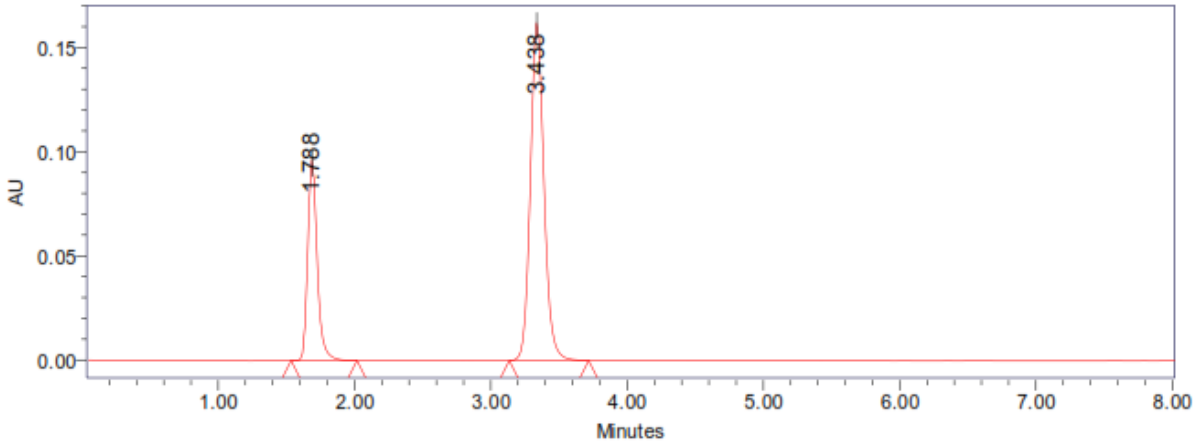


Fig.No.8: Chromatogram showing assay of standard injection -1

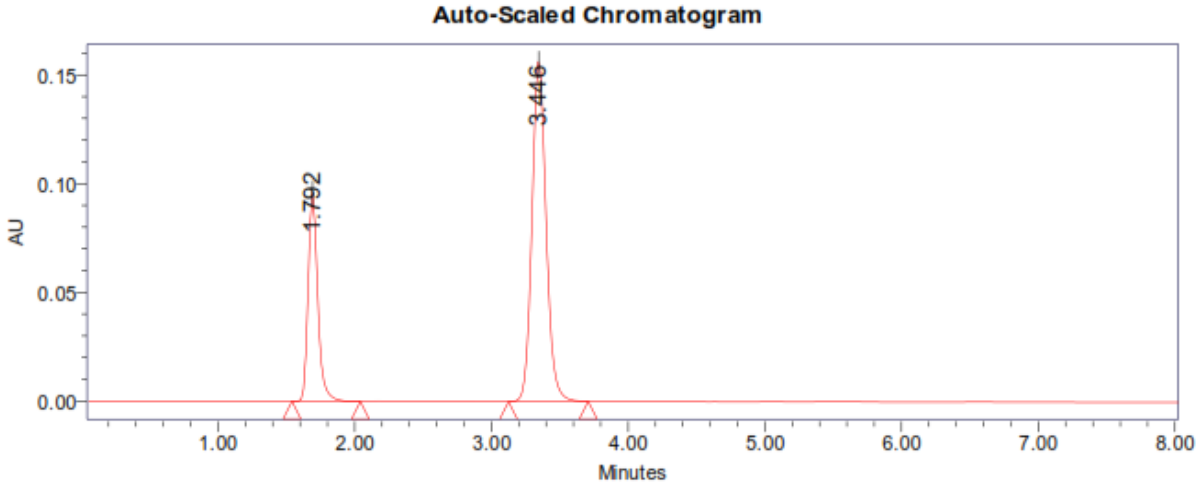


Fig.No.9: Chromatogram showing assay of standard injection -2

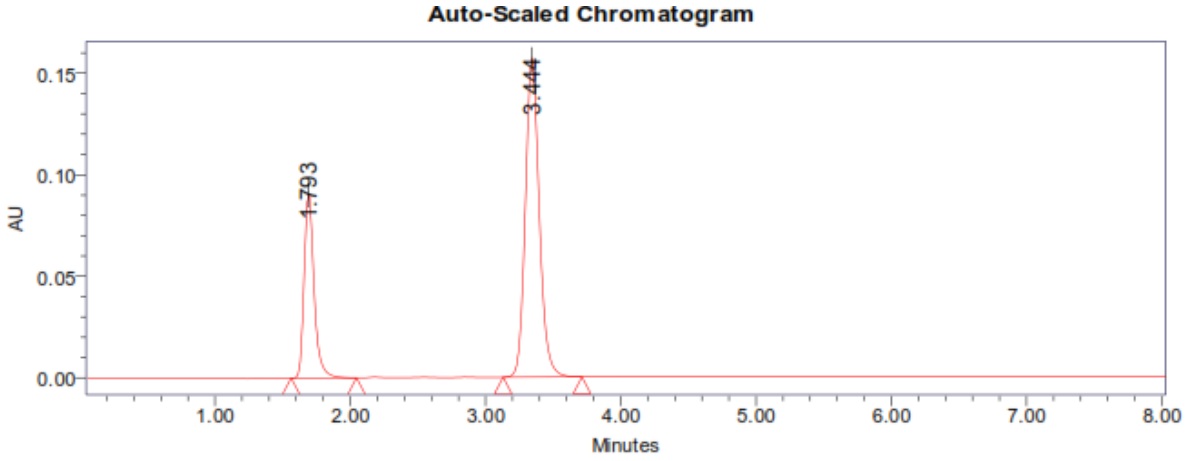
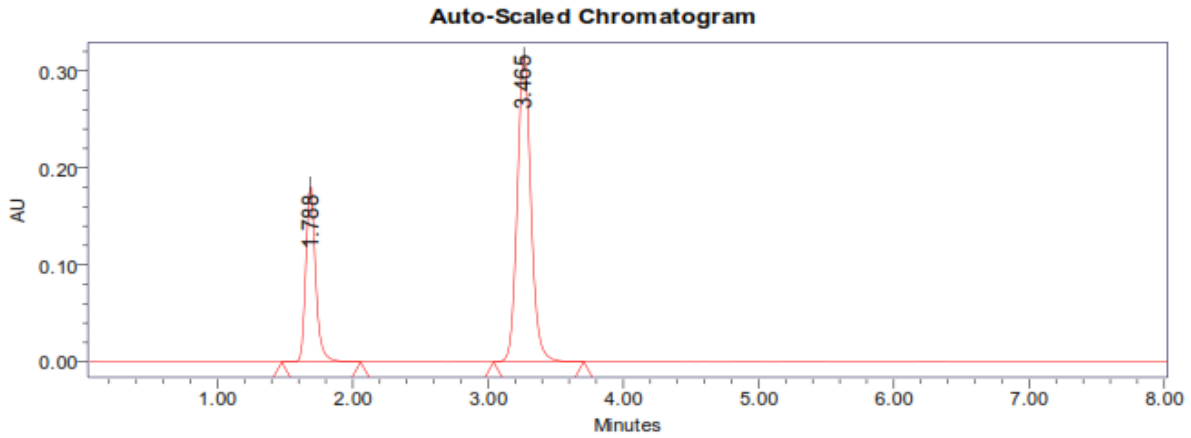


Fig.No.10 : Chromatogram showing assay of standard injection -3



- Fig.No.11: Chromatogram showing assay of standard injection -4

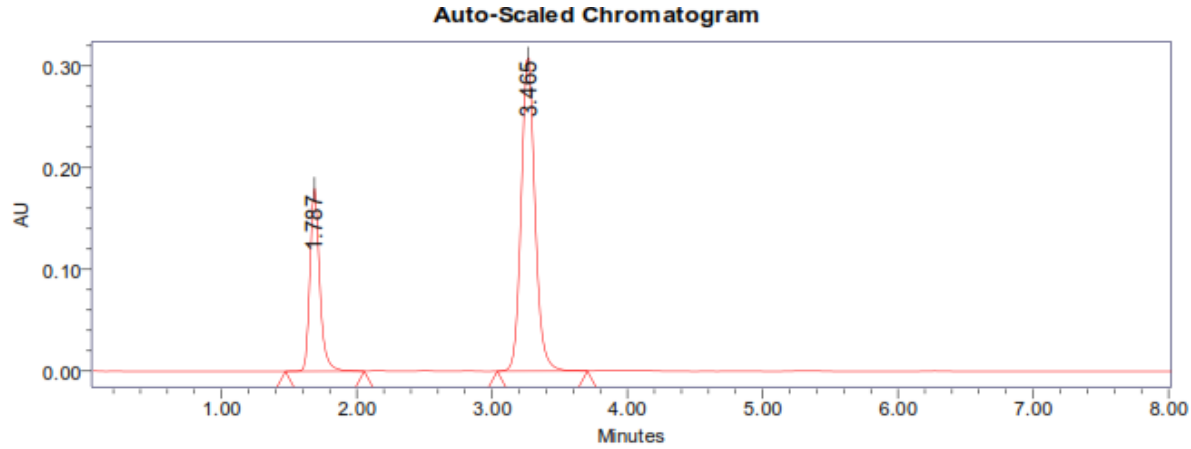


Fig.No12: Chromatogram showing assay of standard injection -5

Table No. 9: Peak results for assay standard of Diosmin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Diosmin	1.788	545698	7458	7595	1.09
2	Diosmin	1.792	548765	7469	7548	1.10
3	Diosmin	1.793	548965	7428	7563	1.09
4	Diosmin	1.788	548783	7495	7592	1.10
5	Diosmin	1.787	548752	7461	7543	1.09
Mean			548192.6			
Std. Dev.			1397.209			
% RSD			0.254876			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Table No.10: Peak results for assay standard of Hesperidin

S.No	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Hesperidin	3.438	7785698	43652	8652	1.12
2	Hesperidin	3.446	7786354	43698	8674	1.13
3	Hesperidin	3.444	7786942	43587	8692	1.13

4	Hesperidin	3.465	7785464	43698	8649	1.12
5	Hesperidin	3.465	7785986	43568	8625	1.12
Mean			7786089			
Std. Dev.			581.3667			
% RSD			0.007467			

Acceptance Criteria:

- %RSD of five different sample solutions should not more than 2.
- The %RSD obtained is within the limit, hence the method is suitable.

Assay (Sample):

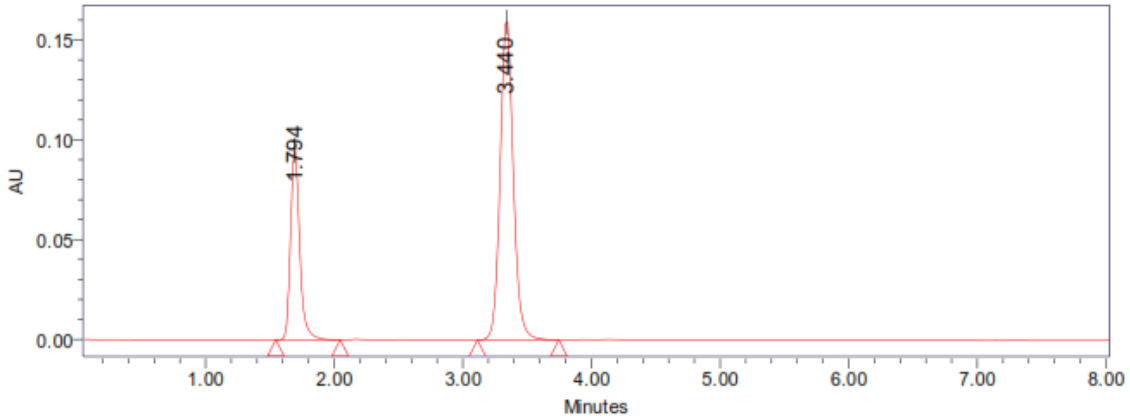


Fig.No.13: Chromatogram showing assay of sample injection-1

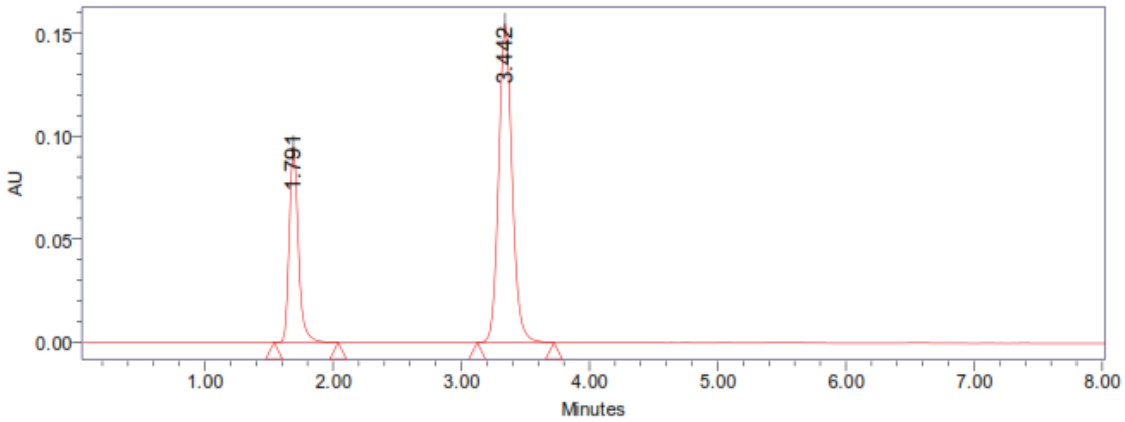


Fig.No.14: Chromatogram showing assay of sample injection-2

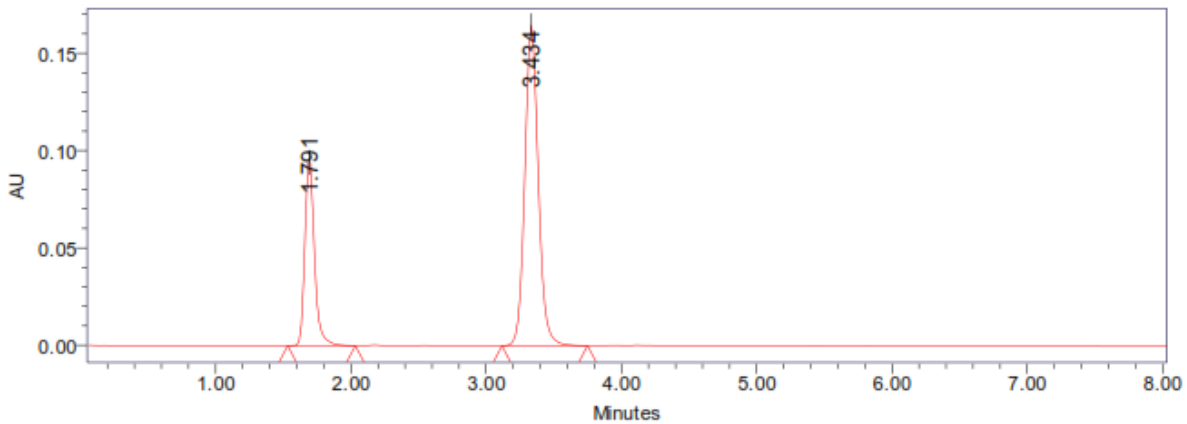


Fig.No.15: Chromatogram showing assay of sample injection-3

Table No.11: Peak results for Assay sample of Diosmin

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count	Injection
1	Diosmin	1.794	556985	75895	1.10	7698	1
2	Diosmin	1.791	558742	75468	1.10	7682	2
3	Diosmin	1.791	559683	75426	1.11	7649	3

Table No.12: Peak results for Assay sample of Hesperidin

S.No	Name	RT	Area	Height	USP Tailing	USP Plate Count
1	Hesperidin	3.440	7856859	44586	1.14	8759
2	Hesperidin	3.442	7826594	44658	1.15	8726
3	Hesperidin	3.434	7854879	44859	1.14	8794

%ASSAY =

$$\frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Diosmin and Hesperidin in pharmaceutical dosage form was found to be 100.154%

LINEARITY

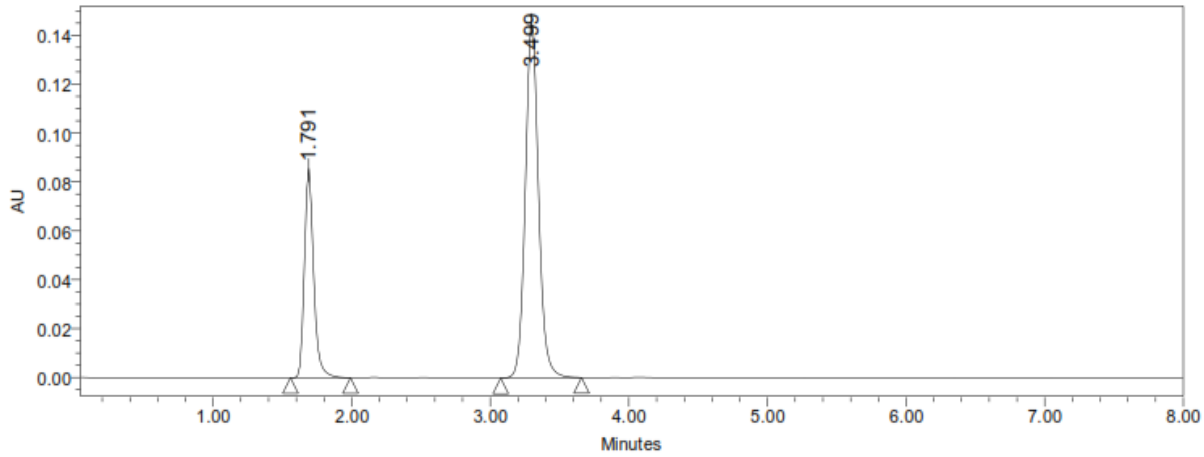


Fig.No.16: Chromatogram showing linearity level-1

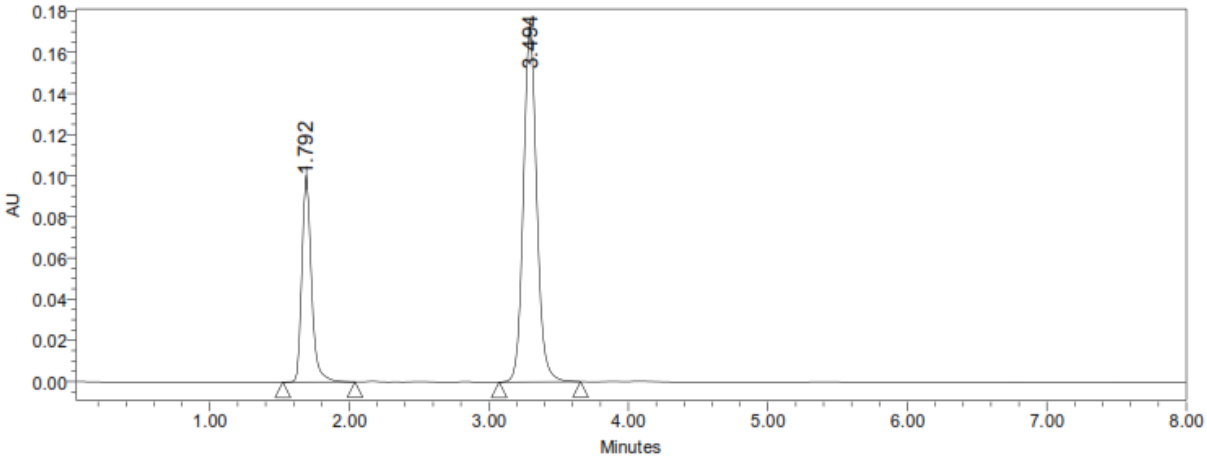


Fig.No.17: Chromatogram showing linearity level-2

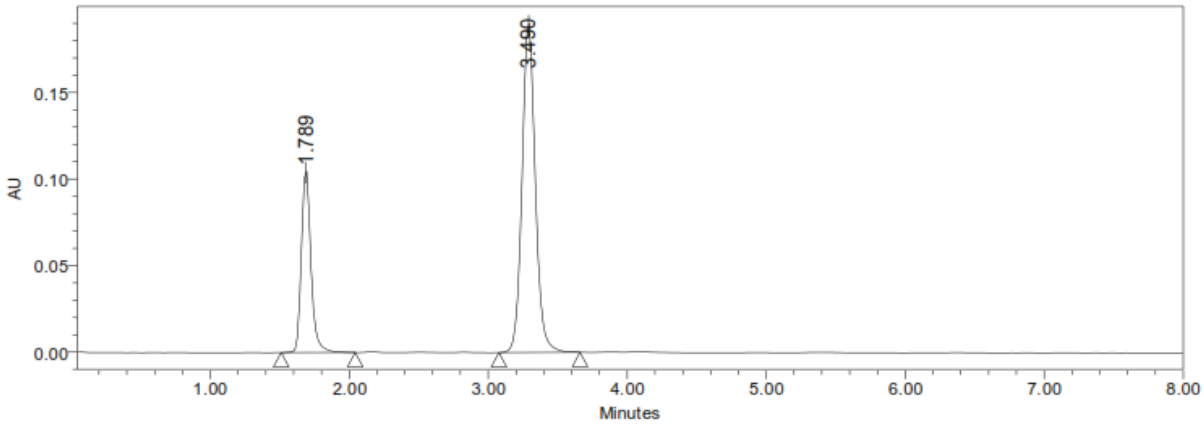


Fig.No.18: Chromatogram showing linearity level-3

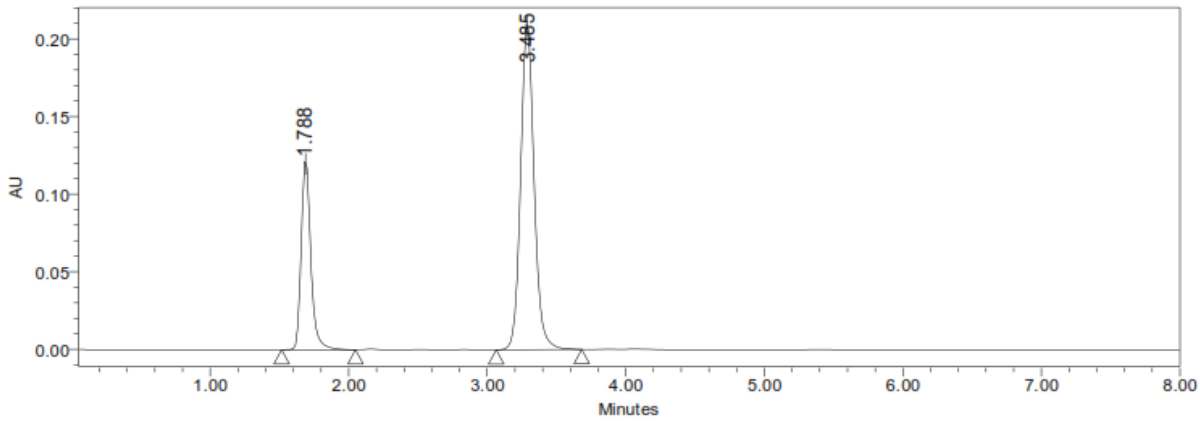


Fig.No.19: Chromatogram showing linearity level-4

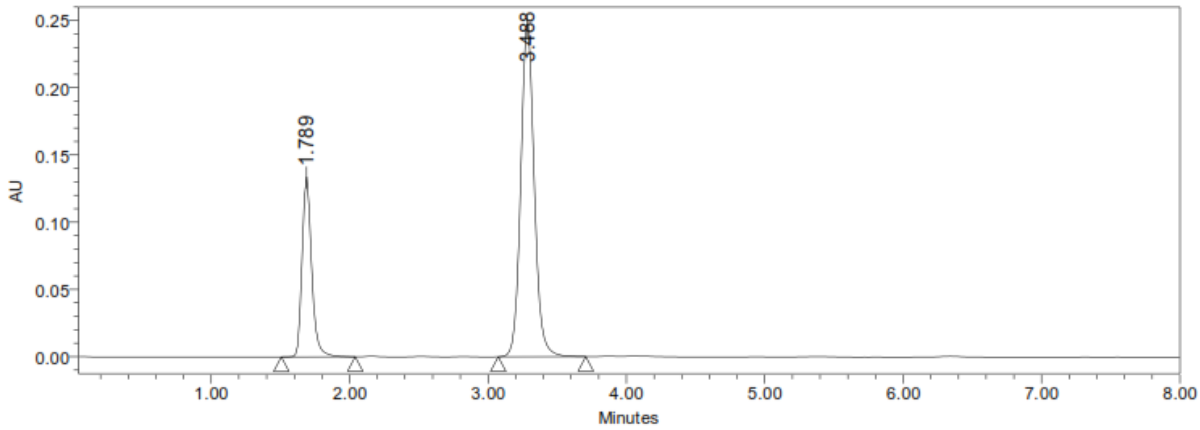


Fig.No.20: Chromatogram showing linearity level-5

Table No-13: CHROMATOGRAPHIC DATA FOR LINEARITY STUDY FOR DIOSMIN:

Concentration µg/ml	**Average Peak Area**
10	292985
15	430752
20	565265
25	693487
30	821584

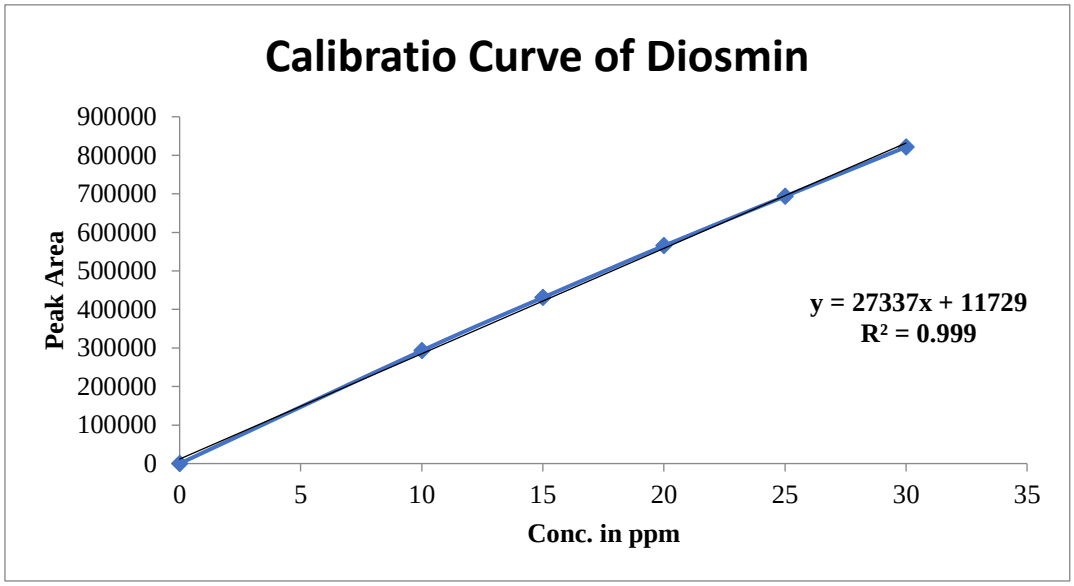


Fig.No.21: Chromatogram showing linearity level

LINEARITY PLOT:

The plot of Concentration (x) versus the Average Peak Area (y) data of Diosmin is a straight line.

Y = mx + c

Slope (m) = 27337

Intercept (c) = 11729

Correlation Coefficient (r) = 0.999

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.

CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 11729. These values meet the validation criteria.

Table No.14: CHROMATOGRAPHIC DATA FOR LINEARITY STUDY FOR HESPERIDIN:

Concentration µg/ml	Average Peak Area
10	2828756
20	5485784
30	7999859
40	10656542
50	13085985

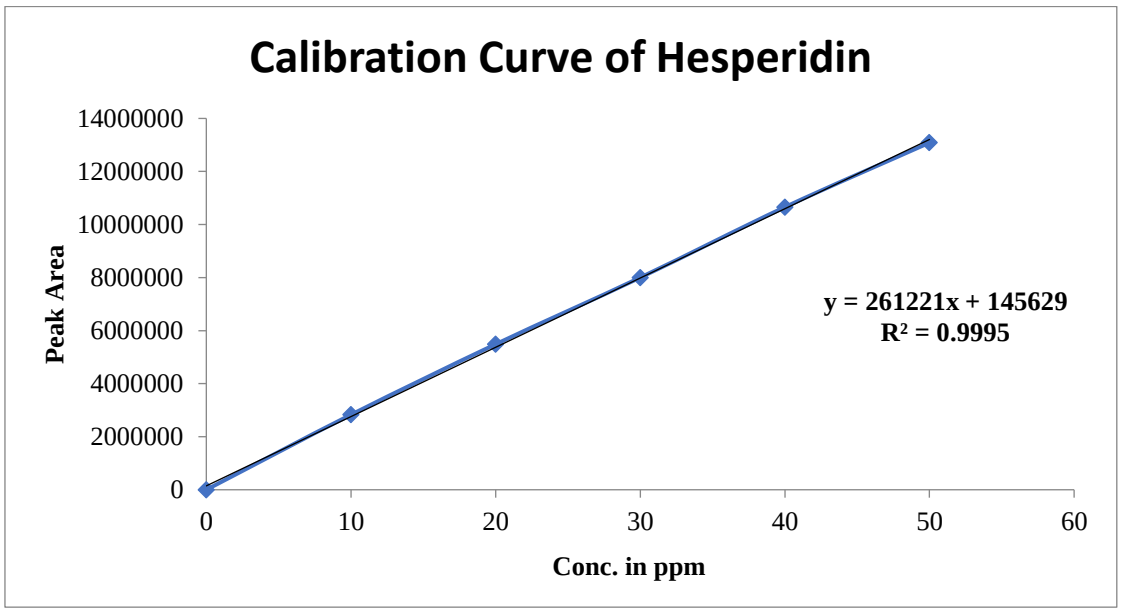


Fig.No.22: Chromatogram showing linearity level

LINEARITY PLOT:
The plot of Concentration (x) versus the Average Peak Area (y) data of Hesperidin is a straight line.
Y = mx + c
Slope (m) = 26122
Intercept (c) = 14562
Correlation Coefficient (r) = 0.9994

VALIDATION CRITERIA: The response linearity is verified if the Correlation Coefficient is 0.99 or greater.
CONCLUSION: Correlation Coefficient (r) is 0.99, and the intercept is 14562. These values meet the validation criteria.

PRECISION:
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

REPEATABILITY
Obtained Five (5) replicates of 100% accuracy solution as per experimental conditions. Recorded the peak areas and calculated % RSD.

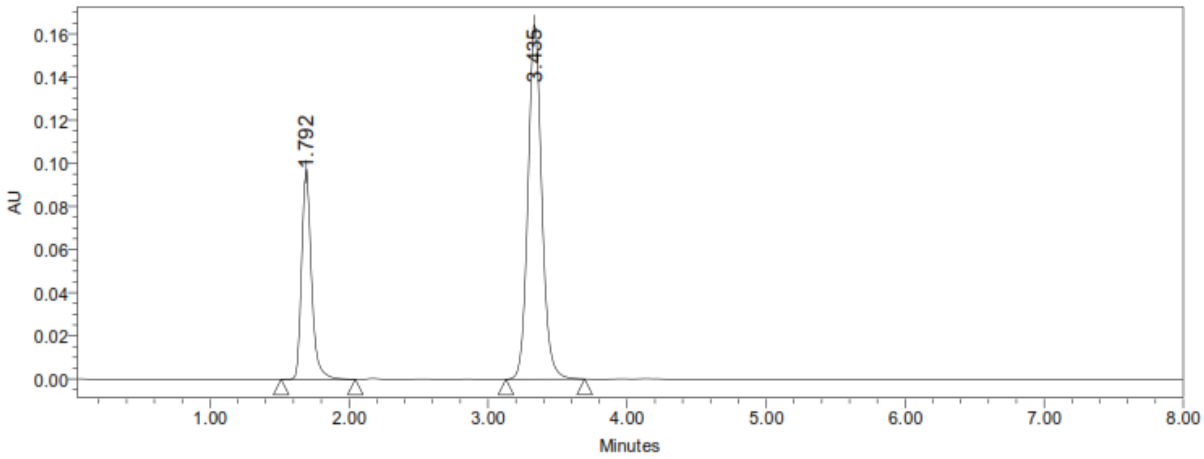


Fig.No.23: Chromatogram showing precision injection -1

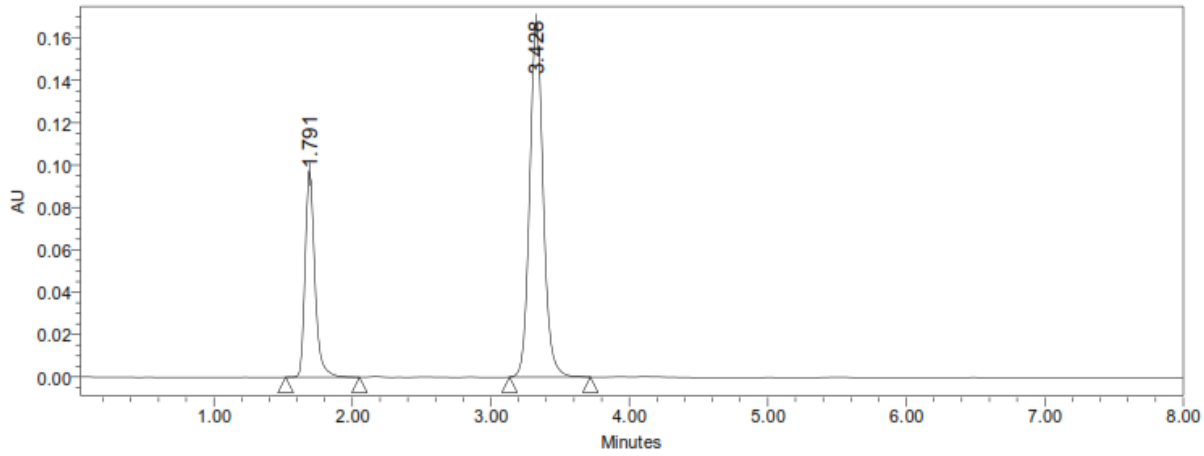


Fig.No.24:Chromatogram showing precision injection -2

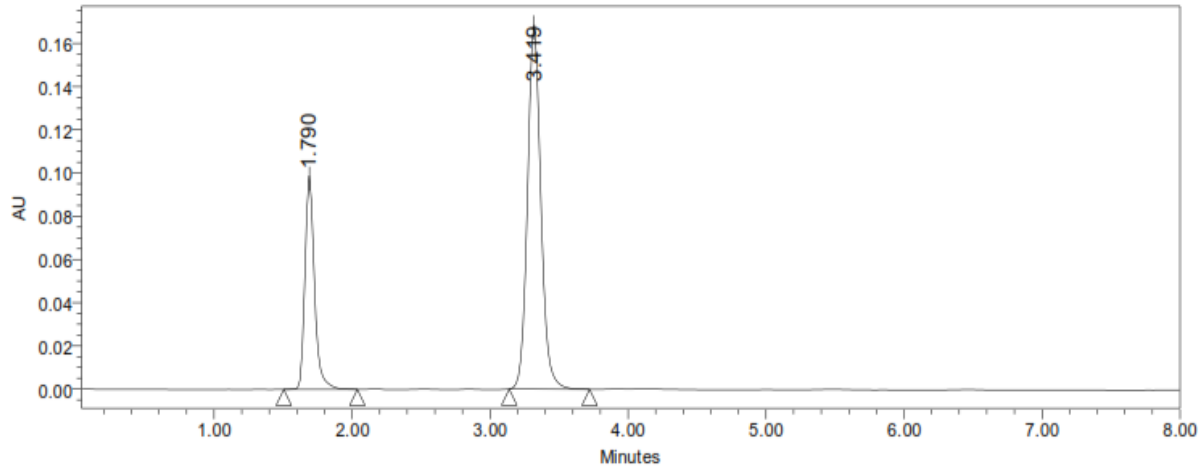


Fig.No.25: Chromatogram showing precision injection -3

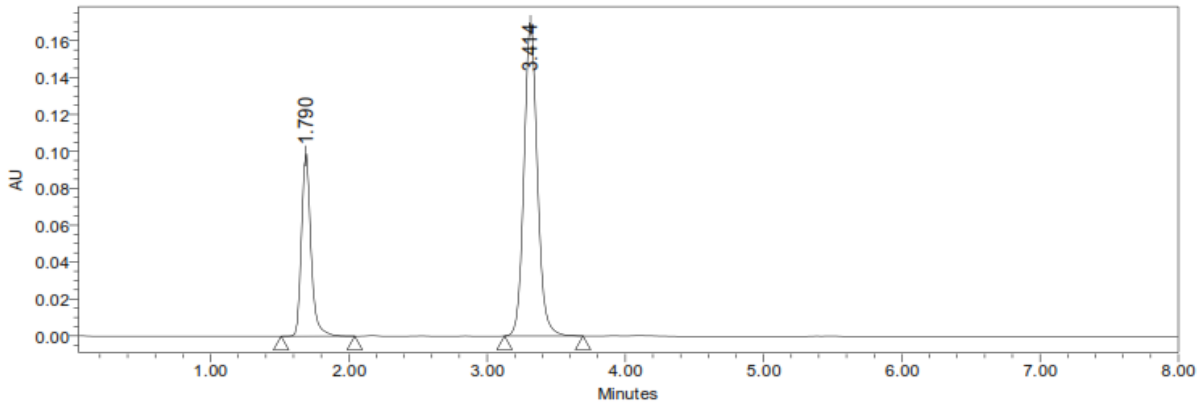


Fig.No.26: Chromatogram showing precision injection -4

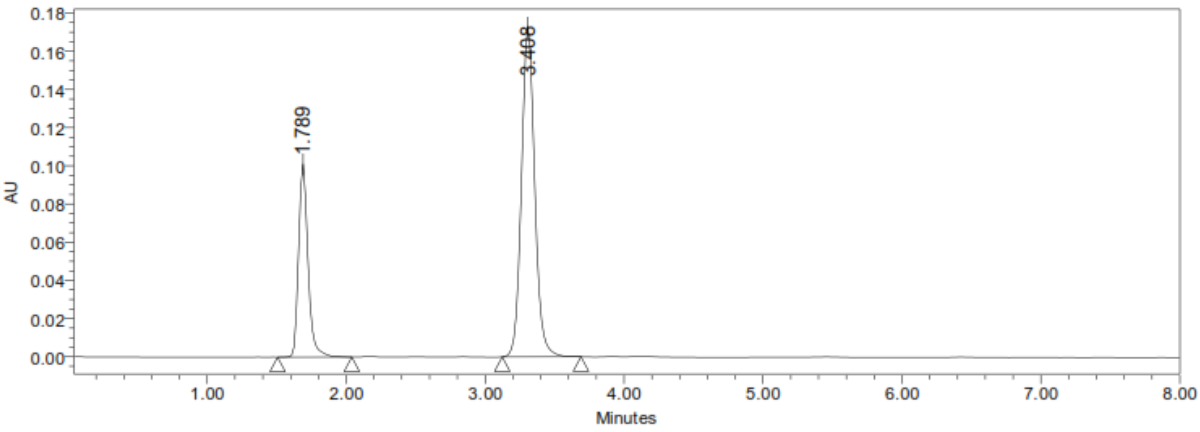


Fig.No.27: Chromatogram showing precision injection -5

Table 15: Results of Repeatability for Diosmin

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Diosmin	1.792	548698	7458	7569	1.10
2	Diosmin	1.791	548955	7485	7546	1.10
3	Diosmin	1.790	548745	7469	7592	1.09
4	Diosmin	1.790	549856	7463	7519	1.10
5	Diosmin	1.789	546587	7495	7535	1.09
Mean			548568.2			
Std.dev			1202.217			
%RSD			0.2191554			

Acceptance Criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Table No. 16: Results of Repeatability for Hesperidin

S. No.	Peak Name	Retention time	Area (µV*sec)	Height (µV)	USP Plate Count	USP Tailing
1	Hesperidin	3.435	7768958	43659	8659	1.12
2	Hesperidin	3.428	7765984	43856	8647	1.13
3	Hesperidin	3.419	7785469	43658	8675	1.12
4	Hesperidin	3.414	7785498	43549	8652	1.12
5	Hesperidin	3.408	7769852	44526	8692	1.13
Mean			7775152			
Std.dev			9539.236			
%RSD			0.122689			

Acceptance Criteria:

- %RSD for sample should be NMT 2.
- The %RSD for the standard solution is below 1, which is within the limits hence method is precise.

Intermediate precision:

Day 1:

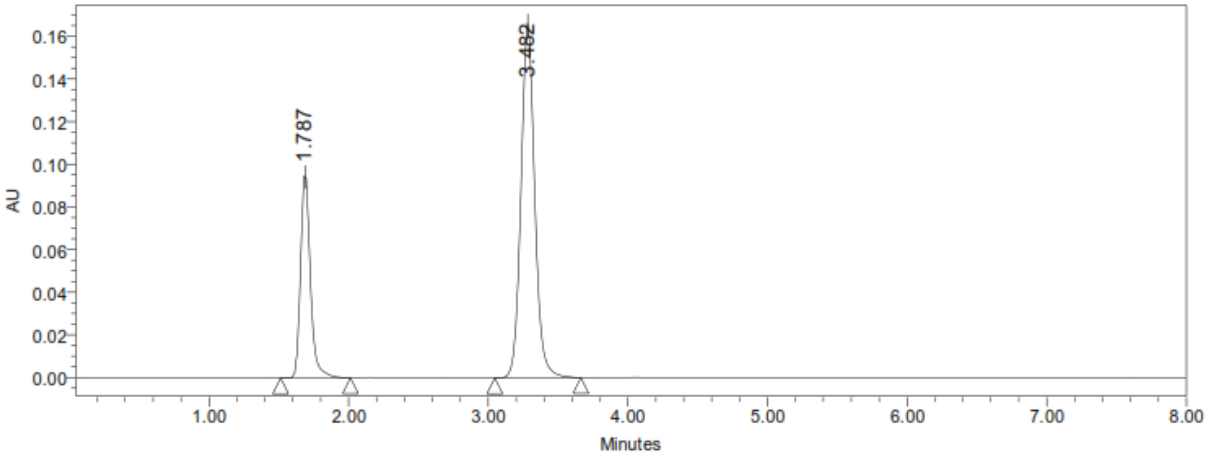


Fig.No.28: Chromatogram showing Day1 injection -1

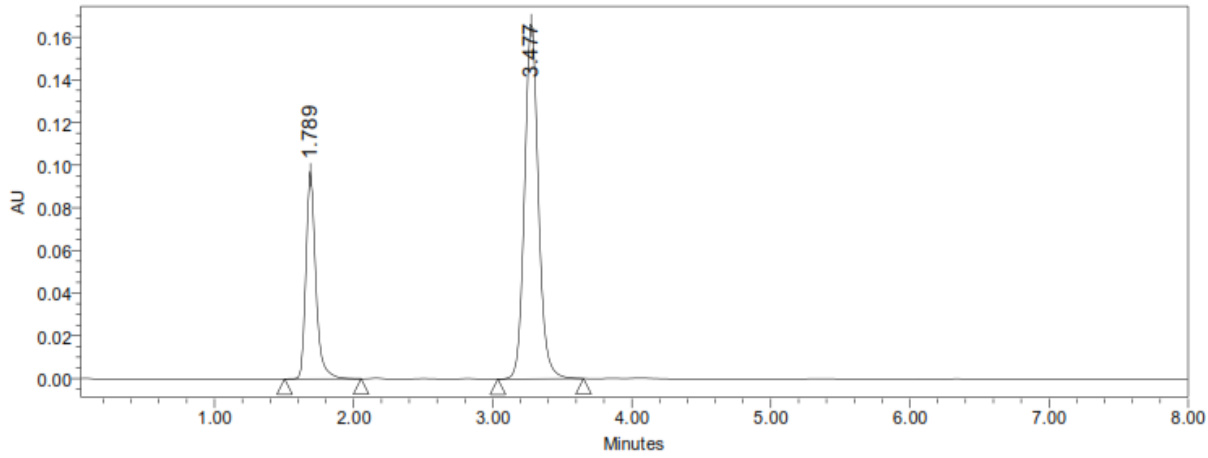


Fig.No.29: Chromatogram showing Day1 injection -2

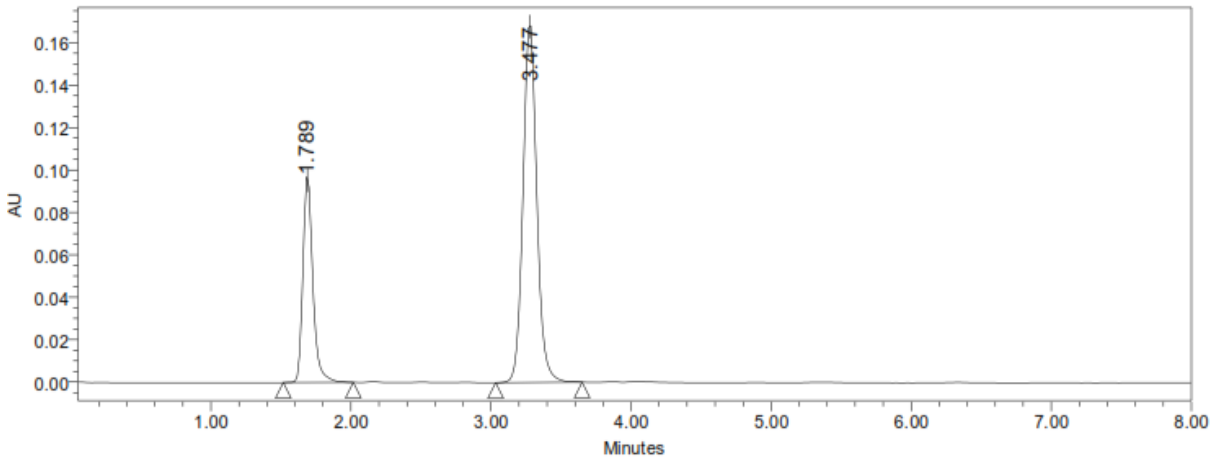


Fig.No.30: Chromatogram showing Day1 injection -3

Table No. 17: Results of Intermediate precision day1 for Diosmin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Diosmin	1.787	556985	75986	7695	1.11
2	Diosmin	1.789	558649	75986	7642	1.12
3	Diosmin	1.789	557847	75689	7683	1.12
Mean			557827			
Std. Dev.			832.1803			
% RSD			0.149183			

Acceptance Criteria:
%RSD of three different sample solutions should not more than 2.

Table No. 18: Results of Intermediate precision day1 for Hesperidin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Hesperidin	3.482	7856982	44586	8758	1.13
2	Hesperidin	3.477	7845285	44758	8769	1.14
3	Hesperidin	3.477	7854633	44986	8728	1.13
Mean			7852300			
Std. Dev.			6187.659			
% RSD			0.078801			

Acceptance Criteria:

- %RSD of three different sample solutions should not more than 2.

Day 2:

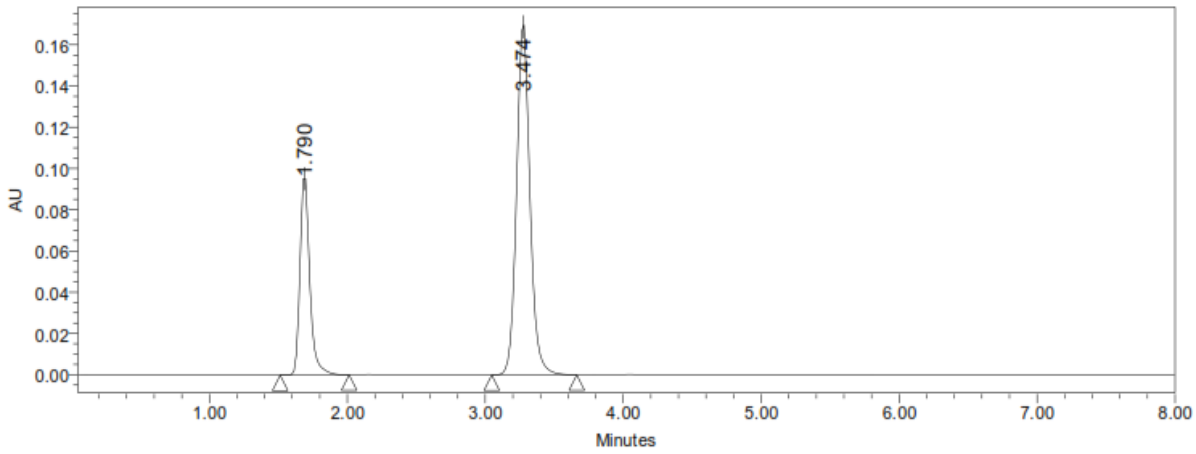


Fig.No.30: Chromatogram showing Day 2 injection -1

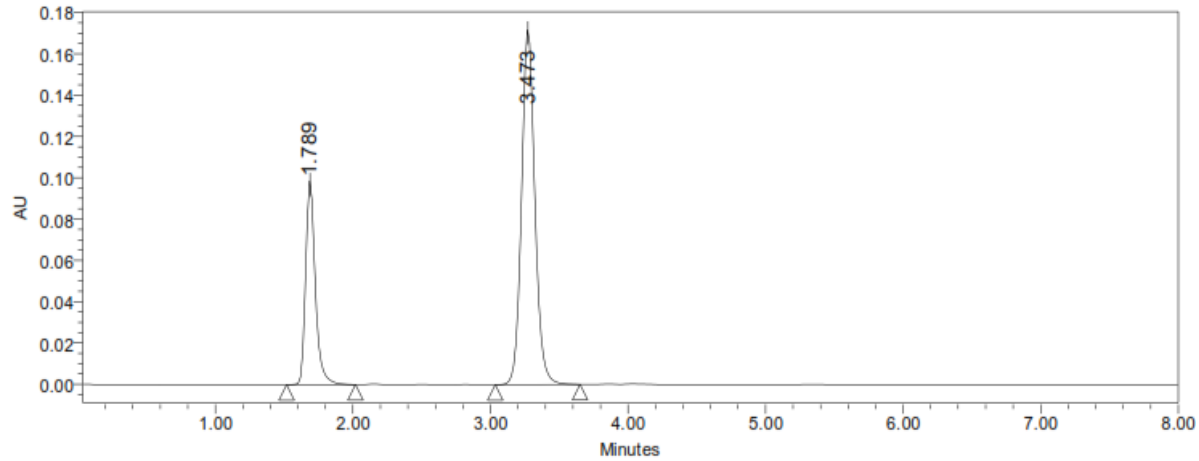


Fig.No.31: Chromatogram showing Day 2 injection -2

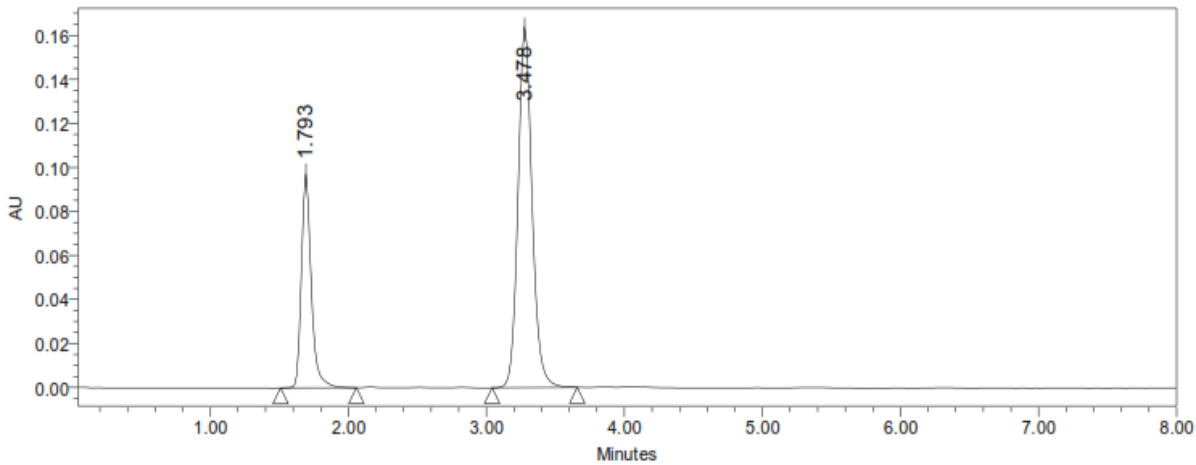


Fig.No.32: Chromatogram showing Day 2 injection -3

Table No. 19: Results of Intermediate precision Day 2 for Diosmin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Diosmin	1.790	536598	7365	7459	1.08
2	Diosmin	1.789	534875	7358	7436	1.07
3	Diosmin	1.793	534698	7349	7482	1.08

Mean			535390.3			
Std. Dev.			1049.608			
% RSD			0.196045			

Acceptance Criteria:
%RSD of three different sample solutions should not more than 2.

Table No. 20: Results of Intermediate precision Day 2 for Hesperidin

S.No.	Peak Name	RT	Area (µV*sec)	Height (µV)	USP Plate count	USP Tailing
1	Hesperidin	3.474	7698521	42568	8582	1.11
2	Hesperidin	3.473	7685985	42698	8546	1.10
3	Hesperidin	3.478	7645897	42365	8574	1.10
Mean			7676801			
Std. Dev.			27487.83			
% RSD			0.358064			

Acceptance Criteria:
- %RSD of three different sample solutions should not more than 2.

ACCURACY:
Accuracy at different concentrations (50%, 100%, and 150%) were prepared and the % recovery was calculated.
Accuracy 50%:

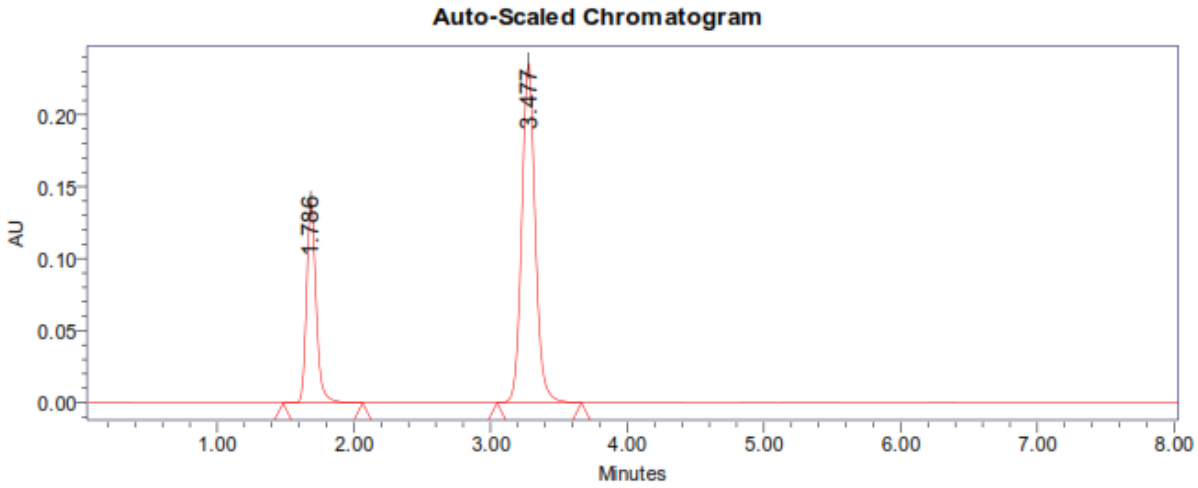


Fig.No.33: Chromatogram showing accuracy-50% injection-1

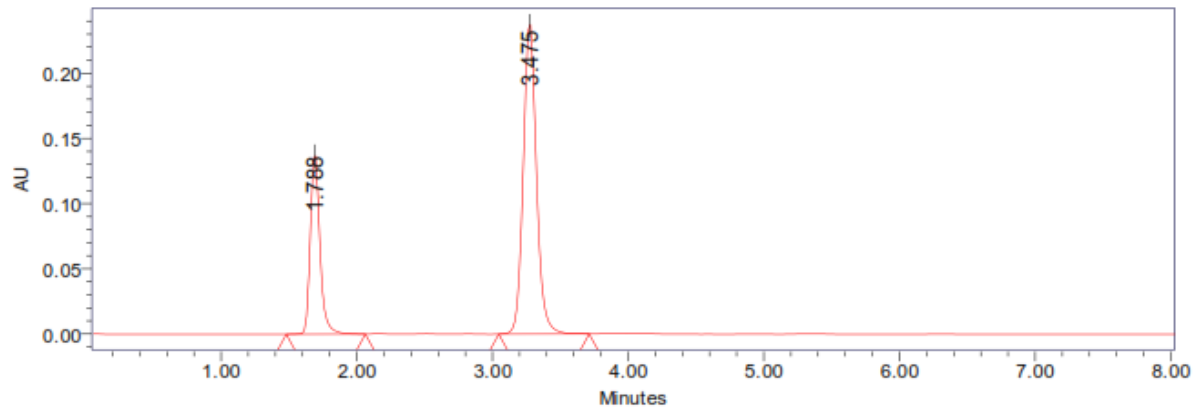


Fig.No.34: Chromatogram showing accuracy-50% injection-2

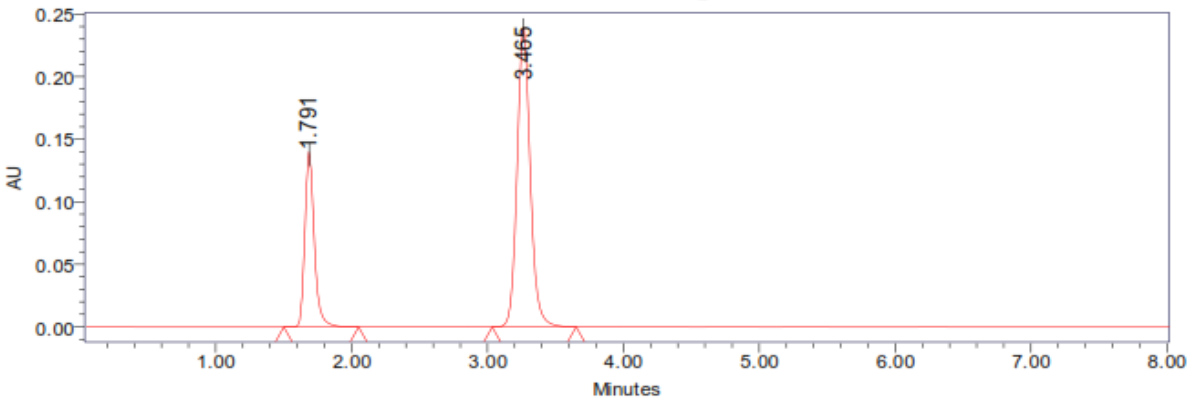


Fig.No.35: Chromatogram showing accuracy-50% injection-3

Accuracy 100%:

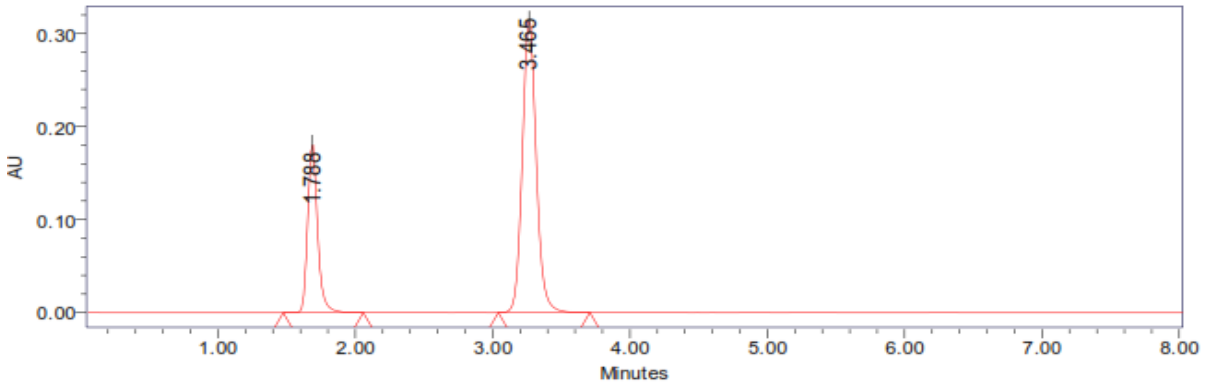


Fig.No.36: Chromatogram showing accuracy-100% injection-1

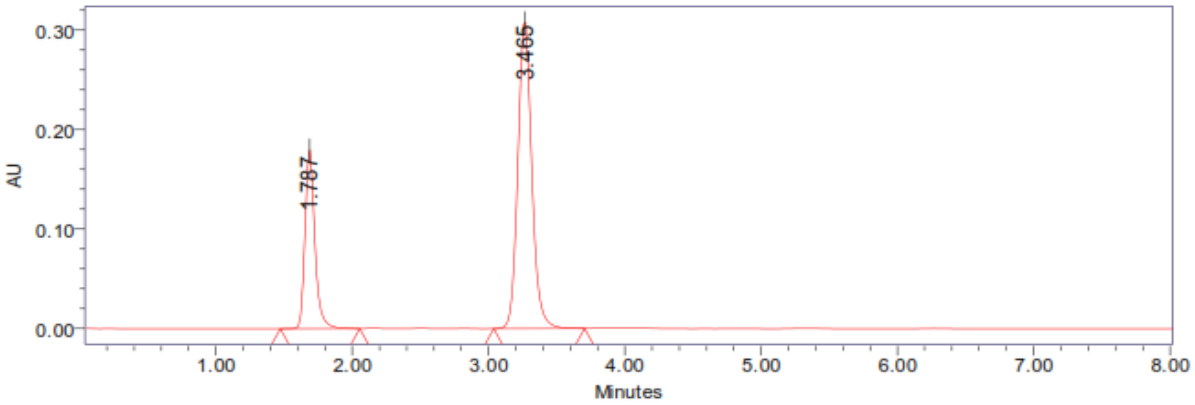


Fig.no.37: Chromatogram showing accuracy-100% injection-2

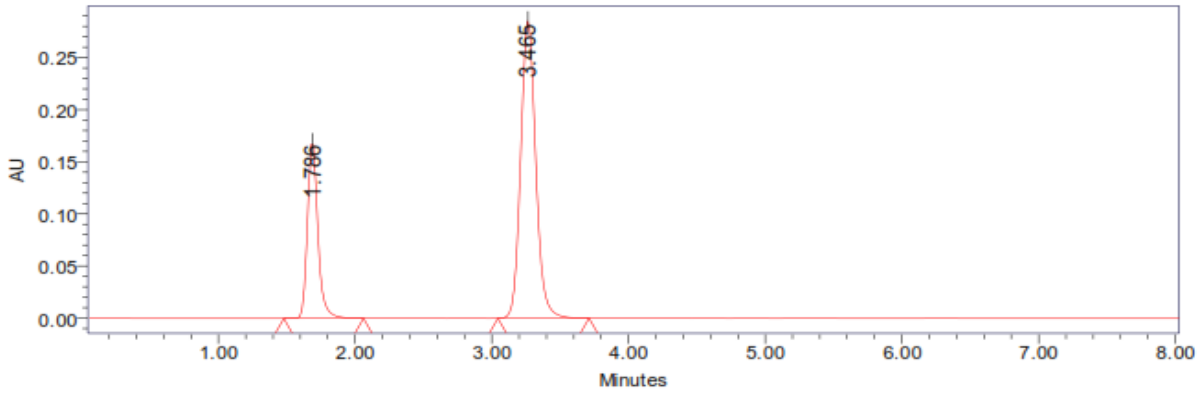


Fig.no. 38: Chromatogram showing accuracy-100% injection-3

Accuracy 150%:

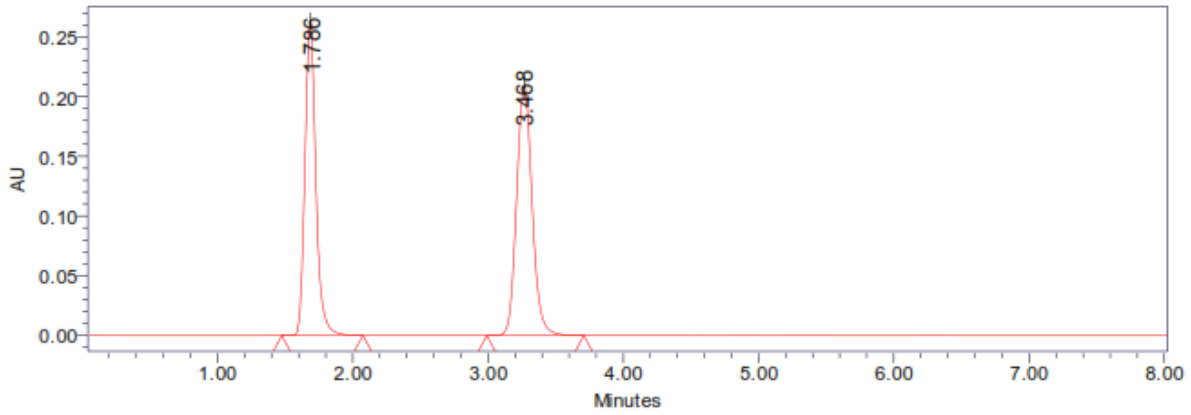


Fig.No.39: Chromatogram showing accuracy-150% injection-1

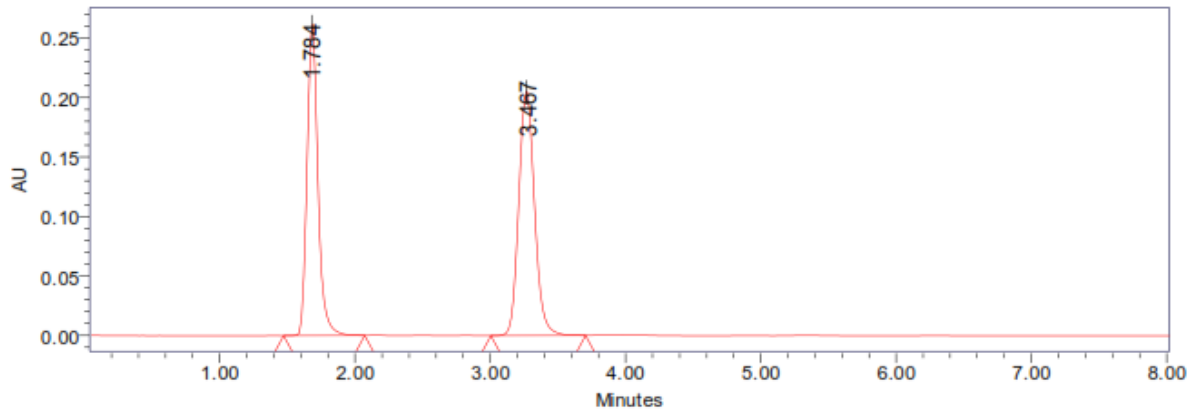


Fig.No.40 : Chromatogram showing accuracy-150% injection-2

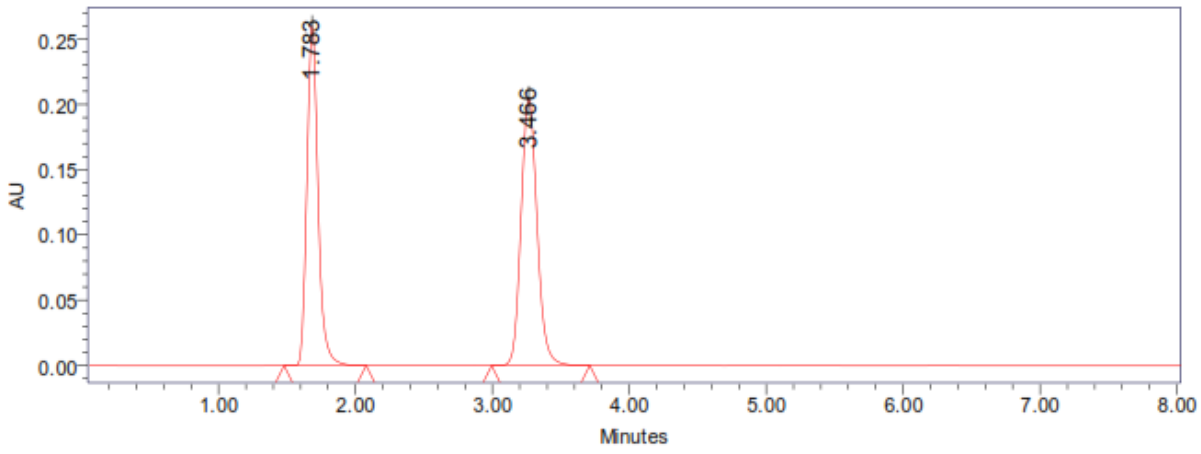


Fig.No.41: Chromatogram showing accuracy-150% injection-3

Table No. 21: The accuracy results for Diosmin

%Concentration (at Specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	286080.7	10.035	10	100.350%	100.291%
100%	561215	20.100	20	100.500%	
150%	833959.7	30.077	30	100.023%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

Table No.22: The accuracy results for Hesperidin

%Concentration (at Specification Level)	Area	Amount Added (ppm)	Amount Found (ppm)	% Recovery	Mean Recovery
50%	408328	15	15.074	100.493%	100.163%
100%	798306.3	30	30.003	100.010%	
150%	1189915	45	44.994	99.986%	

Acceptance Criteria:

- The percentage recovery was found to be within the limit (98-102%).

The results obtained for recovery at 50%, 100%, 150% are within the limits. Hence method is accurate.

LIMIT OF DETECTION FOR DIOSMIN AND HESPERIDIN

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

LOD= 3.3 × σ / s

Where , σ = Standard deviation of the response and S = Slope of the calibration curve

Result:

Diosmin

=0.86µg/ml

Hesperidin

=1.28µg/ml

QUANTITATION LIMIT FOR DIOSMIN AND HESPERIDIN

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined.

LOQ=10×σ/S

Where

σ = Standard deviation of the response

S = Slope of the calibration curve

Result:

Diosmin

=2.58µg/ml

Hesperidin

= 3.84µg/ml

ROBUSTNESS

The robustness was performed for the flow rate variations from 0.9 ml/min to 1.1ml/min and mobile phase ratio variation from more organic phase to less organic phase ratio for Diosmin and Hesperidin. The method is robust only in less flow condition and the method is robust even by change in the Mobile phase ±5%. The standard and samples of Diosmin and Hesperidin were injected by changing the conditions of chromatography. There was no significant change in the parameters like resolution, tailing factor, asymmetric factor, and plate count.

Variation in flow

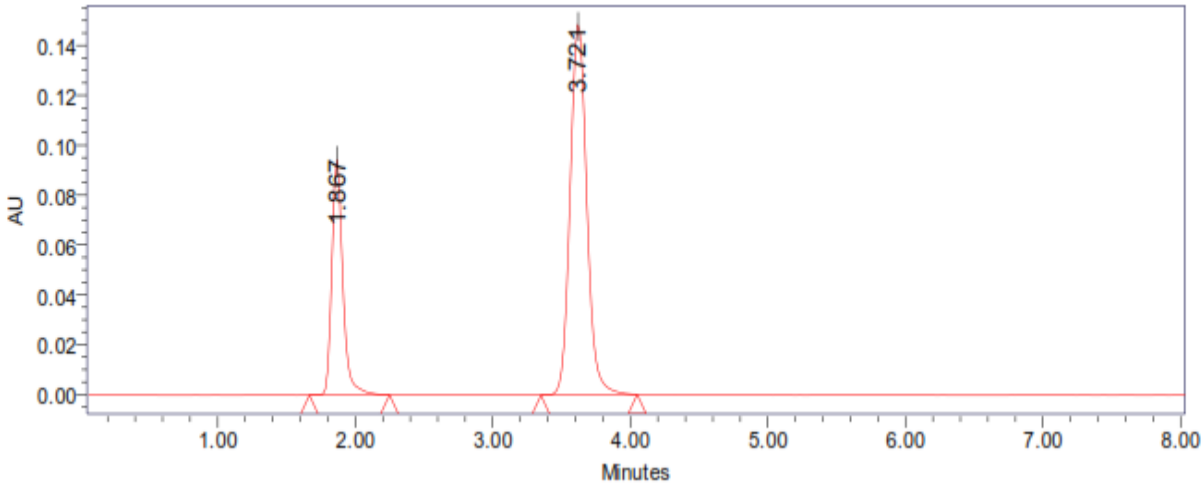


Fig.No.42: Chromatogram showing less flow of 0.8ml/min

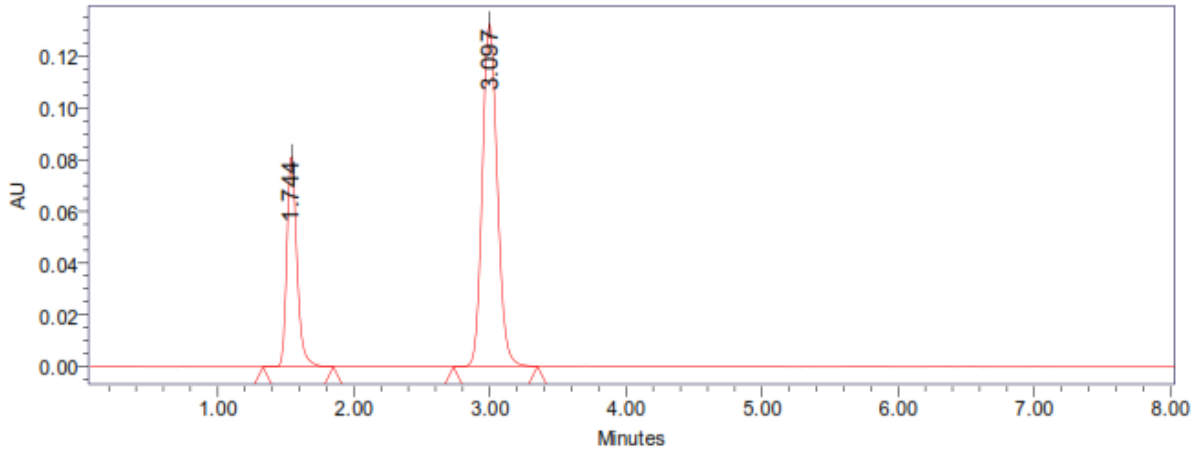


Fig.No.43: Chromatogram showing more flow of 1.0 ml/min

Variation of mobile phase organic composition

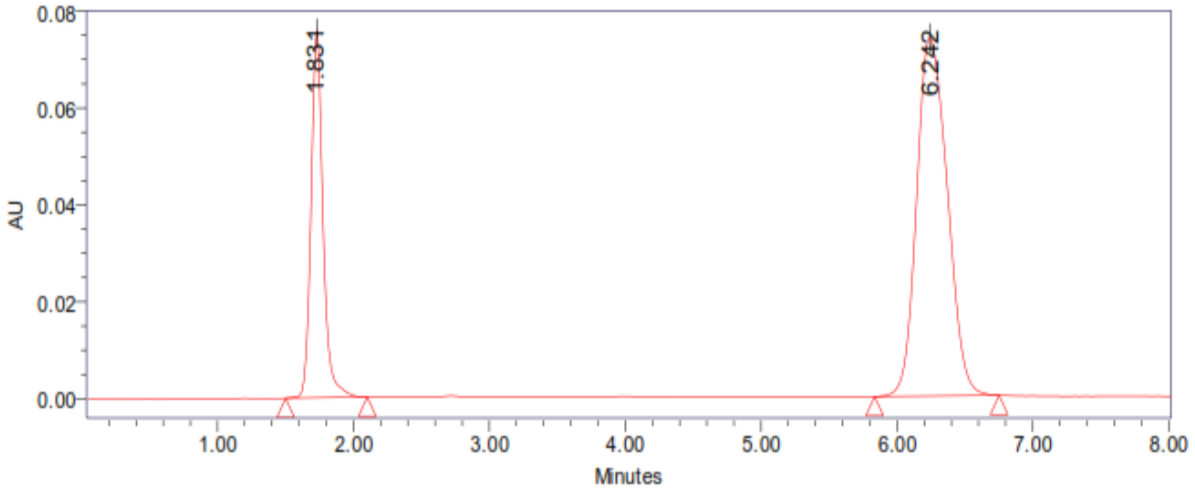


Fig.No. 44: chromatogram showing less organic composition

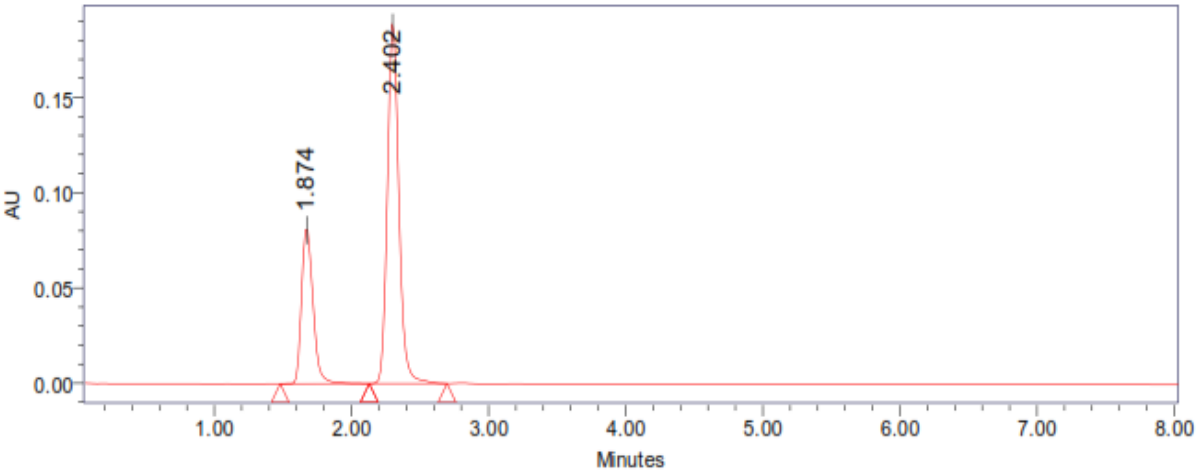


Fig.No.45: chromatogram showing more organic composition

Table No. 23: Results for Robustness -Diosmin

Parameter used for sample analysis	**Peak Area**	**Retention Time**	**Theoretical plates**	**Tailing factor**
Actual Flow rate of 0.9mL/min	545265	1.794	7564	1.09
Less Flow rate of 0.8mL/min	625486	1.867	7856	1.13
More Flow rate of 1.0mL/min More Flow rate of 0.9mL/min	526548	1.744	7425	1.12
Less organic phase (about 5 % decrease in organic phase)	536548	1.831	7265	1.06
More organic phase (about 5 % Increase in organic phase)	514875	1.874	7169	1.08

Table No. 24: Results for Robustness-Hesperidin

Parameter used for sample analysis	**Peak Area**	**Retention Time**	**Theoretical plates**	**Tailing factor**
Actual Flow rate of 0.9mL/min	7768545	3.440	8695	1.12
Less Flow rate of 0.8mL/min	7985695	3.721	8948	1.13
More Flow rate of 1.0mL/min	7458642	3.097	8452	1.12
Less organic phase (about 5 % decrease in organic phase)	7685421	6.242	8365	1.10
More organic phase (about 5 % Increase in organic phase)	7569864	2.402	8254	1.09

Acceptance Criteria:

The Tailing factor should be less than 2.0 and the number of theoretical plates (N) should be more than 2000.By variation of mobile phase i.e. Methanol: Water was taken in the ratio and 70:30, 60:40 instead (65:35), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

IV. SUMMARY AND CONCLUSION

This study focused on the development and validation of a novel RP-HPLC method for the simultaneous estimation of Diosmin and Hesperidin in pharmaceutical formulations. The method development phase involved optimizing mobile phase Acetonitrile: Water in the ratio of 50:50% v/v and the optimised column used is Symmetry column C_{18}(4.6×150mm, 5.0 µm).

Conditions to achieve efficient separation of the two compounds, ensuring adequate sensitivity and resolution. Following method optimization, validation was conducted in accordance with the guidelines outlined by the International Council for Harmonisation (ICH). Specificity studies confirmed the absence of interference from placebo excipients, establishing the method's ability to selectively quantify Diosmin and Hesperidin in the presence of potential matrix components. Linearity assessment over a specified concentration range

demonstrated the method's suitability for quantitative analysis, while accuracy and precision studies provided robust validation data through analysis of spiked samples at different concentrations. Robustness testing evaluated the method's reliability by varying critical parameters such as pH, temperature, and flow rate within defined limits. The developed RP-HPLC method was then successfully applied to analyze commercially available pharmaceutical formulations containing Diosmin and domperidone, demonstrating its practical applicability in pharmaceutical quality control.

In conclusion, the newly developed RP-HPLC method for simultaneous estimation of Diosmin and Hesperidin adheres to ICH guidelines and proves to be specific, precise, accurate, and robust. The method's validation parameters, including specificity, linearity, accuracy, precision, and robustness, all met the acceptance criteria, confirming its reliability for routine analysis in pharmaceutical laboratories. By providing an effective analytical tool for quantifying Diosmin and Hesperidin in pharmaceutical formulations, this study contributes to enhancing quality control measures and ensuring the consistency of drug formulations. Future research directions may include expanding the method's application to different matrices or investigating its compatibility with other drug combinations, thereby further validating its versatility and utility in pharmaceutical research and development.

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