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A Validated UV-Spectrophotometric Method For Fisetin Estimation From Cubosomal Nanoformulation, Marketed Formulation And Strawberry Fruit Extract

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Abstract

Fisetin (3,3′, 4′7-tetrahydroxyflavone) is an active flavanol phytoconstituent, available in the market as a nutraceutical but poor aqueous solubility limits its therapeutic use. Even though sensitive and modern technique based fiestin estimation was reported earlier, there is a need of simple, fast and cost-effective method based on UV-Spectrophotometric technique for routine application. Developed and validated a UV-spectrophotometric method and used for estimation of fisetin from cubosomal nano-formulation, marketed formulation, and strawberry fruits. A standard calibration graph was developed for fisetin using methanol in the 2-10 µg/ml concentration range using λmax 362 nm. The linearity equation for fisetin absorbance was y=0.0947x + 0.005 with a correlation coefficient of 0.9998. The method was accurate as fisetin recovery > 95%, precise and robust as % RSD < 2 for absorbance after a change in analyst, instrument, and wavelength. The fisetin loading and cubosomal entrapment percentage was found to be 99.48±0.23 and 79.83±0.65 respectively. Fisetin estimated in 'Doctor's Best ® Fisetin' was 98.82±0.18 mg per capsule (Label claim 100mg/capsule) and in strawberry 2.59±0.21 µg/gm of its dry powder. We report a new, simple, cost-effective, fast, accurate, and sensitive UV-spectroscopic method for routine quality control of fisetin formulations and other samples.

Keywords: Fisetin, UV-spectroscopy, Fisetin cubosome nanoformulation, Doctor's Best ® Fisetin, Strawberry

1. INTRODUCTION

A plant polyphenolic secondary metabolite Fisetin, is extracted from various plants like strawberry, green tea, apple, kiwi, grapes, chamomile flowers, and lotus root. It is a yellow powder extracted commercially from various plants like Rhus succedanea and Cotinus coggygria Scop and reported in varying amounts. Fisetin attracts more study attention because of the wide pharmacotherapeutic potential in the in vitro and in vivo study reports (1). Four hydroxy groups in the fisetin structure are responsible for considerable antioxidant potential, inhibit many types of angiogenesis by binding

with many anticancer receptor sites, and have anti-inflammatory effect by inhibiting inflammatory cytokines (Figure 1). However, its limited cellular absorption and bioavailability restrict its therapeutic development (2,3). Various pharmaceutical drug delivery strategies have been reported to overcome this problem. Liposomes, Polymeric micelles, Trans and Binary ethosomes, Self-nano emulsions, Polyvinyl alcohol and poly (lactic-co-glycolic acid) nanoparticles have been reported (4-10). Cubosome nanoformulation of fisetin was prepared because of its simple procedure, use of very few formulation ingredients, increased water solubility and bioavailability, and targeted drug delivery effect (11-14).

Figure 1. Fisetin Chemical Structure

Several sensitive analytical methods were used for the estimation of fisetin from plant and biological samples like HPLC (15), HP-TLC, and LC-MS/MS but are expensive, need skill and not used frequently (16).

Earlier we reported a very sensitive and reproducible RP-HPLC method using formic acid and acetonitrile mixture as mobile phase for estimation of fisetin from cubosomal formulation, various plants extract and marketed formulation along with stress degradation study of fisetin (17). UV-visible spectroscopy is a very simple, fast, and sensitive technique for the estimation of fisetin and very few reports on developed and validated methods are available. In many reports on fisetin identification and estimation from plants, the UV-visible spectroscopic method was used without method development and validation therefore its accuracy and reproducibility are questionable.

A single developed and validated UV-visible spectroscopic method was reported using solvent methanol for quantification of FIS in Self-Nanoemulsifying Drug Delivery System but not applied for plant samples and marketed formulation and was used only for their in-house formulation (18). In earlier literatures, validated UV-visible spectroscopic methods were not used for the estimation of fisetin from plant samples and formulations (19,20). Therefore, we developed and validated a UV-visible spectroscopic method for fast and simple estimation of fisetin from various types of samples. The present study aims to develop a simple, better, economical, more sensitive, and reproducible UV-visible spectroscopic method to evaluate the prepared Fisetin cubosome nanoformulation and some plant extracts for its quantification, drug entrapment efficiency, evaluation of marketed FIS formulation. The developed method is cost-effective and used for both the identification and quantification of fisetin with maximum accuracy, precision, and reproducibility from plant samples, marketed formulation, and prepared cubosomal nanoformulation. We report a validated, novel UV-visible spectroscopic method using methanol as solvent which can be used for the estimation of fisetin from different categories of samples.

2. MATERIALS AND METHODS

2.1 Materials

A gift sample of Fisetin was provided by Shaanxi Yi an Biologicals, Technology Co., Ltd. China. The Poloxamer 407 received from BASF, Mumbai, and Glyceryl monooleate from Mohini Organics Pvt. Ltd, Mumbai, were received. The analytical grade methanol, ethanol, and dimethyl sulfoxide were

obtained from Fisher Scientific and Merck, Mumbai, India. The commercial formulation Doctor's Best ® Fisetin was procured containing fisetin capsule from the market.

2.2 Instrument

A double beam scanning UV/Visible spectrophotometer, Shimadzu UV-1800 (Japan) made with Deuterium lamp, a silicon photodiode detector of wavelength range 190-1100 nm, and UV probe software were used (21).

2.3 Preparation of Cubosomal Nanoformulation

A simple top-down method of cubosomal preparation using glycerol mono-oleate (0.8 gm) and poloxamer 407 (0.6 gm) were used (22-24). Fisetin (40 mg) was added in melted glycerol mono-oleate at 65°C, mixed and added to molten mass of poloxamer 407. In this homogeneous mixture, add water dropwise with starring up to 40 ml to get milky dispersion. Homogenization and sonication were performed for the separation of cubosomes and characterized cubosome particle properties (25). To estimate the fisetin entrapped in the cubosome, nanoformulation supernatant was diluted with methanol and find absorbance using the developed method.

2.4 Plant Sample Collection and Preparation

Strawberries were purchased from the local market, chopped, and dried completely. Crushed for size reduction and performed maceration for extraction of fisetin in the dark using methanol and water for three days (26). Filtrate of this was mixed with 30 ml chloroform. Separate chloroform layer, dried and diluted with methanol for fisetin estimation using developed UV Spectrophotometric method(27-29).

2.5 Sample preparation from marketed fisetin formulation (Doctor's Best ® Fisetin)

Ten capsules were taken, a blend of the powder content was prepared and average powder weight was measured. Powder weight equivalent to 10 mg was dissolved in methanol and sonicated. Further diluted with methanol and absorbance was measured. The label claim of Doctor's Best ® Fisetin was 100 mg per capsule (17).

2.6 Stock Solution Preparation

A mother stock solution of 1mg/ml of fisetin was arranged by dissolving 10 mg in 10 ml of methanol and sonicated for 10 minutes for complete dissolution. Take 1 ml of this mother stock solution and dissolve it in 9 ml of methanol to prepare 100 µg/ml working stock solution. Using this stock solution 2, 4, 6, 8, and 10 µg/ml solutions were developed. All dilutions were prepared in umber color volumetric flask and stored at 4°C temperature.

2.7 Method development

For the selection of a suitable solvent and parameter development of a new UV Spectrophotometric method, the solubility of fisetin in different solvents were considered. Among the solvents used, methanol, ethanol, acetone, and dimethyl sulfoxide, methanol was selected for study considering better solubility. Fisetin using solvent methanol was scanned between 200 to 800 nm and found highest absorption at 362 nm.

2.8 Method Validation

Validation of the developed method was done using ICH guidelines Q2(R1), based on parameters accuracy, precision, system suitability, linearity, the limit of quantification (LOQ), and limit of

detection (LOD), ruggedness, and robustness (30,31). For the development of the linearity curve, 0, 2, 4, 6, 8, and 10 µg/ml dilutions were prepared from the working stock solution (100 µg/ml). Absorbance using the developed method was measured for these dilutions and a graph of absorbance versus concentration was prepared and used. The accuracy of the developed UV Spectrophotometric method was assessed by taking the three levels of 50 %, 100%, and 150% of median concentration (5µg/ml) of fisetin. The resultant concentrations of fisetin 2.5, 5, and 7.5 µg/ml were prepared by adjusting the final volume with methanol and absorbance was measured.

Actual % recovery = (Actual concentration recovered/ Theoretical concentration) X100 …(1)

The percentage relative standard deviation measurment for intraday and interday using the absorbance of low, medium, and high concentrations 2.5, 5, and 7.5 µg/ml in triplicate were considered to study the precision of the developed method.

% RSD = (Standard deviation of absorption/ Average absorption) X 100 ……..(2)

The results after deliberate small changes in the selected parameters of the developed analytical method hint about the ruggedness and robustness of the established UV Spectrophotometric method. The absorbance for fisetin concentration of low, medium, and high levels of 5 µg/ml was measured in triplicate for changed parameters. The sensitivity of the developed method will be decided from values of Limit of LOQ and LOD. These parameter values were estimated from the standard deviation (σ) and slope of the linearity graph (S).

$LOD = 3.3 \sigma / S$ …… (3)

$LOQ = 10 \sigma / S$ …… (4)

The stability of the prepared stock solutions over 3 days were studied for 10 µg/ml fisetin concentration and absorbance was measured using this method.

For measuring the fisetin concentration in cubosome nanoformulation, 1 ml of formulation was diluted up to 10 ml using methanol in an umber color volumetric flask. Filter this solution and dilute 1 ml up to 10 ml using methanol and test for percent drug loading.

% Fisetin Loading = (Actual amount of Fisetin present/Total amount of Fisetin added) X 100 ……..(5)

For measurement of fisetin entrapment in cubosomal formulation, high speed centrifugation was performed and supernatant was taken for absorption measurement.

% Fisetin Entrapment = (Actual amount of Fisetin entrapped/Total amount of Fisetin added) X 100 ……..(6)

Fisetin quantification in marketed Doctor's Best ® Fisetin, capsule formulation, and resultant dilution equivalent to 10 µg/ml were processed. Dried chloroform extracts of plants were diluted with methanol and analyzed using the developed method.

3. RESULTS AND DISCUSSION

3.1 Method development

Fisetin is an active plant flevanol, there for extraction methods have been reported from various plants and biological samples using solvents methanol, ethanol, chloroform, and DMSO. We took 10 µg/ml fisetin concentration using methanol, ethanol, and DMSO as solvents and scanned between 200 to 800 nm. for maximum absorption wavelength and found to be 362 nm (Table 1). As methanol gives a sharp and clear peak of fisetin absorption at 362 nm, it was selected as a solvent system and further validated for various parameters of ICH Q2(R1) guiding principles.

Table 1. Method Development

Parameter	Details
Solute drug/Analyte	Fisetin
Solvents used	Ethanol, Methanol, Dimethyl Sulfoxide
Fisetin Absorption max (λ max) for methanol	362 nm
Instrument Used	Double Beam Scanning, Shimadzu UV-1800

3.2 Method Validation

The developed method using solvent methanol was validated for selectivity and specificity, accuracy, precision, ruggedness and robustness, and sensitivity.

Selectivity and Specificity of developed method

There was no interference in the spectrum developed for both fisetin and plane methanol, sharp and clear absorbance peak at 362 nm indicates the selectivity and sensitivity of the developed method. The scanned absorption spectrum of fisetin using selected solvent methanol is represented in figure 2.

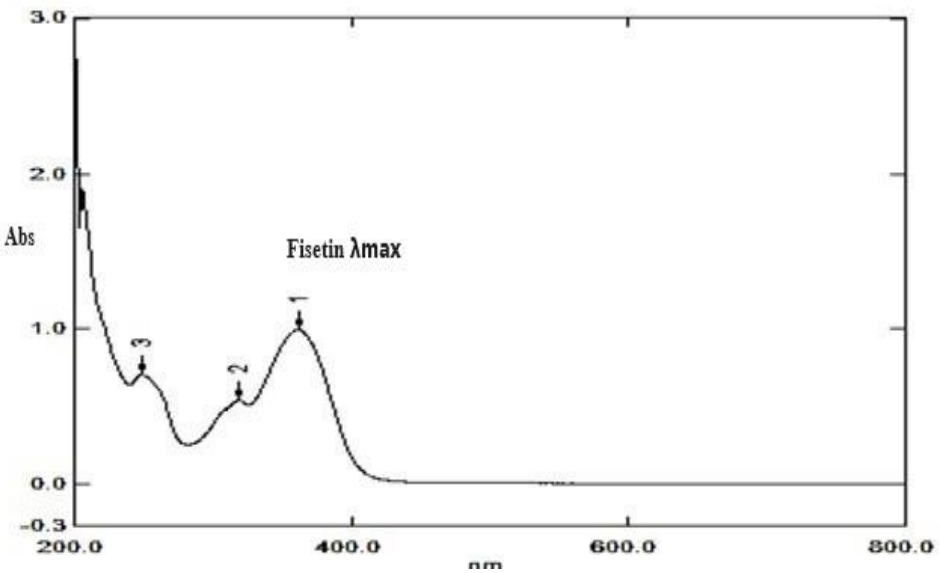

Figure 2. UV- Spectrum of Fisetin showing Absorption max at 362 nm

Calibration Curve Development

The calibration curve was developed using solvent methanol in the range of 2 to 10 µg/ml fisetin concentration in triplicate and recorded absorption at 362 nm (Table 2). The calibration graph used to develop the linearity equation, the correlation coefficient (R^2) was recorded more than 0.9998 and the slope value as 0.0947 (Figure 3).

Table 2. Calibration Curve Development for Fisetin

Sr. No.	Fisetin Conc. (µg/ml)	Fisetin Abs. at 362 nm
1	2	0.197
2	4	0.388
3	6	0.578
4	8	0.756
5	10	0.953

R^2	0.9998
Slope	0.0947
LOD (µg/ml)	0.119
LOQ (µg/ml)	0.250

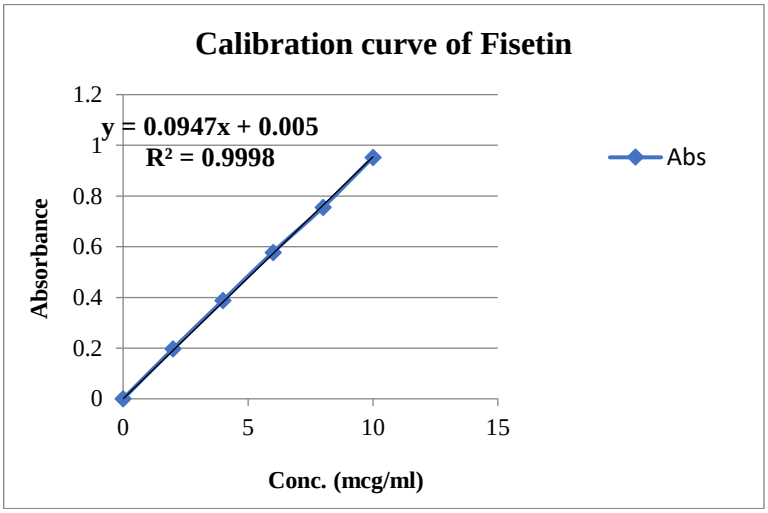

Figure 3. Calibration curve of fisetin

Accuracy

The accuracy of the developed method was assessed using three-level fisetin concentrations within the calibrated concentration range (50, 100, and 150 percent) of the median concentration (5µg/ml). The values of percentage recovered fisetin concentration for 2.5, 5, and 7.5 µg/ml were found to be more than 95%, hence developed method was found to be accurate for fisetin quentification (Table 3).

Table 3. Accuracy data for Fisetin

Level %	Fisetin Conc. (µg/ml)	Absorbance	Amount recovered (µg/ml)	%Mean recovery ±SD
50	2.5	0.239	2.471	99.683± 0.422
		0.24	2.482	
		0.241	2.492	
100	5	0.477	4.984	101.373± 0.921
		0.478	4.995	
		0.485	5.069	
150	7.5	0.713	7.476	98.557± 0.695
		0.714	7.487	
		0.705	7.392	

Precision

The repeatability test of the developed method was studied using three levels as low, medium, and high concentrations were tested for interday and intraday precision. The values for percentage relative standard deviations were calculated for interday and intraday repeatability and found to be less than two, which indicates the established method was found to be precise (Table 4).

Table 4. Precision data for Fisetin

Conc. (µg/ml)	Interday Abs. reading			Intraday Abs. reading	
	Day 1	Day 2	Day 3	Morning	Evening
2.5	0.238	0.237	0.237	0.235	0.237
	0.240	0.235	0.231	0.239	0.231
	0.237	0.235	0.239	0.234	0.234
%RSD	0.641	0.490	1.767	1.121	1.282
5	0.482	0.479	0.475	0.475	0.477
	0.480	0.477	0.481	0.478	0.476
	0.476	0.470	0.475	0.474	0.479
%RSD	0.637	0.994	0.726	0.438	0.320
7.5	0.711	0.712	0.703	0.714	0.719
	0.701	0.703	0.713	0.712	0.716
	0.712	0.716	0.706	0.709	0.718
%RSD	0.859	0.937	0.725	0.354	0.213

Ruggedness and Robustness

For testing the ruggedness of the method, change in analyst and instrument were done and results were measured for three-level concentration within the linearity range. Ruggedness was interpreted from the % RSD values and readings were found to be less than 2. Robustness was tested by making internal minor changes in method parameters like λmax and results were calculated for change in % RSD values. It was found that the % RSD values for the changed parameter λmax were less than two and confirming that the method was robust (Table 5).

Table 5. Ruggedness and Robustness data for Fisetin

Sr. No.	Change in Analyst			Change in Instrument			Change in Wavelength (360nm)			Change in Wavelength (364nm)		
	Fisetin conc. (µg/ml)	Abs.	% RSD	Fisetin conc. (µg/ml)	Abs.	% RSD	Fisetin conc. (µg/ml)	Abs.	% RSD	Fisetin conc. (µg/ml)	Abs.	% RSD
1	2.5	0.235	0.649	2.5	0.233	1.121	2.5	0.233	1.372	2.5	0.238	1.497
2	2.5	0.234		2.5	0.238		2.5	0.238		2.5	0.231	
3	2.5	0.237		2.5	0.237		2.5	0.232		2.5	0.235	
4	5	0.473	0.967	5	0.477	0.963	5	0.479	0.754	5	0.471	1.109
5	5	0.479		5	0.471		5	0.481		5	0.479	
6	5	0.47		5	0.480		5	0.474		5	0.481	
7	7.5	0.708	0.429	7.5	0.714	0.354	7.5	0.711	0.428	7.5	0.709	0.643
8	7.5	0.714		7.5	0.711		7.5	0.717		7.5	0.712	
9	7.5	0.712		7.5	0.709		7.5	0.713		7.5	0.718	

Sensitivity

The LOD and LOQ were estimated using values of standard deviation of response and linearity curve slope. Sensitivity of the developed method was decided based on the results of LOD and LOQ and were found 0.119 and 0.250 µg/ml correspondingly (Table 2). The developed method was found to be more sensitive than earlier reported method (18).

Fisetin Solution Stability Study

The absorbance for 10 µg/ml fisetin freshly prepared and stored for 3 days was measured and % RSD was calculated. The prepared solution was found to be stable during 3 days of storage as the % RSD was less than 2 for both samples (Table 6).

Table 6. Fisetin solution stability study

Sample Type	Fisetin Conc. (µg/ml)	Absorbance	% RSD
Fresh Stock	10	0.808	0.285
	10	0.812	
	10	0.812	
Old Stock	10	0.791	0.622
	10	0.790	
	10	0.799	

3.3 Application of the Method for FIS Estimation in Cubosomal Nanoformulation, Marketed Formulation, and Plant Extracts

The samples of cubosomal nanoformulation, marketed formulation, and strawberry fruit samples were processed for fisetin estimation using the developed UV spectroscopic method. The amount of fisetin loading and entrapment in cubosomes were measured using this method and found to be 99.48±0.23 and 79.83±0.65 percent respectively. The absorption of methanol diluted samples of the marketed formulation 'Doctor's Best ® Fisetin' were measured and fisetin content was found to be 98.82±0.18 mg per unit of capsule. The dried chloroform extract of strawberry was diluted using methanol, absorbance was recorded, and fisetin concentration was calculated from a linearity equation and found to be 2.59±0.21 µg/gm of dried strawberry powder weight.

4. CONCLUSION

The study describes the simple, cost-effective, fast, sensitive, and robust UV-Spectrophotometric method for fisetin estimation. The quantity of fisetin were estimated from cubosomal nanoformulation, Doctor's Best ® Fisetin formulation, and strawberry in a very short time, and easily. Developed and validated method can be widely applicable for fisetin estimation from pure drug, quality control of various types of marketed pharmaceutical dosage forms, plant extracts, and biological samples. Conclusively, the reported UV method has many advantages over previously reported other analytical techniques for fisetin estimation and is found more sensitive than earlier reported UV method.

ABBREVIATIONS

HPLC, High-performance liquid chromatography; HP-TLC, High-performance liquid chromatography; DMSO, Dimethyl sulfoxide; RSD, Relative standard deviation; LOQ, Limit of Quantification; LOD, Limit of detection; ICH, International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use; R2, correlation coefficient

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