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# Development and Validation of an RP-HPLC Technique for Quantifying Fisetin in Rat Plasma

Sukriti Vishwas[1], Monica Gulati[1,2], Kamal Dua[2,3], Sachin Kumar Singh[1,2,4]

[1]*School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India*

[2]*Faculty of Health, Australian Research Centre in Complementary and Integrative Medicine, University of Technology Sydney, Ultimo, NSW 2007, Australia*

[3]*Discipline of Pharmacy, Graduate School of Health, University of Technology Sydney, Ultimo, NSW 2007, Australia*

[4]*School of Medical and Life Sciences, Sunway University, 47500 Sunway City, Malaysia*

**Corresponding author:** Sachin Kumar Singh, School of Pharmaceutical Sciences, Lovely Professional University, Phagwara, Punjab, India, Discipline of Pharmacy, Graduate School of Health, University of Technology Sydney, Ultimo, NSW 2007, Australia. School of Medical and Life Sciences, Sunway University, 47500 Sunway City, Malaysia E-mail ID: singhsachin23@gmail.com

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**Abstract**

Fisetin (FS) is a phyto-flavonoid with antioxidant, neuroprotective, and anticancer properties. A bioanalytical method was developed and validated to estimate FS levels in rat plasma using reverse phase ultra-fast liquid chromatography with a C-18 reverse phase column. Quercetin (Qu) served as an internal standard. The mobile phase consisted of acetonitrile and orthophosphoric acid (0.2% v/v) in a 30:70 v/v ratio, with a flow rate of 1 mL/min, and detection was performed at a wavelength of 362 nm. Protein precipitation was employed to extract the drug from plasma samples. The retention times for FS and Qu were 5.6 min and 10.3 min, respectively. The method demonstrated linearity in the range of 2-10 ng/mL with a regression coefficient ($r^2$) of 0.9994. Validation followed the ICH Q2 (R1) guidelines, with percentage recovery between 98-100%, indicating accuracy. The percentage relative standard deviation was below 2%, showing precision. The limit of detection (LOD) and limit of quantification (LOQ) were 0.03 ng/mL and 0.08 ng/mL, respectively. The method proved robust, showing no significant response changes with variations in flow rate and mobile phase composition. These results indicate that the developed method meets all validation criteria and is suitable for estimating fisetin in rat plasma.

**Keywords:** Fisetin; Bioanalytical method development; Validation; rat plasma; Quercetin

## 1. Introduction

FS exhibited numerous neuroprotective effects for treating AD. It is a polyphenolic flavonoid. Chemically, it is a 2-(3,4-dihydroxyphenyl)-3,7-dihydroxy-4H-1-benzopyran-4-one [1,2]. It possesses antioxidant, anti-inflammatory, reduce mitochondrial dysfunction, endoplasmic reticulum stress and neuronal cell apoptosis. Various scientists have developed different methods for estimating FS concentration in plasma samples. For instance, Kadari et al. administered FS orally to rats as polymeric nanoparticles and used an LC-MS/MS gradient method to measure fisetin levels in blood. This method utilized ammonium formate (10 mM, pH 3.5) and acetonitrile as the mobile phase at a flow rate of 0.4 mL/min [3]. This study aims to develop a simple, economical, sensitive, accurate, precise, and reproducible reverse phase HPLC (RP-HPLC) method for estimating FS in rat plasma. The developed method was validated for accuracy, precision, specificity, and sensitivity, and the results are discussed in the following sections.

## 2. Materials and methods

### *2.1. Material*

#### *2.1.1. Chemicals and equipment*

FS and Quercetin (Purity more that 96% HPLC grade, company Tokyo chemical industries, Japan) was purchased from D.P. Traders, Mohali, Punjab, India. Acetone, methanol, orthophosphoric acid and acetonitrile were purchased from LOBA and Rankem Pvt. Ltd., India. Triple distilled water was used throughout the study. UFLC (LC-20AD, Shimadzu, Japan) was used for quantitative analysis. Vortex mixer and cooling centrifuge were from REMI, India. The HPLC was used Analytical Ptv. Ltd. Gujrat, India.

#### *2.2.2. Procurement and storage of animals*

Male SD rats, weighing between 220-270 g, were obtained from the National Institute of Pharmaceutical Education and Research (NIPER) in Mohali, India. These rats underwent a 10-day acclimatization period in plastic cages filled with rice husk bedding. During this period, they were provided with pellet feed and had unrestricted access to clean water. The housing conditions were maintained at a temperature of 24 ± 1 °C and a relative humidity of 45%. To regulate their circadian rhythm, the rats were exposed to a 12 h light and 12 h dark cycle using artificial lighting [4]. All experimental procedures were conducted in accordance with the

approval of the Institutional Animal Ethical Committee, with the assigned approval number LPU/IAEC/2021/91.

***2.2.3. Chromatographic conditions***

The method was developed by using a C-18 reverse phase column. A combination of acetonitrile and orthophosphoric acid (0.2 % v/v) in the ratio of 30-70 v/v was used as the mobile phase. During HPLC analysis, one mL/min flow rate was used and chromatograms were analysed at 362 nm. The same analytical method was used to validate bioanalytical method development. The method for extraction of the plasma sample is described below [5].

***2.2.4. Collection of blood and extraction of plasma***

To perform the pharmacokinetic study, rat blood was taken through the tail vein method and transferred into radioimmunoassay (RIA) vials. RIA vials containing EDTA crystals as an anticoagulant. The blood sample was gently mixed with EDTA in vial and transferred to a centrifugation tube. The blood sample was centrifuged at 4932 *g* for the duration of 10 minutes. The resulting clear supernatant was plasma obtained which was carefully extracted using a micropipette and stored in a deep freezer at -20 °C for further processing [5].

***2.2.5. Preparation of blank plasma***

A 1 mL plasma sample was extracted from the blood and mixed with 1 mL of methanol. Further, the mixture was vortexed for a duration of 5 minutes to induce the precipitation of plasma proteins. Subsequently, the resulting supernatant was taken by centrifuging a precipitated plasma protein sample at 4932 *g* for 15 minutes. The supernatant was then transferred to a 100 mL volumetric flask, and the volume of methanol was adjusted up to 100 mL [6].

***2.2.6. Preparation of standard stock solution***

For the stock solution with plasma, 10 mg FS was added in 2 mL of plasma sample. FS solution was further vortexed for a duration of 10 minutes, followed by the addition of 2 mL methanol. Subsequently, vertexing was done to form the precipitation of plasma proteins. Samples were then centrifuged at 4932 g for 15 minutes. The resulting supernatant was collected in a volumetric flask, and the volume was adjusted to 100 mL methanol to develop a stock solution with a concentration of 100 μg/mL ≡ 100000 ng/mL. From this solution, 10 mL was taken and diluted to 90 mL of methanol to attain the concentration of 10 μg/mL ≡ 10000 ng/mL.

Furthermore, from this solution 10 mL was taken and diluted to 100 mL of methanol, to attain the concentration of 1 µg/mL ≡ 1000 ng/mL. The final concentration was maintained at 0.01 µg/mL ≡ 10 ng/mL following the same procedure as above [6].

***2.2.7. Preparation of internal standard (IS)***

Quercetin (Qu) (25 ng/mL) was used as the internal standard (IS). Considering the structural homology of Qu with FS, Qu was chosen as the IS. IS plays a crucial role in facilitating the quantification of an analyte, especially in situations with an anticipated loss of sample volume, such as in the case of biological samples with poor recovery from the matrix. To prepare the Qu stock solution, 10 mg of Qu was accurately weighed and added to a 100 mL volumetric flask containing 20 mL of methanol. The solution was subjected to sonication for 10 minutes to ensure complete dissolution of the drug. The final volume was adjusted to 100 mL using methanol, resulting in a stock solution with a 100 µg/mL ≡ 100000 ng/mL concentration. From this solution, 10 mL was taken and diluted to 90 mL of methanol to attain the concentration of 10 µg/mL ≡ 10000 ng/mL. Furthermore, from this solution 10 mL was taken and diluted to 100 mL of methanol, to attain the concentration of 1 µg/mL ≡ 1000 ng/mL. further 10 mL was taken and diluted to 100 mL of methanol to attain a concentration of 0.1 µg/mL ≡ 100 ng/mL. Finally, 25 mL was taken from 100 ng/mL solutions and diluted in 100 mL of methanol to attain the concentration of 0.25 µg/mL ≡ 25 ng/mL [6].

***2.2.8. Method validation***

Dilutions were made in the concentration range of 2-10 ng/mL from the stock solution of 10 µg/mL containing a FS in plasma. 1mL of Qu (25 ng/mL) was added to get the final volume of 10mL of IS Qu. Each dilution was injected into HPLC in five replicates and analyzed at 362 nm. Similarly, the calibration curve of FS was developed in the concentration range of 2-10 ng/mL in the mobile phase [5].

***2.2.8.1. Linearity and range***

The range was selected by injecting minimum concentration of FS (10 ng/mL). Further, different dilutions of FS was injected to HPLC. Total five injections of each concentration of FS were analyzed and the mean area were recorded. Afterwards, a calibration curve was plotted in the range of 2-10 ng/mL. Slope, intercept and correlation coefficient of the calibration curves

(peak area versus concentration) were determined to ensure linearity of the analytical method [7].

***2.2.8.2. System suitability***

In the system suitability study, various parameters were measured. These include peak area, peak hight, tailing factor, theoretical plates/meter, and peak purity index. According to the ICH Q2(R1) guidelines in the system suitability tailing factor, theoretical plates and resolution of the peaks must be under acceptance criteria such as tailing factor must be less than 2, peak purity index should be smaller than 0.5, and theoretical plates/meter should always be smaller than 20000 [8].

***2.2.8.3. Limit of detection (LOD) and limit of quantification (LOQ)***

The LOD and LOQ of FS was calculated from the calibration curve. LOD is the quantity of analyte that can be detected at low concentration whereas, LOQ is the lowest concentration of analyte that can be determined by acceptable accuracy and repeatability [9]. The formula of both LOD and LOQ are mentioned in Eq. 1 and Eq.2.

$$LOD = \frac{3.3\sigma}{S} \qquad Eq.\ (1)$$

$$LOQ = \frac{10\sigma}{S} \qquad Eq.\ (2)$$

***2.2.8.4. Accuracy***

In order to evaluate the accuracy of the developed method three different concentration were taken i.e lower quantified concentration (LQC, 4.8 ng/mL) at 80 % level, middle quantified concentration. (MQC, 6 ng/mL) at 100 % level and higher quantified concentration (HQC, 7.2 ng/mL) at 120 % level and their % mean recovery was calculated. To prepare these samples an aliquot of 4.8 mL, 6 mL and 7.2 mL were taken from 10 ng/mL (solution A) and added separately in 10 mL volumetric flasks. The volume of each sample was made upto 10 mL by using methanol. The prepared samples were run for 5 times on the HPLC system and % recovery of each drug was calculated [9]. The formula to calculate absolute absolute recovery (%) is showed in Eq. 3.

$$Absolute\ recovery\ (\%)\ \frac{Actual\ concentration\ recovered}{Theoritical\ concentration} \times 100 \qquad Eq.\ (3)$$

***2.2.8.5. Precision***

The precision of the developed method was evaluated by checking repeatability and intermediate precision. In order to evaluate repeatability, the samples of 4.8 ng/mL, 6 ng/mL and 7.2 ng/mL were run on HPLC at the same day (intra-day) by following the same experimental conditions. Intermediate precision was determined by injecting six samples of each 4.8 ng/mL, 6 ng/mL and 7.2 ng/mL on different days (inter-day) as well as by different analyst (inter analyst). The average area of each six runs of 4.8 ng/mL, 6 ng/mL and 7.2 ng/mL were recorded and % relative standard deviation (% RSD) was calculated. The formula to calculate % RSD is shown in Eq. 4. Additionally, an inter-analyst study was conducted by having three different analysts each prepare and inject LQC, MQC, and HQC samples six times under identical experimental conditions. The mean data were recorded, and the percentage relative standard deviation (% RSD) was calculated [9].

$$\%\ relative\ standard\ deviation\ (\%\ RSD) = \frac{Standard\ deviation\ of\ peak\ area}{Average\ peak\ area} \times 100 \qquad Eq.\ (4)$$

***2.2.8.6. Robustness***

Robustness of the analytical method was studied by varying flow rate (0.8, 1.0 and 1.2 mL/min), mobile phase ratio (28:72, 30:70 and 32:68) and pH (2.9, 3.1, and 3.3). The six replicates of MQC (6 ng/mL) sample were injected in the HPLC. Their average mean area, % recovery, retention time were recorded. In addition, their % RSD were also calculated [9].

***2.2.8.7. Specificity***

The effect of excipients used in the formulation on FS's peak was checked with the help of specifity study. The specificity study was carried out in order to check any possible interaction of excipients with the drugs. Each solubilizer and excipient used in formulation such as Capmul MCM EP/NF®, Tween 80, and Transcutol P, was diluted either with ethanol or hexane, depending upon their solubility, and injected into HPLC. The specificity of the proposed HPLC method for the determination of FS was established by injecting the mobile phase and placebo NE solution into the HPLC system [9]. Additionally, the black chromatograms of different solubilizers used during solubility study were recorded.

## 3. Results and discussion

Method validation includes several parameters like accuracy, precision, linearity, robustness, SST, specificity, LOD, and LOQ. These all-validation parameters have been performed systematically as per ICH Q2 (R1) guidelines.

### *3.1 Chromatograms of FS and Qu*

The chromatogram of FS and QUE prepared in mobile phase is shown in Fig. 1. The Rts of FS, Qu were found to be 5.6 and 10.3 min respectively.

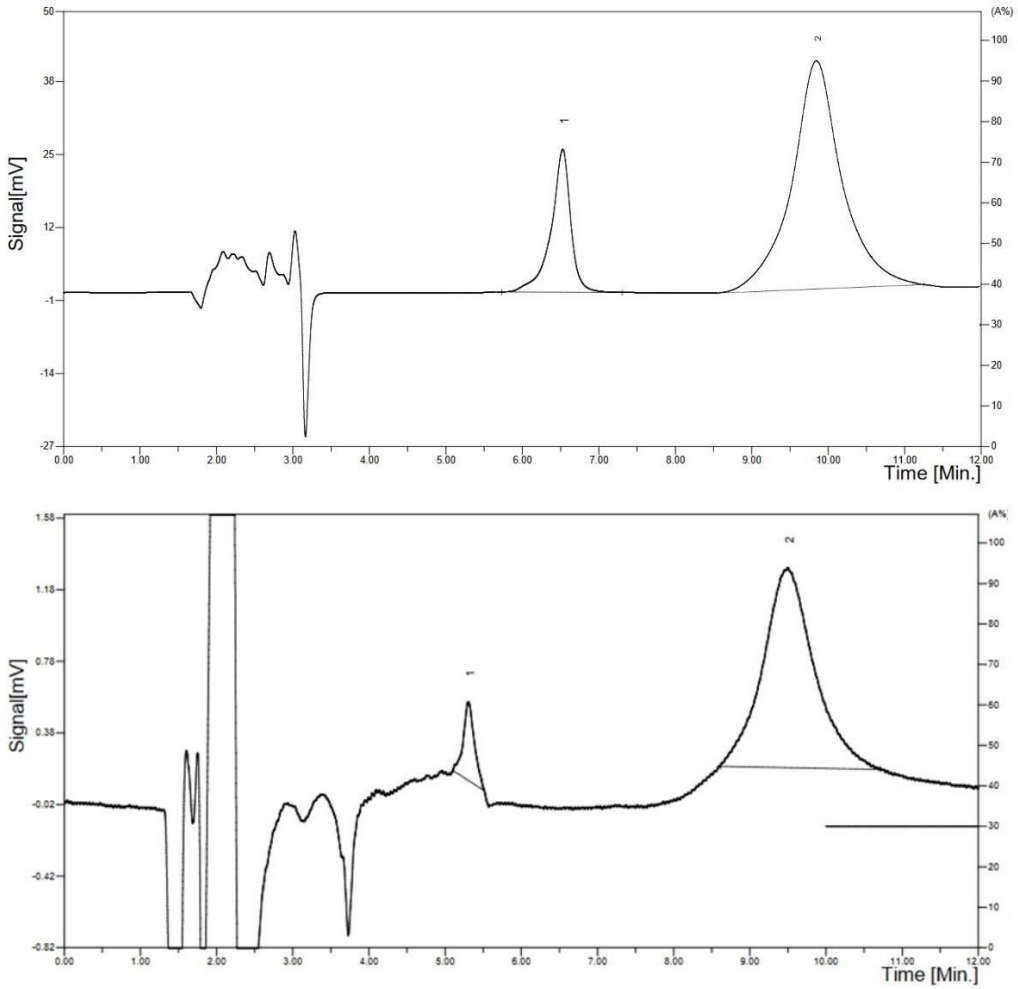


**Figure 1: Chromatograms of FS and Qu**

### *3.2. Specificity studies*

Figure 2 displays the chromatogram of blank plasma. The lack of peaks at the retention times (Rts) of FS and Qu confirms that the plasma matrix does not interfere with the quantitative analysis of these drugs when extracted from blood.

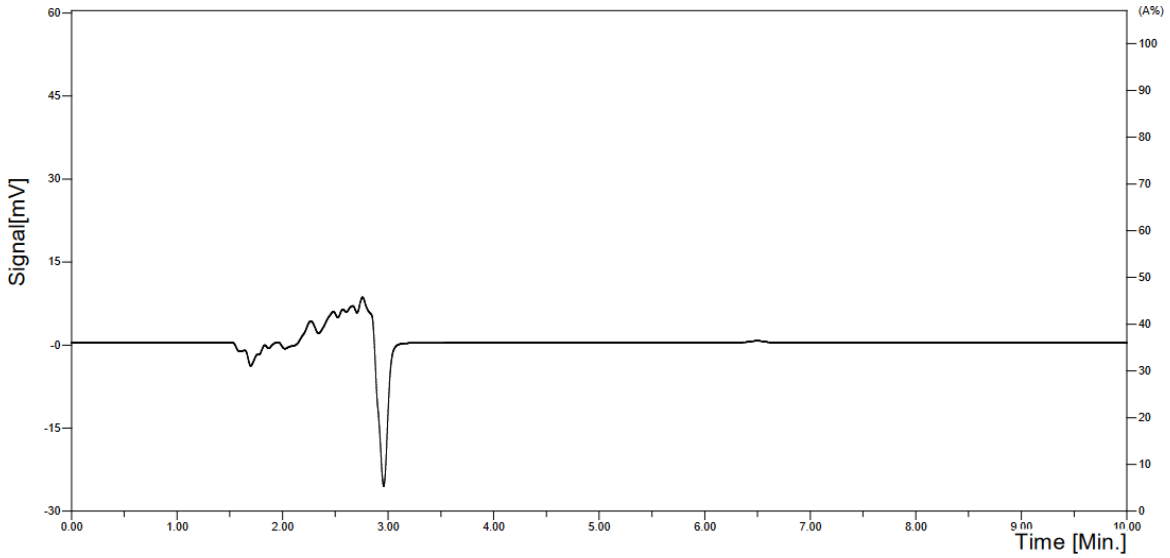


**Figure 2: Chromatograms of blank plasma**

### *3.3. Development of calibration curve*

Five serial dilutions having concentrations of 2, 4, 6, 8 and 10 ng/mL were prepared to determine linearity and range. The data was found linear in this range with the correlation coefficient ($r^2$) of 0.9994. The slope of the curve was found to be 0.0655X and intercept was 0.093. The calibration curve of FS is shown in figure 34.

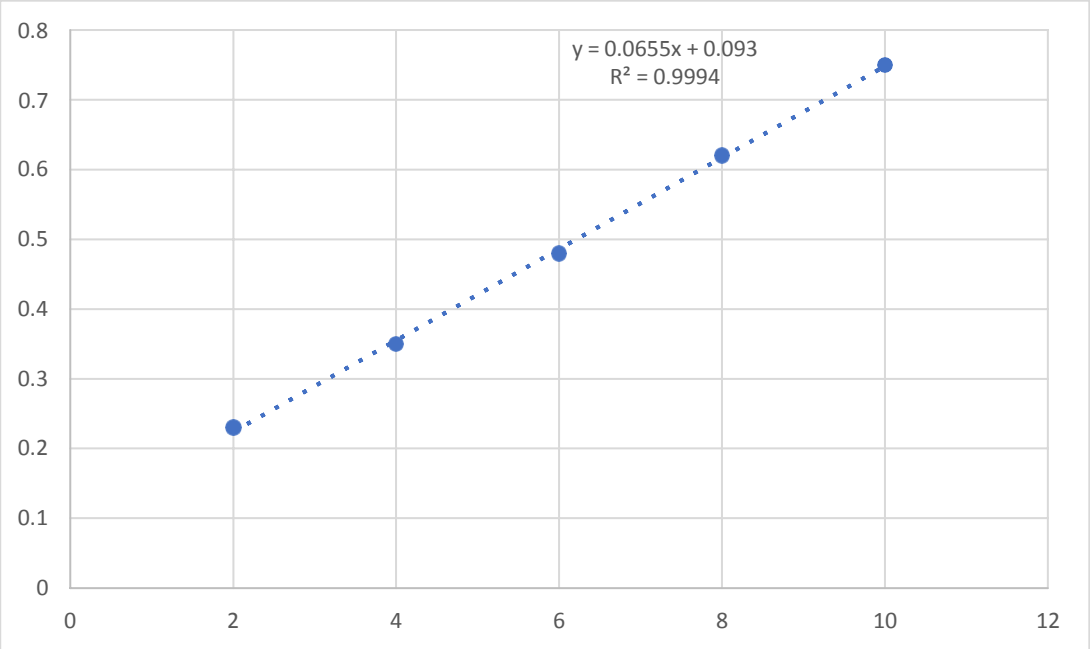


**Fig. 34.** Calibration curve of FS in please sample

### *3.4. Method validation*

#### *3.4.1. Accuracy study*

The study was carried out as per the procedure described in the section of 4.3.1.5.4. The % recovery was found in the range of 98-100 %, indicating about the accuracy of method. Accuracy study of FS is tabulated in Table 1.

**Table 1:** Accuracy study

| **Level** | **Actual concentration of std solution (µg/mL)** | **Concentration of drug recovered (µg/mL), (*N*=5)** | **% recovery** |
|---|---|---|---|
| LQC | 4.8 | 4.74 | 98.76 |
| MQC | 6 | 5.89 | 98.29 |
| HQC | 7.2 | 7.13 | 99.15 |

### *3.4.2. Precision studies*

The intraday, interday, and interanalyst precision, studies were carried out for FS in plasma and mobile phase. The % RSD of the areas recorded for LQC, MQC and HQC samples was less than 2 indicating the precision of the method. (Table 2)

### *3.4.3. Robustness study*

In the robustness study, The % RSD was observed to be less than 2%, which indicated that the method was found to be robust after slight changes in flow rate, mobile phase ratio, and pH. The results of the robustness study are shown in Table 3.

**Table 2:** Results of precision study

| **Parameter** | **Level** | **Concentration (ug/mL)** | **Area** | | | | | | **Mean area** | **Standard deviation** | **% RSD** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | **1** | **2** | **3** | **4** | **5** | **6** | | | |
| **Intra day** | | | | | | | | | | | |
| | LQC | 4.80 | 0.426 | 0.422 | 0.425 | 0.424 | 0.428 | 0.427 | 0.424 | 0.0021 | 0.49 |
| | MQC | 6.00 | 0.514 | 0.518 | 0.519 | 0.517 | 0.523 | 0.520 | 0.517 | 0.0026 | 0.51 |
| | HQC | 7.20 | 0.601 | 0.604 | 0.597 | 0.602 | 0.599 | 0.604 | 0.601 | 0.0035 | 0.58 |
| **Inter-day** | | | | | | | | | | | |
| Day 1 | LQC | 4.80 | 0.424 | 0.426 | 0.427 | 0.424 | 0.421 | 0.425 | 0.426 | 0.0015 | 0.36 |
| | MQC | 6.00 | 0.513 | 0.516 | 0.514 | 0.517 | 0.516 | 0.519 | 0.514 | 0.0015 | 0.30 |
| | HQC | 7.20 | 0.605 | 0.601 | 0.599 | 0.600 | 0.560 | 0.603 | 0.602 | 0.0031 | 0.51 |
| Day 2 | LQC | 4.80 | 0.421 | 0.424 | 0.427 | 0.426 | 0.422 | 0.425 | 0.424 | 0.0030 | 0.71 |
| | MQC | 6.00 | 0.514 | 0.510 | 0.515 | 0.517 | 0.521 | 0.525 | 0.513 | 0.0026 | 0.52 |
| | HQC | 7.20 | 0.601 | 0.604 | 0.597 | 0.602 | 0.599 | 0.596 | 0.601 | 0.0035 | 0.58 |
| Day 3 | LQC | 4.80 | 0.423 | 0.426 | 0.422 | 0.427 | 0.424 | 0.419 | 0.424 | 0.0021 | 0.49 |
| | MQC | 6.00 | 0.515 | 0.512 | 0.516 | 0.518 | 0.526 | 0.529 | 0.514 | 0.0021 | 0.40 |
| | HQC | 7.20 | 0.603 | 0.601 | 0.598 | 0.604 | 0.602 | 0.599 | 0.60 | 0.0025 | 0.42 |
| **Intermediate precision** | | | | | | | | | | | |
| Analyst 1 | LQC | 4.80 | 0.421 | 0.425 | 0.422 | 0.426 | 0.428 | 4.225 | 0.42 | 0.0021 | 0.49 |
| | MQC | 6.00 | 0.513 | 0.510 | 0.514 | 0.516 | 0.520 | 0.518 | 0.51 | 0.0021 | 0.41 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | HQC | 7.20 | 0.601 | 0.604 | 0.597 | 0.602 | 0.599 | 0.596 | 0.60 | 0.0035 | 0.58 |
| Analyst 2 | LQC | 4.80 | 0.422 | 0.425 | 0.423 | 0.424 | 0.421 | 0.425 | 0.42 | 0.0015 | 0.36 |
| | MQC | 6.00 | 0.514 | 0.517 | 0.519 | 0.516 | 0.514 | 0.518 | 0.52 | 0.0025 | 0.49 |
| | HQC | 7.20 | 0.604 | 0.601 | 0.597 | 0.599 | 0.597 | 0.603 | 0.60 | 0.0035 | 0.58 |
| Analyst 3 | LQC | 4.80 | 0.422 | 0.425 | 0.421 | 0.422 | 0.427 | 0.424 | 0.42 | 0.0021 | 0.49 |
| | MQC | 6.00 | 0.514 | 0.512 | 0.516 | 0.516 | 0.519 | 0.517 | 0.51 | 0.0020 | 0.39 |
| | HQC | 7.20 | 0.602 | 0.601 | 0.598 | 0.603 | 0.599 | 0.601 | 0.60 | 0.0021 | 0.35 |

**Table 3:** Robustness results of various parameters

| **Variable** | **Value** | **Conc (ng/mL)** | **Peak area (Mean ± SD) (*N=6)** | **Mean of peak area of three values (*N=3)** | **% Recovery (Mean ± SD) (*N=6)** | **Mean of % recovery of three values (*N=3)** | **Retention time (Mean ± SD) (*N=6)** | **Mean of retention time values of three (*N=3)** |
|---|---|---|---|---|---|---|---|---|
| Flow rate (mL/min) | 0.8 | 6 | 0.53 ± 0.002 | 0.52 | 111.45 ± 0.611 | 109.78 | 7.76 ± 0.135 | 6.708 |
| | 1 | 6 | 0.524 ± 0.003 | SD = 0.02 | 109.75 ± 0.695 | SD =0.582 | 6.69 ± 0.09 | SD = 0.101 |
| | 1.2 | 6 | 0.517 ± 0.002 | % RSD = 0.436 | 107.84 ± 0.43 | % RSD = 0.53 | 5.66 ± 0.071 | % RSD = 1.508 |
| Mobile phase Ratio (A:B) v/v | 28:72 | 6 | 0.519 ±0.002 | 0.52 | 108.312 ± 0.526 | 110.13 | 7.90 ± 0.089 | 6.812 |
| | 30:70 | 6 | 0.525 ± 0.003 | SD = 0.003 | 109.96 ± 0.84 | SD = 0.662 | 6.77 ± 0.064 | SD = 0.084 |
| | 32:68 | 6 | 0.534± 0.002 | % RSD = 0.49 | 112.12 ± 0.616 | % RSD = 0.601 | 5.76 ± 0.100 | % RSD =1.23 |
| pH | 2.9 | 6 | 0.517 ± 0.018 | 0.519 | 107.76 ± 0.509 | 108.369 | 6.88 ± 0.030 | 6.75 |
| | 3.1 | 6 | 0.525 ± 0.003 | SD = 0.008 | 109.96 ± 0.84 | SD =2.01 | 6.77 ±0.039 | SD = 0.05 |
| | 3.3 | 6 | 0.515 ± 0.002 | % RSD = 1.524 | 107.37 ±0.50 | % RSD =1.87 | 6.60 ± 0.088 | % RSD = 0.77 |

### *3.4.4. System suitability*

In the system suitability, the results indicated that the values of all parameters were found in the pharmacopeial limits and method was suitable for method development [10]. System suitability provides us information about the chromatographic system prior to use. The continuous use of chromatographic system affects their performance and affect the reliability of analytical results. The lower values of LOD and LOQ revealed that the method was enough sensitive to detect FS under specified chromatographic conditions. The value of theoretical plate more than 2,000, theoretical plate/meter more than 20,000 and tailing factor less than 1.5 indicated about better column efficiency. The results obtained in the current study suggest excellent system suitability for the developed method. The obtained results of system suitability indicated that method is reliable and suitable for further studies. The results of system suitability are mentioned in Table 4.

**Table 4:** System suitability of FS in plasma

| Parameters | Value | Limits |
|---|---|---|
| Area | 0.77 | -- |
| Height | 0.39 | -- |
| Theoretical plate | 2952.21 | More than 2,000 |
| Theoretical plate/meter | 29520.21 | More than 20,000 |
| Tailing factor | 0.59 | Less than 1.5 |
| Peak purity index | 0.79 | More than 0.5 |
| LOD | 0.03 ng/mL | -- |
| LOQ | 0.08 ng/mL | -- |

## Conclusion

The study aimed to develop an economical, simple, sensitive, accurate, and precise bioanalytical method for quantitatively estimating FS in rat plasma. The percentage recovery within 98-100% indicated excellent recovery of FS from plasma samples. The % RSD of samples with various concentrations used for intraday and intermediate precision studies was found to be below 2%. System suitability studies demonstrated the method's reproducibility and robustness. Additionally, the method showed better drug recovery from plasma samples, lower linearity and range, and lower LOQ and LOD values compared to existing methods for estimating fisetin in biological samples. This developed method can be further utilized for pharmacokinetic and biodistribution studies of FS in its bulk form or in various nutraceutical or pharmaceutical formulations. Moreover, it is also suitable for routine quality control analysis for estimating fisetin in pharmaceutical companies.

**Declaration of Competing Interest**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Authors declare no conflict of interest.

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