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SIMULTANEOUS ESTIMATION OF RUTIN USING HPLC METHOD FROM AYURVEDIC FORMULATION

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Abstract – A precise and reliable High-Performance Liquid Chromatography (HPLC) method was developed and validated for the simultaneous estimation of Rutin in Ayurvedic formulations, including syrup and tablet dosage forms. Rutin was used as a reference standard, and standard stock and working solutions were prepared using methanol. A calibration curve was constructed across a concentration range of 6–18 µg/mL, demonstrating excellent linearity with a correlation coefficient (r^2) of 0.9985. The mobile phase, optimized for sharp peak resolution, consisted of 70% methanol and acetate buffer (pH 4.5) in a 95:5 (v/v) ratio. Sample solutions were prepared by ultrasonic extraction followed by filtration. The method was validated in accordance with ICH Q2(R1) guidelines. Specificity was confirmed by the absence of interference at Rutin's retention time. Precision studies, including system precision, method precision, and intermediate precision, showed %RSD values below 2%, indicating high reproducibility. The LOD and LOQ were determined to be 0.321 µg/mL and 0.921 µg/mL, respectively. Accuracy was established through recovery studies at 80%, 100%, and 120% levels, with mean recoveries ranging from 99.35% to 100.73%. Robustness testing under varied flow rates, detection wavelengths, and column temperatures demonstrated the method's stability and reliability. This validated HPLC method is suitable for routine quality control of herbal and Ayurvedic formulations containing Rutin, ensuring both accuracy and precision in quantification.
Keywords – Simultaneous, Recovery, Validated, Calibration

Introduction – Simultaneous estimation is an analytical technique employed to determine the concentrations of two or more active pharmaceutical ingredients (APIs) present in a combined dosage form without prior separation. This method is particularly advantageous in the pharmaceutical industry, where combination drugs are increasingly developed to enhance therapeutic efficacy, reduce dosing frequency, and improve patient compliance. In such formulations, each component must be quantified precisely to ensure consistent therapeutic activity, stability, and safety[1-3]. Simultaneous estimation serves as a time-saving and cost-effective alternative to individual drug estimations, especially during routine quality control, formulation screening, and pharmacokinetic studies. In the context of the current work, Rutin a bioflavonoid with antioxidant and anti-inflammatory properties was estimated using a validated RP-HPLC method. In future research, the method can be extended for the simultaneous estimation of Rutin along with other bioactives like Quercetin, Gallic acid, or Kaempferol commonly found in polyherbal formulations. This makes the method not only

robust for single- marker analysis but also adaptable for multi-marker quantification, enhancing the quality control process for complex formulations[4].

Methodology

Preparation of Standard Stock Solution

Rutin was used as the reference standard. To prepare the standard stock solution, 10 mg of Rutin was accurately weighed and dissolved in 10 ml of methanol, resulting in a final concentration of 1000 µg/ml (1000 ppm).

Preparation of Working Standard Solution

From the standard stock solution, 1 mL was pipetted and further diluted to 10 mL with methanol in a volumetric flask to obtain a working standard solution with a concentration of 100 µg/mL (100 ppm)[5-6].

Preparation of Calibration Curve

A calibration curve was constructed using seven different concentrations, prepared by serial dilution of the working standard solution to achieve concentrations ranging from 6 to 18 µg/mL[7].

Preparation of Solvents

Solvent A was prepared using 70% methanol, with a water-to-methanol ratio of 30:70. Solvent B consisted of acetate buffer adjusted to pH 4.5, which was filtered and degassed before use[8-9].

Preparation of Mobile Phase

The mobile phase was prepared by mixing Solvent A and Solvent B in a ratio of 95:5 (v/v).

Method Development

Various compositions of the mobile phase were evaluated based on the solubility, stability, and suitability of the standard solution. Individual standards and sample solutions were analyzed using different mobile phase compositions to achieve optimal chromatographic conditions. The optimized method was selected as it provided sharp peaks with good resolution at an absorbance wavelength of 280 nm[10-12].

Preparation of Sample and Test Solutions

Accurately weighed quantities of 100 mg tablet powder and 5000 mg syrup were transferred into separate 50 mL volumetric flasks. Approximately 40 mL of methanol was added to each flask, and the mixtures were sonicated in an ultrasonic water bath for 30 minutes at room temperature. After sonication, the solutions were allowed to cool to room temperature, and the volume was adjusted to the mark with methanol. The resulting solutions were first filtered through Whatman filter paper No. 41, followed by filtration through a 0.45 µm syringe filter. These filtrates were used as the test solutions for analysis[13-16].

Assay Percentage of Standard Solution from Formulations

Both formulations Rutin syrup and Rutin tablet were analyzed to quantify the Rutin content using the HPLC method as described under the specified chromatographic conditions. Each analysis was performed in triplicate, and the results were expressed as percent assay[17-19].

Analytical Method Validation

The developed HPLC method was validated in accordance with the guidelines provided by the International Council on Harmonisation (ICH), Q2(R1) for the validation of analytical procedures: text and methodology. The method was evaluated based on key validation parameters as described below:

Specificity

According to ICH guidelines, specificity is the ability to assess unequivocally the analyte in the presence of components such as diluents, excipients, and other potential interferences. Specificity of the method was confirmed by comparing the retention time and chromatographic profiles of the standard (Rutin) with those of the diluent, placebo, and sample solutions (Rutin syrup and Rutin tablet). This ensured that the method could distinctly identify and quantify Rutin without interference from other components[20-22].

Linearity

As per ICH guidelines, linearity was evaluated using a minimum of seven different concentrations. A calibration curve was constructed by plotting the peak area against the concentration of the standard solutions. The linear relationship was confirmed by calculating the regression coefficient (R^2), indicating the method's ability to produce results that are directly proportional to the concentration of analyte within a given range[23].

Precision

Precision was assessed in terms of system precision, method precision, and intermediate precision:

- System Precision: The same sample was analyzed six times using the developed method. The assay results for each analyte were expressed as the percentage relative standard deviation (% RSD).
- Method Precision: Six replicates of the sample were analyzed using the established procedure. The assay values were reported in terms of % RSD to evaluate repeatability under the same operating conditions.
- Intermediate Precision: To evaluate reproducibility, the analysis was carried out by different analysts on different instruments equipped with UV detectors. Six separate samples of the extract were analyzed, and the results were expressed as % RSD.

Limit of Detection (LOD) and Limit of Quantification (LOQ) The LOD and LOQ were calculated based on the standard deviation of the response and the slope of the calibration curve, in accordance with ICH guidelines. The following formulas were used:

- $LOD = 3.3 \times (\sigma/s)$
- $LOQ = 10 \times (\sigma/s)$

Where σ represents the standard deviation of the response, and s denotes the slope of the calibration curve.

Accuracy

Accuracy is expressed as the percentage recovery of a known quantity of analyte added to the sample. It was evaluated by performing recovery studies at three concentration levels 80%, 100%, and 120% with a total of nine determinations. Known amounts of standard solutions of GA, VS, PTS, and PP were added to the sample matrix, and the percentage recovery was calculated by comparing the obtained results with the expected values.

Robustness

Robustness evaluates the reliability of an analytical method under small, deliberate variations in method parameters. It reflects the method's capacity to remain unaffected under typical usage conditions. To assess robustness, key parameters such as flow rate, detection wavelength, and column temperature were intentionally varied. The method's performance under these conditions confirmed its stability and reliability[24-25].

Result and discussion Specificity Assessment

The specificity of the developed HPLC method for Rutin was demonstrated by ensuring clear separation of the analyte from potential interfering components such as placebo, diluent, and other actives present in the formulation. A 20 µL volume of these components was injected individually, and the chromatograms were examined. No interference was observed at the retention time of 4.5 minutes, which corresponds to Rutin, confirming that the method is specific for Rutin.

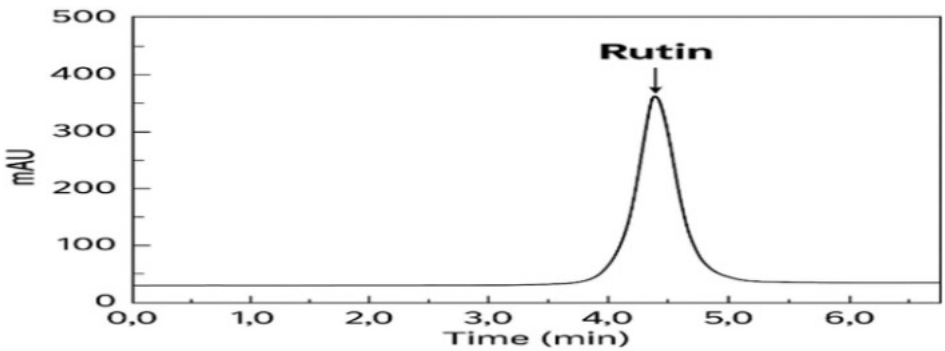

Fig.01 Chromatogram of Standard Rutin

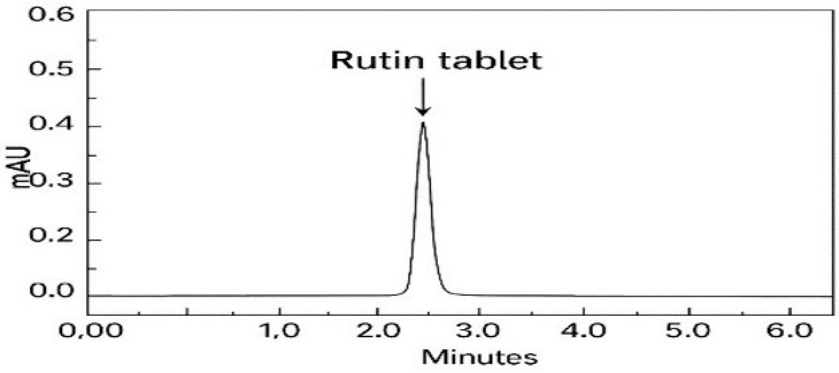

Fig.02 Chromatogram of marketed Rutin tablet

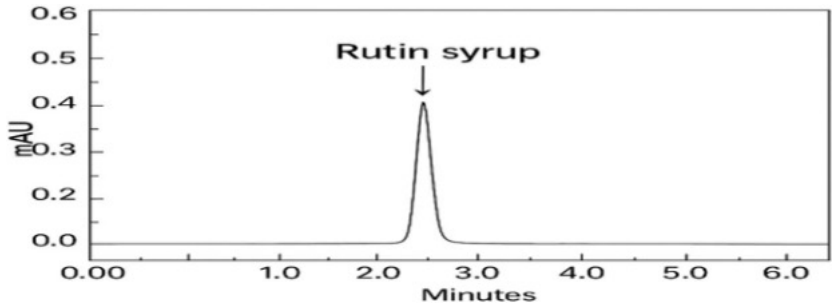

Fig.03 Chromatogram of marketed Rutin Syrup

Linearity Assessment

The linearity and regression parameters for the calibration curve of Rutin were determined using a series of standard solutions within the concentration range of 6–18 µg/mL. The calibration curve was plotted between the concentration of Rutin and the corresponding peak area obtained through HPLC analysis. A strong linear relationship was observed across the tested concentration range, as indicated by the correlation coefficient (r^2) of 0.9985, demonstrating excellent linearity. The linear regression equation obtained from the curve was in the form:

$$Y = 187454x + 54784$$

where Y represents the peak area and x denotes the concentration in µg/mL. The slope (187454) and y-intercept (54784) suggest a consistent and proportional increase in response with increasing concentrations of Rutin, confirming the method's sensitivity. The Limit of Detection (LOD) and Limit of Quantification (LOQ) were found to be 0.321 µg/mL and 0.921 µg/mL, respectively. These low values of LOD and LOQ indicate that the method is capable of detecting and accurately quantifying even very small amounts of Rutin, which is crucial for trace-level analysis in complex Ayurvedic formulations. Overall, the regression analysis confirmed that the developed HPLC method is reliable, sensitive, and reproducible for the simultaneous estimation of Rutin in herbal and Ayurvedic dosage forms. The robustness of the calibration curve lays a strong foundation for further analytical method validation and application in routine quality control.

Table no. 02 Linear regression data for calibrations curve

S.No	Parameter	Rutin
1	Linearity Range	6-18
2	Correlation coefficient	0.9985
3	y-intercept	54784
4	Slope	187454
5	LOD	0.321
6	LOQ	0.921

Precision Assessment

System suitability testing is an essential part of method validation to ensure consistent chromatographic performance. The system suitability parameters for Rutin were evaluated and are summarized in the table below:

- Retention Time (RT) of Rutin was found to be 5.73 minutes, indicating a relatively fast elution with good separation under the chosen chromatographic conditions.
- The mean peak area was recorded as 327200 mAU, reflecting the consistent response of the detector for Rutin at the given concentration.

- The % RSD (Relative Standard Deviation) for six replicate injections was 0.51%, which is well below the acceptable limit of 2%, indicating excellent repeatability and system precision.
- The USP Tailing Factor was found to be 1.12, suggesting symmetrical peak shape, which is critical for accurate quantification.
- The USP Plate Count was 6250, demonstrating a high theoretical plate number and thus good column efficiency.

These parameters confirm that the chromatographic system used for Rutin analysis meets the required criteria for suitability and ensures reliable analytical performance. Method precision, evaluated by repeated analysis of the same sample under the same operating conditions, yielded a % RSD of 0.1472% for Rutin. This low % RSD confirms that the developed HPLC method is highly precise and reproducible. Intermediate precision was determined by evaluating the method across different systems (System-1 and System-2) and analysts on different days. The results showed:

- % RSD for System-1: 0.147%
- % RSD for System-2: 0.150%
- Overall % RSD: 1.003%

These values confirm that the method performs consistently across different conditions, and the overall % RSD is well within the acceptable range (≤2%). This validates the ruggedness of the method, confirming that it is reliable for routine analysis in varying laboratory environments. The developed HPLC method for the estimation of Rutin satisfies all system

suitability criteria and demonstrates high precision and ruggedness. These results support the method's applicability for routine quality control analysis of Ayurvedic formulations containing Rutin.

Table no. 03 System suitability parameter

Sl. No.	Name of Standard	Retention Time (RT, min)	Mean Peak Area (mAU)	% RSD	USP Tailing Factor	Plate Count (USP)
01	Rutin	5.73	327200	0.51	1.12	6250

Table no. 04 Method precision parameter

S.No.	Standard	%RSD
01	Rutin	0.1472

Table no. 05 Intermediate precision parameter

S.No	Standard	% RSD for System-1	% RSD for System-2	Overall % RSD
1	Rutin	0.147	0.150	1.003

Repeatability Assessment

Repeatability is a measure of precision under the same operating conditions over a short interval of time. In this study, the repeatability of the standard solution of Rutin was evaluated by injecting six replicates of a 100 µg/mL solution and analyzing the peak area. The mean peak area was calculated as 2757616, with a standard deviation (SD) of 4552.62, and a % RSD of 0.17%. The low % RSD (<2%) indicates excellent repeatability of the method for the analysis of Rutin. This shows that the method is precise, consistent, and reproducible for standard solutions under identical conditions. The developed HPLC method exhibits high repeatability for Rutin with minimal variation between replicates. This reinforces the reliability and robustness of the method for routine quality control analysis in herbal and Ayurvedic formulations.

Table no. 06 Repeatability of standard solution

S. No.	Concentration (µg/mL)	Peak Area of Standard Solution (Rutin)
1	100	2759481
2		2761423
3		2749865
4		2756378
5		2760934
Mean		2757616
SD		4552.62
% RSD		0.17

Accuracy Assessment

Accuracy of the developed HPLC method was assessed through a recovery study by spiking known quantities of Rutin standard at three concentration levels: 80%, 100%, and 120% of the target concentration. The recovery was evaluated by calculating the percentage of Rutin recovered from the formulation, and the results are tabulated below.

80% Recovery Level:

- The peak areas recorded for the three replicate samples at 80% level were: 158734, 159128, and 160002.
- The corresponding % recoveries were 99.12%, 99.46%, and 99.47%.
- The average recovery at 80% level was found to be 99.35%.

100% Recovery Level:

- Peak areas: 261034, 262518, and 263486.
- % recoveries: 100.42%, 100.71%, and 101.06%.
- Average recovery at 100% level: 100.73%.

120% Recovery Level:

- Peak areas: 351745, 353621, and 354193.
- % recoveries: 99.98%, 100.72%, and 100.95%.
- Average recovery at 120% level: 100.55%.

The overall average recovery across all three levels was calculated as 100.21%, which lies well within the acceptable range of 98–102%, as per ICH guidelines.

These results confirm the high accuracy and reliability of the developed HPLC method for the quantification of Rutin in Ayurvedic formulations. The method is capable of accurately recovering the active compound even when spiked at different levels, which is critical for quality control and dosage uniformity.

Table no. 07 Accuracy Recovery Study

Standard Solutions	Recovery Level	Peak Area	% Recovery	Average % Recovery	Overall Recovery
Rutin	80% - 01	158734	99.12	99.35	100.21
	80% - 02	159128	99.46		
	80% - 03	160002	99.47		
	100% - 01	261034	100.42	100.73	
	100% - 02	262518	100.71		

100% - 03	263486	101.06		
120% - 01	351745	99.98	100.55	
120% - 02	353621	100.72		
120% - 03	354193	100.95		

Robustness Assessment

Robustness of an analytical method refers to its capacity to remain unaffected by small, deliberate variations in method parameters, providing an indication of its reliability during normal usage. In this study, the robustness of the developed HPLC method for Rutin was assessed by making slight changes to flow rate (±0.2 mL/min), detection wavelength (±2 nm), and column temperature (±5°C).

Effect of Flow Rate Variation

The method was tested at three flow rates: 0.3, 0.5, and 0.7 mL/min. The retention time decreased with increasing flow rate (from 6.12 min at 0.3 mL/min to 4.91 min at 0.7 mL/min), which is expected due to faster elution at higher flow rates.

- **The % RSD values were:**
  - 0.4% at 0.3 mL/min
  - 0.52% at 0.5 mL/min
  - 1.28% at 0.7 mL/min
- The average % RSD for flow rate variation was 0.73%, which is well within the acceptable limit (<2%).

This indicates that the method is sufficiently robust with respect to flow rate variations.

Effect of Detection Wavelength Variation

The robustness with respect to detection wavelength was examined at 358 nm, 360 nm, and 362 nm.

- The retention time remained fairly consistent across the range (5.48 to 5.67 min).
- **The % RSD values were:**
  - 0.65% at 358 nm
  - 0.52% at 360 nm
  - 0.82% at 362 nm
- The average % RSD was 0.66%, again showing excellent robustness.

This confirms that minor shifts in detection wavelength do not significantly affect the precision or response of the method.

Effect of Column Temperature Variation

Column temperature was varied at 20°C, 25°C, and 30°C to evaluate thermal robustness.

- The retention time decreased slightly with increasing temperature (from 5.62 min at 20°C to 5.32 min at 30°C).

- The % RSD values were:
  - o 1.29% at 20°C
  - o 0.52% at 25°C
  - o 0.91% at 30°C
- The average % RSD was 0.91%, which remains within acceptable limits.

This shows that the method tolerates small changes in column temperature without significant variation in analytical performance.

Table no. 09 Robustness Assessment

Standard Solutions	Parameter condition	RT	Mean Area	SD	% RSD	Average % RSD
Rutin	Flow Rate (± 0.2)					0.73
	0.3 mL/min	6.12	2456731	9845.65	0.4	
	0.5 mL/min	5.48	2398476	12356.42	0.52	
	0.7 mL/min	4.91	2415623	43120.34	1.28	
Rutin	Detection Wavelength (± 2.0 nm)					0.66
	358 nm	5.67	2513890	16234.56	0.65	
	360 nm	5.48	2398476	12356.42	0.52	
	362 nm	5.51	2467893	18456.78	0.82	
Rutin	Column Temperature (± 5.0°C)					0.91
	20°C	5.62	2334578	30218.45	1.29	
	25°C	5.48	2398476	12356.42	0.52	
	30°C	5.32	2367890	16789.67	0.91	

Conclusion

The specificity of an analytical method is its ability to accurately measure the analyte response in the presence of other potential components such as impurities, degradation products, or matrix excipients. In this study, the specificity of the developed High-Performance Liquid Chromatography (HPLC) method for Rutin was thoroughly evaluated to confirm its ability to selectively analyze the active compound without interference from other substances. To assess specificity, individual injections of diluent, placebo, and marketed formulations (tablet and syrup) were carried out. A 20 µL volume of each component was introduced under the established chromatographic conditions, and the resulting chromatograms were carefully examined. The retention time of standard Rutin was found to be approximately 4.5 minutes. Importantly, none of the interfering components exhibited any peak at or near the retention time of Rutin. This clear resolution of the Rutin peak in the presence of formulation excipients and other matrix components confirms the high specificity of the developed method. The chromatograms of the standard Rutin, marketed Rutin tablet, and Rutin syrup samples displayed well-resolved peaks with no overlapping or co-eluting substances. This signifies that the method can effectively distinguish Rutin from other components present in complex herbal and Ayurvedic formulations. In conclusion, the HPLC method demonstrated excellent specificity for the estimation of Rutin. The absence of interfering peaks at the retention time of Rutin ensures accurate and reliable quantification in the presence of formulation excipients, thus validating the method's applicability for routine analysis and quality control of Rutin-containing products.

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