RESEARCH ARTICLE

Quality by Design Strategy for Development and Validation of an RP- HPLC Method for Marker-Based Standardization of Fisetin in Marketed Herbal Preparations

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Abstract

Introduction: This study underscores the importance of QbD in developing pharmaceutical processes, with a strong emphasis on risk assessment. It demonstrates how QbD principles were applied to create a reliable and cost-effective HPLC method for fisetin capsules, ensuring consistent quality within defined criteria.

Method: The method, developed through a rigorous Design of Experiment approach, employs an isocratic elution method using a Chemsil ODS-C18 column with a mobile phase composition of 70:30 methanol to 0.1% OPA. Detection of peaks was achieved with a UV detector set at 362 nm, and an optimal flow rate of 1.0 mL/min was determined. The column oven was maintained at an ambient temperature, and the injection volume was set at 10 µL.

Result: Thorough validation following ICH Q2 (R1) guidelines verifies the method's reliability. Key parameters evaluated during this process include linearity, which was established over a range of 10 to 50 µg/mL with an r^2 value of 0.997; LoD and LoQ, determined to be 2.903 and 8.797 µg/mL, respectively; and precision and robustness, with %RSD values found to be below 2%. Furthermore, accuracy and assay tests were performed, and the results were within the acceptable range as specified by ICH guidelines.

Discussion and Conclusion: The method is particularly suitable for analyzing fisetin capsules in the pharmaceutical industry due to its remarkable sensitivity, selectivity, precision and accuracy. Designed for routine pharmaceutical quality control, this method effectively distinguishes between marketed drugs and the innovator product.

Keywords: Fisetin, AQbD, RP-HPLC, ICH, QbD.

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Introduction

Historically, developing analytical methods was a laborious process involving the painstaking adjustment of one system characteristic at a time. This approach required multiple experimental runs and subsequent refinements. The pharmaceutical industry has highlighted flaws in this traditional method, particularly in dealing with challenges like out-of-specification (OOS) and out-of-trend (OOT) results. Regulatory bodies have issued quality control (QC) warning letters, emphasizing the urgent need for a more innovative approach. Consequently, the pharmaceutical sector has embraced QbD, as mandated by regulatory authorities such as the US FDA, EMA, and other ICH member countries.[1,2]

This journal article focuses on developing and validating analytical methods for fisetin by utilizing QbD principles, which emphasize precision and reliability. QbD facilitates the understanding of essential method attributes, the identification of potential hazards, and the development of a thorough control strategy by using a methodical and risk-based approach.[3,4] By incorporating these concepts into the lifetime of an analytical method, researchers can maximize efficiency, minimize unpredictability, and guarantee adherence to regulatory standards.

Flavonoids, a group of plant-based polyphenolic chemicals, include compounds like Quercetin, Fisetin, Apigenin, and Resveratrol, known for their diverse health benefits such as anti-inflammatory, anti-allergic, anticancer, and antioxidant properties. Because of its capacity to stop the growth of cancer cells, trigger apoptosis, and obstruct DNA repair mechanisms, fisetin also known by its chemical name, 3,3′,4′,7-tetrahydroxyflavone shows promise as a cancer treatment agent. Fisetin is found in large quantities in fruits and vegetables, including cucumbers, onions, apples, grapes, and strawberries. Its natural occurrence in everyday foods highlights its potential as a safe cancer-fighting agent. Structurally, fisetin consists of two aromatic rings connected by a 3-carbon oxygenated heterocyclic ring, with four hydroxyl group substitutions and one oxo group. Its potent anticancer activity underscores the importance of including flavonoid-rich foods in the diet for overall health.[5-9] Figure 1 is the chemical structure of fisetin.

Developing an HPLC method for fisetin presents challenges due to its limited solubility in water, necessitating

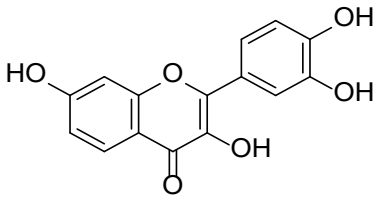

Figure 1: Chemical structure for fisetin

the use of organic modifiers in the mobile phase. Separating fisetin from other flavonoids or plant components requires selecting appropriate stationary and mobile phase conditions for specificity. Additionally, optimizing sensitivity, potentially by adjusting injection volume or detector selection, is crucial for achieving desired detection limits, especially with UV detection.

This study investigates the application of a QbD approach to enhance the chromatographic separation of fisetin, with a focus on regulatory compliance and adaptability to industry changes. Utilizing AQbD principles, a chromatographic method for fisetin was developed and optimized through the design of experiment (DoE). Key variables considered included buffer pH, methanol concentration, flow rate, and oven temperature, with metrics such as retention time and theoretical plate evaluated. Optimization was guided by statistical analyses using counterplots, Pareto charts, and interaction plots.[10-12]

The method, validated according to ICH Q2 (R1) guidelines, underwent rigorous testing for key parameters such as robustness, accuracy, precision, LoQ, and LoD. These evaluations confirm the method's reliability and consistency. Its exceptional sensitivity allows for detecting even trace amounts of substances, and its selectivity ensures precise differentiation between components. Additionally, the method's speed and cost-effectiveness make it highly beneficial for routine pharmaceutical analysis, particularly for fisetin capsules. This thorough validation establishes the method's suitability for high-quality pharmaceutical analysis.

Materials and Methods

Chemicals and Reagents

Analytical and HPLC-grade chemicals and reagents were utilized for method development, validation, and market product assessments. Methanol and orthophosphoric acid were purchased from Merck India. Carbinio of India provided the fisetin reference standards. Centenarians Life Sciences produces innovative fisetin strawberry extract capsules.

Equipment

The analysis was conducted using an HPLC system comprising a WATERS-2695 with a Detector-2998 PDA Detector. A Chemsil ODS-C18 column (250 × 4.6 mm, 5.0 µm particle size) was employed at ambient temperature.

The HPLC system featuring the WATERS-2695 pump and WATERS-2998 PDA detector is a sophisticated analytical setup designed for precise separation, identification, and quantification of compounds. The WATERS-2695 offers both isocratic and gradient elution with accurate flow control and flexibility for various analytical needs. The WATERS-2998 PDA detector provides comprehensive UV-visible spectral data, enhancing compound identification and sensitivity. The system uses a Chemsil ODS-C18 column (250 × 4.6 mm, 5.0 µm) for high-resolution separations of organic compounds based on hydrophobic interactions. This combination of components supports detailed analysis across diverse applications.

Chromatographic conditions

The HPLC method for fisetin utilized a Chemsil ODS-C18 column (250 × 4.6 mm, 5.0 µm particle size) with a mobile phase of Methanol: 0.1% OPA in water (70:30, v/v) at a flow rate of 1-mL/min and ambient column temperature. Detection was performed at 362 nm using a PDA detector. The method optimization involved varying mobile phase composition and flow rate using a CCD approach to achieve satisfactory separation and peak symmetry.

Development of HPLC Method Utilizing the AQbD Framework

The eight sequential steps in developing an HPLC method for fisetin using the AQbD approach were as follows.[13,14]

The HPLC method was developed using the AQbD approach through an eight-step systematic process. Initially, a Fish Bone Diagram was employed to qualitatively define the target profile for the analytical procedure, considering variables such as injection volume, buffer pH, mobile phase, and column type. In the second step, a comprehensive literature search was conducted to gather information on the analyte's molecular structure, weight, pKa, functional groups, presence of chromophores, solubility, and available methods.

In the third step, method parameters were identified during method scouting, taking into account the analyte's physicochemical properties, compatibility with mobile phases, and suitability for stationary phases. The fourth step involved identifying key predictive method parameters both statistically and qualitatively to differentiate between predictable and unpredictable factors. In the fifth step, important procedure parameters were determined over a range using DoE for multivariable interaction analysis.

The sixth step entailed screening and optimization to empirically analyze the interactions between input factors and output responses, ultimately defining an operable design space. The seventh step involved finalizing the feasible strategy, which included stability-indicating experiments and method validation focusing on accuracy, precision, robustness, and solution stability.[15,16]

In the final step of monitoring and lifecycle management, market products and the reference listed drug were evaluated, identifying necessary adjustments for improvements. This comprehensive, organized process ensured that the development of HPLC methods adhered to QbD principles.

Assessing Experimental Results and Determining Optimal Method Parameters

The primary objective was to investigate the impacts and interactions between two different elements on numerous responses, namely retention time and theoretical plate: mobile phase ratio and flow rate. In order to achieve this, the experimental design presented in Table 1 was developed to cover the range of each variable. Much research would generally be required to determine the actual influence of two factors on the two target answers. Design Expert version 13 was used to create a DoE in order to hasten the identification of interaction patterns in a methodical and scientific way. Using a thorough full factorial experimental design, the DoE included three (03) center points in each block.[17] Eleven distinct experimental combinations were identified as a result of this DoE, as shown in Table 2. These combinations were then used to generate different mobile phases using different combinations of organic solvent and buffer. The goal was to carry out a thorough analysis of these characteristics in order to optimize the HPLC method for Fisetin analysis. For this, an ODS-C18 column with a 10 µL injection volume and a 250 × 4.6 mm, 5.0 µm particle size, was used. The HPLC system featured an auto-injector, column oven, PDA detector and operated at 362 nm.[18]

Evaluation of Experimental Outcomes and Identification of Optimal Method Conditions

Various statistical tools, such as Pareto charts, interaction plots, and contour plots, were used to analyze how different factors influenced outcomes during Fisetin analysis (Table 3, Figure 2 a-e). Interaction plots highlighted the relationship between mobile phase and flow rate on theoretical plates and retention time. Methanol concentration and flow rate directly impacted retention time.

DoE results, shown in counterplots, displayed how theoretical plates and retention time varied across different variables. Theoretical plates increased with higher methanol concentrations and flow rates and decreased with lower concentrations and rates. Similarly, shorter retention times were observed at lower flow rates and methanol concentrations. Using Design Expert Software, response

Table 1: Chosen factors and their levels for DoE investigations

Factors				*Response*
% Methanol	Min 60%	Flow Rate	Min 0.8ml/min	Retention Time
	Max 80%		Max 1.2ml/min	Theoretical Plate Count

Table 2: DoE for preliminary assessment in Fisetin pre-method development

Run	*Method Parameter*	
	A: Mobile phase (%)	*B: Flow rate (ml/min)*
1	70	1
2	80	1.2
3	60	1
4	80	0.8
5	70	0.8
6	60	1.2
7	70	1
8	70	1.2
9	80	1
10	70	1
11	60	0.8

Table 3: Responses to DoE central composite design by design expert for fisetin

Run	*Factor 1 A: Mobile phase (%)*	*Factor 2 B: Flow rate (ml/min)*	*Response 1 RT (min)*	*Response 2 TP*
1	70	1	3.837	5346
2	80	1.2	2.582	5406
3	60	1	5.853	5813
4	80	0.8	3.863	6860
5	70	0.8	4.822	5658
6	60	1.2	5.042	5541
7	70	1	3.837	5346
8	70	1.2	3.277	5242
9	80	1	3.095	5660
10	70	1	3.837	5346
11	60	0.8	7.254	6244

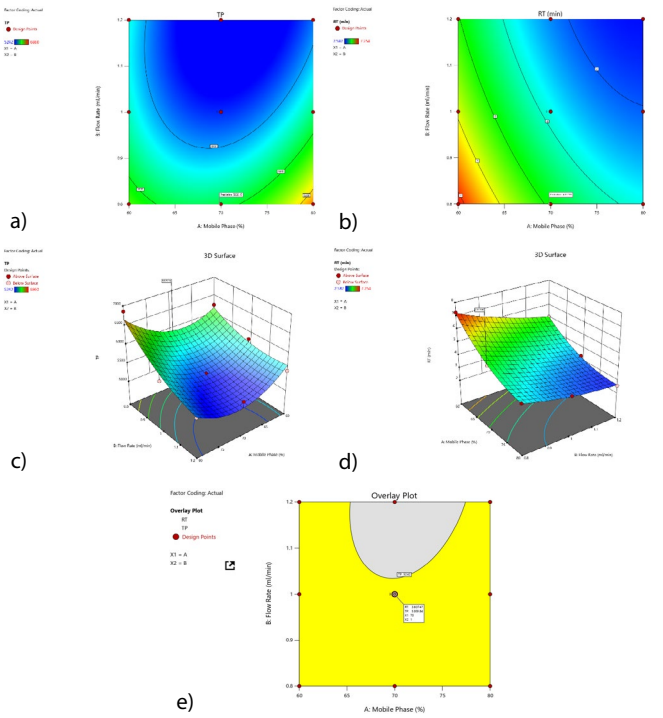

Figure 2: a: Contour plot for RT b: Contour plot for TP c: 3D Response surface plot for RT d: 3D Response surface plot for TP e: Overlay plot

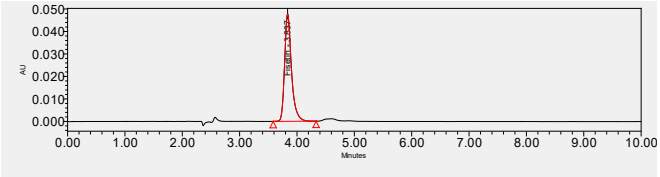

Figure 3: Representative chromatogram of fisetin

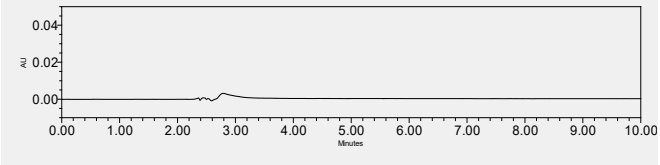

Figure 4: Chromatogram for specificity of fisetin RP-HPLC

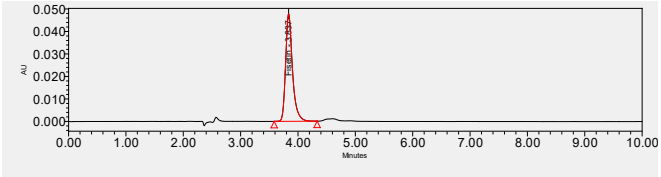

Figure 5: Chromatogram for selectivity of fisetin RP-HPLC

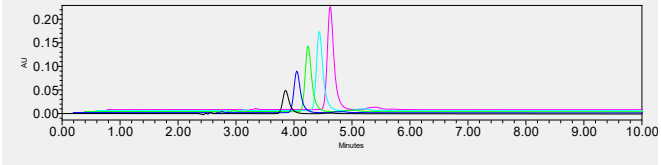

Figure 6: Overlay chromatogram for linearity of 10–50 µg/mL for fisetin RP-HPLC

optimization determined standardized values to achieve the highest composite desirability for the final Chromatographic Method for Fisetin.

The optimized method employed gradient HPLC equipment from multiple manufacturers, including Waters, USA, featuring an auto-injector, column oven, and PDA detectors. It used a 150 × 4.6 mm C18 column with 5 µm particle size. The mobile phase was 70:30 methanol to 0.1% OPA buffer at pH 8.2, and detection occurred at 362 nm using a UV detector with a flow rate of 1.0 mL/min. The injection volume was 10 µL.

Results And Discussion

Method Validation

System suitability

To assess different parameters, a thorough system compatibility test was carried out with a typical chromatogram (Figure 3). The percentage RSD for each of the six replicates was determined to be 0.78%. Theoretical plates 9168, telling factor 1.36, retention time 3.893, and peak area were found to be 81556.

Specificity and Selectivity

At 362 nm, a chromatogram was obtained using a blank working standard solution containing the drug and pure methanol. The chromatograms are displayed in Figure 4 and 5 for selectivity and specificity. The chromatograms of the samples and the blank, respectively, display the compound's selectivity and specificity. The sample chromatogram, which peaks at 3.87 minutes, is not coincident with the blank chromatogram's peak. Figure 4 and 5 show specificity and selectivity.

Linearity

The calibration curve for fisetin showed excellent linearity across concentrations ranging from 10 to 50 µg/ml. Table 4 and Figure 6 summarizes the analysis of five standard solutions at concentrations of 10, 20, 30, 40, and 50 µg/ml. The

Table 4: Data for Linearity of Fisetin RP-HPLC

Sr. No.	*Concentration (µg/ml)*	*Peak Area*
1.	10	393370
2.	20	672255
3.	30	994019
4.	40	1307226
5.	50	1677468
r^2		0.9977
Regression equation		y = 32032x + 47918

Table 5: Summary results of accuracy

Level	*50%*	*100%*	*150%*	*Mean*	*%RSD*
Fisetin	99.25%	100.36%	98.19%	98.45	0.611%

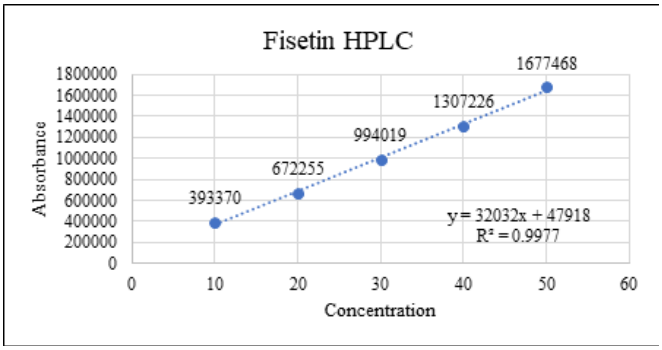

Figure 7: Calibration curve for fisetin RP-HPLC

relationship between peak area and concentration (Figure 7) was used to calculate a correlation coefficient of 0.9977.

Precision and repeatability:

In the evaluation of fisetin at concentrations of 10, 30, and 50 µg/ml over three repetitions, the percent RSD for repeatability was found to be less than 2% for both intraday and interday precision.

Table 6: Data for Quantification of Fisetin

Name of Molecule	*% Assay value (Fisetin Strawberry extract 500mg)*
Fisetin	11.63%

Accuracy

A recovery study employing sample solutions spiked at 50%, 100%, and 150% of the nominal concentration of 0.1 mg/mL was carried out in order to assess the accuracy percentage. Details on the recovery percentage figures obtained with the recommended HPLC method are given in Table 5. The correctness of the developed approach is confirmed by the mean recovery of 99.96% and the percentage recovery lying between 98 and 102%, both of which meet the requirements of ICH Q2 (R1).

Robustness and Ruggedness Studies

At a sample concentration of 30 µg/mL, changes in mobile phase ratio, detector wavelength, and flow rate were studied to understand their impact on retention time and peak area of the main analyte. The procedures were deemed robust, with a RSD of less than 2%, indicating consistent responses even with variations in these method parameters.

Acceptable levels were observed for the detection and quantitative limitations, which were 2.903 µg/ml and 8.797 µg/ml, respectively. In accordance with ICH rules, the detection limit was lower than the quantitative limit.

Quantification

The recognized techniques were successfully applied to evaluate the identification and assay values of a well-known brand that was on the market. The exceptionally clear chromatographic separations demonstrated that the excipients had not interfered. Table 6 contains quantitative data.

Discussion

Using a central composite design, the Quality by Design approach was used to complete the RP-HPLC. Fisetin was analyzed by measuring absorbance at 200–800 nm wavelengths, with the greatest absorption detected at 362 nm. The range of Beer-Lambert's concentration for linearity was determined to be 10-50 µg/ml. After computation, the correlation coefficient was discovered to be 0.997. The system's appropriateness was verified by a %RSD for absorbance of less than 2%. After calculations, the LOD and LOQ were determined to be 2.903 and 8.797 g/ml, respectively. A robustness test that included parameter modifications and %RSD within allowable bounds was conducted. High accuracy was demonstrated by the intra- and inter-day precision, which was confirmed by the percentage recovery at 50%, 100%, and 150% concentrations. When Fisetin strawberry extract capsules were quantified, 11.63% was discovered.

Conclusion

In developing the HPLC method for fisetin, the application of AQbD principles significantly advanced the process compared to previous studies. The method utilized a detailed multivariate analysis of key parameters, such as mobile phase composition and flow rate, through Design of Experiments (DoE) software. This approach allowed for optimized chromatographic conditions and a deeper understanding of how these variables affect separation. Unlike earlier methods, which often relied on less systematic approaches, this study consistently met acceptable standards and demonstrated improved robustness, ruggedness, linearity, accuracy, precision, and specificity.

The use of QbD principles and automated software, specifically Design Expert 13, provided a more comprehensive grasp of method variables and minimized errors during validation and transfer. This contrasted with manual development processes that were more prone to variability and less efficient. Statistical analysis of the developed method highlighted its superior robustness, accuracy, selectivity, and reproducibility, particularly in distinguishing between marketed products, including those similar to the innovator product. This advancement makes the method ideally suited for routine quality control in the pharmaceutical industry, offering a more reliable and efficient solution compared to previous HPLC methods for fisetin analysis.

Source of Funding

None.

Conflict of Interest

None.

Reference

1. Peris-Vicente J, Esteve-Romero J, Carda-Broch S. Validation of analytical methods based on chromatographic techniques: An overview. Analytical separation science. 2015 Dec 7:1757-808.
2. Guideline IH. Pharmaceutical development. Q8 (2R). As revised in August. 2009 Aug 23;23.
3. Raman NV, Mallu UR, Bapatu HR. Analytical quality by design approach to test method development and validation in drug substance manufacturing. Journal of chemistry. 2015;2015(1):435129.
4. Ojha A, Bhargava S. International council for harmonisation (ICH) guidelines. InRegulatory affairs in the pharmaceutical industry 2022 Jan 1 (pp. 47-74). Academic Press.
5. Ullah A, Munir S, Badshah SL, Khan N, Ghani L, Poulson BG, Emwas AH, Jaremko M. Important flavonoids and their role as a therapeutic agent. Molecules. 2020 Nov 11;25(22):5243.
6. Imran M, Saeed F, Gilani SA, Shariati MA, Imran A, Afzaal M, Atif M, Tufail T, Anjum FM. Fisetin: An anticancer perspective. Food science & nutrition. 2021 Jan;9(1):3-16.

7. Khan N, Syed DN, Ahmad N, Mukhtar H. Fisetin: a dietary antioxidant for health promotion. Antioxidants & redox signaling. 2013 Jul 10;19(2):151-62.
8. Antika LD, Dewi RM. Pharmacological aspects of fisetin. Asian Pacific Journal of Tropical Biomedicine. 2021 Jan 1;11(1):1-9.
9. Imran M, Saeed F, Gilani SA, Shariati MA, Imran A, Afzaal M, Atif M, Tufail T, Anjum FM. Fisetin: An anticancer perspective. Food Sci. Nutr. 2021 Jan;9(1):3-16.
10. ul Alam AM, Mamun IR, Nahar N, Shoeb M. A Quality by Design (QBD) approach for the development and validation of RP-HPLC method for the quantification of linagliptin tablets.
11. Alam AMU, Nahar N, Husain M, Shilpi S. Constituents of The Essential Oil from Aquilaria Agallocha Roxb By Gc-Ms and Its Cytotoxic Activities. Eur J Pharma Med Res. 2020;7(12):105–9.
12. ul Alam AM, Naha N, Mamun MIR, Shoeb M. A QBD based RP-HPLC Method Development and Validation for Quantification of Pregabalin Capsules . Int J Sci Res. 2023;12(11):948–55.
13. United States Pharmacopoeia, USP <1225>, Validation of Compendial Methods. Available from: http://www.uspbpep.com/usp29/v29240/usp29nf24s0_c1225.html.
14. US FDA Guidance for Industry, Dissolution Testing of Immediate Release Solid Oral Dosage Forms; 1997. Available from: https://www. fda.gov/regulatory-information/search-fda-guidance-documents/dissolution-testing-immediate-release-solid-oral-dosage-forms.
15. ul Alam AM, Nahar N, Husain M, Shilpi S, Mosihuzzaman M. Constituents of the essential oil of Aquilaria agalocha Roxb, poster presented & abstracts published in Workshop on Medicinal plants, spices and Other Natural Products (ASOMPS X). *Eur J Pharm Med Res*. 2000;7(12):105–9.
16. Kazemipoor M, Lorestani MA, Ansari M. Extraction and determination of biomarkers in Ficus carica L. leaves from various species and different cultivars by HPLC. J. Liq. Chromatogr. Relat. technol. 2012 Jan 1;35(20):2831-44.
17. Lakshmi B, Reddy TV. A novel RP - HPLC method for the quantification of linagliptin in formulations. J Atoms Mol 2012;2(2):155–64.
18. Ramzia I, El-Bagary EF, Elkady BM. Spectrophotometric Methods for the Determination of Linagliptin in Binary Mixture with Metformin Hydrochloride and Simultaneous Determination of Linagliptin and Metformin Hydrochloride using High Performance Liquid Chromatography. Int J Biomed Sci. 2013;9(1):41–7.

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