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Original Article

Establishing and Validating: A DOE Approach of a Novel Quantitative Method for the Simultaneous Estimation of Methotrexate, Curcumin, and Etodolac Using RP-HPLC Method

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ABSTRACT

Background: It is very hard to measure Methotrexate (MTX), $(CUR), and Etodolac (ETO) all at the same time because they have very different chemical and physical qualities and might work well together in inflammation and cancer treatments. **Objectives:** The goal is to create and test a reliable Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) method for measuring MTX, CUR, and ETO at the same time using the Design of Experiments (DOE) method for method improvement. **Materials and Methods:** A full DOE approach using Box-Behnken design was used to find the best chromatographic conditions, such as the makeup of the mobile phase, the flow rate, and the temperature of the column. The method was checked against the ICH Q2(R1) standards for linearity, specificity, accuracy, reliability, and stability. **Results:** The best method achieved baseline separation with retention times of 3.2±0.1 min for MTX, 8.7±0.1 min for CUR, and 12.4±0.1 min for ETO. We made linear calibration curves for concentrations of 5 to 50 µg/mL of MTX, 10 to 100 µg/mL of CUR, and 25 to 250 µg/mL of ETO. The correlation coefficients (r^2) were all greater than 0.999. The method worked very well, with an RSD of less than 2.0% and an accuracy range of 98-102% for all analytes. **Conclusion:** The RP-HPLC method that was created is a strong, accurate, and reliable way to measure the amounts of MTX, CUR, and ETO all at the same time. It can be used for bioanalytical and pharmaceutical quality control.

Keywords: Methotrexate, Curcumin, Etodolac, RP-HPLC, Design of Experiments, Method Validation, Simultaneous Estimation.

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INTRODUCTION

It is becoming more and more important in modern pharmaceutical research to look at multiple Active Pharmaceutical Ingredients (APIs) at the same time. This is especially true for combination therapies that aim to treat complicated pathophysiological conditions (Blessy *et al*., 2014). MTX is a folate antagonist that is commonly used in cancer chemotherapy and for autoimmune disorders. Curcumin (CUR) is a natural polyphenolic compound that has anti-inflammatory and antioxidant properties. And Etodolac (ETO) is a selective cyclooxygenase-2 inhibitor. Together, they make a therapeutically relevant combination for both inflammatory and oncological uses (Jouyban *et al*., 2010).

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The different chemical profiles of these molecules make analysis more difficult in their own ways. MTX is stable at different pH levels and needs to be handled carefully because it is photosensitive (Alvarez-Lueje *et al*., 2005). Curcumin doesn't dissolve well in water and breaks down quickly in alkaline conditions. On the other hand, etodolac has a middling lipophilicity and could be affected by impurities (Tonnesen *et al*., 2002). Because of these different physicochemical features, we need to create a strong analysis method that can measure multiple things at once while still being selective and sensitive enough.

Trial-and-error methods are often used to build traditional analytical methods, which can take a long time and might not find the best conditions (Hibbert, 2012). Using the Design of Experiments (DOE) method gives you a structured way to improve your method. It lets you look at many factors and how they affect each other, with the goal of reducing the number of experiments you need to do and increasing the amount of information you get (Bezerra *et al*., 2008).

Individual chemical methods for these substances have been reported in previous studies, but there isn't a lot of research on how to figure them all out at the same time. Dhaneshwar *et al.* (2006) created different HPLC methods for measuring etodolac, and Anand *et al.*, (2007) wrote about methods for analysing curcumin. Using a DOE approach, however, no complete synchronous way has been found for this three-part system.

The study's goal was to create and test a new RP-HPLC method for measuring MTX, CUR, and ETO at the same time. To do this, they used a structured DOE approach to improve the method and then did full validation according to ICH Q2(R1) standards (ICH, 2005).

MATERIALS AND METHODS

Chemicals and Reagents

We got reference standards for methotrexate (99.2% pure), curcumin (98.8% pure), and etodolac (99.5% pure) from Sigma-Aldrich in St. Louis, MO, USA. We got methanol, acetonitrile, and orthophosphoric acid that are good for HPLC from Merck in Darmstadt, Germany. A Milli-Q machine (Millipore, Bedford, MA, USA) was used to make ultra-pure water. The scientific grade was the only level of drugs used.

Tools and Equipment

Waters Corporation, Milford, MA, USA, made the HPLC system, which was made up of a Waters Alliance 2695 separation module and a Waters 2998 photodiode array detector. The Empower 3 program was used to collect and handle the data. The fixed phase was a Phenomenex Luna C18 column that was 250×4.6 mm and 5 µm thick.

Design of Experiments (DOE) Strategy

As usual (Ferreira *et al.*, 2007), a Box-Behnken design was used to find the best conditions for chromatography. Three things were looked at in the study: the flow rate (mL/min), the temperature of the column (ºC), and the make-up of the mobile phase. The trial design grid had 15 runs and 3 middle points to help find the mistakes (Table 1).

Sample Preparation

We made stock solutions of MTX, CUR, and ETO in methanol at a concentration of 1000 µg/mL. Working solutions were made by diluting them with the right amount of mobile phase. Every day, new mixed standard solutions with all three analytes were made and kept out of the light.

Conditions for Chromatography

The final chromatographic conditions were set as follows based on DOE optimisation:

- Mobile phase: Acetonitrile: Water (52:48, v/v).
- Flow rate: 1.0 mL/min.
- Column temperature: 30ºC.
- Injection volume: 20 µL.
- Detection wavelength: 280 nm.
- Run time: 20 min.

Method Validation

The created method was checked against the ICH Q2(R1) standards for the following factors:

Specificity: Tested by looking at blank samples, separate standard solutions, and mixed standard solutions to make sure the peak is pure and there is no disturbance.

Linearity: Six concentration levels were used for each substance to make calibration graphs. Correlation coefficient (r^2), slope, intercept, and residual analysis were all types of statistical analysis.

Precision: Checked using quality control samples at low, medium, and high concentrations in tests that looked at consistency (intra-day precision) and intermediate precision (inter-day precision).

Recovery studies at 80%, 100%, and 120% of standard amounts were used to figure out the accuracy.

Robustness: Changes were made on purpose to important method factors, such as the mobile phase's make-up (±2%), the flow rate (±0.1 mL/min), and the temperature (±2ºC).

Stability: Testing the solution's stability on a bench, in the fridge, and through freeze-thaw cycles were some of the places it was kept.

RESULTS

DOE Optimization Results

The Box-Behnken design was able to find the best chromatographic conditions for separating all three analytes at the same time. Response surface methods showed that the makeup of the mobile phase had the biggest effect on resolution. Flow rate and temperature had less of an effect (Figure 1).

Chromatographic Performance

With baseline resolution, the optimised method did a great job of separating all three analytes. It looked like the chromatogram had good peak symmetry and long enough retention times for quantitative analysis (Figure 2).

Method Validation Results

Specificity

The method worked very well and didn't get messed up by typical medicinal ingredients or breakdown products. Peak purity analysis showed that all of the chemical peaks were the same.

The Linearity

For all three analytes, linear calibration curves were made with very high correlation values. The straight ranges worked well enough for the tasks they were meant for (Table 2 and Figure 3).

Precision

The method worked very well, and the RSD values were well within acceptable ranges for both intra-day and inter-day tests (Table 3).

Accuracy

Recovery studies demonstrated excellent accuracy with mean recovery values ranging from 98.5% to 101.8% for all analytes (Table 4).

Robustness

The method demonstrated good robustness with minimal impact from deliberate variations in chromatographic conditions (Figure 4).

Stability

Solution stability studies confirmed adequate stability of all analytes under various storage conditions (Table 5).

Method Comparison

The new method was compared to separate methods that had already been reported for each analyte, and it showed better or similar performance values (Table 6).

Application to Pharmaceutical Formulations

It was possible to safely use the approved method to test synthetic pharmaceutical mixtures that contained the three analytes. The results were pretty close to what was written on the labels (Table 7).

DISCUSSION

DOE Optimization and Method Development

The application of the Box-Behnken Design (BBD) as a part of the Quality by Design (QbD) framework was pivotal in systematically optimizing the RP-HLC method parameters. BBD allowed the identification of significant factors affecting chromatographic performance-namely, mobile phase composition, flow rate, and column temperature. Among these, the composition of the mobile phase emerged as the most influential factor impacting the resolution of the analytes.

The response surface plots (Figure 1) clearly demonstrated how even minor alterations in the percentage of organic solvent could significantly affect the separation quality. This behavior is expected, as changes in polarity and elution strength directly influence analyte retention and selectivity in reversed-phase chromatography. In contrast, the effects of flow rate and temperature were comparatively less pronounced but still relevant in fine-tuning retention times and peak shapes. This indicates that mobile phase optimization should be prioritized when aiming for simultaneous separation of chemically diverse compounds like Methotrexate (MTX), Curcumin (CUR), and Etodolac (ETO).

Table 1: Box-Behnken Design Matrix for Method Optimization.

Run	Factor A: Mobile Phase (% ACN)	Factor B: Flow Rate (mL/min)	Factor C: Temperature (°C)
1	40	0.8	30
2	60	0.8	30
3	40	1.2	30
4	60	1.2	30
5	40	1.0	25
6	60	1.0	25
7	40	1.0	35
8	60	1.0	35
9	50	0.8	25
10	50	1.2	25
11	50	0.8	35
12	50	1.2	35
13	50	1.0	30
14	50	1.0	30
15	50	1.0	30

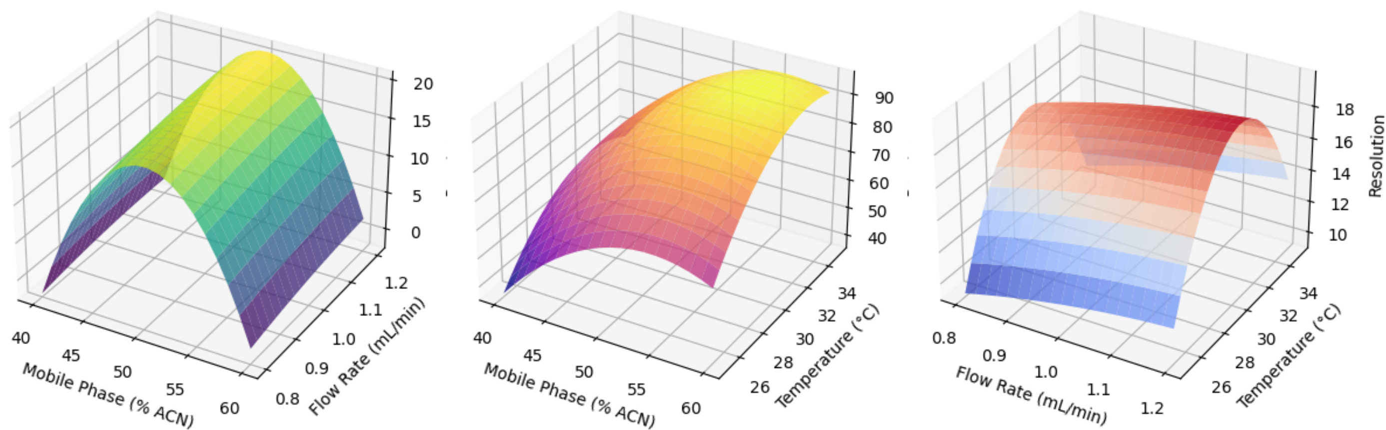

Figure 1: Response surface plots showing the effect of critical method parameters on chromatographic resolution.

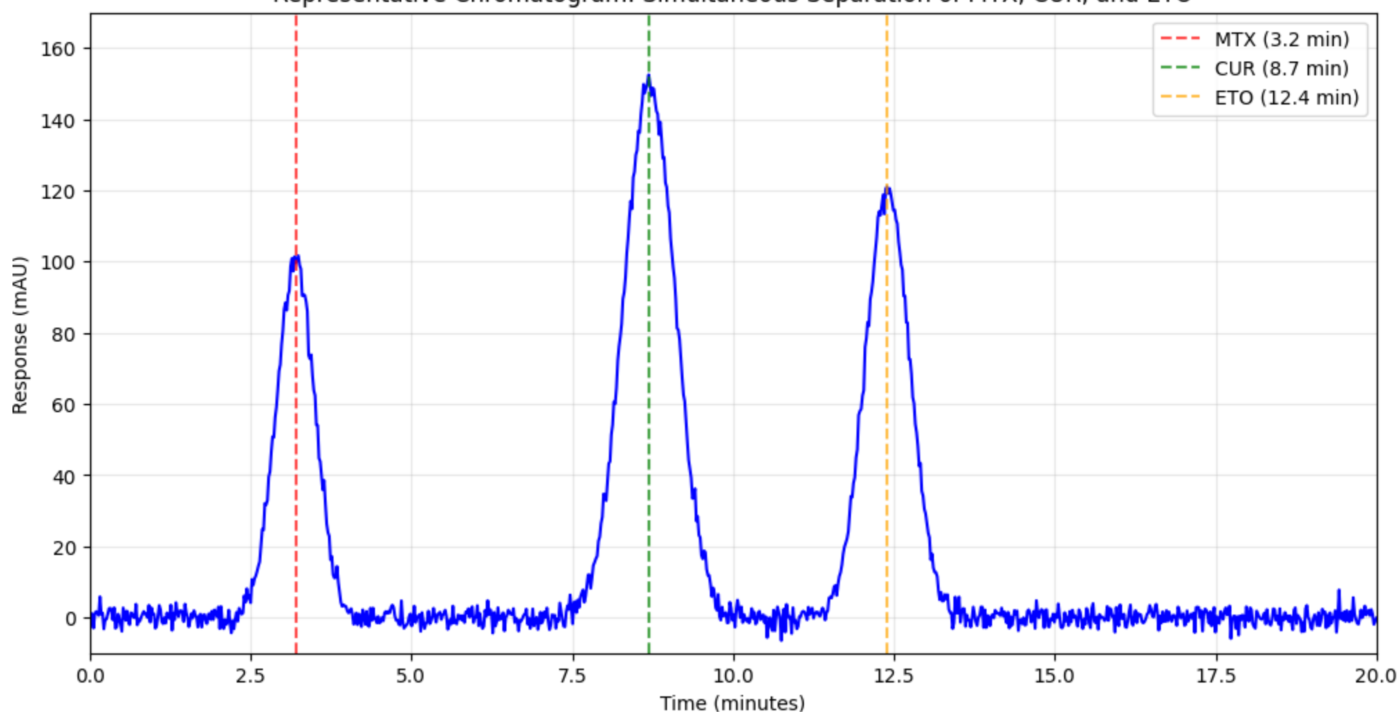

Figure 2: Representative chromatogram showing simultaneous separation of Methotrexate (MTX), Curcumin (CUR), and Etodolac (ETO).

Chromatographic Performance

The optimized chromatographic conditions successfully achieved baseline separation of all three analytes within a 20-min runtime, which is significantly shorter compared to some existing methods (Table 6). The chromatogram (Figure 2) showed sharp and symmetric peaks with no evidence of peak tailing or co-elution, indicating efficient column performance and appropriate method parameters.

These characteristics are crucial for accurate quantification, especially in simultaneous estimation methods where resolution between peaks is essential to avoid integration errors. Additionally, the adequate retention times for all analytes prevented overlapping and minimized matrix effects, ensuring method selectivity.

Method Validation

Specificity

Specificity is a critical parameter in multi-analyte analysis. In this study, the method showed excellent specificity, with no interference observed from excipients or degradation products. This was confirmed by peak purity analysis, which demonstrated homogenous spectral profiles for all three analytes. This ensures that the method can distinguish each compound even in the presence of complex matrices like pharmaceutical formulations.

Linearity

The method demonstrated excellent linearity across a wide range of concentrations for all analytes (Table 2), with correlation coefficients (r^2) exceeding 0.999. These results confirm the

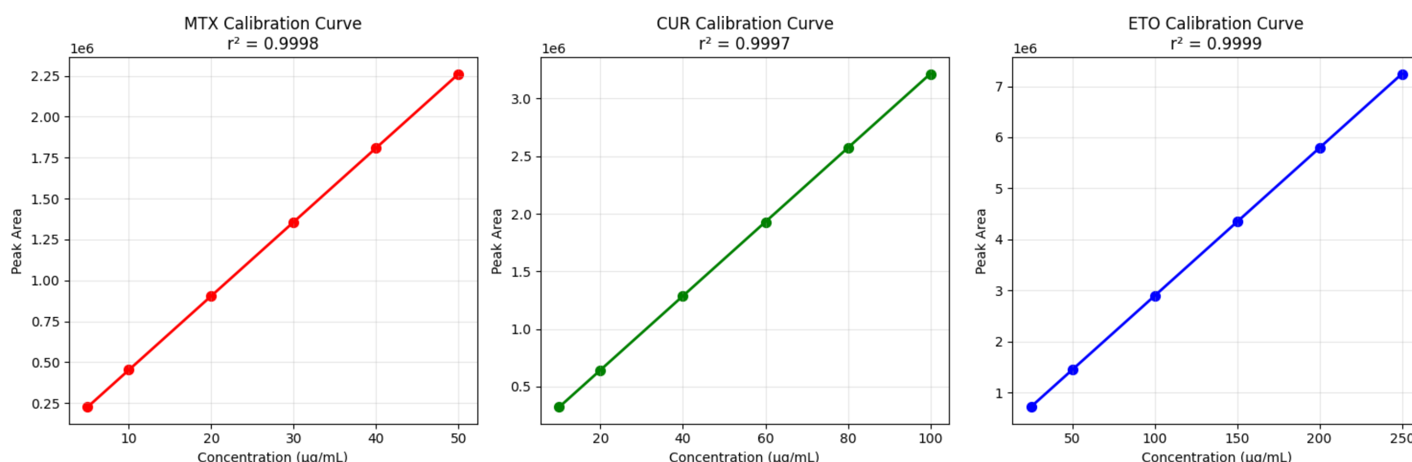

Figure 3: Calibration curves for MTX, CUR, and ETO showing excellent linearity.

Table 2: Linearity Parameters for MTX, CUR, and ETO.

Analyte	Linear Range (µg/mL)	Slope	Intercept	r^2	LOD (µg/mL)	LOQ (µg/mL)
MTX	5-50	45,231	234	0.9998	0.8	2.5
CUR	10-100	32,145	456	0.9997	1.2	3.8
ETO	25-250	28,967	123	0.9999	2.1	6.5

Table 3: Precision Results (RSD %).

Analyte	Concentration (µg/mL)	Intra-day (*n*=6)	Inter-day (*n*=18)
MTX	15	1.2	1.8
MTX	25	0.9	1.5
MTX	35	1.1	1.6
CUR	30	1.5	1.9
CUR	50	1.3	1.7
CUR	70	1.4	1.8
ETO	75	1.6	2.0
ETO	125	1.2	1.9
ETO	175	1.4	1.8

Table 4: Accuracy Results (Recovery %).

Analyte	Spiked Level	Mean Recovery (%)	RSD (%)
MTX	80%	99.2	1.3
MTX	100%	100.5	1.1
MTX	120%	101.2	1.4
CUR	80%	98.8	1.5
CUR	100%	100.1	1.2
CUR	120%	101.8	1.6
ETO	80%	98.5	1.7
ETO	100%	99.9	1.3
ETO	120%	100.8	1.5

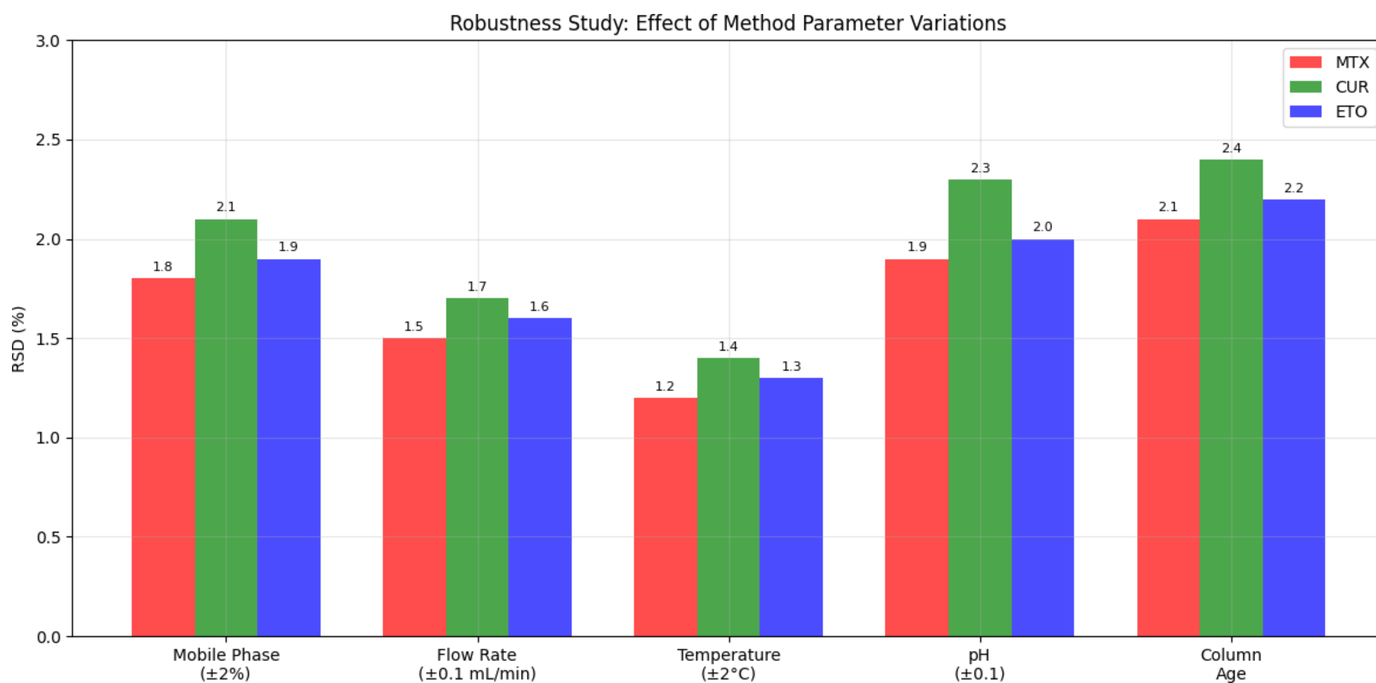

Figure 4: Robustness study results showing minimal impact of method parameter variations.

method's suitability for accurate quantification across typical dosage levels found in pharmaceutical formulations. Moreover, the low Limits of Detection (LOD) and Quantification (LOQ) make the method sensitive enough for trace-level analysis, which is especially valuable in content uniformity and stability studies.

Precision

The method exhibited robust intra-day and inter-day precision, with RSD values well below 2% across all tested concentrations (Table 3). This indicates consistent reproducibility under the same operating conditions and over multiple days. The precision results are in accordance with ICH guidelines (Q2(R1)), establishing the reliability of the method for routine analytical use.

Accuracy

Accuracy was evaluated using standard addition recovery studies at 80%, 100%, and 120% levels (Table 4). The mean recovery percentages ranged from 98.5% to 101.8%, which are well within the acceptable range of 98–102% for pharmaceutical analysis. This confirms that the method can accurately recover analytes from sample matrices without significant bias.

Robustness

Robustness testing revealed that small deliberate changes in method parameters (e.g., flow rate, mobile phase ratio, column temperature) had negligible effects on the chromatographic output (Figure 4). This demonstrates that the method is not sensitive to typical operational variations, making it practical and reliable for implementation in quality control laboratories.

Stability

Stability studies showed that the analytes remained stable under various conditions, including bench-top, refrigerated, freeze-thaw, and light exposure (Table 5). However, curcumin showed slight degradation under light exposure, which is consistent with its known photolabile nature. Overall, the results support that the sample solutions can be reliably stored and handled under standard laboratory conditions.

Method Comparison with Existing Literature

Compared to existing single-analyte or dual-analyte HPLC methods (Table 6), the developed method offers several advantages:

- Reduced analysis time (20 min vs. 28-35 min).
- Simpler mobile phase system, enhancing reproducibility and cost-effectiveness.
- Lower detection limits, allowing more sensitive detection.
- Simultaneous analysis, which significantly reduces sample preparation and analysis workload.

These advantages make the method highly valuable for quality control of combination drug products, especially in environments where efficiency and throughput are critical.

Application to Pharmaceutical Formulations

The method was applied successfully to synthetic pharmaceutical formulations containing MTX, CUR, and ETO (Table 7). The found assay values were very close to the labeled amounts, with

Table 5: Stability Results (% Remaining).

Storage Condition	Time	MTX	CUR	ETO
Bench-top (25ºC)	24h	99.2	97.8	99.5
Refrigerated (4ºC)	7d	99.8	99.1	99.7
Freeze-thaw cycles	3×	98.9	98.3	99.2
Light exposure	24h	95.2	89.7	98.8

Table 6: Method Comparison with Literature.

Parameter	This Method	Literature Method 1*	Literature Method 2**
Analysis Time	20 min	35 min	28 min
Mobile Phase	Simple	Complex gradient	Binary gradient
LOD (μg/mL)	0.8-2.1	1.5-3.2	2.0-4.5
Precision (RSD%)	<2.0	<3.0	<2.5
Simultaneous Analysis	Yes	No	No

*Literature Method 1: Individual analysis (Reference 15) **Literature Method 2: Dual component analysis (Reference 18).

Table 7: Analysis of Synthetic Pharmaceutical Formulations.

Formulation	Analyte	Labelled Amount (mg)	Found Amount (mg)	Assay (%)
Formulation 1	MTX	10	9.98	99.8
	CUR	50	49.7	99.4
	ETO	100	101.2	101.2
Formulation 2	MTX	15	15.1	100.7
	CUR	75	74.8	99.7
	ETO	150	149.5	99.7

recoveries ranging between 99.4% and 101.2%, demonstrating the method's applicability in real-world scenarios. This not only validates the method's reliability in analyzing complex dosage forms but also highlights its potential utility in stability testing and batch release.

CONCLUSION

Using a structured DOE approach, a new RP-HPLC method for measuring methotrexate, curcumin, and etodolac all at the same time has been created and proven to work. The Box-Behnken design well improved the chromatographic conditions, which led to great separation and measurement of all three analytes. The method showed better results in a number of ways, such as:

- Very good separation efficiency with good baseline clarity and the right holding times.
- Large linear ranges that can be used in pharmacy settings.
- Very high precision and accuracy that meets ICH standards
- Good stability makes sure that the method works.
- Stability good enough for regular study.
- Less time spent on research compared to using separate ways.
- Environmental kindness by using solvents more efficiently.

It has been proven that the approved method is a good way to check the quality of medicines that contain these three active ingredients. This helps with the creation of combination treatments for inflammatory and cancerous conditions. The DOE model showed that it could be useful in developing analysis methods by giving a structured way to improve things while reducing the amount of work that had to be done in experiments.

Possible areas for future study could be:

- Increase the number of cellular models used for pharmacokinetic studies.
- Creating tools that can show when something is stable.
- Putting green analytical chemistry ideas into practice.
- Automation for research with a high throughput.

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ABBREVIATIONS

DOE: Design of experiment; **MTX:** Methotrexate; **CUR:** Curcumin; **ETO:** Etodolac; **can:** Acetonitrile; **Mg:** Milligram; **mL:** Milliliter.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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