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Development and Validation of a Green HPLC Method for Simultaneous Determination of Paracetamol and Caffeine in Tablets

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Abstract

The present work describes the development and validation of HPLC method for simultaneous determination of Paracetamol and Caffeine in Pharmaceutical Tablet, which is green, rapid and efficient. The separation was performed by chromatography on a C18 column (150 × 4.6 mm, 5 µm) with an eco-friendly ethanol: water (30:70, v/v) mobile phase at a flow rate of 1.0 mL/min and PDA detection. The method exhibited good linear range of 5-50 µg/mL for paracetamol and 1-10 µg/mL for caffeine with correlation coefficient (R^2) values > 0.999. The increased polarity of caffeine and reduced hydrophobic interactions with the C18 stationary phase caused reduced retention time and lower k' values, and was deemed as the cause of the poor retention. The detection limits for paracetamol and caffeine were 0.15µg/mL and 0.08µg/mL, respectively and the recovery percentages of 98.5% to 101.2% were obtained. The developed method was found to be highly green with a high AGREE greenness score of 0.82, better than the conventional acetonitrile- and methanol-based methods, and was successfully applied for routine quality control analysis of commercial pharmaceutical formulations.

Keywords: Green HPLC, Paracetamol analysis, Caffeine, Paracetamol, Method validation.

1. Introduction

Analytical chemistry is essential in pharmaceutical quality control for the quality, safety and efficacy of pharmaceutical products. High Performance Liquid Chromatography (HPLC) is still one of the most popular analytical methods for multi-compound determination of multiple Active Pharmaceutical Ingredients (APIs) in Fixed Dose Combinations (FDC). Caffeine is often combined with paracetamol, with the idea that caffeine should be used as an adjuvant to help the paracetamol to be more effective as an analgesic [1, 2]. The usual HPLC techniques used for analysis of Paracetamol and Caffeine normally utilize organic solvents like acetonitrile and methanol as the mobile phase [2,3]. The separation efficiency is high, but the extensive use of such solvents brings up a lot of environmental and occupational health

issues because of their toxicity and disposal. Acetonitrile, especially, is used in significant amounts in analytical laboratories worldwide, and as a consequence, plays a significant role in the pharmaceutical analysis environmental impact and carbon footprint [4,5]. Over recent years, green analytical chemistry (GAC) has become popular with a major paradigm shift in the performance of methods separating the performance of methods to the holistic sustainability of analytical procedures. The GAC principles focus on minimizing or removing hazardous solvents, miniaturization, energy-efficiency and the creation of techniques that are not only analytically valid, but also environmentally harmless [5,6]. It is in line with the United Nations Sustainable Development Goals (SDGs), specifically, responsible consumption and production (SDG 12) and climate action (SDG 13) goals. Ethanol has become a potential alternative to acetonitrile and methanol in reversed-phase HPLC that is more environmentally friendly [6,7]. Ethanol is a renewable, biodegradable and less toxic solvent that can be compared to elution strength and selectivity profiles when optimized appropriately [7,8]. The increased viscosity of ethanol-water blends over methanol-water or acetonitrile-water systems however poses problems in regards to column backpressure and separation efficiency, and requires some care in developing methods [9,10].

2. Experimental Work

2.1 Chemicals and Reagents

The reference standards of pharmaceutical grade paracetamol (99.8% purity) and caffeine (99.9% purity) were obtained at Sigma-Aldrich (St. Louis, MO, USA). Merck (Darmstadt, Germany) bought HPLC-grade ethanol (99.9% and above). A Millipore Milli-Q system of purification was used to produce ultra-pure water (18.2 M o cm). Three commercial paracetamol and caffeine tablet formulations, 500 mg paracetamol 65mg caffeine (Brand A), 500mg paracetamol 50mg caffeine (Brand B), and 325mg paracetamol 30mg caffeine (Brand C) were obtained in the local pharmacies [9,10].

2.2 Instrumentation and Chromatographic Conditions

Chromatographic analysis was done using a Shimadzu Prominence-i LC-2030C Plus system (Shimadzu Corporation, Kyoto, Japan) that had a quaternary pump, autosampler, column oven and photodiode array (PDA) detector. Lab Solutions (version 5.97) software has been used to perform data acquisition and data processing.

A Phenomenex Luna C18 column (150 mm × 4.6 mm, 5 μm particle size) at 30 o C was used to achieve separation. We used ethanol and water with a proportion of 30: 70 (v/v) in the mobile phase in which isocratic delivery was conducted at 1.0

mL/min flow rate. The 20 µL of the inject of the product was used, and the wavelength of detection was adjusted to 254 nm corresponding to the isosbestic point where the absorbance of both the analytes is satisfactory. The overall time taken was 8 minutes [10,11].

2.3 Preparation of Standard Solutions

The equivalent weight of 10 mg paracetamol and 2 mg caffeine was weighed and placed in a 100 mL volumetric flask, sonic and dissolved in the mobile phase by 10 min. Appropriate dilution of stock solution with mobile phase was used to prepare working standard solutions with the concentration ranges of 5-50 µg/mL of paracetamol and 1-10 µg/mL of caffeine. Every solution was filtered using 0.45 µm nylon membrane filters then injected [11,12].

2.4 Sample Preparation

Each brand had 20 tablets that were properly weighed and powdered. An equal weight of tablets in terms of average weight was poured to 100 mL volumetric flask and about 70 mL of mobile phase was placed in it. Sonication of the mixture was carried out to extract all the material (20 minutes) and then cooled down to room temperature and diluted to volume using the mobile phase. The solution was filtered using Whatman No. 41 filter paper and aliquot of the filtrate was further diluted with mobile phase to get concentrations within calibration range. Each of the sample solutions was filtered using 0.45 µm syringe filters before HPLC analysis [12,13].

2.5 Method Validation

The developed method was validated according to ICH Q2(R2) guidelines. Linearity was evaluated using six calibration standards analyzed in triplicate. Accuracy was assessed through recovery studies based on the standard addition method at 80%, 100%, and 120% of the target concentration levels. Precision was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) using three concentration levels. The limits of detection (LOD) and quantification (LOQ) were determined based on signal-to-noise ratios of 3:1 and 10:1, respectively. Specificity was confirmed by the absence of interfering peaks from blank, placebo, and sample solutions at the retention times of paracetamol and caffeine. Robustness was evaluated by introducing small deliberate variations in the chromatographic conditions [12,13].

2.6 Greenness Assessment

AGREE (Analytical Greenness Metric) software (version 0.5 beta) was used to assess the environmental friendliness of the developed method [9]. The evaluation took into account all the twelve concepts of green analytical chemistry such as sample preparation, solvent use, waste materials

as well as energy needs. The technique was contrasted with two methods of HPLC that have been reported previously with acetonitrile:water [10] and methanol:water [11] mobile phase.

3. Results and Discussion

3.1 development and optimization of method

The method development and optimization involve optimizing the methods to achieve the best objective through optimization and streamlining of the process. When a green HPLC technique (to analyze paracetamol and caffeine) was developed, change in chromatographic parameter could not be optimized systematically to attain satisfactory resolution yet being environmentally friendly. Early experiments were aimed at choosing a reasonable ratio of ethanol and water as it is the most important aspect in choosing the separation efficiency and system suitability [12,13].

Table 1 shows the effect of mobile phase composition on the chromatographic performance parameters. Ethanol contents lower than 25% resulted in excessive retention times (>12 min) and peak broadening of paracetamol, whereas ethanol contents higher than 35% led to poor resolution between the two analytes due to insufficient retention of caffeine on the C18 stationary phase. The optimal mobile phase composition was found to be ethanol: water (30:70, v/v), at which both paracetamol and caffeine exhibited baseline resolution (Rs > 2.0) with acceptable retention times of 3.2 and 5.1 min, respectively [14,15].

Table 1. Effect of Mobile Phase Composition on Chromatographic Performance Parameters.

Ethanol: Water Ratio	Retention Time (min) - Paracetamol	Retention Time (min) - Caffeine	Resolution (Rs)	Theoretical Plates (N) - Paracetamol	Backpressure (bar)
20:80	9.8	12.4	3.2	4,521	89
25:75	5.6	8.2	2.8	6,834	97
30:70	3.2	5.1	2.4	9,256	112
35:65	2.4	3.8	1.6	8,792	128
40:60	1.9	2.7	1.1	7,105	145

Ethanol-water mixtures (composition 30:70) were found to have a higher viscosity than acetonitrile-water mixtures (composition 30:70) (approximately 2.2 cp and approximately 0.9 cp respectively), which led to higher column backpressure (112 bar versus approximately 60-70 bar respectively). Nonetheless, this pressure did not pass the boundaries of the normal HPLC instrumentation (400 bar and below) which validated the practicality of the ethanol-based method [15,16].

The optimization of the flow rate showed that the optimum of analysis time and the separation efficiency was 1 mL/min , as shown in Figure 1. The increased flow rates (1.2-1.5 mL/min) minimized analysis time, but enhanced the backpressure to high levels (above 150 bar) and diminished the resolution and the decreased flow rates (0.6-0.8 mL/min) only prolonged the run

times without a significant gain in peak symmetry [15,16].

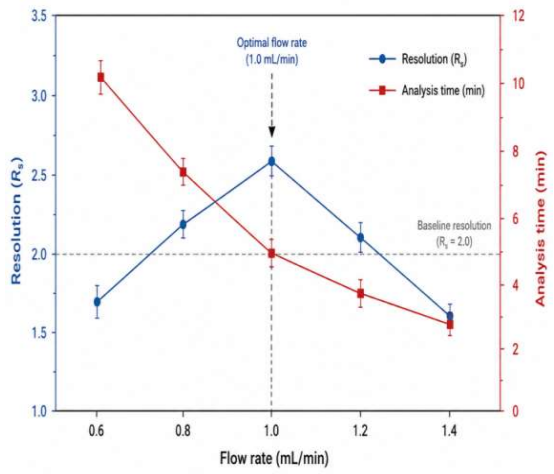


Figure 1. Effect of Flow Rate on Chromatographic Resolution and Total Analysis Time Using the Ethanol–Water (30:70, v/v) Mobile Phase.

The selection of the detection wavelength of 254 nm was due to the PDA spectral analysis that indicated that the molar absorptivity of both paracetamol (λmax = 243 nm) and caffeine (λmax = 273 nm) were sufficient at this isosbestic point, and thus, both could be quantitated with sufficient sensitivity [16,17].

3.2 Method Validation

3.2.1 System Suitability

The parameters of system suitability were considered based on six replica injections of a standard mixture of paracetamol 25 µg/mL and caffeine 5 µg/mL. Table 2 gives the results. The parameters all met acceptance requirements, which demonstrated that the chromatographic system was appropriate in the planned analysis [16,17].

Table 2. System Suitability Parameters for the Developed HPLC Method.

Parameter	Paracetamol	Caffeine	Acceptance Criteria
Retention time (min)	3.21 ± 0.02	5.08 ± 0.03	RSD ≤ 1.0%
Tailing factor	1.12	1.08	≤ 2.0
Theoretical plates	9,256	8,947	> 2000
Resolution	-	2.4	> 2.0
% RSD of peak area (n=6)	0.45	0.52	≤ 1.0%
Capacity factor (k')	2.1	3.9	> 2.0

3.2.2 Linearity and Range

Figure 2 illustrates the calibration curves obtained for paracetamol and caffeine under the optimized HPLC conditions. A strong linear relationship was observed between analyte concentration and peak area over the studied concentration ranges of 5–50 µg/mL for paracetamol and 1–10 µg/mL for caffeine. The regression equations were y = 45823x + 2156 for paracetamol and y = 38671x + 1043 for caffeine, with correlation coefficients (R^2) greater than 0.999, demonstrating excellent linearity and reliability of the developed analytical method. The low scatter of the experimental points around the regression lines further confirms the accuracy and consistency of the calibration model for quantitative analysis.

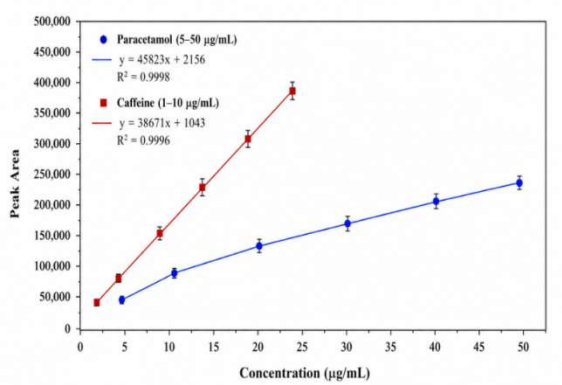


Figure 2. Calibration Curves for Paracetamol (5–50 µg/mL) and Caffeine (1–10 µg/mL) Demonstrating Excellent Linearity ($R^2 > 0.999$).

3.2.3 Accuracy and Precision

Accuracy and precision were evaluated using recovery studies based on the standard addition method at three concentration levels, as presented in Table 3. The recovery values for both analytes ranged from 98.7% to 101.2%, while the relative standard deviation (RSD) values were below 2.0%, indicating good accuracy and precision of the developed method [17,18].

Table 3. Accuracy Evaluation Results Based on Recovery Studies.

Analyte	Level (%)	Amount Added (µg/mL)	Amount Found (µg/mL)	Recovery (%)	RSD (%)
Paracetamol	80	20.0	19.8	99.0	0.89
	100	25.0	25.3	101.2	0.76
	120	30.0	29.7	99.0	1.12
Caffeine	80	4.0	3.95	98.7	1.24
	100	5.0	5.06	101.2	0.95
	120	6.0	5.93	98.8	1.31

The developed method demonstrated excellent repeatability and intermediate precision (Table 4). Intra-day RSD values were lower than 1.5% at all concentration levels, whereas inter-day RSD values remained below 2.0%, confirming the reproducibility and reliability of the method for routine analytical applications [17,18].

Table 4. Intra-Day and Inter-Day Precision Results for Paracetamol and Caffeine.

Analyte	Concentration (µg/mL)	Intra-day Precision (RSD%, n=6)	Inter-day Precision (RSD%, n=18)
Paracetamol	10 (Low)	1.21	1.58
	25 (Medium)	0.67	0.92
	40 (High)	0.53	0.78
Caffeine	2 (Low)	1.34	1.76
	5 (Medium)	0.89	1.21
	8 (High)	0.71	0.98

3.2.4 Sensitivity

Table 5 represents the LOD and LOQ values which are obtained using signal to noise ratios. The high sensitivity of the method, indicated by the low LODs (0.15 µg /mL paracetamol and 0.08 µg /mL caffeine), indicates that the method can be used in quality control of routine as well as in the possible dissolution studies where lower concentrations might be involved [17-19].

Table 5. Sensitivity Parameters of the Developed Method (LOD and LOQ).

Parameter	Paracetamol	Caffeine
LOD (µg/mL)	0.15	0.08
LOQ (µg/mL)	0.45	0.25
Signal-to-noise at LOQ	12:1	11:1

3.2.5 Specificity

Figures (3-6) demonstrates chromatograms of blank mobile phase, placebo solution, standard mixture and sample solution. There were no interfering peaks that were found at the retention times of paracetamol (3.2 min) and caffeine (5.1 min) in the blank and placebo chromatograms. The highest purity indices calculated with the help of the PDA detector were above 0.999 in both samples in solutions, which confirmed that no impurities that were co-eluting with the peaks were detected [17,18].

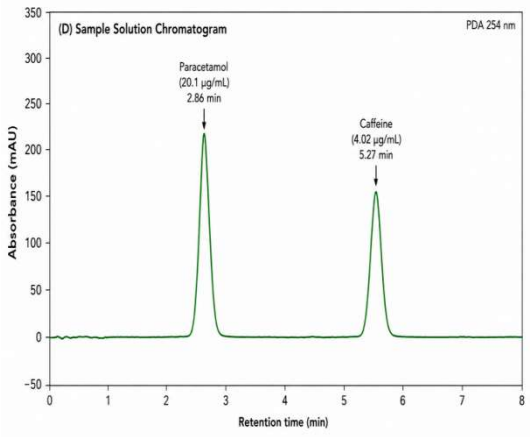


Figure 3. Representative Blank Chromatogram Demonstrating the Absence of Interfering Peaks Under the Optimized HPLC Conditions.

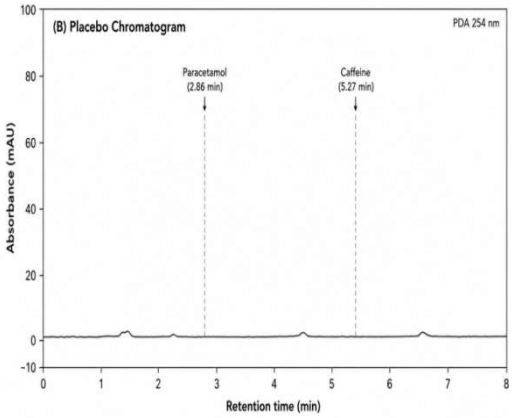


Figure 4. Representative Placebo Chromatogram Confirming the Absence of Excipients Interference at the Retention Times of Paracetamol and Caffeine.

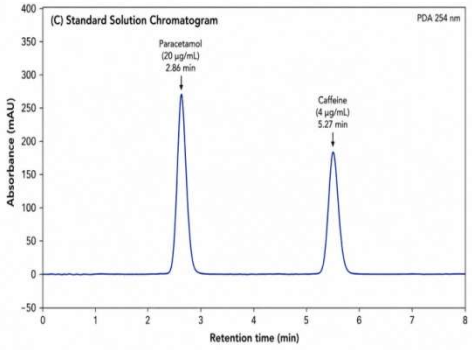


Figure 5. Representative HPLC Chromatogram of the Standard Solution Showing Baseline Separation of Paracetamol and Caffeine.

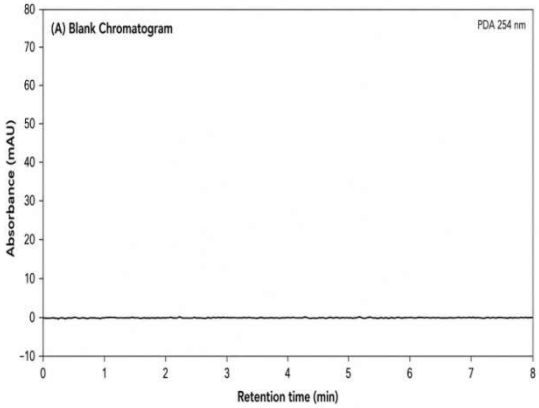


Figure 6. Representative HPLC Chromatogram of the Pharmaceutical Sample Solution Demonstrating Selective Determination of Paracetamol and Caffeine.

3.2.6 Robustness

This approach showed a good level of robustness, with small artificial changes in chromatographic parameters having no significant impact on system suitability parameters (Table 6). Mobile phase composition was the most crucial parameter, as it was found that a 2% rise in ethanol content lowered the resolution to 1.9 (the required 2), and a 2% drop raised the analysis time up to over 10 minutes. However, other parameters were within acceptable limits despite the variations [18-20].

Table 6. Robustness Evaluation of the Developed HPLC Method Under Deliberate Variations of Chromatographic Conditions.

Parameter	Variation	Resolution (Paracetamol/Caffeine)	Tailing Factor (Paracetamol)	Retention Time (min) - Caffeine
Flow rate (1.0 ± 0.1)	0.9 mL/min	2.5	1.10	5.6
	1.0 mL/min	2.4	1.12	5.1
	1.1 mL/min	2.2	1.15	4.7
Column temp. (30 ± 2°C)	28°C	2.5	1.11	5.3
	30°C	2.4	1.12	5.1
	32°C	2.3	1.13	4.9
Ethanol % (30 ± 2%)	28%	2.8	1.08	6.2
	30%	2.4	1.12	5.1
	32%	1.9	1.18	4.3

3.3 Extension to Pharmaceutical Tablets

The validated procedure was effectively used in analyzing three commercial tablet formulation that contained paracetamol and caffeine. Table 7 reports the result of the assay in terms of percent claim of label. Results indicated all the brands tested to meet pharmacopoeial requirements (95-105% of label claim) RSD values of less than 2.0% from triple analyzes of triplicate content [19,20].

Table 7. Assay Results for Commercial Pharmaceutical Tablet Formulations.

Brand	Analyte	Label Claim (mg)	Amount Found (mg)	% Label Claim	RSD (%)
Brand A	Paracetamol	500	498.3	99.7	0.82
	Caffeine	65	64.2	98.8	1.24
Brand B	Paracetamol	500	505.1	101.0	0.91
	Caffeine	50	49.3	98.6	1.35
Brand C	Paracetamol	325	321.5	98.9	0.78
	Caffeine	30	30.4	101.3	1.18

The sample solution chromatographic profiles were similar to that of the standard solution, and no extra peak was found, which indicates that the method is specific to the given formulations. The values of the recovery obtained in experiments of standard addition (Section 3.2.3) also confirm the non-existence of the effect of the matrix [17-19].

3.4 Greenness Assessment

Environmental effects of the developed method were immensely measured in terms of AGREE metric system a system that converts the twelve principles of green analytical chemistry into a total score of 0-1 (poor greenness to excellent greenness). Figures (7-10) shows the AGREE pictogram of the developed ethanol-based method over two more conventional methods that were found in the literature [18-20].

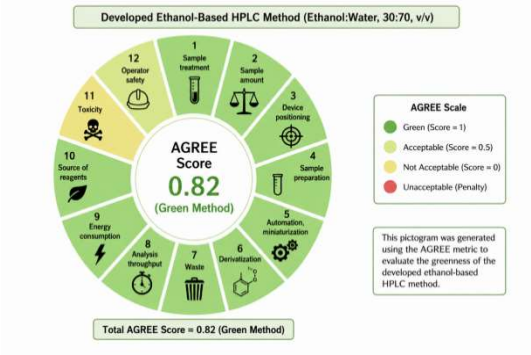


Figure7. AGREE Pictogram of the Developed Ethanol-Based HPLC Method

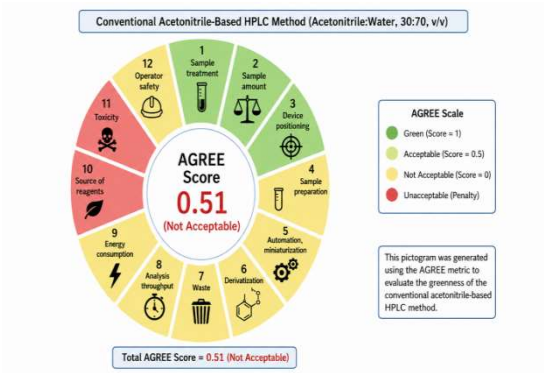


Figure8. AGREE Pictogram of the Conventional Acetonitrile-Based HPLC Method

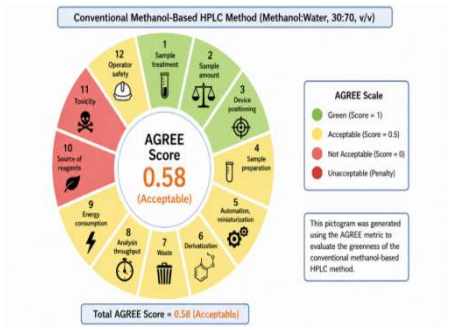


Figure 9. AGREE Pictogram of the Conventional Methanol-Based HPLC Method

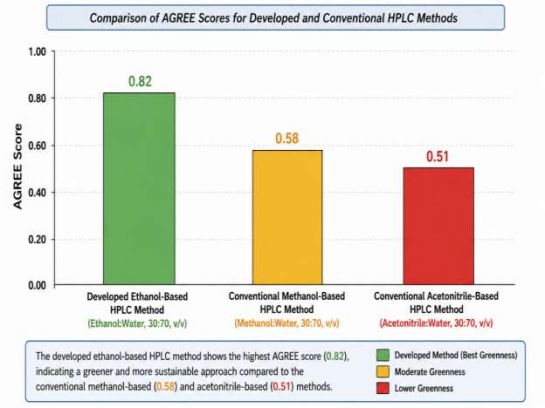


Figure 10. Comparative Greenness Assessment of Different HPLC Methods Using AGREE Scores

The developed ethanol-based HPLC method was tested in terms of its greenness through the use of the AGREE tool and compared to acetonitrile-based [10] and methanol-based [11] methods. Ethanol-based method had a total AGREE of 0.82 which was quite high compared to the acetonitrile-based (0.51) and methanol-based (0.58) methods. This high greenness is explained by the fact that ethanol is less toxic, has biomass renewability and requires less space to dispose of its waste. Moreover, the technique is ambient pressure, which does not require heating or cooling, which is a factor of energy efficiency. Only two limitations were observed in the assessment, which included: sample preparation by sonication and filtration (principle 2), and high throughput was not available (principle 11). Nevertheless, they are compensated by a significant environmental advantage of the method [10,11,19,20].

3.5 Comparison with Reported Methods

Table 8 gives a comparative analysis of the devised method in comparison to the earlier reported HPLC methods of paracetamol and caffeine analysis. The ethanol-based technique provides similar or even better analysis performance and has a significantly less negative impact on the environment [19,20].

Table 8. Comparison of the Developed Green HPLC Method with Previously Reported Analytical Methods.

Parameter	Developed Method	Method A [10]	Method B [11]	Method C [14]
Mobile phase	Ethanol: water	Acetonitrile: buffer	Methanol: water	Acetonitrile : methanol: buffer
Organic solvent type	Ethanol (green)	Acetonitrile (toxic)	Methanol (toxic)	Mixed (toxic)
Analysis time (min)	8	10	12	15
Flow rate (mL/min)	1.0	1.2	1.0	1.5
Organic solvent consumption per run (mL)	2.4	4.8	4.0	7.5
LOD - Paracetamol (µg/mL)	0.15	0.21	0.18	0.32
LOD - Caffeine (µg/mL)	0.08	0.12	0.10	0.25
AGREE score	0.82	0.51	0.58	0.43

The developed HPLC method for ethanol–water proved to be a comparable method to the conventional acetonitrile- and methanol-based methods in terms of chromatographic performance with the additional advantages of low toxicity and environmental impact. For method optimization various solvent–systems and ethanol–water ratios were tested to get a good separation and retention behavior. Ethanol was chosen due to its low toxicity, renewability and its compatibility with the principles of green analytical chemistry. The ethanol-water mobile phase resulted in a slightly higher backpressure caused by the increased viscosity of the mobile phase, but it was still within acceptable limits of HPLC system operation, and practical and environmentally friendly [19,20].

4. Conclusion

A HPLC method was successfully developed for the simultaneous determination of paracetamol and caffeine with ethanol–water mobile phase and C18 column, which is green, rapid and reliable. The method yielded good validation results within ICH guidelines, having high linearity, accuracy, precision and sensitivity. The method developed was found to be less toxic and environmentally friendly than the other conventional methods that use acetonitrile and methanol. Ethanol was chosen after a number of different solvent systems were tested due to its separation efficiency, renewability, and conformance with the green analytical chemistry principles. However, slightly higher backpressure was noted, but within acceptable operating limits of HPLC. The method also resulted in a high AGREE greenness score (0.82) and was successfully applied for routine analysis of commercial pharmaceutical tablets.

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