Applied Chemical Engineering (2026) Volume 9 Issue 2 ISSN: 2578-2010 (O)
doi: 10.59429/ace.v9i2.5937

ORIGINAL RESEARCH ARTICLE

Optimization and validation of a rapid RP-HPLC method for the simultaneous quantification of codeine and diazepam in bulk and pharmaceutical dosage forms

Rulla Sabah[1,*], Nisreen Kais Abood[1], Mustafa Hammadi[2]

[1] *Department of Chemistry, College of Science, Mustansiriyah University, Baghdad, 10011, Iraq*

[2] *Department of Chemistry, College of Education for Pure Sciences, University of Diyala, Baqubah, 32001, Iraq*

***Corresponding author:** Rulla Sabah, rulla_sabah77@uomustansiriyah.edu.iq

ABSTRACT

This study establishes a high-performance liquid chromatography (HPLC) method that is precise and expeditious for the existing separation and quantification of codeine and diazepam, while reducing analysis time and separation efficiency. An RP-Sunfire C18 column (5 µm, 4.6 × 250 mm) was used to separate the samples. The mobile phase consisted of acetonitrile and a phosphate buffer and detection were performed at 230 nm. The approach separated the two drugs in less than 7 minutes using an isocratic elution mode. Codeine and diazepam had retention times of 4.19 and 6.09 minutes, respectively, and the resolution was a good value (Rs = 4.22). The results showed that the column was highly efficient, with theoretical plate counts passing 2579 for codeine and 2373 for diazepam. The very symmetrical peaks, with symmetry factor values ranging from 1.05 to 1.08. The method showed an excellent linearity within the range of concentration (5-25 µg. mL^{-1}) with high Coefficient of Determination ($R^2 \geq 0.9998$) and low limits of detection, indicating its high sensitivity. High recovery rates (99.46-100.27%) further confirmed the method's accuracy and lack of pharmaceutical interference. Separation conditions were optimized using response surface morphology (RSM), with analysis of variance (ANOVA) showing high significance for the quadratic model ($p = 0.0057$) and no significance for the non-conformity test ($p = 0.2700$). Optimal conditions were determined using a mobile phase of acetonitrile and phosphate buffer in a 40:60 (v/v) ratio at pH 4.0, with a flow rate of 1.0 $mL.min^{-1}$. These conditions confirm the method's efficiency and suitability for routine pharmaceutical laboratory applications.

Keywords: codeine; diazepam; optimization; quantification; RP-HPLC; surface morphology (RSM)

ARTICLE INFO

Received: 11 March 2026
Accepted: 6 May 2026
Available online: 8 June 2026

1. Introduction

Codeine is an alkaloid (3-methylmorphine) extracted from poppy seeds (Papaver somniferum) and is manufactured by methylating morphine, **Figure 1a**. It is used to treat mild to moderate pain, such as chronic cancer pain, and it can also help with coughing, stress, and diarrhea. Some codeine is broken down in the body to make morphine[1]. Codeine acts through a mechanism that is still not fully understood. Like morphine, it can connect to opioid receptors in the brain and activate signaling processes in the brain and the rest of the body.Codeine also makes you sleepy, which helps lessen the pain[2,3]. It has also been used with acetaminophen or aspirin to get better pain relief. **Figure 1a** shows the chemical structure of codeine There are a number of different types of codeine salts and hydrates, including codeine phosphate, codeine hydrobromide, codeine N-oxide, codeine monohydrate,codeine phosphate sesquihydrate, codeine hydrochloride, and codeine phosphate hemihydrate[3-5] Codeine monohydrate is a

colorless, odorless base that dissolves easily in ethanol but not as well in water. Codeine phosphate is a common ingredient in pharmaceuticals[6,7]. It is a crystalline powder with no scent that dissolves in water and a little in ethanol. Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one) is a benzodiazepine primarily used to help individuals sleep, calm them, and relax their muscles[8]. Diazepam is frequently administered as the primary treatment for acute convulsions and prolonged status epilepticus[9]. **Figure 1b** shows its chemical structure. The liver's cytochrome P450s break it down into three main active metabolites: N-desmethyldiazepam (also known as nordiazepam), oxazepam, and temazepam[8,10]. These are then conjugated and eliminated, mostly as glucuronides, in the urine. Diazepam is used to treat a variety of conditions[11]. Owing to its abuse ability, forensic and clinical toxicological research has to focus on finding reliable ways to detect diazepam in biological matrices[11,12]. There are several methods for analyzing diazepam and other 1,4-benzodiazepines using chromatography, including thin-layer chromatography (TLC), gas chromatography (GC), and gas chromatography-mass spectrometry (GC-mass)[13]. There have also been reports of other high-performance liquid chromatographic (HPLC) methods for finding diazepam. Laboratories often employ HPLC to measure the number of drugs in clinical samples and pharmaceutical dosage forms[14,15]. It can handle more types of analytes than GC, including ones that aren't volatile and are sensitive to heat. Therefore, HPLC analysis doesn't require any additional steps to change the analyte; because HPLC can separate substances, it has been able to find many opioid compounds with similar structures or effects at the same time, such as heroin, morphine, codeine, and Diazepam[16,17]. HPLC has been coupled with various detectors, including mass spectrometry (MS) and optical detectors such as the photomultiplier tube (PMT) and photodiode array (PDA)[18,19].

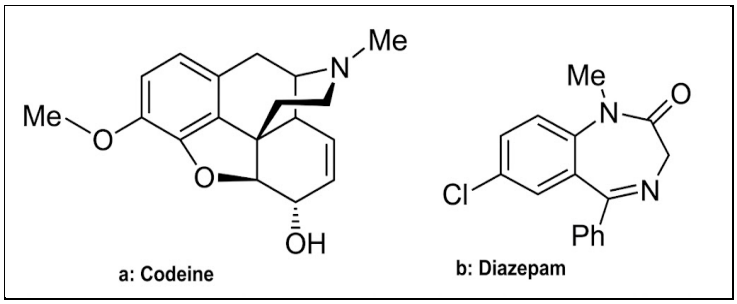

Figure 1. Chemical structure for a: Codeine, b: Diazepam.

The objectives of this work were to develop and improve a rapid, effective analytical method using reversed-phase high-performance liquid chromatography (RP-HPLC) to simultaneously quantify and qualitatively analyze two drugs, codeine and diazepam. The study specifically focused on investigating the effect of mobile phase pH and flow rate to achieve optimal operating conditions for accurate separation in record time. The study also aimed to validate the developed method according to international standards (ICH) in terms of linearity, accuracy, and sensitivity (LOD and LOQ), while evaluating the purity of the chromatographic peaks using a diode array detector (PDA) to ensure the peaks were free from spectral interference. The study aimed to apply this well-established method to determine the concentrations of active ingredients in their crude state and in various commercial pharmaceutical preparations.

2. MATERIAL AND METHODS

2.1. Apparatus

A Shimadzu HPLC system from Kyoto, Japan, with an RP-Sunfire C18 (5 µm, 4.6 × 250 mm) column is employed. A PTFE tube equipped with a 0.45 µm stainless steel mesh filter was used to filter the mobile phase. The Shimadzu injection valve integrated into the HPLC system facilitated manual sample injection. The sample loop may hold 10 or 20 µL of liquid. The HPLC system's DGU-20A5 online degasser unit uses a fluoroethylene membrane to quickly remove gas from HPLC mobile phases. The internal volume capacity is

only 0.4 ml. The SPD-20A UV-VIS detector is very sensitive and stable, and it can measure wavelengths from 190 to 700 nm. Shimadzu LC/LabSolution software was used to gather and process the chromatographic data. The Agilent 1260 Infinity with Diode Array Detector (DAD).

2.2. Optimal Wavelength Selection

Prepared separate standard solutions of Codeine and diazepam, then used a UV-Vis spectrophotometer to scan them over 200–400 nm. By overlaying the absorption spectra, 230 nm was identified as the optimal detection wavelength. This wavelength was very sensitive to the analytes, proving that a stable baseline with less interference from the mobile phase components. An HPLC system with a DAD detector was used for the analysis. This system could monitor multiple wavelengths at once, which allowed for the selection of the optimal wavelength.

2.3. Separation conditions and mobile phase

A mobile phase composed of Acetonitrile and phosphate buffer at pH 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5 was used to identify the medicines. The LC software runs in isocratic mode in flow mode, with the column temperature maintained at 25° C. The organic modifier mobile phase was a mix of Acetonitrile and phosphate buffer in varying ratios (20:80, 30:70, 40:60, 50:50, 60:40, 70:30) at flow rates of 0.7, 0.8, 0.9, 1.0, 1.1, and 1.2 mL min^{-1}. Drugs were filtered through a 0.45 µm pore size filter with a 25mm diameter. Under the experimental conditions, a Hamilton micro-syringe injected 20 µL of the sample into the HPLC apparatus. The detector is set to 230 nm. This study employed the recorded peak area and peak height.

3. Chemicals and materials

3.1. Preparation of Diazepam and Codeine stock solutions

A 100 $\mu g.mL^{-1}$ standard solution of Codeine and Diazepam by dissolving 0.001 g in 2mL of acetonitrile then complete the volume to 10 mL by using mobile phase , and then diluted solutions were prepared by serial dilutions with mobile phase, the range was (0.5-50) $\mu g.mL^{-1}$ for each drug. Stock solutions were prepared freshly.

3.2. Preparation of buffer solution

The phosphate buffer (0.025 M) was prepared in a 1000mL volumetric flask by dissolving 3.40 g of potassium dihydrogen phosphate (KH_2PO_4) in approximately 800mL of de-ionized water and then completing the volume to the mark It. pH Adjustment: Add phosphoric acid (10%) drop-wise until the pH reaches various target values (3.5, 4.0, 4.5, 5.0, 5.5), with maintaining continuous stirring. Then, complete the volume to 1000 mL with de-ionized water[20]. The buffer solution degassed by ultra-sonication and was filtered by using a 0.45 µm membrane filter before its use in the HPLC system.

3.3. Validation of the HPLC method

The optimized chromatographic method was verified by checking its specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ). The ICH Guidelines were followed when validating the method[21].

3.4. Examination of Commercial Tablets

Standard analytical procedures were employed to process commercial codeine and diazepam products. This involved the precise weighing and homogenization of twenty tablets to ensure the accuracy of subsequent quantification a volume of the powder equal to the weight of one tablet was placed in a 100 mL volumetric flask, dissolved in the mobile phase, and sonicated for 20 minutes. The fluid that was collected was filtered through a 0.45 µm membrane filter. After that, the aliquots were diluted to the linear range. Each Sample was injected three times and used the regression equation we had previously created to determine the concentration.

3.5. Peak purity assessment

The peak purity assessment was performed software-based by using a diode array detector (DAD). The detector was set to perform a continuous spectral scan in the 200 – 400 nm wavelength range at a sampling rate of 1.25 Hz. After separating the spectra, the instrument software was used to compare them at several positions along the peak (Start, Apex, and End) to ensure there was no spectral interference[22].

4. Result and discussion

4.1. HPLC separation

Serial experiments were conducted to optimize the operating parameters for a rapid, simple, and effective separation method and determination of Codeine and Diazepam in pure solutions (standard) and in pharmaceutical form, both individually and as a mixture, with the shortest time of analysis. To optimize the variables that affect Isocratic elution by employing 10 µg. mL^{-1} of each drug at a wavelength of 230 nm. This study developed a new simultaneous HPLC method for the separation and quantification of Codeine and Diazepam in pure solutions. A RP-Sunfire C18 (5 µm, 4.6 mm × 250 mm) column was maintained at 25ºC for all types of organic mobile phase changes; the phosphate buffer is extensively used. The optimum mobile phase ratio Acetonitrile: phosphate buffer was 40:60 at a flow rate of 1mL.min^{-1} pH 4 since a short retention time for Codeine and Diazepam was achieved and a short retention time for Codeine and Diazepam was completed so it decreased to 3.05 and 9.86 min, when mobile phase ratio Acetonitrile: phosphate buffer was 20:80 and pH 4, **Figure 2**, retention time were 4.19 to 6.09 minutes for Codeine and Diazepam respectively (**Figure 3**), The mobile phase ratio (40:60) and pH 4 were chosen to balance separation efficiency (Resolution, Rs) and minimize backpressure, reflecting the advantage of acetonitrile in reducing mobile-phase viscosity and accelerating analysis time. also produced sharp, symmetrical peaks with good sensitivity for Codeine Phosphate and Diazepam, respectively, which were ideal for analysis and determination of drug concentrations in Bulk.

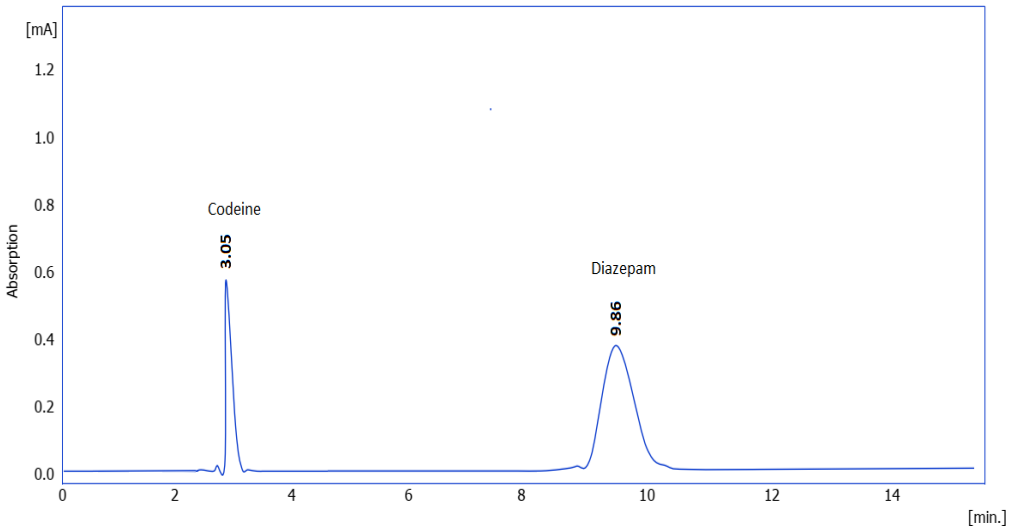

Figure 2. Chromatogram of a 10 µg.mL^{-1} combination of codeine and diazepam on a C18 column with a flow rate of 1.0 mL.min^{-1} and a mobile phase ratio. The acetonitrile: phosphate buffer ratio was 20:80, pH of 3.5.

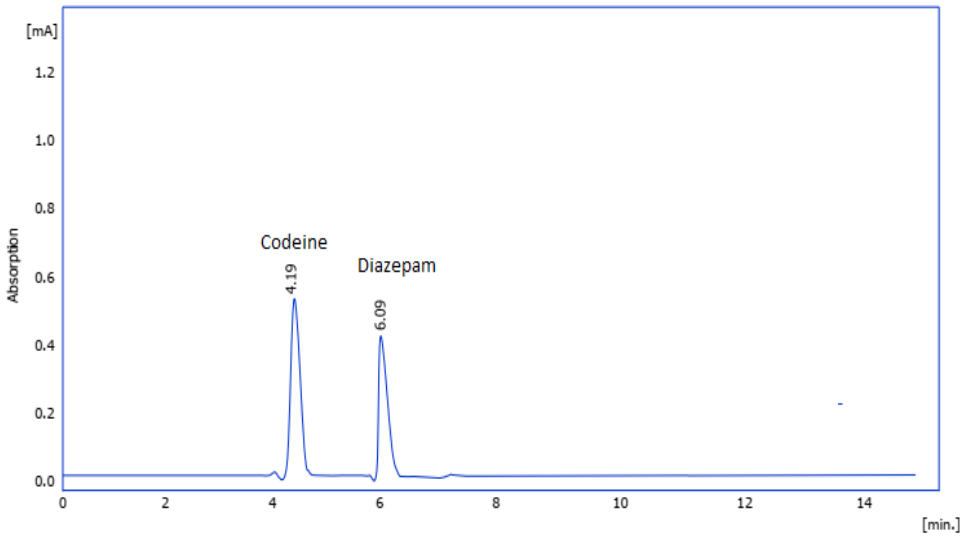

Figure 3. Chromatogram of a 10 µg.mL^{-1} combination of codeine and diazepam on a C18 column with a flow rate of 1.0 mL^{-1} min and an optimum mobile phase ratio of acetonitrile: phosphate buffer ratio was 40:60, at a pH of 4.

pH4 was selected as the optimal pH for the mobile phase after evaluating the effect of several pH values on separation efficiency. At this value, an ideal retention time and peak shape was achieved for both drugs. The impact of mobile phase pH (3.0 – 5.5) on achieving optimal chromatographic separation was investigated. pH 4.0 was found to be optimal for several technical and chemical reasons. At pH values less than 4.0, e.g., 3.0, the probability of hydrolysis of diazepam increased, that affecting on accuracy of quantitative determination[23-26]. For that reason, pH 4.0 was chosen as the optimal pH that confirms substance stability, achieves ideal selectivity, and conserves the efficiency of the chromatographic column, providing peak symmetry and a good resolution (Rs = 4.22).

The effect of flow rate (0.7-1.2 mL.min^{-1}) was studied to obtain an optimal analytical time and separation efficiency. Increasing the flow rate decreased the retention times of the two substances, which led to a observe increase in system pressure. A flow rate of 1.0 mL. min^{-1} was the optimal while providing complete separation of the codeine and diazepam peaks with high resolution (Rs = 4.22) in a time less than 7 minutes, maintaining a stable, safe pressure on the chromatographic column[27,28]. This achieved sharp, symmetrical peaks, facilitating quantitative analysis.

4.2. Chromatographic functions

The evaluation of efficiency and performance of the developed HPLC method involved chromatographic parameters under the optimal conditions, which were a Mobile phase ratio of 40:60 acetonitrile: phosphate buffer at pH 4.0 and a flow rate of 1 mL.min^{-1}. These parameters, including resolution (Rs), selectivity (α), retention factor (k), theoretical plates (N), and symmetry factor (As), are important to improve the suitability of the method for the determination of Codeine and Diazepam. The dead time (t_0) was 2.9 min, performed in a linear velocity (u) of 86.2 mm/min, which confirms efficient mass transfer across the 250 mm Sunfire C18 column. The selectivity factor was 2.5, indicating high chemical distinction between Codeine and Diazepam; also, a high resolution (Rs) of 4.22 was obtained, improving a complete baseline separation between the two substances and the column efficiency, indicated by the number of theoretical plates (N), was establish to be 2,579 for Codeine and 2,373 for Diazepam, that the platelet count for codeine (2579) is a bit higher than for diazepam (2373); this is normal because codeine appears first, the chance of peak diffusion reducing within the column which reflecting the sharpness of the eluted peaks. The required number of theoretical plates (N_{req}) was intended to determine the minimum efficiency required for separation. It was found to be about 380 plates for Codeine and 360 plates for Diazepam. The actual plates provided by the system (N) exceeded 2300, which

proves the high efficiency of the developed method, verifying dependence separation even under potential column aging conditions. The results, as summarized in **Table 1**, demonstrate high column efficiency and excellent separation between the two analytes, conforming to the international standards for pharmaceutical analysis.

Table 1. Chromatographic functions obtained via HPLC method.

Column Length, L, 250 mm
Dead Time, t_0, 2.9 min
Linear Velocity, u, 86.2 mm/min

Drugs	Retention Time,t_R,min	Retention Factor k′	Selectivity, α	resolution Rs	theoretical plates N	N_{effe}	N_{req}	symmetry factor (As)
Cod	4.19	0.44	2.5	4.22	2579	240	380	1.05
Diaz	6.09	1.10			2373	650	365	1.07

Under the studied experimental conditions, linearity for each drug was tested by injecting Codeine and Diazepam mixtures containing drugs in the range of concentration between 0.5-25 µg.mL^{-1}. A typical chromatogram for linearity for the two drugs under optimized HPLC conditions at different concentrations was obtained, **Figure 4**.

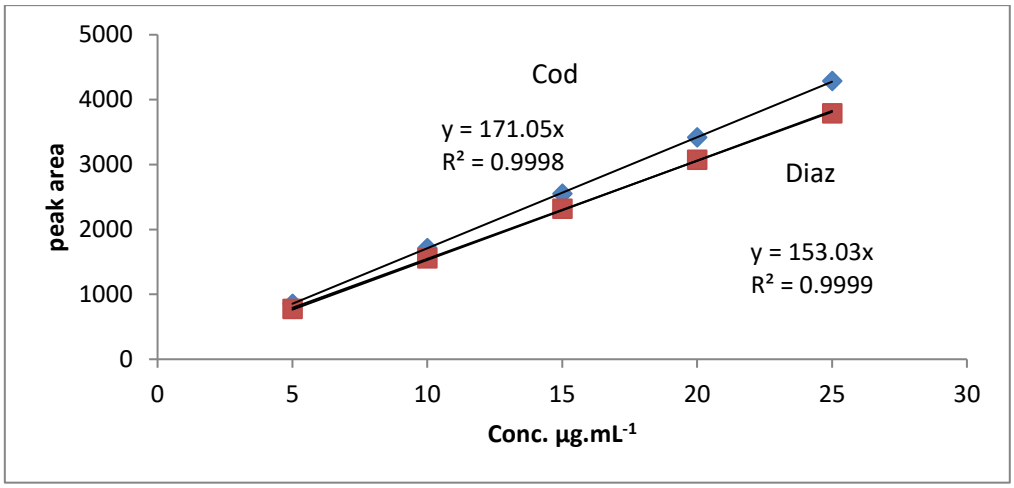

Figure 4. Calibration curve for Codeine and Diazepam using the developed HPLC method.

The calibration curve for Codeine exhibited a highly linear relationship between concentration and detector response within the range of concentration between 0.5-25.0 µg.mL^{-1}. The high value of the determination coefficient ($R^2 = 0.9998$) indicates the excellent precision of the HPLC system and the suitability of the chosen wavelength 230 nm for quantification. The new HPLC method for Codeine was tested by finding the Limit of Detection (LOD) and the Limit of Quantitation (LOQ). The LOD and LOQ were determined to be 0.15 µg.mL^{-1} and 0.45 µg.mL^{-1}, respectively, using the standard deviation of the response and the slope of the calibration curve. These low values indicate that the approach is highly sensitive and can accurately detect and quantify Codeine, even at low levels. The calibration curve for diazepam exhibited excellent linearity over five concentrations ranging from 5.0 to 25.0 µg.mL^{-1}. The regression analysis showed an excellent Determination coefficient ($R^2 = 0.9999$), the sensitivity of the method was further confirmed by the low values of LOD and LOQ, calculated as 0.18 µg.mL^{-1} and 0.54 µg.mL^{-1}, respectively. These results indicate to highly sensitive and reliable for the quantitative analysis of Diazepam in a pharmaceutical mixture by using the developed HPLC method, **Table 2**.

Table 2. Data analytical determination of Codeine and Diazepam.

Statistical Parameters	Codeine	Diazepam
Dynamic range, µg.ml^{-1}	5.0 - 25.0	5.0 - 25.0
Regression Equation	y=171.8x	y=150.84x
Coefficient of Determination, R^2	0.9998	0.9999
Coefficient of Correlation, r	0.9999	0.9999
LOD , µg.ml^{-1}	0.15	0.18
LOQ , µg.ml^{-1}	0.45	0.54

4.3. Precision and Accuracy

A symmetrical peak with no tailing was obtained by repeating the analysis three times. The t_R stayed the same each time. Relative error percentage (RE%) and relative standard deviation percentage (RSD%) for the drugs used to evaluate the accuracy of the proposed method. Three experiments were done on each drug at different concentrations (4, 8, 15, 25, and 35 µg.mL^{-1}) that were within the linearity range. The findings indicate that the proposed approach is highly accurate and precise at the examined concentrations, **Table 3**.

Table 3. Precision and accuracy of standard Codeine and Diazepam solutions via RP-HPLC method.

Drug	Initial Conc., µg.ml^{-1}	*Found conc., µg.ml^{-1} Mean±SD	Er%	RSD%	Recovery %
Codeine	4	3.99±0.0058	-0.320%	0. 1448%	99.65%
	8	7.99±0.0117	-0.125%	0. 1461%	99.87%
	15	14.98±0.0400	-0.133%	0. 2670%	99.86%
	25	25.03±0.0015	+0.120%	0. 0610%	100.20%
	35	34.97±0.0053	-0.085%	0. 0180%	99.91%
Diazepam	4	3.96±0.0305	-0.100%	0. 7682%	99.40%
	8	8.02±0.0578	+0.241%	0. 7217%	100.24%
	15	14.95±0.0603	-0.333%	0. 4030%	99.66%
	25	24.93±0.0177	-0.282%	0. 0700%	99.72%
	35	34.98±0.0262	-0.057%	0. 0749%	99.94%

*Avarege of three determinations

Evaluation efficiency and reliability of the suggested method for routine analysis, it was applied to the quantification of codeine and diazepam in selected commercial samples (tablets). The analysis was performed by comparing the extracted results with the design power recorded on the packaging, with a focus on recovery accuracy and repeatability. To ensure the absence of spectral interference from excipients, a diode-array detector (DAD) was also used, ensuring that the obtained peaks were for the substances under study[29,30]. The following table shows the accuracy and selectivity of the method.

Table 4. Assessment results of codeine and diazepam in pharmaceutical tablets.

Product	Ingredients	Label value mg per tablet	* Found conc., µg.ml^{-1} Mean±SD	Er %	RSD %	Recovery %
Pharmaceutical tablets	codeine	15 mg	14.934±0.0117	- 0.440	0.0781	99.56
		30 mg	30.081±0.0097	+0.270	0.0322	100.27
	diazepam	5 mg	4.963±0.0123	- 0.740	0.2474	99.26
		10 mg	9.946±0.0090	- 0.540	0.0898	99.46

*Average of three determinations

The average of three separate determinations (n = 3) for each value. The low Er% and RSD% values prove that the developed HPLC-DAD method is highly precise and accurate for assessing the quality of these tablets. The results in **Table 4** show that the method we developed is quite effective at detecting codeine and diazepam in their tablet forms. The approach achieved recovery rates of 99.46%- 100.27%, with a very low relative error (Er%), demonstrating that the data were correct and met the British Pharmacopeia (BP 2024) standards[30].

The reliability of these figures is further enhanced by the use of peak purity analysis via DAD detector. Although commercial tablets contain excipients such as starch and binders, UV fingerprint analysis of each peak proved the complete absence of any spectral or chemical interference (co-elution). The alignment of the purity angle with the programmed threshold confirms that the calculated areas in the table belong exclusively to the active ingredients, making this method an ideal tool for quality control to ensure the safety and quality of pharmaceutical products on the market[31,32].

4.4. Peak Purity assessment:

A peak purity assessment was performed using the DAD detector to ensure that there were no co-eluting contaminants or interference from excipients[32,33]. The purity angle for both Codeine and Diazepam was less than the purity threshold, indicating that each chromatographic peak signifies a distinct component. This validates that the developed method is highly specific for analyzing pharmaceutical formulations.

Table 5. A peak purity and purity threshold assessment.

active ingredient	Purity Angle	Purity Threshold	Result
Codeine	0.122	0.450	Pure
Diazepam	0.085	0.380	Pure

The peak purity results shown in **Table 5** provide verification of the chromatographic separation efficiency and the selectivity of the developed method. Technically, when the purity angle is smaller than the purity threshold, it indicates mathematical and spectroscopic evidence that the peak is only one chemical substance. The purity angle for codeine was 0.122, which is lower than the threshold of 0.450, and the purity angle for diazepam was 0.085 versus the threshold of 0.380. This considerable difference proves that the DAD detector did not detect any spectral variation across the entire peak from beginning to end, thus avoiding any interference from additives in commercial addition, such as starch, lactose, citrus, or any degradation products generated during preparation. These results prove the ability of the method for the detection and determination of pharmaceuticals quantity and quality.

4.5. Model validation

Table 6 presents the Design of Experiments (DOE) matrix used to examine how independent factors affect the efficiency of chromatographic separation using HPLC. The variables examined comprised the pH of the mobile phase (A), the acetonitrile (ACN) percentage in the mobile phase (B), and the flow rate (C). Resolution and retention time were used as replies to see how well the chromatographic technique worked. 20 trials were conducted, each with a different range for each variable, to find the ideal conditions for achieving the best separation of the chemicals we were studying while keeping analysis time as short as possible.

Table 6. Box-Behnken experimental design based on independent variables and experimental and predicted results of the chromatographic separation using HPLC.

Run	A:pH	B:ACN	C:Flow Rate	Resolution	Retention time
		%	mL/min		min
1	4.25	100	0.85	0.8	8.9
2	6.3522	50	0.85	2.5	12.3
3	5.5	80	0.5	1.1	2.1
4	5.5	20	0.5	3.8	7.5
5	4.25	50	1.43863	3.2	10.2
6	4.25	50	0.85	5.15	6.1
7	4.25	50	0.26137	4.5	6.08
8	4.25	50	0.85	5.1	3.5
9	4.25	50	0.85	5.2	7.9
10	3	80	1.2	1.2	4.2
11	3	80	0.5	1.9	6.12
12	5.5	80	1.2	0.7	4.9
13	4.25	50	0.85	5.05	1.2
14	2.14776	50	0.85	2.8	6.05
15	5.5	20	1.2	3.1	6.15
16	4.25	45	0.85	1.5	6.07
17	4.25	50	0.85	5.12	8.8
18	3	20	0.5	4.2	28
19	4.25	50	0.85	5.08	5.6
20	3	20	1.2	3.4	2.9

Analysis of variance (ANOVA) as shown in **Table 7** that the second-order statistical model was highly significant ($F = 15.56$, $p < 0.0001$), indicating its suitability for describing the relationship between the tested operational variables and the studied response within the scope of the adopted design. However, analysis of individual effects revealed a clear variability in the contribution of the different factors. The mobile phase pH (A: pH) did not show an independent significant effect ($p = 0.2708$), indicating that its impact on separation efficiency was limited within the studied range. In contrast, the percentage of acetonitrile in the mobile phase (B: %ACN) emerged as the most influential factor, showing strong statistical significance ($p = 0.0005$). This underscores the decisive role of mobile phase composition in dominating the retention mechanism and refining the accuracy of chromatographic separation. The flow rate (C: Flow rate) showed a less significant effect ($p = 0.0500$), proving the sensitivity of the system to minimum changes in this parameter, although its effect was less than that of the ratio of organic solvent. Between the factors (AB, AC, BC), the bilateral interaction coefficients did not show statistical significance, proving the absence of significant synergistic or antagonistic effects between the factors within the operating range experimental. This suggests that the system's response was primarily due to the singular effects of each factor and the quadratic terms (A^2, B^2, C^2) indicated high statistical importance, particularly B^2 ($p < 0.0001$), proving clear nonlinear behavior and justifying the adoption of a second-order model to accurately model the system under study[34].

Table 7. Model Significance Evaluation and R-squared Fit Quality Criteria for Chromatographic Separation Accuracy.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	47.33	9	5.26	15.56	< 0.0001	significant
A-pH	0.4593	1	0.4593	1.36	0.2708	
B-ACN	8.50	1	8.50	25.16	0.0005	

C-Flow Rate	1.68	1	1.68	4.96	0.0500	
AB	0.0450	1	0.0450	0.1331	0.7228	
AC	0.0200	1	0.0200	0.0592	0.8127	
BC	0.0200	1	0.0200	0.0592	0.8127	
A^2	10.80	1	10.80	31.94	0.0002	
B^2	28.08	1	28.08	83.07	< 0.0001	
C^2	2.81	1	2.81	8.30	0.0163	
Residual	3.38	10	0.3381			
Lack of Fit	3.37	5	0.6733	238.19	< 0.0001	significant
Pure Error	0.0141	5	0.0028			
Cor Total	50.71	19				
Std. Dev.	0.5814		R^2	0.9333		
Mean	3.27		Adjusted R^2	0.8733		
C.V. %	17.78		Predicted R^2	0.4951		
			Adeq Precision	12.8318		

df: Degrees of freedom 2. statistical significance 3 Indicates by p-value < 0.05. Adeq Precision: A measure of the signal-to-noise ratio; values > 4 are desirable

4.6. Model Diagnostics for Resolution Response

The trihedral and contour plots show the effect of three variables of pH value (A), percentage of acetonitrile (ACN%) (B), and flow rate (C) on resolution. The surfaces display a clear curvature, reflecting nonlinear effects and significant interactions between the variables. The red areas show the highest resolution values, that obtained at the midpoint of pH, ACN percentage, and flow rate. The elliptical contour plots also expose a common optimal range for the studied variables, supporting that resolution modification depends on their simultaneous interaction rather than the individual effect of each variable. Residual analysis indicates that the statistical model used is suitable and dependable for expected system behavior and optimization of the separation conditions, as shown in **Figure 5**. The histogram exhibits the distribution of accuracy values from the different experiments. The values are closely distributed without sharp deviations or irregular dispersion, indicating data homogeneity and the absence of significant outliers. This distribution suggests that the experimental design adequately covered the study range and that the response results are reliable[35]. Most of the points are near the straight line in the normal distribution of the residuals. This means that the residuals follow a normal distribution. This shows that the model's statistical assumptions are correct and that it is a good way to describe the relationship between the variables under study and the response. It also indicates the absence of significant deviations or systematic errors in the data. As shown in **Figure 6**.

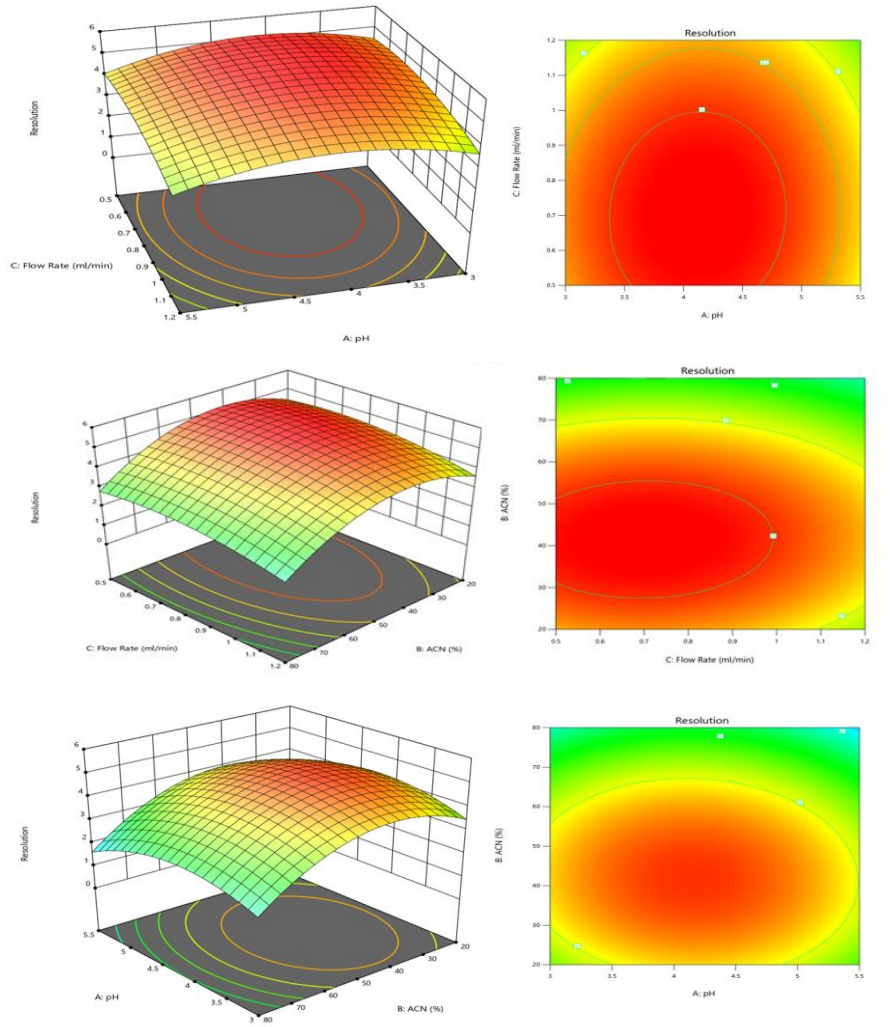

Figure 5. Response surface methodology plots demonstrating the interaction effects on chromatographic responses:(a) Acetonitrile ratio with Flow rate(b) Acetonitrile with pH, and(c) pH with Flow rate.

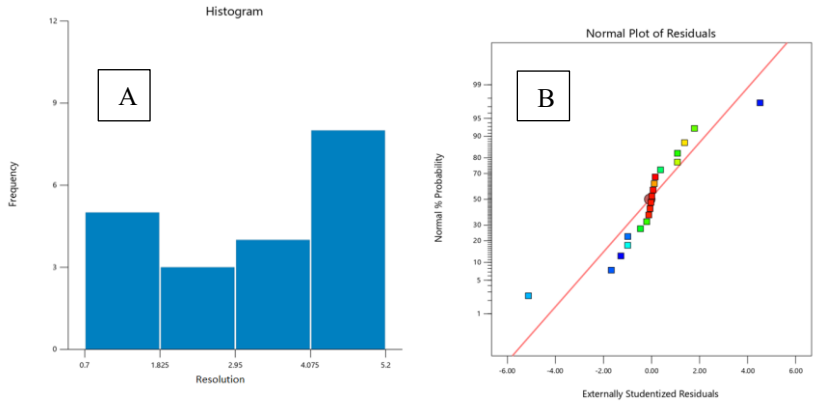

Figure 6. Model Diagnostics Plots A) Histogram of Residuals B) Normal plot of Residuals Analysis of Variance (ANOVA) for response Retention Time.

The analysis of variance (ANOVA) of the applied statistical model showed **Table 8**. The model's p-value was 0.0172, which is below the standard significance level (0.05). This clearly indicates that the model is statistically significant, meaning that the listed factors (pH, ACN ratio, and flow rate) and their interactions have a real, non-chance effect on the separation efficiency of codeine and diazepam. The F-value of 6.19 supports the model's predictive power. The Lack of Fit test yielded a p-value of 0.6750, indicating no significant lack of fit. This is a very strong sign for scientific study, since it suggests that the model fits the experimental data well and that there isn't too much variation that the model can't explain. The coefficient of

determination (R^2) was 0.9306, meaning that the model is able to explain approximately 93% of the variances in the separation accuracy (resolution). Adequate Precision: The ratio reached 11.83. Since it is much greater than the reference value (4), this confirms that the model signal is strong enough to be used for navigating within the Design Space and accurately determining optimal conditions[37,38].

Table 8. Model Significance Evaluation and R-squared Fit Quality Criteria for Chromatographic Separation Accuracy.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	550.96	13	42.38	6.19	0.0172	significant
A-pH	19.53	1	19.53	2.85	0.1422	
B-ACN	4.00	1	4.00	0.5850	0.4734	
C-Flow Rate	8.49	1	8.49	1.24	0.3081	
AB	24.26	1	24.26	3.54	0.1088	
AC	101.32	1	101.32	14.80	0.0085	
BC	93.37	1	93.37	13.64	0.0102	
A^2	18.92	1	18.92	2.76	0.1475	
B^2	4.33	1	4.33	0.6328	0.4566	
C^2	8.76	1	8.76	1.28	0.3010	
ABC	45.27	1	45.27	6.61	0.0422	
A^2B	59.72	1	59.72	8.72	0.0255	
A^2C	64.77	1	64.77	9.46	0.0218	
AB^2	65.01	1	65.01	9.50	0.0216	
Residual	41.07	6	6.85			
Lack of Fit	1.56	1	1.56	0.1979	0.6750	not significant
Pure Error	39.51	5	7.90			
Cor Total	592.04	19				
Std. Dev.	2.62		R^2	0.9306		
Mean	7.23		Adjusted R^2	0.7803		
C.V. %	36.20		Predicted R^2	0.3218		
			Adeq Precision	11.8319		

df: freedom Degrees 2. p-value < 0.05: Indicates statistical significance 3. Adeq Precision: A measure of the signal-to-noise ratio; values > 4 are desirable

4.7. Model Diagnostics for Retention Time Response

The results of the response surface model show that the percentage of acetonitrile (%ACN) is the most influential factor on retention time. A higher percentage leads to a significant decrease in retention time due to increased mobile phase strength and reduced interaction of the compound with the stationary phase. An increased flow rate also reduces retention time because it decreases the sample's residence time in the column. In contrast, the pH effect was less recognizable and is attributed to changes in the ionization state of the substances, which reflects on their interaction strength with the stationary phase. The contour maps and 3D surfaces demonstrate an interaction between the variables under study, particularly between %ACN and flow rate, which indicates the suitability of the statistical model used, as shown in **Figure 7**. The retention time histogram proves that the data are reasonably distributed without significant skewness or obvious outliers. The normal plot of the residuals shows that most values lie close to a straight line, suggesting the residuals are rather evenly distributed. **Figure 8** shows that the normality assumption, the most important requirement for the regression model to be valid and satisfied.

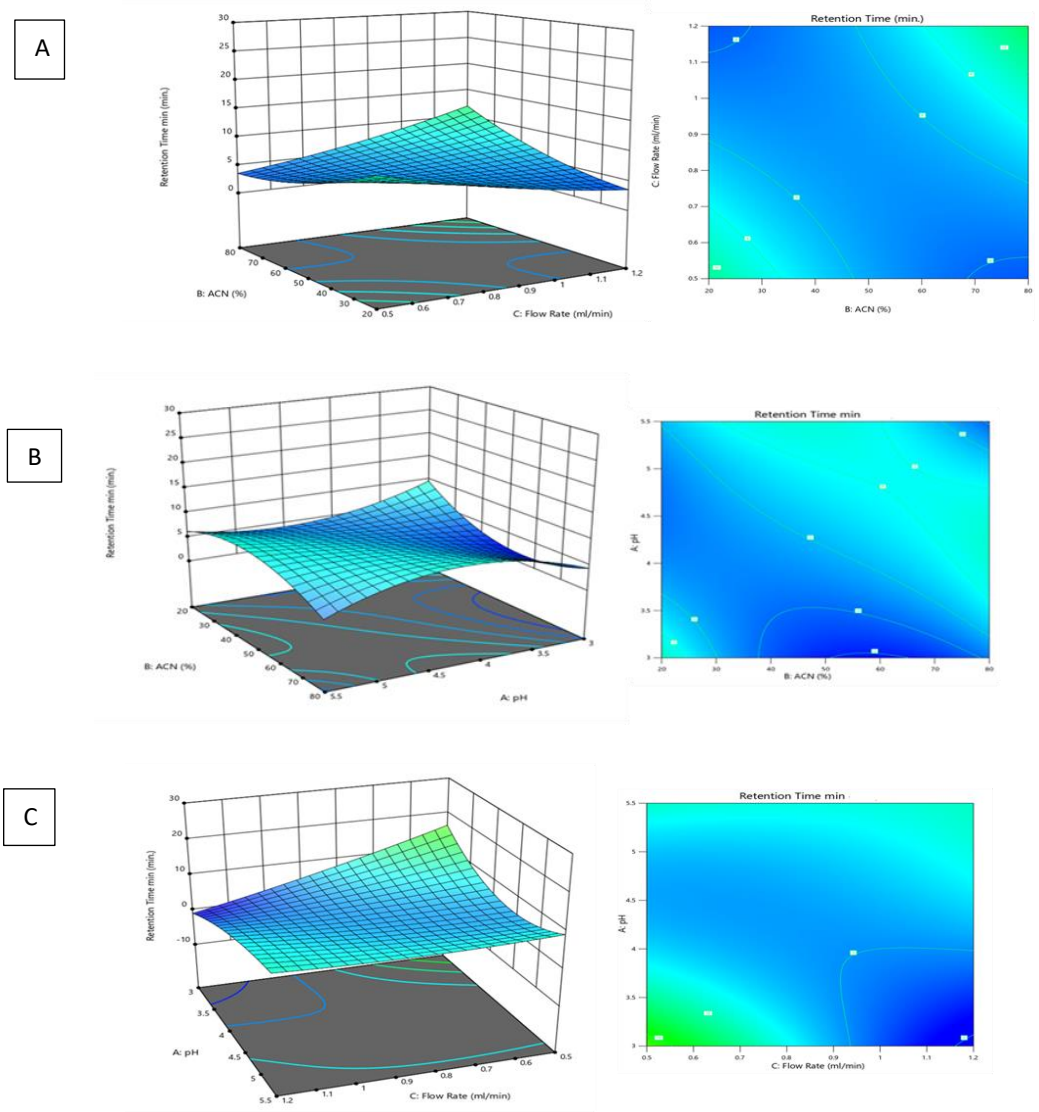

Figure 7. Response surface methodology plots demonstrating the interaction effects on chromatographic responses: (a) Acetonitrile ratio with Flow rate (b) Acetonitrile ratio with pH, and (c) pH with Flow rate.

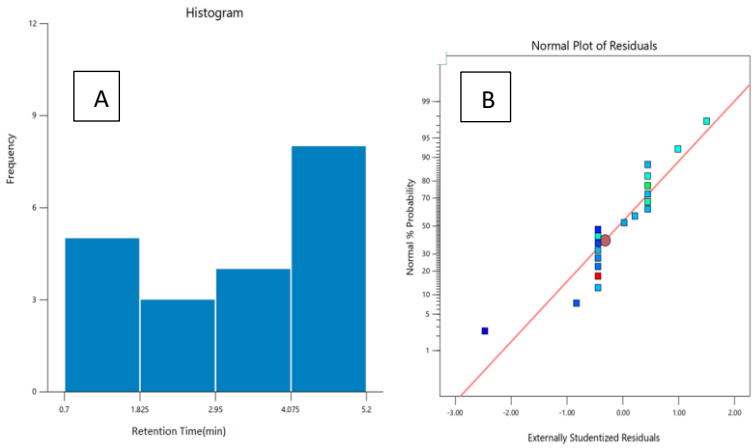

Figure 8. Model Diagnostics Plots A) Histogram of Residuals B) Normal plot of Residual.

5. Conclusion

The results of this study, which used HPLC with a DAD detector to simultaneously quantify codeine and diazepam, indicate that the developed method is very effective in separating the two substances in record time, less than 7 minutes, with an ideal separation degree (Rs = 4.22). This saved time and potential in routine analysis. The calibration curves were highly significant linear (R2 > 0.999), and the recovery rates were about 100%, proving that the developed method is highly accurate and adheres to pharmacopoeias. The method accurately determines the active substance using the peak purity test, without interference from other substances or additives which were commonly found in commercial tablets. The low LOD and LOQ values also demonstrated the approach's sensitivity and its ability to detect extremely small amounts. This suggests that the proposed method is a viable, efficient, and cost-effective approach for analyzing pharmaceutical formulations to detect both codeine and diazepam simultaneously. The Response Surface Model (RSM) was successfully applied to optimize chromatographic separation conditions. The results demonstrated that the quadratic model was statistically accurate and significant ($p < 0.05$) in predicting the behavior of the compounds. Through numerical optimization, an optimal point was achieved, ensuring the highest degree of separation (Rs = 4.22) in a record time of less than 7 minutes. This enhances the efficiency of the proposed method in routine analysis by saving time and organic solvents, while maintaining the highest standards of accuracy and reliability.

Conflict of interest

The authors declare no conflict of interest.

References

1. Ravindran, A., Sharma, T., & Sankhla, M. S. (2024). Comprehensive Analysis of Detection Methods for Over-the-counter Codeine: A Systematic Review. Journal of Forensic Science and Medicine, 10(4), 309-327.
2. Oguche, E., Musa, S. A., Agbon, A. N., Magaji, M. G., & Danborno, A. M. (2024). The Effect of Heinsia crinita Aqueous Leaf extract on Codeine-induced Changes of Brain Antioxidant Parameters, TNF-α and Interleukin-6 in Male Wistar rats. Biological Sciences, 4(3), 746-753.
3. Moradi, R., Khalili, N. P., Septiani, N. L. W., Liu, C. H., Doustkhah, E., Yamauchi, Y., & Rotkin, S. V. (2022). Nanoarchitectonics for abused‐drug biosensors. Small, 18(10), 2104847.
4. Singh, D. S., & Pflugmacher, S. (2026). Ecotoxicological assessments of over-the-counter NSAID (diclofenac, ibuprofen, aspirin) and antipyretic (acetaminophen) pharmaceuticals: a review of their toxicity effects on aquatic crustaceans. Integrated Environmental Assessment and Management, 22(1), 47-83.
5. Ezzati, A., Fanning, K. M., Reed, M. L., & Lipton, R. B. (2023). Predictors of treatment‐response to caffeine combination products, acetaminophen, acetylsalicylic acid (aspirin), and nonsteroidal anti‐inflammatory drugs in acute treatment of episodic migraine. Headache: The Journal of Head and Face Pain, 63(3), 342-352.
6. Zarin, M. K. Z., Dehaen, W., Salehi, P., & Asl, A. A. B. (2023). Synthesis and modification of morphine and codeine, leading to diverse libraries with improved pain relief properties. Pharmaceutics, 15(6), 1779.
7. Pratiwi, R., Noviana, E., Fauziati, R., Carrão, D. B., Gandhi, F. A., Majid, M. A., & Saputri, F. A. (2021). A review of analytical methods for codeine determination. Molecules, 26(4), 800.
8. Mohsen, E. M., Mahood, A. M., & Al Hindawi, A. M. (2022). Spectrophotometric method for the determination of chlordiazepoxide, diazepam and alprazolam drugs by using oxidation coupling reaction. International journal of health sciences, 6(S9), 1260-1276.
9. Di Capua, A., Reale, A., Paolino, M., Chemi, G., Brogi, S., Cappelli, A., ... & Anzini, M. (2020). Design, synthesis and biological evaluation of 7-substituted 4-phenyl-6H-imidazo [1, 5-a] thieno [3, 2-f][1, 4] diazepines as safe anxiolytic agents. European Journal of Medicinal Chemistry, 200, 112405.
10. da Silva, A. V., Meneghetti, S. M., & Meneghetti, M. R. (2021). Benzodiazepines: Drugs with chemical skeletons suitable for the preparation of metallacycles with potential pharmacological activity. Molecules, 26(9), 2796.
11. Ayyed, Z. T., & Wadee, S. A. Effect Of Diazepam And Tramadol On Some Biochemical Parameters In Male Rabbits. Journal of Advanced Sciences and Nanotechnology, 2(1), 182-187.

12. Mahjoub, M., & Alizadeh, N. (2023). Rapid spectrophotometric method for Diazepam quantification in pharmaceutical formulations using ion pair formation with Methyl orange and Bromophenol blue. Journal of Applied Spectroscopy, 90(3), 676-685.
13. Tsvetkova, D., Ivanova, S., & Chaneva, M. (2023). UV-Spectropectrophotometry for Determination of Diazepam by Comparative Estimation of Methods of Calibration Curve and Reference Standard. International Journal of Pharmaceutical Research and Allied Sciences, 12(2-2023), 108-115.
14. Tomić, M., Božinovska, M., Anevska-Stojanovska, N., Lazova, J., Petkovska, R., Anastasova, L., & Nakov, N. (2023). Eco-friendly RP-HPLC method for determination of diazepam in coated tablet: Green RP-HPLC for diazepam determination. Macedonian Journal of Chemistry and Chemical Engineering, 42(2), 153-163.
15. Sabah, R., Al-Ameri, S. A., & Al-Kazazz, F. F. (2019). SIMULTANEOUS HPLC ESTIMATION OF METHAMPHETAMINE AND TRAMADOL ILLICIT DRUGS IN IRAQI HUMAN ADDICTS. *J ATMPH*, *Annals of Tropical Medicine & Public Health*, 22(12).
16. Dural, E., & Kaya, B. N. (2022). Determination of diazepam in human plasma by developed and validated a high-performance liquid chromatographic ultraviolet method. İstanbul Journal of Pharmacy, 52(1), 37-46.
17. Darwish, H. W., Ali, N. A., Naguib, I. A., El Ghobashy, M. R., Al-Hossaini, A. M., & Abdelrahman, M. M. (2021). Development and validation of a stability indicating RP-HPLC-DAD method for the determination of bromazepam. PLoS One, 16(3), e0244951.
18. Buratti, E., Cippitelli, M., Mietti, G., Scendoni, R., Froldi, R., Cerioni, A., & Cingolani, M. (2023). Validation of an HPLC‐HR-MS method for the determination and quantification of six drugs (morphine, codeine, methadone, alprazolam, clonazepam and quetiapine) in nails. Journal of Analytical Toxicology, 47(5), 488-493.
19. [19] Putri, A. L., & Mansur, U. (2022). The Development of Derivative Method analysis 1, 4 Benzodiazepines in Biological Matrix using High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography-Mass. Pharmaceutical and Biomedical Sciences Journal (PBSJ), 4(1), 1-12.
20. Snyder, L. R., Kirkland, J. J., & Dolan, J. W. (2011). Introduction to modern liquid chromatography. John Wiley & Sons.
21. Ermer, J., & Nethercote, P. W. (Eds.). (2025). Method validation in pharmaceutical analysis: A guide to best practice. John Wiley & Sons.
22. Waters Corporation. (2020). Understanding Peak Purity Analysis in HPLC with Photodiode Array Detection. technical note. Milford, MA: Waters Corp.
23. Chakraborty, S., Sharmin, S., Rony, S. R., Ahma, S. A. I., & Sohrab, H. (2018). Stability-indicating UV/Vis Spectrophotometric Method for Diazepam, Development and Validation. Indian Journal of Pharmaceutical Sciences, 80(2).
24. Johnson-Davis, K. L. (2018). Opiate & benzodiazepine confirmations: to hydrolyze or not to hydrolyze is the question. The Journal of Applied Laboratory Medicine, 2(4), 564-572.
25. Uddin, M. N., Samanidou, V. F., & Papadoyannis, I. N. (2014). An overview on total analytical methods for the detection of 1, 4-benzodiazepines. Pharm. Anal. Acta, 5(6), 1000303.
26. Stoeva-Grigorova, S., Hvarchanova, N., Gancheva, S., Eftimov, M., Georgiev, K. D., & Radeva-Ilieva, M. (2025). Differentiation of Therapeutic and Illicit Drug Use via Metabolite Profiling. Metabolites, 15(11), 745.
27. Yang, M., Mei, G., Huang, D., Zhang, X., & He, P. (2025). Quantitative Analysis of Diazepam Residues in Aquatic Products Using Magnetic Solid-Phase Extraction Combined with Ultra-High-Performance Liquid Chromatography‐Tandem Mass Spectrometry. Foods, 14(23), 4087.
28. Shinde, D., Karubothula, B., Gupta, P., Tadala, R., Almansoori, A., AlQaydi, M., ... & Brudecki, G. (2025). Advances in the Analysis of Contaminants, Illicit Drugs, Pharmaceuticals, and Hormones for Wastewater-Based Epidemiology (WBE) Using Automated Extraction and Detection by LC and GC-MS/MS: Evaluation of Method Eco-Friendliness and Practicality.
29. Pandit, R. K., & Pandey, V. I. V. E. K. (2025). Development and Validation of an Rp‐HPLC Chromatographic Method for the Determination of Related Substances in a Polypharmaceutical Oral Suspension With Ion Exchange Resin‐Based Taste Masking. International Journal of Applied Pharmaceutics, 17(4), 216-230.
30. Akhila, B., & Kumar, S. T. (2024). PHARMACOPOEIA. International Journal of Life Sciences Biotechnology and Pharma Sciences, 20(2), 1-19.
31. Sravanthi, G., Gandla, K. S., & Repudi, L. (2023). New analytical method development and validation for estimation of molnupiravir in bulk and tablet dosage form by RP-HPLC method. Cellular, Molecular and Biomedical Reports, 3(3), 130-136.
32. Tyagi, I., Nagarajan, K., & Bhardwaj, S. (2025). Analytical Method Development and Validation of Efavirenz by Using RP-HPLC. International Journal of Pharmaceutical Investigation, 15(1).

33. Chagarlamudi, K., Maddala, V. K. S., & Gandla, K. (2025). A Quality by Design Approach to Greener Ultrahigh‐Performance Liquid Chromatography Method Development for the Quantitative and Impurity Analysis of Favipiravir. Separation Science Plus, 8(11), e70153.
34. Akash, M. S. H., & Rehman, K. (2025). Comprehensive Insights into Pharmaceutical Analysis. In Essentials of Pharmaceutical Analysis (pp. 1-62). Singapore: Springer Nature Singapore.
35. Mohamed, A. A. (2024). Development of Response Surface Approach for Determination⋯ Journal of Chromatographic Science
36. nyder, L. R., Kirkland, J. J., & Glajch, J. L. (2012). Practical HPLC Method Development. John Wiley & Sons.
37. awhari, A. H., & Mohamed, Z. H. (2025). Developing and Validating a Robust RP-HPLC Method⋯ International Journal of Analytical Chemistry.
38. Rulla Sabah, Nisreen Kais Abood, Mustafa Hammadi.(2026), Extraction of bioactive phenolic compounds from yerba mate leaves using ultrasound: HPLC profiling and statistical analysis of variables Applied Chemical Engineering (2026) Volume 9 Issue 1.