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Analytical Method Development and Validation: Calcium Butyrate

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Abstract

This study was designed for the analytical method development and validation for Calcium butyrate (CAB) using High-Performance Liquid Chromatography (HPLC). ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared (FTIR) spectroscopy analysis were carried out to examine the molecular structure. For HPLC analysis, a blend of acetonitrile and phosphoric acid solution (0.1%) (20:80) (*v*/*v*) was used as a mobile phase. A C18 column (5 µm, 250 × 4.6 mm) was used as a stationary phase. The eluent of CAB was monitored with a UV detector at a wavelength of 206 nm and a flow rate of 1 mL/min. According to the results, FTIR and NMR spectra were consistent with the structural characteristics of CAB, and the expected proton and carbon signals were observed. During the HPLC analysis, due to the ionization of CAB, three distinct retention times were observed in the chromatograms at 3.4, 4.5, and 7.4 min. The validation was performed according to ICH guidelines. The obtained results demonstrated that the analytical method was successfully validated, with LOD values of 1.211, 0.606, and 1.816 µg/mL, and LOQ values of 3.670, 1.835, and 3.676 µg/mL. The assay displayed a linear range of 5–1000 µg/L concentration and was found suitable for further formulation content analysis.

Keywords: calcium butyrate; HPLC; method development; validation

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1. Introduction

It is of great importance to develop and validate specific analytical methods for each substance for precise and accurate quantification of active substances in pharmaceutical products [1]. These studies, which are carried out both in research and development studies and by the units responsible for quality in the pharmaceutical industry, are closely related to the physicochemical properties of substances and play essential role in the correct amount, efficacy and safety of drugs [2]. HPLC is the primary pharmaceutical and biomedical analysis method used today due to its ability to provide high-efficiency separations and, in most cases, high detection sensitivity. Compared to other methods, HPLC offers numerous advantages, including specificity, speed, accuracy, sensitivity, and ease of automation. Due to the limitations of methods such as LC-MS/MS in terms of sensitivity, specificity, and efficiency parameters, which are closely related to each other, as well as their high cost and the need for qualified personnel, HPLC analyses may be preferred in the pharmaceutical field where multi-component dosage forms are involved [3,4].

Butyric acid is recognized as a prebiotic substance that regulates metabolism and serves as an organic acid source. It is produced in colon microbiota by fermentation of nutritional contents, controls the metabolism and motility, and more importantly, protects from infection and mediates inflammatory response. Due to its low molecular weight, it reduces fat accumulation in some farm animals through a down-regulation mechanism via free fatty acid binding receptors [5,6].

Moreover, short-chain fatty acids, such as butyric acid, play a role in promoting immune function through butyrate, achieving this by decreasing pro-inflammatory cytokines and diminishing the proliferation and activation of lymphocytes through inhibition of the NF-κB pathway [5].

Calcium butyrate is a fatty acid salt whose chemical structure can be seen in Figure 1, which serves as nutrition for colonocytes and promotes the development of epithelial cells and their viability [7]. Since 10–20% of the bacteria in the body belong to butyrate-producing families, the biosynthesis of butyrate is a distinctive metabolic activity that supports health. These are mainly revealed because of the complex carbohydrates, which could not be digested initial intestinal part through pyruvate and acetyl-coenzyme A [8].

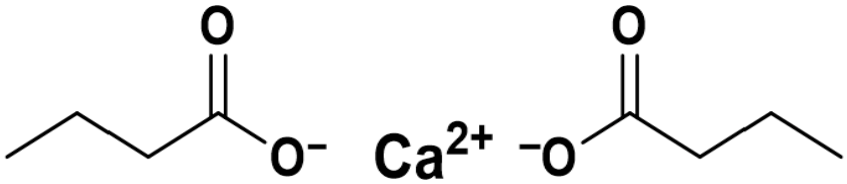


Figure 1. Chemical structure of calcium butyrate.

Various clinical studies and activity assessments, which have mostly focused on butyric acid, have demonstrated the anti-inflammatory effects of both butyric acid and CAB. However, it is crucial to develop a validated analytical method for determining characteristics such as quantitative determination, in vitro release determination, and ex vivo permeation, which are critical processes for formulation development stages [7,9–11]. Although numerous studies have reported the analysis of short-chain fatty acids [12–16] and a limited number have focused specifically on sodium butyrate [17,18], research on the development and validation of analytical methods for CAB remains insufficient. Non-acidic mobile phases were used in the analyses developed for sodium butyrate. This suggests that sodium butyrate and CAB, with pKa values between 4 and 5, cause ionization. The present study aimed to develop and validate an HPLC-based analytical method for CAB in accordance with the International Council for Harmonisation (ICH) guidelines. The validated method is expected to serve as a reliable tool for application in future formulation studies.

2. Experimental

2.1. Materials

Acetonitrile (HPLC grade) and o-phosphoric acid were procured from Merck, Darmstadt, Germany. CAB (95% purity) was gifted from Medera Nutrition BV, Utrecht, The Netherlands, Izmir, Türkiye. Ultrapure water was procured from the Direct-Q® Water Purification System, Merck, Darmstadt, Germany. Throughout the study, the chemicals and solvents used were of analytical or HPLC grade.

2.2. FTIR Analysis

The sample was scanned between 4000–600 cm^{-1} through an FTIR (Shimadzu IRSpirit-X, Kyoto, Japan) spectrophotometer, and the wavelengths at which the peaks occurred were determined [19].

2.3. NMR Analysis

NMR spectra of CAB were saved with the help of a Bruker Avance-III 400 Fourier Transform spectrometer (Karlsruhe, Germany) operating at 400 MHz for ^{1}H and ^{13}C NMR equipped with a 5 mm probe, in deuterium oxide (D2O), with all shifts referring to internal tetramethyl silane (TMS). Baseline correction and imaging studies of the ^{1}H NMR spectra were carried out using the software Mestrenova v14.1.2–25024 (Mestrelab Research SL, Santiago de Compostela, Spain).

2.4. Instrumentation

An instrument including a gradient pump, thermostable column section, and a UV detector (Agilent 1200, Santa Clara, CA, USA) was used for HPLC analysis. A temperature-controlled C18 column (5 µm, 250 × 4.6 mm) (Develosil, Seto, Japan) was utilized in this system. UV detectors were used throughout the analysis. ChemStation v4.03.016 software was used for the analysis conditions and all results obtained.

2.5. Chromatographic Conditions

The optimum wavelength was selected by following a previous study carried out [20] for the determination of CAB and to prevent the undesired noise.

The mobile phase was prepared as a mixture of acetonitrile and o-phosphoric acid solution (0.1%) (20:80) (*v*/*v*) and pumped at a 1 mL per minute flow rate. The temperature of the column was fixed at 30 °C over the analyses, and the injection volume of the sample was set to 20 µL. Before sample injection into the device, the column was equilibrated by passing the mobile phase through it for at least 40 min. All prepared samples were filtered through a membrane filter with a 0.45 µm pore size prior to analysis. The run time was set to 10 min for each sample.

2.6. Preparation of Stock Solution and Standard Sample Solutions

Stock solutions (1000 µg/mL) of CAB were prepared by dissolving 250 mg of the drug in 250 mL of the mobile phase mixture. The prepared standard solutions were stored at 4 °C in amber-colored glass volumetric flasks. Concentrations of 5, 10, 25, 50, 100, 200, 400, 600, 800, and 1000 µg/mL were selected for the creation of calibration curves. These working solutions were freshly prepared before the analyses. All samples were filtered using a 0.45 µm pore size membrane filter before being injected into the device.

2.7. HPLC Method Validation

The validation of the method was examined in terms of linearity, LOD, LOQ, precision, accuracy, specificity and robustness according to ICH guidelines Q2(R1) [21].

2.7.1. Linearity

The linearity between the peak areas in the chromatograms obtained from the analysis of the samples and the concentration was analyzed using a calibration curve created in the range of 5 to 1000 µg/mL.

2.7.2. Specificity

Specificity was examined by analyzing the mobile phase and solutions of CAB, which will be used to prepare formulations.

2.7.3. Precision

To verify the precision parameter, 200 µg/mL of CAB solution was injected into the device ten times, and the mean, standard deviation (SD), and coefficient of variation (CV%) values of the series were determined.

The repeatability of the method was tested using six different samples on consecutive days. Afterwards, intermediate precision was tested by two different analysts with six different levels.

2.7.4. Accuracy

The three prepared standard solutions (200, 400, and 1000 µg/mL) were analyzed five times at different levels as test samples using the device.

2.7.5. Robustness

The robustness of the developed method was determined by the peak area values of the prepared solutions, which were tested at various time points (30, 60, 120, and 240 min, as well as 24 and 48 h) in accordance with the guidelines.

2.7.6. Determination of Detection Limit and Quantification Limit

The LOD value of the developed method for CAB is calculated by dividing the SD of the data obtained from the minimum concentrations in the sampling series by the slope of the calibration curve and multiplying by 3.3; the LOQ is calculated by multiplying by 10.

3. Results and Discussion

3.1. FTIR Analysis

The spectrum of CAB was examined between 4000 and 600 cm^{-1} with a resolution of 4 cm^{-1}. The stretching between 3000 and 2800 cm^{-1}, especially in 2962 cm^{-1}, exhibited C-H stretching of methyl groups of the structure, similar to the literature [22]. Moreover, the strong bands in 1541 cm^{-1} and 1411 cm^{-1} demonstrate the stretching vibrations of the COO^{-} group. The band at 1022 cm^{-1} was evaluated as the C–O stretching vibrations belong to the molecule [23]. The spectrum of CAB is shown in Figure 2.

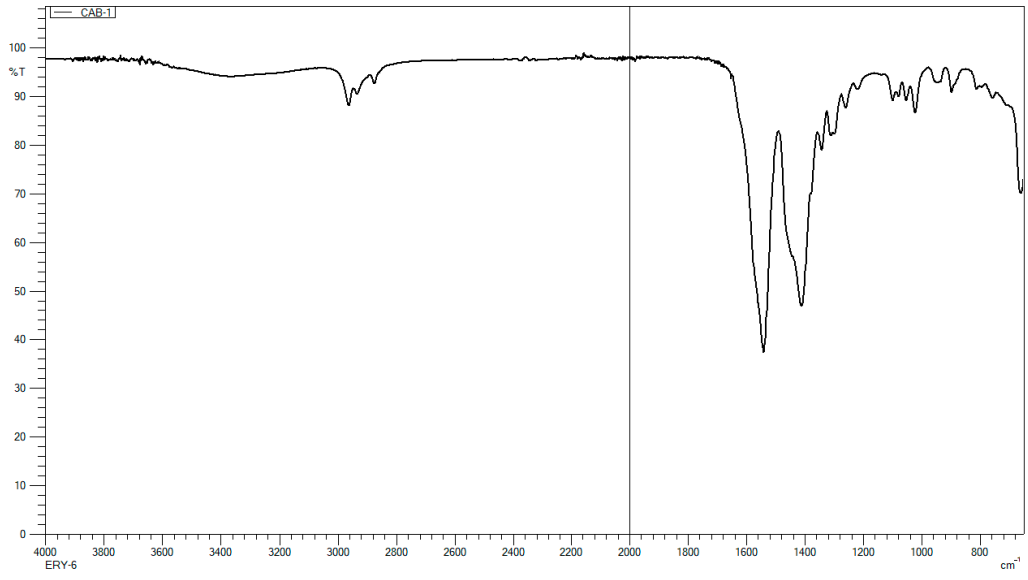


Figure 2. FTIR spectrum of calcium butyrate.

3.2. NMR Analysis

^{1}H NMR (δ, ppm, solution) and ^{13}C NMR (δ, ppm, solution) analysis findings are given in Figures 3 and 4. The obtained spectra confirm that it is an aliphatic compound. Signals in the 0.6 to 2.4 ppm range in the ^{1}H spectrum and in the 10–40 ppm region in the ^{13}C spectrum prove the presence of aliphatic carbon signals, especially terminal -CH3. When examining the ^{1}H spectrum, the sharp singlet peak observed at 1.788 ppm is attributed to the -CH3

group. The multiplets observed between 0.7–0.95 ppm and 1.40–1.50 ppm are signals from branched alkane methyl groups and, consequently, from the -CH_2-CH_2- groups in the butyrate structure. The multiplets observed in the 2.0–2.1 range are interpreted as a signal originating from the carbonyl group adjacent to the alkyl group [24,25]. Considering the ^{13}C spectrum, signals between 180–190 ppm, especially 181.899 ppm, belong to the carbonyl group. Additionally, signals of similar intensity observed in the 23.259 ppm, 19.246 ppm, 39.490 ppm and 13.243 ppm indicate the presence of multiple equivalent carbons as in aliphatic groups (CH_3-CH_2-CH_2-) of butyrate [26]. Both spectra demonstrate that they belong solely to CAB.

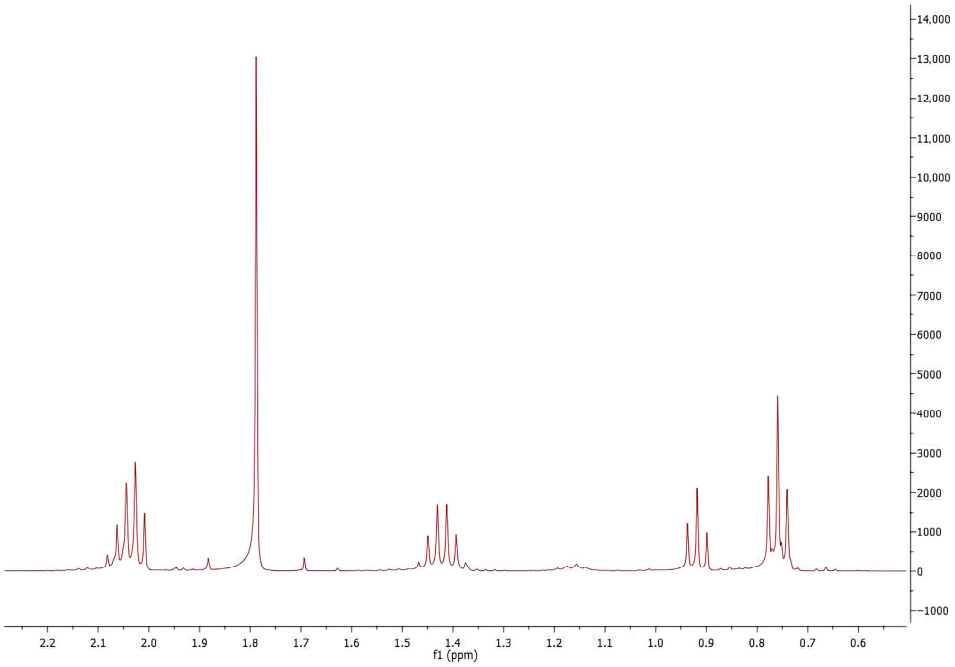


Figure 3. ^{1}H NMR Spectrum of Calcium Butyrate.

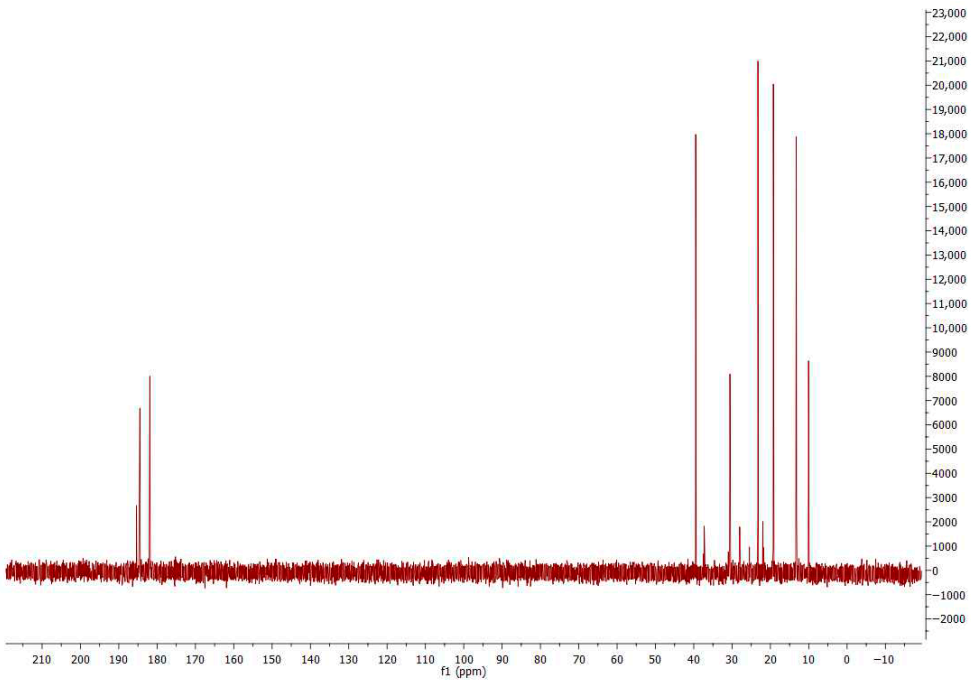


Figure 4. ^{13}C NMR Spectrum of Calcium Butyrate.

3.3. HPLC Analysis and Validation

The analyses were performed using UV light at a low wavelength, such as 206 nm. The choice of low wavelength is due to the absence of chromophore groups in the molecular structures of short-chain fatty acids, as well as the UV spectrophotometer absorbance values of calcium butyrate [16]. During the analysis, the chromatograms displayed three different peaks in 3.4, 4.5 and 7.4min as shown in Figure 5B. Each peak showed area values directly proportional to the concentration given.

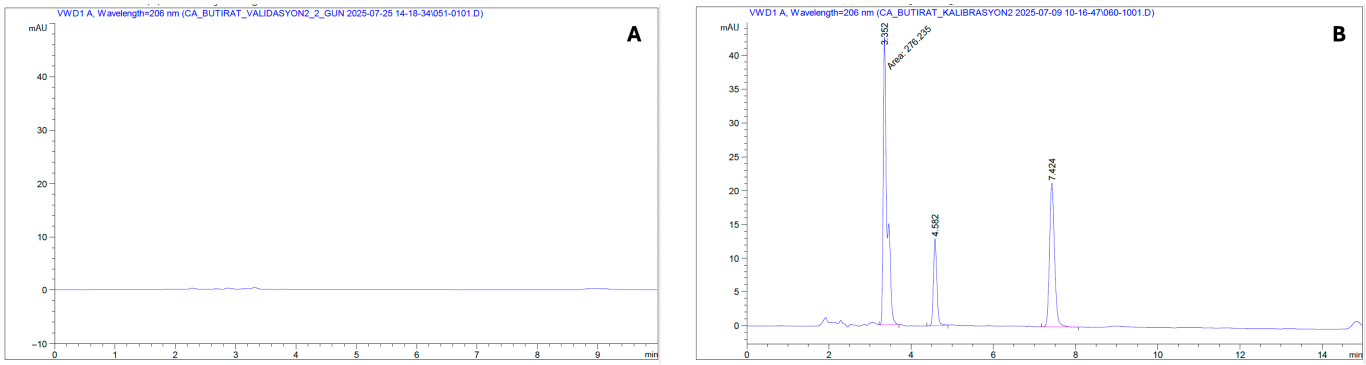


Figure 5. The chromatograms of (**A**) a sample which does not contain calcium butyrate and (**B**) calcium butyrate analysis (The bases of the peaks are marked with colored lines to indicate the areas).

As seen in the results obtained, CAB does not behave as a single elute in the mobile phase with an acidic aqueous environment used in the method. Considering the pKa value (~4–5), the analyte exists in equilibrium as multiple ionic forms (protonated butyric acid and butyrate anion) and calcium counterion binder/ion pairs. These coexisting species differ in terms of hydrophobicity and ion pairing interactions and therefore exhibit distinct retention on the column, yielding three reproducible peaks (3.4, 4.5, 7.4 min). The absence of these peaks in blank readings, the proportional increase in each peak area with CAB concentration in the range of 5–1000 µg/mL ($R^2 \geq 0.998$), the near 100% precision, and the low %RSD confirm the analytical results for CAB. While we were unable to perform additional mass-based chromatographic analyses (e.g., LC–MS) or peak purity tests at this stage, which we recognize as a limitation, the reproducible chromatographic data together with FTIR and NMR analyses provide structural insight into CAB and support our explanation of the three peaks. The obtained results are consistent with HPLC analyses of short-chain fatty acids performed at low wavelengths such as 210 nm. In such compounds, the pH of the mobile phase, ionization state, and ion pairing can affect retention on the column and lead to separate, reproducible peaks for different types of the same substance. However, when these conditions are carefully controlled, this situation does not adversely affect the accuracy of quantitative analyses. De Baere and colleagues demonstrated the applicability of non-derivatized HPLC for short-chain fatty acids and emphasized the role of mobile phase conditions on retention [16]. A similar result was also observed by Low et al. During the analysis of catecholamines, sodium heptane sulfonate (ion-pair reagent) and sodium sulfate (neutral salt) were used in different proportions in the C18 column and mobile phase. Acetic acid was used to maintain a pH of around 3, ensuring the protonation of all compounds. HPLC analyses of ephedrine showed that the peaks were split into two and three, with the first peak belonging to the ephedrine–heptane sulfonate ion pair, as seen in GC-MS analyses. Here, the formation of ion pairs, their adsorption onto the hydrophobic phase, and the dynamic ion exchange mechanism, whereby the ion-pair reagent adsorbs onto the column surface and forms a temporary ion exchange surface, are explained as the mechanisms causing the formation of three peaks. Therefore, it is assumed that peak splitting is a physical phenomenon observed in reverse-phase columns and that

the separation of ion pairs by HPLC is based on a mixed mechanism [27]. Additionally, Jiang et al. demonstrated in their study on tobacco-specific N-nitrosamines that the stability of the E/Z isomers of these molecules changes depending on their protonation state and that mobile phase pH and temperature affect peak separation [28].

A calibration curve was created in the concentration range of 5 to 1000 µg/mL, and three independent determinations were performed to determine the area values corresponding to each concentration. As shown in Figure 6, the data examined for each different peak vary linearly in the concentration range studied for CAB. Additionally, the standard deviations of the slopes and intercepts were found to be low. The value R^2, the determination coefficient for the regression line, is 0.998 at each peak. The slopes and intercepts of three different peaks are as follows:

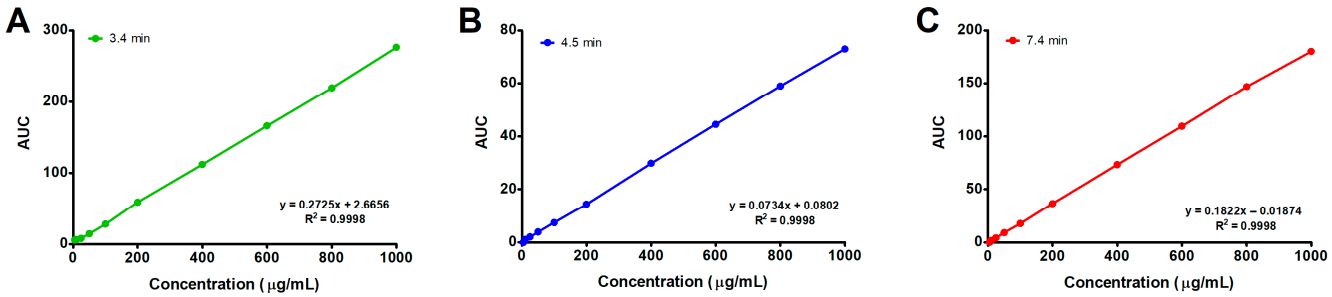


Figure 6. The regression line for calcium butyrate (**A**) 1st peak at 3.4 min, (**B**) 2nd peak at 4.5 min, (**C**) 3rd peak at 7.4 min.

Min 3.4: y = 0.2725x + 2.6656

Min 4.5: y = 0.0734x + 0.0802

Min 7.4: y = 0.1822x − 0.1874

System suitability was assessed at the beginning of each analytical batch (n = 10 injections, 200 µg/mL). The mean backpressure ranged from 119.47 ± 1.26 to 120.23 ± 1.01 bar. The relative standard deviation (RSD) of retention time was ≤0.5% for each of the three CAB peaks, while the peak area RSD remained ≤2%. Theoretical plate counts were 12,060.70 ± 166.84, 15,058.90 ± 743.55, and 19,264.70 ± 364.97 for the peaks eluting at 3.4, 4.5, and 7.4 min, respectively. Retention time variation across 10-sample sequences was within ±0.051, ±0.009, and ±0.022 min for the 3.4, 4.5, and 7.4 min peaks, respectively. The resolutions of these peaks were 9.342 ± 0.098, 7.739 ± 0.118, and 15.584 ± 0.188, while their selectivity values were 1.480 ± 0.000, 1.303 ± 0.005, and 1.620 ± 0.000, respectively.

The system suitability parameters obtained demonstrated excellent chromatographic performance and high method reproducibility. The mean backpressure values (119.47–120.23 bar) indicated stable system operation throughout the injection series, confirming the robustness of the chromatographic conditions. The low retention time RSD (≤0.5%) for all three CAB peaks reflected high precision and stability of the analytical system, while the peak area RSD (≤2%) further supported the repeatability of detector response.

Theoretical plate numbers for the peaks at 3.4, 4.5, and 7.4 min (12,060.70 ± 166.84, 15,058.90 ± 743.55, and 19,264.70 ± 364.97, respectively) were well above the minimum acceptable limit of 2000, indicating good column efficiency and sharp peak symmetry. The minimal variation in retention times across 10-sample sequences (±0.051, ±0.009, and ±0.022 min) demonstrated excellent run-to-run stability and negligible drift in chromatographic performance.

In addition, the resolution values (9.342 ± 0.098, 7.739 ± 0.118, and 15.584 ± 0.188) confirmed complete separation between adjacent peaks, exceeding the commonly accepted criterion of Rs > 2. The selectivity factors (1.480, 1.303, and 1.620) were consistent with well-resolved analyte interactions under the optimized conditions. Collectively, these results

confirmed that the method fulfilled all ICH and USP requirements for system suitability, ensuring reliability for quantitative analysis of CAB and its related components.

The specificity of the analyte and the developed method was investigated in the case of the presence and absence of CAB. The results obtained from the chromatogram, as can be seen in Figure 5A, showed that the method was specific for CAB.

The precision of an analytical method is the proximity of coherence between a series of analyses procured from identical sample levels under the same circumstances. In order to evaluate method precision, in the concentration of 200 µg/mL, solutions were injected ten times into the HPLC system, and the percentage of recovery was calculated. As presented in Table 1, the means of the percentage of recovery values were 100.633 ± 0.393, 105.214 ± 1.409 and 105.434 ± 0.402 for the three different peaks obtained from the analysis. Given that the recoveries were close to 100% and the SD values of the series were less than the acceptance criteria, which is 2%, the analysis method is confirmed to be valid for precision. The average of 105% obtained in the precision study, when evaluated together with the RSD value of <2%, which is an indicator of intra-method repeatability, demonstrates that the method provides consistent and reliable results. Therefore, the average value is within acceptable bias limits, and it is understood that the precision parameter of the method is satisfied.

Table 1. The percentage of recovery, coefficient of variations and SD of 200 µg/mL calcium butyrate solution for precision study.

Test Solution	1st Peak Recovery (%)	2nd Peak Recovery (%)	3rd Peak Recovery (%)
1	100.151	105.013	104.984
2	99.781	107.684	105.265
3	100.707	107.684	105.828
4	100.707	105.681	104.984
5	100.522	105.013	105.547
6	100.893	103.678	105.828
7	101.078	104.346	105.547
8	100.893	104.346	106.109
9	100.707	104.346	105.265
10	100.893	104.346	104.984
Average	100.633	105.214	105.434
SD	0.393	1.409	0.402
RSD	0.390	1.339	0.381

Repeatability is a parameter that reveals whether the method is consistent under the same conditions inter days [29]. Accordingly, 200 µg/mL concentration samples were analyzed over two consecutive days, and the results showed that all RSD values were below 2%, as listed in Table 2, indicating successful repeatability.

Intermediate precision studies are conducted to demonstrate the repeatability of the method under possible variations during laboratory work [30]. To evaluate whether the intermediate precision parameter was met, six different samples of the same concentration were prepared and analyzed by two analysts under the same conditions. The results obtained clearly demonstrated that the same concentrations can be determined even if different analysts prepared them, as can be seen in Table 3 with the required RSD value (less than 2%).

Table 2. Interday Precision.

Test Solution	1st Peak Recovery (%)		2nd Peak Recovery (%)		3rd Peak Recovery (%)	
	1st Day	2nd Day	1st Day	2nd Day	1st Day	2nd Day
1	100.430	103.733	102.996	100.952	100.679	100.953
2	103.366	99.329	102.996	100.271	100.405	99.856
3	101.348	103.182	103.677	102.315	100.130	99.856
4	100.063	102.265	102.996	102.315	100.405	100.404
5	102.081	101.898	103.677	100.952	100.130	100.679
6	102.081	102.448	102.996	103.677	100.130	100.130
Average	101.562	102.143	103.223	101.747	100.313	100.313
SD	1.108	1.396	0.321	1.250	0.224	0.448
RSD	1.091	1.367	0.311	1.228	0.223	0.447

Table 3. The intermediate precision results of the samples.

Test Solution	1st Peak Recovery (%)		2nd Peak Recovery (%)		3rd Peak Recovery (%)	
	1st Analyst	2nd Analyst	1st Analyst	2nd Analyst	1st Analyst	2nd Analyst
1	100.121	100.893	101.676	103.678	97.954	104.422
2	99.595	101.078	100.340	101.008	102.172	101.047
3	100.522	100.522	99.005	103.011	98.798	103.859
4	99.039	99.966	99.673	102.343	99.641	103.578
5	99.410	100.707	101.676	101.008	100.204	103.016
6	100.707	100.522	100.340	100.340	100.766	103.016
Average	100.121	100.615	100.452	101.898	99.922	103.156
SD	0.919	0.384	1.069	1.313	1.488	1.163
RSD	0.917	0.382	1.065	1.288	1.489	1.127

The validation of an analytical method's accuracy is expressed as the ratio of the obtained concentration values to the true values or reference values and the percentage of recoveries [31]. The results obtained in this study, as shown in Table 4, indicate that the average recovery data for CAB in the sample ranged from 100.575% to 103.842%. The average % R.S.D. ranged from 0.245% to 1.721%, which, as previously mentioned, is within acceptable limits. Therefore, the accuracy of the method has been confirmed.

The robustness test is a parameter that demonstrates the stability of the developed method even when changes are made within certain limits. This study serves as a valuable guide for demonstrating stability in formulation studies, where such changes are often unavoidable. Thus, it establishes the reliability and applicability of the method in the pharmaceutical field [32]. The robustness of CAB in standard solutions at a concentration of 200 µg/mL was examined over a total period of 48 h. Freshly prepared solutions were stored at room temperature (24 ± 1 °C) and injected into the HPLC system at specific times for measurement. When the obtained data were compared to each other, it was observed that the solutions remained stable for 48 h. Thus, it demonstrates that CAB is stable in standard solutions at room temperature for at least 2 days and maintains its stability. The results are in Table 5.

Table 4. Recovery of calcium butyrate samples.

Concentration (μg/mL)	Test Solution	1st Peak Recovery (%)	2nd Peak Recovery (%)	3rd Peak Recovery (%)
200	1	102.448	101.008	100.405
	2	102.265	102.343	100.679
	3	102.005	101.008	100.953
	4	101.263	102.343	100.130
	5	101.819	103.011	100.953
	Mean	101.960	101.943	100.624
	SD	0.410	0.801	0.320
	RSD	0.402	0.786	0.318
400	1	101.225	101.238	103.814
	2	101.503	105.577	102.970
	3	101.873	101.906	103.814
	4	101.132	100.571	103.111
	5	101.688	101.572	105.501
	Mean	101.484	102.173	103.842
	SD	0.277	1.759	0.900
	RSD	0.273	1.721	0.867
1000	1	101.906	100.308	102.830
	2	101.498	100.575	103.674
	3	101.239	100.442	102.774
	4	101.350	100.308	102.099
	5	101.757	101.243	102.380
	Mean	101.550	100.575	102.752
	SD	0.249	0.348	0.533
	RSD	0.245	0.346	0.519

Table 5. Robustness values of 200 μg/mL samples.

Time	0 min	30 min	60 min	120 min	24 h	48 h
1st peak Recovery (%)	99.039	101.263	100.707	100.893	99.329	102.265
	100.151	100.522	100.707	100.707	101.449	101.078
	101.008	100.522	100.522	100.893	101.228	101.502
Mean	100.066	100.769	100.645	100.831	100.669	101.615
SD	0.806	0.349	0.087	0.088	0.952	0.491
RSD	0.806	0.347	0.087	0.087	0.945	0.483
2nd peak Recovery (%)	105.681	105.681	107.684	104.346	103.677	105.721
	105.013	108.351	105.681	104.346	105.681	105.681
	104.346	108.351	105.013	104.346	104.346	104.703
Mean	105.013	107.461	106.126	104.346	104.568	105.368
SD	0.545	1.259	1.135	0	0.833	0.471
RSD	0.519	1.171	1.069	0	0.797	0.447
3rd peak Recovery (%)	104.422	105.828	105.828	106.109	104.141	104.422
	104.984	105.265	104.984	105.265	105.013	104.346
	105.828	105.828	105.547	104.984	105.828	105.828
Mean	105.078	105.640	105.453	105.453	104.994	104.865
SD	0.578	0.265	0.351	0.478	0.689	0.681
RSD	0.550	0.251	0.333	0.453	0.656	0.650

LOD and LOQ values of the developed method were evaluated following the ICH guidelines, and the obtained results of each peak were below the investigated concentration range. The results are in Table 6.

Table 6. Limits of detection and quantification of calcium butyrate.

	1st Peak	2nd Peak	3rd Peak
Limit of detection (µg/mL)	1.211	0.606	1.816
Limit of quantification (µg/mL)	3.670	1.835	3.676

4. Conclusions

In this study, the molecular analysis of CAB was performed using FTIR and NMR studies, and then a simple and effective HPLC method was developed and validated for its quantitative determination. The spectra of the substance confirmed the specific structure of the molecule. In the developed analytical method, the substance was observed to give peaks at three different times, and the method was validated for each area value. In the validation studies conducted according to the ICH guideline, the method was validated by demonstrating good linearity with a determination coefficient of 0.9998 for each, high accuracy, precision, adequate LOD and LOQ values, selectivity, and stability. Owing to the developed method, it has been possible to determine CAB using an aqueous mobile phase with a method that is as environmentally friendly, economical, and practical as possible in terms of analysis time. In conclusion, the developed and validated method has been confirmed as simple, cost-effective, and efficient, providing detailed information for the quantitative analysis of CAB, including drug content, in vitro release, and in vivo distribution studies, in pharmaceutical dosage forms for future studies.

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