

Validated RP-HPLC Method for
Identification and Simultaneous
Quantitation of Calcium Gluconate and
Calcium Phospholactate in Combined
Dosage FormSebaey Mahgoub^{1,2*}¹Research and Development, Unipharma, El-Obour City, Egypt²Food Analysis Laboratory, Ministry of Health, Zagazig, Egypt

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Keywords RP-HPLC; Calcium gluconate; Calcium phospholactate; Method validation

Abstract

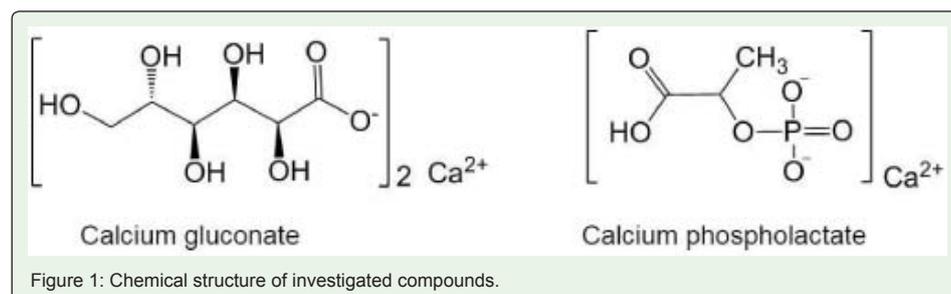
A simple, precise and accurate reversed phase high performance liquid chromatography (RP-HPLC) method was developed and validated for identification and simultaneous estimation of calcium gluconate and calcium phospholactate in a tablet dosage form. Detection and quantitation were performed by a Shimadzu Nexera LC-30AD system. Separation of the two analytes was achieved within 5 minutes by using Inertsil C18-3 column (4.6 mm x 150 mm, 5 μ m) and isocratic elution of aqueous solution of 1% (v/v) phosphoric acid and methanol by (90: 10, v/v) ratio at a flow rate of 1.0 mL/min. Detection was monitored at 210 nm. The linearity for calcium gluconate was in the range of 1.92-2.88mg/ mL with correlation coefficient of 0.9982 and linearity for calcium phospholactate was in the range of 2-3mg/ mL, with correlation coefficient of 0.9981, respectively. Stability indicating capability was established by forced degradation experiments. The proposed method was validated for linearity, repeatability, accuracy, specificity, robustness, Limit of Detection (LOD) and Limit of Quantification (LOQ). The developed method was successfully applied in analysis of a tablet formula.

Introduction

Calcium is one of the most important nutrients in the human diet, due to its implication in bone health and other physiological functions and enzymatic reactions [1]. Calcium gluconate can be used effectively for treatment of hydrofluoric acid burn [2] and sometimes as a calcium supplement [3]. Solubility of Calcium gluconate makes it appropriate for incorporation into foods [4]. Its empirical formula is C₁₂H₂₂CaO₁₄ and its chemical structure is shown in (Figure 1).

Phosphorus is essential for life and plays multiple roles that sustain cellular vitality and as a key structural component of DNA and RNA–sugar phosphates forming the helical structure of every molecule. It is also critical to ATP (adenosine-5'-triphosphate), phospholipids and cell membranes as well as the formation and maintenance of bones and teeth in all vertebrates [5]. Calcium phospholactate (calcium lactophosphate) have been used in drug formulations since 1869 [6]. It's used as a calcium and/or phosphorus dietary supplement [3]. Its empirical formula is C₃H₅CaO₆P and its chemical structure is shown in (Figure 1).

To the best of my knowledge, no stability indicating HPLC method has been reported yet for simultaneous estimation of calcium gluconate and calcium phospholactate in a combined formula, they are often determined as calcium or phosphorous. Several methods were reported for determination of calcium and/or phosphorous in pharmaceutical dosage forms such as derivative spectrophotometry [7] and potentiometric titration [8]. The most widely used technique is atomic absorption spectrometry [9-12] and these methods can't be used as stability indicating methods because they depend on determination of calcium or phosphorous content not the whole molecule.



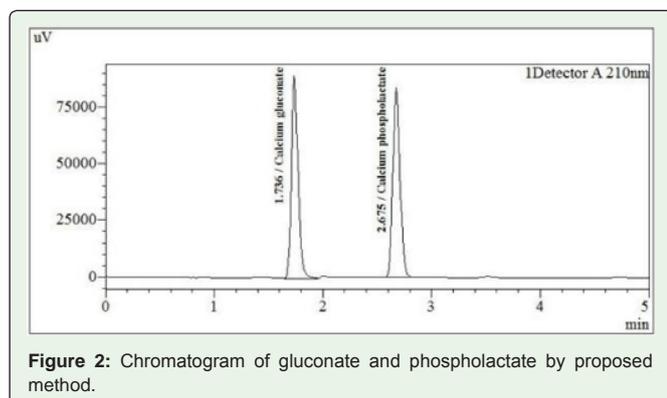


Figure 2: Chromatogram of gluconate and phospholactate by proposed method.

In this paper the first RP-HPLC method for the simultaneous determination of the mixture of calcium gluconate and calcium phospholactate in tablet dosage form is reported. The method was able to separate each component as well as other unknown degradation products within a run time of 5 minutes. The study was performed in accordance with established ICH guidelines [13] and was successfully applied for simultaneous determination of the combined formula of these two components.

Experimental Section

Chemicals and reagents

HPLC-grade methanol and phosphoric acid were manufactured by Fischer Scientific, UK and purchased from their local agent. Standard materials used were working standard available at our laboratories. The tested formula was a new product developed for registration.

Preparation of Mobile Phase

10 mL of phosphoric acid was added to 950 mL of purified water, then completing the volume to 1000 mL using purified water giving a concentration of 1% (v/v) phosphoric acid. Mobile phase composed of a filtered and degassed mixture of 1% (v/v) phosphoric acid and methanol by (90: 10, v/v) ratio.

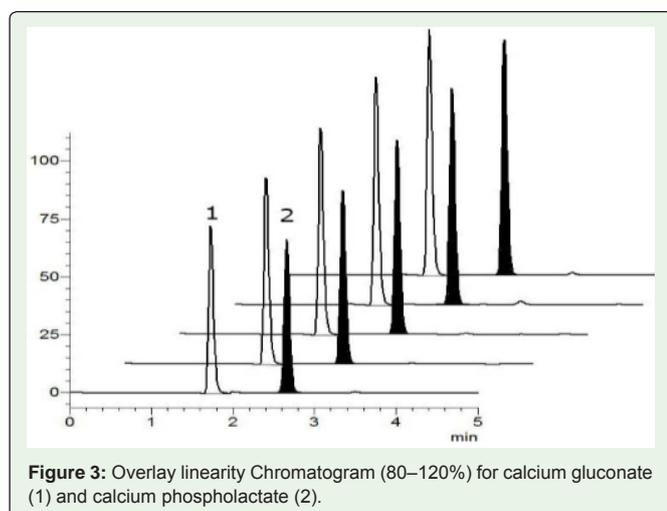


Figure 3: Overlay linearity Chromatogram (80–120%) for calcium gluconate (1) and calcium phospholactate (2).

Chromatographic system

The analysis was performed on a Shimadzu Nexera LC-30AD system (Shimadzu Corporation, Kyoto, Japan) consisting of LC-30 AD-LPGE pump, SPD-20A UV detector and DGU-20A5 Degasser. A Shimadzu SIL-30AC autosampler was used to inject 10 μ L of the samples on an Inertsil C18-3 column (4.6 mm x 150 mm, 5 μ m), GL Sciences, Japan), isothermally at 40°C. The detection was carried out at 210 nm. Mobile phase was filtered through a 0.22 μ m membrane filter (Chromatech, UK or equivalent), then degassed ultrasonically for 5 min and delivered at a flow rate of 1.0 mL/min.

Solutions preparation

Standard solution: Standard solution was prepared by dissolving standard substances in water, then warming gently till complete dissolving, sonicated for 5 min and diluted up to the mark with water to obtain a solution containing, 2.4 mg/mL of calcium gluconate and 2.5 mg/mL of calcium phospholactate.

Sample solution: Twenty tablets were grinded to fine powder. An accurately weighed portion of the powder equivalent to 24 mg of calcium gluconate and 25 mg of calcium phospholactate per tablet were taken in 10 ml volumetric flask. About 5 ml of water was added to this flask and the flask was warmed gently then sonicated in an ultrasonic bath. The volume was completed to the mark using water then filtered through a 0.22 μ m syringe filter.

Results and Discussion

Method development and optimization

The main target of the chromatographic method was achieving a method for simultaneous assay for calcium gluconate and calcium phospholactate. The combined standard solution (2.4 mg/mL of calcium gluconate and 2.5 mg/mL of calcium phospholactate) was injected. For method optimization, different chromatographic conditions were experimented to achieve better efficiency of the system. After many trials the method was applied using an Inertsil C18-3 column (4.6 mm x 150 mm, 5 μ m) as a stationary phase and mobile phase consisting of 1% (v/v) phosphoric acid and methanol by ratio (90: 10, v/v) giving good separation, acceptable retention time, peak shape, plate's count and good resolution. Chromatogram obtained for the combination upon using final conditions is shown in (Figure 2). Stability indicating capability was established by forced degradation experiments.

Method validation

System suitability: Numerous approaches may be used to set the limits for system suitability tests. This depends on experience with the method, material available and personal preference [14]. System suitability was performed on six replicate injections of mixed standard solution. The relative standard deviation (%RSD) values, tailing factor and number of theoretical plates were the chromatographic parameters selected for the system suitability test. The following requirements were fulfilled, % RSD of peak responses due to each component for the six replicate injections must be less than or equal to 1.0 %, tailing factor must be less than 2.0 %, resolution must be more than 2 and theoretical plates count must be more than 2000. System suitability parameters were calculated and are presented in Table 1.

Table 1: System suitability parameters.

No.	Parameter	Calcium gluconate	Calcium phospholactate
1.	Retention time	1.736	2.675
2.	USP resolution	--	7.087
3.	Tailing factor	1.290	1.148
4.	No. of theoretical plates	3019	5969
5.	Area [RSD (%), n=6]	0.184	0.178

Linearity: In chromatography, peak parameters are related to analyte concentration via standardization procedures. This relationship is then used to convert a sample's peak parameter to its apparent analyte concentration. A linearity assessment establishes the nature of the peak parameter relative to analyte concentration. The linearity assessment determines the procedure's ability to obtain test results which are proportional to the concentration of the analyte in the sample within a given range either directly or via a well-defined mathematical transformation [15]. It was studied by preparing standard solution of five concentration levels ranging from 80 % to 120 % of test concentration (1.92-2.88 mg/mL of calcium gluconate and 2-3 mg/mL of calcium phospholactate) and analyzed in triplicate per level (Figure 3). The graphs were plotted using peak responses of each component on Y-axis and the corresponding concentration on X-axis. The peak area ratios of calcium gluconate and calcium phospholactate were linear with respect to the concentrations of the two analytes. A good correlation was found between the observed peak area ratios (y) and the theoretical concentration (x). Least-squares regression analysis provided typical regression lines: $y = 166.6x - 13194$ ($r^2 = 0.9982$) for calcium gluconate and $y = 162.3x - 16567$ ($r^2 = 0.9981$) for calcium phospholactate.

Precision: Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (%RSD) for a statistically significant number of samples [16]. The precision of the method was established by carrying out the analysis of 100% concentration of the analyte for six times. The obtained low value of standard deviation showed that the method is precise. The results obtained are presented in Table 2.

Accuracy: Accuracy is the degree of agreement between the experimental value, obtained by replicate measurements, and the accepted reference value. It has been pointed out that the accuracy is

Table 2: Method precision results.

No.	Calcium gluconate	Calcium phospholactate
	377098	379743
	377134	378561
	376269	378421
	376027	378568
	375774	378186
	375353	377769
Mean	376275.8	378541.3
SD	718.03	660.66
RSD%	0.191	0.175

the most crucial aspect that any analytical method should address [17]. The accuracy of the method was verified by adding different amounts of known standards to the sample solution then determining the percentage recovery of each concentration with respect to real values. The accuracy of the assay method was evaluated in triplicate at three concentration levels 80%, 100% and 120% of the label claim. Amount of drug recovered was quantified and % recovery was calculated. The results of the accuracy study are reported in Table 3. For the two drugs, at the different concentration levels, good recoveries were obtained meeting the acceptance criteria of $100 \pm 2\%$. These results revealed the good accuracy of the proposed methods and providing the working range for the method.

Specificity: Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [18]. Forced degradation studies were performed to demonstrate selectivity of the method using 0.1M HCl, 0.1M NaOH and thermal (80°C). The degradation samples were prepared by taking suitable aliquots of the drug solution, and then undertaking the respective stress testing procedures for each solution. After the fixed time period the treated drug solutions were diluted with water. For every stress condition drug solution was prepared as 2400 µg/ml of calcium gluconate and 2500 µg/ml of calcium phospholactate. The specific stress conditions are described as follows. Acidic degradation was carried out by adding 1.0 mL of 0.1M HCl, and after 30 min neutralizing the mixture by adding 0.1M NaOH. Alkali degradation was carried out by adding 1.0 mL of 0.1M NaOH, and after 30 min neutralizing the mixture by adding 0.1M HCl. Thermal degradation was performed by heating the drug solutions at 80°C on a water bath for 45min.

After the degradation process, samples were allowed to cool at room temperature and diluted, if necessary, to the same concentration as that of the standard solution, after being neutralized. Calcium gluconate and calcium phospholactate were found to be sensitive to acid, base and thermal degradation (Figure: 4a-d). The figures show that there is no interference at the retention time of calcium gluconate and calcium phospholactate due to placebo or degradation products, indicating that the method is selective.

Robustness: Robustness of the developed method was determined by analyzing the samples after some small but deliberate changes in the method parameters such as change in flow rate (± 0.2 mL/min), change in organic composition of mobile phase by ($\pm 5\%$). Changes in chromatographic parameters such as theoretical plates count and tailing factor were evaluated for the studies and are shown in Table 4. The method is robust for all tested parameters.

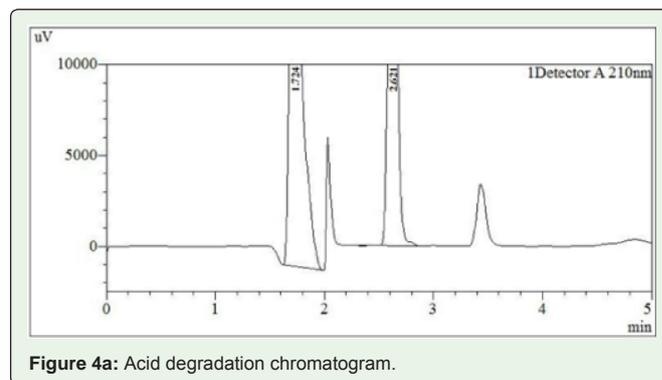


Figure 4a: Acid degradation chromatogram.

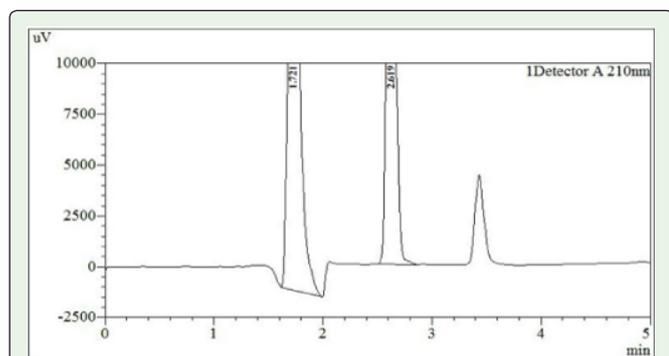


Figure 4b: Base degradation chromatogram.

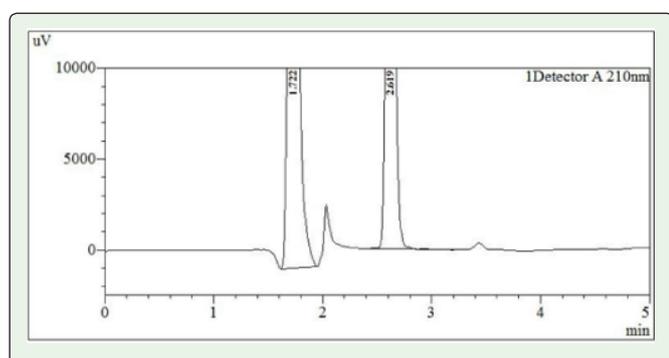


Figure 4c: Thermal degradation chromatogram.

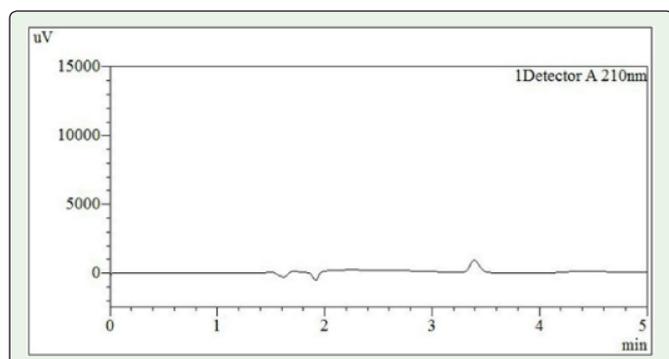


Figure 4d: Placebo chromatogram.

Limit of Detection (LOD) and Limit of Quantitation (LOQ): Sensitivity of the method was proved by establishing the Limits of Detection (LOD) and Quantitation (LOQ) for calcium gluconate and calcium phospholactate in pure and in dosage forms. The LOD and LOQ were determined by visual inspection using signal to noise approach, by injecting a series of dilute solutions with known concentrations. The concentration (in $\mu\text{g/mL}$) with signal to noise ratio of at least 3 was taken as LOD and concentration with signal to noise ratio of at least 10 was taken as LOQ. The limit of detection of calcium gluconate and calcium phospholactate was 32.7 and 27.6 $\mu\text{g/mL}$, respectively. The limit of quantification was 96.6 and 78.4 $\mu\text{g/mL}$, respectively.

Stability of standard solution, sample solution and mobile phase: The solution stability was checked by leaving the both the solutions of sample and standard in tightly capped volumetric flasks

Table 3: Results of the Accuracy study.

Component	Spiked level (% n=3)	Spiked conc. ($\mu\text{g}/\text{ml}$)	Recovery (%)
Calcium gluconate	80	1920	100.59
	100	2400	100.79
	120	2880	99.71
Calcium phospholactate	80	2000	100.50
	100	2500	99.85
	120	3000	100.75

Table 4: Robustness evaluation of the method.

Chromatographic changes	%RSD (Peak Area) n = 6	Tailing factor	Theoretical plate count
Calcium gluconate			
Flow rate (mL/min)			
0.8	0.15	1.33	3489
1.0	0.16	1.30	2993
1.2	0.18	1.35	2989
Organic composition (%)			
5	0.24	1.36	2841
10	0.15	1.31	3135
15	0.17	1.29	2933
Calcium phospholactate			
Flow rate (mL/min)			
0.8	0.11	1.17	7393
1.0	0.13	1.16	5888
1.2	0.12	1.14	6931
Organic composition (%)			
5	0.13	1.16	6595
10	0.12	1.16	5909
15	0.14	1.13	6399

at room temperature for 2 days. The same sample solutions were analyzed after 2 days. The mobile phase stability was also carried out by assaying the freshly prepared sample solutions against freshly prepared standard solution for 2 days. Mobile phase was kept constant during the study period. There is no significant change in the solutions up to a period of 2 days; all the drugs had enough stability up to 2 days after preparation.

Conclusion

A novel stability indicating RP-HPLC method was successfully developed and validated for simultaneous determination calcium gluconate and calcium phospholactate in tablets dosage form. All the drugs were found to be well resolved in a run time of 5 min. No interference was found with the degradants and excipients. Validation results have proved that the method is selective, precise, accurate, robust and stability indicating which can be successfully applied for the routine analysis as well as for stability studies.

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