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UTILIZATION OF THE STABILITY-INDICATING RP-HPLC METHOD FOR DIETRY SUPPLEMENT CALCIUM OROTATE QUANTIFICATION IN CAPSULE DOSAGE FORM

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A rapid, accurate, and precise HPLC approach for quantifying calcium orotate in capsule dosage form was developed and validated. As optimum conditions for analysis, a C_8 column (250 mm x 4.6 mm, 5µ) was used as the stationary phase, with a mobile phase of 65:35 v/v acetonitrile and 25 mM potassium dihydrogen phosphate buffer (pH 4.7) at a flow rate 1 ml/min and detected at 254 nm. The method showed a 0.999 correlation and an excellent linear response throughout a concentration range of 15–90 µg/ml. Retention lasted for 3.186 minutes. Validation of the method done as per the requiremnts of ICH and subjected to oxidation, photolysis, heat, acid, alkali hydrolysis, and water stress. Degradation byproducts did not affect the ability to detect calcium orotate, hence the approach is stability indicating. The approach may be used to determine calcium orotate in pharmaceutical capsule dose form since the findings of the research were within the parameters of ICH standards.

Keywords: *Calcium orotate; HPLC; stability-indicating*

INTRODUCTION

The calcium salt of orotic acid is called calcium orotate (Fig. 1). Tetrahydro-1,2,3,6-2,6-dioxo-4-pyrimidinecarboxylic acid is what orotic acid is chemically. With weak water solubility, it is a white to virtually white crystalline powder[1]. Its chemical formula is $C_{10}H_6CaN_4O_8$ and having a molecular weight 350.25 g/mol. This salt is mostly used as a calcium supplement to those who do not get enough calcium via their normal diet[2&3]. Additionally, it is used as a drug to treat ailments brought on by low calcium levels, including osteoporosis, rickets, osteomalacia, hypoparathyroidism, and a particular muscular disorder called latent tetany[3]. It may also be used to ensure that certain patients are receiving adequate calcium, such as those who are postmenopausal, pregnant, breastfeeding, or using phenytoin, phenobarbital, or prednisone.

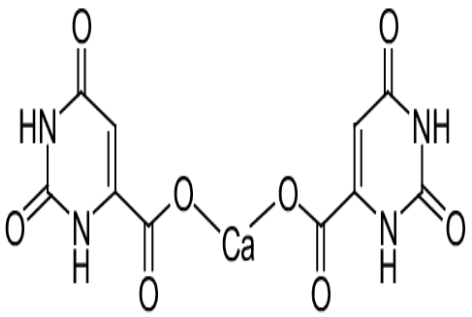

Fig.1: Structure of Calcium orotate.

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It is much superior to all other currently used conventional calcium salts as a source of calcium supplement[4]. For instance, calcium orotate has no adverse effects whatsoever. When used in osteoporosis with concurrent abdominal aortic arteriosclerosis, conventional calcium salts present certain issues. On the other hand, calcium orotate guards against arteriosclerosis in the body. Because calcium orotate may pass through the intricate cell membranes, it can make up for a disruption in calcium transport across these membranes. Additionally, when it is metabolized, calcium orotate has an unique affinity for bradytrophic tissue (such as cartilage)[5]. Parallel investigations have shown the significant pathogenetic importance of a faulty calcium transport via the cell membrane. Therefore, this material is a highly effective drug for recalcifying metastatic skeletal system abnormalities as well as other calcium shortage syndromes.

A literature review finds that only a few analytical techniques, such as UV spectrophotometry[6] and HPLC[7&8], have been published for the quantification of calcium orotate in dosage forms. One LC-MS[9] technique for estimating calcium orotate in dried blood spots. All medications must undergo a stability-indicating assay technique test before being approved for use, in accordance with current good manufacturing practices[10]. Literature review suggested only one stability indicating method reported for the estimation of calcium orotate but method didn't provide the peak purity basis stability assay, so there is a need of stability indicating LC method based on peak purity. So in the present research a peak purity based stability-indicating liquid chromatography (LC) method[11] was developed for the determination of calcium orotate as a bulk drug and as pharmaceutical dosage forms using the conditions recommended by the International Conference on Harmonization[12] (ICH) (hydrolysis, oxidations, photolysis, and thermal stress). As a result, the main goal of this work was to create a novel, inexpensive, accurate, repeatable, and stability-indicating RP-HPLC technique for the measurement of calcium orotate in pharmaceutical dosage forms and bulk medication.

MATERİALS AND METHODS

Chemicals

Purity > 99.8 % calcium orotate pure drug was received as a free sample from pharmazell Ltd. (Vizag,India). Acetonitrile and water of HPLC quality were supplied by MERCK India Ltd. Methanol of HPLC quality was provided by Hyderabad's Standard Reagent Pvt Ltd. The analytical grade HCl, NaOH, and H_2O_2 were acquired from SD Fine chemicals in Mumbai, India. The 0.2 µm and 0.45 µm nylon membrane filters came from PALL Life Sciences in Mumbai, India.

Equipment

Electronic balance (Apex, India), sonicator (LAB india Ltd. Mumbai, 3.5 L), hot air oven (Accumax, India), and digital pH meter were the equipment used during the study (Elico LI 120). HPLC (Waters, E2695/2996) used Empower software for monitoring and integration, UV Spectrophotometer (Perkinelmeyer, India, Pvt, Ltd). Additionally, syringe ["Hamilton" (Rheodyne-20 µL)] and syringe filter ["Himedia Syringe-driven filters" (0.22 µm)] were used.

Chromatographic conditions

The Shimadzu HPLC chromatographic system with a PDA detector and an auto sample injector was used to develop and validate the technique. "LC solutions" was used to monitor and integrate the output signal. The Rheodyne injector 7725i, which was built with a 20 µL loop was utilized in the experiment and data was collected. At room temperature, separation was carried out using a Waters RP-8 (250 × 4.6 mm i.d., 5 µm). For the best chromatographic separation of Calcium orotate, a combination of 25 mM potassium dihydrogen phosphate buffer (pH 4.7) and acetonitrile in a ratio of 65:35 v/v was determined to be the most acceptable mobile phase. Prior to use, the solvent combination was sonicated and filtered through a 0.45 micron membrane filter. It was forced through the column at a rate of 1.0 milliliters per minute under pressure. The column was kept at room temperature, and 20 µL was injected. After pumping the mobile phase through the column for at least 30 min, the column was equilibrated in preparation for the

injection of the drug solution. At 254 nm, the drug's detection was seen. The 10 min runtime was chosen. Table 1 displays the ideal chromatographic conditions.

Preparation of mobile phase

The mobile phase was made by combining 650 mL of 25 mM potassium dihydrogen phosphate buffer pH 4.7 with 350 mL of HPLC grade acetonitrile (prepared by dissoving 1.36 g of potassium hydrogen phosphate into a suitable container containing 1000 mL of HPLC grade water , mixed and pH adjusted to 4.7 with ortho-phosphoric acid). A 0.45 µm membrane filter was used to filter the mobile phase after being sonicated for 10 minutes.

Preparation of standard stock solutions

By blending 50 mg of the pure drug with 50 mL of the mobile phase in a volumetric flask, the standard stock solution of the medication at 100 µg/mL was created. After more diluting with mobile phase to get a final concentration of 100 µg/mL, the solution was refrigerated. Standard stock solution aliquots were added to a volumetric flask of capacity 10 mL and adjusted with mobile phase until the desired concentration was reached. As a result, the drug's final concentrations ranged from 15 to 90 µg/mL.

Preperation of sample solution

To assess the calcium orotate content in capsule dose form. The capsule powder containing 10 mg of calcium orotate (each capsule contains 85 mg calcium orotate) was dissolved in 50 mL of mobile phase. The resulting solution (3 mL) was delivered to a volumetric flask with a capacity of 10 mL and volume marked with mobile phase to the desired concentration. The finished solution was filtered using an injection filter and a 0.45 micron membrane filter. The filtrate was introduced into the chromatographic system in the amount of 20 µL. A linear regression equation generated from the calibration curve was used to compute the concentration using the peak area of the calcium orotate.

Method validation

The proposed method's “linearity, accuracy, precision, robustness, ruggedness, detection limit, quantification limit, and stability” were all evaluated. Except for the quantification limit, which was set at 2 % as indicated in the literature, variation coefficients and relative errors of lower than 2% were regarded acceptable.

System Suitability Test

Prior to undertaking validation testing, a system suitability test (SST) must be conducted to confirm that the HPLC system and procedure are able to provide data of adequate standard. SST was achieved by assessing the peak areas' capacity factor, tailing factor, theoretical plate number, resolution, and relative standard deviation (RSD).

Stability

The stability of QC standard solutions was determined by evaluating them after 48 hours at room temperature. The obtained findings were examined as recovery values and contrasted to newly prepared solutions.

Linearity

Using mobile phase, an calcium orotate concentration of 1000 µg/mL was created. From it, several concentrations in the range of 15 to 120 µg/ml were prepared and then inserted into the HPLC system. It was revealed that the chosen medication showed linearity between 15-90 µg/mL. Replicate study (n= 9) at all concentration levels produced the calibration plot (peak area of calcium orotate vs calcium orotate concentration), and the linear connection was assessed using the least square technique inside the Microsoft Excel® application.

Accuracy

One set of several standard addition techniques was used to test the method's accuracy at concentration levels of 80%, 100%, and 120%, and a comparison was made between the difference between the spike value (theoretical value) and the actual value obtained.

Precision

The precision of the procedure was evaluated by measuring the peak area of six replicates of a constant amount of the drug (60 µg/mL) in order to estimate the standard deviation. On three distinct days, intra- and

inter-day variations in the peak areas of a series of drug solutions were used to evaluate the assay's precision. Relative standard deviation (RSD) was used to measure the variations in peak area of the drug solution over the day(intra-day) and between days(inter-day).

Robustness

The robustness of the suggested approach for calcium orotate was tested by varying the flow rate, pH, wavelength, and mobile phase ratio slightly. Calcium orotate's % recovery and RSD were recorded.

Ruggedness

The test solutions were produced according to the test technique and injected under variable conditions. Different analysts investigated the method's ruggedness.

Detection limit and quantification limit

Based on the calibration curve parameters, the limits of detection (LOD) and quantification (LOQ) were determined using the following formulas:

LOD= 3.3σ/s , LOQ= 10σ/s, where s is the calibration curve's slope and σ is the standard deviation of the regression line's y-intercept.

Forced degradation studies

Forced degradation experiments on the sample employing oxidative, thermal, photolytic, acid, alkaline, and UV degradations was used to show the method's specificity. Demonstrating that the technique effectively isolated degradation products from pure active ingredient, the sample was subjected to these conditions, and the peak purity of the primary peak was examined.

Hydrolytic degradation

The goal of hydrolytic stress testing was to gradually expose the drug material to neutral, acidic, and basic environments while forcing the drug substance to degrade to its principal breakdown products. To initiate hydrolytic research, a preliminary solubility test of the pharmaceutical was conducted. Solubility of at least 1 mg/mL in neutral, acidic, and basic conditions was recommended for stress testing. If solubility is a problem, a concentration of less than 1 mg/mL may be employed. To get the desired concentration, a co-solvent could be essential in certain circumstances. When a suitable co-solvent was selected, special consideration should be paid to the drug substance's structure.

Oxidative degradation

It is commonly known that medication ingredients oxidize throughout the pharmaceutical formulation process. Although the precise mechanisms promoting the interaction between the drug ingredient and molecular oxygen included in dosage form are not completely known, such reactions are often considered to fall under the category of auto-oxidation processes.

“Autoxidation, or radical-mediated oxidation”, “peroxide-mediated oxidation”, and “photochemically induced oxidation” are the three main routes. Pharmaceuticals have historically been subjected to oxidative stress testing using diluted aqueous peroxide solutions. Aside from the auto-oxidation procedures, there may also be oxidative deterioration caused by peroxide that is difficult to detect with a radical indicator. Oxidation depends on the kind of substance it contacts and the quantity of oxygen present in the air. The process of true oxidation is molecular in nature. Only when oxygen causes free radicals on the surface to disperse can we see the consequences on a broad scale.

Photolytic degradation(Sunlight)

The medication was directly exposed to the sun to cause photolytic breakdown. The medicine was administered in an adequate quantity and placed in a closed petri dish with sunshine. To gauge the degree of drug degradation, the drug was removed at various time intervals, properly diluted, and then put into HPLC.

UV-degradation

Studies on UV-induced drug degradation aim to accelerate drug substance disintegration by UV and fluorescent conditions over time in order to identify the main degradation products. When an absorption band partially coincides with the energy of the incoming light and an excited valence electron exists in the appropriate environment, a molecule absorbs light.

Thermal degradation

Evaluated temperatures (such as 50 °C and 80 °C) in the solid state and/or in solutions may be utilized to assess thermolytic processes. Above 80 °C, several substances started to break down via various processes, producing degradation products. The utilization of high and low humidity environments with the assessed temperature is adequate for solid-state stressing. Stress conditions were chosen in order to assess stability at the aforementioned temperatures using a conservative approximation of the Arrhenius expression, which describes the link between reaction rate and temperature quantitatively using average activation energy.

$$K_{obs} = A\exp^{-Ea/RT}$$

RESULTS AND DİSCUSSİON

Optimization of chromatographic conditions

The nature of the medication, the complexity of the sample, and the intended usage are just a few examples of the considerations that go into choosing an analytical technique. In this investigation, the physicochemical characteristics of calcium orotate had an impact on the chromatographic conditions (column, mobile phase, and flow rate). The mobile phase was tuned to give enough selectivity and sensitivity in a brief separation time in order to produce the optimal chromatographic conditions. Because it is suited for separating the excipients of capsules and calcium orotate with acceptable peak qualities, a combination of acetonitrile: 25 mM phosphate buffer pH 4.7 (35:65) was chosen. The tailing factor of the peak was 1.04, the column efficiency for calcium orotate was around 5386 theoretical plates, and the capacity factor was 5.2. A PDA detector was utilized to choose the appropriate wavelength for detection. At the detection wavelength, there was no interference from the sample solvent, contaminants, or excipients in the dosage form (254 nm). As shown in Fig. 2, a representative chromatogram produced by the suggested LC approach illustrates the symmetrical peak corresponding to calcium orotate. The optimized chromatographic conditions are shown in Table 1.

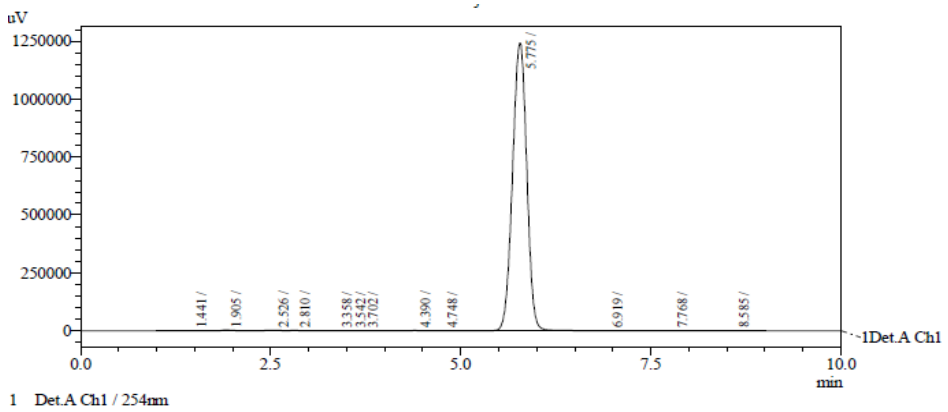

Fig 2: Chromatogram of calcium orotate .

Table 1: Optimized chromatographic conditions.

Parameters	Conditions
Stationary Phase(Column)	C_8 (250 × 4.6 mm i.d.,5µ)
Mobile Phase	Acetonitrile: 25 mM phosphate buffer p^H 4.7(65:35,v/v)
Flow rate(ml/min)	1.0 mL/min
Run time(min)	10 min
Column temperature (°C)	ambient
Volume of injection loop(µL)	20
Detection wavelength(nm))	254 nm
Retention time(min)	5.775

Method validation

System Suitability Test

The system suitability parameters for the developed approach were calculated once the ideal circumstances had been established, and they were compared to suggested limits. Six injections were used in the investigation using a standard solution at a concentration of 60 µg/ml to ascertain the parameters. Table 2 shows the method's system suitability parameters. The approach was determined to be appropriate for the analysis based on the results which showed that all of the system suitability parameters were within the suggested ranges.

Table 2: Results of system suitability test (n = 6).

Parameter	Criteria	Result
Capacity factor(k')	$k' > 2$	5.824
Tailing factor (T)	T < 2	1.042
Theoretical plates (N)	N> 2000	5429
% RSD (peak area)	% RSD ≤1	0.74

Stability

By injecting the same solution at 0/12/24/48 hrs, the stability of the sample solution was examined.The developed approach did not show the same change. Additionally, the findings (RSD< 2) were judged to be within acceptable bounds. The data presented in Table 3.

Table 3 : Stability data of Calcium orotate (standard solutions).

Time (hr)	Assay(%)	% Difference
Initial	100.08 ---	----
After 12 hrs	100.02	0.05
After 24 hrs	99.87	0.21
After 36 hrs	99.16	0.92
After 48 hrs	98.32	1.76

Linearity and sensitivity

A linearity analysis was carried out using calibration standards with concentrations of 15, 30, 45, 60, 75, and 90 µg/ml. Triplicate injections of the standards were performed. By correlating the peak areas to the target concentrations, calibration curves were generated. The determination coefficient was used to assess the calibration curve. The calibration curves' determination coefficient (R^2) was 0.9999. As a result, it was discovered that the calibration curve for calcium orotate was linear within the concentration range of 15-90 µg/ml. The calibration graphs were used to compute the regression equations. Limits of detection (LOD) and quantitation were used to assess the analytical method's sensitivity (LOQ). Table 4 provides the LOD and LOQ values. The sensitivity of the technique is shown by the low values of LOD and LOQ.

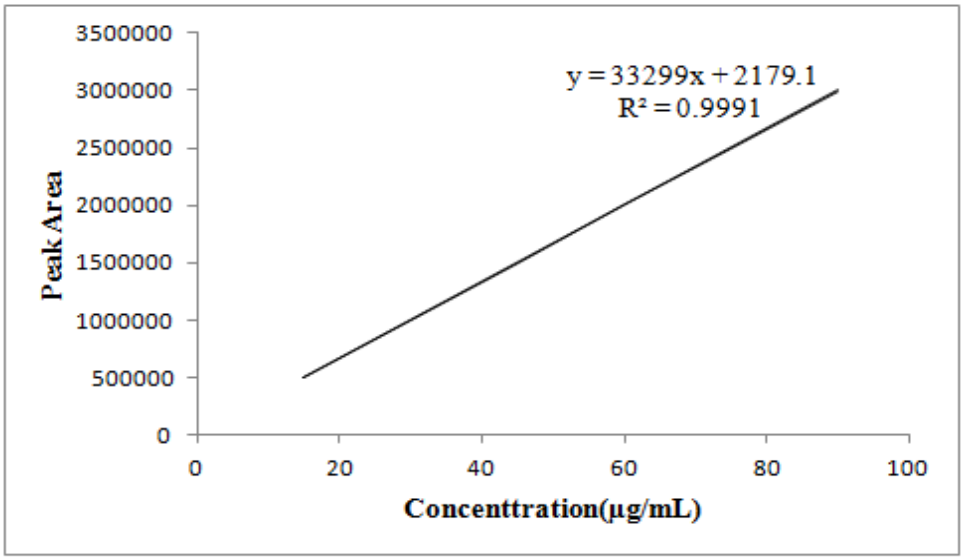

Fig . 3: Calibration curve of calcium orotate.

Table 4: Spectral and statistical data for determination of Calcium orotate by proposed RP-HPLC method.

Parameter	Result
Detection wavelength (nm)	254
Linearity range (µg/ml)	15-90
Coefficient of determination (r^2)	0.9991
Regression equation (Y^a)	Y= 33299x+2179.1
Slope (m)	33299
Intercept (c)	2179.1
Limit of detection, LOD (µg/ml)	0.02
Limit of quantitation, LOQ (µg/ml)	0.08

[a]$Y = mx + c$, where x is the concentration (µg/ml).

Accuracy

Recovery tests were conducted to examine the method's reliability, applicability, and accuracy. The placebo was mixed with known amounts of the pure medication to create samples at concentrations of 0%, 100%, and 150%, which were then measured using the suggested technique. Table 5 shows the results of the accuracy calculation as a percentage of recovery.

Precision

Repetition, intermediate precision, and reproducibility (between laboratories' precision) were the three levels at which the precision was shown. Three sequential replicate injections at three different concentrations—30, 60, and 90 µg/mL—were used to test each level of precision.

Using the relative standard deviation (RSD) or coefficient of variation, the precision was described. Table 6 displays the outcomes of three precision levels.

Table 5: Accuracy Data.

% Level	Concentration(µg/mL)		Recovery(%)	Statistical Results		
	Formulation	Pure drug		Mean	SD	%RSD
50	60	30	98.4			
50	60	30	98.1	99.1	1.47	1.48
50	60	30	100.8			
100	60	60	100.5			
100	60	60	101.9	101.3	0.72	0.71
100	60	60	98.8			
150	60	90	99.4			
150	60	90	99.7	99.8	0.51	0.52
150	60	90	100.4			

Table 6: Precision data.

Precision	Results		
	Concentration(µg/mL)	RSD of Peak area	RSD of Retention Time
Repeatability	30	0.89	0.021
	60	1.21	0.088
	90	1.11	0.123
Intermediate precision	30	1.42	0.087
	60	0.75	0.066
	90	0.67	0.062
Reproducibility	30	1.64	0.111
	60	0.78	0.17
	90	0.85	0.094

Robustness and ruggedness

By purposefully changing analytical parameters like flow rate (± 0.1 mL/min) , pH levels, mobile phase composition and column temperature the robustness of the method was investigated. Tables 7. Pictorial presentation was shown in Fig 4. Different analysts carried out the procedure's ruggedness. Table 8 presents the outcomes. Pictorial presentation was shown in Fig 5.

Table 7 : Robustness data.

Parameter	Variation	Observed value			
		RSD % of area	RSD% of R.T	Tailing factor(T)	Theroteical plates(N)
Flow rate(mL/min)	0.9	0.47	0.097	1.02	5112
	1.1	0.65	0.076	1.03	5225
M.Phase Composition	70% methanol	0.79	0.044	1.04	5187
	60 % methanol	0.81	0.132	1.05	5302
Wavelength	277 nm	0.66	0.076	1.02	5221
	273 nm	0.92	0.026	1.01	5107
P^H	4.9	0.89	0.092	1.09	5378
	4.5	0.91	0.064	1.05	5218
Column Temperature	25 °C	0.94	0.097	1.03	5189
	35 °C	0.82	0.091	.1.02	5451

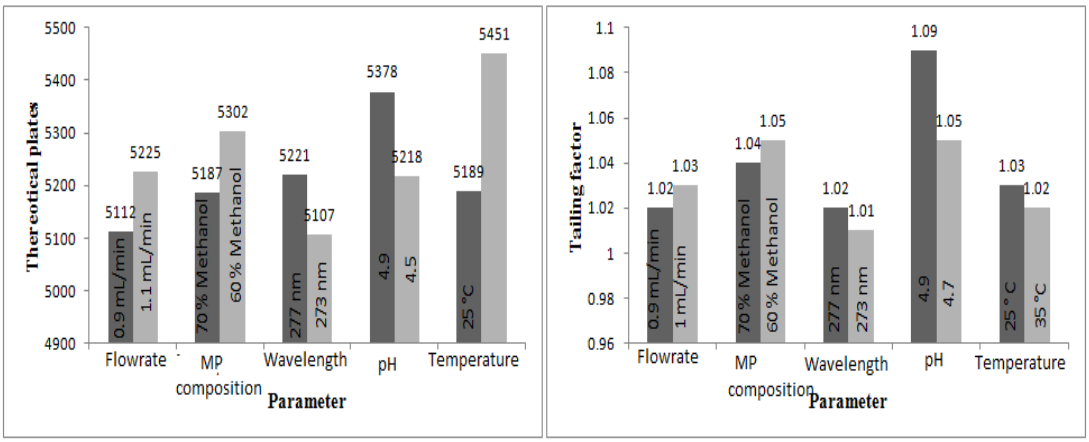

Fig .4 : Robustness data.

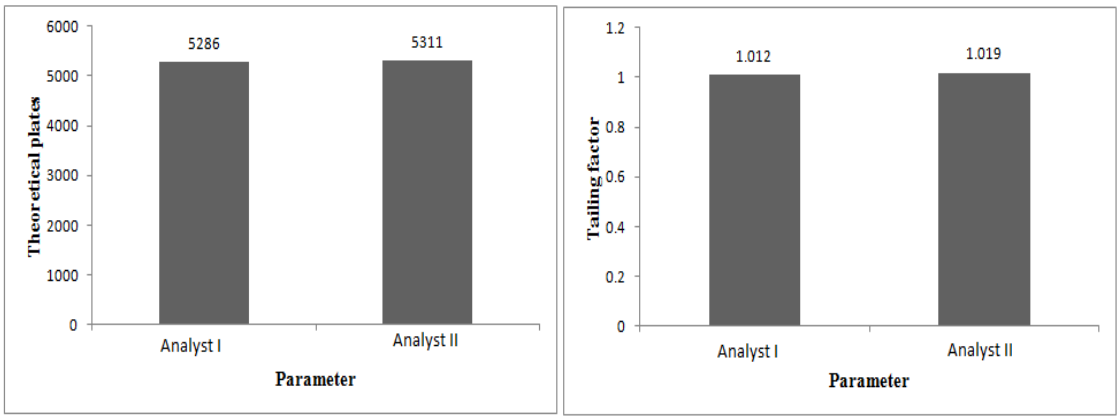

Fig .5 : Rugedness data.

Table 8 : Ruggedness data.

Analyst	Observed value			
	RSD % of area	**RSD% of R.T**	**Tailing factor(T)**	**Theroteical plates(N)**
Analyst I	0.45	0.078	1.012	5286
Analyst II	0.52	0.066	1.018	5311

Mobile phase stability

The mobile phase was kept at 4 to 8 °C for a week in order to test its stability. A freshly prepared mobile phase and an older one were compared. At 4–8 °C, the mobile phase remained stable for up to a week.

Forced degradation studies

Degradation in neutral condition

Using pure water, calcium orotate underwent neutral degradation. Accurately weighing ten micrograms of calcium orotate mass, it was then put into a tidy volumetric flask of capacity 10 mL. The volumetric flask's contents were mixed with distilled water of 5 mL. The volumetric flask was then heated to 80 °C in a water bath. For various time periods, including 0 min, 30 min, 1 hr, 2 hrs, and 4 hrs, samples were produced. Several sample solutions were collected at various periods, and then 5 mL of HPLC-grade methanol was added to each . After sonicating the sample solution for 5 minutes, it was diluted to a concentration of 60 µg/mL . After filtering it via a 0.22 µm filter, 20 µL was added for analysis in the HPLC. The acquired chromatogram was checked for any degradation that could have happened over time. Fig. 6 present the findings.

Degradation in acidic condition

Using 0.1 M HCl, calcium orotate was acidically degraded. Accurately weighing ten micrograms of calcium orotate mass, it was then put into a clean volumetric flask of capacity 10 mL. The volumetric flask's contents were dissolved in 5 mL of 0.1 M HCl and heated to 80 °C on a water bath. For various time periods, including 0 min, 30 min, 1 hr, 2 hrs, and 4 hrs, samples were produced. At different periods, various sample solutions were withdrawn, and 5 mL of HPLC-grade methanol was subsequently added. The sample solution was then subjected to a 5-minute sonication , and diluted to a concentration of 60 µg/mL. After that, it was put into the HPLC after being filtered with a 0.22 µm filter. The resultant chromatogram was evaluated for any degradation happening throughout the period. Fig.7 present the findings.

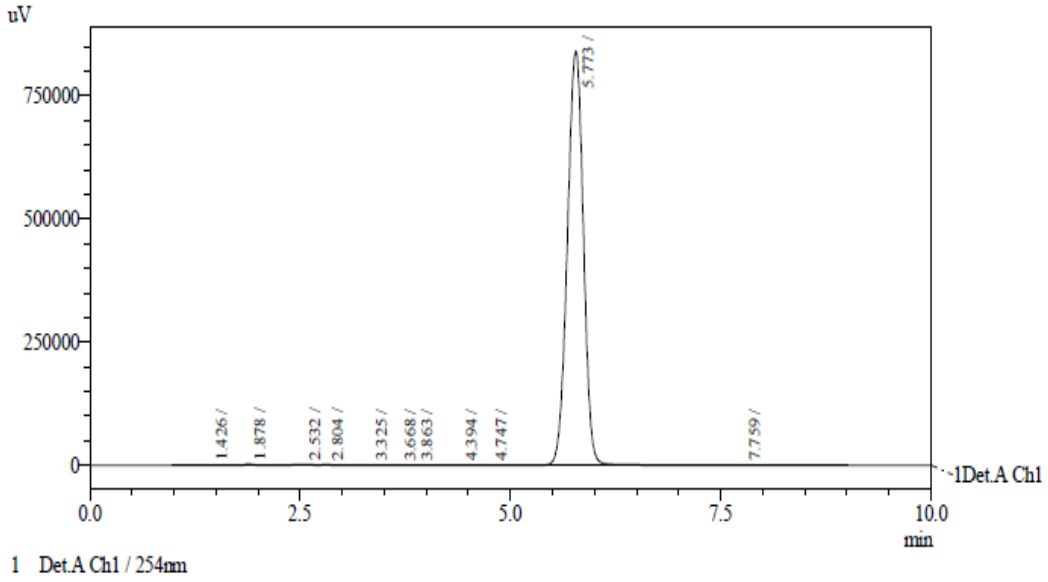

Fig.6 : Chromatogram for neutral degradation.

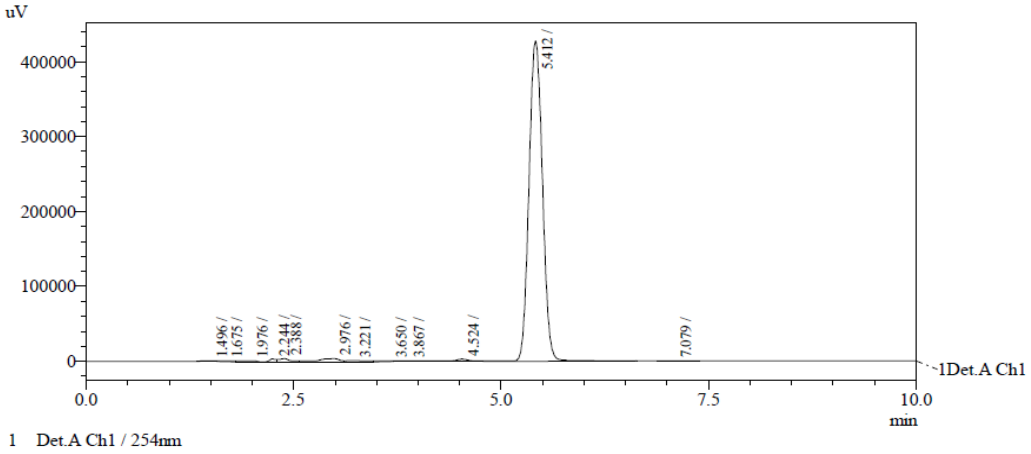

Fig.7 : Chromatogram for acid degradation**.**

Degradation in basic condition

With 0.1 M NaOH, calcium orotate was subjected to an alkaline degradation process. Accurately weighing ten micrograms of calcium orotate mass, it was then put into a tidy volumetric flask of capacity 10 mL. The volumetric flask's contents were dissolved in 5 mL of 0.1 M NaOH and heated to 80 °C on water bath. For various time periods, including 0 min, 30 min, 1 hr, 2 hrs, and 4 hrs, samples were produced. At different periods, various sample solutions were withdrawn, and 5 mL of HPLC-grade methanol was subsequently added. The sample solution was then subjected to a 5-minute sonication , and diluted to a concentration of 60 µg/mL. After that, it was put into the HPLC after being filtered with a 0.22 µm filter. The generated chromatogram was examined for any deterioration that could have occurred over time. Fig. 8 present the findings.

Oxidative degradation

Hydrogen peroxide (3 % concentration) was the agent used for oxidation. Accurately weighing ten milligrams of calcium orotate mass, it was then put into a tidy volumetric flask of capacity 10 mL. The contents of the volumetric flask added to 5 mL of H_2O_2. It was then allowed to degrade at room temperature. For various time periods, including 0/30/60/120/240 min samples were produced. At different periods, various sample solutions were withdrawn, and 5 mL of HPLC-grade methanol was subsequently added. The sample solution was then subjected to a 5-min sonication , and diluted to a concentration of 60 µg/mL. After that, it was put into the HPLC after being filtered with a 0.22 µm filter. The acquired chromatogram was examined for any deterioration that could have occurred throughout the allotted period. Fig. 9 present the findings.

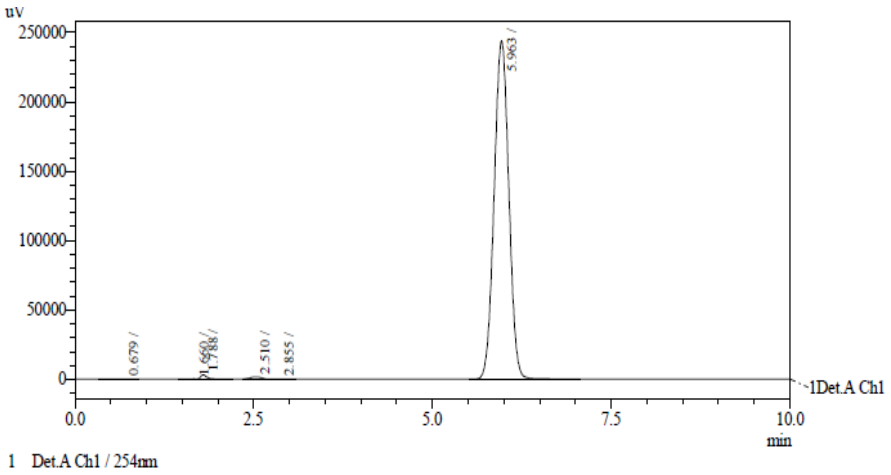

Fig.8 : Chromatogram for base degradation.

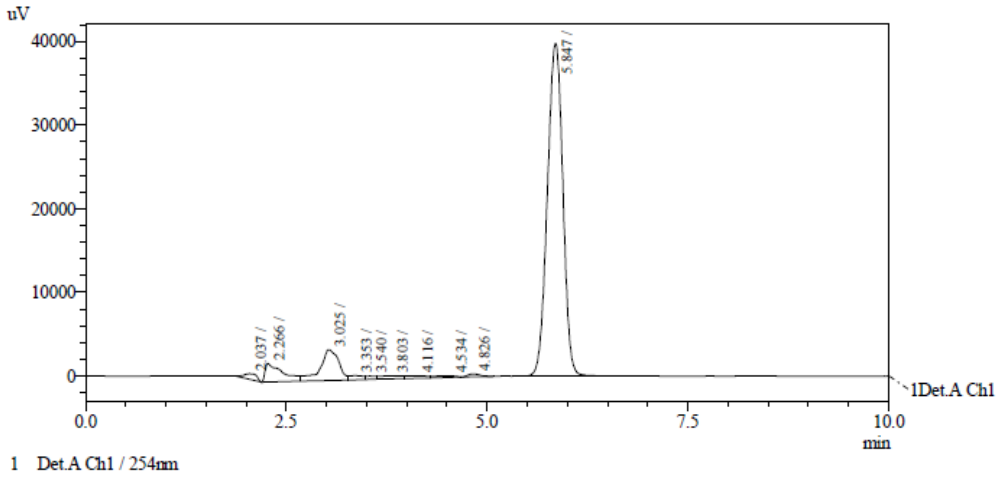

Fig.9 : Chromatogram for oxidative degradation**.**

Photolytic degradation

Carefully weighing 100 mg of calcium orotate mass and placing it in a clean petridish for photolysis. The closed petridish was then exposed to direct sunshine to degrade. At various time periods, 10 mg of material was extracted. It was used to make a 1000 µg/mL stock solution. It was then subjected to a 5-minute sonication and diluted to create a workable solution of 60 µg/mL in the mobile phase. Following that, it was filtered via a 0.22 µm filter and put into the HPLC. The resulting chromatogram was analyzed for any degradation that occurred throughout the course of the experiment. Fig. 10 show the findings.

UV-degradation

Calcium orotate, weighed to the 100 mg, was placed in a clean petri dish and exposed to ultraviolet light. After that, the petridish was placed in a UV chamber 30 cm away from the UV light. The petridish's lid was removed to allow for deterioration. The UV light was turned off after 3 hrs, and 10 mg of material was extracted. A stock solution of 1000 µg/mL was created using the mobile phase, from which a working solution of 60 µg/mL was obtained. It was sonicated , filtered through a 0.22 µm filter and feed into HPLC(20 µL). Fig. 11 show the breakdown of calcium orotate in the presence of UV radiation.

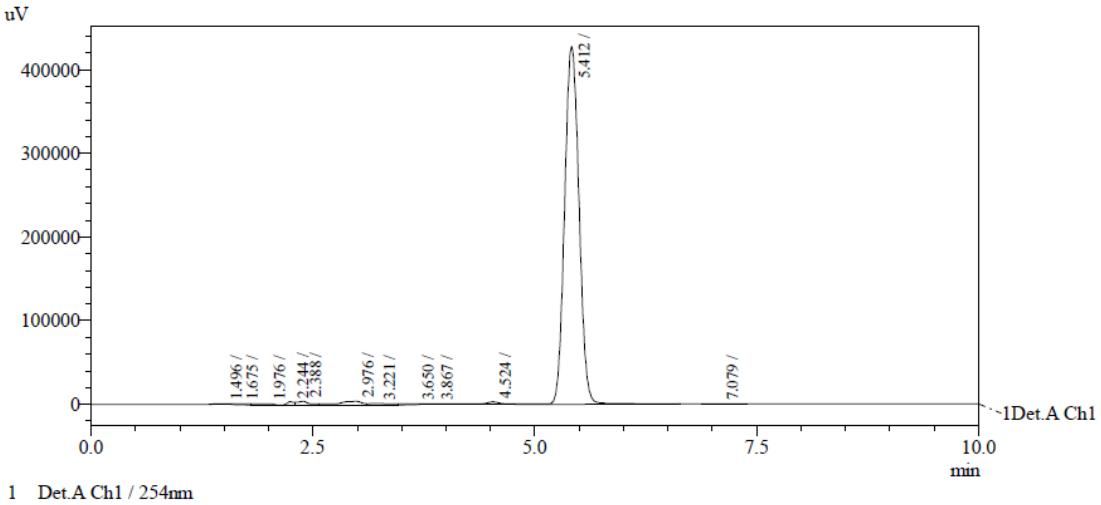

Fig.10 : Chromatogram for photolytic degradation.

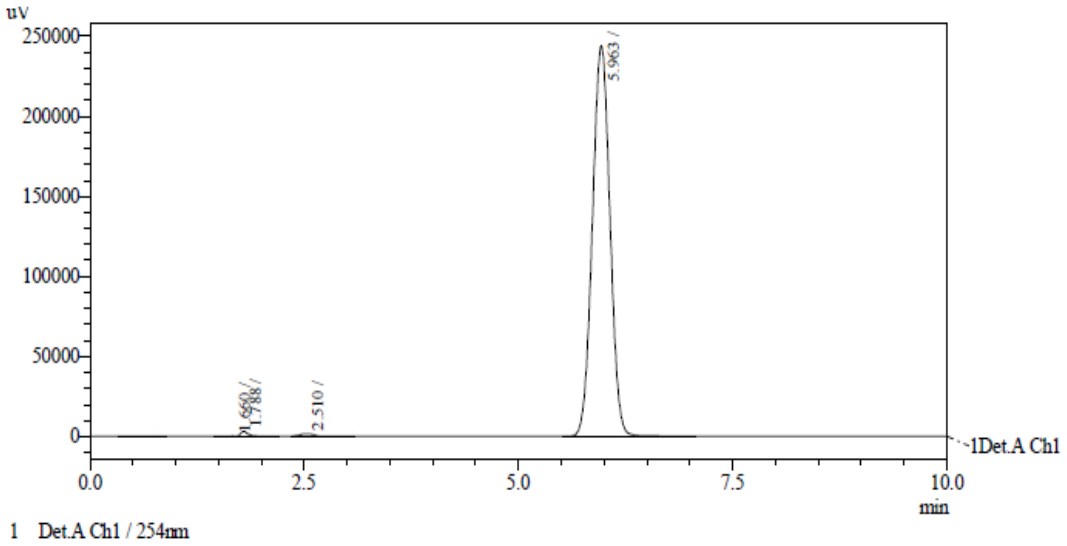

Fig.11 : Chromatogram for UV degradation.

Thermal degradation

The calcium orotate mass was thermally degraded by putting it in a hot air oven set to 40 °C. At periodically, samples were obtained. The weighted sample was sonicated for 5 minutes after being combined with 5 mL of HPLC grade methanol. The standard stock solution (at 1000 µg/mL) was made in methanol. The stock was diluted with mobile phase to provide a working standard solution at a concentration of 60 µg/mL. It was put through a 0.22 µm filter after being sonicated and fed into the HPLC in 20 µL batches. The chromatogram obtained was evaluated for any deterioration that occurred over time, and the findings are shown in Fig. 12.

The HPLC findings demonstrated that when stress conditions were applied to calcium orotate, there was no interaction between the tested drug and the degradation products, demonstrating the specificity and stability indicating of the procedure. The medication degraded extensively under oxidative conditions, UV, and heat conditions. The chromatograms obtained following deterioration under various stress settings are shown in Figs. 6-12. The percentage of degradation was shown in Fig.13. The peak purity was presented in Table 10.

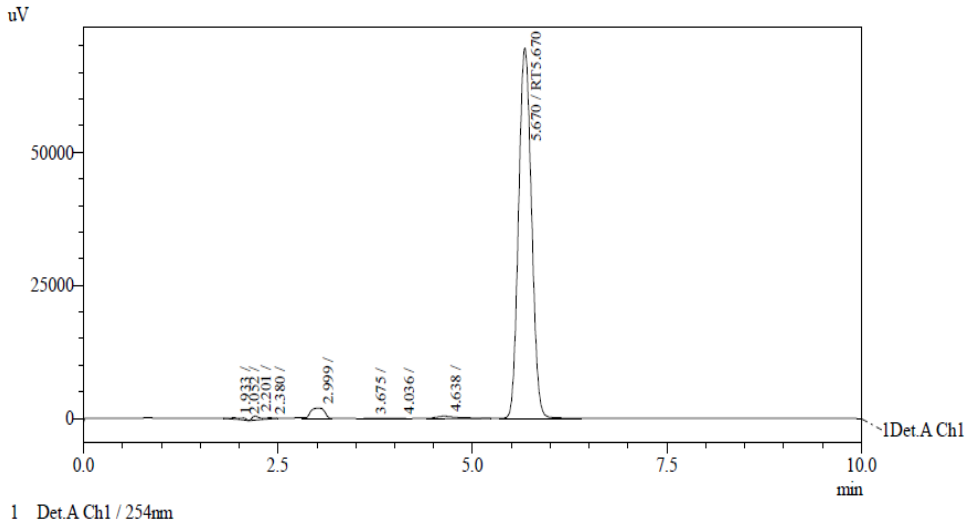

Fig.12 : Chromatogram for thermal degradation**.**

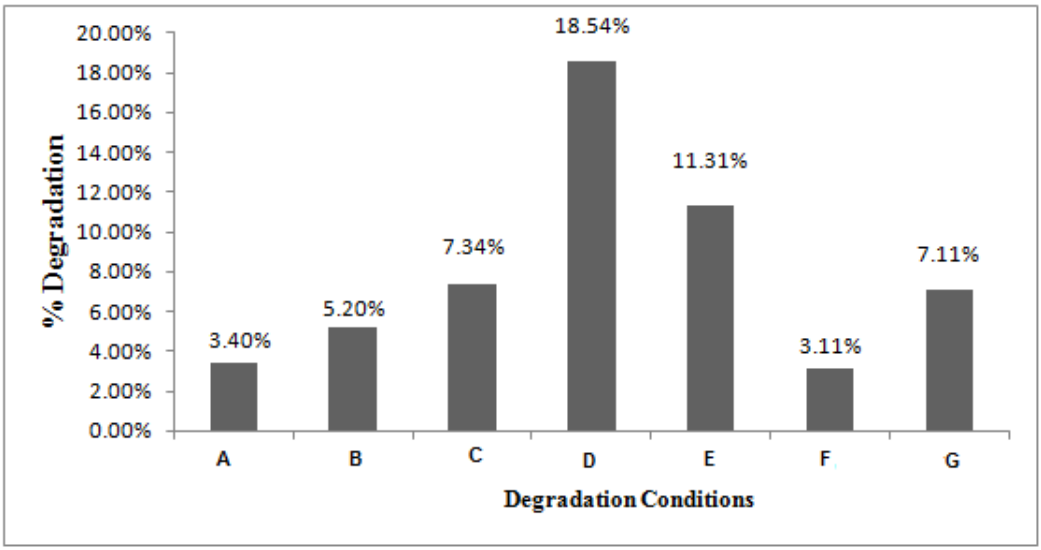

Fig.13 : Data for calcium orotate in various degradation conditions- A(Neutral),B(Acid),C(Base),D(Oxidative),E(Thermal),F(Photolytic)G(UV).

Sample Analysis

The developed and validated method was used to the analysis of calcium oratate – containing capsule formualtion. The material was examined three times. Using a calibration curve, the analysis outcomes were reviewed. Estimates of calcium orotate concentrations in the samples were calculated using the calibration curve equation, and values for recovery and relative standard deviation were also calculated. The analytical findings are shown in Table 9. The recoveries were consistent with the claims made on the label. It was determined that the approach could be effectively used to the analysis of calcium orotate in capsule dosage form. The same were also ana-lysed by the reference method[6] for comparison. The reference method is UV method. The results revealed that there is close agreement between the results obtained by the proposed methods and those of the reference method. Indeed, the results are close to the label claim. When the results were statistically evaluated by applying Student's *t*-test for accuracy and variance ratio *F*-test for precision, the calculated *t*- and *F*-values did not exceed the tabulated values at the 95% confidence level and for four degrees of freedom . This suggested that the new methods and the reference method have similar accuracy and precision.

Table 9: Results of analysis of tablets by the proposed methods and statistical comparison of the results with the reference method**.**

Capsule brand name	Nominal amount (mg)	Found[a] (% of nominal amount ± SD)	
		Refrence method	Proposed method
SWANSON ULTRA	85 mg calcium orotate	99.21±0.46	99.78±0.26 t= 2.346 F= 1.01

aMean value of five determinations.

(Tabulated t-value at the 95% confidence level and for four degrees of freedom is 2.78).

(Tabulated F-value at the 95% confidence level and for four degrees of freedom is 6.39).

Table 10: Peak purity data.

S.No	Degradation condition	Rention time of calcium orotate(min)	Peak Purity	
			Purity Angle	Purity Threshold
1	Neutral	5.773	0.025	0.217
2	Acidic	5.412	0.023	0.216
3	Basic	5.963	0.025	0.217
4	oxidative	5.866	0.024	0.217
5	Thermal	5.670	0.025	0.217
6	Photolytic	5.413	0.023	0.216
7	UV	5.961	0.025	0.217

Conclusion

For the estimation of calcium orotate in capsule dosage forms in the presence of degradation products, a simple, selective, repeatable, economical, stability indicating RP-HPLC technique has been designed and validated in accordance with ICH requirements. The percentage recovery was observed within the acceptance criteria, so it indicated method was accurate. Calcium orotate was subjected to a variety of stresses in order to undertake forced degradation tests to ascertain the stability-indicating nature of the analytic approach. Stability demonstrating forced degradation established studies revealed findings that there is no influence from any degraded products and it did not interact with ingredients in the formulation. The detection of calcium orotate and the conducted procedure are therefore specific stability-indicating. The technique is excellent for routine quantification of calcium orotate with high precision and accuracy because to its broad linearity range, sensitivity, accuracy, short retention period, and easy mobile phase.

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استخدام طريقة كروماتوجرافيا السوائل ذات الأداء العالى كتحاليل دالة على الثبات لمكملات الحمية الغذائية الكمية لأورتات الكالسيوم فى الكبسولات كشكل صيدلى

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تم فى هذا البحث تطوير طريقة RP- HPLC سريعة ودقيقة لقياس كمية الكالسيوم الموجودة فى كبسولات وتم التحقق من صحة هذه الطريقة . و كظروف مثلى للتحليل تم استخدام عمود C_8 (٢٥٠ مم X 4.6 مم ، 5µ) كمرحلة ثابتة ومادة متحركة مكونة من ٦٥: ٣٥ v/v أسيتونيتريل و ٢٥ مللى مول من فوسفات ثنائى هيدروجين البوتاسيوم (الرقم الهيدروجينى ٤,٧) بمعدل تدفق ١ مل / دقيقة عند طول موجى ٢٥٤ نانوميتر . وأظهرت الطريقة درجة إرتباط قيمتها ٠,٩٩٩ وإستجابة خطية ممتازة عبر نطاق تركيز يتراوح بين ١٥– ٩٠ ميكروجرام / مل ووقت الإحتفاظ كان ٣,١٨٦ دقيقة . وتم التحقق من صحة الطريقة وفقا لمتطلبات ICH وتعرضها للأكسدة والتحلل الضوئى والحرارة والحمض والتحلل المائى القلوى والإجهاد المائى . ولم تؤثر المنتجات الثانوية للتحلل على قدرة الطريقة على تقدير مادة أورتات الكالسيوم وبالتالى فان الطريقة تشير إلى الثبات . وتوصل البحث أنه يمكن إستخدام هذه الطريقة لتعيين مادة أورتات الكالسيوم الموجودة فى كبسولات كشكل صيدلى وأن نتائج البحث كانت خاضعة لمعايير ICH .