(*)Development of A New HPLC Method for Separation and Determination 25(OH)- Cholecalciferol (Vitamin D_3).

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ABSTRACT:

Vitamin D_3 was found interference with other fat soluble vitamins (especially vitamin A and E) that are commonly present with vitamin D, in Serum & pharmaceutical preparation.

In the present study, a rapid, simple and economical reversed phase-HPLC procedure has been fixed optimization of condition and developed for the separation and determination of vitamin D_3 in mixture standard of Vitamin $D_3$and Vitamin A.

Anew method were developed in HPLC ,based method with a UV detector by examining various conditions including mobile phase, flow rate, volume inject and temperature, we have also proposed a very simple method of isolation of these vitamins. The vitamins were separated isocratically on a Knauer C18 column (250×4.6mm, 5µm particle size) with a mobile phase consisting of isocratic acetonitrile and methanol (75:25, v/v) operated at 40ºC with retention times less than 10 min. and the detection limit of our developed HPLC method is 0.01µg/ml. The eluted vitamins were identified and monitored on a UV-Detector at 265 nm with optimum flow rate 1.5 mL/min and 50µL Injection volume. The linearity of the method was excellent (r_2 > 0.999), over the concentration range of 10-200ng/ml.

Key Words: 25(OH) Cholecalciferol (Vitamin D_3) , HPLC.

Chemistry Classification QD 71-142

*(*)This research is a part of an Ph.D. Dissertation in the case of the third researcher

INTRODUCTION:

Vitamin D (vit.D) is not a single compound but is a family of compounds that exhibit vit.D activity. The most important forms of the vit.D compounds are vitamins D_2& D_3 where the most important of them is vitamin D_3 (cholecalciferol) **[1]**. Both vitamins are absorbed from the diet and vitamin D_3 (vit.D_3) is also synthesized biologically in the skin from 7-dehydrocholesterol as a result of the action of UV radiation **[2, 3].**

Vitamin D is very important fat soluble vitamins in human and animal diets. It plays a vital role to the maintenance of normal levels of calcium and phosphorus in the blood stream and is essential for the proper development and maintenance of bone and may also have roles in the control of muscle activity, cardiovascular, colon and cellular health **[4, 5].** Without vit. D, bones can become thin, brittle or misshapen, furthermore its prevents rickets in children and osteomalacia in adults (the two forms of skeletal diseases that weaken bones). In

Many methods required expensive materials, including competitive protein binding assay (CPBA), radioimmunoassay (RIA), and high-performance liquid chromatography (HPLC) **[10]** and very sophisticated sample preparation and extraction procedures such as solid phase extraction **[11],** liquid- liquid extraction [12] and supercritical fluid extraction **[13]** while our procedure does not include any pre-treatment, extraction .Purification steps for vitamin D hadn't any recovery problem with this method. The previous methods also required long time analysis; 40min. **[13]** and16 min **[10]** but in our methods the analysis time was less than 10 min. In addition, the detection limit of our developed HPLC method is 0.01µg/ml while 7.5-30µg/ml **[9]**, 50 µg/ml and 4.1 µg/ml **[13]**. For other previous methods, which means our method has better detection limit than others.

The aim of this study is to apply, a simple, rapid, relatively low cost and straight forward HPLC method for determination of vitamin D3 in a commercial pharmaceutical preparations without purification. The described method addition, vit. D plays role in muscle contraction, nerve conduction, maintain a healthy immune system and help to maintain regular cell growth and help to maintain regular cell growth and differentiation, the process that determines what a cell is to become**[4-6]**.

Vitamin D in serum has been determined by many methods such as chromatographic that classified into biological and chemical determination **[7, 8]**. Most of these methods include complex stages, time consuming and inability to discriminate between vit.D forms. In addition to its lacking of selectivity, precision and accuracy because of ingredients in the formulation. However, these methods were widely used for analysis of lipid soluble vitamins from a sample **[8]**.

In the last decade, high performance liquid chromatography (HPLC) was announced as the most suitable technique for determination of vit.D in food, infant formula, serum and pharmaceutical preparation **[9].**
could also be applied for determination other derivatives of vitamin D and fat soluble vitamins after extraction from their samples.

Materials, apparatus and reagents:
Materials:

Vitamin D_3(25(OH)-cholecalciferol), 99.3% 50mg standard and Vitamin A (Retinol) 99%, 1 mg standard was obtained from Sigma-Aldrich/ Germany. All the solvents used were of HPLC grade, (methanol, acetonitrile) supplied by (Sigma-Aldrich/ Germany) and water (Scharlau/Spain).

Chromatographic conditions:

The computerized HPLC system consisted of a quaternary pump ((Knauer 1000S with pump head 10mL, Germany), UV detector (Knauer 2500S with flow cell 10mm), oven (Knauer Jet stream 25-80 Co), and an Auto sampler (Knauer 39505 with loop 100 µL). Evaluation and quantification were made on clarity chrom

(Vr.5 XX) which controls the whole liquid chromatographic system.

A 5 µm particle size RP C-18 column (250×4.6 nm) (Merck) was used through-out. The column eluent was monitored at 265 nm.

Stock Solution:

A 0.01 g of each of vitamin D3 and vitamin A were accurately weighed and dissolved in methanol in two 10 ml volumetric flasks. The volume was then made up to the mark with methanol in each flask, and stored at -20 °C.

Standard Working Solution:

Standard working solutions were prepared individually in methanol for vitamin D_3 and vitamin A. Flasks containing stock and working solutions were covered with aluminum foil to protect them from light. Aliquots from each working solution were combined and diluted with methanol to yield a solution with final concentrations of 200 $ng.mL^{-1}$ vitamin D_3 and 500 $ng.mL^{-1}$ vitaminA.

Procedure:

HPLC analysis was performed by isocratic elution with a flow rate of 1.5 ml/min. The mobile phase composition was methanol-acetonitrile (75: 25) (v:v). The solvents were filtered through a 0.45 µm Millipore filter before use and degassed in an ultrasonic bath. Volumes of 50 µL prepared solutions and samples were injected into the column. Quantification was effected by measuring at 265 nm. The chromatographic run time was 10 min. throughout the study, the suitability of the chromatographic system was monitored by calculating the capacity factor (k'), the resolution (R), the selectivity (α) and peak asymmetry (T).

Calibration graph. The six concentrations of

Calibration Curve:

Mixed standard solutions containing vitamin D_3 in a concentration range of (10-200 $ng.mL^{-1}$) vitamin D_3 and vitamin A in a concentration range of (150-1200 $ng.mL^{-1}$), was prepared in methanol. Triplicate 50 µL injections were made for each standard solution to see the reproducibility of the detector response at each concentration level. The peak area and peak height of each vitamin was plotted against the concentration to obtain the

Each compound was subjected to regression analysis to obtain the calibration equation and correlation coefficients.

Results and Discussion:

UV-Spectrum Selection:

Figure (1) shows the overlapped UV spectra of vitamin D_3 and vitamin A. 265 nm was selected for monitoring and quantification the studied vitamins via HPLC, since at this wavelength vitamin D_3 shows maximum absorption.

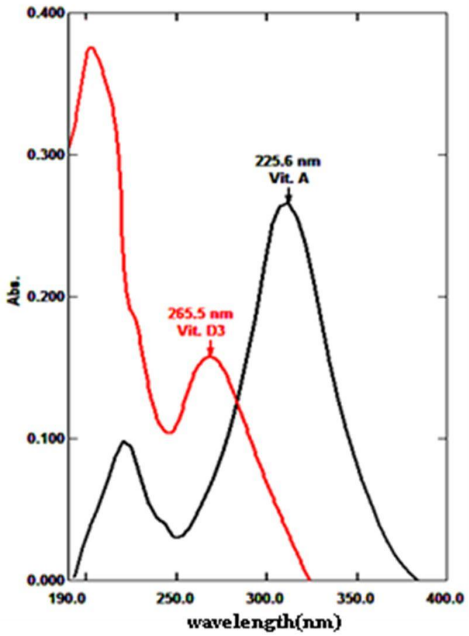

Figure (1): Overlaid UV-spectra of the vitamins D3 and A against methanol.

Flow Rate Effect:

The two vitamins were subjected to chromatographic analysis using different mobile phase's compositions, flow rates, Injection volumes, and column temperatures. The changes in the retention time, capacity factor and resolution of both analytes were noted as a function the studied parameters.

Initially different mobile phases including water with methanol and acetonitrile as organic modifiers were tried to affect the retention mechanism, but incomplete separation of peaks was observed. Later methanol and acetonitrile mixture with different ratios were tried, the best results found 25:75 v/v (methanol: acetonitrile), (Figure 2).

The study was extended to include the effect of mobile phase flow rate, injection volume column temperature. Flow rates between 0.5 and 2.0 $mL.min^{-1}$ were studied. A flow rate of 1.5 $mL.min^{-1}$ gave an optimal signal to noise ratio with a reasonable separation time, (Figure2).

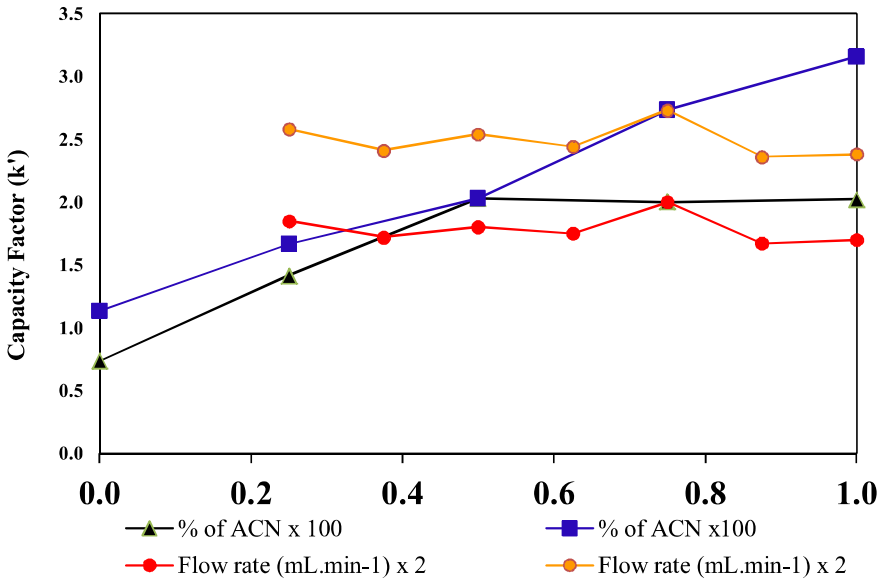

Figure (2): Effects of mobile phase composition and flow rate of the values of capacity factors of the separated vitamins.

Effect of injection volume:

The injected sample volume should be adequate such that the peak area of the smallest concentration is easily measured **[14]**. The optimum volume of the two vitamins mixture was studied over the range 5-70 µL (Figure 3), 50 µL was chosen to be the best volume injection since it gave highest peak area and peak height (Table 1).

Table (1): Effect of injection volume on the peak areas and peak heights of vitamin D3 and vitamin A.

Injection volume(µL)	Vitamins	t_R (min)	Peak area (mm)	Peak Height (mm)
5	A	4.58	268.8	22.62
	D3	5.67	130.5	10.2
10	A	4.55	655.2	49.44
	D3	5.72	375.72	20.46
20	A	4.55	1263.6	99.00
	D3	5.73	703.62	41.7
30	A	4.53	2142.3	161.28
	D3	5.73	1098	65.94
40	A	4.52	2905.08	220.98
	D3	5.67	1566.24	94.08
50	A	4.52	3769.38	291.84
	D3	5.67	199.4	120.86
60	A	4.52	4465.8	336.9
	D3	5.67	2428.8	145.56
70	A	4.52	4636.2	342.2
	D3	5.67	2484	146.28

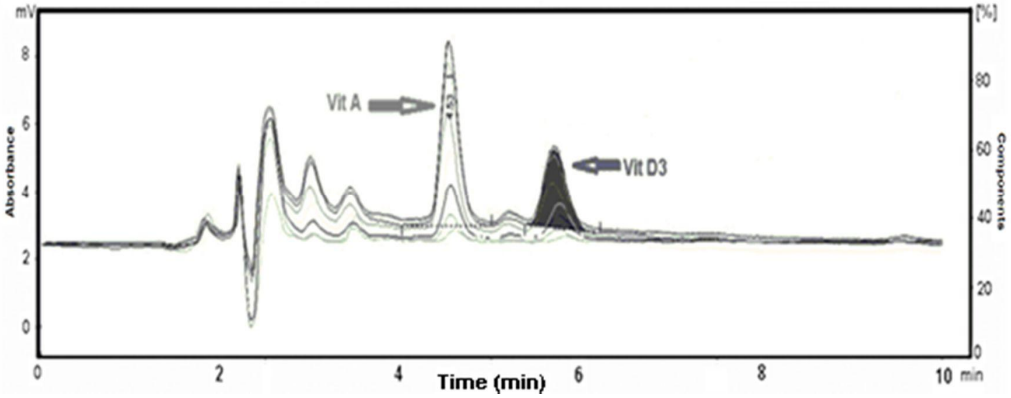

Figure (3): Overlaid chromatogram of the two vitamins D_3 and A at different sample injection volumes (5, 10, 20, 30,40,50,60 and 70 µL).

Column Temperature Effect:

Generally increasing column temperature in RP–chromatography decreases the t_R of the separated bands and increases column efficiency by decreasing mobile phase viscosity, which in turn lowering the column head pressure **[15].** Therefore, the effect of column temperatures in the range of 25 to 50 °C on the separation of the investigated compounds was studied. Figure (4) shows that optimum column temperature was found to be 40° C. At this temperature the best resolution value for the separated bands was attained (Table 2) with a well-defined shape bands.

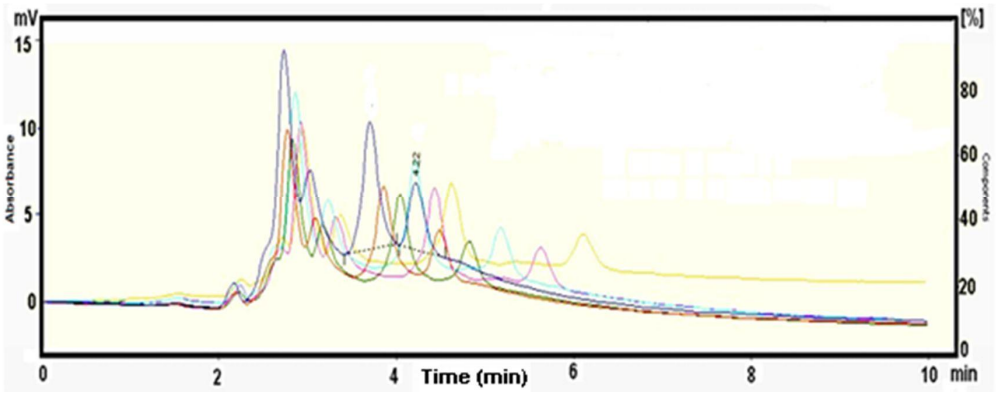

Figure (4): The overlaid chromatograms of the separation of the two vitamins at different temperatures.

Table (2): Effect of column temperature on the suitability of the chromatographic system used for separation of vitamins D_3 and A.

Temper-ature °C	Vitamin	Retention Time (t_R)	Capaci-ty factor (k')	Selectivity Factor (α)	Efficiency (N)	Resolution (Rs)
25	A	4.73	1.993	1.460	2660.9	2.99
	D3	6.18	2.911		2594.6	
30	A	4.54	1.837	1.377	2789.0	2.47
	D3	5.65	2.531		2519.8	
35	A	4.30	1.687	1.340	2493.2	2.09
	D3	5.22	2.262		2251.6	
40	A	4.13	1.613	1.290	2334.7	2.03
	D3	4.87	2.082		2048.4	
45	A	3.95	1.500	1.240	2248.6	1.45
	D3	4.52	1.860		1918.3	
50	A	3.80	1.405	1.211	2130.4	1.40
	D3	4.27	1.702		1905.2	

Applying the optimum experimental conditions, the chromatogram at 265 nm showed a complete resolution of all peaks (Figure 5).

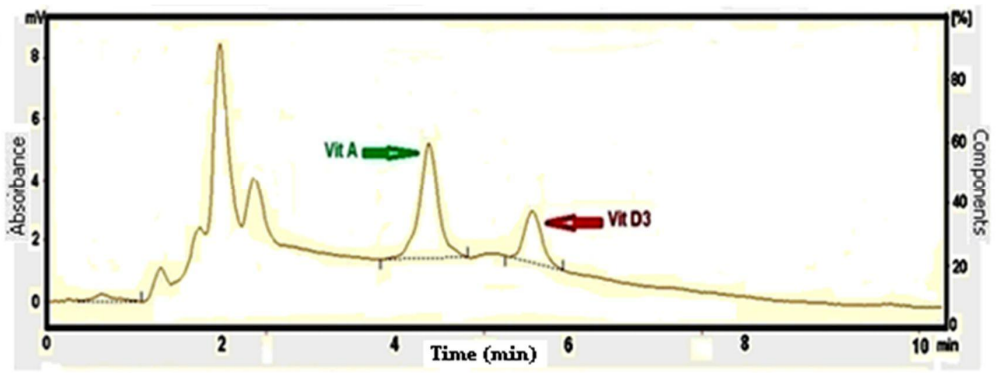

Figure (5) Chromatogram for separation of two vitamins (A&D_3) under the optimized of HPLC conditions.

Calibration Curve :

Calibration plots for vitamin D_3 and vitamin A were constructed by plotting the peak area and peak height against respective concentrations as shown in Figure 6. Table 3 presents the equation of the regression line, correlation coefficient (r^2), lower limit of detection (LOD), and lower limit of quantification (LOQ). Excellent linearity was obtained for the compound between both the peak areas and peak height vs concentrations in concentration range of 1-200 $ng.mL^{-1}$ with r^2 values of 0.9990 and 09996 respectively for Vit D_3 and 150-1200 $ng.mL^{-1}$ with r^2 values of 0.9991 and 09988 respectively of Vit A.

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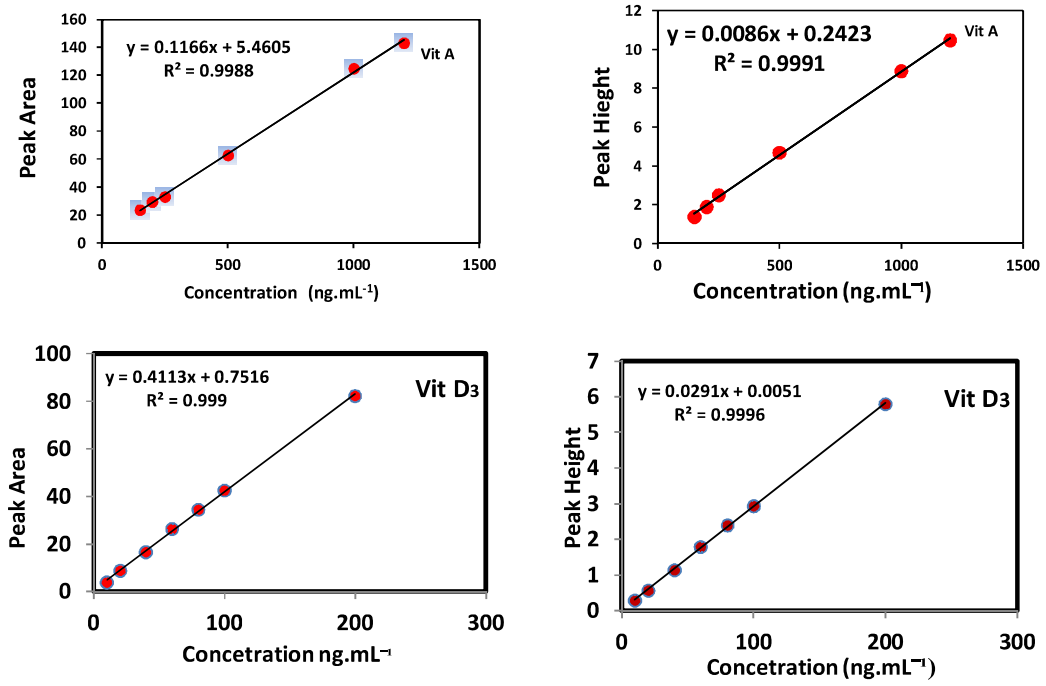

Figure (6): Calibration graphs, concentration (ng/mL) vs. peak area and peak height for Vitamins D3 &A.

Table (3): Calibration data for the analysis of vitamin D3 & vitamin A.

Analyte	Mode	Linearity range ($ng.mL^{-1}$)	Regression equation	r^2	LOD ($ng.mL^{-1}$)	LOQ ($ng.mL^{-1}$)
Vit. A	Peak area	150-1200	y=0.01166 x+5.461	0.9988	46.53	155.12
	Peak height		y=0.0086 x+0.2423	0.9991	39.57	131.89
$Vit.D_3$	Peak area	10-200	y=0.411+0.751	0.999	6.22	20.74
	Peak height		y=0.029x+0.005	0.9996	5.79	12.65

Mobil Phase Effect:

Methanol - acetonitrile mixtures have approximately 2.5 times lower viscosity than the corresponding methanol-water mixtures; this enables using 2.5 faster flow rates with acetonitrile as organic modifier and develops faster separation methods **[16].**

It was found that when the percent of acetonitrile less than 50% , Vit D3 eluted before Vit A , with asymmetrical peak ,poor separation, resolution and tailing problems, when the percent of acetonitrile at 50%, leads to completely interference between these peak.

The best separation with good resolution ,symmetrical peaks and shorter time analysis was observed when the percent of acetonitrile at became to 75% , Vit D3 eluted after than Vit A, 100% acetonitrile mobile phase lead to good separation and resolution but longer time analysis and may be interference with other fat soluble especially in serum.

Flow Rate Effect:

The aim of choosing the optimum flow rate is to obtain short analysis time and preventing solute band diffusion (i.e. band boarding), which in turn leads to high column efficiency **[17]**, Flow rate of mobile phase has an important effect on the analysis retention time. Higher flow rates lead to a shorter retention time because the eluent carries the vitamins through the column faster after desorption and vice versa.
1.5 ml/min was chosen to be the optimum since separation of the studied vitamins was obtained the best resolution (Rs) values and a reasonable analysis time.

Volume injection Effect:

Volume injection appeared significant difference in peak area and peak height, this is related to the amount of analyst passed through the column. The injection volume should be adequate such that the peak area of the smallest concentration is easily measured **[14]**, therefore; 50 µL was chosen to be the best volume injection since it gave highest peak area and peak height. The study shows that changing sample injection volume has no significant effect on the retention times of the separated bands.

Temperature Effect

Generally the depression in k' values at elevated temperature is attributed, as mentioned before, to decrease in viscosity of the mobile phase, as well as the transfer of the analyte from the mobile phase to the stationary phase is usually exothermic **[15]** .The optimum column temperature was found to be 40C°. At this temperature good shape and good resolution values of the separated bands of the Vitamins were obtained.

Reliable measurement of serum 25(OH)D can present some difficulties. The first problem is connected with the structure of 25(OH)D, which characterized with a high hydrophobic, so it can result in interference with serum components. Another difficulty is the presence of vitamin D metabolites which differ in biological activity and affinity for liver enzymes, binding proteins and VDR (vitamin D receptor) **[18].**

Conclusion:

In this study, we developed a new HPLC-based method:

- With a UV detector(at 265nm) by examining and
- Then establishing various conditions including mobile phase (methanol: acetonitrile v:v), flow rate(at1.5 $mL.min^{-1}$), volume inject(50 µL)and temperature (40C°).
- In result we proposed a very simple method for determination and isolation vitamins Aand D.

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(*)تطوير طريقــة جديدة في كروماتوكرافيا السائل عالي الأداء لفصل وتقدير

(D3) فيتامين 25-(OH)-Cholecalciferol

تاريخ الاستلام 2016/3/20 تاريخ القبول 2016/5/22

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الخلاصة :

يتواجد فيتامين D_3 متداخلا مع بقية الفيتامينات الذائبة في الدهون (خاصة مع كل من فيتامين A و E) الموجودة في مصل الدم والمستحضرات الدوائية . أذ تم في هذه الدراسة الحالية استخدام طريقة سريعة وبسيطة واقتصادية وحساسة هي كروموتوغرافيا السائل عالي الاداء ذو الطور العكوس reversal HPLC وتثبيت ظروف قياسية مثلى جديدة لفصل وتقدير فيتامين D_3 في مزيج يحوي محاليل قياسية لكل من فيتامين A وD_3 .

وشملت الدراسة الظروف المثلى التي ثبت بها الفصل ضمن مدى من الاطول الموجية تراوحت مابين (200-400) nm ، الطور المتحرك، سرعة الجريان، حجم النموذج المحقون ودرجة الحرارة أذ تم فصل هذه الفيتامينات بطريقة سهلة وبأستخدام عمود فصل ذو مواصفات C18 column (250×4.6mm, 5µm particle size) والمجهز من شركة Knauer/Germany وطور متحرك مكون من مزيج الايثانول: الاسيتونايترايل بنسبة حجمية (25:75)ودرجة حرارة 40ºC وبزمن تظهير اقل من 10دقائق وحد كشف عند0.01µg/mL. وتم تشخيص ومراقبة الفيتامينات المعزولة بأستخدام مكشاف الاشعة فوق البنفسجية UV عند طول موجي 265 nm و بمعدل سرعة جريان مثلى 1.5 mL/min و كان حجم النموذج المحقون 50 µl.

الكلمات المفتاحية: 25- هيدروكسي كوليكاليسفيرول-كروموتوغرافيا السائل عالي الاداء.

(*) البحث مستل من اطروحة دكتوراه للباحث الثالث .