Development of High-Performance Liquid Chromatographic Method for Vitamin D_3 Analysis in Pharmaceutical Preparation

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ABSTRACT

In the present study, a rapid, simple and economical reversed phase-HPLC procedure has been developed for the determination of vitamin D_3 and vitamin D_2 in pharmaceutical preparations. The vitamins were separated isocratically on a Teknokroma C18 column (150×4.6mm, 3.5µm particle size) with a mobile phase consisting of isocratic acetonitrile and methanol (75:25, v/v) operated at 40ºC with retention times less than 12 min. The eluted vitamins were identified and monitored on a diode-array detector (DAD) at 265nm. The linearity of the method was excellent ($r^2 > 0.999$), over the concentration range of 0-10µg/ml. The statistical evaluation of the method was examined performing intra-day and inter-day calibrations and were found to be satisfactory, with 2.600 ± 0.078% and 6.267 ± 0.050% accuracy and 0.001 ± 0.002% to 2.144 ± 0.050% precision results. Mean recovery of vitamin D_3 was 98.2 ± 0.349% for different spiking levels. Detection limit was found to be 25ng/ml. No interference was found from other fat soluble vitamins (vitamin A, vitamin E and vitamin E acetate) that are commonly presents with vitamin D pharmaceutical preparation. The method was applied for the analysis of sixteen solid samples of pharmaceutical preparation (calcium with vitamin D). The results show only 31.25% of the tested samples give satisfactory agreement with the declared values.

Keywords: Vit. D, HPLC, DAD and Pharmaceutical Preparation.

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. Vit.D sufficiency prevents rickets in children and osteomalacia in adults (two forms of skeletal diseases that weaken bones). In addition, vit.D plays role in muscle contraction, nerve conduction, maintain a healthy immune system and help to maintain regular cell growth and differentiation, the process that determines what a cell is to become [(4, 5, 6)].

Because vit.D might be lost during processing and

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storage of fortified foods through chemical reactions and inadequate level of cholecalciferol (due to reduction in either absorption or metabolism by the liver), it is important to take vit.D supplement so as to compensate the possible deficiency of this vitamin in our bodies. For these reasons, it takes part in many pharmaceutical preparations, but its concentration in that preparation is always very low. This has stimulated research on accurate, sensitive and efficient analyses of vit.D for the quality control of this vitamin in its pharmaceutical or other preparation.

Vitamin D in pharmaceutical products has been determined by many methods (chromatographic and other technique) 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 it's 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 [7, 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 and pharmaceutical preparation [9-16]. Many of these HPLC methods required expensive materials [11, 13, 14, 16] and very sophisticated sample preparation and extraction procedures such as solid phase extraction [9, 14], liquid- liquid extraction [11, 13] and supercritical fluid extraction [16] while our procedures does not include any pre-treatment, extraction and sample purification steps for vitamin D. Thus, there isn't any recovery problem with this method. The previous methods also required long time analysis; 40min [15] and 16 min [13] but in our methods the analysis time was less than 12 min. In addition, the detection limit of our developed HPLC method is 0.02µg/ml while 7.5-30µg/ml [10], 50 µg/ml [15] and 4.1 µg/ml [16] for other previous methods, which means our method has better detection limit than others.

In this paper, a simple, rapid, relatively low cost and straightforward HPLC method is described for determination of vitamin D_2 (vit.D_2) and vit.D_3 in their commercial pharmaceutical preparations without purification. The described method could also be applied for determination of other vitamin D derivatives and fat-soluble vitamins after extraction from their samples.

METHODS AND MATERIALS

Apparatus

The HPLC system used through out this study consisted of a quaternary pump (Agilent 1100, G1311A /USA), a manual sample injector (Rheodyne, Cotati, CA, USA) with a 20µl sample loop, a degasser (Agilent 1100, G1322A /USA) and photodiode array detector (Agilent 1100, G1315A /USA). Evaluation and quantification were made on HP ChemStation software (Rev. A 06.03) which control the whole liquid chromatographic system. The column used was a reversed phase C18 column (150x 4.6 mm, 3.5µm particle size), purchased from Teknokroma/Barcelona, Spain**.** Mobile phase was acetonitrile/methanol (75:25%) delivered at a flow rate of 1.0 ml/min and held constant at 40 ºC. The results were monitored at three different wavelengths; 265, 280 and 325 nm, that have been found the λ_{max} of different vitamins D, E and A; respectively.

Materials

Chemicals and solvents used through out this study such as methanol (BDH/UK) and acetonitrile (BDH/UK) were of high quality and HPLC grade. Vit.D_3, 99% was obtained from Acros Organics, USA and vit.D_2 was obtained as a gift from the United Pharmaceuticals / Amman-Jordan. All vit.D_3 tablets used in this study were purchased from a local drug store in Amman.

Standard Preparation

A stock solution mixture of vit.D_3 standard and the internal standard (I.S) vit.D_2 was prepared (1mg/ml each) in methanol and stored at -20˚C. Then a working solution mixture was prepared by diluting with the mobile phase. Flasks containing stock and working solutions were covered with aluminum foil and the whole experiment was performed in the dark.

Sample Preparation

Each sample was analysed with the addition of 200µl of 25µg/ml I.S. After being powdered in a mortar and transferred into 20 ml glass tube. The sample was vigorously shaken twice with 5ml mobile phase for at least 10min in rotary mixer, centrifuged at 4000rpm for 5min., the supernatant was taken and filtered, then evaporated to dryness under a steam of N_2. Finally, the residue was dissolved in 0.5ml mobile phase and was injected into the HPLC column.

RESULTS AND DISCUSSION

A) Optimization of the chromatographic separation

The Experiments were conducted on two C18 columns using the previous mentioned HPLC system. Table (1) illustrates varieties of mobile phases with different constituents which were prepared and used to select the one which the best separation (resolution) of the vitamins (D_3& D_2) with an acceptable retention time. The optimum mobile phase was found to be 75:25% (v/v) acetonitrile: methanol and the column was C18 column (150mm ×4.6mm, I.D, 3.5µm, particle size).

Table 1: The HPLC column packing materials and mobile phases investigated (vit.D_3, vit.D_2, vit.E, vit.E acetate and vit.A at 265, 280 and 325nm at flow rate 1ml/min and column temperature 25°C).

Packing material (stationary phase)	Mobile phase	Note
Eurosher-100, C18 (250mm×4.6mm,I.D, 5µm, particle size)	Gradient elution:100% MeOH for 4min. then THF + MeOH (13%+87%, v/v) for 2min., finally THF + MeOH (50%+50%).	Asymmetrical peaks, poor separation with tailing problems and long time analysis (>20min.).
Eurosher-100, C18 (250mm×4.6mm,I.D, 5µm, particle size)	Gradient elution :100% MeOH for 4min. then THF + MeOH (5%+95%, v/v) for 5min., finally THF + MeOH (50%+50%).	Asymmetrical peaks, poor separation with improve tailing problems and long time analysis (>20min.).
Eurosher-100, C18 (250mm×4.6mm,I.D, 5µm, particle size)	Gradient elution :100% MeOH for 4min. then MeOH + ACN (70%+30%, v/v) for 10min., finally ACN + MeOH (50%+50%).	Symmetrical peaks, Poor separation of vitamins D_2 & D_3 and shorter time analysis.
Eurosher-100, C18 (250mm×4.6mm,I.D, 5µm, particle size)	MeOH and ACN gradient from 0 to 100%	Poor separation of vitamins D_2 & D_3.
Eurosher-100, C18 (250mm×4.6mm,I.D, 5µm, particle size)	Isocratic elution: (MeOH+ACN, 70%+30%)	Poor separation of vitamins D_2 & D_3.
Eurosher-100, C18 (250mm×4.6mm,I.D, 5µm, particle size)	Isocratic elution (MeOH:ACN,50%+50%,v/v)	Poor separation of vitamins D_2 & D_3.
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Isocratic elution (MeOH:ACN,50%+50%,v/v)	Poor separation of vitamins D_2 & D_3.
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Isocratic elution (MeOH:ACN,80%+30%,v/v)	Poor K & Rs for vitamins D_2 & D_3.what is K??
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Isocratic elution (MeOH:ACN,90%+10%,v/v)	Good K but poor Rs.
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Isocratic elution (MeOH:ACN,95%+5%,v/v)	Good separation of vitamins D_2 & D_3 and short retention time, but poor separation of vit.D_3 with vit.E.

Packing material (stationary phase)	Mobile phase	Note
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Gradient elution :100% H_2O for 2min., then H_2O + ACN(70%+30%) for 10min., finally H_2O+ ACN(50%+50%)	Poor separation of all vitamins (E, D_2 & D_3) and vit.A give two peaks.
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Gradient elution Start with (50%+50%) H_2O + ACN and end after 20min.at H_2O + ACN (20%+80%)	Very Poor separation of all vitamins (E, D_2 & D_3) & vit.A give two peaks.
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Gradient elution ACN:MeOH:H_2O(90%: 4%: 6%) for 5min., followed by gradient elution in order to obtain ACN:MeOH (40%: 60%) in 6min., and hold 4 min.	Poor separation of all vitamins (E, D_2 & D_3) & vit.A give two peaks.
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Isocratic elution (MeOH:ACN,40%+60%,v/v)	Poor separation of vitamins (E, D_2 & D_3).
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Isocratic elution (MeOH:ACN,30%+70%,v/v)	Poor K & Rs of all vitamins (E, E acetate, A, D_2 & D_3).
Teknokroma, C18 (150mm ×4.6mm,I.D, 3.5µm, particle size)	Isocratic elution (MeOH:ACN,25%+75%,v/v)	Good separation, K & Rs of all vitamins (E, E acetate, A, D_2 & D_3).

The effect of different temperatures (25°C, 30°C, 35°C and 40°C) was evaluated in order to find out the optimum separation, using the resolution factor; the symmetry factor and the peaks area are as basic criteria. Temperature of 40°C was found to give the best results.

Under the following optimum conditions the retention times were 9.27 and 9.80 min. for vit.D_2 & vit.D_3 respectively. Figure 1 shows the chromatogram of a standard mixture solution containing vit.D_3 & vit.D_2.

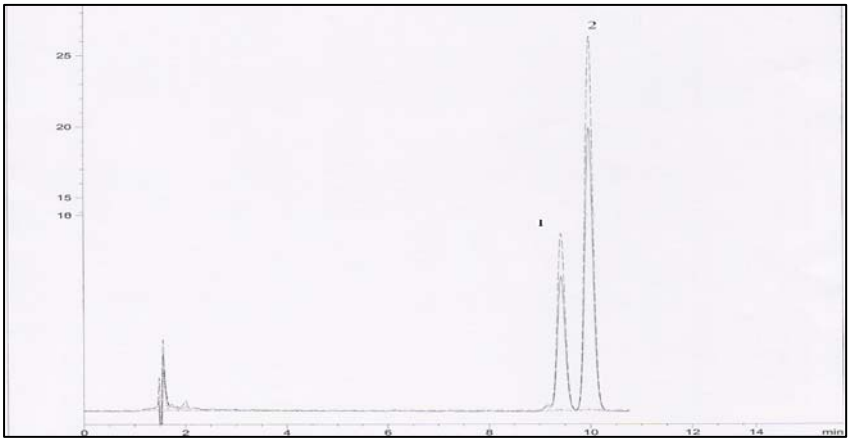

Figure 1: Chromatogram of a standard mixture solution containing vit.D_2; 25µg/ml (as internal standard) (1), vit.D_3; 10 µg/ml (2). Using the optimum HPLC conditions, at different λ_{max} 280, 325 and 265 nm, C18 column (150mm ×4.6mm,I.D, 3.5µm, particle size) and mobile phase consist from acetonitrile: methanol (75:25,v/v) at column temperature 40˚C.

B) Recovery of vit.D_3 optimization from tablets:

Different solvents were tested to find the best recovery of vit.D, after dissolving from pharmaceutical tablet. The mobile phase (75: 25%, v/v) mixture of acetonitrile / methanol was the solvent, because it shows the highest ability to dissolve vit.D_3, was easily to evaporate and gave the best recovery (99.2 ± 0.349%) as shown in table (2).

Table 2: Determination of vit.D_3 percent recovery from tablets for different solvents at concentration 10µg/ml.

Solvent	% Recovery
Methanol	94.7 ± 0.389%
Ethanol	81.2 ± 0.758 %
Chloroform	66 ± 0.967%
Acetone	123 ± 1.722%
Mobile phase (75:25%; ACN:MeOH)	99.2 ± 0.349 %

C) Method Validation

1) Calibration Curve (Linearity)

Calibration Curve was prepared by plotting vit.D_3 concentrations (µg/ml) versus the area ratio of vit D_3 to I.S (vit D_2). For this purpose a mixture of 0.0, 1.0, 3.25, 5.5, 7.75 and 10.0 µg/ml vit.D_3 with 25µg/ml vit.D_2 were prepared by diluting the stock solutions with mobile phase. Each of these standard solutions was injected twice onto the HPLC-column and the peak area ratios were calculated.

It was necessary to demonstrate a linear response for the procedure to enable quantitative analysis of vit.D_3 in pharmaceutical preparations. Linearity of the calibration curve was excellent ($r^2 > 0.999$) for the concentration range 0 – 10 µg/ml.

2) Recovery:

The percentage recoveries were determined by comparing the peak area ratios obtained from the standard and from the spiked matrix. Recoveries of vit.D_3 were determined to be between 97.62 ± 0.001 %and 98.99 ± 0.050 %as shown in table 3.

Table 3: Determination of the % recovery of vit.D_3 in the mobile phase.

Concentration range	1µg/ml	2.5µg/ml	5µg/ml
Recovery	98.03 ± 0.641%	98.99 ± 0.050%	97.62 ± 0.001%

3) Accuracy and Precision :

The accuracy was estimated carrying out the measurements of three matrix samples spiked with 1, 2.5 and 8 µg/ml of vit.D_3 and three measurements of the same matrix sample without spiking. Then the difference between the found average concentration in both spiked and non-spiked samples, is considered as the added concentration. The accuracy expressed as % bias in the estimation of the added concentration, was found to be between 2.600 ± 0.078% and 6.267 ± 0.050% as shown in table 4.

Table 4: Determination of accuracy:

Parameters	Actual Conc. (1µg/ml)	Actual Conc. (2.5µg/ml)	Actual Conc. (8µg/ml)
Measured Con.	0.959 ± 0.006	2.343 ± 0.050	7.793± 0.078
% Bias	4.10 ± 0.006	6.28 ± 0.050	2.59 ± 0.078

Table 5: Determination of the intraday and interday precision

Vit.D_3 conc.	Intraday C.V % ± SD	Interday C.V % ± SD
1µg/ml	0.641 ± 0.006	0.644 ± 0.006
2.5µg/ml	2.144 ± 0.050	0.001 ± 0.002
8µg/ml	1.002± 0.078	0.399 ± 0.031

Method precision was determined by measuring repeatability and intermediate precision (between day precision and time-different intermediate precision) for vit.D_3 during 3 days at the same concentration levels. The

variation coefficient range was from 0.001 ± 0.002% to 2.144 ± 0.050 % as shown in table 5.

4) Detection limit and Quantification limit:

Limit of detection for vit.D_3 was evaluated by determining the average concentrations that gives the signal three times the background noise which was 25ng/ml, while Limit of quantification for vit.D_3 was evaluated by determining the average concentrations that gives the signal ten times the background noise which was 60 ng/ml.

5) Specificity test:

The possible interference with the retention times of the vitamins under investigation was evaluated for the vitamins most frequently present in the pharmaceutical preparation using the same HPLC conditions. No interferences were observed as shown in table 6 and figure 2.

Table 6: The retention times of the possible interfereing vitamins, on HPLC, C18, using the optimized mobile phase acetonitrile: methanol (75:25%, v/v). While the retention times of vit.D_3 and vit.D_2 were 9.80 and 9.27 min. respectively.

Tested Vitamins	Retention time (min.)
Vitamin A	3.14
Vitamin E	11.54
Vitamin E acetate	13.41

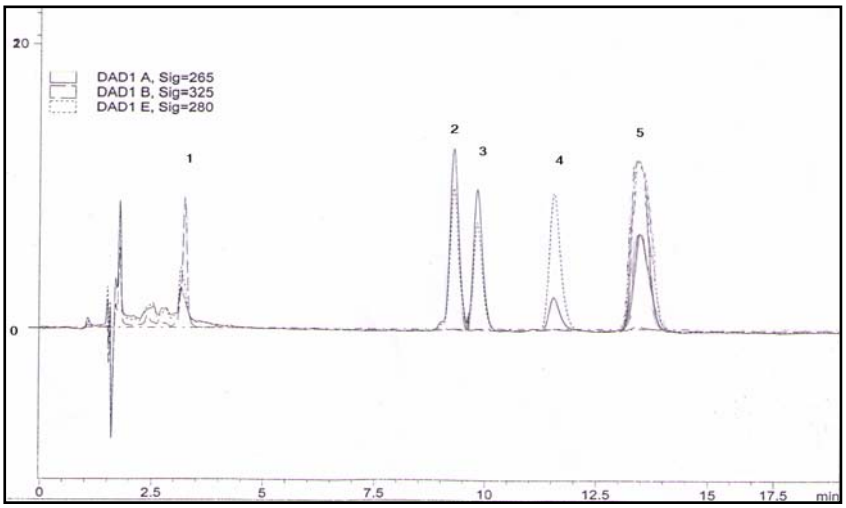

Figure 2: Chromatogram of a standard mixture solution containing (1) vit.A; 25µg/ml (2) vit.D_2, 20 µg/ml (3) vit.D_3; 5 µg/ml (4) vit.E; 50 µg/ml and (5) vit.E acetate; 50 µg/ml, Using the optimum HPLC conditions, at different λ_{max} 280, 325 and 265 nm, C18 column (150mm ×4.6mm,I.D, 3.5µm, particle size) and mobile phase consist from acetonitrile: methanol (75:25,v/v) at column temperature 40˚C.

D) Identification and determination of vit.D in authentic samples:

The developed method was applied to authentic samples (tablet and caplet of vit.D_3 with calcium). In all experiments the mobile phase was used as the dissolving solvent before starting in the HPLC analysis. Analyses were performed in duplicate. The results are shown in table 7, which show that 31.25% of the tested samples gave satisfactory results, qualitative and quantitative results were good, and in agreement with the declared values. While 68.75% of the tested samples did not agree with the declared values. This may refer to the shelf life of vit.D. Figure 3 shows the chromatogram obtained from one of the samples analyzed with the proposed method.

Table 7: Vitamin D_3 concentration in the samples obtained from the Jordanian market (n=16).

Sample number	Weight (g)	Measured conc. (µg/ml)	Labeled Conc. (µg/ml)	% Remaining	% Loss
1	1.462	2.33 ± 0.08	2.5	93.20	6.80
2	1.626	4.70 ± 0.11	5	94.00	6.00
3	1.816	4.33 ± 0.07	5	86.60	13.4
4	2.229	4.40 ± 0.01	5	88.00	12.00
5	1.219	1.21 ± 0.05	25	4.84	95.16
6	1.588	4.31 ± 0.11	5	86.20	13.8
7	1.983	4.14 ± 0.16	5	82.80	17.20
8	0.963	3.57 ± 0.06	10	35.70	64.30
9	1.824	4.50 ± 0.13	5	90.00	10.00
10	1.577	4.23 ± 0.05	5	84.50	15.40
11	1.100	3.94 ± 0.03	5	78.80	21.20
12	1.717	1.25 ± 0.15	3.125	40.00	60.00
13	1.608	2.78 ± 0.09	3.125	88.96	11.04
14	1.648	4.52 ± 0.03	5	90.40	9.60
15	1.377	2.22 ± 0.14	3.125	71.04	28.96
16	1.686	4.27 ± 0.07	5	85.40	14.60

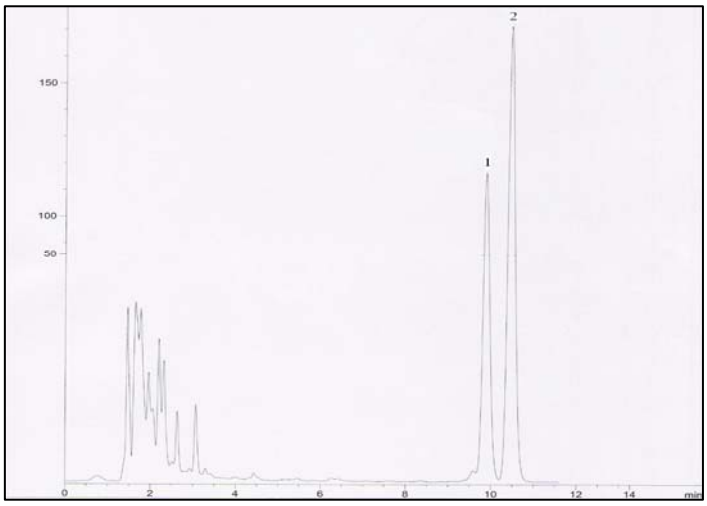

Figure 3: Chromatogram of sample, vit.D_2 (1) and vit.D_3 (2), using the optimized HPLC method at 265nm.

CONCLUSION

The separation of fat-soluble vitamins under different chromatographic conditions has been investigated. The best separation was achieved on octadecyl-bonded stationary phase using methanol-acetonitrile (75:25% v/v) as mobile phase and the DAD at $\lambda_{max.}$ = 265 nm. The validation criteria, which have been examined and discussed, show that the procedure is reliable for the

intended application.

The developed method can be considered relatively simple, rapid, and economic for routine determination and quality control of vit.D_2 and vit.D_3 in pharmaceutical products and analysis of fat-soluble vitamins in pharmaceutical preparation.

The analytical method avoids the complication of gradient separation and multi-step sample preparation. Sample preparation is very simple and because the analysis time is relatively short, the method can be used in the industrial quality assurance laboratory.

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تطوير طريقة لتحليل فيتامين (د3) في جهاز الكروماتوغرافيا السائلة ذات الكفاءة العالية في المستحضرات الصيدلانية

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ملخـص

توصلت الدراسة إلى تطوير طريقة سهلة، وسريعة، واقتصادية لتحليل فيتامين د في المستحضرات الصيدلانية بواسطة الطور الثابت (C18, 150×4.6mm, 3.5µm). كما تم استخدام الميثانول والاسيتونيترال بنسبة 25: 75 كطور متحرك بنسبة تدفق تساوي 1 مليلتر لكل دقيقة عند درجة حرارة 40°م، ودرجة امتصاص 265 نانوميتر خلال فترة زمنية تقل عن 12 دقيقة . وقد تم الحصول على نتائج مرضية لعملية تقييم دقة الطريقة المطورة وضبطها، اذ تراوحت نتائج حساب دقة الطريقة المطورة مابين 0.078% ± 2.600 - 0.050 ± 6.267%. كما تم اختبار ضبط الطريقه المطوره من خلال حساب معامل التغير خلال يوم وعدة أيام وكانت النتيجه مابين0.002± 2.144% - 0.001% ±0.050. كما كان خط المعايرة مستقيماً لكل من التراكيز 0 إلى 10 مايكروجرام / مليلتر لفيتامين د3 . ونسبة الشوائب تساوي 3 أو أقل . كما أن الفيتامينات: أ، وهـ، وهـ أسيتيت لم تتداخل أو تتأثر مع فيتامين د. وقد تم تطبيق هذه الطريقة المطورة على ست عشرة عينة صيدلانية صلبة تحتوي على فيتامين د مع الكالسيوم، قد أظهرت النتائج أن 31.25 % من العينات المختبرة أعطت قيماً موافقة لتلك القيم المصرح عنها في تلك الأدوية .

الكلمـات الدالـة: فيتامين د، جهاز الكروماتوغرافيا السائلة ذات الكفاءة العالية، الكشاف ذو المصفوفة الدايودية والمستحضرات الصيدلانية.

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