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# Separation and Assay of Cholecalciferol in Vitamin A + D Ointment

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**Summary.** In this paper two methods are presented. One involves the separation of cholecalciferol from a topical ointment. The other involves the assay of cholecalciferol in this ointment. The study was performed with normal-phase high performance liquid chromatography using a NP-L3 column and UV detection. Applying a mobile phase mixture containing *n*-hexane:2-propanol (99:1) a total separation was achieved within 15 min. For isolation and assay of cholecalciferol from an ointment (vitamin A + D), dissolution in *n*-hexane gave the highest recovery (>95%). The isolation and assay process can be performed within 3.5 h.

**Keywords.** Vitamin D; Cholecalciferol; Vitamin A; Methyl paraben; NP-HPLC.

## Introduction

Vitamin D sufficiency is required for optimal health. The conditions with strong evidence for a protective effect of vitamin D include several bone diseases, muscle weakness, multiple sclerosis and type 1 diabetes mellitus [1, 2]. The skin is the major source of vitamin $D_3$ (cholecalciferol) and ultra violet light (UV) is critical for its formation [3]. Vitamin D and its metabolites may be categorized into two families of secosteroids, the cholecalciferols and ergocalciferols. Solar UVB radiation is an initiator of vitamin $D_3$ production in the human skin [4]. Cholecalciferol, the parent compound of the naturally occurring family, is produced in the skin on exposure to sunlight. The UVB (280–320 nm) portion of sunlight converts 7-dehydrocholesterol to previtamin $D_3$. Previtamin $D_3$ changes by isomerization to form vitamin $D_3$. Vitamin $D_3$ is translocated from the skin to the blood with the help of vitamin D-binding protein (DBP) [5]. It is a fat-soluble steroid that is essential for maintaining normal calcium metabolism.

Vitamin $D_3$ is a prohormone produced in skin through ultraviolet irradiation of 7-dehydrocholesterol. It is biologically inert and must be metabolized to 25-hydroxyvitamin $D_3$ in the liver and then to 1-$\alpha$,25-dihydroxyvitamin $D_3$ in the kidney before function. The hormonal form of vitamin $D_3$, *i.e.*, 1-$\alpha$,25-dihydroxyvitamin $D_3$, acts through a nuclear receptor to carry out its many functions, including calcium absorption, phosphate absorption in the intestine, calcium mobilization in bone, and calcium reabsorption in the kidney. It also has several non-calcemic functions in the body [6].

In vitamin D deficiency, calcium absorption is insufficient and cannot satisfy the body's needs. Consequently, parathyroid hormone production increases and calcium is mobilized from bones and reabsorbed in the kidneys to maintain normal serum calcium levels [7]. Determination of vitamin $D_3$ in different materials such as foods [8–11], cod-liver oil [12–14], drugs [15, 16], and blood plasma [17] has been performed by HPLC methods (normal or reversed phase). In this study, a separation procedure was developed using

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normal phase high performance liquid chromatography (NP-HPLC). The mobile phase composition was optimized by testing *n*-hexane with different ratios of other solvent such as isopropanol and isoamyl alcohol. An isolation method for cholecalciferol in a commercially available ointment (A + D) was explored.

## Results and Discussion

The aim was finding a procedure for separation of vitamin $D_3$ (cholecalciferol) from other ingredients in A + D ointment and assay of vitamin $D_3$ in this product.

### *Separation of Cholecalciferol from other Ingredients in the Ointment*

A search for a suitable mobile phase composition began with monitoring at 265 nm with a flow rate of 1 $cm^3$/min. The mobile phase optimization began with *n*-hexane because of its good extracting behavior in the ointment. The result showed that the separation of peaks would be better if the solvent becomes more polar, so several mobile phases were tried to increase separation of peaks between vitamin $D_3$ and butyl paraben. The mixtures of isopropanol: *n*-hexane and isoamyl alcohol:*n*-hexane were tested, and *n*-hexane:isopropanol gave better results for separation [4]. The results show that good separation for cholecalciferol and butyl paraben was achieved with *n*-hexane:isopropanol (99:1) (Fig. 1).

### *Limit of Detection*

The relative sample sensitivity of a detector is defined as the minimum concentration of solute which can be detected and is evaluated by using the signal to noise (S/N) ratio for this purpose. A 200 $\mu g/cm^3$ stock of cholecalciferol was prepared by dissolving necessary amount in *n*-hexane and then solutions of different concentration from 200 to 20 $\mu g/cm^3$ include (200, 100, 80, 50, 40, and 20 $\mu g/cm^3$) were made after injection of these solutions. The concentration of 100 $\mu g/cm^3$ was found to be minimum detectable concentration by the detector. The separation of 40 $\mu g/cm^3$ was not detected with certainty. The S/N ratio was 2.5 at a preferred detection wavelength of 265 nm and a flow rate of 1 $cm^3$/min. Results showed that cholecalciferol can be easily detected at known concentration.

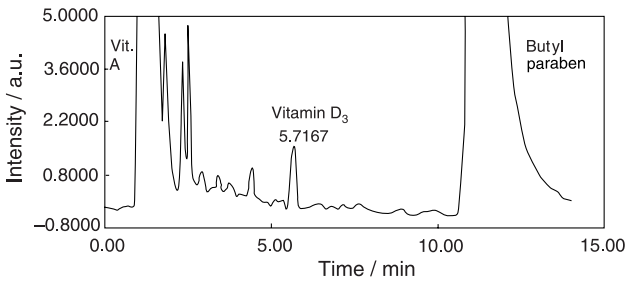


**Fig. 1.** Chromatogram of a sample of the vitamin A + D ointment containing the peaks of vitamin A, vitamin $D_3$, and butyl paraben on a Partispher silica (L3) column using *n*-hexane:isopropanol (99:1, *v*/*v*) mobile phase. Flow rate 1 $cm^3$/min; wavelength 265 nm, loop volume 20 $mm^3$, injection concentration 0.4 $\mu g/cm^3$

### *Linearity*

The linearity of an analytical method is its ability to elicit results that are directly proportional to the concentration of analyte in samples within a given range. For the establishment of linearity, a minimum of five concentrations normally is used. The relationship obtained between peak area and amount of cholecalciferol injected is linear from the selected range of 320–480 ng/$cm^3$ with a suitable correlation coefficient of 0.9991.

### *Determination of Cholecalciferol in a Pharmaceutical Preparation*

The selection of solvent for the isolation of cholecalciferol from its pharmaceutical preparation was based on finding the solvent which could dissolve only cholecalciferol or minimum of the ingredient from the ointment. Several solvents were tested by dissolving 10 g of ointment in 20 $cm^3$ of solvent and shaking for 20 min. The solvents tested were methanol, ethanol, chloroform, diethyl ether, and *n*-hexane. Among these solvents only chloroform and *n*-hexane liquefied the ointment and other solvents even after prolonged shaking did not have any effect on the ointment. However, using TLC for separation of cholecalciferol from other ingredients, we found that chloroform dissolves all the chemicals in the ointment but *n*-hexane gave better separation of cholecalciferol from other materials. So, the turbid mixture produced after shaking in *n*-hexane was centrifuged in order to separate the solidified fats and insoluble materials. Removing of other saponifiable compounds by washing with aqueous acid and base solutions were made. However, after six times extraction with *n*-hexane and centrifugation, it was injected onto the column. We observed three chromatograms

for butylated hydroxytoluene, cholecalciferol, and butyl paraben with retention times of 2, 5.8, and 12 min. The signal of vitamin A in the ointment overlapped with a retention time of 2 min with the signal of butylated hydroxytoluene.

### *Precision and Repeatability*

The precision of an analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of a homogenous sample. Determination of cholecalciferol in the ointment was performed with six samples prepared from a homogenous ointment. Tests for repeatability of injections of sample solutions showed an overall relative standard deviation of less than 2%. The recovery from six separate determinations were found to be greater than 98%. The complete procedure from isolation to recovery can be performed within 3.5 h. The HPLC method is suitable for routine quality control analysis of vitamin A + D ointment.

### *Conclusion*

A normal phase high performance liquid chromatography method is introduced for determination of cholecalciferol in vitamin A + D ointment. The isolation and recovery can be performed within 3.5 h. The method described, is simple, sensitive, repeatable and can be used on a daily basis as part of routine analysis.

## Exprimental

*Materials*

Standard sample of cholecalciferol as a pharmaceutical grade was provided by Roche (Basel, Switzerland), *n*-hexane and other analytical grade chemicals were purchased from E. Merck (Darmstadt, Germany). Vitamin A + D ointment was manufactured by Darou Pakhsh Pharmaceuticals (Tehran, Iran).

*Equipment*

NP-HPLC analysis was performed on a Partisphere 5-$\mu$m silica (L3) $4.6 \times 125$ mm column (Whatman, USA), a Younglin SP930D pump with a Younglin 730D UV spectrophotometer detector, and an Autochro 2000 integrator (Korea). The software used in Younglin HPLC was Atochrom 2005, version 18. The flow rate was 1 $cm^3$/min for separation and assay recovery. The UV detection wavelength was 265 nm for separation and assay. The loop volume was 20 $mm^3$. A Labcon speep model at 85 vibrations per minute was used for shaking during the isolation of a mixture containing the ointment and solvent. All centrifugation were performed using Clements GS200 (England).

*Solutions*

Stock solutions containing 1000 and 200 $\mu g/cm^3$ of cholecalciferol were prepared by dissolving the necessary amounts in *n*-hexane, which were then diluted to make 20 and 0.4 $\mu g/cm^3$ solutions. The other solutions of cholecalciferol with concentration of 40–100 $\mu g/cm^3$ in *n*-hexane were made for injection. Sample solutions were kept in amber-colored bottles and stored under +10°C until use.

*Determination of Cholecalciferol in the Ointment*

The amount of 10 g of the ointment containing 850 IU cholecalciferol was dissolved in 20 $cm^3$ *n*-hexane, shaken for 20 min, then centrifuged with 6000 rpm for 10 min. The clear portion was removed and 20 $cm^3$ *n*-hexane were added to the residual solid phase and again shaken for 20 min and centrifuged as above. This treatment was repeated four times, after which 2 $cm^3$ of the solution were removed and centrifuged again. The clear part of the solution was ready for injection.

*Recovery*

The recovery was computed as the peak area of cholecalciferol from the ointment divided by the corresponding peak area of a reference solution (standard) containing cholecalciferol at a concentration of 0.4 $\mu g/cm^3$ in *n*-hexane.

## References

[1] Cirunay JJN, Heyden YV, Plaizier-Vercammen J (1998) J Chromatogr Sci **36**: 417
[2] Grant WB, Holick MF (2005) Med Rev **10**: 94
[3] Bikle DD, Oda Y, Xie Z (2005) J Steroid Biochem Mol Biol **97**: 83
[4] Turnbull DJ, Parisi AV, Kimlin MG (2005) J Steroid Biochem Mol Biol **96**: 431
[5] Burtis CA, Ashwood ER (2001) Tietz Textbook of Clinical Chemistry, 5th ed, WB Saunders Co., Washington, DC
[6] Deluca HF (2004) Am J Clin Nutr **80**: 1689S
[7] Staud R (2005) Curr Rheumatol Rep **7**: 356
[8] Walker GA, Carpenter BE, Tuescher DL (1980) J Pharm Sci **69**: 846
[9] Monard AM, Berthels J, Nedelkovitch G, Draguet M, Bouche R (1986) Pharm Acta Helv **61**: 205
[10] Stancher B, Zonta F (1983) J Chromatogr **256**: 93
[11] Pask-Hughes RA, Calam DH (1982) J Chromatogr **246**: 95
[12] Bognar A, Lebensm Z (1992) Lebensm Unters Forsch **194**: 469
[13] Johnsson H, Halen B, Hessel H, Nyman A, Thorzell K (1989) Int J Vitam Nutr Res **59**: 262
[14] Muniz JF, Wehr CT, Wehr HM (1982) J Assoc Off Anal Chem **65**: 791
[15] Olkowski AA, Aranda-Osorio G, McKinnon J (2003) Int J Vitam Nutr Res **73**: 15
[16] Upreti P, Minstry VV, Warthesen JJ (2002) J Dairy Sci **85**: 3173
[17] De Vries EJ, Zeeman J, Esser RJE, Borsje B, Mulder FJ (1979) J Assoc Off Anal Chem **62**: 1285