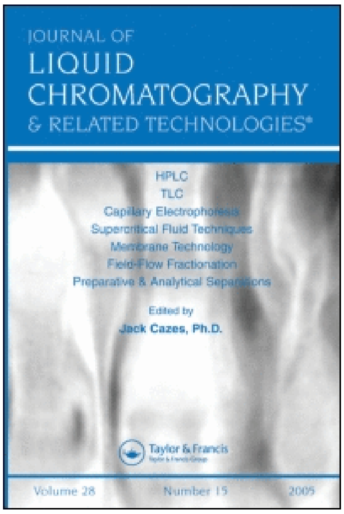

**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# Determination of Vitamin D In Multivitamin Preparations by HPLC

P. A. Lotfy[a]; H. C. Jordi[a]; J. V. Bruno[a]
[a] Waters Associates Inc., Milford, Massachusetts

**To cite this Article** Lotfy, P. A. , Jordi, H. C. and Bruno, J. V.(1981) 'Determination of Vitamin D In Multivitamin Preparations by HPLC', Journal of Liquid Chromatography & Related Technologies, 4: 1, 155 — 164

**To link to this Article: DOI:** 10.1080/01483918108064805

**URL:** http://dx.doi.org/10.1080/01483918108064805

JOURNAL OF LIQUID CHROMATOGRAPHY, 4(1), 155-164 (1981)

# DETERMINATION OF VITAMIN D IN MULTIVITAMIN PREPARATIONS BY HPLC

P.A. Lotfy, H.C. Jordi and J.V. Bruno
Waters Associates Inc.
Maple Street
Milford, Massachusetts 01757

## ABSTRACT

A selective extraction for vitamin D was achieved from oils, dry concentrates and multivitamins without the need for saponification. This was accomplished by utilizing SEP-PAK cartridges as an alternative to the pre-column clean-up required in the standard vitamin D assay. The final LC analysis was streamlined by using Radial Compression Technology in place of the conventional analytical LC column.

## INTRODUCTION

Use of High Performance Liquid Chromatography has been investigated as an approach to the determination of vitamin $D_2$ ($D_3$) in various feedstuffs and vitamin preparations. HPLC provides a greatly improved vitamin D assay by making it more rapid and specific than conventional methods; however, nearly every method relies on a complicated, time-consuming extraction and sample preparation procedure prior to injection onto the HPLC column.

In general, extraction of vitamin D out of its matrix has been a major problem, as vitamin D is present in very small amounts in formulations containing large amounts of vitamin A and, also, substantial amounts of other fat soluble vitamins.

Any method for vitamin D analysis requires accurate quantitation that is applicable to all types of multivitamin preparations including resins, oils, dry concentrates and gelatin protected beadlets.

Recent applications of HPLC to the determination of vitamin D include analysis from gelatin-protected beadlets (1). Vitamin D has been separated from its photochemical isomers (2), from inactive forms out of resins, oils and dry concentrates by Hofsass et al. (3), Oscada et al. (4), and deVries et al. (5).

Analysis of vitamin D has been accomplished from livestock feeds by HPLC (6, 7). Various reverse phase systems have been reported for analyzing vitamins A, D and E simultaneously from multivitamin tablets (8, 9) and from animal feeds (10).

Although HPLC has been found to be the most specific method for determining bioactive isomers only (3), not all systems distinguish active from inactive isomers. The presence of intermediate irradiation products in many preparations may result in higher than actual vitamin D potency in cases where there is no distinction made between them and active vitamin D. A comparison of the A.O.A.C. chemical method and the A.O.A.C. biological method shows this, indeed, to be the case (2).

A method has been developed which involves dissolution of vitamin D by homogenization and followed by extraction from other sample components by means of SEP-PAK cartridges. A normal phase HPLC system is used in the final analysis as in the work of deVries et al. (5) which separates pre-D and cis-D from all inactive isomers. This allows quantitation based on the sum of these two forms providing there are no compounds in the sample matrix with similar extraction and retention characteristics.

An interference, determined to be an impurity from vitamin A, was sometimes encountered when wavelength ratioing of the 280nm to 254nm detector response revealed an incorrect ratio in the cis-D peak. This interference was removed by employing a different solvent and simple extraction procedure that will be discussed below.

## MATERIALS

### Reagents and Solvents

LC grade n-hexane, tetrahydrofuran and methanol were used (Waters Associates, Milford, MA). Spectrograde ethyl acetate and

LC grade diemthyl sulfoxide were obtained from Burdick and Jackson, Muskegon, Michigan. Ethanol (85%), completely denatured, and normal amyl alcohol, AR, obtained from Mallinckrodt, St. Louis, Missouri.

Crystalline butylated hydroxy toluene (BHT), cholcalciferol and ergocalciferol were obtained from Sigma Chemical Company, St. Louis, Missouri. SEP-PAK $C_{18}$ and SEP-PAK Silica cartridges (Waters Associates, Milford, MA).

System suitability standard used to check column efficiency was an oil containing approximately 2mg each of vitamin $D_3$ and tachysterol and 0.2mg each of pre-vitamin $D_3$ and 5,6-trans vitamin $D_3$ per gram of oil. System suitability standard and Δ4,6=cholestadienol were obtained from Philips-Duphar, Amsterdam, Holland.

## Preparation of Standard Solutions

a. Vitamin $D_3$ Standard Solution - 50mg of cholcalciferol was dissolved in 100ml hexane and 10ml of this solution was diluted to 100ml with hexane in volumetric flask.

b. Internal Standard Solution - 15mg of Δ4,6-cholestadienol was placed in 200ml volumetric flask and diluted to volume with hexane.

   1. Procedure A: 50mg of Δ4,6-cholestadienol was placed in a 50ml volumetric flask and diluted to volume with 50:50 Methanol/THF.
   2. Procedure B: 10ml of internal standard solution was diluted to 100ml with hexane.

## Determination of $D_3$ Response Factor

Pipet 4.0ml of $D_3$ standard solution and 10ml of internal standard solution into a 100ml volumetric flask and dilute to volume with hexane. Inject 200ul and determine peak heights of the Vitamin $D_3$ and internal standard. Calculate vitamin $D_3$ response factor by the equation:

$$F_D = (P_{ir} \times W_r \times V_{ir}) \div (P_r \times W_{ir} \times V_r)$$

Where $P_{ir}$ = internal standard peak height

$P_r$ - vitamin $D_3$ peak height

$W_{ir}$ = weight of internal standard in mg (15)

$W_r$ = weight of vitamin $D_3$ in mg (50)

$V_{ir}$ = final volume in ml of internal standard (2,000 ml)

$V_r$ = final volume in ml of vitamin $D_3$ (25,000 ml)

## Determination of pre-$D_3$ Response Factor

Pipet 5.0ml of $D_3$ standard solution into a 100ml amber volumetric flask, add 20mg BHT crystals, replace air by nitrogen, heat 3/4 hour in subdued light in a $90^oC$ waterbath and cool. Add 10ml internal standard solution and dilute to volume with hexane. Inject 200ul and measure peak heights of pre-$D_3$, $D_3$ and internal standard.

Calcualte $D_3$ content (in % amount in unheated soln) $(F_D \times P_D \times V_r \times W_{ir}) \div P_{ir} \times V_{ir} \times W_r) \times 100 = q\%$. Calculate pre-vitamin D content by 100 - q% = p%. Calculate pre-D response factor as follows:

$F_{pre} = (P \times (P_{ir} \times V_{ir} \times W_r) \div 100 \times P_{pre} \times V_{pre} \times W_{ir})$

Where $F_D$ = Vitamin D response factor

$P_D$ = peak height of vitamin $D_3$ in heated solution

$P_{pre}$ = peak height of pre-vitamin D in heated solution

$P_{ir}$ = peak height of internal standard in heated solution

$W_r$ = weight $D_3$ in mg (50)

$W_{ir}$ = weight internal standard in mg (15)

$V_r$ = final volume in ml vitamin D in heated solution (20,000 ml)

$V_{ir}$ = final volume in ml internal standard in heated solution (2,000 ml)

$V_{pre}$ = final volume in pre-D in ml preD in heated solution (20,000 ml)

## Calculate Conversion Factor

$F = F_{pre}/FD$

This conversion factor must be determined for each new column and should be checked routinely.

## HPLC Chromatographic Procedure

All analyses were performed with a Waters Associates Model ALC/GPC 244 liquid chromatograph equipped with a Model 440 UV detector monitoring at 254nm and 280nm simultaneously. Waters' Model 730 Data Module was used to integrate all peaks and calculate results.

All separations were achieved using a RCM-100 Radial Compression Module (Waters Associates, Milford, MA) equipped with a Radial-PAK B normal phase chromatographic cartridge. The cartridge was equilibrated with n-hexane containing 0.35% n-amyl alcohol for approximately 2 hours at a constant flow of 5.0 ml/min before analysis.

The system suitability standard was injected to check column efficiency before any analyses were run. Resolution of $\geq$ 0.8 of pre-vitamin D from trans-vitamin D and $\geq$ 1.0 of cis-D from tachysterol were requirements to assure accurate quantitation of bioactive isomers only. Figure 1 shows a chromatogram of the system suitability standard obtained with the Radial-PAK B cartridge used. Retention of pre-D should be approximately 7 minutes and cis-D approximately 13 minutes. This test was repeated on a weekly basis and each time a new cartridge was used.

Sample Preparation

Dry concentrates, resins and oils - an amount of sample containing 50,000 I.U. vitamin D is accurately weighed.

Multivitamins from 5-10 tablets, accurately weighed, are coarsely ground.

Procedure A: Sample was transferred to a homogenization flask with the aid of 20ml ethanol, homogenized 5-10 minutes (time depends on type of sample matrix), centrifuged and a 2ml aliquot was transferred into a vial and 75ul of internal standard solution #1 was added followed by 1ml water. This mixture was pumped

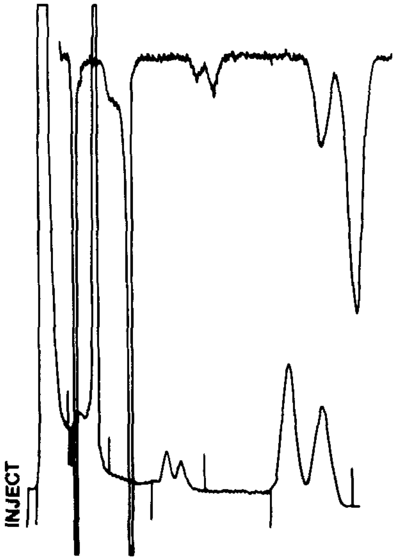

Figure 1. HPLC of system suitability standard on the Radial-PAK B cartridge used.

through a prepared $C_{18}$ SEP-PAK, which was then flushed with 10ml of a 25/25/50 Methanol/THF/$H_2O$ solution. Vitamin D, pre-D and internal standard were then eluted with 10ml methanol (100%) and the resultant eluate evaporated to dryness over sodium sulfate. The residue was redissolved in 5ml hexane and 200ul were injected. Figure 2 shows an oil analyzed in this manner.

Procedure B: Sample was transferred to a homogenization flask with the aid of 20ml DMSO, homogenized 5-10 minutes, centrifuged and 2ml were then transferred to a vial, 5ml of internal standard solution #2 was added and shaken vigorously for 30 seconds. The vial was placed in ice-cooled water and 1ml of ice-cooled water was added to the vial. After 1 minute the vial was shaken vigorously for another 30 seconds, 2ml of the hexane layer was separated and pumped across a silica SEP-PAK and the SEP-PAK was flushed with 3ml of 85/15 hexane/ethyl acetate. Vitamin D, pre-D and internal standard were eluted with 5ml of 80/20 hexane/ethyl acetate and evaporated to dryness. The residue was redissolved in 2ml hexane and 200ul was injected. Figure 3 shows an oil analyzed as described.

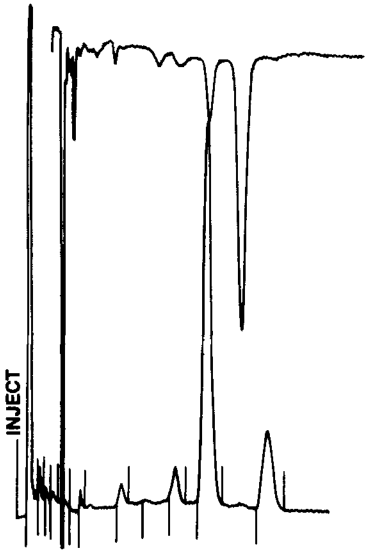

Figure 2. HPLC of an oil containing 40,000 I.U./gram Vitamin D prepared by Procedure A.

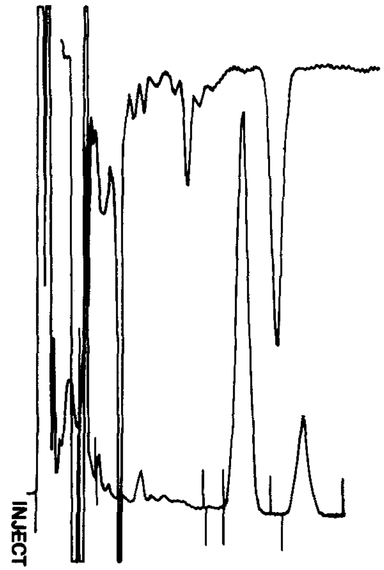

Figure 3. HPLC of an oil containing 40,000 I.U./gram Vitamin D prepared by Procedure B.

Calculation

D potency in I.U./gram in sample is calculated using the following equation:

$$P_D + (P_{pre} \times F) \times F_D \times W_{ir} \times V_s \times 40{,}000 \div (P_{ir} \times W_s \times V_{ir})$$

Where $P_{ir}$ = peak height of internal standard in sample
$P_D$ = peak height of pre-vitamin D in sample
$P_{pre}$ = peak height of pre-vitamin in sample
F - conversion factor
$F_D$ = response factor of vitamin $D_3$
$W_{ir}$ = weight internal standard in mg (15)
$W_s$ = weight of sample in grams
$V_s$ = final volume in ml of sample solution (50ml)
$V_{ir}$ = final volume in ml of internal standard (20,000ml)
40,000 - number of I.U. vitamin D per mg in USP reference std.

## RESULTS AND DISCUSSION

The HPLC conditions used permit the resolution of vitamin $D_3$ ($D_2$) from all inactive isomers which may be present, such as irradiation by-products or breakdown products formed as a result of accelerated storage conditions (i.e. elevated temperature or direct light).

Results of analyses performed on three different vitamin types (dry concentrate, oil, gelatin encapsulated multivitamin) by Procedure A compated very well with those obtained by the proposed A.O.A.C. method (Fig. 4). A problem was encountered, however, in the analysis of certain types of dry multivitamin formulations, which gave unusually high vitamin D potency. Employing wavelength ratioing of the 280nm - 254nm detector response, an incorrect ratio revealed an interference in the cis-D peak. (The proper wavelength ratio for the vitamin D standard should be determined for each set of filters and be less than that at 254nm resulting in a ratio < 1.0.) This interference, which appears to be an impurity in the vitamin A, has a larger absorbance at 280nm giving a ratio > 1.0.

This difficulty with multivitamin analysis resulted in the development of Procedure B using DMSO as the dissolution solvent which does not extract the interfering substance and results in the correct wavelength ratio in the final analysis.

Because no saponification step is necessary, the actual ratio of pre-vitamin D to vitamin D is preserved. This method is also applicable to oils, dry concentrates, gelatin encapsulated or dry multivitamins.

**Results of AOAC Method
and Radial Compression Techniques**

| Type of Sample | Concentration Range | AoAc Method | Radial Compression Technique |
|---|---|---|---|
| Concentrated Oil | 40,000 I.U./gm (± 30%) | 31,666<br>35,310 | 41,042<br>42,319 |
| Dry Powder | 100,000 I.U./gm (± 30%) | 88,833<br>90,504 | 90,624<br>94,331 |
| Dry Powder | 50,000 I.U./gm (± 30%) | 49,629<br>48,942 | 49,296<br>48,238 |
| Multi-Vitamin | 400 I.U./Cap. (± 50%) | 752<br>677 | 576<br>646 |

Figure 4. Results of AOAC method and Radial Compression Techniques

Time required for smaple preparation has been reduced from 2-3 hours to 15 minutes, enabling 20-30 samples to be run per day by either procedure. A problem remains, however, in the quantitation of pre-vitamin D in some types of multivitamins. Figure 5 shows a typical multivitamin analyzed by Procedure B. The vitamin D is well separated and may be accurately quantitated in all cases; however, the pre-vitamin D region of the chromatogram is crowded with compounds which obscure the pre-D peak. Work is currently in progress on a method to separate pre-D from these interferences.

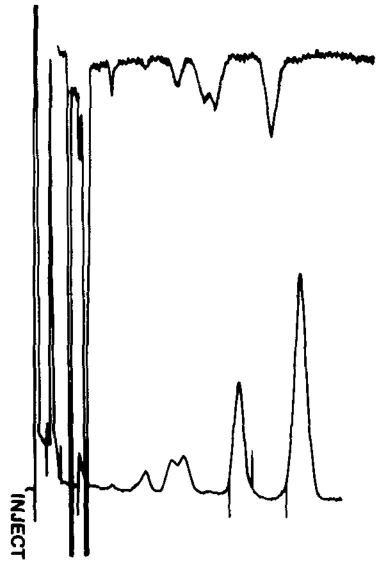

Figure 5. HPLC of a typical multivitamin analyzed by Procedure B.

## REFERENCES

1. Tompkins, D.F., Tscherne, R.J., Anal. Chem. 46, 1602-1604, 1974.
2. Tartirita, K.A., Sciarello, J.P., Rudy, B.C., J. Pharm. Sci. 65, 1024-1027, 1976.
3. Hofsass, H., Grant, A., Alicino, J.J., and Greenbaum, S.B., JAOAC, 59, 253-260, 1976.
4. Oscada, M., and Araujo, M., JAOAC, 60, 993-997, 1977.
5. deVries, E.J., Zeeman, J., Esser, R.J.E., Borsje, B., and Mulder, F.J., JAOAC, 129-135, 1979.
6. Ray, A.C., Dwyer, J.N., and Reagor, J.C., JAOAC, 60, 1296-1301, 1977.
7. Cohen, H., and Lapointe, M., J. Chromatogr. Sci., 17, 510-513, 1979.
8. Eriksson, M., Eriksson, T., and Sorensen, B., Acta. Pharm. Suec., 15, 274-281, 1978.
9. Barnett, S.A., and Frick, L.W., 51, 641-645, 1979.
10. Cohen, H., and Lapointe, M., Anal. Chem., 26, 1210-1213, 1978.
11. Vanhaelen-Fastre, R., and Vanhaelen, M., J. Chrom., 153, 219-226, 1978.