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High Pressure Liquid Chromatographic Determination of Vitamin D in Dietetic Products*

Introduction

Vitamin D determination in dietetic products and infant formulas, as in food in general, is known to be quite difficult because of their relatively low vitamin D content and of the mostly unfavourable ratio vitamin D/fat.

In a previous report (1) a chemical determination method was described, based on the colorimetric reaction, and routinely applied at our laboratory. But this method requires three days for a duplicate determination, and as mentioned in that report, high pressure liquid chromatography (HPLC) offered the possibility of a more rapid and accurate determination.

Several publications have reported HPLC determination methods applied to concentrates (2, 3) and multivitamin preparations (4—6).

A few publications have dealt with vitamin D determination in fortified feed (5, 7, 8) and in milk (9, 10), describing more or less time-consuming clean-up. *J. N. Thompson* (9) performs clean-up of the extract on a Sephadex column before straight-phase HPLC. *S. K. Henderson* (10) carries out clean-up on an alumina column and uses reversed-phase HPLC.

The first step of our study was to develop a HPLC method for routine determination of vitamin D in fortified infant milks at concentrations of 200—400 IU (5—10 µg) per 100 g, with a minimum sample handling, i. e. without preliminary purification. It is the object of this first communication.

The procedure described in this paper involves fat extraction, saponification and direct analysis by straight-phase HPLC.

Experimental

Apparatus and Reagents

High pressure liquid chromatographic system

Pump: Constametric II G, LDC
Detector: UV III Monitor, wavelength 254 nm LDC

* Presented as poster.

Recorder:	Perkin Elmer 56
Injector:	Rheodyne model 7120 with 20 µl sample loop
Column:	Spherisorb-Si 5 µm; 3.2 x 250 mm
Solvents:	2-propanol, n-hexane; LiChrosolv quality, Merck Darmstadt
Mobile phase:	1% 2-propanol in hexane
Vitamin D-standard:	cholecalciferol, vitamin D_3 cryst. for biochemistry, Merck Darmstadt Stock solution: 500 µg/ml in hexane

Method

Sample preparation

1. Fat extraction
   Dissolve 10 g product in 10 ml water at 45 °C. Transfer into a 500 ml separating funnel with about 10 ml water. Add 5 ml NH_4OH and shake vigorously. Add 25 ml absolute alcohol and shake again vigorously. Extract with 4 portions of 50 ml ethyl ether and 50 ml petroleum ether, by adding the latter each time after shaking with ethyl ether.
   Evaporate the solvents of the combined extracts under reduced pressure (temperature $\leqslant$ 40 °C).

2. Saponification and extraction of the unsaponifiable matter
   Add to the extracted fat 5 ml 60% KOH, 10 ml absolute alcohol and a spatula tip of hydroquinone. Saponify for 15 min at 50 °C under a slight nitrogen current. Transfer the saponified mixture with a minimum amount of water into a 250 ml separating funnel. Extract the unsaponifiable matter with 5 portions of 50 ml petroleum ether. Wash the combined extracts with two or more 100 ml portions of water. The last washwater should be neutral against phenolphthalein. Filter the washed extract through phase separating paper to eliminate residual water. Evaporate the extract to dryness under reduced pressure, then dissolve the residue in 5 ml mobile phase to obtain a minimum concentration of 4—8 IU (100—200 ng) vitamin D/ml for HPLC.

3. High pressure liquid chromatography
   Chromatograph 20 µl of the extract solution under following conditions: Flow rate 1—1.2 ml/min; detector set at 254 nm, 0.008 AUFS; recorder range 10 mV; chart speed 5 mm/min.
   The retention time for vitamin D is 6.5 min. The vitamin D content is calculated from peak height by direct comparison with chromatogram of standard solution.

Results and Discussion

A chromatogram of an infant formula sample is shown in figure 1.

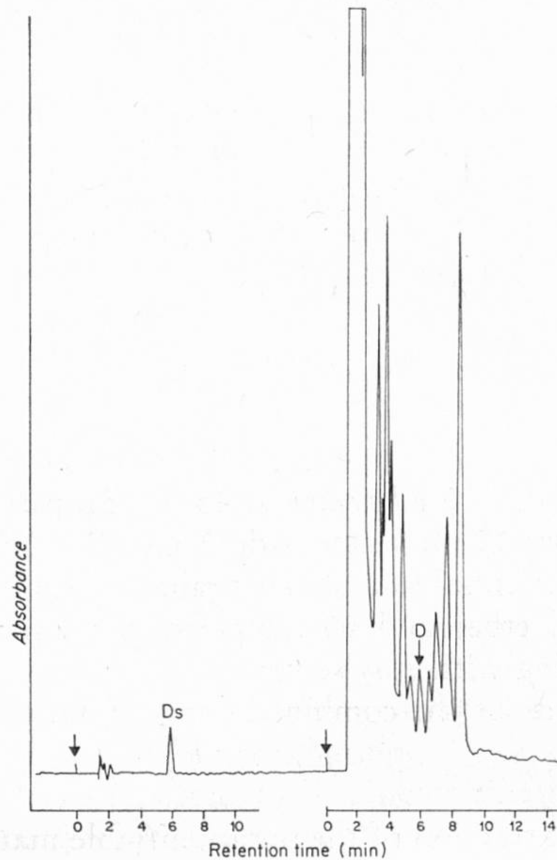

Fig. 1. Chromatogram of an infant formula (400 iu vitamin D/100 g)
D = position of vitamin D; D_s = standard, injection 4 ng vitamin D

The 254 nm wavelength has been retained after observing that the peak height of a solution of 40 IU vitamin D/ml differed only by 7% from that measured at the maximum absorption of vitamin D. This wavelength is advantageous because it is available on most UV detectors with fixed wavelength, and it allows the quantitative determination of both vitamins A and E too. Besides, a detector with fixed wavelength has a higher light energy than one with variable wavelength.

Two detectors have been compared, the Perkin Elmer LC 55 and the UV III Monitor of LDC. The former has shown not to be sensitive enough for our working conditions (i. e. 0.08—0.16 IU injected) and the signal-to-noise ratio is rather low. This ratio is 10 times higher with the UV III Monitor.

The chromatograms of figure 2 show the influence of the ID of the column on the height of the vitamin D peak. With a column of 3.2 mm ID one obtains peaks 2—2.5 times higher than with one of 4.6 mm ID.

The chromatographic system has been tested as follows:

1. By injecting a standard solution of 8 IU (200 ng) vitamin D/ml, at a rate of 0.16 IU (20 µl = 4 ng) per injection.
   With 6 x 10 injections we obtained a mean coefficient of variation of 1.93%.

2. By injecting the solution of unsaponifiable matter from the sample, to which were added 8 IU vitamin D/ml.
The mean recovery with 6 x 10 injections was 99.7% ($SD = 3.5\%$).

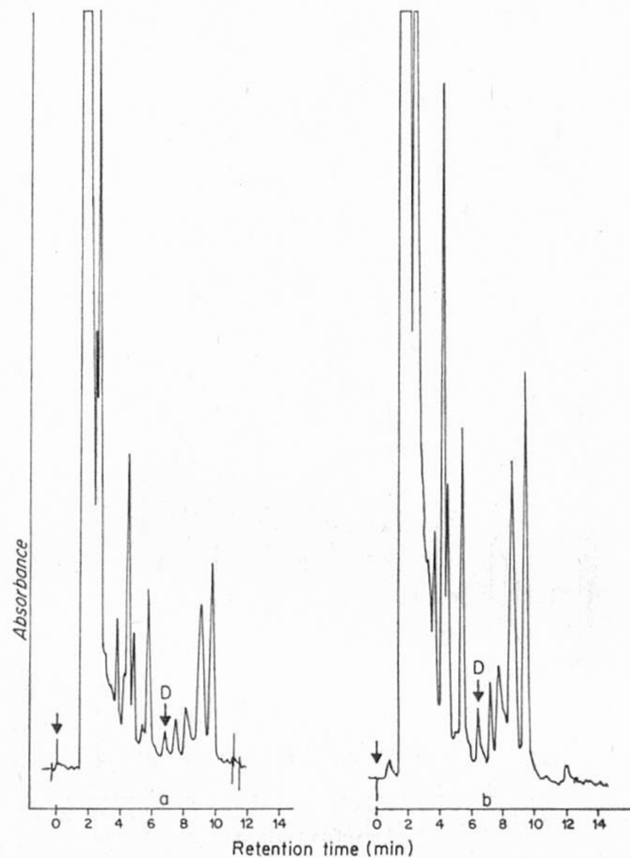

Fig. 2. Chromatograms of a dietetic milk
a) with a column of 4.6 mm ID; b) with a column of 3.2 mm ID
D = position of vitamin D

To verify sample preparation, saponification tests with recovery were carried out first on the standard solution, then on a product. Saponification at 50 °C for 15 min gave approximately full recovery of vitamin D (table 1).

Table 1. Influence of the saponification
A. on 400 IU vitamin D (standard)
B. on a product 1. on the same fat extract
2. average of two determinations

Saponification	30 min 100 °C	30 min 70 °C	15 min 50 °C
A. Standard, recovery	88.1% 88.6%	90.6% 90.4%	100.6% 99.2%
B. Infant formula, content/100 g	1. 335 IU 2. 299 IU	1. 363 IU 2. 312 IU	1. 377 IU 2. 326 IU

The chromatograms from two different saponification procedures (fig. 3) show the formation of previtamin D. In the conditions applied in this method, its presence is negligible.

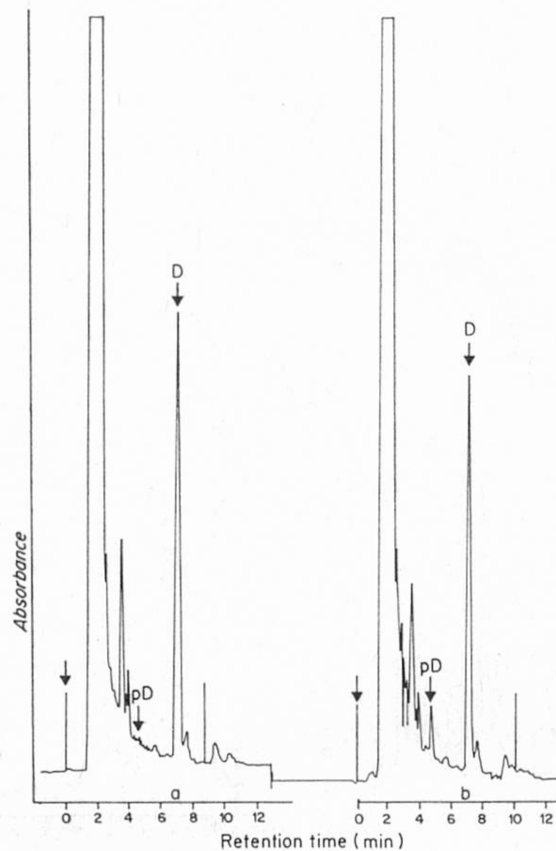

Fig. 3. Chromatograms showing the formation of previtamin D (pD) after saponification at a) 15 min 50 °C; b) 30 min 100 °C
D = position of vitamin D

Two extraction procedures of the unsaponifiable matter have been compared:

1. Extraction according to *E. de Vries* et al. (11)
2. Extraction as described in this method.

With the procedure according to 2. we found 106.75% of the amount obtained with 1. (average of 6 analyses).

Recovery with the proposed method applied to 3 samples was 99.2%, 40 IU (1 µg) vitamin D having been added to the fat extract before saponification.

The coefficient of variation, s %, calculated on the basis of 6 consecutive determinations, was 3.3%.

This method has been compared with other methods and the results are shown in table 2.

Acknowledgement

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Table 2. Comparison of the HPLC Vitamin D determination with other methods (mean of duplicate determinations)

	HPLC method	Colorimetric method (1)
Infant formula I	330 IU	335 IU/100 g
Dietetic milk	505 IU	495 IU/100 g
	HPLC method	**Biological method**
Infant formula II	270 IU	250 IU/100 g
Infant formula III	200 IU	210 IU/100 g

Summary

A high pressure liquid chromatographic (HPLC) method for the determination of vitamin D in dietetic milks and infant formulas is described.

After fat extraction, saponification and extraction of the unsaponifiable matter, an aliquot of the extract obtained is directly analysed by HPLC (straight-phase) on a Spherisorb-Si column, using as mobile phase 2-propanol 1% in n-hexane. Detection at 254 nm.

The method has been tested on infant formulas containing 200—400 IU (5—10 μg) vitamin D per 100 g. The vitamin D peaks obtained by injecting sample volumes with amounts of only 0.08—0.16 IU (2—4 ng) are well resolved and easily measurable.

The recovery of added vitamin D was 99.2%. The coefficient of variation calculated on 6 consecutive determination, *s* %, was 3.3%.

Zusammenfassung

Eine Methode mittels Hochdruck-Flüssigkeitschromatographie (HPLC) zur Bestimmung von Vitamin D in diätetischen Milchprodukten und Kindermilchpräparaten wird beschrieben.

Nach Fettextraktion, Verseifung und Extraktion des Unverseifbaren wird ein aliquoter Teil des erhaltenen Extraktes direkt durch HPLC (straight-phase) mit einer Spherisorb-Si-Säule und 2-Propanol 1%ig in n-Hexan als mobile Phase analysiert. Nachweis bei 254 nm.

Die Methode wurde auf Kindermilchpräparaten geprüft, die 200—400 IE (5—10 μg) Vitamin D pro 100 g enthielten. Die Vitamin-D-Peaks von eingespritzten Extraktionsvolumina, die nur 0,08—0,16 IE (2—4 ng) enthalten, sind gut getrennt und leicht meßbar.

Die Zurückgewinnung von hinzugefügtem Vitamin D war 99,2%. Der Variationskoeffizient, auf 6 konsekutiven Bestimmungen berechnet, *s* %, war 3,3%.

Résumé

Une méthode de dosage par chromatographie liquide à haute pression (HPLC) de la vitamine D dans les laits diététiques et pour nourrissons est décrite.

Après extraction de la graisse, saponification et extraction de l'insaponifiable, une partie aliquote de l'extrait obtenu est directement analysée par HPLC (straight-phase), sur une colonne de Spherisorb-Si, avec pour phase mobile du 2-propanol 1% dans du n-hexane. Détection à 254 nm.

La méthode a été testée sur des laits pour nourrissons contenant 200—400 ui (5—10 μg) de vitamine D par 100 g. Les pics de vitamine D obtenus à partir de volumes d'extrait injectés ne contenant que 0,08—0,16 ui (2—4 ng) sont bien résolus et facilement mesurables.

La récupération de vitamine D ajoutée était de 99,2%. Le coefficient de variation calculé sur 6 dosages consécutifs, *s* %, était de 3,3%.

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