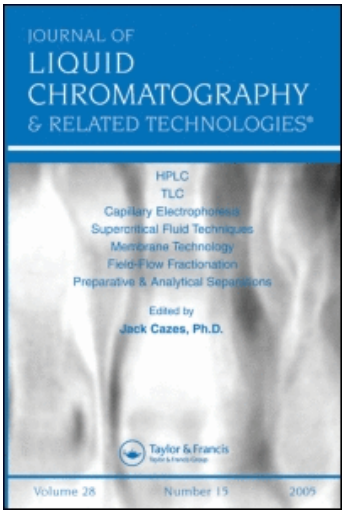

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HIGH PERFORMANCE LIQUID CHROMATOGRPHY OF FAT-SOLUBLE VITAMINS. III. DETERMINATION OF VITAMIN D_3 IN PHARMACEUTICAL PREPARATIONS AND IN BLOOD

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ABSTRACT

A reversed phase high-performance liquid chromatographic method (HPLC) is described for separation and determination of colecalciferol(Vitamin D_3) in Vitamin preparations and in biological materials. Vitamin D_3 is extracted from the formulations and from the blood in a fully automated electronically controlled extraction apparatus. For HPLC a column of lichrosorb RP18 and methanol as eluent are used. The extraction, separation and determination of vitamin D_3 needs about 10-20 minutes. The described extraction and HPLC methods allow the detection of 1-2 ng per injection and are well reproduced with a maximum coefficient of variation of $< 3,5$ %. Vitamin A-acetate is used as internal standard

INTRODUCTION

Vitamin D_3 is classified as a Fat-soluble vitamin (1) and is one of the essential vitamins. The efficiency of HPLC for separation and determination of water-and Fat-soluble Vitamins especial Vit. D is well documented[2,11]. The most of the described methods are suitable for vit. D_3-determination in single vitamin preparations, but they have desadvantages if they applied in multi-vitamin preparations or if they used for the determination of vitamin D_3 in biological fluids as the time required for the HPLC of Vit. D_3 with the reported methods was 30-50 min (3,5) while with the present method the time needed, for the whole assay was 10-20 min. Also the present procedure which allows the determination of 1-2 ng is much more sensitive than the previously published methods (2,5,6,9-11). The presence of a low concentration in nanogram range of vitamin D_3 with a large excess of pharmaceutical excipients or of biological material makes the separation and determination of this drug almost impossible. In this report simple, specific and sensitive methods are developed which enable the extraction of vitamin D_3 from the pharmaceutical preparations and from biological materials and its separation and determination in the nanogram range within 10-20 minutes.

Experimental :

Apparatus and Conditions :

Electronically controlled extraction apparatus :

(Produced by W. Krannich K. G., 3400 Gottingon, Ellihauser Weg 17, West Germany.).

The apparatus is composed of the glass set for simultaneous extraction of 3 samples as described in a previous report (12). The good reproducibility of the extraction of many active substances especial vitamins

from pharmaceutical preparations using this apparatus is demonstrated (12-16). For Vitamin D_3 three minutes were sufficient to extract the vitamin from the formulations.

High-performance Liquid Chromatography (HPLC) :

Reversed phase HPLC was carried out using a Knauer compact apparatus with a variable Wave Length spectrophotometer detector and a syringe-loaded loop injection valve with an internal volume of 50 µl. A stainlesssteel column 100 x 4.6 mm i.d. packed with vertex lichrosorb RP 18, 7 µm and Methanol as eluent were used.
The chromatograms were recorded on a Knauer recorder with a 100 mv span set.

CONDITIONS

The following conditions were maintained : Detection wave-length = 270 nm, pressure = 70 bar, flow rate = 2 ml. $min.^{-1}$, temperature = 22-25°C, injection volume = 10-20 µl, chart speed = 1 cm. min^{-1} and detector sensitivity = 0.08 AUFS.

MATERIALS AND REAGENTS

Vitamin D_3 and vitamin A-acetate standard substances were obtained from Pfizer Pharmaceutical Co. (Cairo, Egypt). The following investigated pharmaceutical preparations were purchased locally.

Preparation A : Capsules With 750 I. E. = 15 mg vitamin D_3 per capsule.

Preparation B : Capsules containing 1500 I. E. = 30 mg vit. D_3

Heparinzed blood : 6 samples of 20 ml heparinized blood with 0,1 mg vitamin D_3 per sample.

PROCEDURE

Preparation of Standard Solutions :

25 mg of vitamin D_3 and vitamin A-acetate were weighed to 0,01 mg and dissolved in 100 ml calibrated flask and diluted to volume with methanol. 1 ml of this solution was pipetted accurately into 100-ml calibrated flask and diluted to volume with methanol.

Sample Preparations :

The contents of one capsule was pulverized and transfered accurately into a fully automated extraction apparatus and extracted with methanol. A solution of vit. A-acetate was added as internal standards such that the final concentration of every substance was 2,5 $\mu g\ ml^{-1}$

Heparinized Blood :

The blood samples were centrifuged for 5 min. at 1260g. The plasma decanted from the coagulum and the coagulum again centrifuged for 5 min. at 1260g after mixing with 5 ml. 0,9% aqueous sodium chloride solution. After decanting, the collected solution was transfered accurately into the extraction apparatus and was treated with 100 ml aceton-methanol (1:1) as described previously (12). The deproteinized plasma was filtered and after retransfer in a new interchangeable filter funnel in the extraction apparatus the organic solvent mixture was drawn off in vacuum at room temperature. After addition

of 0,1 mg vitamin A-acetate as internal standard the two vitamins were extracted from the remaining deproteinized plasma 3 times with a total volume of 100 ml chloroform-Methanol (1:1).

Calculation :

The percentage recovery of vitamin D_3 in pharmaceutical preparation and in blood can be calculated by using either the calibration line method or the internal standard method according to the following equation :

$$\text{Recovery of vit. } D_3\ (\%) = \frac{Ps/PI \quad (\text{sample})}{Ps/PI \ (\text{standard})} \times 100$$

Where Ps is the peak area for the active substance (vit. D_3) and PI is the peak area of vit. A-acetate (internal standard).

RESULTS

Figures (1) and (2) show the HPLC separation pattern of vitamin D_3 from the pharmaceutical preparation A and from heparinized blood. (The separation pattern of vitamin D_3 from preparation B is exactly the same as in Fig. (1) "or as from preparation A"). Table (1) illustrates the reproducibility of the HPLC determination of vitamin D_3. The standard deviation and coefficient of variation based on six determination of vit. D_3 are summarized in this table. Table (2) presents the vitamin D_3 determination in pharmaceutical preparations and in heparinized blood.

DISCUSSION

By taking advantage of the combination of the extraction apparatus and HPLC, vitamin D_3 was determined in pure

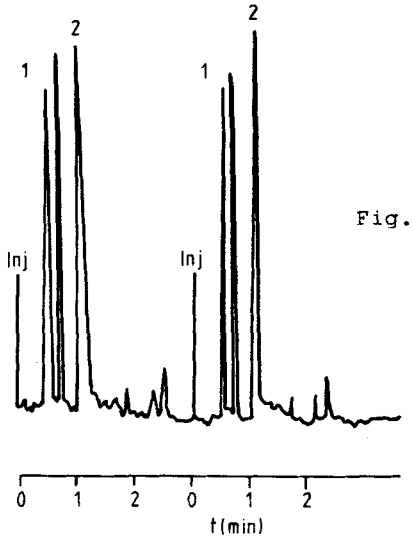

Fig. (1)

Fig.1: Typical HPLC chromatogram of vitamin D_3 and vit. A-acetate (internal standard) separation of preparation A.
Amount injected = 25 ng 1 = vit D_3;
2 = vit. A-acetate.
Conditions :
Column : vertex lichrosorb RP 18; Pressure= 80 bar. Flow rate = 2 ml. min^{-1}.
Detector : 1 = 270 nm; 2 = 320 nm.
Sensitivity 1 = 0,16 AUFS; 2 = 0,04 AUFS
Eluent = methanol.

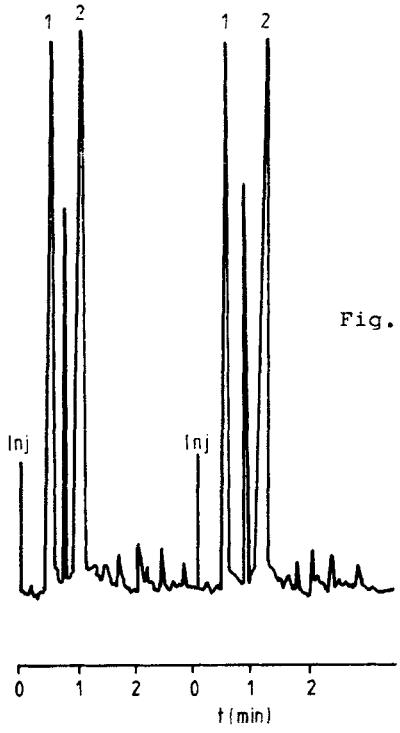

Fig. (2)

Fig. (2) : Typical HPLC chromatogram of vitamine D_3 separation of heparinized blood.
Amount injected = 50 ng. 1 = vit. D_3;
2 = vit. A-acetate.
Conditions :
Column : Vertex lichrosorb RP 18.
Pressure : 80 bar; Flow rate : 2 $ml.min^{-1}$
Detector : 1 = 270 nm; 2 = 320 nm.
Sensitivity : 1 = 0,16 AUFS; 2 = 0,04 AUFS
Eluent = methanol.

TABLE (1) : Reproducibility of HPLC Determination of Vitamin D_3 Carried Out With Pure Substance.

	VITAMIN D_3
Amount injected (ng)	50
Arithmetic mean (peak area cm^2) $\bar{X}$	0,48
Standard deviation of single value (cm^2) SD	0,015
Coefficient of variation VK (%)	3,1

TABLE (2) : Quantitative HPLC Determination of Vitamin D_3 in Preparations A, B and in Heparinized Blood. (6 extracts)

Preparations	Vitamin D_3 Amount present in injected preparations (ng)	Amount of vitamin D_3 found		
		Mean Value (ng) $\bar{X}$	SD (ng)	VK (%)
A	15mg/cap 25	26,0	0,64	2,5
B	30mg/cap 50	51,70	1,7	3,3
Heparinized blood	0,1 mg/20ml 10	9,25	0,34	3,7

substance, pharmaceutical preparations such as capsules as well as in biological materials such as blood in nanogram range in a 10-20 minutes. Direct analysis of Vitamin D_3 in pharmaceutical preparation or biological materials without prior extraction are not possible because the pharmaceutical exciprents and the biological materials contain components the chromatographic peaks of which coincide with the peak of vitamin D_3. Furthermore some of these excipients tend to accumulate on the column and thus irreversibly alter the separation pattern of the HPLC system. However, by using the extraction apparatus in combination with a reversed phase HPLC system, there was no accumulation of excipients and an excellent reproducible separation of vitamin D_3 from pharmaceutical preparations as well as from biological materials in less than 1 min. was noted. The described extraction and HPLC methods are rapid, accurate, sensitive and enable the determination of 1-2 ng vitamin D_3 with coefficient of variation of $< 3,5\%$ within 10-20 min. while the published methods needed only for HPLC separation of the vitamin 30-50 min. (3,5). Furthermore the sensitivities of such reported methods were inadequate as the lowest limit was more than 10 ng of Vit. D_3 (4,5,11). The present methods including extraction step and HPLC measurement are suitable for analysis of vitamin D_3 in blood and can be used in the pharmacokinetic and bioavailability studies of this drug.

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