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Journal of Chromatography B, 660 (1994) 49–55

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Simultaneous determination of vitamins D_3, E and K_1 and retinyl palmitate in cattle plasma by liquid chromatography with a narrow-bore column

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First received 21 February 1994; revised manuscript received 20 May 1994

Abstract

A reversed-phase high performance liquid chromatographic method is described for the simultaneous determination of vitamins D_3, E and K_1 and retinyl palmitate in plasma. Narrow-bore columns are recommended because this alternative provides a good separation efficiency, plus greater economy and sensitivity. Detection limits for individual vitamins range from 0.42 to 2.8 ng. All vitamins were separated in less than 9 min. Recovery studies showed good results for all solutes (88.8–100.3%) and the intra-day coefficients of variations ranged from 1.0 to 4.5%. This method permits the simple determination of fat-soluble vitamins using 1 ml of cattle heparinized plasma.

1. Introduction

The role of fat-soluble vitamins has been reported from many different aspects. Many physiological functions in animals and man are affected by the liposoluble vitamin concentration and a large number of pathological lesions appear with a deficiency or an overdose of vitamins.

Several workers [1,2] have reported that the frequency of certain types of cancer is inversely proportional to the serum level of retinol (vitamin A) and carotenoids (provitamin A). Likewise, the fundamental role of these vitamins in the photochemical process associated with vision is well documented [3].

Many of the diverse deficiency syndromes observed in animals deprived of vitamin E, a generic name which represents four methyl-substituted derivatives of tocol (α-, β-, γ- and δ-tocopherol) and four corresponding tocotrienol structures, can be explained by the vitamin acting as an antioxidant in stabilizing unsaturated fats in biological membranes [4]. In the same way, α-tocopherol may act as a cancer-chemopreventive agent [5].

The measurement of vitamin D metabolites, a group of antirachitic substances derived from the irradiation of sterols whose main forms are

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0378-4347/94/$07.00
SSDI 0378-4347(94)00279-E

cholecalciferol (D_3) and ergocalciferol (D_2), provides important information about the pathogenesis of diseases involving disturbances of calcium and phosphorus [6]. Finally, vitamin K, the generic name for a series of 2-methyl-1,4-naphthoquinone derivatives, shows antihemorragic activity through its involvement in the biosynthesis and regulation of prothrombin and three blood-clotting factors [7].

To understand vitamin interactions in different diseases, it is necessary to test the vitamins' serum and plasma levels. Hence, methods are needed that allow precise and rapid determination. The development of techniques for measuring fat-soluble vitamins in plasma has greatly contributed to the understanding of the factors that influence the functions of vitamins in animals and humans [8,9].

In recent years, many HPLC assays have been published, the principles of which have been summarized [10,11]. However, methods currently available for determining physiological concentrations of vitamins in biological samples by HPLC require several preliminary steps of clean-up and, sometimes, of preconcentration because the methods do not possess sufficient sensitivity. Some works [12–14] have attempted to resolve this problem by establishing the appropriate analytical methodology for some fat-soluble vitamins. However, few reports, to our knowledge, have been published on the use of HPLC for the simultaneous determination of all of these vitamins, except for some multivitamin premixes [15] and specially prepared formulae for infants [16].

We have developed a practical, simple and sensitive method for the simultaneous determination of fat-soluble vitamins from animal plasma by HPLC using narrow-bore columns (2.1 mm I.D.) packed with octadecylsilane, with emphasis on the comparison of the results obtained by this method and those provided by ordinary chromatographic columns (4 mm I.D.).

The proposed method is specifically suitable for the determination of plasma levels of vitamins D_3, E and K_1 and retinyl palmitate (Fig. 1) in animals, particularly cattle.

2. Experimental

2.1. Reagents

Methanol and tetrahydrofuran were of HPLC grade and were used as received. Ultrapure water was obtained through a Milli-Q system (Millipore, Milford, MA, USA). Analytical-reagent grade retinyl palmitate, DL-α-tocopherol, cholecalciferol and vitamin K_1 (Merck, Darmstadt, Germany) were used. Butylated hydroxytoluene (BHT) was purchased from Sigma (St. Louis, MO, USA). Absolute ethanol and hexane

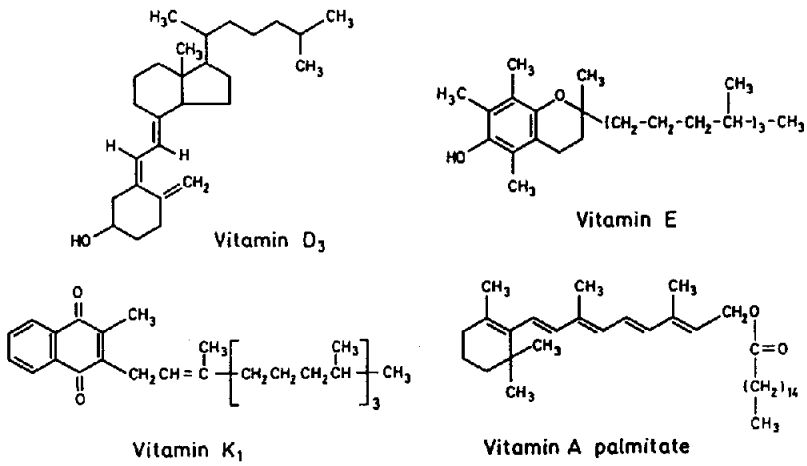

Fig. 1. Structures of the fat-soluble vitamins studied.

were purchased from Romil (Loughborough, UK).

2.2. Blood samples

All blood samples were drawn directly into 10-ml glass tubes using Venojet needles. Tubes containing lithium heparin (anticoagulant) (1 mg) and butylated hydroxytoluene (0.25 mg) to protect oxidizable substances during clarification were used. All tubes were protected from light. Immediately following this, tubes were centrifuged at 2000 *g* at 4°C for 8 min, and the plasma was transferred into polypropylene freezer tubes, which were handled under diffuse light. All tubes were closed and kept frozen in liquid nitrogen for transport to the laboratory until needed.

2.3. Sample preparation

Bovine plasma (1 ml) was transferred into a 10-ml glass centrifuge tube and 2 ml of ethanol were added to initiate protein precipitation. After vortex-mixing for 3 min, 3 ml of hexane were added, followed by vortex-mixing for 5 min. The solution was then centrifuged at 1500 *g* for 10 min. The organic layer was transferred and the extraction process was repeated with 3 ml of hexane. The two organic layers were combined and transferred by glass pipette into a centrifuge tube and washed with 2 ml of methanol–water (9:1). The organic upper layer was separated and passed through a 0.45-μm filter. This was then evaporated under nitrogen until dryness, the residue was reconstituted in 33.4 μl ethanol (20–50 μl of ethanol can be used) and a 5-μl aliquot of this solution was injected into the HPLC system.

2.4. Stock and working standard solutions

Individual stock standard solutions of each vitamin were prepared in ethanol containing 0.025% of BHT to provide a concentration of 5 mg/ml for retinyl palmitate and vitamin K_1 and 2.5 mg/ml for DL-α-tocopherol and cholecalciferol. These solutions were degassed with helium and stored in dark glass flasks at −20°C.

Working standard solutions were prepared by appropriate dilution of the stock standard solution and filtered through a 0.45-μm membrane (Millex-HV_{13}, Millipore) before being injected into the system.

2.5. Apparatus and conditions

The experiments were carried out using an HPLC system equipped with two Kontron (Milan, Italy) Model 422 pumps; a Rheodyne Model 7125 injector with a 20-μl injection loop or a Rheodyne Model 8125 injection valve with a 5-μl loop. A Kontron Model 430 UV–Vis detector with a 3-μl flow cell and a data station with Data System 450 software were used. The wavelength of the detector was set at 328, 265, 284 and 250 nm for retinyl palmitate, cholecalciferol, α-tocopherol and vitamin K_1, respectively.

The columns used were LiChrospher 100 RP-18 (125 × 4 mm I.D., 5 μm) and Spherisorb ODS-2 (100 × 2.1 mm I.D., 3 μm). An ODS guard column was used to protect the analytical column.

The mobile phase was pumped at a flow-rate of 1.5 ml/min with an ordinary column (4 mm I.D.) or 0.2 ml/min with a narrow-bore column (2.1 mm I.D.), in both isocratic (100% methanol) and gradient [A = methanol–water (99:1); B = methanol–tetrahydrofuran (70:30)] modes. As a result of optimization, the gradient run conditions were programmed as follows: 0–2 min, 0% B; 2–5.5 min, 95% B; 5.5–10 min, 100% B; 10–12 min, 0% B. Before use, the mobile phase was vacuum filtered through a 0.45-μm nylon filter and degassed with helium. The chromatographic experiments were carried out at room temperature (20 ± 2°C).

2.6. Stability of samples

The stability of plasma samples was tested by using different plasma pools and by measuring the life of the vitamins. When the plasma was stored at −20°C in the dark, the vitamin concentrations decreased markedly with time, par-

ticularly the most labile, retinyl palmitate. As a result, the storage of samples was also carried out under liquid nitrogen.

The stability of plasma under laboratory conditions during the sample preparation was tested. The results indicate that the vitamin concentrations were not significantly modified.

3. Results and discussion

Using previous studies as a basis, we investigated the effect of the elution conditions, particularly flow-rate and mobile phase composition, on the resolution of those fat-soluble vitamins which are present in cattle plasma. This was achieved with normal and narrow-bore columns.

Fig. 2 shows the variation of the capacity factor (k') of each vitamin with the mobile phase composition run in the isocratic mode. Obviously, it is desirable to obtain k' less than 8 in order not to prolong the retention times unreasonably, particularly for vitamin A palmitate. Further, k' must be greater than 2 to avoid any overlapping with the elution front that presumably could appear in the samples. The use of methanol–water mobile phases in the isocratic mode provides retention times which are too long, although it is possible to reach the aforementioned k' using 100% methanol as mobile phase. However, in this case, and as can be seen in Fig. 3, which illustrates the chromatograms obtained with the standards for vitamin and a plasma sample, there is overlapping of vitamin D_3 and the elution front.

The addition of tetrahydrofuran to the mobile

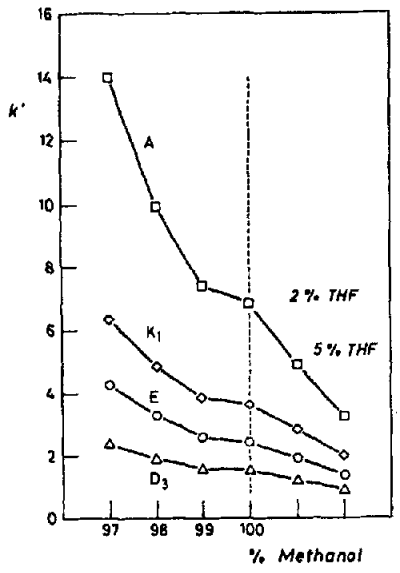

Fig. 2. Effect of the mobile phase composition on the capacity factor of the fat-soluble vitamins. Column, 100 × 2.1 mm I.D., 3-μm Spherisorb ODS-2; flow-rate, 0.2 ml min^{-1}

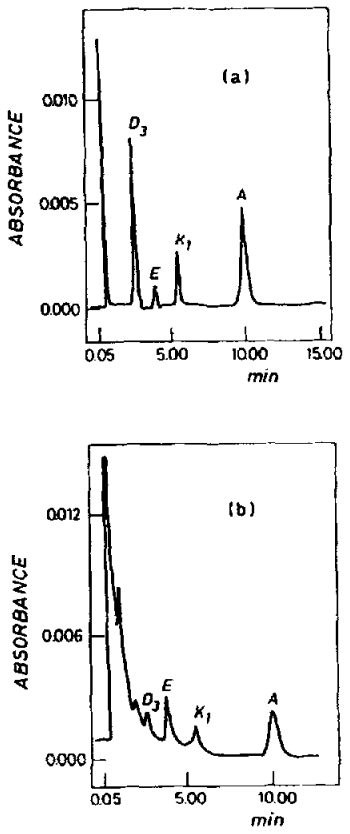

Fig. 3. Separation in isocratic mode of the fat-soluble vitamins. (a) In a standard mixture. Amounts: 10 ng for vitamins D_3 and E and 5 ng for vitamin K_1 and retinyl palmitate (A). (b) In a plasma sample. Concentrations: 16.7, 86.7, 20.0 and 16.5 ng ml^{-1} for vitamins D_3, E and K_1 and retinyl palmitate (A), respectively. Column, 100 × 2.1 mm I.D., 3-μm Spherisorb ODS-2; mobile phase, 100% methanol; flow-rate 0.2 ml min^{-1}.

phase decreases the retention time of the last vitamins eluted, but it increases the problems of overlapping.

For the purpose of decreasing the retention times, another approach is to use the gradient mode beginning with a low elution power to obtain about a 5-min delay and then to increase this power gradually.

As can be seen in Fig. 4, which shows the chromatograms obtained with normal and narrow-bore columns, both of these provided an adequate resolution and similar total retention time. However, by using the narrow-bore column it is clear that less mobile phase solvent is consumed and, therefore, the analytical cost is

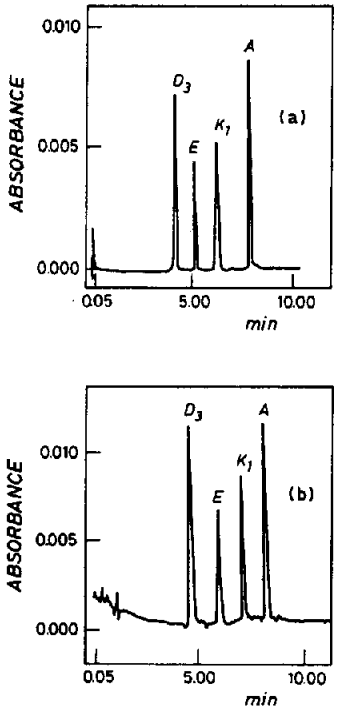

Fig. 4. Chromatograms obtained in the gradient mode from a standard solution of vitamins D_3, E and K_1 and retinyl palmitate (A). (a) Column, 125 × 4 mm I.D., 5-μm LiChrospher 100 RP-18; flow-rate, 1.5 ml min^{-1}. Amounts: 80 ng for vitamin D_3, 0.24 μg for vitamin E and 60 ng for vitamin K_1 and retinyl palmitate (A). (b) Column, 100 × 2.1 mm I.D., 3-μm Spherisorb ODS-2; flow-rate, 0.2 ml min^{-1}. Amounts: 15 ng for vitamin D_3, 20 ng for vitamin E, 7.5 ng for vitamin K_1 and 5 ng for retinyl palmitate (A). For gradient programme, see text.

Table 1
Detection limits of fat-soluble vitamins determined by using narrow-bore and normal-bore columns.

Vitamin	Detection limit (ng)	
	Normal-bore	Narrow-bore
D_3	19.80	0.44
E	106.30	2.80
K_1	6.40	0.42
A	11.80	0.49

Normal-bore column, 125 × 4 mm I.D., 5-μm Lichrospher 100 RP-18; flow-rate, 1.5 ml/min; injection volume, 20 μl. Narrow-bore column, 100 × 2.1 mm I.D., 3-μm Spherisorb ODS-2; flow-rate, 0.2 ml/min; injection volume, 5 μl; Mobile phase, solvent A: methanol–water (99:1), solvent B: methanol–tetrahydrofuran (70:30). Gradient conditions as in text; detection at 328, 265, 284 and 250 nm for vitamins D_3, E and K_1 and retinyl palmitate (A), respectively.

less. Further, in order to compare the detection limits, based on a signal-to-noise of 3:1, obtained with the two types of columns (Table 1) it can be observed that the limits obtained with narrow-bore column are much lower than those provided by the ordinary column. This result is significant for the analysis of samples (such as in bovine cattle plasma, particularly calves) in which vitamins occur in small amounts. Hence this sensitivity appears to be adequate for the determination of the vitamin levels usually encountered in cattle plasma.

In order to demonstrate the applicability of reversed-phase HPLC using narrow-bore columns for the separation of fat-soluble vitamins in biological material, several different pool plasma samples were analysed. Fig. 5 illustrates a typical chromatogram of the vitamins in a plasma sample obtained using the operating conditions specified. Identification was achieved by comparing the retention times with those of the standards and obtaining UV spectra to confirm peak identity. Four vitamins were present in the sample and these were all determined.

The determination of vitamins was achieved by using the external standard method owing to the difficulty encountered in selecting an adequate internal standard for all vitamins and

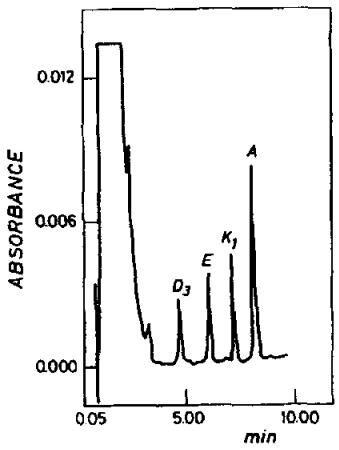

Fig. 5. Typical chromatogram of the fat-soluble vitamins in an extracted plasma pool sample. Column, 100 × 2.1 mm I.D., 3-μm Spherisorb ODS-2; flow-rate, 0.2 ml min^{-1}. Gradient conditions as in the text. Concentrations: 33.4, 93.3, 22.3 and 16.7 ng ml^{-1} for vitamins D_3, E and K_1 and retinyl palmitate (A), respectively.

because it was necessary to switch the detection wavelengths in order to select the absorption maximum for each vitamin. The calibration graphs constructed daily from peak areas versus vitamin concentrations were linear ($r > 0.9996$) from the determination limit up to at least 4 ppm for vitamins D_3 and K_1 and retinyl palmitate and up to at least 7 ppm for vitamin E.

To study the accuracy of the method, recovery experiments were performed. Known amounts of each vitamin were added to a variety of samples and the resulting spiked samples were subjected to the entire analytical sequence. Each solute was spiked at three different concentrations and recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples. All analyses were carried out in triplicate. The results for a cattle plasma are given in Table 2.

The average recoveries obtained, which ranged between 88.8 and 100.3%, testify to the accuracy of the proposed method. Thus, the recovery study demonstrates that difference concentrations of vitamins do not influence the quantitative extraction.

The precision of the method was investigated using both standards and samples. Five repeated injections of the standard gave a relative standard deviation (R.S.D.) of 3.1%. The R.S.D.s for the sample were determined using three different pool plasma and by analysing each sample in triplicate, and were always less than 4.5%.

Table 2
Recovery studies on fat-soluble vitamins added to the various extracted plasma samples

Vitamin	Concentration in plasma (μg/l)	Concentration added (μg/l)	Concentration found (μg/l)	Recovery (%)
D_3	64.0	21.7	80.5	95.1 ± 3.00
		43.7	107.7	100.3 ± 2.04
		65.3	126.8	98.9 ± 4.01
E	128.0	50.0	167.7	95.3 ± 3.76
		66.7	187.8	97.5 ± 4.47
		83.3	195.5	93.4 ± 3.16
K_1	17.6	16.7	33.8	94.3 ± 1.03
		33.3	52.6	99.7 ± 3.08
		50.0	66.9	96.7 ± 3.15
A	13.0	16.7	27.8	88.8 ± 1.25
		33.3	43.8	91.3 ± 1.93
		50.0	60.4	93.3 ± 1.39

Column, 100 × 2.1 mm I.D., 3-μm Spherisorb ODS-2; flow-rate, 0.2 ml/min injection volume, 5 μl. Other conditions in Table 1.

4. Conclusion

Reversed-phase HPLC with narrow-bore columns packed with 3-μm particles provides a rapid, simple and economic alternative for the separation and determination of fat-soluble vitamins. The proposed method is particularly suitable for determining vitamins D_3, E and K_1 and retinyl palmitate in bovine cattle plasma, but it can also be applied to other plasma types.

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