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Rapid and sensitive high-performance liquid chromatographic method for simultaneous determination of retinol, α-tocopherol, 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 in human plasma with photodiode-array ultraviolet detection

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Abstract

A new rapid and sensitive high-performance liquid chromatographic method using 0.5 ml of plasma has been developed for the simultaneous determination of retinol (vitamin A), α-tocopherol (vitamin E), 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3. The eluate was monitored with a photodiode-array detector with two fixed wavelengths (267 nm for vitamin D, 292 nm for α-tocopherol and retinol). For all compounds, including internal standards, the method provides extraction recoveries greater than 81%. Detection limits were equal to or lower than 1.5 μg/l for the 4 vitamins. Linearity of standards was excellent ($r>0.999$ in all cases). Intra-day and inter-day precision were generally acceptable; the intra-day-assay C.V. was $\frac{3}{4}$ 7.7 for all compounds and the inter-day-assay C.V. was <9.2% except for the lower concentrations of 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2 and α-tocopherol (10.8, 11.8 and 11.9, respectively). The important properties of the present method are its ease of use, its rapidity, since sample preparation was achieved in 15 min and all the compounds were eluted in less than 15 min, and its small sample volume required (=0.5 ml), which enables it to be used in pediatric practice.

Keywords: Vitamins; Retinol; α-Tocopherol; 25-Hydroxyvitamin D

1. Introduction

Due to their fat-solubility, vitamins A (retinol), D and E (α-tocopherol) have a common mechanism of absorption from the intestine. These vitamins are absorbed together with fat and deficiency occurs when fat intake is too low or when fat malabsorption is present. The decreased levels of these vitamins in plasma could lead to eye diseases for vitamin A, rickets for vitamin D, cancer or coronary disease for vitamin E [1,2]. Moreover, vitamins A and D can reach toxic levels when ingested in large quantities [3,4]. So, over the last decade, nutritionists and clinicians have been increasingly interested in measuring these fat-soluble vitamins in human plasma in order to evaluate vitamin status, fat malabsorption disorders or toxic levels.

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Many methods have been published that described the simultaneous determination of vitamins A, D and E in food [5–10] or in tablets [11,12]. In human plasma or serum, the recent published methods usually allow the simultaneous determination of only retinol and tocopherol [13–17]. For vitamin D, it is important that the method allows the determination of the 25-hydroxylated metabolite since it is the main circulating form of this vitamin. Moreover, two forms of vitamin D exist (vitamin D_2 found in vegetables and vitamin D_3 naturally produced by human skin under the influence of UV irradiation). It is important to measure both forms of 25-hydroxyvitamin D in order to monitor the effect of supplemented vitamin D_2 on the total vitamin D status. Plasma or serum 25-hydroxyvitamin D has been determined by using a different extraction method and either HPLC [18–20], competitive protein binding assay [19] or radioimmunoassay [21]. Other methods describe the simultaneous determination of vitamins D_2, D_3 and 25-hydroxyvitamin D_3 without determination of 25-hydroxyvitamin D_2 [22] or need large quantities of plasma or serum [23–25]. One published method allows the simultaneous determination of vitamin A, E and 25-hydroxyvitamin D_3 without determination of 25-hydroxyvitamin D_2 and requires 5 ml of human serum [24]. Finally, only one study that allows the simultaneous determination of human serum vitamin A, E, 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 using high-performance liquid chromatography and ultraviolet detection has been published [26]. In this method, the four vitamins were eluted in 30 min using a mobile phase at a flow rate of 2.5 ml/min and the extraction recoveries of the vitamins from serum were not reported.

This report describes a new rapid and sensitive method for quantifying the four vitamins simultaneously in less than 15 min with satisfactory extraction recovery. The use of 0.5 ml or less of human plasma allows its use in pediatric practice.

2. Experimental

2.1. Chemicals

All-*trans* retinol was purchased from Aldrich–Sigma (Steinheim, Germany), α-tocopherol was supplied by Roche (Neuilly sur Seine, France), 25-hydroxyvitamin D_3 by Roussel Uclaf (Romainville, France) and 25-hydroxyvitamin D_2 was a generous gift from Dr. Richard (St Germain Hospital, France). Tocopherol acetate and 1α-hydroxyvitamin D_3 (used as internal standards) were supplied by Roche (Neuilly sur Seine, France) and Leo (St Quentin en Yvelines, France), respectively. Human serum albumin was from LFB (Les Ulis, France). Methanol and acetonitrile were obtained from Carlo Erba (Val de Reuil, France), ethanol from Prolabo (Paris, France), *n*-hexane from Fischer-Scientific (Loughborough, UK) and dichlormethane from E. Merck (Darmstadt, Germany). All reagents used were of HPLC grade.

Stock standard solutions of retinol (2.5 g/l), α-tocopherol (6.7 g/l), 25-hydroxyvitamin D_3 (0.5 g/l) and 25-hydroxyvitamin D_2 (0.5 g/l) were prepared in ethanol and stored at −20°C. A working solution was prepared every 2 weeks by an appropriate dilution of concentrated stock standard solutions in ethanol and containing the four vitamins at the following concentrations: retinol, 5 mg/l; α-tocopherol, 100 mg/l; 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2, 0.5 mg/l of each. The internal standard solution was prepared in ethanol every month at a concentration of 0.8 mg/l of 1α-hydroxyvitamin D_3 and 1.3 g/l of tocopherol acetate.

2.2. Chromatography

Chromatography was performed using a quaternary gradient pump P1000XR Spectra System with a 100 μl fixed volume injector (Thermoquest, Les Ulis, France) coupled with an auto-injector (AS 3000 Spectra System) and a photodiode array-detector (Spectra System 6000 LP), with two fixed wavelengths: 267 and 292 nm. The Thermoquest Chromquest software was used for system control, data acquisition and process. The separation column (250×4.0 mm I.D.) was a 5-μm Lichrospher 100 RP-18 (Merck) maintained at 40°C. The device was completed with a precolumn (C_{18}, 5 μm, 4×4.0 mm I.D., Merck).

Elution was performed with acetonitrile/methanol/water (90/4/6) for 5 min, followed by a gradient elution in order to obtain acetonitrile/methanol (40/60) in 6 min, and hold for 4 min. The equilibration time between two consecutive samples was set

at 2 min. The mobile phase was used at a constant flow rate of 1.5 ml/min.

2.3. Sample preparation

All the sample preparation was carried out with protection from light. Plasma sample 0.5 ml was placed in polypropylene 15-ml tubes and spiked with 20 μl of the internal standard solution. After vortex-mixing, 400 μl of ethanol were added, and the tubes were vortex-mixed for 10 s. The mixture was extracted with 2 ml of *n*-hexane/dichlormethane (90/10, v/v) by vortex-mixing for 60 s and centrifugation at 3000 *g* for 5 min. The *n*-hexane/dichlormethane layer was carefully transferred to a 13× 75-mm disposable glass test tube and evaporated to dryness under a stream of N_2. The sample was dissolved in 250 μl acetonitrile and transferred to a micro-vial for auto-sampler (250 μl, 30×5 mm, Thermoquest), and 100 μl were injected by the auto-sampling injector.

2.4. Calibration curves

The standard curves were prepared using six concentrations of each compound (including a 0 point). To prepare standard curves, appropriate amounts of working solution were added to 0.5 ml of human serum albumin. The high-level concentration standard solution (standard 6) contained retinol, 1000 μg/l; α-tocopherol, 20 mg/l; 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2, 50 μg/l. The other standard curves used consisted in standards containing all compounds at concentrations of 0% (standard 1), 10% (standard 2), 20% (standard 3), 50% (standard 4) and 75% (standard 5) of those of high-level concentration standard point. Quantification was performed by calculating the peak-area ratios of retinol and α-tocopherol to the tocopherol acetate and the peak-area ratios of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 to the 1α-hydroxyvitamin D_3.

3. Results and discussion

3.1. Chromatogram

The chromatographic separations obtained from standard curves and from human plasma are shown, respectively, in Figs. 1 and 2. All the peaks are completely resolved without any interference from endogenous compounds. Retention times for retinol, 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2, 1α-hydroxyvitamin D_3, tocopherol acetate and α-tocopherol are respectively 4.0, 4.8, 5.2, 7.3, 13.5 and 14.8 min. The elution was twice as rapid as in the previous published method that allows the simultaneous determination of the four vitamins [26] and permits its routine use in high series.

3.2. Extraction recovery

In order to check the validity of the assay, plasma samples ($n=12$) with and without addition of different known amounts of standard were analyzed. The amount of analytes measured (obtained by measuring the amount of the analytes in spiked plasma and subtracting the amount measured in the plasma without the addition) and the amount added were very close for all compounds (between 81 and 105%, Table 1). In the only previous procedure reported in the literature that allows the simultaneous determination of the four vitamins [26], the extraction recoveries were reported only for 25-hydroxyvitamin D_3 (77.2±3.9), and the two internal standards (retinyl acetate: 80.1±3.0 and α-tocopheryl acetate: 79.1±2.0) without any mention of retinol, α-tocopherol and 25-hydroxyvitamin D_2.

3.3. Linearity and limit of detection

The calibration curves were obtained with 5 determinations in 1 day. The mean slopes, the mean intercept, the back calculated concentrations of the highest and lowest standard curves with their percentages of error (bias%) and their coefficient of variation (C.V.%) are reported in Table 2. For all compounds, the mean correlation coefficients were 0.999. Linearity of the method with acceptable criteria of precision has been tested between 2 and 100 μg/l for vitamin D, 10 and 1000 μg/l for vitamin A and 1 and 30 mg/l for vitamin E.

The detection limits under the described conditions were less than 1.5 μg/l for all compounds with a signal-to-noise ratio of 3. These limits of detection are comparable to or better than those reported

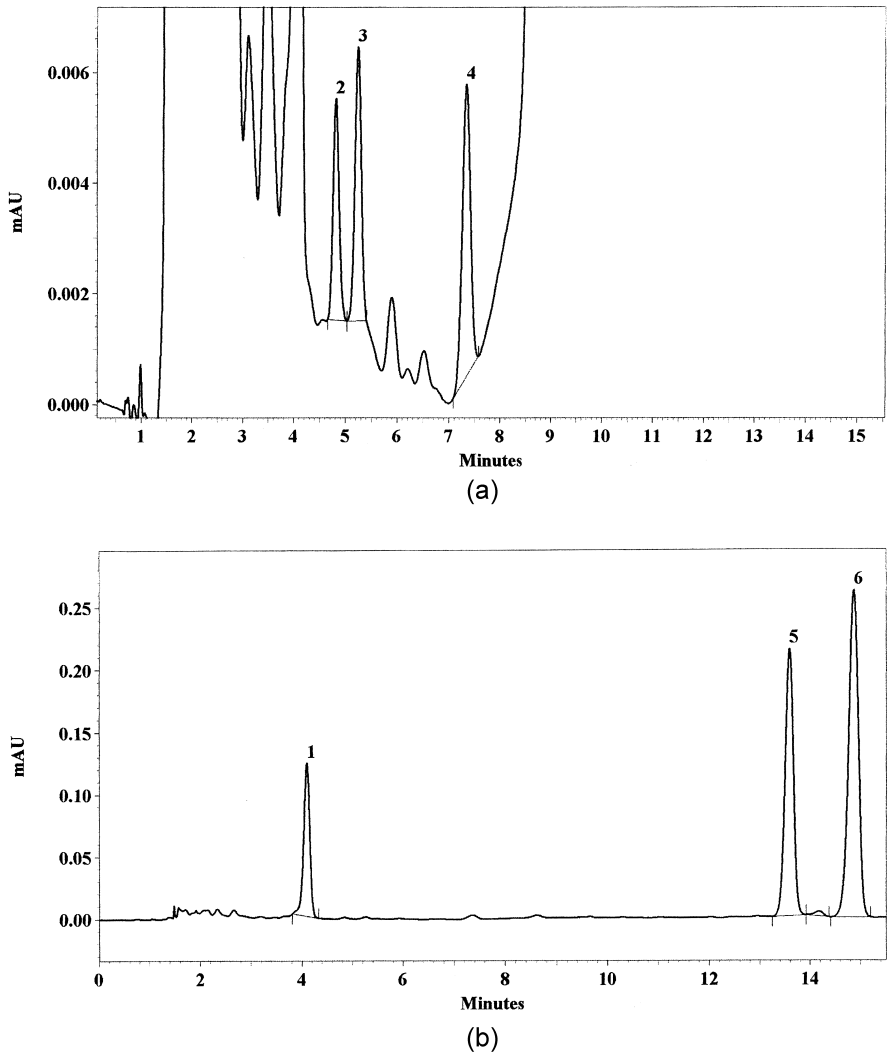

Fig. 1. Chromatogram of a standard solution containing retinol (1), 500 μg/l; 25-hydroxyvitamin D_3 (2), 25 μg/l; 25-hydroxyvitamin D_2 (3), 25 μg/l; 1α-hydroxyvitamin D_3 (4), 32 μg/l (as the internal standard for 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2), α-tocopherol (5), 10 mg/l and tocopherol acetate (6), 52 mg/l (as the internal standard for retinol and α-tocopherol). (A) Channel 1 from detector set at 267 nm; (B) Channel 2 from detector set at 292 nm.

earlier [26] and allow the use of 250 μl of plasma in our method, notably for pediatric samples.

3.4. Intra-day and inter-day repeatabilities

The study for evaluating validation criteria of the method was carried out over 3 days. Each day, 2 calibration curves with 6 determinations of 3 spiked plasma were analyzed. The values obtained were analyzed using variance analysis (ANOVA), which separated the intra-day-assay and inter-day-assay standard deviation and consequently the corresponding coefficients of variation (C.V.). The intra-day-assay C.V. took into account the variability of the six replicates each day for 3 days and the inter-day-assay C.V. the variability of the days of analysis. The results are presented in Table 3. For all compounds at the 3 levels of concentration, the intra-day-assay

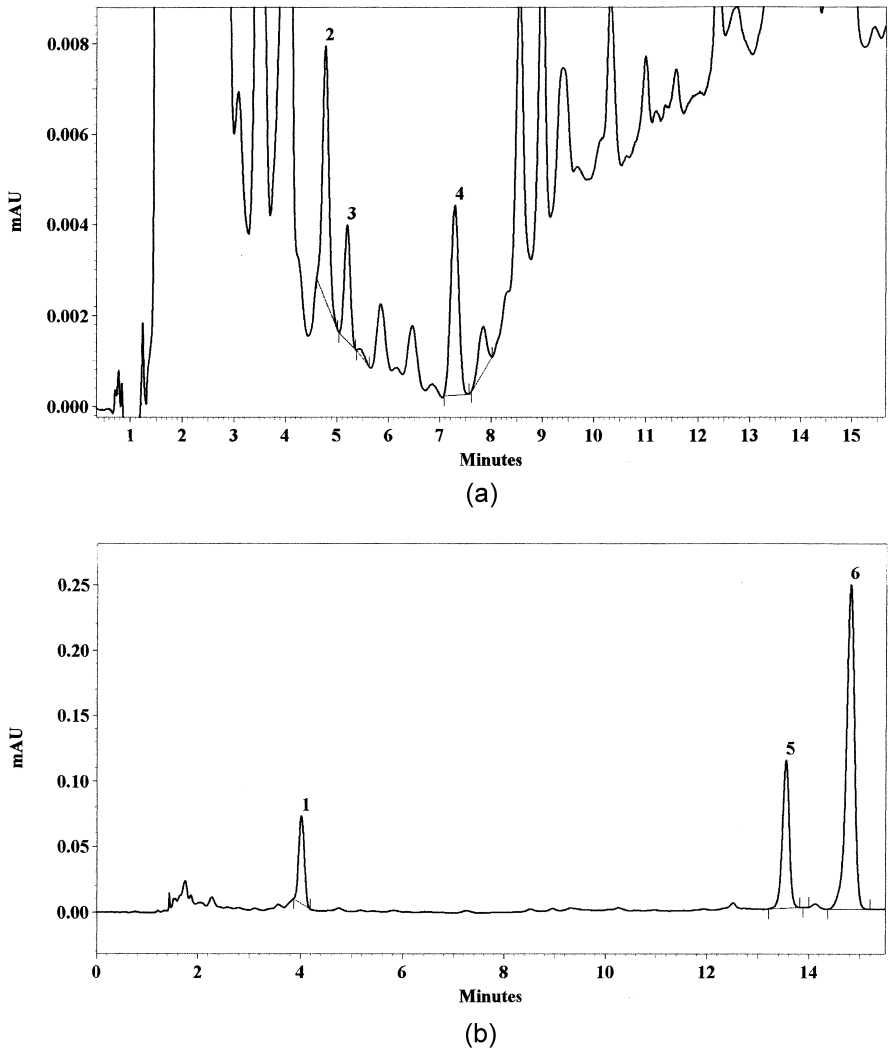

Fig. 2. Chromatogram of an extract of 0.5 ml of plasma with a measured concentration of 285 μg/l of retinol (1), 45 μg/l of 25-hydroxyvitamin D_3, 15 μg/l of 25-hydroxyvitamin D_2 and 5.5 mg/l of α-tocopherol. (A) Channel 1 from detector set at 267 nm; (B) Channel 2 from detector set at 292 nm.

C.V. were less than 7.7%. The inter-day-assay variability was less than 9.2% except for the lower concentrations of 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2 and α-tocopherol (10.8, 11.8 and 11.9, respectively).

3.5. Stability

Plasma stored at −20°C and protected from light during storage was stable for at least 6 months. The acetonitrile phase extract remains stable for 3 days when stored at +4°C.

3.6. Applications

This HPLC method is routinely used in our laboratory for the simultaneous determination of the four vitamins. The photodiode-array detector can be replaced by a multi-wavelength UV detector. Moreover, as shown in Fig. 3, due to much higher plasma

Table 1
Extraction recovery of the analytical method, determined by comparing peak area ratios of extracts with those obtained by direct injection of the same amount of compound ($n=12$)

	Recovery (%) (mean±SD)
Retinol	105±7.5
25-OHD_3	88.0±5.1
25-OHD_2	81.5±4.7
1-OHD_3	93.0±7.9
Tocopherol acetate	94.0±3.8
Tocopherol	84.0±8.6

levels of vitamins A and E (whose maximum absorption is at 292 and 325 nm, respectively) compared to 25-hydroxyvitamin D, the absorption at 267 nm with a classic UV detector coupled with an integrator is high enough to allow sensitive determination of the four vitamins. In that case, since absorption at 267 nm of the internal standard tocopherol acetate is much higher than those of retinol and α-tocopherol, it is preferable to prepare internal standard solutions at a concentration of 0.1 g/l instead of 1.3 g/l of tocopherol acetate. Our normal limit in adults, determined in 10 subjects (5 women and 5 men, 40.2±10.0 years old) is 10–50 μg/l for 25-hydroxyvitamin D (25-hydroxyvitamin D_2+25-hydroxyvitamin D_3), 300–700 μg/l for retinol and 7–15 mg/l for α-tocopherol.

Table 3
Intra- and inter-assay validation[a]

Concentrations	Intra-assay (C.V.%)	Inter-assay (C.V.%)
Retinol (μg/l)		
60	3.9	7.1
270	2.1	4.6
900	3.4	5.4
25-Hydroxyvitamin D_3 (μg/l)		
9	7.7	10.8
20	4.1	9.2
45	4.6	1.2
25-Hydroxyvitamin D_2 (μg/l)		
6	6.1	11.8
25	3.9	8.3
50	3.2	4.5
α-Tocopherol (mg/l)		
1	1.6	11.9
4	2.5	4.5
12	1.2	2.2

[a] Each day for 3 days, 2 calibration curves with 6 determinations of 3 spiked serums were measured. The values obtained were analyzed using variance analysis (ANOVA).

4. Conclusion

The HPLC–UV method described here enables retinol, 25-hydroxyvitamin D_3, 25-hydroxyvitamin D_2 and α-tocopherol to be determined with accept-

Table 2
Linearity and detection limits[a]

Calibration ($n=5$)	Range	Slope: mean±SD (C.V.)	Intercept: mean±SD	Coefficient of correlation: mean	Back calculated concentrations: mean (C.V.%; bias%)	Limit of detection (μg/l)
Retinol (μg/l)	94–940	1367±140 (10.3)	5.8±1.1	>0.999		0.l
94					100.7 (3.4; 7.2)	
940					945.2 (0.4; 0.5)	
25-OHD_3 (μg/l)	5–50	97.6±5.9 (6.0)	−1.5±0.5	>0.999		1.5
5					5.1 (12.4; 3.6)	
50					50.1 (0.8; 0.2)	
25-OHD_2 (μg/l)	5–50	63.3±4.5 (7.1)	1.0±0.7	>0.999		1.5
5					5.3 (6.9; 6.0)	
50					51.3 (0.6; 2.6)	
α-Tocopherol (mg/l)	2–20	13.4±1.0 (7.8)	0.6±0.2	>0.999		1.0
2					2.1 (12.0; 7.2)	
20					20.0 (0.3; 0.1)	

[a] The calibration curves were obtained with 5 determinations in 1 day.

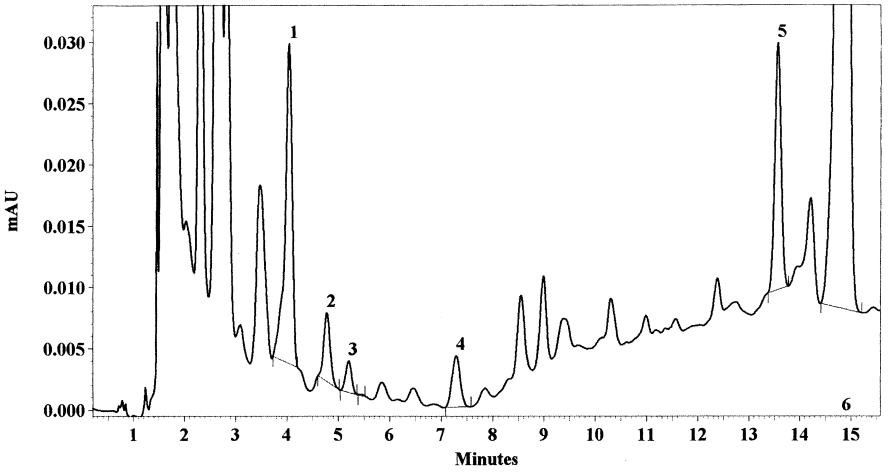

Fig. 3. Same chromatogram as in Fig. 2 (A) at a lower sensibility (0.03 mAU full-scale) showing the possibility to detect all the compounds at 267 nm with a classic UV detector coupled with an integrator.

able validation performance and extraction recoveries. Its ease of use and its rapidity make it useful for routine use in any laboratory. Finally, the small volume of plasma required in this method allows its use in pediatric practice.

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