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Automated method for the determination of fat-soluble vitamins in serum☆

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Abstract

A new fully automated high-performance liquid chromatography (HPLC) method using 1 ml of serum has been developed for the determination of retinol (Vitamin A), α-tocopherol (Vitamin E), 25-hydroxyvitamin D_3 and 24 R,25-hydroxyvitamin D_3. The eluate was monitored with a photodiode-array detector at three wavelengths—namely: 265 nm for Vitamin D_3, 291 nm for Vitamin E and 325 nm for Vitamin A. The detection limits were equal to or lower than 1 ng ml^{-1} for all vitamins. The linearity obtained with serum samples (standard addition method) gives correlation coefficients (r^2) ranging between 0.999 and 0.996 in all cases, with standard deviation of the slope between 3.2 and 1.6%. The repeatability was between 4.0 and 6.0% and the within-laboratory reproducibility was lower than 10% in all cases. The most outstanding features of the present method are its ease of use, its rapidity and fully automation, which enables its use for routine analysis. The time required per sample was 30 min, because the overlapped development of the steps. This method was used for the determination of normality range of these vitamins in healthy people in the 18–80-year-old interval.

Keywords: Vitamins; HPLC; Retinol; α-Tocopherol; 24 R,25-$(OH)_2$ hydroxyvitamin D_3; 25-(OH) hydroxyvitamin D_3

1. Introduction

Osteoporosis is a skeletal disorder characterised by compromised bone strength predisposing to an increased risk of fractures [1]. This is a major public health problem because of the high incidence of fragility fractures, particularly hip and vertebral fracture. Bone strength reflects the integration of two main features: bone density and bone quality [1].

Skeletal bone mass is determined by a combination of endogenous (genetic, hormonal) and exogenous (nutritional, physical activity) factors. Nutrition plays a key role in bone health. The two essential nutrients for bone health are calcium and Vitamin D [2]. The status of the latter is usually assessed by measuring the serum 25-hydroxyvitamin D (25(OH)D) [3] mainly by competitive protein binding (CPB) assay with or without prior purification by high-performance liquid chromatography (HPLC) or radioimmunoassay [4,5].

There are consistent evidences that other fat-soluble vitamins also play a significant role in human bone health. In fact, Vitamin K can increase bone mineral density but also actually reduces fracture rates [6] depresses circulating levels of 25(OH)D and Vitamin K concomitantly and co-operatively participates in osteopenia in elderly people, which may reflect the aetiology of the type 2 moiety of involutional osteoporosis [7].

Moreover, an excess of Vitamin A stimulates bone resorption, inhibits bone formation and produces bone loss; thus, contributing to osteoporosis development [8]. In fact, unappreciated or subclinical hypervitaminosis A may increase the risk of fracture [9]. Vitamin A antagonises the serum calcium response to Vitamin D [10], consistent with the hypothesis of an antagonistic relationship between these vitamins.

Although the influence of Vitamin E on bone metabolism is less clearly established, an insufficient dietary intake of this vitamin may increase substantially the risk of hip fracture, whereas a more adequate intake seems to be protective, at least in current smokers [11].

The knowledge of the impact of Vitamin D and other fat-soluble vitamins on the mineral metabolism and other important biological functions [12] makes their simultaneous quantitation a challenge for researchers in the metabolism field.

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There are few methods for the simultaneous determination of fat-soluble vitamins [13]. These methods are cumbersome, slow, time consuming and very difficult to automate. The necessity for routine analysis of these vitamins calls for the development of fully automated methods for their simultaneous determination.

In this work, a fast, automated and sensitive high-performance liquid chromatography method using 1 ml serum for the simultaneous determination of the 24,25-$(OH)_2D_3$, 25-$(OH)D_3$, A and E Vitamins and the normality range of them in people 18–65-year-old is reported.

2. Material and methods

2.1. Apparatus and instruments

The experimental set-up consists of a Zymate II Plus robot with the necessary peripherals, described below, and the following modules: a ProStar 410 autosampler equipped with a 100 μl sample loop (Varian, Palo Alto, CA, USA) connected on-line with a liquid chromatograph (Varian 240 pump; Varian PDA detector 330). Data processing was carried out using a Star chromatography Workstation version 6.0 software running on a personal computer.

An Ultrabase C_{18} column (250 mm × 4.6 mm; 5.0 μm particle size, Scharlau Science, Barcelona, Spain), an Ultrabase C_{18} guard columns (10 mm × 4.0 mm; 5 μm particle size; Scharlau Science) were used. Solutions and reagents, dichloromethane, hexane, isopropanol, methanol and ethanol (Panreac, Barcelona, Spain) were HPLC grade. Demineralised water was purified in a Milli-Q filtration system to obtain water of HPLC grade.

Individual standard solutions of 40 μg ml^{-1} 24,25-$(OH)_2$ Vitamin D_3, 25-(OH) Vitamin D_3, Vitamin A and Vitamin E (Sigma–Aldrich, St. Louis, MO, USA) were prepared separately by dissolving the content of each vial in methanol.

Retinyl acetate (Sigma, St. Louis, MO, USA) was used as internal standard and the solution prepared by dissolving the amount necessary for a concentration of 500 μg ml^{-1} in 1:1, methanol:water.

The blood samples were supplied by a local hospital. Then, they were centrifuged for 20 min to 4500 × *g* and the serum collected was stored at −20 °C until use. No decrease in the concentration of the target analytes was observed when the samples were stored at −20 °C for 3 months. The methanol standard solutions were stored for 6 months at −20 °C without degradation. The standard dilutions in methanol were prepared daily.

2.2. Sample pre-treatment

All sample preparation was carried out with protection from light. One millilitre serum was placed in 10 ml glass tubes, spiked with 20 μl of the internal standard solution, and located in rack 1. The robot, fitted with a syringe hand, withdraw 800 μl of ethanol to each sample. Then, it changes to an all-purpose hand and takes the tube from rack 1 to place it in the vortex rack, where, after the rack has been filled with tubes, agitation is applied at 200 rpm for 10 s in order to precipitate the proteins. Next, the robot changes the hand to a syringe hand in order to withdraw 4 ml of *n*-hexane/dichloromethane to each tube for extracting the analytes. When the mixture is added to all tubes, agitation at 130 rpm for 60 s is applied. Then, the robot changes the syringe hand to all-purpose hand and takes the tube from the vortex rack to place it in the centrifuge, where, after filling all positions, centrifugation is applied at 4500 × *g* for 5 min. After this, the robot takes the tube from the centrifuge to place it in the liquid–liquid extractor and the *n*-hexane-dichloromethane layer is transferred to other glass tube and the tube placed in rack 2. This process was repeated, and the tube with the two extracts is placed in the evaporator rack where, after the evaporator rack has been filled with tubes, the extracts are evaporated to dryness under a stream of N_2. The robot takes the tube from the evaporator rack to put it in the vortex rack. When the latter has been filled, the robot changes the all-purpose hand to the syringe hand in order to put 250 μl of methanol to dissolve the residue. Then, agitation is applied at 130 rpm for 60 s. The reconstituted extract is transferred to an insert (0.1 ml; 5 mm) for the auto-sampler and 100 μl is injected by the auto-sampling injector [14].

2.3. Chromatographic separation and detection

The initial mobile phase was 90:10, methanol:water and the flow rate 1.0 ml min^{-1}. A linear gradient in 4 min to obtain 90:10, methanol:isopropanol was programmed. At minute 15 a second linear gradient was programmed in 3 min to obtain a 50:50, methanol:isopropanol mixture with a flow rate of 1.2 ml min^{-1} until the end of the run. Finally, 5 min was necessary for re-establishing and equilibrating the initial conditions. The total analysis time was 30 min. The eluate was monitored photometrically with a diode-array detector at the maximum absorption wavelength for each analyte.

2.4. Applications

This fully automated method was routinely used for the simultaneous measurements of the of retinol, α-tocopherol, 25-hydroxyvitamin D_3 and 24 R,25-hydroxyvitamin D_3 and the determination of normality range of these vitamins in 120 healthy subjects of both sexes in the 18–80-year-old interval divided in four group. The study was approved by the ethical Investigation Committee of the University Hospital "Reina Sofia", Córdoba, Spain. After giving informed consent morning fasting samples of venous blood were taken from blood donors. Serum samples were kept frozen at −80 °C until processing.

Table 1
Features of the method

Analyte	Limit of detection	Limit of quantitation	Linear range	Correlation coefficient
24,25-$(OH)_2$ Vitamin D_3 ($ng\,ml^{-1}$)	0.1	0.5	1.0–100	0.999
25-(OH) Vitamin D_3 ($ng\,ml^{-1}$)	0.08	0.3	1.0–100	0.998
Vitamin A ($ng\,ml^{-1}$)	0.2	1.0	1.0–1000	0.998
Vitamin E ($mg\,ml^{-1}$)	0.01	0.05	0.1–20	0.996

3. Results

3.1. Linearity

For the study of linearity, calibration curves were run covering the concentration range of the analytes usual in serum samples. Regression analysis was performed using the analytes area/internal standard area ratio versus concentration of each analyte. After routine use of the method, the relative standard deviations (R.S.Ds.) of the slope were 1.6% for 24,25-$(OH)_2$ Vitamin D_3, 3.3% for 25-(OH) Vitamin D_3, 3.5% for Vitamin A and 2.7% for Vitamin E (10 calibration curves obtained from different samples and days) and the correlation coefficients (r^2) were between 0.999 and 0.996 (Table 1).

3.2. Precision

To evaluate the precision of the proposed method, within-laboratory reproducibility and repeatability were estimated in a single experimental set-up with duplicates [15]. The experiments were carried out using a serum pool. The optimal values obtained for the variables were used in all experiments. Two measurements of each analyte signal per day were carried out on 7 days. The repeatability, expressed as R.S.D., was between 2.02 and 4.3%; meanwhile, the R.S.D. for within-laboratory reproducibility was between 2.8 and 9.5% for low concentration (Table 2).

3.3. Accuracy

The accuracy was estimated carrying out six measurements of a serum pool spiked with $50\,ng\,ml^{-1}$ for the metabolites of Vitamin D_3, $500\,ng\,ml^{-1}$ for Vitamin A and $10\,\mu g\,ml^{-1}$ for Vitamin E, and six measurements of the same serum pool without spiked. Then, the difference between the average concentration in both, the spiked and non-spiked serum is the added concentration [15]. The accuracy, expressed as relative error (RE) in the estimation of the added concentration, was between 1.1 and 2.0% (Table 2).

3.4. Detection and quantification limits

For the purpose of this method, the limits of detection and quantification are defined as 3 s_b/slope and 10 s_b/slope (where s_b is the standard deviation of 11 blank measurements). In this case, the blank is the noise measured before each analysis (Table 1).

4. Discussion

The liquid–liquid extraction was optimised with an experimental design with three variables—namely: time of agitation, volume and proportion of the hexane–dichloromethane mixture and three centre points. The optimum value of time was 1 min, because larger values did not increase the extraction and lower values decreased the extraction. The volume range was 4–8 ml. The optimum value was 8 ml. The *n*-hexane-dichloromethane proportion was studied in the range 10–30% of dichloromethane. The optimum mixture was 90:10 *n*-hexane-dichloromethane, because for percentages of dichloromethane larger than 10% the extraction of vitamins was lower. This optimisation was done with 1 ml of serum, because for lower volumes the 24,25-$(OH)_2$ Vitamin D_3 was not detected.

The optimum volume of methanol for reconstituting the sample was 250 μl, which was the lowest volume required for injection.

We used as analytical column an Ultrabase C_{18} (250 mm × 4.6 mm; 5 μm) [16]. The mobile phase was optimised to obtain the best separation of the analytes in the shortest time. The experimental variables were composition

Table 2
Precision and accuracy

Analyte	Repeatability[a]	Within-laboratory reproducibility[a]	Accuracy[b]
24,25-$(OH)_2$ Vitamin D_3	2.25	9.5	2.0
25-(OH) Vitamin D_3	4.3	9.2	1.8
Vitamin A	2.95	5.27	1.3
Vitamin E	2.02	2.8	1.1

[a] Expressed as relative standard deviation (%).
[b] Expressed as relative error (%).

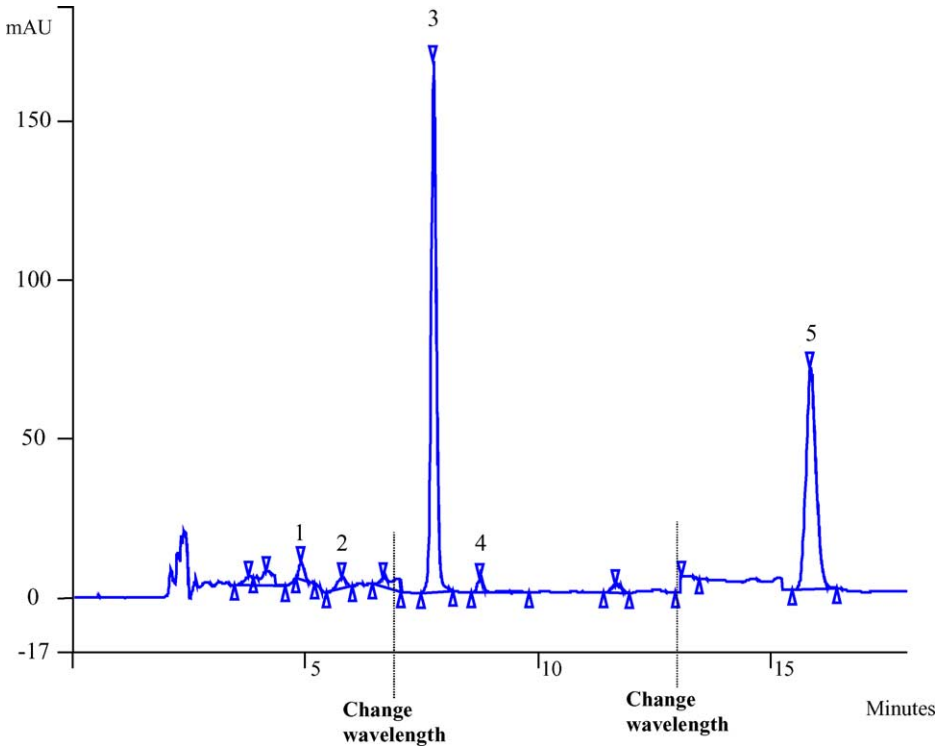

Fig. 1. Chromatogram from non-spiked serum: (1) 24,25-$(OH)_2$-Vitamin D_3; (2) 25-(OH)-Vitamin D_3; (3) Vitamin A; (4) internal standard and (5) Vitamin E.

of the mobile phase and flow rate. Several mobile phases (namely, methanol, acetonitrile, water, isopropanol) were assayed. The best results were obtained with the sequence explained in under experimental.

The eluate was monitored at 265 nm for both 24,25-$(OH)_2$ Vitamin D_3 and 25-(OH) Vitamin D_3; 292 nm for Vitamin E and 325 nm for both Vitamin A and internal standard (retinyl acetate) [17]. These are the maximum absorption wavelengths for the analytes.

Under the optimum working conditions, the chromatogram obtained from serum is that shown in Fig. 1.

Human intervention is minimised in this method thus making easy to apply it and allowing improvement of both reproducibility and repeatability, as compared with previous contributions [14,16] where these parameters ranged between 5.3 and 9.7%. The described method is fast and sensitive to determine with acceptable performance and extraction recoveries the target analytes.

This high-performance liquid chromatography method is the only fully automated method proposed so far that allows the determination of these fat-soluble vitamins in a single analysis. The real time required for the routine process is 30 min per sample, because overlapping between steps of the successive samples or batches. As can be seen in Table 3, the values of the target vitamins found by the proposed method are within of the normality range [14,18,19]. This method will constitute a useful tool for epidemiological studies in the fields of osteoporosis and nutrition.

Table 3
Average and normal range of concentration of analytes

Age	24,25-Hydroxyvitamin D_3 (ng ml^{-1})		25-Hydroxyvitamin D_3 (ng ml^{-1})		Vitamin A (ng ml^{-1})		Vitamin E (μg ml^{-1})	
	Range	Average	Range	Average	Range	Average	Range	Average
Men								
18–27	1.2–20	6.06	7.8–33	16.3	498–1057	683	2.4–8.0	4.7
28–37	1.38–18	7.14	3–38	20	175–896	575	1.4–10	5.2
38–47	1.2–18	6.2	2.5–35	21	509–1029	719	1.2–10.6	5.9
48–65	2–15	7.1	3–28	11.3	102–962	656	2.6–9.8	6.4
Women								
18–27	1.4–19	5	5.4–32	14.5	270–747	513	2.1–6.4	4.0
28–37	0.4–15	5.6	2–31	13.0	330–1182	578	1.4–11.5	5.4
38–47	1.4–12	6.0	2–29	15.4	350–770	570	1.8–7.0	4.9
48–65	1.3–9.2	3.7	1.8–25	12.2	354–782	536	2.9–9.8	5.7

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