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Journal of Chromatography B, 805 (2004) 289–296
JOURNAL OF CHROMATOGRAPHY B
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Development of a validated liquid chromatography method for the simultaneous determination of eight fat-soluble vitamins in biological fluids after solid-phase extraction

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Received 20 October 2003; received in revised form 19 January 2004; accepted 8 March 2004

Available online 15 April 2004

Abstract

In the present study, a simple and rapid reversed-phase HPLC procedure has been developed for the simultaneous determination of eight fat-soluble vitamins (retinol, menadione, menaquinone, δ-tocopherol, cholecalciferol, α-tocopherol, α-tocopherol acetate and phylloquinone) in biological fluids: blood serum and urine. The analytical column, Phenomenex Luna C_{18} (150 mm × 4.6 mm) 3 μm, was operating at ambient temperature. Mobile phase consisted of a mixture of CH_3OH–CH_3CN delivered using a linear gradient, starting with a composition of 50–50% v/v and ending at 30–70% at a flow rate of 1.3 ml/min. Xanthophyll was used as internal standard (2 ng/μl). Detection and identification was performed using a photodiode array detector. Eluent monitoring was achieved at 280 nm for vitamins and 450 nm for the internal standard. However, quantitation was performed at maximum wavelength for each vitamin. Detection limits were found in the range of 1.4–6.6 ng per 20-μl injected samples, while linearity held up to 25 ng/μl. The statistical evaluation of the method was examined performing intra-day ($n = 6$) and inter-day calibration ($n = 7$) and was found to be satisfactory, with high accuracy and precision results. The biological fluids were treated using solid-phase extraction cartridges, to remove all endogenous interferences from sample matrix. The solid-phase extraction protocol was optimized in terms of retention and elution. High extraction recoveries from biological matrices: blood serum and urine, (average recovery ranging between 95 and 97.6% for blood serum and between 94.2 and 95.8% for urine) were achieved for the eight fat-soluble vitamins, using Cyclohexyl J.T. Baker SPE cartridges with methanol as eluent, requiring small volumes, 100 μl of blood serum and 100 μl of urine.

Keyword: Vitamins

1. Introduction

Vitamins are a broad group of organic compounds that are minor, but essential, constituents of food, required for the normal growth, self maintenance and functioning of human and animal bodies. Their presence in the human body is of vital importance, since vitamins have a catalytic function in anabolic and catabolic pathways. Fat-soluble vitamins that are studied here comprise Vitamins A, D, E, and K, whose biological activities are attributed to a number of structurally related compounds known as vitamers. Vitamin A or retinol is stored in the liver and secreted into the bloodstream when needed. It is considered to be necessary for vision, growth, tissue differentiation, reproduction and maintenance of the immune system. Vitamin D consists of two different compounds, Vitamins D_2 and D_3 both absorbed from the diet. Certain human populations depend on dietary sources of Vitamin D because of insufficient biosynthesis of this particular vitamin due to inadequate skin exposure to sunlight. Naturally occurring Vitamin E is also composed of a number of tocopherol analogues (α-, β-, γ-, δ-) and their corresponding unsaturated tocotrienols. Finally Vitamin K, which is essential for the activation of specific proteins involved in blood clotting and bone metabolism, appears in three different forms, two naturally occurring K_1 (phylloquinone) and K_2 (menaquinone) and a synthetic one K_3 (menadione) [1,2].

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doi:10.1016/j.jchromb.2004.03.009

Fat-soluble vitamins have been determined so far, in many different samples, by a variety of techniques. Among them the most widely applied are the chromatographic techniques mainly HPLC (both normal and reversed-phase), which provides rapid, sensitive and accurate methods for vitamin determination and has the advantages of solvent economy and easy coupling with other techniques. It also requires small amounts of sample. However, the already reported methods measure a few vitamins at a time. Only one reported study determines simultaneously eight fat-soluble vitamins in plasma: retinoic acid, retinal, retinal, ergocalciferol, cholecalciferol, menadione, phylloquinone, and menaquinone, which are separated in 18 min [3].

A continuous clean-up pre-concentration procedure coupled on-line with liquid chromatography-UV detection has been reported for six vitamins (retinol, ergocalciferol, cholecalciferol, α-tocopherol, menadione, and phylloquinone). Total analysis time was 27 min [4].

Individual vitamins can be determined chromatographically using isocratic eluting systems. The same stands true for combinations of two or three vitamins [5,6]. However, the simultaneous determination of more complicated mixtures often requires gradient elution programs. Mobile phases consisting of mixtures of three or four types of solvents or phosphate buffers have been reported [7–12].

Detection modes involved in the determination of fat-soluble vitamins include UV, diode array [9,13] fluorimetric [14–16], and electrochemical [17–19], as well as particle beam MS detection [20].

Concerning sample preparation, it is recommended to use short time and gentle extraction methods, sometimes in a darkened place, since vitamins are unstable during common procedures such as boiling for deproteinization, alkali- or acid-treatment). Deproteinization of the plasma is mainly achieved by ACN, EtOH, MeOH, and liquid–liquid extraction for sample clean-up is performed by *n*-hexane or heptane [9,17,21–29]. Sometimes, sample preparation requires perchloric acid or trichloroacetic acid deproteinization of the plasma followed by centrifugation and filtration through a disposable filter [12]. Solid-phase extraction has been also

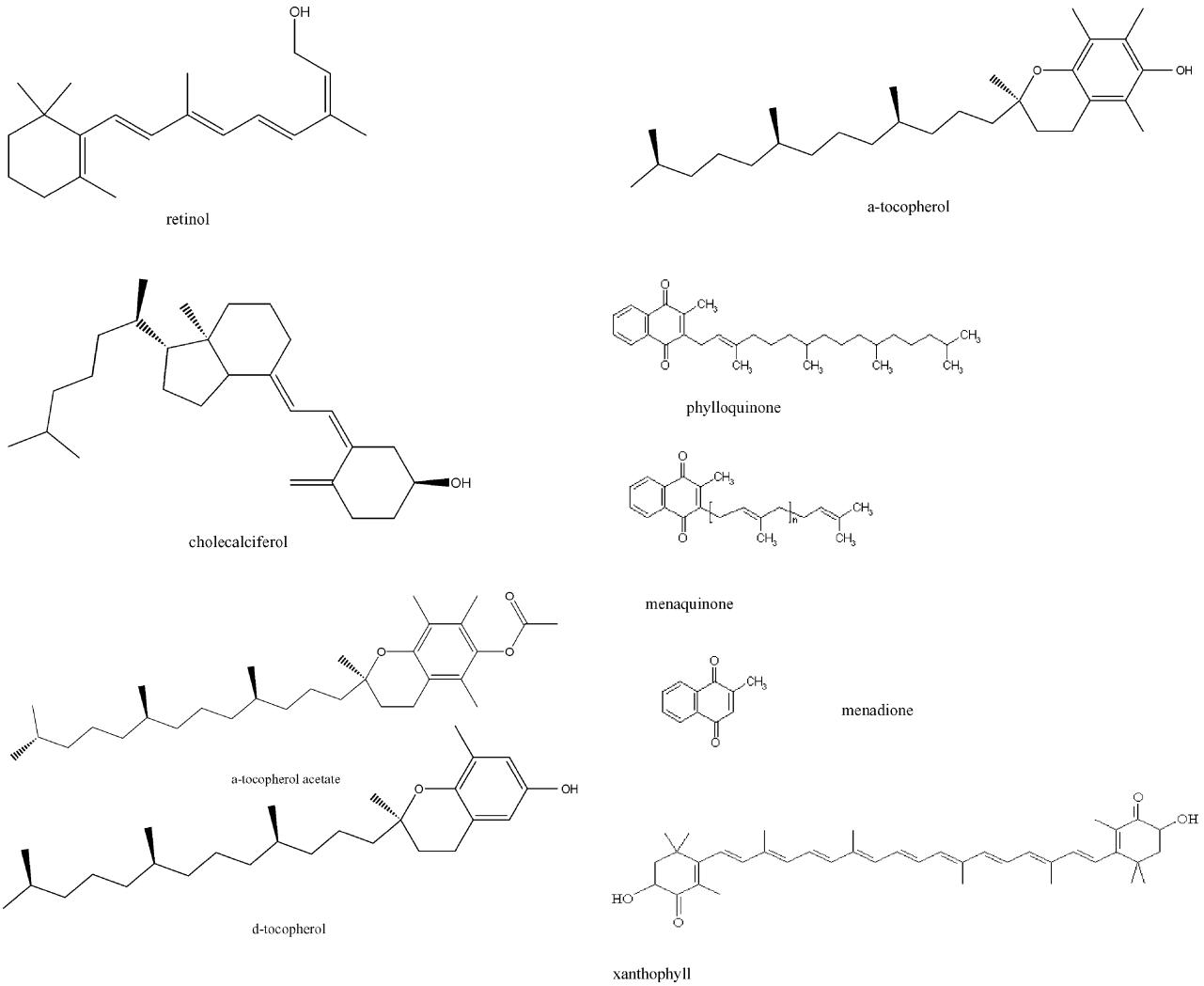

Fig. 1. Chemical structures of examined fat-soluble vitamins and internal standard.

used for the recovery of vitamins from biological fluids [30,31].

The authors in a previous study had developed a method for the simultaneous determination of fat-soluble vitamins and water-soluble vitamins. However, only three fat-soluble vitamins were separated and studied: retinal, α-tocopherol, and α-tocopherol acetate [31].

In the present work, a simple, fast and accurate simultaneous determination of eight fat-soluble vitamins: retinol (A), cholecalciferol (D_3), α-tocopherol (E), δ-tocopherol (E), α-tocopherol acetate (E-acetate), phylloquinone (K_1), menaquinone (K_2), and menadione (K_3) in biological fluids is proposed. The chemical structures of all examined fat-soluble vitamins are shown in Fig. 1.

2. Experimental

2.1. Instrumentation and chemicals

A Waters (Milford, MA, USA) liquid chromatograph consisted of a Waters 600E system controller, a Waters 991 Photo Diode Array detector, a Waters 717 plus autosampler and a 5200-printer/plotter was used.

All solvents were of HPLC grade. Methanol was obtained by Rathburn (Walkerburn, Scotland), whereas HPLC grade acetonitrile by J.T. Baker (Deventer, The Netherlands). Vitamins A, D_3, E, E-acetate, K_1, K_2, K_3, and δ-tocopherol were all of analytical grade and were supplied by Sigma (Sigma-Aldrich Chemie BV, The Netherlands). Xanthophyll, the internal standard used, was obtained by Fluka (Buchs, Switzerland). The SPE columns used for sample clean-up, Bakerbond (SPE) Cyclohexyl (C_6H_{11}) 500 mg/3 ml, were obtained by J.T. Baker. Other SPE cartridges investigated in this study include C_8 (500 mg/2.8 ml) by Alltech (Deerfield, IL, USA), C_4 by J.T. Baker (500 mg/6 ml), Oasis HLB (60 mg/3 ml) by Waters (Waters Corporation, MA, USA) and LC-SAX by Supelco (500 mg/3 ml) (Bellefonte, PA, USA).

2.2. Chromatographic conditions

For the separation of the fat-soluble vitamins, a Phenomenex (Torrance, CA, USA) column, type Luna 3μ C_{18}(2) (150 mm × 4.60 mm, 3 μm) coupled with a Phenomenex security guard pre-column, at room temperature was used. The mobile phase of the HPLC system consisted of acetonitrile (solvent A) and methanol (solvent B). A simple linear gradient was used, starting from 50/50 (solvent A/solvent B) and ending after 20 min at 70/30 (solvent A/solvent B). The mobile phase flow rate was 1.3 ml/min and the injection volume each time was 20 μl. The chromatographic conditions were chosen in terms of peak shape, column efficiency, chromatographic analysis time, selectivity, and resolution.

2.3. Preparation of standard solutions

The stock methanolic solutions of the fat-soluble vitamins were prepared every week. The working standards were prepared every day by appropriate dilution of the concentrated stock standard solutions. All solutions were stored at −20 °C, covered with aluminum foil in order to protect them from light.

2.4. Method validation

The method described has been validated with respect to accuracy, within-day ($n = 6$) and between-day precision for 7 consecutive days, linearity and sensitivity. Linearity was studied by a series of mixed standards of fat-soluble vitamins, covering the entire working range; each solution was injected three times. Regression analysis revealed calibration equations with the respective correlation coefficients.

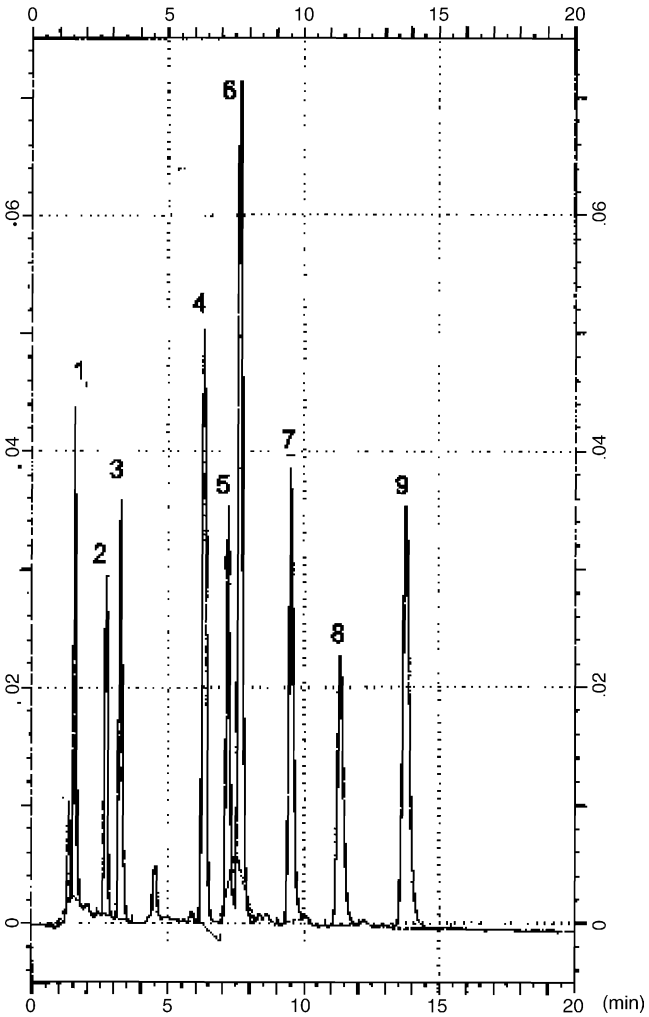

Fig. 2. Typical HPLC chromatogram of fat-soluble vitamins: (1) menadione; (2) retinol; (3) xanthophyll (IS); (4) menaquinone; (5) δ-tocopherol; (6) cholecalciferol; (7) α-tocopherol; (8) α-tocopherol acetate; (9) phylloquinone.

The detection limits were assessed in the presence of the internal standard. Those are considered to be the quantities that are producing a signal of peak height three times the size of the background noise.

2.5. Sample preparation—solid-phase extraction procedure

SPE optimization was performed in terms of retention and elution. Several different SPE cartridges were tested. Different washing and elution solvents were assessed. Optimum SPE conditions include Cyclohexyl (C_6H_{11}) cartridges (500 mg/3 ml), conditioned immediately prior to use with 1 ml of methanol, followed by 1 ml of deionized water. After sample application the fat-soluble vitamins were retained on the sorbent and subsequently eluted by passing 2 ml of methanol. The eluent was evaporated to dryness, using a Speedvac evaporator. Finally the dry residue of the fat-soluble vitamins fraction was reconstituted in 100 μl of a methanolic solution of xanthophyll (internal standard, 2 ng/ml) and 20 μl of each solution was injected in the HPLC column.

Aliquots of 100-μl pooled human blood serum (blank or spiked with 100 μl of water-soluble vitamin solutions, at concentration levels of 1, 2, 5, 10, 15 ng/μl) were treated with 500 μl of CH_3CN in order to precipitate proteins. After vortex mixing for two minutes, the sample was centrifuged at 4000 rpm for 15 min. The supernatant was subsequently applied to the solid-phase cartridge.

Same procedure was also used for urine samples, though an aliquot of 100 μl of CH_3CN was sufficient for sample pre-treatment.

3. Results and discussion

3.1. Chromatography

The gradient acetonitrile–methanol changing from 50/50 (v/v) to 70/30 (v/v), achieved an ideal separation of examined compounds in less than 14 min (Fig. 2). Retention times are as follows: menadione $t_R = 1.6$ min, retinol $t_R = 2.75$ min, xanthophyll (IS) $t_R = 3.31$ min, menaquinone $t_R = 6.37$ min, δ-tocopherol $t_R = 7.2$ min, cholecalciferol $t_R = 7.7$ min, α-tocopherol $t_R = 9.5$ min, α-tocopherol acetate $t_R = 11.38$ min, phylloquinone $t_R = 13.81$ min. Resolution factors were greater than unity indicating a sufficient separation.

The use of the PDA gave the opportunity to measure each of examined vitamins at its wavelength of maximum absorbance. The sample peaks in biological fluids were identified by comparing both the relative retention times and the UV-Vis spectrum of each one with those of the standard reference vitamins. Their quantification was carried out using the internal standard method. Xanthophyll, a carotenoid, mostly found in plant material, proved to be an adequate solution for this problem. Due to its spectral characteris-

Table 1
Regression analysis data of the eight fat-soluble vitamins with xanthophyll 2 ng/μl as internal standard

Parameter	K_3	A	K_2	δ-Tocopherol	E	E-acetate	K_1	D_3
Retention time (min)	1.63 ± 0.03	2.76 ± 0.03	6.36 ± 0.03	7.21 ± 0.04	9.49 ± 0.05	11.39 ± 0.04	13.81 ± 0.05	7.72 ± 0.03
LOD (ng/μl)	0.33	0.07	0.33	0.33	0.17	0.33	0.03	0.17
Range (ng/μl)	1–25	0.2–25	1–25	1–25	0.5–25	1–25	0.1–25	0.5–25
Slope (ng^{-1})	0.03371 ± 0.00081	0.03662 ± 0.00099	0.07202 ± 0.00168	0.26596 ± 0.00692	0.00877 ± 0.00019	0.04315 ± 0.00025	0.00723 ± 0.00184	0.06500 ± 0.00377
Intercept	0.10836 ± 0.01018	0.70556 ± 0.00216	0.29889 ± 0.02281	1.10573 ± 0.11375	0.46574 ± 0.00494	0.02655 ± 0.00077	0.18043 ± 0.02918	0.95216 ± 0.01394
R	0.997	0.999	0.998	0.998	0.999	0.999	0.997	0.996
Serum								
Slope (ng^{-1})	0.01421 ± 0.00023	0.01478 ± 0.00059	0.02145 ± 0.00031	0.01971 ± 0.00031	0.02341 ± 0.00038	0.01732 ± 0.00018	0.01082 ± 0.00020	0.01931 ± 0.00022
Intercept	0.09647 ± 0.01431	0.64344 ± 0.02196	0.27268 ± 0.02892	1.03492 ± 0.03247	0.43423 ± 0.01628	0.02438 ± 0.01235	0.17133 ± 0.01173	0.79773 ± 0.01421
R	0.998	0.999	0.997	0.999	0.998	0.997	0.999	0.999
Urine								
Slope (ng^{-1})	–	0.01422 ± 0.00052	0.02432 ± 0.00019	0.01822 ± 0.00022	0.02248 ± 0.00015	0.01901 ± 0.00012	0.01261 ± 0.00016	0.01781 ± 0.00031
Intercept	–	0.64091 ± 0.02512	0.26873 ± 0.02991	1.03108 ± 0.03102	0.43321 ± 0.01581	0.02381 ± 0.01178	0.16737 ± 0.01167	0.80417 ± 0.01901
R	–	0.999	0.999	0.999	0.999	0.999	0.999	0.999

tics monitoring of the internal standard was performed at 450 nm.

3.2. Method validation

3.2.1. Linearity and sensitivity

Calibration curves were obtained by least-squares linear regression analysis of the peak area ratio of analyte to internal standard versus analyte concentration. The method was linear up to 25 ng/μl. Correlation coefficients ranged from 0.996 to 0.999. All calibration data are presented in Table 1.

The LOD was defined as the compound concentration that produced a signal-to-noise ratio greater than three. Respective LOD values for each compound based upon this criterion are shown in Table 1. These values were observed for 10 samples. The limit of quantitation of the assay was evaluated as the concentration equal to 10 times the value of the signal-to-noise ratio. Limits of detection ranged from 1.4 to 6.6 ng.

3.3. Precision and accuracy

The precision of the method based on within-day repeatability was assessed, by replicate injections ($n = 6$) of three standard solutions covering different concentration levels: low, medium, and high, namely 1.0, 3.0, and 5.0 ng/μl, where peak areas were measured, in comparison to the peak area of the internal standard. Statistical evaluation revealed relative standard deviations, at different values. Results are shown in Table 2.

The reproducibility (day-to-day variation) of the method was established using the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of 7 consecutive days. Reproducibility results are illustrated in Table 2. The measured concentrations had R.S.D. values <3%. Accuracy was determined by replicate analysis of three different levels. Expressed as relative error can be calculated by the equation:

$$\text{Relative error (\%)} = \frac{\text{mean determined value} - \text{theoretical (added amount)}}{\text{theoretical}} \times 100$$

Relative error (inaccuracy) lays in the range of −3.1 to 4.0%.

3.4. Solid-phase extraction

Recovery rates determined by comparing observed analyte concentration in extracted sample to those of non-processed standard solutions, using different sorbents (C_8 by Alltech, C_4 by J.T. Baker, Cyclohexyl by J.T. Baker,

Table 2
Data day-to-day (over a period of 7 consecutive days) and within-day ($n = 6$) precision and accuracy study for the determination of fat-soluble vitamins

Vitamin	Intra-day ($n = 6$)				Inter-day ($n = 7$)			
	Added (ng)	Found (ng) ± S.D.	R.S.D.	R.E. (%)	Added (ng)	Found (ng) ± S.D.	R.S.D.	R.E. (%)
K_3	19.80	20.19 ± 0.32	1.6	1.9	19.60	19.89 ± 0.35	1.8	1.5
	59.20	59.74 ± 0.44	0.7	0.9	59.70	60.07 ± 0.53	0.9	0.6
	99.60	99.13 ± 1.61	1.6	−0.5	99.10	98.13 ± 1.22	1.2	−1.0
A	20.40	20.81 ± 0.23	1.1	2.0	20.70	21.31 ± 0.29	1.4	2.9
	59.40	58.84 ± 0.57	1.0	−1.0	59.20	59.81 ± 0.62	1.0	−1.0
	100.40	101.73 ± 1.22	1.2	1.3	99.40	101.23 ± 0.92	0.9	1.8
K_2	20.20	20.67 ± 0.28	1.3	2.3	21.20	20.88 ± 0.44	2.1	−1.5
	60.60	60.13 ± 0.47	0.8	−0.8	60.90	60.34 ± 0.19	0.3	−0.9
	99.80	100.56 ± 0.78	0.8	0.7	99.20	100.18 ± 1.17	1.2	−1.0
δ-Tocopherol	21.00	20.33 ± 0.29	1.4	−3.1	19.40	20.11 ± 0.22	1.1	3.6
	60.80	61.21 ± 0.82	1.3	0.7	59.70	59.31 ± 0.45	0.8	−0.7
	100.20	101.87 ± 1.81	1.8	1.7	101.50	101.99 ± 1.23	1.2	0.5
D_3	19.00	19.77 ± 0.36	1.8	4.0	19.30	18.87 ± 0.51	2.7	−2.2
	58.80	58.34 ± 0.71	1.2	−0.7	59.30	59.38 ± 0.64	1.1	0.1
	100.60	101.72 ± 1.48	1.4	1.1	101.60	102.14 ± 1.27	1.2	0.5
E	20.80	20.16 ± 0.39	1.9	3.1	19.60	20.23 ± 0.36	1.8	3.2
	59.20	58.84 ± 0.88	1.5	−0.6	59.80	59.44 ± 0.82	1.4	−0.6
	99.00	97.37 ± 1.53	1.6	−1.6	99.40	98.73 ± 1.35	1.4	−0.7
E-acetate	18.80	19.21 ± 0.18	0.9	2.2	19.10	20.01 ± 0.41	2.0	4.8
	60.20	59.62 ± 0.39	0.7	−0.9	61.30	60.62 ± 0.25	0.4	−1.1
	99.40	98.83 ± 0.85	0.9	−0.5	101.40	101.68 ± 1.17	1.2	0.3
K_1	19.60	19.97 ± 0.24	1.2	1.9	19.80	20.17 ± 0.21	1.0	1.9
	59.80	58.61 ± 0.67	1.1	−2.0	59.40	59.67 ± 0.41	0.7	0.5
	101.00	99.54 ± 1.23	1.2	−1.4	100.20	101.54 ± 1.08	1.1	1.3

Table 3
Comparison of different sorbents for their recovery rates of the eight fat-soluble vitamins

Vitamin	Cartridge				
	Alltech C_8 500 mg	J.T. Baker C_4	J.T. Baker Cyclohexyl	Waters Oasis HLB	Supelco LC-SAX
K_3	80.4	91.3	93.2	101.1	87.3
A	85.3	90.4	95.4	91.4	82.2
K_2	91.6	79.1	94.1	89.3	88.1
δ-Tocopherol	93.5	90.2	97.7	93.1	90.9
D_3	62.4	73.4	88.2	87.3	79.2
E	89.8	83.3	97.7	92.2	92.1
E-acetate	93.3	79.0	94.4	100.6	93.0
K_1	90.1	91.2	97.3	87.1	89.1

Sample volume: 100 μl; conditioning: 1 ml MeOH, 1 ml H_2O; elution: 2 ml MeOH; reconstitution: 100 μl methanolic solution of IS (2 ng/μl).

Oasis HLB by Waters, and LC-SAX by Supelco) and elution protocols are summarized in Table 3. As shown in this table, Cyclohexyl cartridges provide higher efficiency in vitamins extraction.

The precision and accuracy studies of SPE of fat-soluble vitamins from standard solutions were conducted at five concentration levels (1.0, 2.0, 5.0, 10.0, and 15 ng/μl). Results are shown in Table 4.

3.4.1. Biological fluids: human blood serum and urine

The precision and accuracy studies of SPE of fat-soluble vitamins from biological samples were conducted by spiking blood serum samples, with five known concentrations: 1.0, 2.0, 5.0, 10.0, and 15.0 ng/μl. Regression equations revealed correlation coefficients ranging between 0.997 and 0.999 as shown in Table 1. Relative recovery rates from serum sample were determined at five different concentrations by comparing the peak area ratios for extracted vitamins from serum and the respective values derived from the serum calibration curve. Results of recovery studies are given in Table 5. These are mean values from five concentration levels and six measurements for each level.

Identification was performed by UV spectra comparison using a photodiode array detector. High performance liquid chromatogram of fat-soluble vitamins extracted from human blood serum is shown in Fig. 3A. No interference was observed in blood serum samples.

Six serum samples were prepared and analyzed in order to check the applicability of the method in real clinical samples. Concentrations found after triplicate analysis are summarized in Table 6. Regarding Vitamin D the samples were pre-concentrated 10 times.

Urine samples were analyzed using a similar solid-phase extraction protocol. Calibration curves were constructed by spiking pooled urine sample.

Regression equations revealed the same correlation coefficient namely 0.999 as shown in Table 1.

A blank chromatogram of urine sample is shown in Fig. 3B. High performance liquid chromatogram of

Table 4
SPE results of standard solutions using the protocol described in text

Vitamin	Added (ng)	Found (ng) ± S.D.	R.S.D.	Recovery (%)
K_3	19.40	19.11 ± 0.27	1.4	98.5
	40.70	40.14 ± 0.62	1.5	98.6
	100.10	91.13 ± 1.44	1.6	91.0
	199.60	198.34 ± 3.21	1.6	99.3
	299.40	289.40 ± 2.27	0.8	96.6
A	20.20	19.81 ± 0.32	1.6	98.0
	39.20	36.92 ± 0.51	1.4	94.2
	99.80	99.18 ± 1.32	1.3	99.4
	200.40	201.87 ± 2.43	1.2	100.7
	301.20	289.42 ± 3.21	1.1	96.0
K_2	21.60	19.18 ± 0.37	1.9	88.8
	40.70	41.13 ± 0.74	1.8	101.1
	100.20	92.09 ± 1.34	1.5	91.2
	198.20	197.34 ± 2.71	1.4	99.6
	299.60	301.13 ± 3.79	1.3	100.5
δ-Tocopherol	18.90	19.51 ± 0.28	1.4	103.2
	40.30	38.79 ± 0.56	1.4	96.2
	99.50	98.79 ± 1.51	1.5	99.3
	201.40	188.12 ± 2.19	1.2	93.4
	298.30	292.19 ± 3.47	1.2	97.9
D_3	21.10	19.76 ± 0.32	1.6	93.6
	38.30	40.27 ± 0.58	1.4	105.1
	100.20	98.41 ± 2.32	2.3	98.2
	201.50	190.55 ± 3.21	1.6	94.6
	300.20	287.21 ± 3.77	1.3	95.6
E	19.10	20.23 ± 0.36	1.8	105.9
	40.60	38.44 ± 0.82	2.1	94.7
	99.90	95.73 ± 1.35	1.4	95.8
	200.40	192.93 ± 1.58	0.8	96.3
	299.30	279.02 ± 3.71	1.3	93.2
E-acetate	20.30	18.91 ± 0.37	2.0	93.2
	41.30	38.92 ± 0.46	1.2	94.2
	100.70	98.14 ± 1.39	1.4	97.4
	200.40	189.27 ± 1.63	0.9	94.4
	299.20	285.91 ± 3.81	1.3	95.5
K_1	18.60	19.23 ± 0.37	1.9	103.3
	40.40	38.81 ± 0.52	1.3	96.1
	100.50	101.21 ± 1.78	1.8	100.7
	198.20	202.32 ± 2.21	1.1	102.1
	301.50	288.87 ± 3.54	1.2	95.8

Table 5
Mean recovery values of fat-soluble vitamins from serum and urine samples

Compound	Blood serum (%) ± S.D.	R.S.D.	Urine (%) ± S.D.	R.S.D.
K_3	95.6 ± 3.0	3.1	–	–
A	95.6 ± 2.5	2.6	95.2 ± 5.3	5.6
K_2	97 ± 4.2	4.3	95.6 ± 2.6	2.7
δ-Tocopherol	95.8 ± 3.3	3.4	95.4 ± 2.7	2.8
D_3	95 ± 4.1	4.3	95.8 ± 1.3	1.4
E	95.4 ± 3.1	3.2	95.2 ± 3.1	3.3
E-acetate	96.2 ± 3.5	3.6	95.4 ± 4.7	4.9
K_1	97.6 ± 4.0	4.1	95.4 ± 4.2	4.4

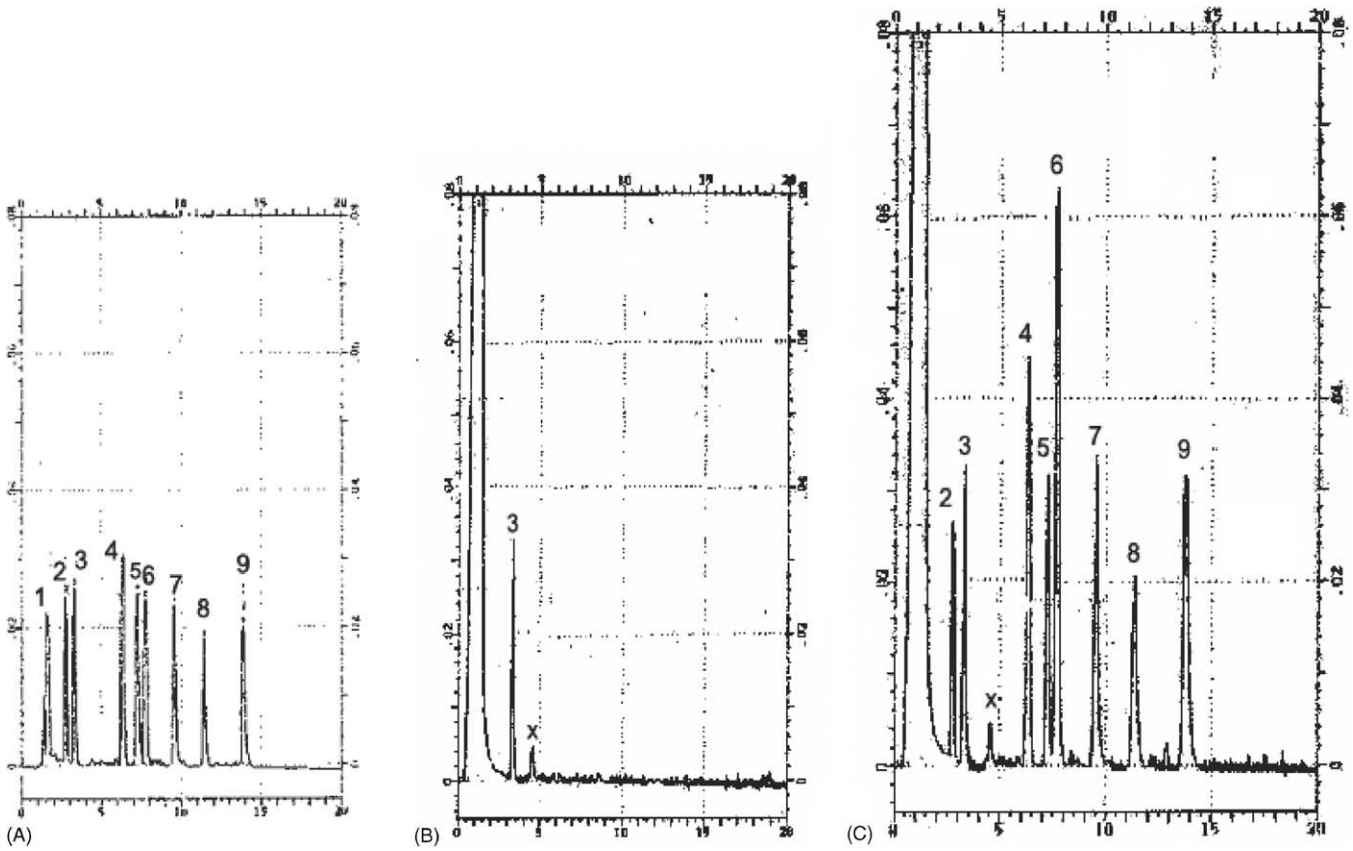

Fig. 3. High performance liquid chromatogram of fat-soluble vitamins in (A) spiked human blood serum, (B) blank urine, and (C) spiked urine sample, after SPE using the conditions described in text: (1) menadione; (2) retinol; (3) xanthophyll (IS); (4) menaquinone; (5) δ-tocopherol; (6) cholecalciferol; (7) α-tocopherol; (8) α-tocopherol acetate; (9) phylloquinone. X: endogenous compound.

fat-soluble vitamins in spiked human urine is shown in Fig. 3C. The quantitation of menadione in urine was impaired by polar endogenous compounds that interfere.

Relative recovery from urine was determined at five different concentrations by comparing the peak area ratios for extracted vitamins and the respective values derived from the urine calibration curve.

Mean recovery rates regarding extraction of fat-soluble vitamins from urine samples are summarized in Table 5.

Adequate biological samples clean-up was achieved by the developed SPE protocol. With the exception of matrix interference on menadione determination in urine samples, no further endogenous interference was noticed.

Table 6
Measured concentrations of fat-soluble vitamins in serum samples

Vitamin	Range of measured concentrations (ng/μl)[a]
A	0.3–1.1
K_2	ND
δ-Tocopherol	1.0–1.4
E	2.2–6.5
K_1	ND
D_3	0.03–0.05[b]

[a] Triplicate analysis of six serum samples.
[b] After pre-concentration 10:1.

4. Conclusions

In the present work, a new method for the separation, determination and quantification of eight fat-soluble vitamins is presented. The advantage of the proposed method is that with a single run completed in less than 15 min a screening of the fat-soluble vitamins potentially present in biological samples is achieved. In this way the proposed method could be considered as a universal one for the examined fat-soluble vitamins. Detectability of the method lays at the same levels to those reported in the literature.

Solid-phase extraction proved to be an adequate way, for the separation of the eight fat-soluble vitamins from interferences in the samples used, with the exception of menadione, which could not be separated from urine matrix. The latter though is not a significant disadvantage as this vitamin is a synthetic one. The accuracy of this method was tested obtaining an average recovery ranging from 95 to 97.6% for blood serum and from 94.2 to 95.8% for urine.

Detection limits, the good sensitivity and resolution and the short analysis time, combined with the simplicity of the

procedure should make this method a useful tool for the determination of fat-soluble vitamins in clinical samples.

Acknowledgements

The authors would like to express their acknowledgments to Professor R. Verpoorte, Pharmacognosy Department, Leiden University (LACDR) for his help, support and fruitful discussions during method development.

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