Determination of vitamins D_2, D_3, K_1 and K_3 and some hydroxy metabolites of vitamin D_3 in plasma using a continuous clean-up–preconcentration procedure coupled on-line with liquid chromatography–UV detection

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A semi-automatic procedure for the continuous clean-up and concentration of several fat-soluble vitamins prior to their separation by HPLC and UV detection is reported. The procedure is based on the use of a minicolumn packed with aminopropylsilica as sorbent located prior to the chromatographic detection system. The overall process was developed and applied to the main liposoluble vitamins (A, D_2, D_3, E, K_1, K_3) and several hydroxy metabolites of vitamin D_3 [25-(OH)-D_3, 24,25-$(OH)_2$-D_3 and 1,25-$(OH)_2$-D_3]. All the analytes were monitored at a compromise wavelength of 270 nm. Calibration graphs were constructed between 0.01 and 100 ng ml^{-1} for vitamin D_2 and D_3 and their hydroxy metabolites, between 0.1 and 100 ng ml^{-1} for vitamin A, K_1 and K_3 and between 1 and 100 ng ml^{-1} for vitamin E, with excellent regression coefficients ($\geq$0.9901) in all cases. The precision was established at two concentration levels with acceptable RSDs in all instances (between 3.6 and 8.7%). The method was appropriate for the determination of vitamin D_2, D_3, K_1 and K_3 and the 24,25-dihydroxy and 25-hydroxy metabolites of vitamin D_3 in human plasma. The method was applied to plasma samples spiked with the target analytes and the recoveries ranged between 78 and 109%.

The quantification of fat-soluble vitamins at low levels is one of the demands in diagnostic and metabolism units. However, the simultaneous determination of these vitamins in biological fluids and foods is difficult because of (i) the low concentration of circulating vitamins in human fluids, (ii) the presence of a number of metabolites which exhibit similar chemical behaviours, (iii) the presence of other associated compounds such as lipids and proteins and (iv) the poor stability of some of their chemical structures under the influence of UV radiation and heat.[1]

The decisive influence of the sample matrix on the methods proposed for the simultaneous determination of fat-soluble vitamins in clinical samples makes pre-treatments based on several clean-up–preconcentration steps mandatory.

The importance of the determination of fat-soluble vitamins is well documented.[2,3] Vitamin D and, in particular, its hydroxy metabolites play an important role as hormonal regulators of calcium metabolism and their quantification is widely used as a means of clinical testing for several pathophysiological states (*e.g.*, parathyroid gland disorders, renal failures, vitamin dependent rickets and sarcoidosis).[4,5] The so-called retinoids, which comprise a large number of related compounds which are designated vitamin A, play a decisive role on reproductive function and are also related to vision processes, to osteoblastic activity and to resistance associated with infections.[6–8] Vitamin E (tocopherol) acts as an antioxidant and its deficiency gives rise to muscular degradation.[9–11] Vitamin K shows antihaemorrhagic activity through its involvement in the biosynthesis and regulation of several coagulation factors, such as prothrombin and factors II, VII, IX and X.[12–16]

The aim of this research was the development of a semi-automatic method for the clean-up and preconcentration of several fat-soluble vitamins [D_2, D_3, 25-(OH)-D_3, 24,25-$(OH)_2$-D_3, K_1, K_3, A and E] from clinical samples using a continuous approach located prior to a liquid chromatograph and UV absorbance detector.

Experimental

Reagents and solutions

All solutions were prepared using doubly distilled water [obtained using a Millipore (Bedford, MA, USA) Milli-Q system]. Bond-Elut NH_2 packed sorbent [500 mg/2.8 ml from Varian (Palo Alto, CA, USA)] was used for packing the minicolumn. All organic solvents used were of HPLC grade. Acetonitrile–phosphate buffer (50 mmol l^{-1}, pH 6.5) (20 + 80) was used as the initial mobile phase. A linear gradient was programmed in order to obtain a propan-2-ol–methanol (10 + 90) mobile phase in 1.7 min, then stabilised for 30 min.

Standard solutions of vitamin A (all-*trans*-retinoic acid, R-2625), vitamin E [(+)-α-tocopherol, T-3251], vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone, V-3501), vitamin K_3 (2-methyl-1,4-naphthoquinine, M-5625), vitamin D_2 (ergocalciferol, E-5750) and vitamin D_3 (calciferol, C-5750) were supplied by Sigma–Aldrich (Madrid, Spain). Standard solutions of 24,25-$(OH)_2$-vitamin D_3, 1,25-$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3 (Solvay Duphar, The Netherlands) were prepared by dissolving separately the contents of a vial of each in methanol. Other solutions were prepared by dilution in 50 mmol l^{-1} phosphate buffer adjusted to pH 6.5.

Blood samples

Plasma specimens were stored at 4 °C for a maximum of 10 d, then centrifuged at 6500 *g* for 5 min before analysis. Samples from both hospitalised patients and healthy volunteers were used.

Apparatus and instrumentation

A Vac Elut sps24 (Varian Iberica, Madrid, Spain) vacuum station connected to an Eyel4 A-3S (Tokyo, Japan) evaporator was used. A modular Hitachi (Tokyo, Japan) liquid chromatograph consisting of an L-6200A high-pressure ternary gradient pump, a Rheodyne (Cotati, CA, USA) Model 7125 high-pressure manual injection valve (the loop of which was replaced by a 100 cm × 0.25 mm id stainless-steel tube), an L-4250 UV/VIS spectrophotometric detector and a D-2500 integrator was used. A Gilson (Villiers-le Bel, France) Minipuls-2 low-pressure peristaltic pump, a Rheodyne Model 5041 low-pressure injection valve, a Rheodyne Model 5010 low-pressure selection valve, an Omnifit (Cambridge, UK) 50 mm long glass minicolumn and Teflon tubing of 0.5 mm id and different lengths were also used for constructing the flow injection (FI) manifold. An Ultrabase C_{18} column (250 × 4.6 mm id, 5.0 μm; Scharlau Science, Barcelona, Spain) was used as the analytical column.

Sample pre-treatment

In order to minimise the effect of related neutral lipids and other macromolecules present in the samples, the following liquid–liquid extraction procedure was used: 2 ml of plasma were first extracted by vortex mixing four times with 1 ml of propan-2-ol, centrifuged for 5 min, collected and evaporated under a flow of nitrogen at room temperature in order to reduce the volume to 1 ml. This solution was mixed four times with 2 ml of hexane, vortex mixed and centrifuged. The four hexane aliquots were mixed and evaporated to dryness under a flow of nitrogen at room temperature. After this treatment, the residue could be kept refrigerated at 4 °C for 2 weeks without appreciable degradation. The residue was dissolved in 7 ml of 10 mmol l^{-1} phosphate buffer (pH 6.5), and the solution was then ready for aspiration into the continuous clean-up–preconcentration and separation system.

Manifold and procedure

Fig. 1 shows the integrated system for continuous clean-up–preconcentration and HPLC separation. The clean-up–preconcentration subsystem permits the simultaneous clean-up of the sample and concentration of the target analytes prior to their injection into the separation subsystem. The aminopropylsilica preconcentration minicolumn inserted in the sample loop of a low-pressure injection valve was conditioned by sequential washing with methanol, 50 mmol l^{-1} phosphate buffer (pH 6.5) and water for 2 min. The pre-treated sample was then passed through the minicolumn (SV in the position for sample aspiration), and both the target analytes and interferents with similar features were retained. After a 20 min preconcentration time, the minicolumn was sequentially washed in 2 min cycles with 50 mmol l^{-1} phosphate buffer (pH 6.5) and methanol–water (70 + 30) in order to remove the interferents by changing manually the position of SV. The analytes were then eluted with a methanol solution by switching manually the low-pressure injection valve and driven to the injection valve of the chromatograph. This valve was modified by replacing the conventional loop with a 100 cm length of tubing of 0.25 mm id. The methanol containing the eluted analytes was trapped by switching the HPLC injection valve 95 s after switching the low-pressure injection valve and the analytes were thus introduced into the column. The gradient started simultaneously with injection and changed the initial mobile phase of acetonitrile–phosphate buffer (50 mmol l^{-1}, pH 6.5) (20 + 80) to propan-2-ol–methanol (10 + 90) in 1.7 min, then stabilised for 30 min. The analytes were removed from the column as a function of their relative polarity and monitored spectrophotometrically at 270 nm, followed by data treatment.

Results and discussion

A preliminary study was made to select the wavelength for monitoring the analytes. With this aim, the absorption spectra of the analytes were recorded from solutions containing similar concentrations of each analyte in methanol. The individual UV spectrum of each analyte revealed that vitamin A, K_1, K_3, D_2 and D_3 and the metabolites 24,25-$(OH)_2$-D_3, 1,25-$(OH)_2$-D_3 and 25-(OH)-D_3 exhibit absorption maxima within the range 250–290 nm. The maximum of vitamin A appears at longer wavelength (325 nm), but it also absorbs appreciably at 270 nm, so this value was selected as a compromise.

All the steps of the overall procedure were studied using the univariate method. Table 1 gives the variables, ranges studied and the optimum values found in each case.

Extraction step

In order to establish the optimum conditions for the extraction of the fat-soluble vitamins, different organic solvents (dichloromethane, hexane, propan-2-ol and propan-2-ol–hexane) were tried. Finally, an extraction procedure previously reported by the authors[17] was selected.

Clean-up–preconcentration step

The main variables affecting this step (sorbent nature, pH and washing and elution solutions) were checked. Sorbents such as silica, aminoalkyl, diol, cyanopropyl, phenyl, ethyl, octyl and

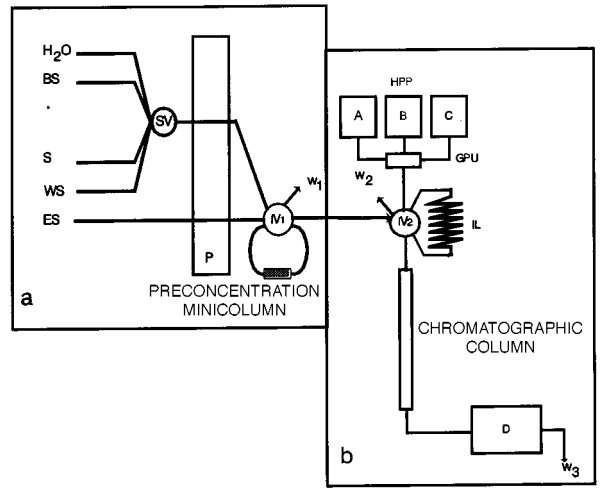

Fig. 1 Integrated continuous assembly for the determination of vitamin D_3 hydroxy metabolites. (a) Clean-up–preconcentration flow subsystem; (b) modular chromatograph for separation and UV detector. P, Peristaltic pump; SV, selection valve; BS, buffer; S, sample; WS, washing solution; ES, eluent solution; IV_1, low-pressure injection valve; HPP, high-pressure pump; A, B, C, solvent reservoirs; GPU, gradient programmable unit; IV_2, high-pressure injection valve; IL, injection loop; D, detector; and w, waste.

octadecyl types were tested. Finally, aminopropylsilica was selected as it provided the highest retention capacity. A pH of 6.5 yielded the best sorbent conditions for retention of the analytes. Binary and ternary mixtures of acetonitrile, methanol, propan-2-ol and water were tried for the selective removal of potential interferents retained in the minicolumn with minimal losses of the target analytes (washing step). Concentrations of methanol higher than 70% gave rise to partial elution of the target analytes with the interferents; when the methanol content was lower than 40% only partial removal of the interferents occurred. Methanol–water (70 + 30) was selected as the optimum. After the rinsing step, methanol effected quantitative elution of the vitamins from the aminopropylsilica support.

High flow rates decreased the residence time of a given sample plug in the packed minicolumn, thus reducing the sorbent–analyte contact time; low flow rates reduced the sampling frequency. A flow rate of 0.32 ml min^{-1} was used as a compromise between efficiency and sample throughput. After fixing the flow rate, a 2 cm minicolumn length proved to be sufficient for efficient retention of the analytes. Depending on the sample volume available, different preconcentration times were used, which obviously influenced the sensitivity of the method. The selected flow rate and a final sample volume of 7 ml (after conditioning), which required a preconcentration time of 20 min, were adopted as a compromise between sensitivity and sampling frequency.

Temperature was not an influential variable on the continuous clean-up and preconcentration procedure, which was thus developed at room temperature.

Individual separation–detection step

The interface for quantitative transfer of the analytes from the flow injection (FI) manifold to the chromatograph was a stainless steel tube of 0.25 mm id that connected the end of the preconcentration system and the injection valve of the chromatograph; 100 cm was the minimum length of the tube necessary for connection. In order to ensure trapping of the eluate containing the analytes in the loop of the HPLC valve, a stainless steel tube of 0.25 mm id and a volume of 100 μl replaced the conventional loop of the high-pressure injection valve. A delay time of 95 s between switching the preconcentration and high-pressure injection valves was the optimum for catching the whole volume of the eluate containing the analytes in the modified loop.

In order to obtain a good separation of the target analytes, the gradient and the composition of the mobile phase were optimised. The influence of a polarity modifier was studied using methanol, acetonitrile and propan-2-ol mixtures. A propan-2-ol–methanol mixture provided an excellent separation of the target analytes. Acetonitrile–phosphate buffer (50 mmol l^{-1}, pH 6.5) (20 + 80) was used as the initial mobile phase, and a linear gradient was programmed in order to obtain a propan-2-ol–methanol (10 + 90) composition of the mobile phase in 1.7 min. The system was finally stabilised with propan-2-ol–methanol (10 + 90) for 30 min. Under these conditions, the analytes were separated in 28 min. Fig. 2(a) shows the chromatogram obtained from a solution containing 1 μg ml^{-1} of each analyte.

Features of the method

After optimisation, two experiments were carried out to check the efficiency of the clean-up and preconcentration procedure by injecting solutions containing 100 ng ml^{-1} of each analyte both directly into the chromatograph and through the continuous clean-up–preconcentration system. Fig. 2(b) and (c) depict the chromatograms thus obtained. Table 2 summarises the chromatographic parameters [retention time (t_R), peak width at half-height ($W_{1/2}$), plate number (N), capacity factor (k'), separation factor (α)] and the preconcentration factor (ϕ) achieved in each case. The preconcentration factor was established as the ratio of the peak areas obtained using the solution as in Fig. 2(b) and (c). As can be seen, the retention capacity, expressed as the preconcentration factor, is in the range 55.2% [for 1,25-$(OH)_2$-D_3] to 8.1% (for vitamin K_3), depending on the nature of the analyte.

Calibration graphs were constructed using both the integrated FI–HPLC approach and the optimum values of the variables listed in Table 1. Standard solutions of the analytes were used in order to prepare solutions at concentrations between 0.001 and 200 ng ml^{-1} of each analyte, which were processed as required. Table 3 summarises the features of the overall continuous method (equations, regression coefficients, linear ranges and RSD values). As can be seen, the clean-up–preconcentration step endows the method with sufficient sensitivity for the

Table 1 Study of variables

Type	Variable	Range studied	Optimum value
Physical	Temperature/°C	20–50	25
Clean-up–preconcentration			
	Mode	—	Single
	Sorbent	—	NH_2
	Particle size/μm	15–63	63
	pH	4–12	6.5
	Washing solution:		
	Methanol (%)	40–70	70
	Water (%)	30–60	30
	Elution solution:		
	Methanol (%)	80–100	100
	Flow rate/ml min^{-1}	0.1–0.6	0.32
	Minicolumn length/cm	0.5–5.0	2
	Stainless steel tubing length/cm (0.25 mm id)	50–200	100
	Injection volume/μl	50–500	100
HPLC	Type of chromatography	—	Reversed-phase gradient
	Column	—	C_{18}, 5 μm, 250 × 4.6 mm id
	Flow rate/ml min^{-1}	0.5–2.0	1.2
	Initial mobile phase:		
	Acetone–phosphate buffer	20 + 80	—
	Final mobile phase:		
	Propan-2-ol–methanol	10 + 90	—
	Gradient time/min	1.7	—
	Stabilisation time/min	30	—

determination of vitamin D_2, D_3, 24,25-$(OH)_2$-D_3, 25-(OH)-D_3, K_1 and K_3 in human plasma, at their normal levels (even at concentrations much lower than the limits at which a deficiency of these vitamins can be considered, for most of them, as can be seen in Table 3). However, the sensitivity is insufficient for the quantification of 1,25-$(OH)_2$-vitamin D_3 (normal values in plasma < 60 pg ml^{-1}). In short, the method exhibits linear ranges between 0.005 and 100 ng ml^{-1} for vitamin D_2 and D_3 and the D_3 hydroxy metabolites, between 0.1 and 100 ng ml^{-1} for vitamin A, K_1 and K_3 and between 1 and 100 ng ml^{-1} for vitamin E, with excellent regression coefficients ($\geq$0.9901). As can be seen, the method is not appropriate for the quantification of vitamin A and E at the normal levels in plasma. However, it constitutes an acceptable tool for screening the main fat-soluble vitamins in human plasma at levels lower than the deficiency limit in this fluid.[18]

The precision of the method was studied by determining the repeatability using a pool of plasma spiked with standard solutions of each analyte at concentration levels within the linear range of the calibration graphs. Five solutions prepared at concentrations of 1 and 10 ng ml^{-1} of each analyte were processed in triplicate (n = 15). The repeatability of the method yielded acceptable RSD values in all instances (see Table 3). The sample throughput under the optimum working conditions for the overall method was 2 h^{-1}; this means that 18 individual analytes were determined per hour.

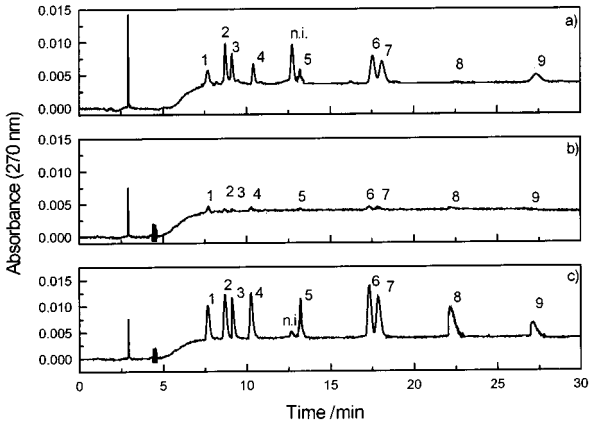

Fig. 2 Chromatograms obtained for (a) 1000 ng ml^{-1} of each analyte without a preconcentration step, (b) 100 ng ml^{-1} without a preconcentration step and (c) 100 ng ml^{-1} after a preconcentration step. Peaks: 1 = vitamin K_3; 2 = 24,25-$(OH)_2$-vitamin D_3; 3 = 1,25-$(OH)_2$-vitamin D_3; 4 = 25-(OH)-vitamin D_3; 5 = vitamin A; 6 = vitamin D_2; 7 = vitamin D_3; 8 = vitamin E; 9 = vitamin K_1; n.i. = not identified.

Table 2 Chromatographic and preconcentration parameters

Analyte	t_R/min	$W_{1/2}$	N	k'	α	ϕ
Vitamin K_3	7.65	0.47	1732.6	6.65	1.15	8.12
24,25-$(OH)_2$-vitamin D_3	8.67	0.45	2427.6	7.67	1.05	29.97
1,25-$(OH)_2$-vitamin D_3	9.10	0.34	4684.9	8.10	1.14	55.20
25-(OH)-vitamin D_3	10.23	0.45	3379.9	9.23	1.32	27.28
Vitamin A	13.19	0.32	11111.6	12.19	1.34	18.97
Vitamin D_2	17.30	0.47	8860.8	16.30	1.03	20.73
Vitamin D_3	17.86	0.62	5426.9	16.80	1.26	20.93
Vitamin E	22.17	0.69	6751.6	21.17	1.23	17.42
Vitamin K_1	27.07	0.69	10065.9	26.07	—	36.20

t_R denotes retention time; $W_{1/2}$, peak width at half-height; N, plate number; k', capacity factor; α, separation factor between consecutive peaks; and ϕ, preconcentration factor [calculated as the ratio of peak areas in chromatograms in Fig. 2(b) and (c)].

Application of the method

The proposed method was validated by applying it to the determination of the analytes in plasma in two ways: (a) determination of the analytes in 10 plasmas from a hospital and (b) study of the recovery afforded after addition of two standard solutions containing 1 and 10 ng ml^{-1} of each analyte in three plasma pools. Fig. 3 depicts the chromatogram from a plasma sample after continuous clean-up–preconcentration and HPLC separation–UV detection. Table 4 summarises the concentrations found and the recoveries achieved by the standard additions method. As can be seen, acceptable recoveries, between 78 and 109%, were obtained in all instances. Table 5 also gives the concentrations found and the standard deviations achieved in this study. As can be seen, the method provides

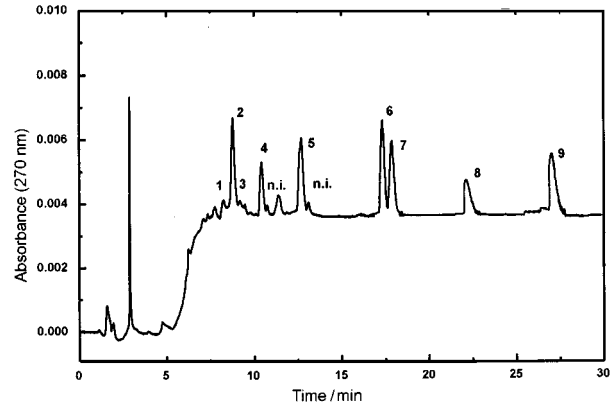

Fig. 3 Chromatogram from a plasma sample after continuous clean-up–preconcentration and HPLC separation–UV detection. Peaks: 1 = vitamin K_3; 2 = 24,25-$(OH)_2$-vitamin D_3; 3 = 1,25-$(OH)_2$-vitamin D_3; 4 = 25-(OH)-vitamin D_3; 5 = vitamin A; 6 = vitamin D_2; 7 = vitamin D_3; 8 = vitamin E; 9 = vitamin K_1; n.i. = not identified.

Table 3 Features of the methods

Analyte	Equation[a]	r^2	Linear range/ ng ml^{-1}	Deficiency limit[18]/ ng ml^{-1}	RSD (%)[b]	
					Low level	High level
Vitamin K_3	$y = 1.56 \times 10^{-4} + 1.19 \times 10^{-5} x$	0.9925	0.1–100	0.4	6.4	4.8
24,25-$(OH)_2$-vitamin D_3	$y = -3.16 \times 10^{5} + 1.56 \times 10^{-5} x$	0.9919	0.005–100	15	6.6	5.2
1,25-$(OH)_2$-vitamin D_3	$y = 9.34 \times 10^{-6} + 9.08 \times 10^{-6} x$	0.9911	0.005–100	0.001	8.5	5.4
25-(OH)-vitamin D_3	$y = 1.68 \times 10^{-6} + 1.53 \times 10^{-5} x$	0.9907	0.005–100	8	6.8	3.8
Vitamin A	$y = 1.48 \times 10^{-6} + 0.79 x$	0.9999	0.1–100	100	7.6	8.7
Vitamin D_2	$y = -4.24 \times 10^{-6} + 1.37 x$	0.9995	0.005–100	1	4.5	3.6
Vitamin D_3	$y = -4.55 \times 10^{-5} + 1.99 x$	0.9919	0.005–100	1	5.3	4.9
Vitamin E	$y = -2.04 \times 10^{-5} + 1.34 x$	0.9902	1–100	2000	8.7	7.4
Vitamin K_1	$y = -2.66 \times 10^{-5} + 9.99 \times 10^{-6} x$	0.9901	0.1–100	0.24	4.8	4.1

[a] y denotes area in arbitrary units and x concentration in ng ml^{-1}. [b] For 1 and 10 ng ml^{-1} as low and high level, respectively.

Table 4 Application of the method

Sample No.	Parameter	Vitamin							
		K_3	24,25-$(OH)_2$-D_3	1,25-$(OH)_2$-D_3	25-(OH)-D_3	A	D_2	D_3	E	K_1
1	Concentration found ng ml^{-1}	5.6	15.0	—[b]	12.8	47.0	0.56	0.33	73.2	0.26
	1st addition:[a] recovery (%)	95	105	80	92	104	95	88	87	100
	2nd addition:[a] recovery (%)	97	101	92	88	100	101	100	100	98
2	Concentration found/ng ml^{-1}	35.8	12.1	—[b]	22.6	26.5	0.63	0.28	16.4	0.16
	1st addition:[a] recovery (%)	85	90	89	95	98	96	97	90	82
	2nd addition:[a] recovery (%)	82	91	78	99	99	99	90	97	97
3	Concentration found/ng ml^{-1}	13.2	9.5	—[b]	13.8	72.1	0.40	0.14	14.3	0.33
	1st addition:[a] recovery (%)	105	101	87	95	100	99	99	100	97
	2nd addition:[a] recovery (%)	109	95	89	101	98	93	87	98	100

[a] Additions of 1 and 10 ng ml^{-1}. [b] Lower than the quantification limit.

Table 5 Determination of fat-soluble vitamin in plasma

Sample No.	Vitamin[a]								
	K_3	24,25-$(OH)_2$-D_3	1,25-$(OH)_2$-D_3[b]	25-(OH)-D_3	A	D_2	D_3	E	K_1
1	5.60 ± 0.36	15.00 ± 0.99	—[c]	12.80 ± 2.91	47.00 ± 3.57	0.56 ± 0.03	0.33 ± 0.02	73.20 ± 6.37	0.26 ± 0.01
2	35.38 ± 2.26	12.10 ± 0.80	—	22.70 ± 5.62	26.30 ± 2.00	0.63 ± 0.03	0.28 ± 0.01	16.40 ± 1.43	0.16 ± 0.02
3	13.20 ± 0.84	9.50 ± 0.63	—	13.80 ± 0.94	72.10 ± 5.48	0.40 ± 0.02	0.14 ± 0.02	14.30 ± 1.24	0.33 ± 0.02
4	20.20 ± 0.13	5.16 ± 0.34	—	5.78 ± 1.75	19.84 ± 1.51	0.18 ± 0.01	0.23 ± 0.03	10.70 ± 0.93	0.14 ± 0.02
5	40.26 ± 2.58	16.27 ± 1.07	—	4.19 ± 1.64	8.78 ± 0.67	—	0.33 ± 0.02	115.00 ± 8.5	0.18 ± 0.07
6	22.58 ± 1.45	9.61 ± 0.63	71.80 ± 1.00	2.93 ± 2.24	36.61 ± 2.78	0.11 ± 0.01	0.24 ± 0.01	87.70 ± 7.63	0.12 ± 0.01
7	22.64 ± 1.46	10.96 ± 0.72	83.87 ± 2.03	3.45 ± 2.07	19.60 ± 1.49	0.10 ± 0.06	0.28 ± 0.01	65.30 ± 5.65	0.35 ± 0.02
8	23.69 ± 1.52	20.77 ± 1.37	—	10.85 ± 2.1	58.61 ± 4.45	0.12 ± 0.02	0.24 ± 0.05	10.90 ± 0.95	0.18 ± 0.03
9	26.54 ± 1.70	5.12 ± 0.34	62.43 ± 1.91	6.51 ± 4.25	11.61 ± 0.88	0.13 ± 0.03	0.23 ± 0.04	85.90 ± 7.47	0.31 ± 0.03
10	40.34 ± 2.58	5.44 ± 0.36	—	4.71 ± 2.90	18.65 ± 1.42	0.74 ± 0.03	0.32 ± 0.03	105.60 ± 9.19	0.14 ± 0.01

[a] Mean concentrations ± standard deviation in ng ml^{-1}. [b] Concentration in pg ml^{-1}. [c] —, Lower than the quantification limit.

sufficient information for the quantification of fat-soluble vitamins in human plasma, in particular vitamin D_2, D_3, 24,25-$(OH)_2$-D_3, 25-(OH)-D_3, K_1 and K_3.

Conclusions

A continuous method for the clean-up and preconcentration of fat-soluble vitamins in plasma using a continuous clean-up–preconcentration procedure coupled on-line with HPLC separation and UV detection has been developed. The method shows the following advantages in comparison with other methods proposed so far:

(i) Shorter manual sample treatment and conditioning prior to the continuous clean-up–preconcentration step[17] in comparison with other previous methods.[19,20] Continuous clean-up and preconcentration of the target analytes.

(ii) Miniaturisation of the clean-up step based on continuous solid-phase extraction, which reduces the sorbent consumption as the minicolumn can be re-used at least 200 times without a decrease in capacity and/or deterioration.

(iii) Preconcentration of the target analytes, which makes possible the determination of vitamin D_2, D_3, 24,25-$(OH)_2$-D_3, 25-(OH)-D_3, K_1 and K_3 in human plasma samples at the usual concentrations in healthy people and also at lower levels. The quantification of 1,25-$(OH)_2$-vitamin D_3 at levels higher than usual in plasma is also feasible. The method provides a useful tool for screening fat-soluble vitamins at levels much lower than the limits at which a deficiency of these vitamins can be considered. The method could also be extended to the determination of vitamin A and E at the normal levels in human samples using a diode array detector, monitoring at the maximum absorption of each analyte.

(iv) Easy adaptation to commercial devices, which facilitates automation *via* coupling to chromatographs, low- and high-pressure valves, *etc.*

(v) Reduction of derivatisation costs compared with other methods based on mass spectrometry and radioimmunoassay.

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