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HPLC determination of Vitamin D_3 and its metabolite in human plasma with on-line sample cleanup

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Abstract

A HPLC method with automated column switching and UV-diode array detection is described for the simultaneous determination of Vitamin D_3 and 25-hydroxyvitamin D_3 (25-OH-D_3) in a sample of human plasma. The system uses a BioTrap precolumn for the on-line sample cleanup. A sample of 1 ml of human plasma was treated with 2 ml of a mixture of ethanol–acetonitrile (2:1 (v/v)). Following centrifugation, the supernatant was evaporated to dryness under a stream of dry and pure nitrogen. The residue was reconstituted in 250 μL of a solution of methanol 5 mmol l^{-1} phosphate buffer, pH 6.5 (4:1 (v/v)), and a 200 μl aliquot of this solution was injected onto the BioTrap precolumn. After washing during 5 min with a mobile phase constituted by a solution of 6% acetonitrile in 5 mmol l^{-1} phosphate buffer, pH 6.5 (extraction mobile phase), the retained analytes were then transferred to the analytical column in the backflush mode. The analytical separation was then performed by reverse-phase chromatography in the gradient elution mode with the solvents A and B (Solvent A: acetonitrile–phosphate buffer 5 mmol l^{-1}, pH 6.5; 20:80 (v/v); solvent B: methanol–acetonitrile–tetrahydrofuran, 65:20:15 (v/v)). The compounds of interest were detected at 265 nm. The method was linear in the range 3.0–32.0 ng ml^{-1} with a limit of quantification of 3.0 ng ml^{-1}. Quantitative recoveries from spiked plasma samples were between 91.0 and 98.0%. In all cases, the coefficient of variation (CV) of the intra-day and inter-day-assay precision was ≤2.80%. The proposed method permitted the simultaneous determination of Vitamin D_3 and 25-OH-D_3 in 16 min, with an adequate precision and sensitivity. However, the overlap of the sample cleanup step with the analysis increases the sampling frequency to five samples h^{-1}. The method was successfully applied for the determination of Vitamin D_3 and 25-OH-D_3 in plasma from 46 female volunteers, ranging from 50 to 94 years old. Vitamin D_3 and 25-OH-D_3 concentrations in plasma were found from 4.30–40.70 ng ml^{-1} (19.74 ± 9.48 ng ml^{-1}) and 3.1–36.52 ng ml^{-1} (7.13 ± 7.80 ng ml^{-1}), respectively. These results were in good agreement with data published by other authors.

Keywords: Vitamin D_3; 25-OH-D_3; Plasma samples; HPLC; Solid-phase extraction; Column switching; On-line sample cleanup

1. Introduction

Humans derive their Vitamin D (both Vitamins D_3 and D_2) from two sources. Vitamin D_3 is biosynthesized in the skin during exposure to UV light, whereas Vitamin D_2 is absorbed from the diet. The amount of skin biosynthesis per day is affected by many factors, such as latitude, sun (UV) exposure, clothing, season of the year, aging and skin melanin pigmentation [1–3]. Vitamin D_3 is hydroxylated in the liver to 25-hydroxyvitamin D_3 (25-OH-D_3), which is further metabolized in the kidney either to 1α, 25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-D_3] or to other compounds. Although about 40 metabolites have been determined, 25-OH-D_3 is the major circulating metabolite and its levels together with Vitamin D_3 levels are an indicator of Vitamin D status in humans [4,5]. The determination of Vitamin D_3 and its metabolites is a difficult challenge. There are many reasons for this consideration; the low level at which the metabolites circulate in plasma, the number of different metabolites and their similarity to one another, and finally the instability of their chemical structures in the presence of

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UV light and heat. Various methods for the determination of Vitamin D_3 and its metabolites in human fluids have been reported and reviewed in the literature [6–8]. These methods are based on saturation analysis (protein binding assays) [9–14], gas–liquid chromatography [15–17] and high performance liquid chromatography with several detection systems, photometric [18–27], electrochemical [28,29] and fluorimetric [30]. Generally, all these methods include a purification step prior to their determination, based on either liquid–liquid extraction [18–21] or solid-phase extraction [24–26], which are both slow and time-consuming operations. However, the use of automated solid-phase extraction procedures is an alternative that minimizes the amount of time spent in the sample preparation step. In an effort to reduce the time required for the sample preparation step, recently, Ortiz-Boyer et al. [31–35] have improved a clean up batch procedure in order to incorporate it into a continuous flow injection manifold for cleanup and concentration of hydroxyvitamin D_3 metabolites prior to their separation by HPLC and UV-detection.

As an alternative to these methodologies, the present work describes a simple, reproducible and accurate column-switching high performance reversed-phase liquid chromatographic method for the simultaneous determination of 25-OH-D_3 and Vitamin D_3 in human blood plasma samples. The method is less time-consuming with lower costs, in comparison with other methods proposed so far.

2. Experimental

2.1. Reagents and standards

All solvents and chemicals used were HPLC or analytical reagent grade. Acetonitrile, methanol, ethanol, isopropanol, potassium dihydrogen phosphate and potassium hydrogen phosphate were purchased from J. T. Baker (Phillipsburg, NJ, USA). Tetrahydrofuran was furnished from OmniSolv (NJ, USA). High purity water was obtained through a Millipore Milli-Q system (Bedford, MA, USA). Vitamins A, E, K_2, D_3 (cholecalciferol) and 25-hydroxyvitamin D_3 were supplied from Sigma (St. Louis, MO, USA).

Individual stock standard solutions of Vitamin A (238 $\mu g\,ml^{-1}$), Vitamin E (686 $\mu g\,ml^{-1}$), Vitamin K (214 $\mu g\,ml^{-1}$), Vitamin D_3 (250 $\mu g\,ml^{-1}$) and 25-OH-D_3 (500 $\mu g\,ml^{-1}$) were prepared in methanol and stored at −20 °C. Working solutions were prepared every week by an appropriate dilution of concentrated stock standard solutions in methanol.

2.2. Blood samples

For the development of this work, a pool of 200 ml of normal human plasma from healthy adult volunteers was used. In addition, 46 blood samples from healthy women, ranging from 50 to 94 years of age were analyzed using the proposed method in order to establish normal ranges for Vitamin D_3 and 25-OH-D_3. In all of the cases, an informed consent was obtained from each person. Blood samples (10 ml) were drawn from a forearm vein into heparinized polyethylene tubes. All tubes were protected from light and centrifuged at 3000 rpm for 10 min. Plasma was then separated and analyzed immediately or stored at −20 °C until analyzed.

2.3. Sample pretreatment

The effect of analytes protein-bound in the samples was reduced using a previous extraction procedure with organic solvents, which, summarizing is as follows: 1 ml of human plasma was transferred into glass centrifuge tube and 2 ml of ethanol–acetonitrile (2:1 (v/v)) were added. After vortex-mixing for 2 min, the solution was centrifuged at 3000 × *g* for 10 min. The supernatant was separated and evaporated to dryness under a stream of dry pure nitrogen. The residue was reconstituted in 250 μl methanol–5 mmol l^{-1} phosphate buffer, pH 6.5 (4:1 (v/v)), and a 200 μl aliquot of this solution was then ready to be injected into column-switching HPLC system.

2.4. Instrumentation

The experiments were performed using a liquid chromatographic system equipped with a Waters Alliance 2690 HPLC system (Milford, MA, USA), connected to a Waters 996 photodiode array (PDA) detector, and then to a Venturis Fx 51665 control station with a MILLENIUM^{32} software for instrument control and to collect and process the data. Detection was carried out at 265 nm. Injections were made with a Rheodyne type 7125 six-port valve equipped with 200 μl loop. For the column-switching purposes, a column switching six-port valve (Waters) controlled by the workstation was used, along with an additional Knauer 64 pump (Berlin, Germany) to deliver the extraction mobile phase.

2.5. Chromatographic conditions

The extraction precolumn was a BioTrap 500 C_{18} column (20 mm × 4 mm i.d., Chromtech Ltd., UK) and the extraction mobile phase consisted of 6% acetonitrile in 5 mmol l^{-1} phosphate buffer, pH 6.5, being pomped at a flow rate of 0.8 ml min^{-1}. A C_{18} column (25 cm × 4.6 mm i.d.; 5 μm; Jones Chromatography) was used as analytical column to provide further separation of analytes from other endogenous plasma components before detection. Two solvents (A): acetonitrile–phosphate buffer 5 mmol l^{-1}, pH 6.5 (20:80 (v/v)) and (B): methanol–acetonitrile-tetrahydrofuran (65:20:15 (v/v)) were used in gradient elution mode as the mobile phase for the transfer and separation of analytes. Before used, the mobile phases were vacuum filtered through a 0.45 μm nylon filter and degassed. All the chromato-

graphic experiments were carried out at room temperature (22 ± 2 °C).

2.6. Analytical procedure

The coupled-column analysis can be subdivided into three different steps as follows:

2.6.1. Sample loading and processing

With the switching valve in load position, a volume of 200 μl of treated human plasma sample (see Section 2.3) was injected onto the extraction precolumn using the extraction mobile phase. While the endogenous compounds were flushed to waste, the analytes were retained by the hydrophobic inner surface of BioTrap precolumn. During the sample cleanup step (5 min), the analytical column was equilibrated with the analytical mobile phase.

2.6.2. Analytes transfer

After 5 min, the switching valve was switched to the inject position and the precolumn and analytical column were series-connected. Thus, the retained analytes were swept by the analytical mobile phase from the BioTrap precolumn to the top of the analytical column in a backflush mode during 4 min.

2.6.3. Analytes separation

Four minutes later, the switching valve was changed back to the load position to recondition the BioTrap precolumn with the extraction mobile phase to be ready for the next injection. In the meantime, the analytes were separated in the analytical column under gradient mode elution and detected by UV-DAD at 265 nm.

3. Results and discussion

3.1. Optimization of the extraction and cleanup step

The on-line sample extraction was performed with a BioTrap 500 C_{18} precolumn. This precolumn was designed to permit continuous direct injections and extraction of plasma and other biological matrices. BioTrap 500 C_{18} is a silica-based extraction column with a biocompatible external surface and hydrophobic internal surface (C_{18} groups). The biocompatibility has been obtained by attachment of the plasma protein α_1-acid glycoprotein (AGP) on the external surface of the particles. The pore sizes of the particles have been chosen so that plasma proteins and other large molecules will be excluded from the pores. The separations of analytes from proteins are based on a combination of size exclusion and reverse-phase partitioning. Small analytes molecules enter the pores and are retained by the C_{18} groups on the inner phase. The largest protein molecules are washed away with the continuously flowing extraction mobile phase [36]. To determine the elution profile of the sample matrix and analytes, the BioTrap extraction precolumn was first directly coupled to the UV diode-array detector set at 265 nm and the composition and flow-rate of the extraction mobile phase were studied. In this way, it has been found that an extraction mobile phase consisting of an aqueous buffer is favorable to remove serum proteins using a pH which is close to the physiological pH (7.0). On the other hand, an organic solvent should be added to the extraction mobile phase in order to improve the washing of the extraction column and to displace the analytes from the plasma protein binding. Thus, different composition of organic solvents such as acetonitrile, methanol and isopropanol with phosphate buffer solutions were assayed. As a result, low recoveries of Vitamin D_3 and 25(OH)D_3 were obtained probably due to the high protein-binding with the analytes. In order to overcome this effect and to obtain best recoveries of analytes in the solid-phase extraction step, a previous extraction procedure with organic solvents was assayed. In this way, to study the recovery efficiency several solvents were tried, such as methanol, ethanol, acetonitrile and acetone. Table 1 shows that the best results were obtained with an ethanol–acetonitrile (2:1 (v/v)) at a 2:1 (v/v) ratio of organic solvent to plasma. Finally, the liquid–liquid extraction procedure reported in Section 2.3 was selected prior to the sample cleanup in the column-switching system.

3.2. Retention behavior on the analytical column

A high selectivity is required to separate the analytes from endogenous compounds in particular some other fat-soluble vitamins. Methanol, acetonitrile, tetrahydrofuran and buffer phosphate were used as the basis for the different mobile phases preparation. Various proportions of these solvents were tested. However, separation was achieved using the following binary lineal gradient: from 70 to 95% of B within 3 min, then to 100% B in 1 min and held for 8 min to 100% B. After completion of the chromatographic run, the pump was programmed to regain its initial conditions within 2 min

Table 1
Recoveries obtained from the batch extraction procedure with organic solvents

Solvent	Concentration added (ng ml^{-1})	25-OH-D_3 (%)	Vitamin D_3 (%)
Methanol	15.0	67.1	39.3
	30.0	64.6	37.5
Acetonitrile	15.0	97.7	42.9
	30.0	98,7	42.7
Ethanol	15.0	77.0	45.0
	30.0	78.3	43.1
Acetone	15.0	90.0	35.2
	30.0	92.4	36.3
Ethanol:acetonitrile (2:1 (v/v))	15.0	94.7	93.5
	30.0	96.3	97.1

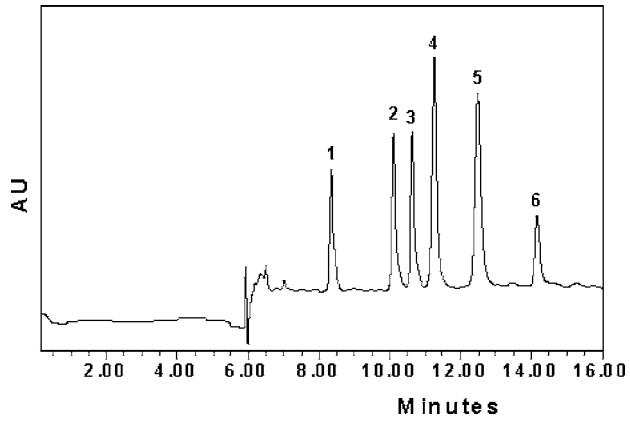

Fig. 1. Chromatogram obtained from a standard solution of the fat-soluble vitamins and Vitamin D_3 metabolites (optimized conditions: see text). (1) 1,25-$(OH)_2D_3$, retention time (t_r) 8.40 min; (2) 25-OH-D_3, t_r: 10.25 min; (3) Vitamin A, t_r: 10.60 min; (4) Vitamin K, t_r: 11.20 min; (5) Vitamin D_3, t_r: 12.60 min and (6) Vitamin E, t_r: 14.30 min.

and 2 more minutes as reconditioning time. The optimum flow rate for the mobile phase was found to be 1.4 ml min^{-1}, resulting in a maximum run time of 16 min. Fig. 1 shows the chromatogram obtained under optimized conditions for a standard solution of the fat-soluble vitamins. We can see an adequate resolution within the analytes in an acceptable analysis time.

3.3. Switching times

Table 2 summarized the optimized switching times for the proposed column-switching method. As it can be seen, the equilibration of both the BioTrap extraction precolumn and the analytical column after analysis can be achieved within a few minutes, thus, the total analysis time is ca. 16 min. However, the overlap of sample preparation, analysis and reconditioning of the precolumn increases the overall sample throughput to one injection every 12 min.

Representative chromatograms obtained in the coupled-column system under the proposed conditions (see Table 2) for a standard solution of 25$(OH)D_3$ and Vitamin D_3 (A), a blank of human plasma (B) and a spiked human plasma sample with both analytes at 15 ng ml^{-1} (C) are shown in Fig. 2(A–C). By comparing these figures, it can be concluded that there are not interfering endogenous compounds at the retention time of 25$(OH)D_3$ and Vitamin D_3. On the other hand, the diode array UV-spectra taken from each peak at the retention time of 10.25 and 12.60 min confirmed the solely presence of 25$(OH)D_3$ and Vitamin D_3, respectively.

3.4. Features of the method

To study the accuracy of the method, recoveries of analytes from pooled human plasma samples were estimated. A blank of human pooled plasma and several human plasma samples spiked with known amounts of 25$(OH)D_3$ and Vitamin D_3 were subject to the entire analytical sequence. Each analyte was spiked at three different concentrations. The results are summarized in Table 3. Recovery values for all cases were obtained superior to 91.2%. These values are quantitative and demonstrate the extraction efficiency of the BioTrap precolumn and in the batch extraction procedure.

Table 2
Optimized conditions of the on-line sample extraction, cleanup and HPLC separation steps

Step	Component system	Condition
Sample injection ($t = 0$ min)	Rheodyne 7125 valve: inject position Switching valve: load position	Injection volume: 200 μl
Sample extraction ($t = 5$ min)	Precolumn: BioTrap 500 C_{18} 20 mm × 4 mm	Extraction mobile phase: 6% de acetonitrile in 5 mmol l^{-1} phosphate buffer, pH 6.5 Flow rate: 0.8 ml min^{-1}
Transfer of analytes ($t = 9$ min)	Switching valve: inject position	Analytical mobile phase: solvent A: 5 mM phosphate buffer pH 6.5–acetonitrile (80:20 (v/v)) Solvent B: methanol–acetonitrile–tetrahydrofuran (65:20:15 (v/v)) Flow rate: 1.4 ml min^{-1}
Analytes separation ($t = 17$ min)	Switching valve: load position	Reversed-phase gradient: 70–95% of B within 3 min, then to 100% B in 1 min and held for 8 min to 100% B
Next injection ($t = 12$ min)	Analytical column: Spherisorb C_{18} 250 mm × 4.6 mm, dp: 5 μm	Flow rate: 1.4 ml min^{-1} Detection UV: 265 nm

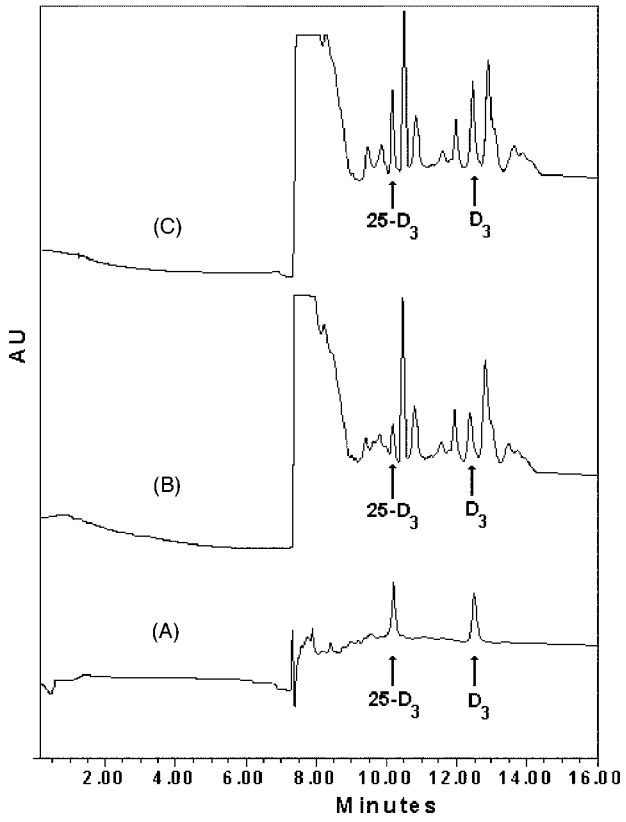

Fig. 2. Typical chromatograms obtained after injection of 200 μl of: (A) standard solution of 25-OH-D_3 and Vitamin D_3 at 15 ng ml^{-1}; (B) human blood plasma blank; and (C) human blood plasma spiked with 15 ng ml^{-1} of 25-OH-D_3 and Vitamin D_3. Optimized experimental conditions given: in Table 2. t_r 25-OH-D_3: 10.25 min and t_r Vitamin D_3: 12.60 min.

Calibration curves over the range from 3.0 to 32.0 ng ml^{-1} were constructed for aqueous standards and for human plasma samples spiked with both analytes. The curves were prepared using six concentrations of each compound and a blank and injected in triplicate into the column-switching HPLC system. Table 4 shows the regression equations of the peak area as a function of 25(OH)D_3 and Vitamin D_3 concentration obtained from aqueous standard and from spiked human plasma samples. For all regression lines, the correlation coefficients (r) are larger than 0.9943. On the other hand, the slope of the calibration using aqueous standard solutions were not statically different ($P < 0.05$) from those for 25(OH)D_3 and Vitamin D_3 additions to real plasma samples, hence, the standard calibration technique with aqueous standards could be used for the determination of 25(OH)D_3 and Vitamin D_3 in the human plasma samples investigated in this work.

Table 3
Recoveries of 25-OH-D_3 and Vitamin D_3 from spiked human plasma

Compound	Added (ng ml^{-1})	Found (ng ml^{-1})	Recovery (%)	CV (%) ($N = 5$)
25-OH-D_3	8.0	7.3	91.3	3.00
	16.0	14.6	91.2	1.08
	32.0	31.2	97.5	1.87
Vitamin D_3	8.0	7.4	92.5	3.83
	16.0	15.6	97.5	2.95
	32.0	30.0	93.8	1.30

CV: coefficients of variation ($n = 5$).

Table 4
Linear correlations between peak areas and concentrations of 25-OH-D_3 and Vitamin D_3

Compound	Matrix	Equation[a]	r[b]	CV slope[c] (%)
25-OH-D_3	Aqueous standard	$A = 265188.96C$	0.9966	1.50
	Plasma	$A = 260421.56C$	0.9943	3.00
Vitamin D_3	Aqueous standard	$A = 293400.38C$	0.9990	3.00
	Plasma	$A = 291996.95C$	0.9992	3.40

[a] A: peak area; C: concentration of each compound.
[b] r: correlation coefficient.
[c] CV: coefficients of variation of the slope ($n = 3$).

The lowest concentration that can be quantified with acceptable accuracy and precision for all analytes was 3.0 ng ml^{-1}. Furthermore, the limit of detection for both analytes defined as signal-to-noise (S/N) ratio of 3:1 was 0.5 ng ml^{-1}. As it can be seen, the method is sufficient sensitivity for the determination of Vitamin D_3 (normal values in plasma, 0.5–25.0 ng ml^{-1}) and 25-OH-D_3 (normal values in plasma, 10.0–50.0 ng ml^{-1}) in human plasma, at their normal levels [1,6].

The intra-day-assay precision of the method was examined by replicate analysis ($n = 5$) of aqueous standards and human plasma spiked with known amounts of analytes. The precision expressed by coefficient of variation (CV) is given in Table 5. The CV was <2.80% for all tested concentrations, showing that the method provided good reproducibility. The inter-day-assay precision was also determined by assaying aqueous standards and spiked plasma samples on five different days. The CV did not exceed 2.75% in all concentrations, demonstrating the good stability and repeatability of this described system.

Table 5
Intra- and inter-assay validation

Compound	Matrix	Concentration (ng ml^{-1})	Intra-assay (CV, %)[a]	Inter-assay (CV, %)[a]
25-(OH)-D_3	Aqueous standard	30.0	1.63	1.93
		60.0	1.55	1.78
		130.0	1.59	2.10
	Plasma	7.0	2.00	1.97
		15.0	1.85	2.54
		30.0	2.80	2.64
Vitamin D_3	Aqueous standard	30.0	0.95	1.37
		60.0	1.52	1.73
		130.0	1.87	1.55
	Plasma	7.0	2.30	3.15
		15.0	1.47	2.75
		30.0	1.96	2.43

[a] CV: coefficients of variation ($n = 5$).

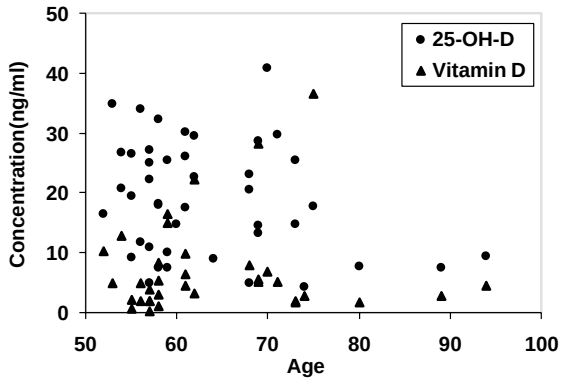

Fig. 3. Individual human plasma concentrations of 25-OH-D_3 and Vitamin D_3 related to age.

3.5. Application and endogenous concentration in human plasma

To test the clinical applicability of the proposed method further, we examined plasma samples from 46 healthy female volunteers ranging from 50 to 94 years of age. Vitamin D_3 and 25-OH-D_3 concentrations in plasma were found from 4.30 to 40.70 ng ml^{-1} (mean ± S.D.; 19.74 ± 9.48 ng ml^{-1}) and from 3.1 to 36.52 ng ml^{-1} (7.13 ± 7.80 ng ml^{-1}), respectively. These values are in good agreement with those previously published by other authors [37–40]. Fig. 3 shows the individual plasma concentrations of both analytes related to age. As it can be seen, the concentrations of Vitamin D_3 and 25-OH-D_3 in the plasma of normal subjects show wide variation. This could be explained by differences in ageing, exposure to sunlight and in dietary Vitamin D_3 intake. On the other hand, we observed that in all cases Vitamin D_3 concentrations were lower than the concentrations of 25-OH-D_3. This behavior was expected if one considers that once formed, Vitamin D_3 is metabolized in the liver to 25-OH-D_3. In addition, the results showed that plasma 25-OH-D_3 concentrations tend to fall with age, it has been shown that this is due to an age-related decline in the production of 7-dehydrocholesterol and Vitamin D_3 in the skin [41,42]. Other variables that might affect plasma 25-OH-D_3 concentrations, such as transport of cholecalciferol to the liver and its subsequent conversion to 25-OH-D_3 and rate of conversion to 1,25-$(OH)_2$-D_3 or 24,25-$(OH)_2$-D_3, could also affect blood concentrations [41].

4. Conclusions

The present method represents a great simplification of the determination of circulating concentrations of 25-OH-D_3 and Vitamin D_3 in a column-switching HPLC system with UV-diode array detection. The pretreatment of plasma samples by ethanol–acetonitrile (2:1 (v/v)) was very simple and fast to perform and gave good and reproducible recoveries. The use of on-line solid-phase extraction to perform sample cleanup has the potential to increase sample throughput to five samples h^{-1}. The method has adequate sensitivity to measure the analytes in 1.0 ml of normal human plasma. The main advantage of this procedure over previous published ones is the possibility of performing sample cleanup and determination in a completely automatized system. On the other hand, it is important to emphasize that the BioTrap precolumn was found to have a long life span, although during the method development and application, about 50 ml of treated human blood plasma were injected the pressure was not raised in this study. This indicates that the efficiency of the clean-up process was satisfactory.

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