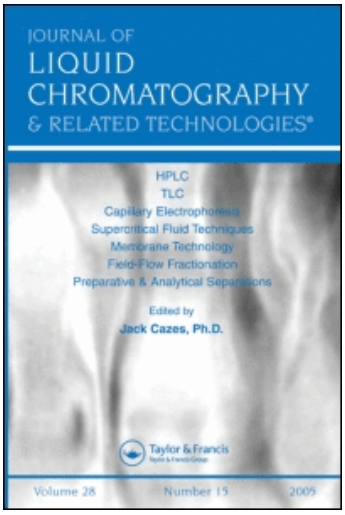

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IMPROVED PROCEDURE FOR THE DUAL CARTRIDGE CLEANUP OF HYDROXYVITAMIN D_3 METABOLITES IN PLASMA

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ABSTRACT

A modified cleanup procedure of 25-(OH)-vitamin D_3 from a previous method based on the use of C_{18} and Si cartridges is proposed. The monitoring of the optimisation study was carried out by individual separation of the target analytes by HPLC and UV-detection. The improvement of the original procedure allows a dramatic reduction of plasma especies, which results in a more clean chromatogram in which other two hydroxymetabolites of vitamin D_3 (namely, 24-R,25-$(OH)_2$-vitamin D_3 and 1-I,25-$(OH)_2$-vitamin D_3) can be determined. A closer to quantitativeness recovery of the target analytes (at least 4 times higher than in the original method) is achieved.

INTRODUCTION

The knowledge of vitamin D metabolism and actions have undergone a fast growth in the last two decades. New actions not directly related to calcium homeostasis have been reported.[1] Calcitriol and its analogues with low calcemic actions are used both in metabolic bone diseases (osteoporosis and asteomalacia) and in oncology and dermatology on the basis of its actions on differentiation, proliferation, and also as immunomodulator.

These new prospects in biological actions and therapeutic use of calcitriol and its analogues, together with the low concentration levels of these metabolites in human fluids in the presence of other species with similar structures and properties, call for the development of more sensitive and selective methods for their determination.

The methods available at present for measurement of vitamin D_3 and its metabolites in human fluids, reviewed by Makin et al.[2,3] and more recently by Shimada et al.,[4] are based on protein saturation,[5-7] radioimmunoassay,[8] mass-spectrometry,[9] and high performance liquid chromatography with photometric,[10,11] fluorimetric,[12,13] radiochemical,[14-16] or mass-spectrometry[17,18] detection.

It is remarkable that thermal cyclation of these metabolites by high temperature makes non available their direct determination by gas-chromatography;[3] nevertheless, GC-MS method have recently been reported.[19]

The methods for the determination of these species described in the literature unfaingly include a purification step prior to their determination in biological fluids. Usually, these steps are based on either solid-phase extraction[20-24] or liquid-liquid extraction,[20,25] which are slow, time-consuming operations which provide results far from satisfactory.

Solid-phase extraction procedures are usually based on either a single cleanup step with reverse-phase C_{18} cartridges[23] or a dual step using both reverse-phase and normal-phase silica[19] or aminoalkyl[25] cartridges.

The aims of this work were as follows: a) to accomplish a more effective cleanup of biological samples than that previously reported for 25-OH-vitamin D_3;[19] b) to make the clean extract useful for the determination of other vitamin D_3 metabolites; c) to minimise losses of the target analytes by a more selective removal of the interferents. With this purpose HPLC-UVdetection at 270 nm was used in order to monitor the optimisation study.

MATERIALS

Reagents

All solutions were prepared using bidistilled water (Millipore Milli-Q-System). Bond-Elut cartridges of C_{18} octadecyl 500 mg, 2.8 mL (n. 01210-2028) and Si silica 500 mg, 2.8 mL (n. 01210-2037) from Varian SPP were used. All organic solvents were HPLC grade. A mixture of 50:0:50 acetonitrile:methanol:phosphate buffer (10 mmol L^{-1}, pH 5.0) was used as initial mobile phase. Linear gradients were programmed in order to obtain a 0:100:0 ratio in 3 min, then stabilized for 9 min.

Standard solutions of 24-R,25-$(OH)_2$-vitamin D_3, 1-α,25-$(OH)_2$-vitamin D_3, and 25-(OH)-vitamin D_3 were prepared by dissolving, separately, the content of a vial of each (Solvay Duphar B.V., The Netherlands) in methanol. Other solutions were prepared by dilution in 10 mmol L^{-1} phosphate buffer adjusted to pH 7.0.

Plasma samples of normal and sick individuals from a local hospital were used.

Apparatus and Instruments

A Vac elut sps24 vacuum station incorporated to an Eyel4 A-3S evaporator was used. A modular Hitachi liquid chromatograph consisting of an L-6200A high pressure ternary gradient pump, a Rheodyne 7125 manual injection valve, an L-4250 UV-vis spectrophotometric detector and a D-2500 integrator was used. An ultrabase C_{18} column (4.6x25 mm; 5.0 μm, Sharlau Science) was also used.

METHODS

Original Cleanup Procedure

A method recently proposed by Coldwell et al.,[19] one of the most effective for cleanup of plasma samples prior to the determination of 25-OH- vitamin D_3 by MS, consists of the following steps: 2-mL plasma is extracted by vortexing with 2 mL acetonitrile and centrifuging for 10 min. The supernatant is mixed with 400 mmol L^{-1} phosphate buffer pH 10.5 to make 65 % solution which is

applied to a Bond-Elut C_{18} cartridge and washed with 3 mL H_2O, 3 mL 40:60 methanol-water and then the analytes are eluted with 3 mL methanol. The eluate is evaporated to dryness under vacuum at 37°C. The residue is dissolved in 300 μL isopropanol/hexane (1:99) and then applied to a Bond-Elut silica cartridge and eluted with 10 mL isopropanol/hexane (3:97). The extract is dried under vacuum at 37°C and reconstituted in 100 μL methanol.

Modified Cleanup Procedure

The cartridges were conditioned before using as follows: the Bond-Elut C_{18} cartridge was washed with 3 mL methanol, dried under vacuum, and then washed with 3 mL of 400 mmol L^{-1} phosphate buffer pH 10.5. The Bond-Elut silica cartridge was also conditioned by washing it with 3 mL isopropanol/hexane (1:99). Then, 2 mL of plasma is extracted by vortexing with 2 mL acetonitrile and centrifuging for 10 min. The supernatant is mixed with 400 mmol L^{-1} phosphate buffer pH 10.5 to make 65 % solution which is applied to a Bond-Elut C_{18} cartridge. The cartridge is washed with 3 mL of phosphate buffer and 3 mL methanol/water (70:30), and finally eluted with 3 mL methanol/water (90:10). The eluate is evaporated to dryness under N_2 flow at room temperature.

The residue is dissolved in 1 mL isopropanol/hexane (1:99) and applied to the Bond-Elut silica cartridge, which is washed with 10 mL isopropanol/hexane (3:97), and then the analytes are eluted with 5 mL isopropanol/hexane (25:75). The extract is dried under N_2 flow at room temperature and reconstituted in 100 μL methanol.

Chromatographic Separation-Detection

Figure 1 shows the modular high performance liquid chromatograph used. A mixture of 50:0:50 acetonitrile:methanol:phosphate buffer (10 mmol L^{-1}, pH 5.0) is pumped along the system, as initial mobile phase. The loop of the injection valve is filled with 100 mL of the sample-methanol solution and injected into the chromatograph.

A linear gradient is established in order to reach a 0:100:0 ratio in 3 min, which is stabilized for 9 min more. The analytes are separated on the column as a function of their relative polarity and monitored photometrically at 270 nm.

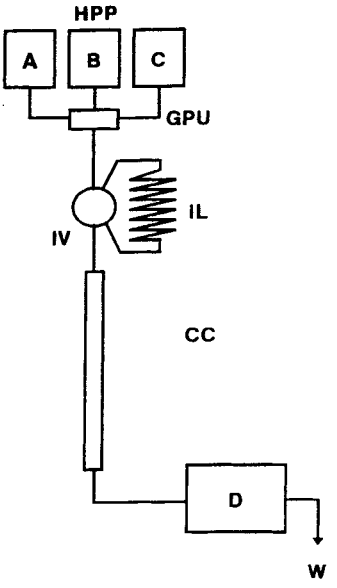

Figure 1. Modular chromatograph for the separation-UV detection of hydroxymetabolites of vitamin D_3. HPP denotes, high presure pump; GPU, gradient programmable unit; IV, injection valve; IL, injection loop, D, detector and W, waste.

RESULTS AND DISCUSSION

A two-step cleanup procedure using both non-polar and polar sorbent materials was considered to be the most efficient way for removal of polar and non-polar interferents usually present in plasma. With this aim, the method previously reported by Coldwell et al.,[19] based on dual solid-phase extraction with C_{18} and silica cartridges, was chosen as starting point after checking by HPLC-UV detection the presence of a number of species in the chromatogram which had not been detected when MS was used after this cleanup procedure.

So, UV-detection was used through the optimisation process in order to check that the pursued removal of as many components of plasma as possible was achieved with minimal losses of the target analytes. Owing to the lack of sensitivity of photometry as regards to the low concentration of vitamin D_3 metabolites in plasma, this matrix spiked with the targets analytes was used in order to detect their potential losses in the cleanup step, to know their position in the chromatogram, and to use them as reference to improve the cleanup step.

The Coldwell method was tested using four solutions (namely: blank, standard, sample, and spiked sample) which were prepared according to the procedure described.

The blank and standard solutions consisted of addition of ethanol, in the same proportion as in the samples, to 16 mmol L^{-1} sodium chloride used as plasma substitute.

Figure 2 depicts the chromatograms obtained. Chromatogram (a) corresponds to a standard solution prepared by diluting 500 ng mL^{-1} of each analyte in the sodium chloride solution. This recording enabled the retention times of the analytes (namely, 10.79, 11.34, and 13.28 min for 24-R,25-$(OH)_2$-vitamin D_3, 1-α,25-$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3, respectively) to be known, after their individual injection.

Chromatogram (b) corresponds to the blank solution after extraction, in which a series of peaks appeared due to prior lack of conditioning of the column. These peaks also appear in the chromatogram of the extracted standard solution (c), where the peaks corresponding to the analytes are smaller than in the chromatogram (a); thus indicating losses of the analytes in the cleanup step. A plasma sample, after the cleanup step, provided the chromatogram (d), in which only the peak of 25-OH-vitamin D_3 can be identified (t=13.28 min).

When 500 ng mL^{-1} of the three target analytes were added to the plasma, the chromatogram obtained after cleanup (e) showed the presence of the three metabolites, but all them have been partially removed in the cartridges, as compared with chromatogram (a), but particularly 1-α,25-$(OH)_2$ vitamin D_3.

Figure 2 justifies why the original cleanup procedure was only recommended for the determination of the 25-hydroxy derivative by LC-UV detection.[10,11] These chromatograms also show a strong contribution of the cleanup cartridges to the appearance of new chromatographic peaks, which can be due to lack of the conditioning step, as described by the authors.[19]

Modification of the Conventional Procedure

In order to improve the efficiency of the cleanup step some modifications on the original procedure were assayed. The changes were focused to: a) conditioning of the cartridges; b) optimisation of both the washing and elution steps.

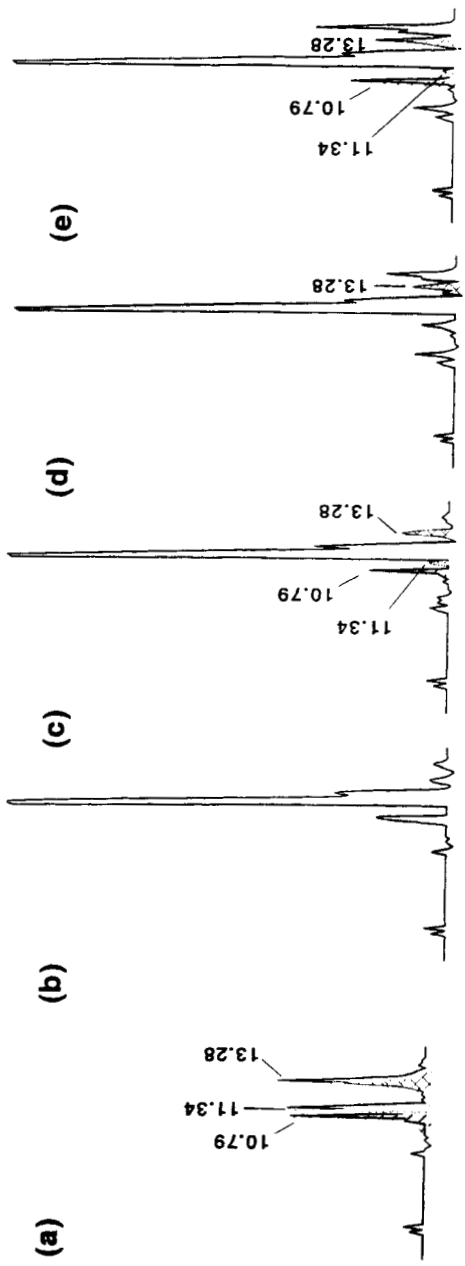

Figure 2. Chromatograms obtained by the Coldwell method.[12] a) by direct injection of a standard solution containing 500 ng ml^{-1} of each analyte, without solid-phase extraction. b) Blank solution after extraction. c) Standard solution after solid-phase extraction. d) Plasma sample and e) Plasma spiked with 500 ng mL^{-1} of each analyte. Peaks at retention times of 10.79, 11.34 and 13.28 min correspond to 24-R,25-$(OH)_2$vitamin D_3, 1-α,25-$(OH)_2$-vitamin D_3, and 25-(OH)-vitamin D_3, respectively.

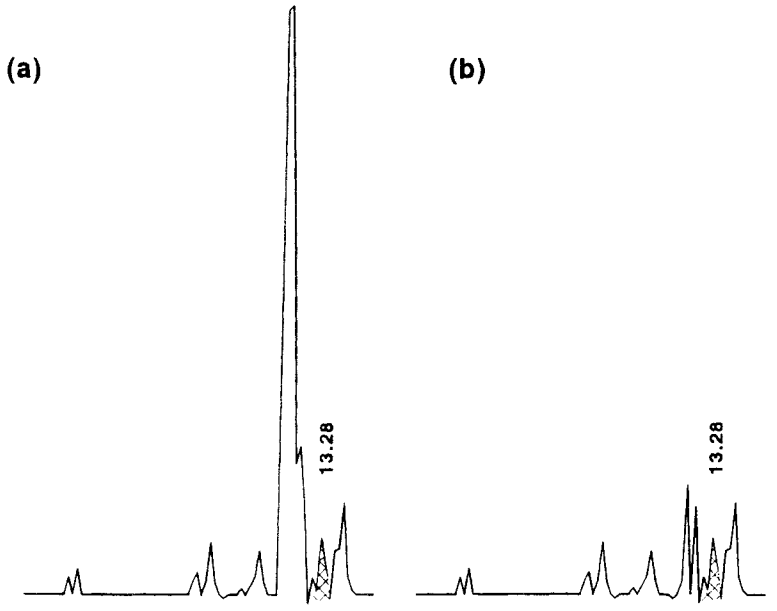

Figure 3. Chromatograms of plasma samples after cleanup with non-conditioned (a) and conditioned (b) cartridges.

Conditioning of the Cartridges

The C_{18} cartridges were conditioned by washing them with aliquots of methanol up to 3 mL; then, the cartridge was dried under vacuum and washed with 3 mL of 400 mmol L^{-1} phosphate buffer pH 10.5. The silica cartridges were also conditioned by washing them with 3 mL isopropanol:n-hexane (1:99).

The efficiency of the conditioning step was tested by extraction of two plasma samples according to the conventional procedure and using the Bon-Elut cartridges with and without conditioning. Figure 3 shows the results obtained.

As can be seen, the chromatogram obtained before conditioning shows a dual peak which appears at the retention times of 1,25 $(OH)_2D_3$ and 25$(OH)D_3$, as can be inferred by comparing Figures 2 and 3. When the cartridges were conditioned the dual peak was dramatically diminished.

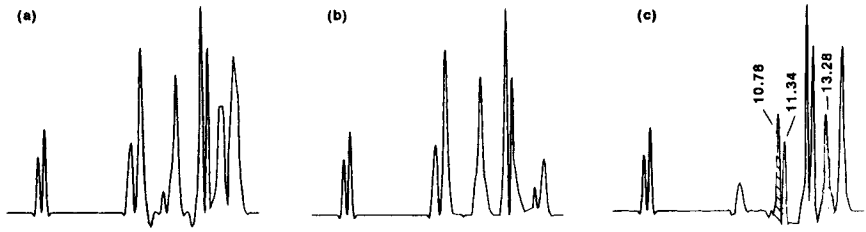

Figure 4. Modification of the C_{18} solid-phase extraction step of the original procedure.[12] (a) Coldwell procedure. (b) Washing with buffer solution and elution with 70:30 methanol-water. (c) Washing with buffer solution, interference removal with 70:30 methanol-water and analytes elution with 90:10 methanol-water (the Si cartridge step as in the Coldwell procedure in all instances).

Optimisation of the washing and elution steps with the C_{18} cartridge

The aim of this step was to remove polar species present in plasma with minimal removal of the target analytes. After application of the spiked plasma to the cartridge the procedure for removal of the polar species was as follows: elution with 3 mL of 400 mmol L^{-1} phosphate buffer, pH 10.5 (which was checked as much more effective than pure water) and subsequent elution of the interferents with 3 mL of 70:30 methanol:water instead of using the same volume of a 40:60 mixture. Percentages of methanol between 40 and 70 were assayed. Amounts of organic solvent higher than 70% gave rise to partial elution of the hydroxymetabolites, together with the inteferents; when the methanol content was lower than 40% only partial interference removal was achieved.

Figure 4a shows a chromatogram obtained after the cleanup steps proposed by Coldwell et al., while only a washing step followed by elution with 70:30 methanol-water prior to dryness was carried out in chromarogram (b). Finally, chromatogram (c) was obtained after treatment of the C_{18} cartridge with buffer, 70:30 methanol-water, and elution on passage of a 90:10 methanol:water mixture.

As can be seen, the analytes are eluted with this last mixture and a major part of the interferents are removed with the 70:30 mixture. The last eluate was always dried, reconstituted, and transferred to the Si cartridge in which the Coldwell procedure was applied in all the experiments.

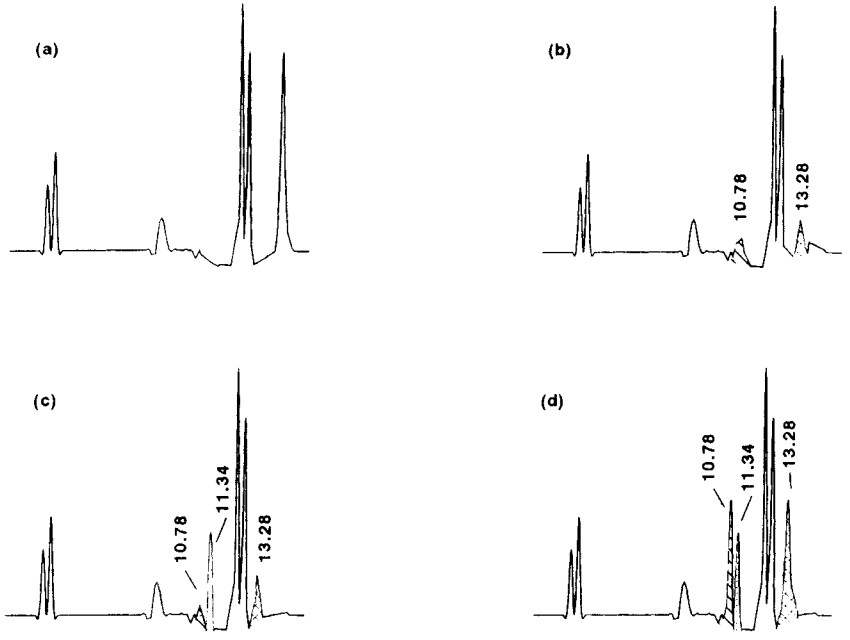

Figure 5. Modification of the original procedure on the the silica cartridge: (a) elution with 5 mL isopropanol:hexane (3:97). (b) elution with 5 mL isopropanol:hexane (10:90). (c) elution with 10 mL isopropanol:hexane (10:90). (d) elution with 5 mL isopropanol:hexane (25:75).

Optimisation of the washing and elution steps with the silica cartridge

The removal of the non-polar interferents was critical in this support due to the also non-polar nature of the target analytes. The dried sample from the optimised previous step was reconstituted in 1 mL of 1:99 isopropanol-hexane instead of 300 μL in order to accomplish a more homogenous passage of the solution through the Si cartridge. Different isopropanol-hexane ratios were assayed as washing solutions. Figure 5 shows the results obtained. Both the volume and nature of the eluent were crucial to achieve selective elution of interferents and analytes.

After applying the sample reconstituted in isopropanol-hexane (1:99), the cartridge was washed with 10-mL portions of the iso-propanol-hexane mixture in increased proportion of isopropanol from 1:99 to 25:75, which gave rise to increased polarity of the solvent mixture. Most of the non-polar interferents

Table 1

HPLC Variables

Type	Variable	Range Studied	Optimum Value
Physical	Temperature, °C	20 - 50	25
HPLC	Type of chromatography	----	reverse phase
	Mode	----	gradient
	Column	----	C_{18}, 5μm, 250x4.6
	Flow-rate, mL min^{-1}	0.5 - 2.0	1.2
	Injection volume,μL	20 - 200	100
	Phosphate buffer, $mmol^{-1}$	10 - 100	10
	pH	4.0 - 12.0	10.5

Table 2

Features of the Chromatographic Step

	24,25-$(OH)_2$-D_3	**1,25-$(OH)_2$-D_3**	**25-(OH)-D_3**
Equation (1)	$A = 79.7 \cdot C + 87$	$A = 62.8 \cdot C + 2009$	$A = 96.8 \cdot C + 205.6$
r^2	0.9994	0.9998	0.9983
Linear range, ng mL^{-1}	10 - 1000	100 - 1000	10 - 1000
Detection limit, ng mL^{-1}	10	50	10
Quantitation limit, ng mL^{-1}	20	100	20
rsd % (2)	6.9	6.5	6.0

(1) A denotes peak area, C analytes concentration in ng mL^{-1}.
(2) For 500 ng mL^{-1} of each analyte.

were eluted with a 3:97 isopropanol-hexane ratio (see Figure 5a). Finally, the elution of the analytes was achieved using 5 mL of 25:75 isopropanol:n-hexane, shown in Figure 5d, while chromatograms, b and c show a higher efficiency of the elution step as the percent of isopropanol in the eluent increased.

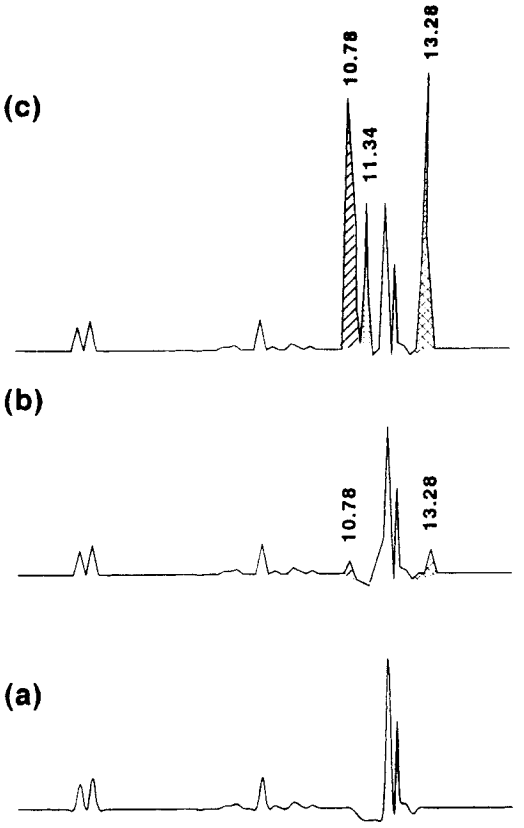

Figure 6. Chromatograms obtained with the modified procedure: (a) blank. (b) plasma. (c) sample spiked with 500 ng mL^{-1} of 24,25-$(OH)_2$-vitamin D_3, 1,25-$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3.

Features of the Chromatographic Separation

Prior to validation of the extraction procedure the features of the individual separation method were established. Calibration graphs were run using the optimum values of the variables listed in Table 1. Standard solutions of 24,25-$(OH)_2$-vitamin D_3, 1,25-$(OH)_2$-vita-min D_3 and 25-(OH)-vitamin D_3 were mixed at concentrations between 10 and 1000 ng mL^{-1} of each analyte and injected in triplicate into the chromatograph. Table 2 summarises the features of the method (equation, regression coefficient and rsd %).

Table 3

Validation of the Extraction Procedure

	24,25-$(OH)_2$-D_3	1,25-$(OH)_2$-D_3	25-(OH)-D_3
Precision study (1)			
$\bar{x}$	39937.28	34461.66	48633.87
σ_{n-1}	2781.45	2505.05	3211.90
rsd %	6.9	7.3	6.6
Standard addition (2)			
C	4.6	n.d. (3)	35
r %	94.6	101	95

(1) $\bar{x}$ denotes, signal average (n=6), σ, standard deviation and rsd% percent of relative standard deviation.

(2) C, analyte concentration in ng mL^{-1} and r recovery percent after addition of 500 ng mL^{-1} of each analyte.

(3) n.d. not detected.

As previously reported[10,11] UV detection is not sufficiently sensitive for the determination of hydroxyderivatives of vitamin D_3 in biological fluids. Nevertheless, it is an excellent tool to monitor plasma cleanup studies after HPLC separation, providing the plasma samples have been spiked with appropriate amounts of the target analytes.

The poor determination limit obtained make mandatory the use of a preconcentration procedure prior to application of HPLC, with UV detection method, for the determination in plasma samples. However in order to validate the extraction procedure spiked samples were used.

Validation of the Modified Cleanup Procedure

The validation of the cleanup steps was carried out using the standard addition method. Six plasma samples from a hospital were collected, cleaned, and injected into the chromatograph for the determination of the analytes. Figure 6 shows the chromatograms obtained. Chromatogram 6b was obtained from non-spiked plasma, and the concentration of 1,25-$(OH)_2$-vitamin D_3 was

not detected. When the sample was spiked with sufficient amounts of each analyte, the modified cleanup procedure provided both a clean chromatogram and an excellent recovery of the target species. Six aliquots from a plasma pool were spiked with 500 ng mL^{-1} of each analyte, cleaned and injected into the chromatograph. Table 3 summarised the precision provided by the proposed modified cleanup procedure and the recovery obtained after addition of the analytes to the samples. The precision of the extraction procedure, expressed as percent of standard deviation, was acceptable (lower than 7.3 % in all instances). The recovery achieved for the spiked samples (recoveries of 94.6 and 95 % for $24,25(OH)_2D_3$ and $25(OH)D_3$, respectively) was also acceptable.

CONCLUSIONS

A simple modification of a previous cleanup procedure is proposed based on both slight modifications of the eluents and conditioning of the C_{18} and silica cartridges. The improvements thus achieved can be summarised as follows:

a) A drastic decrease in interferents in the analytes-eluate, which is shown in a more clean chromatogram.

b) The removal of matrix interferences allows the determination 24-R,25-$(OH)_2$- and 1-alfa,25$(OH)_2$-vitamin D_3 in addition to 25-(OH)-vitamin D_3; this last being the sole species which could be determined in the original cleanup procedure[19] (see Figures. 4 and 6).

c) Closer to a quantitative separation of the analytes than in the previous method was also achieved (see Figure 4).

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