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Continuous cleanup/preconcentration procedure of hydroxyvitamin D_3 metabolites in plasma as an alternative to batch solid-phase extraction

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Abstract

A continuous automatable cleanup procedure coupled on-line with a liquid chromatograph and UV detector for hydroxyvitamin D_3 metabolites [24,25-$(OH)_2$, 1,25-$(OH)_2$ and 25-(OH)] as an alternative to batch solid-phase extraction is reported. The method, based on continuous solid-phase cleanup/preconcentration of the analytes, requires only the passage of the sample through a single minicolumn, which also results in a preconcentration effect which increases the sensitivity. The proposed method is also compared with a conventional batch, two-step solid-phase extraction method previously improved by the authors. The method has been checked by applying it to plasma samples spiked with the target analytes (linear range between 0.05 and 100 ng/ml with coefficient of variation values lower than 6.5%) and acceptable recoveries ranging between 94.6 and 101% have been obtained. The sampling frequency was 4 h^{-1}. © 1997 Elsevier Science B.V.

Keywords: Hydroxyvitamin D_3; Vitamins

1. Introduction

Knowledge of vitamin D metabolism and action has undergone a fast growth in the last two decades [1–7]. New actions not directly related to calcium homeostasis have been reported [2]. Vitamin D_2 and D_3 analogues with low calcemic actions are used both in metabolic bone diseases (osteoporosis and osteomalacia), in oncology and dermatology on the basis of their actions on differentiation, proliferation and also as immunomodulators [4]. The low concentration levels of vitamin D hydroxymetabolites in human fluids, together with the presence of other clinical species with similar chemical structures and properties, makes the development of new analytical methods with higher sensitivity and selectivity mandatory. The methods available at present for measurement of vitamin D_3 and its metabolites in human fluid, reviewed by Makin and coworkers [8,9] and more recently by Shimada and Kobayashi [10] have so far been based on high-performance liquid chromatographic (HPLC) separation coupled with protein binding assays [11,12] with the use of photometric [11,13], fluorimetric [14,15] or radiochemical [16,17] detection and GC–MS [18,19]. Nevertheless, these methods do not improve selectivity and sensitivity by either new sample cleanup and/or preconcentration procedures.

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The methods for the determination of these species in biological fluids described in the literature unfailingly include a purification step prior to determination. Usually, these procedures are based on solid-phase [12,17,20,21] or liquid–liquid extraction [21], which are slow, time-consuming operations and do not provide information on the simultaneous determination of several hydroxymetabolites. Recently, the authors have modified and improved a cleanup procedure for hydroxymetabolites of vitamin D_3 with subsequent HPLC separation and UV detection [22]. As UV detection is not sufficiently sensitive for the determination of these hydroxymetabolites in biological fluids, the use of a concentration step prior to application of HPLC–UV detection is mandatory.

In this work, a monochannel continuous flow-injection cleanup/preconcentration system as alternative to the dual solid-phase conventional methods for extraction of the hydroxyvitamin D_3 metabolites from human plasma is proposed. The approach was coupled on-line with a liquid chromatograph–UV detector and applied to the determination of 24,25-$(OH)_2$-vitamin D_3, 1,25-$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3 in plasma.

2. Experimental

2.1. Solutions and reagents

All solutions were prepared by using bidistilled water (Millipore Milli-Q system). Bond-Elut cartridges of C_{18} octadecyl 500 mg/2.8 ml and Si 500 mg/2.8 ml from Varian SPP were used. All organic solvents used were of HPLC grade. A acetonitrile–phosphate (20:80) buffer (50 mmol/l, pH 6.5) mixture was used as initial mobile phase. A linear gradient was programmed in order to obtain a acetonitrile–methanol (20:80) mobile phase in 3 min then stabilized for 9 min.

Standard solutions of 24,25-$(OH)_2$-vitamin D_3, 1,25-$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3 were prepared separately by dissolving the contents of a vial of each (Solvay Duphar, Netherlands) in methanol. Other solutions were prepared by dilution in 50 mmol/l phosphate buffer adjusted to pH 6.5. Plasma samples of normal and sick individuals from a local hospital were used.

2.2. 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 high-pressure manual injection valve (whose loop was exchanged by a 100-cm stainless-steel tubing of 0.25 mm I.D.), an L-4250 UV–Vis spectrophotometric detector and a D-2500 integrator was used. A Gilson Minipuls-2 low pressure peristaltic pump, a Rheodyne 5041 low-pressure injection valve, a Rheodyne 5010 low-pressure selecting valve, an Omnifit 50 mm length glass minicolumn and PTFE tubing of 0.5 mm inner diameter and different lengths were also used. An Ultrabase C_{18} (250×4.6; 5.0 μm, Scharlau Science) was used as analytical column.

2.3. Batch solid-phase extraction/separation–detection procedure

Following the method reported by Coldwell et al. [19], the cartridges were conditioned before use 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 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 was extracted by vortexing with 2 ml acetonitrile and centrifuged for 10 min. The supernatant was mixed with 400 mmol/l phosphate buffer pH 10.5 to make a 65% solution which was applied to a Bond-Elut C_{18} cartridge which was washed with 3 ml phosphate buffer and 3 ml methanol–water (70:30) and, finally, eluted with 3 ml methanol–water (90:10). The eluate was evaporated to dryness under an N_2 stream at room temperature. The residue was dissolved in 1 ml isopropanol–hexane (1:99) and applied to the Bond-Elut silica cartridge, which was then washed with 10 ml isopropanol–hexane (3:97), and the analytes eluted with 5 ml isopropanol–hexane (25:75). The extract was dried under a N_2 flow at room temperature and reconstituted in 100 μl methanol.

A modular HPLC/photometric detector was used for the separation and detection step. An acetonitrile–phosphate buffer (20:80) (50 mmol/l, pH 6.5) mixture was pumped along the system as initial

mobile phase. The loop of the injection valve was filled with 100 μl of the sample-methanol solution and injected into the chromatograph. A linear gradient was established in order to reach an acetonitrile–methanol ratio (20:80) in 3 min, which was then stabilized for 9 min. The analytes were removed from the column as a function of their relative polarity and monitored photometrically at 270 nm.

2.4. Continuous cleanup/preconcentration procedure

Fig. 1 shows the integrated continuous cleanup/preconcentration–HPLC separation system. The cleanup/preconcentration subsystem permits simultaneous cleanup of the sample and preconcentration of the target analytes prior to their injection into the separation subsystem. The C_{18} preconcentration minicolumn inserted in the sample loop of a low pressure injection valve was conditioned by sequential washing with methanol, 50 mmol/l phosphate buffer of pH 6.5 and water for 2 min. 2 ml of plasma was extracted by vortexing with 4 ml acetonitrile, centrifuged for 10 min and evaporated under a N_2 flow at room temperature in order to reduce the volume to 1 ml. This solution was mixed with 7 ml of 50 mmol/l phosphate buffer pH 6.5 and passed through the C_{18} 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 phosphate buffer of pH 6.5 and methanol–water (70:30) solution in order to remove the interferents by changing manually the position of SV. Then, the analytes were eluted by a methanol–acetonitrile (95:5) solution by switching manually the low-pressure injection valve and thus drove to the injection valve of the chromatograph. This valve had been modified by changing the conventional loop for a 100-cm length tubing of 0.25 mm I.D. The volume of the methanol–acetonitrile solution 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 was started simultaneously with injection. The analytes were removed from the column as a function of their relative polarity, monitored photometrically at 270 nm and the data integrated for presentation.

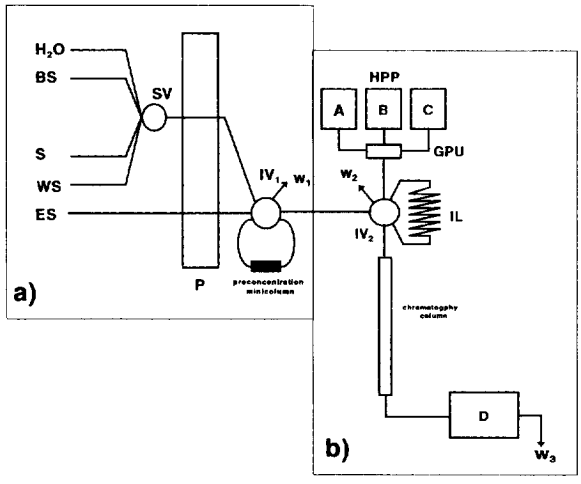

Fig. 1. Integrated continuous cleanup/preconcentration flow injection–HPLC manifold. (a) Cleanup/preconcentration flow injection subsystem, (b) modular chromatograph for the separation–UV detection. P denotes, peristaltic pump; SV, selecting valve; BS, buffer; S, sample; WS, washing solution; ES, eluent solution; IV_1, low-pressure injection valve; HPP, high pressure pump; GPU, A, B, C, solvents; GPU, gradient programmable unit; IV_2, high pressure injection valve; IL, injection loop; D, detector; and w_1, w_2 and w_3 wastes.

3. Results and discussion

The batch cleanup procedure proposed by Coldwell et al. [19] and based on the use of solid-phase sorbents has for years been demonstrated as an efficient way for removal of interferents present in plasma prior to the determination of vitamin D_3 hydroxymetabolites by GC–MS. Recently, we have demonstrated that the extract obtained by using this cleanup procedure contains a number of plasma species which strongly overlap the peaks of the analytes obtained by photometric detection after LC separation thus making this type of detection unsuitable. An improvement of this procedure provides a sufficiently clean extract to enable the determination of the main vitamin D_3 hydroxymetabolites by UV detection after chromatographic separation [22]. However, the poor determination limit obtained with this detection makes mandatory the development of a concentration step prior to introduction of the extract into the chromatograph. With this aim, our modified

batch procedure was chosen as starting point for the development of a continuous cleanup/preconcentration procedure for removal of as many plasma components as possible with minimal losses of the target analytes. This study was accomplished using a flow injection approach for the development of a single cleanup step, with an in-line minicolumn packed with a sorbent material. In order to check the efficiency of the continuous extraction procedure, parallel studies were performed using its batch counterpart.

3.1. Optimization of the continuous cleanup/preconcentration subsystem

The influence of the variables affecting the continuous procedure, classified into hydrodynamic and chemical, were studied by the univariate method. Table 1 shows the variables and ranges studied and the optimum values found in each case.

After checking *sorbents* such as silica, aminoalkil, diol, cyanopropyl, phenyl, ethyl, octyl and octadecyl, C_{18} was selected as the best. Small particles of this sorbent (15–40 and 40–60 μm particle size) caused overpressure in the low-pressure manifold due to increased compactness. A diameter greater than 63 μm of the C_{18} bonded silica beads provided optimal performance.

The *pH and ionic strength* as modifiers of the retention capacity were studied and a higher retention was found when the value of both increased. However, pH values greater than 10 cause an irreversible increase of the support compactness. A pH of 6.5 was selected as compromise. The ionic strength was adjusted with sodium chloride in the range 5 to 200 mmol/l, and values over 150 mmol/l decreased the retention efficiency between 20 and 30% depending on the target analyte. After extraction with acetonitrile the ionic strength was adjusted to the optimum value.

Binary and ternary mixtures of acetonitrile, methanol, isopropanol and water were assayed for selective removal of potential interferents present in plasma with minimal losses of the target analytes

Table 1
Study of variables

Type	Variable	Range studied	Batch	FI/HPLC
Physical	Temperature (°C)	20–50	25	25
Cleanup/preconcentration	Mode	–	Dual	Single
	Sorbent	–	C_{18}/Si	C_{18}
	Particle size (μm)	15–63	–	63
	pH	4–12	10.5	6.5
	Washing solution		70	–
	Methanol (%)	40–70	30	100
	Water (%)	30–60		
	Elution solution			
	Methanol (%)	40–70	90	95
	Water (%)[a]	30–60	10	5
	Flow-rate (ml/min)	0.1–0.6	–	0.32
	Minicolumn length (cm)	0.5–5.0	–	2
	Stainless steel (cm) (0.25 mm I.D.)	50–200	–	100
	Injection volume (μl)	50–500	–	100
HPLC	Type of chromatography	–	RP gradient	RP gradient
	Column	–	C_{18}, 5 μm, 250×4.6	C_{18}, 5 μm, 250×4.6
	Flow-rate (ml/min)	0.5–2.0	1.2	1.2
	Injection volume (μl)	20–200	100	100
	Phosphate buffer (mmol/l)	10–100	10	50
	pH	4–12	10.5	6.5

[a] In the FI/HPLC system water is replaced by acetonitrile.
RP denotes reversed-phase.

(washing step). Levels of methanol greater than 70% gave rise to partial elution of the hydroxymetabolites together with the interferents; when the methanol content was lower than 40% only partial interferents removal was achieved. A methanol–water (70:30) mixture was selected as optimal. A methanol–acetonitrile (95:5) mixture provided quantitative removal of the hydroxymetabolites from the C_{18} support.

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

The interphase for quantitative transfer of the analytes from the FI manifold to the chromatograph was a metallic tube of 0.25 mm I.D. which connected the end of the preconcentration system and the injection port of the chromatograph. 100 cm was the minimal 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 metallic tube of 0.25 mm I.D. and a total volume of 100 μl was substituted for the conventional loop of the high pressure injection valve. Finally, a delay time of 90 s between switching the preconcentration and high pressure injection valves was optimal to catch the whole volume of the eluate containing the analytes in the modified loop.

3.2. Features of the chromatographic determination

Prior to validation of the continuous cleanup procedure the features of the determination step were established for each analyte. 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-$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3 were mixed at concentrations between 1 and 100 ng/ml of each analyte and injected in triplicate into the chromatograph. Table 2 summarises the

Table 2
Features of the methods

	24,25-$(OH)_2$-D_3	1,25-$(OH)_2$-D_3	25-(OH)-D_3
(a) Batch method			
Equation[a]	$A=79.7C+87$	$A=62.8C+2009$	$A=96.8C+205.6$
r^2	0.9994	0.9998	0.9983
Linear range (ng/ml)	10–1000	100–1000	10–1000
Detection limit (ng/ml)[b]	10	50	10
Quantitation limit (ng/ml)[c]	20	100	20
C.V. (%)[d]	6.9	6.5	6.0
(b) Continuous method			
Equation[a]	$A=41.2C-193.5$	$A=17.3C-62.9$	$A=10.1C-43.1$
r^2	0.9911	0.9939	0.9917
Linear range (ng/ml)	0.05–100	0.05–100	0.05–100
Detection limit (ng/ml)[b]	0.01	0.01	0.01
Quantitation limit (ng/ml)[c]	0.05	0.05	0.05
C.V. (%)[e]	5.2	5.3	6.4

[a] A denotes peak area, C analyte concentration in ng/ml.
[b] Expressed as 3σ of the blank signal.
[c] Expressed as 10σ of the blank signal.
[d] For 500 ng/ml of each analyte.
[e] For 50 ng/ml of each analyte.

features of the method [equations, regression coefficients, linear ranges, detection and quantitation limits and coefficient of variation (C.V.) values]. As previously reported [13,21,22] UV detection is not sufficient sensitive for the determination of hydroxy-derivatives of vitamin D_3 in biological fluids without preconcentration step. Nevertheless, it is an excellent tool for monitoring the plasma cleanup studies after HPLC separation, providing the plasma samples have been spiked with appropriate amounts of the target analytes.

3.3. Features of the overall method

Calibration graphs were run using both the integrated FI/HPLC approach and optimum values of the variables listed in Table 1. Standard solutions of the analytes were mixed at concentrations between 0.01 and 500 ng/ml of each analyte and processed. Table 2 summarises the features of the overall continuous method (equations, regression coefficients, linear ranges, detection and quantitation limits and C.V.s). Fig. 2 shows the chromatogram corresponding to three situations: (a) injection of a blank solution, (b) injection of a standard solution containing 500 ng/ml of each analyte without preconcentration and (c) injection of a standard solution of 10 ng/ml of each analyte after preconcentration. The sensitivity, expressed as detection limit, was increased 100-fold by the continuous cleanup/preconcentration system in comparison with the batch solid-phase extraction procedure.

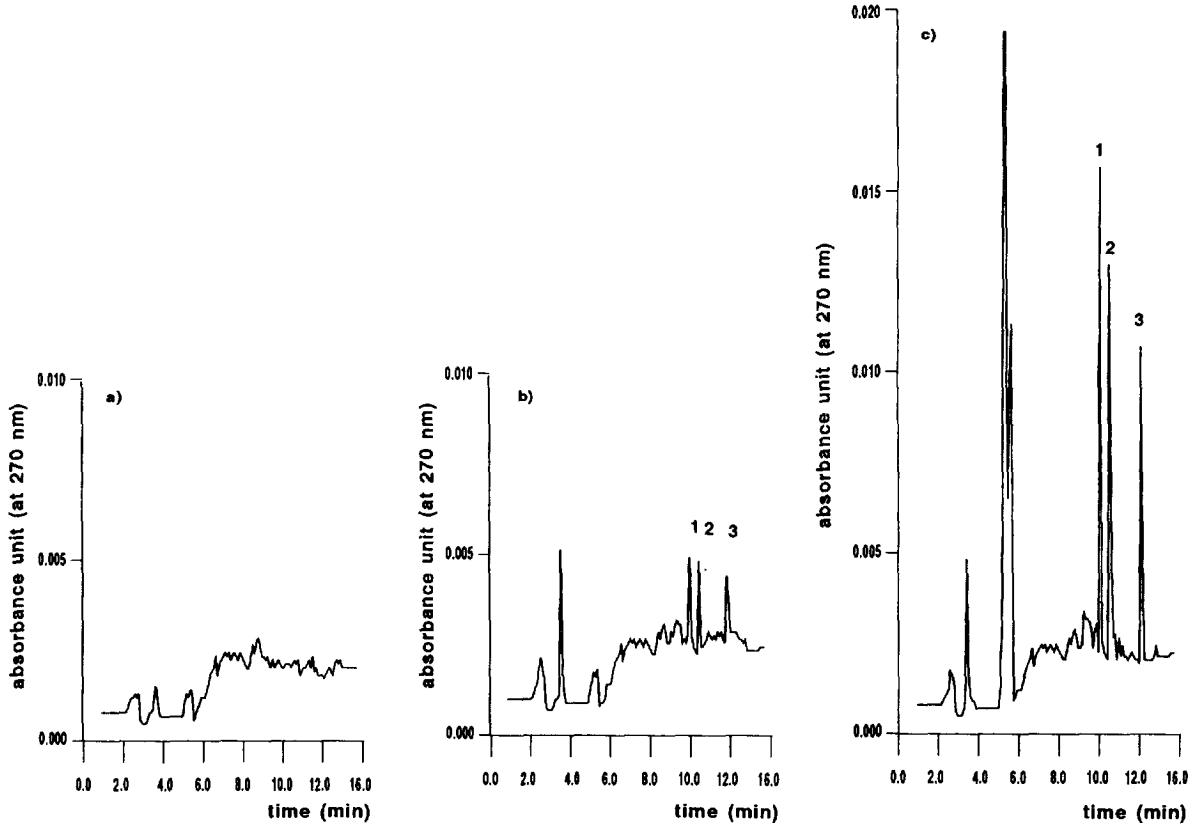

Fig. 2. Chromatograms obtained after injection of (a) a blank solution, (b) a standard solution with 500 ng/ml of each analyte without preconcentration and (c) a solution of 10 ng/ml of each analyte after preconcentration. 1, 2 and 3 denote, 24,25-$(OH)_2$-vitamin D_3, 1,25-$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3, respectively.

Seven solutions of 50 ng/ml of each analyte were assayed in triplicate. The repeatability of the method yielded an acceptable C.V. in all instances (see Table 2).

3.4. Sampling frequency

The sampling frequency achieved under the optimal working conditions for the overall cleanup/preconcentration–separation/determination was 4 h^{-1}. Taking into account that three species are determined in each sample a determination rate of 12 determination/h is achieved.

3.5. Application of the method

The proposed method was validated by applying it to the determination of 24,25-$(OH)_2$-vitamin D_3, 1,25$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3 in plasma in comparison with the batch extraction/HPLC method in two ways, namely: (a) determination of the analytes and (b) study of the recovery afforded after addition of a standard solution of each analyte. A comparison between the proposed and the batch method was performed. The addition of standards was different according to the sensitivity of the method: concentration of 500 and 50 ng/ml were added for the batch solid-phase extraction and the continuous cleanup/preconcentration methods, respectively. Table 3 summarises the concentrations found and the recoveries obtained and chromatograms of both plasma and spiked plasma are shown in Fig. 3. As can be seen, an excellent agreement between both methods and recoveries of 98 and 99% for 24,25-$(OH)_2$-vitamin D_3 and 25-(OH)-vitamin D_3 were obtained.

4. Conclusions

In comparison with the methods published so far, that proposed here presents the following advantages: (i) a single cleanup step versus 2 or 3 steps usually necessary. This improvement involves lower costs and shorter manipulation time. (ii) Miniaturization of the cleanup step; this means a smaller amount of sorbent material (0.2 g versus 2 g in the previous methods). In addition, the minicolumn can be re-used for least 200 times without loss of capacity. (iii) Drastic preconcentration factor, which enables the determination of the target analytes in very diluted samples as plasma using a conventional spectrophotometer. (iv) Simple automation of the experimental setup (using an automated chromatograph and automatic low-pressure injection valve) can be implemented. A better control of the injection time would be achieved in this way, thus improving precision. (v) Dramatic reduction of the derivatization cost as compared with other techniques such as MS and radioimmunoassay (RIA). In addition the latter gives rise to cross-reactions and does not enable simultaneous determination of two analytes.

Acknowledgments

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Table 3
Application of the batch and continuous method

	24,25-$(OH)_2$-D_3	1,25-$(OH)_2$-D_3	25-(OH)D_3
Batch method			
Concentration found (ng/ml)	4.6	n.d[a]	35
Recovery (%)	94.6	101	95
Continuous method			
Concentration found (ng/ml)	4.4	n.d.[a]	36
Recovery (%)	98	100	99

[a] Concentration lower than the quantitation limit.

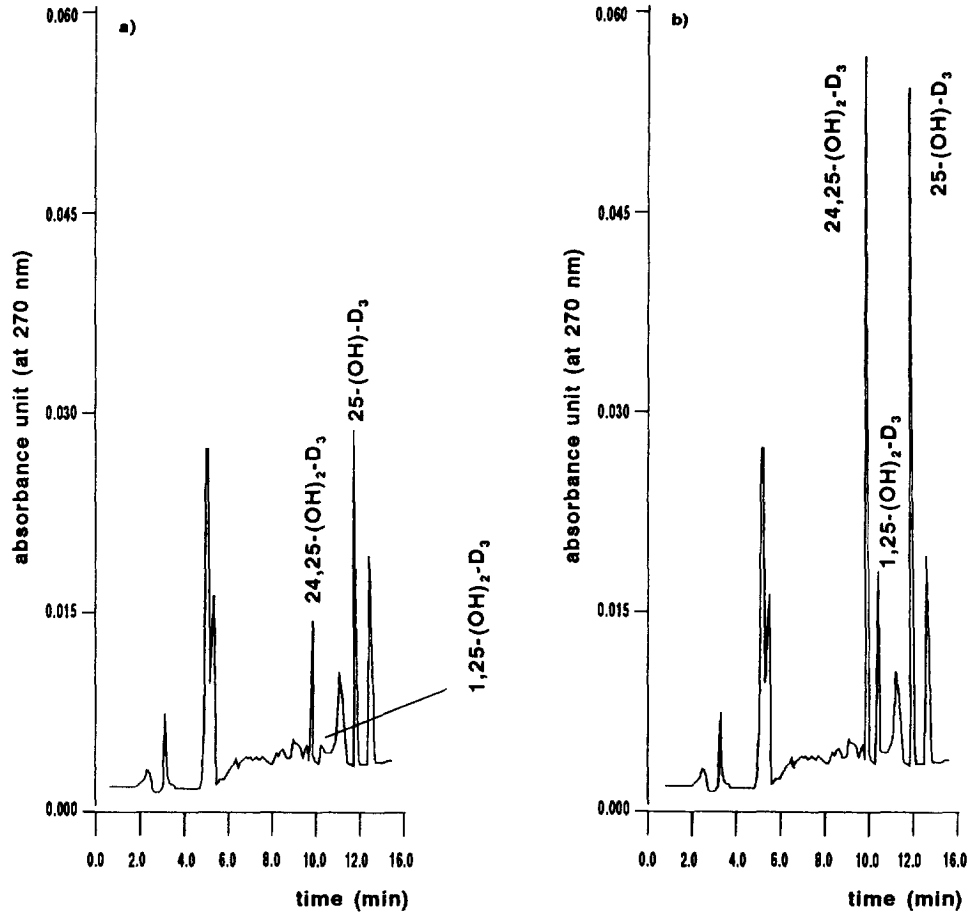

Fig. 3. Chromatograms obtained after injection of (a) human plasma and (b) the same plasma spiked with 50 ng/ml of each analyte.

vay Duphar, Weesp, Netherlands) for standard solutions.

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