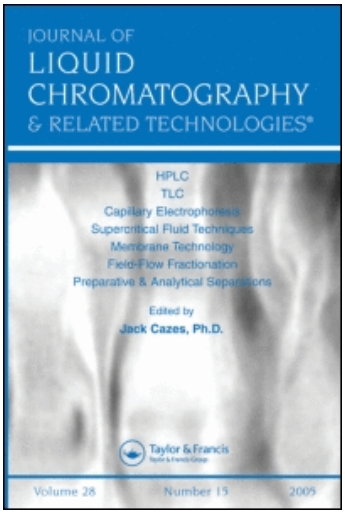

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF DIHYDROXYLATED VITAMIN D_3 METABOLITES USING MOBILE PHASE CONTAINING CYCLODEXTRIN

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF DIHYDROXYLATED VITAMIN D_3 METABOLITES USING MOBILE PHASE CONTAINING CYCLODEXTRIN

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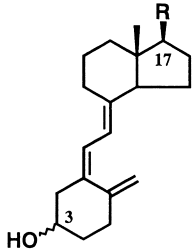
ABSTRACT

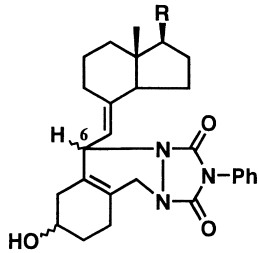
The separation of side chain dihydroxylated vitamin D_3 metabolites by inclusion high performance liquid chromatography using cyclodextrin (CD) as the mobile phase additive was examined. 24,25-Dihydroxyvitamin D_3 and its 3-epimer were clearly separated by the addition of γ-CD to the mobile phase using acetonitrile as the organic modifier. The separation of the 25-epimers of 25,26-dihydroxyvitamin D_3 has been done, to a certain extent, with the mobile phase using methanol and dimethylated β-CD as the organic modifier and the additive, respectively. These separation methods were applied to the detection of the metabolites in the plasma of the rat administered 25-hydroxyvitamin D_3. These metabolites were also identified by liquid chromatography/tandem mass spectrometry after derivatization.

INTRODUCTION

Vitamin D_3 (D_3) is metabolized to 25-hydroxyvitamin D_3 [25(OH)D_3] (Figure 1, **1**), which is a circulating form, in the liver and, subsequently, to the active form, 1,25-dihydroxyvitamin D_3 in the kidneys, and has important func-



D_3 metabolite



PTAD derivative

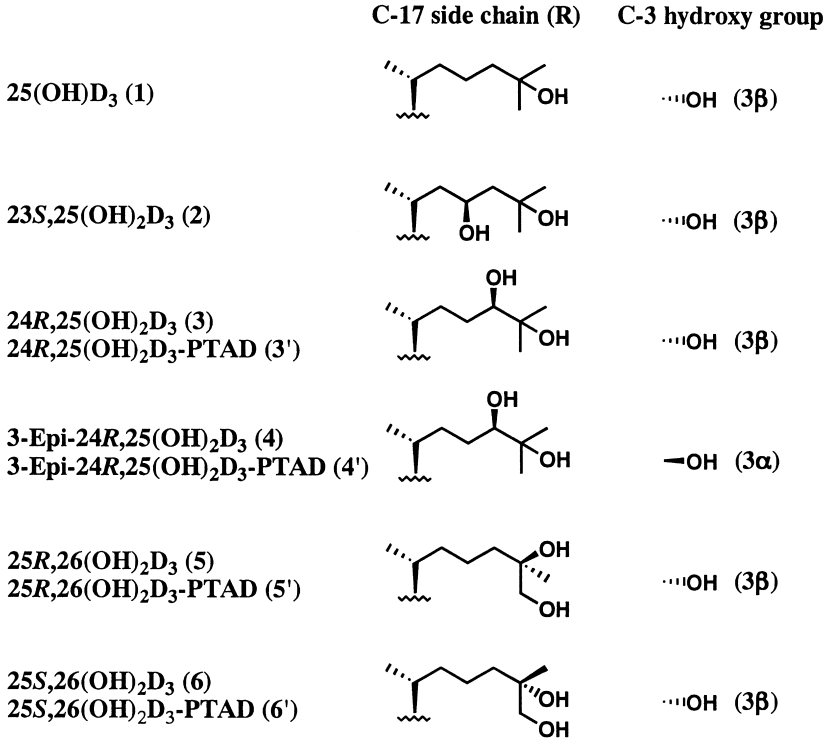


Figure 1. Structures of D_3 metabolites and their PTAD derivatives.

tions, such as the regulation of calcium metabolism and bone formation. On the other hand, when the serum calcium level is greater than 90 mg/L, 25(OH)D_3 is hydroxylated at the C-23, 24, and 26 positions on its side chain. In mammalians, 23,25-dihydroxyvitamin D_3 [23,25$(OH)_2D_3$] (**2**) and 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] (**3**) have a single configuration, 23*S* and 24*R*, respectively. On the other hand, 25,26-dihydroxyvitamin D_3 [25,26$(OH)_2D_3$] (**5** and **6**) is reported to exist as a mixture of the 25*R* and 25*S* isomers.[1] Recently, we reported that 3-epi-24*R*,25$(OH)_2D_3$ (**4**) was one of the major plasma metabolites of rats administered 24*R*,25$(OH)_2D_3$ and it was formed through the 3-oxo-form as an intermediate by a liver cytosol enzyme.[2] The biological significance of these metabolites and the relationship between the physiological conditions and the hydroxylated position on the side chain are still poorly understood. A profile analysis of these metabolites in a single sample using high performance liquid chromatography (HPLC) is expected to be helpful in clarifying these subjects.

The regio-isomers, 23,25-, 24,25-, and 25,26-$(OH)_2D_3$, are easily separated from each other during normal-phase HPLC,[3] but this mode is not always sufficiently versatile because the biological samples, such as plasma, contain polar components. A reversed-phase HPLC overcomes this problem, but this mode gives poor results with respect to their separation. Furthermore, the stereoisomers, the C-25 isomers of 25,26$(OH)_2D_3$ (**5** and **6**), and the C-3 isomers of 24*R*,25$(OH)_2D_3$ (**3** and **4**), can not be separated by the usual normal- and reversed-phase HPLC without derivatization; the separation of the former and latter pairs was achieved after conversion to the (+)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) esters[1] and the 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) adducts,[4] respectively. However, the separation of the MTPA esters required a long time (*ca*. 100 min) and two normal-phase columns connected in tandem.

Based on these results, development of a separation method of the side chain dihydroxylated D_3 metabolites using reversed-phase HPLC was considered to be important. In the present study, the effect of cyclodextrin (CD) as the mobile phase additive in the separation of the metabolites and the application of the developed method to the detection of the metabolites in plasma of the rat administered 25(OH)D_3 were examined. The identification of the metabolites using liquid chromatography/mass spectrometry (LC/MS) after derivatization was also reported.

EXPERIMENTAL

Materials and Reagents

25(OH)D_3 and 24*R*,25$(OH)_2D_3$ were obtained from Wako Pure Chemical Co. (Osaka, Japan) and Duphar B. V. Co. (Amsterdam, The Netherlands), respectively. 23*S*,25$(OH)_2D_3$ is prepared from its 23-glucuronide obtained

from rats administered 25(OH)D_3 by enzymic hydrolysis with β-glucuronidase.[4] 25*R*,26- and 25*S*,26-$(OH)_2D_3$ were synthesized in our laboratory according to the known method.[5] 3-Epi-24*R*,25$(OH)_2D_3$ was prepared by us.[4] 24*S*,25 $(OH)_2D_3$ was kindly provided by Kureha Chemical Co. (Tokyo, Japan). PTAD was synthesized from 4-phenylurazole (Nakalai Tesque, Kyoto, Japan) and purified by sublimation.[6] Heptakis-(2,6-di-*O*-methyl)-β-CD (Me-β-CD) and γ-CD were donated by Kao Co. (Tokyo) and Nihon Shokuhin Kako Co. (Tokyo), respectively. Isolute C18 (EC) cartridges (500 mg, International Sorvent Tech., Ltd., Hengoed, U.K.) were obtained from Uniflex (Tokyo). All other reagents and solvents were of analytical grade.

Apparatus

HPLC was performed using a Hitachi L-7110 chromatograph (Tokyo) equipped with a Shimadzu SPD-10A UV (265 nm) detector (Kyoto).

LC/MS was performed using a Finnigan MAT LCQ (San Jose, CA, U.S.A.) liquid chromatograph/ion trap-mass spectrometer connected to a JASCO PU-980 (Tokyo) chromatograph, and atmospheric pressure chemical ionization (APCI) was used in the positive-ion mode. The heated capillary temperature was set at 225°C. The sheath gas flow rate was set at 80 units with a vaporizer temperature of 400°C. The source current, the capillary voltage, and the tube lens offset were 5 mA, 1 V, and 10 V, respectively. For the MS/MS analysis, helium was used as the collision gas and the relative collision energy was set at 20%. A J'sphere ODS H-80 column (4 μm, 15 X 0.46 cm i.d.)(YMC, Kyoto) was used at a flow rate of 1 mL/min at 30°C for both the HPLC and LC/MS. The column oven temperature was set at 30°C and 40°C for the HPLC and LC/MS, respectively.

Plasma Samples from Rats

A male Wistar strain rat (*ca*. 170 g, 7 w, Japan S.L.C., Hamamatsu, Japan) was starved for 10 h prior to the administration of 25(OH)D_3. A suspension of 25(OH)D_3 (0.5 mg) in dimethylsulfoxide (0.1 mL) with saline (0.7 mg) and Tween 80 (0.2 mL) was orally given to the rat. Twelve hours after dosing, blood was collected from the aorta descendens under anesthesia with diethyl ether. Sodium heparin was immediately added to the blood (2% v/v of blood volume) and the sample was centrifuged at 1500*g* (15 min, 4°C). The separated plasma was stored at –20°C prior to use.

Pretreatment of Plasma Samples

The plasma sample (1 mL) was mixed with MeCN (1 mL) and subjected to centrifugation at 1,500*g* for 10 min. To the supernatant was added H_2O

(3 mL) and the sample was then passed though an Isolute C18 (EC) cartridge. After washing with H_2O (4 mL) and 70% MeOH (4 mL), the steroids were eluted with MeOH (3 mL), which was evaporated under a N_2 gas stream. The residue was redissolved in EtOH and subjected to preparative HPLC [MeCN-H_2O (3:2, v/v), retention time (t_R) 10.5-13.5 min]. After dilution with four volumes of H_2O, the obtained fraction was applied to an Isolute C18 (EC) cartridge in the manner described above. The MeOH eluate was evaporated, redissolved in EtOH (15 μL) and p art of this (10 μL) was subjected to HPLC. The other plasma sample (1 mL) was similarly pretreated and analyzed by LC/MS after derivatization with PTAD.

Derivatization with PTAD

Standard D_3 metabolites (*ca*. 50 ng) or the pretreated plasma sample was dissolved in AcOEt (50 μL) containing PTAD (5 μg) and kept at room temperature for 1 h. MeOH (50 μL) was added to decompose any excess reagent, and the solvent was evaporated under a N_2 gas stream. The residue was redissolved in EtOH (50 μL) and part of this was subjected to LC/MS.

RESULTS AND DISCUSSION

Separation of Dihydroxylated D_3 Metabolites by Inclusion HPLC

First, the separation of the regio-isomers [23*S*,25$(OH)_2D_3$ (**2**), 24*R*, 25$(OH)_2D_3$ (**3**) and 25*R*,26$(OH)_2D_3$ (**5**)] by the usual reversed-phase HPLC was examined. When MeCN-H_2O (11:9, v/v) was used as the mobile phase, the latter two were not separated [t_R: 23*S*,25$(OH)_2D_3$; 14.3 min, 24*R*,25$(OH)_2D_3$; 13.6 min and 25*R*,26$(OH)_2D_3$; 13.2 min]. On the contrary, when MeOH-H_2O (3:1, v/v) was used, the former two gave the same t_R (18.2 min) and only 25*R*,26$(OH)_2D_3$ was separated from them (t_R; 22.5 min). These results prompted us to use CD as the mobile phase additive, which has been proved to be a great advantage in the separation of isomeric steroids.[7,8] Me-β-CD and γ-CD were used based on their solubility in organic solvents[9] and inclusion effect for steroids. The combination of organic modifiers and CDs were systematically examined, and two mobile phase systems shown in Figures 2a and b were found to give a good separation of **2**, **3**, and **5**.

The relationship between the position of the hydroxy group and the inclusion effect is summarized in Table 1. The use of Me-β-CD and γ-CD showed a similar tendency and the relative capacity factor (Rk') values decreased in the following order; 25,26- > 24,25- > 23,25-$(OH)_2D_3$. These results indicated that the hydroxy group closer to the end of the side chain had an effect on the inclusion.

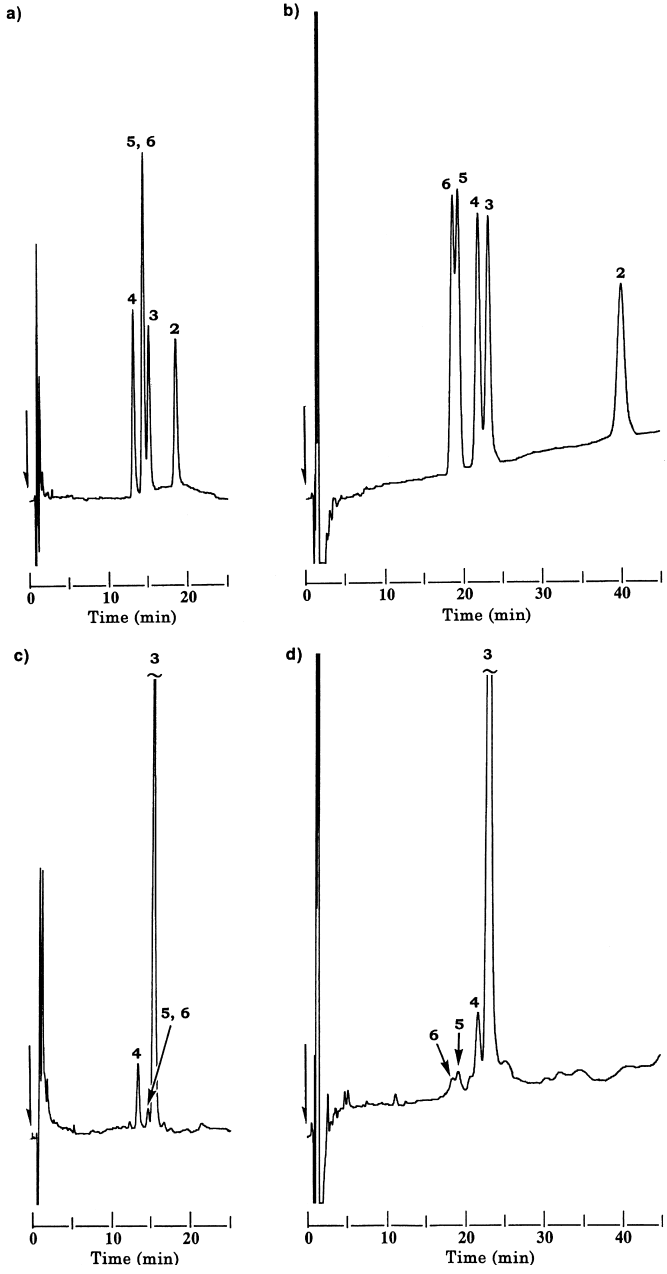


Figure 2. Chromatograms of dihydroxylated D_3 metabolites. a) and b) Authentic samples. c) and d) From rat plasma. Mobile phase; a) and c) MeCN-H_2O (1:1, v/v) containing 5 mM γ-CD, b) and d) MeOH-H_2O (3:2, v/v) containing 10 mM Me-β-CD. The numbers refer to the structures as shown in Figure 1.

Table 1

Relationship Between the Position of the Hydroxy Group and the Inclusion Effect

CD	Rk'[a]		
	23*S*,25(OH)$_2$D$_3$	**24*R*,25(OH)$_2$D$_3$**	**25*R*,26(OH)$_2$D$_3$**
Me-β-CD (3 mM)	0.75	0.59	0.48
γ-CD (3 mM)	0.83	0.81	0.74

[a] The capacity factor (k') values obtained without CD were taken as 1.0 for the calculation of the Rk' values. Mobile phase; MeCN-H_2O (1:1, v/v) containing CD as indicated. $NaNO_3$ was used for the measurement of t_0 = 1.27 min.

Second, our effort was directed toward the separation of the C-3 isomer of 24*R*,25(OH)$_2$D$_3$ (**3** and **4**), which could not be achieved by the usual reversed-phase HPLC. As shown in Figure 2a, the addition of γ-CD to the mobile phase using MeCN as an organic modifier significantly improved the separation of 24*R*,25(OH)$_2$D$_3$ and its C-3 epimer. Although both compounds were fairly separated in the MeOH containing Me-β-CD system [Figure 2b, resolution (Rs) = 1.27], both the γ-CD in MeOH and Me-β-CD in MeCN systems were ineffective with respect to their separation. Further investigations on the separation of the C-3 isomer of 24*R*,25(OH)$_2$D$_3$ using the γ-CD in MeCN system were performed, and the results are shown in Figure 3. The Rk' of 3-epi-24*R*,25(OH)$_2$D$_3$ was more remarkably influenced than 24*R*,25(OH)$_2$D$_3$, with increasing concentration of γ-CD in the mobile phase, which was the reason why their Rs values increased.

As mentioned in the introduction, it was reported that the C-25 isomers of 25,26(OH)$_2$D$_3$ (**5** and **6**) were derivatized to MTPA esters and then separated after 100 min using two normal-phase columns in series.[1] In the present study, the separation of the isomers in the shorter time using inclusion HPLC without derivatization was examined. Although various mobile phase systems were examined, their complete separation could not be achieved. The most effective separation (Rs = 0.54) was obtained in the use of MeOH-H_2O (3:1, v/v) containing 10 mM Me-β-CD in the examined systems (Figure 2b). The proposed method is not effective in separately quantifying the isomers, but considered to be helpful for detecting them in biological fluids. This mobile phase system achieved the separation of not only each regio-isomer but also 24*R*,25(OH)$_2$D$_3$ and its 3-epimer.

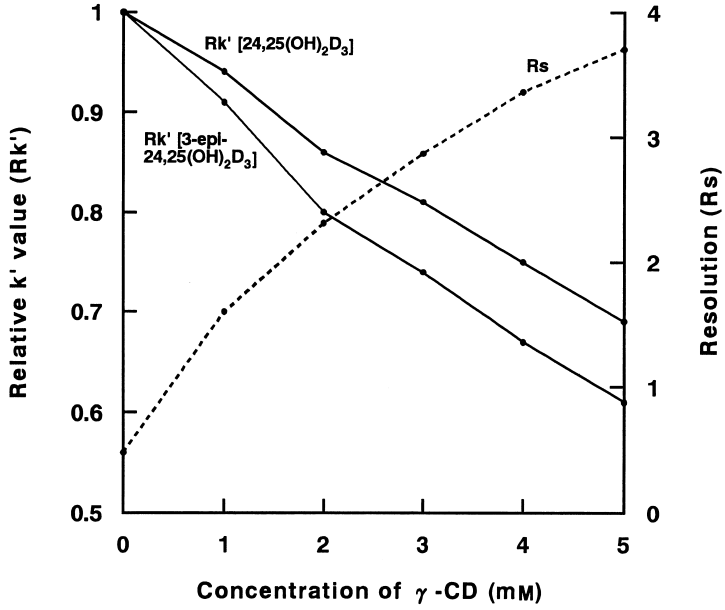


Figure 3. Effect of γ-CD on the retention and separation of 24*R*,25(OH)$_2$D$_3$ (**3**) and its C-3 epimer (**4**). The k' values obtained without CD [24*R*,25(OH)$_2$D$_3$; 15.9, 3-epi-24*R*,25(OH)$_2$D$_3$; 15.4, t_0=1.27 min ($NaNO_3$)] were taken as 1.0 for the calculation of the Rk' values. Mobile phase; MeCN-H_2O (1:1, v/v) containing γ-CD as indicated.

Incidentally, 24*R*,25(OH)$_2$D$_3$ and 24*S*,25(OH)$_2$D$_3$, the latter of which is the metabolite formed in only birds,[10] were completely separated using MeCN-H_2O containing 5 mM γ-CD (Rs = 3.87).

Application of Inclusion HPLC to Detection of Dihydroxylated D$_3$ Metabolites in Rat Plasma

The plasma sample obtained from a rat dosed with 25(OH)D$_3$ (**1**) was subjected to inclusion HPLC using MeCN-H_2O containing 5 mM γ-CD and MeOH-H_2O containing 10 mM Me-β-CD as the mobile phases (Figures 2c and d). In both systems, peaks corresponding to 24*R*,25(OH)$_2$D$_3$ (**3**) and its C-3 epimer (**4**) were distinctly observed, but that of 23*S*,25(OH)$_2$D$_3$ (**2**) was not detected. We isolated 23*S*,25(OH)$_2$D$_3$ 23-glucuronide, 24*R*,25(OH)$_2$D$_3$ 24-glucuronide, and 3-epi-24,25(OH)$_2$D$_3$ 24-glucuronide from the bile of rat administered 25(OH)D$_3$ in the previous study, and the yield of 23*S*,25(OH)$_2$D$_3$ 23-glucuronide was the largest of

the three.[4] However, in contrast with 24*R*,25$(OH)_2D_3$ and 3-epi-24*R*,25$(OH)_2D_3$, 23*S*,25$(OH)_2D_3$ was not detected in the plasma, and a similar phenomenon was also reported by Napoli et al.[11] These results indicated that this metabolite was rapidly conjugated or further metabolized. The peaks corresponding to the epimers of 25,26$(OH)_2D_3$ (**5** and **6**) were observed in Figure 2d, and that of the mixture was also observed in Figure 2c. However, these peaks were so small that their structures were confirmed by LC/MS.

LC/MS Analysis of PTAD Adducts of Dihydroxylated D_3 Metabolites

To identify the formed dihydroxylated D_3 metabolites in the plasma more reliably, an LC/MS analysis was performed. As mentioned above, the examined D_3 metabolites could not be separated by the mobile phase without CDs, but CDs could not be applied to the LC/MS because they are nonvolatile and decrease the sensitivity of the MS detector.

We reported that the derivatization with PTAD improves the separation of 24*R*,25$(OH)_2D_3$ and its C-3 epimer, and supplies the characteristic product ion derived from the cleavage of the C-6–7 bond (*m/z* 298) in the MS/MS mode.[2,4] Furthermore, the PTAD adducts of vitamin D compounds give two peaks due to the epimers at the C-6 position caused by the fact that the reagent attacks at the *s*-cis diene of vitamin D from the α- and β-sides (Figure 1), which are very useful in identifying the compounds.[12] These data prompted us to use the derivatization with PTAD in the present study.

Based on the data from the inclusion HPLC (Figures 2c and d), the 23*S*,25$(OH)_2D_3$ derivative was not investigated. In addition, because the chromatographic and mass separation of the PTAD adducts of 25*R*,26$(OH)_2D_3$ (**5'**) and 25*S*,26$(OH)_2D_3$ (**6'**) could not be achieved under various conditions, the 25*R*-epimer was used in the following experiments; therefore, the PTAD adducts of 24*R*,25$(OH)_2D_3$ (**3'**), 3-epi-24*R*,25$(OH)_2D_3$ (**4'**), and 25*R*,26$(OH)_2D_3$ (**5'**) were investigated in the present study.

Under the conditions described in the experimental section, every adduct gave the ion at *m/z* 574 $[M+H-H_2O]^+$ as a base ion, and provided the product ion at *m/z* 298 when the above ion was used as the precursor ion in the MS/MS mode. Incidentally, when the capillary temperature was below 200°C, all these adducts gave the protonated ion (*m/z* 592) as the base ion, and the main product ion obtained from it was the *m/z* 574 ion; this method was less effective than the above one (precursor ion; *m/z* 574 and product ion; *m/z* 298) for the improvement of selectivity. The LC/MS/MS chromatogram of these adducts is shown in Figure 4a, in which MeCN was used as an organic modifier. The MeOH system was superior in sensitivity, but gave poor results for the separation of the adducts of 3-epi-24*R*,25$(OH)_2D_3$ and 25,26$(OH)_2D_3$.

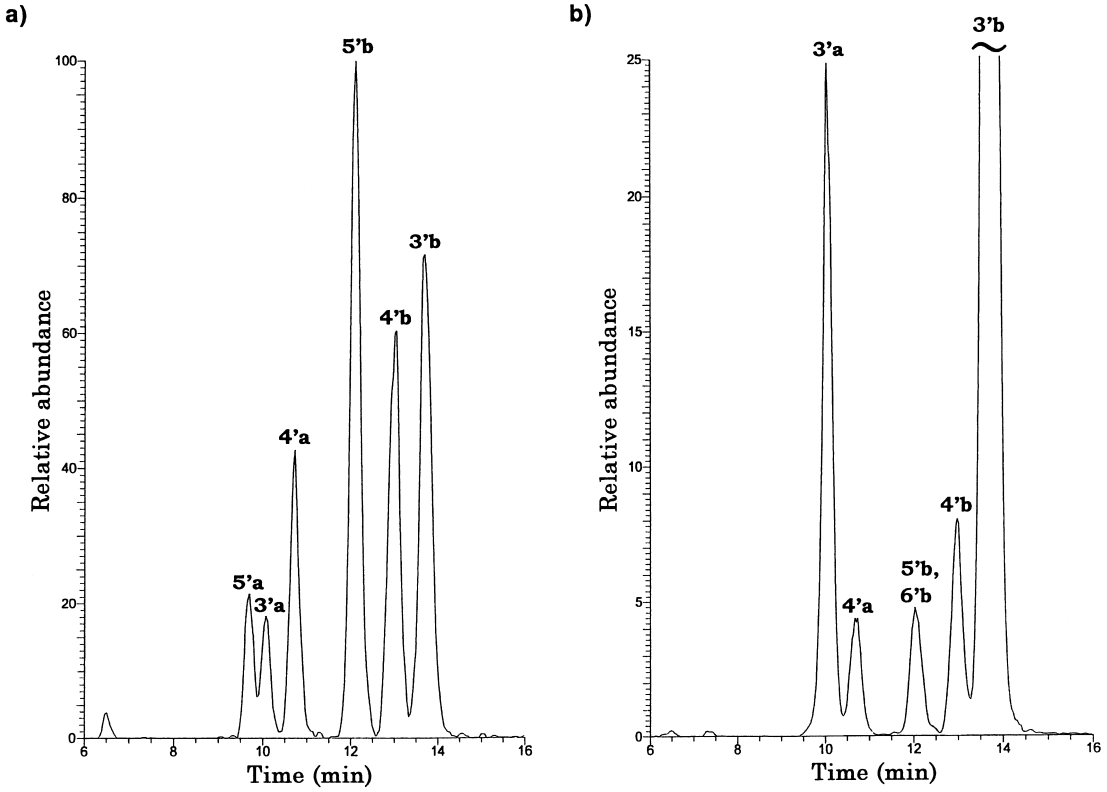


Figure 4. LC/MS/MS chromatograms of PTAD adducts of dihydroxylated D_3 metabolites. a) Authentic samples. b) From rat plasma. Conditions, mobile phase; MeCN-H_2O (9:11, v/v), monitoring ion; *m/z* 298 (precursor ion; *m/z* 574). **3'a** and **3'b**; 24*R*,25$(OH)_2D_3$-PTAD, **4'a** and **4'b**; 3-epi-24*R*,25$(OH)_2D_3$-PTAD, **5'a** and **5'b**; 25*R*,26$(OH)_2D_3$-PTAD, **6'b**; 25*S*,26$(OH)_2D_3$-PTAD. The derivatization with PTAD gave two adducts as previously described, which were denoted as **a** and **b**.

In the LC/MS/MS chromatogram of the plasma specimen (Figure 4b), although the peak corresponding to the minor epimer of the adduct of 25,26$(OH)_2D_3$ (**5'** and **6'**) (t_R 9.7 min) was not detected due to overlapping with that of the 24*R*,25$(OH)_2D_3$ derivative (**3'**), the major peak (t_R 12.1 min) was clearly observed, together with the peaks derived from 24*R*,25$(OH)_2D_3$ (**3'**) and 3-epi-24*R*,25$(OH)_2D_3$ (**4'**). Furthermore, their mass and product ion mass spectra agreed with those of the synthetic standards. Based on these results, it was concluded that 25,26$(OH)_2D_3$ existed in the plasma as a minor metabolite, and it was considered to be a mixture of C-25 epimers based on the inclusion HPLC data (Figure 2d). It was found that 3-epi-24,25$(OH)_2D_3$ was also detected in the plasma of the rat administered a large dose of 25$(OH)D_3$, which indicated that C-3 epimerization is one of the important pathways in D_3 metabolism during D_3 intoxicated state.

In conclusion, the separation of the dihydroxylated D_3 metabolites using CD as a mobile phase additive was investigated. When Me-β-CD was added to the mobile phase using MeOH as an organic modifier, the five examined metabolites were satisfactorily separated. This method was useful in identifying the metabolites in biological fluids.

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