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# Analysis of stereoisomeric $C_{27}$-bile acids by high performance liquid chromatography with fluorescence detection[1]

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**Abstract**

A method for differentially measuring the 24-hydroxylated stereoisomeric intermediates (3α,7α,12α,24-tetrahydroxy- and 3α,7α,24-trihydroxy-5β-cholestan-26-oic acids) and related $C_{27}$-bile acids in β-oxidation of bile acid biosynthesis has been developed by high performance liquid chromatography with fluorescence detection. The method involved the derivatization of the above intermediable $C_{27}$-bile acids into fluorescent esters with 3-(4-bromomethylphenyl)-7-diethylaminocoumarin, a newly synthesized labeling reagent for carboxylic acids. The fluorescent derivatives were subjected to a short silica gel column to eliminate interfering products prior to analysis by high performance liquid chromatography. The separation of the 16 kinds of bile acids containing stereoisomers was carried out using a reversed-phase Inertsil C8-column by gradient elution and detected with a fluorometer (Ex. 400 nm, Em. 475 nm). The linearity of calibration curve for each bile acid was from 0.5 to 250 pmol ($r = 0.999$) and the detection limits were about 15 fmol at a signal-to-noise ratio of 3. The method was applied to the determination of intermediates in β-oxidation of bile acid biosynthesis using rat liver homogenate. The results showed that two stereoisomers of 24-hydroxylated $C_{27}$-bile acids were predominantly produced, indicating the formation of the isomers by the *cis*-hydration with water. © 1997 Elsevier Science B.V.

*Keywords:* Bile acid biosynthesis; $C_{27}$-Bile acid; Stereoisomer; β-Oxidation; HPLC; Fluorescent labeling; Rat liver homogenate

## 1. Introduction

It is known that primary bile acids (3α,7α,12α-trihydroxy- and 3α,7α-dihydroxy-5β-cholan-24-oic acids, CA and CDCA) are synthesized from cholesterol via intermediable $C_{27}$-bile acids (3α,7α,12α-trihydroxy- and 3α,7α-dihydroxy-5β-cholestan-26-oic acids, THCA and DHCA) by the side chain degradation (β-oxidation) [1–3]. The degradation reaction by β-oxidation system involves dehydrogenation, hydration and final oxidative cleavage of the side chain between the C-24 and C-25 positions. The intermediates in the side

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chain degradation, 3α,7α,12α,24-tetrahydroxy- and 3α,7α,24-trihydroxy-5β-cholestan-26-oic acids (VA and DVA), are synthesized from THCA and DHCA *via* 3α,7α,12α-trihydroxy- and 3α,7α-dihydroxy-5β-cholest-24-en-26-oic acids (24*E*-THCA and 24*E*-DHCA), respectively [4,5]. The peroxisomal enzymatic system has been reported to catalyze the above reaction [6,7]. Another possible pathway was also reported in microsomal fraction of rat liver homogenate [8]. Une et al. reported that the only one isomer of VA was produced in rat liver homogenates using THCA or DHCA as substrates [9,10]. On the other hand, Kobayashi et al. reported the production of all possible four isomers of VA, and they concluded that VA was synthesized non-stereoselectively in rat liver homogenates [11]. The above stereochemical findings seemed to reflect the results by the catalytic reaction of different enzyme systems. Thus, the stereochemistry of the products in each pathway has not been clearly established yet. It is required for the development of quantitative analytical method to clarify the stereochemistry of the intermediates for the side chain degradation system. This paper deals with a simultaneous quantitative determination of the stereoisomeric intermediates in the side chain degradation of bile acids using high performance liquid chromatography (HPLC) with novel sensitive fluorescence detection.

## 2. Experimental

### 2.1. Chemicals

All $C_{27}$-bile acids and 3-(4-bromomethylphenyl)-7-diethylaminocoumarin (MPAC-Br) were synthesized in our laboratory as previously reported methods [12–14]. CA and CDCA were purchased from Sigma (St. Louis, MO, USA). All the solvents and reagents used were of analytical grade. The internal standard, 3α,7α,12α-trihydroxy-26a,26b-dihomo-27-nor-5β-cholestan-26b-oic acid, was synthesized as follows; The solution of 3,7,12-triformyloxy-homo-CA (5.4 mmol in 100 ml of tetrahydrofuran) prepared from homo-CA [15], was first treated with triethylamine (6 mmol) and ethyl chloroformate (6 mmol) at 0°C for 30 min. The solution of sodium borohydride (25 mmol in 5 ml of water) was added dropwise to the above stirred solution. The mixture was stirred for 30 min at 0°C. After usual work-up, the resulting alcohol was subjected to Swern oxidation to give triformyloxy aldehyde. The aldehyde was then treated with triphenylphosphoranylideneacetic acid ethyl ester to give unsaturated ester. The ester was hydrogenated with 10% Pd on carbon under hydrogen atmosphere. The hydrolysis of the ester with 2M KOH in methanol gave the carboxylic acid as an internal standard (38% from homo-CA), mp 175–177°C (ethyl acetate), $[\alpha]_D$: +33.2° ($c = 1.00$, MeOH). $^1$H-NMR (pyridine-$d_5$): 0.84 (3H, *s*, 18-Me), 1.02 (3H, *s*, 19-Me), 1.21 (3H, *d*, $J = 6.4$ Hz, 21-Me), 3.73 (1H, *m*, 3-H), 4.11 (1H, *brs*, 7-H), 4.27 (1H, *brs*, 12-H). $^1$H-NMR spectrum of the above compound was recorded at 400 MHz with a JEOL JNM-EX 400 spectrometer (JEOL, Tokyo, Japan) and the abbreviations used are *s* = singlet, *d* = doublet, *brs* = broad singlet and *m* = multiplet. Anal. Calcd for $C_{28}H_{48}O_5$: C, 72.37; H, 10.41. Found: C, 72.21; H, 10.52.

### 2.2. Apparatus and chromatographic conditions

HPLC apparatus consisted of an LC 6A system equipped with RF-535 spectrofluorometer (Shimadzu, Kyoto, Japan). The chromatographic separation was performed on an Inertsil C8 column (250 × 4.6 mm i.d., GL Science, Kyoto, Japan) using gradient elution mode at a flow rate of 1.0 ml min$^{-1}$. The column temperature was ambient. The mobile phases were 20 mM tris-acetate buffer (pH 7.5)/methanol (22:78, v/v, mobile phase A) and 20 mM tris-acetate buffer (pH 7.5)/methanol (10:90, v/v, mobile phase B). The gradient program was as follows; an isocratic elution with mobile phase A for 20 min, a linear gradient elution to 35% of a mobile phase B over a period of 30 min, then set to 65% of mobile phase B and a linear gradient elution to 90% of mobile phase B over a period of 20 min. The fluorescence intensity of the eluent was monitored with an emission wavelength at 475 nm and an excitation wavelength at 400 nm.

### 2.3. Derivatization procedure

The sample was mixed with 150 μl of 0.2% MPAC-Br in acetonitrile, 150 μl of 0.4% 18-crown-6 in acetonitrile saturated with $KHCO_3$. (This saturated solution was prepared as follows; addition of large excess solid $KHCO_3$ to 0.4% 18-crown-6 solution, sonication for 10 min at room temperature and centrifuging for 10 min at $1000 \times g$. The separated solution was used for reaction.) The mixture was warmed to 60°C for 20 min and then the solvent was evaporated off by centrifugal evaporator at room temperature. The residue was dissolved in chloroform (0.5 ml) and subjected to a silica gel column (55 × 6 mm i.d.). The column was washed with 6 ml of chloroform/methanol (200:1, v/v) and the labeled compounds were then eluted with 5 ml of chloroform/methanol (8:1, v/v). After evaporation of the solvent by centrifugal evaporator, the residue was dissolved in an appropriate volume of methanol, and applied to HPLC analysis.

### 2.4. Preparation of rat liver light mitochondrial fraction

Male Wistar rats weighing around 200 g were sacrificed by decapitation. The livers were minced and homogenized in 9 volumes (w/v) of 0.25 M sucrose to a liver weight by two strokes (1000 rpm) in a potter-Elvehjem homogenizer. Cellular debris and nuclei were removed by centrifuging at $800 \times g$ for 10 min. The supernatant solution was then centrifuged at $3000 \times g$ for 15 min. The separated supernatant solution was again centrifuged at $12\,500 \times g$ for 20 min. The pellets were suspended in an appropriate volume of 0.25 M sucrose and were used as a partially purified light mitochondrial (LM) fraction. Protein content was determined by the Lowry method [16] using bovine serum albumin as a standard.

### 2.5. Calibration curves

Each 100 μl of the standard bile acid solution of different concentration (*ca.* 1.0–500 μg in 10 ml of methanol) and 100 μl of the internal standard solution (25 μg in 10 ml of methanol) were mixed and evaporated. The residue was derivatized and purified as described above. The labeled bile acids were dissolved in 200 μl of methanol and 5 μl was injected into HPLC. The calibration curves were constructed by plotting the peak area ratio of each labeled compound to the internal standard against the ratios of sample concentration to that of the internal standard.

### 2.6. Recovery

The relative recoveries were measured by two different concentrations using 100 μl of the bile acid standard solutions (10 and 100 μg in 10 ml of methanol). A mixture of the standard solutions, 250 μl of heat-treated LM fraction and 250 μl of 0.2 M phosphate buffer (pH 7.6) containing ATP (11 mM), EDTA (20 mM), $MgCl_2$ (40 mM) and CoA (3 mM) was incubated for 60 min at 37°C. The reaction mixture was then cooled with ice-bath and 100 μl of the internal standard solution (25 μg in 10 ml of methanol) was added to the mixture. Ethanol (5 ml) was added to the above mixture and stored at 0°C for 30 min. The mixture was then centrifuged at $1000 \times g$ for 20 min. The supernatant solution was evaporated by centrifugal evaporator. The residue was treated with 0.2 M NaOH (0.5 ml) at 50°C for 5 min and acidified with 0.2 M HCl at 0°C. The aqueous solution was extracted with ethyl acetate (5 ml) and the extract was washed with water (1 ml) twice. The solvent was evaporated off by centrifugal evaporator. The residue was dissolved in 90% ethanol (0.5 ml) and the solution was subjected to piperidinohydroxypropyl Sephadex LH-20 column (45 × 6 mm i.d.). After washing with 90% ethanol (2 ml), the free bile acid fraction was eluted with 0.15 M acetic acid in 90% ethanol (6 ml) [17]. The eluate was evaporated and the residue was derivatized and purified as described above. The labeled sample was dissolved in 200 μl of MeOH and 5 μl was injected into HPLC. The relative recovery of each bile acid was calculated from the peak area ratio to that of the internal standard.

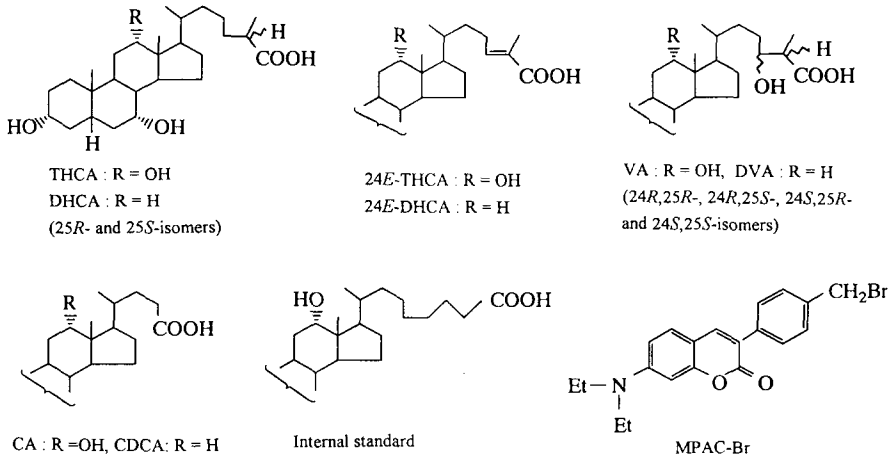


Fig. 1. Structures and abbreviated name of the bile acids and labeling reagent.

### 2.7. Incubating conditions

The mixture of each substrate (100 μg), LM fraction (250 μl, *ca.* 1.2–1.8 mg of protein) and 0.2 M phosphate buffer (pH 7.6, 250 μl) containing cofactors as above was incubated for 60 min at 37°C. After the addition of the internal standard (1 μg), the mixture was worked up and the free bile acids were derivatized into MPAC esters as described above. The fluorescence labeled compounds were then dissolved in 200 μl of methanol and analyzed by HPLC.

## 3. Results and discussion

### 3.1. Development of the analytical method

Fig. 1 shows the structure of intermediates of bile acid biosynthesis, the internal standard and the labeling reagent (MPAC-Br) used in this study. The possible stereoisomers of these bile acids were also indicated. THCA and DHCA are the starting compounds of side chain degradation ($\beta$-oxidation) for CA and CDCA. Both 25*R*- and 25*S*-isomers of these $C_{27}$-bile acids were used as substrates for the study of $\beta$-oxidation. 24*E*-THCA and 24*E*-DHCA are biologically converted from THCA and DHCA by the catalytic dehydrogenation with acyl CoA oxidase. The 24-hydroxylated $C_{27}$-bile acids, VA and DVA, are derivatized from the above 24-unsaturated compounds (24*E*-THCA and 24*E*-DHCA) by the catalytic hydration with acyl CoA hydratase. There should be four stereroisomers due to the two asymmetric centers at the C-24 and C-25 positions in the 24-hydroxylated compounds, namely, 24*R*,25*R*-, 24*R*,25*S*-, 24*S*,25*R*- and 24*S*,25*S*-isomers. CA and CDCA are the final products of $\beta$-oxidation.

These 16 kinds of the compounds were first converted into fluorescence derivatives with MPAC-Br (Fig. 1), which was recently reported as a highly sensitive labeling reagent for carboxylic acids [14]. The reaction conditions (temperature and time) for derivatization of the bile acids were first investigated. When the reaction was carried out at 60°C, the conversion rates of bile acids into labeled compounds reached to a plateau for 15 min. While the time of the derivatization reaction required over 2 h at room temperature. From the above results, the reaction temperature and reaction time were set at 60°C and 20 min, respectively.

Prior to the analysis, the pre-treatment of the reaction mixture was examined because the large excess reagent and its decomposition products interfered with the determination of bile acid derivatives. A good result was obtained, when a silica gel short column was used to eliminate

interfering compounds by initial washing with chloroform/methanol (200:1, v/v) and elution with chloroform/methanol (8:1, v/v).

The conditions for the separation of labeled compounds were then investigated using C-18 and C-8 reverse-phase columns and various combinations of solvents. The use of C-18 column resulted the incomplete separation of the stereoisomers of VA and DVA (data not shown). On the contrary, C-8 column gave a good separation. Fig. 2 shows the separation of the labeled standard bile acids using a C-8 reversed-phase column (Inertsil C8) by the gradient elution of tris-acetate buffer/methanol. The biologically intermediable $C_{27}$-bile acids and $C_{24}$-bile acids were separated within 70 min. The stereoisomers of $C_{27}$-bile acids were also separated. The peaks 1–4 and 6–9 were the four stereoisomers of VA and DVA attributed to the 24*R*,25*S*-, 24*S*,25*R*-, 24*R*,25*R*- and 24*S*,25*S*-isomers, respectively. The peak 10, 11 and 15, 16 were the two stereoisomers (25*S*- and 25*R*-isomers) of THCA and DHCA, respectively. On the chromatogram there appeared some peaks due to decomposition of the reagent before 10 min, but all the labeled bile acids were eluted after 20 min, where no obstructive peak was observed. Table 1 shows the $k'$ value of each labeled compound. It was indicated that the determination of each peak could be easily achieved under the conditions described above. The relative sensitivity of labeled bile acids for the internal standard were 0.92–1.06 which also indicated the quantitative determination of each bile acid could be readily carried out with the almost same sensitivities. The calibration curves of bile acids showed the good linearity ($y = 0.86 \sim 1.02x \pm 0.003 \sim 0.01$) between 0.5–250 pmol per injection. The correlation coefficients ($r$) for all compounds were over 0.999. The detection limits of these bile acids were estimated about 15 fmol per injection at a signal to noise ratio of 3.

The relative recoveries were measured using standard bile acids with a rat liver LM fraction under the same conditions as incubating experiment. The rat liver LM fraction was heated to eliminate the enzymatic activities before use. The

Table 1
Capacity ratios ($k'$) and relative fluorescence sensitivities of the labeled bile acids

| Bile acid | $k'$ value[a] | Sensitivity[b] |
|---|---|---|
| (24*R*,25*S*)-VA | 7.6 | 0.87 |
| (24*S*,25*R*)-VA | 8.0 | 0.89 |
| (24*R*,25*R*)-VA | 8.9 | 0.92 |
| (24*S*,25*S*)-Va | 10.3 | 0.94 |
| CA | 11.8 | 0.92 |
| (24*R*,25*S*)-DVA | 12.7 | 0.91 |
| (24*S*,25*R*)-DVA | 13.4 | 1.00 |
| (24*R*,25*R*)-DVA | 15.1 | 0.98 |
| (24*S*,25*S*)-DVA | 16.3 | 0.89 |
| (25*S*)-THCA | 17.9 | 1.07 |
| (25*R*)-THCA | 19.0 | 0.98 |
| CDCA | 19.7 | 1.09 |
| 24*E*-THCA | 21.1 | 1.03 |
| (25*S*)-DHCA | 23.6 | 1.13 |
| (25*R*)-DHCA | 24.3 | 1.18 |
| 24*E*-DHCA | 25.7 | 1.17 |

[a] $T_0 = 2.5$ Min.
[b] Represented as the relative sensitivities to the internal standard, 3α,7α,12α-trihydroxy-26a, 26b-biohomo-27-nor-5β-cholsestan-26b-oic acids (1.00).

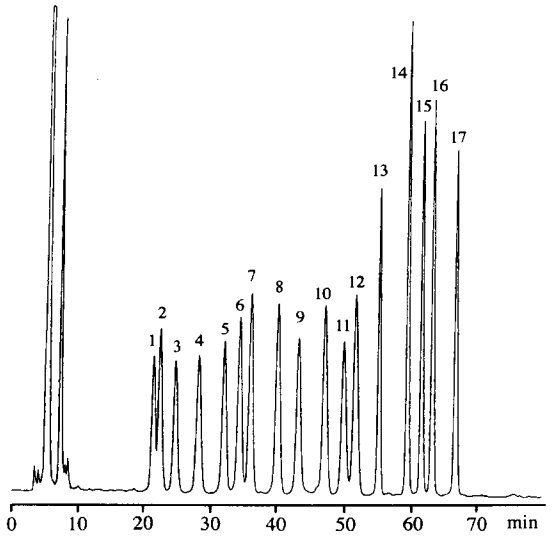


Fig. 2. Chromatogram of the fluorescence derivatives of standard bile acids. 1: (24*R*,25*S*)-VA, 2:(24*S*,25*R*)-VA, 3: (24*R*,25*R*)-VA, 4: (24*S*,25*S*)-VA, 5: CA, 6: (24*R*,25*S*)-DVA, 7: (24*S*,25*R*)-DVA, 8: (24*R*,25*R*)-DVA, 9: (24*S*,25*S*)-DVA. 10: (25*S*)-THCA, 11: (25*R*)-THCA, 12: CDCA, 13: 24*E*-THCA, 14: Internal standard, 15: (25*S*)-DHCA, 16: (25*R*)-DHCA, 17: 24*E*-DHCA.

recovered bile acids were labeled with MPAC-Br, and the fluorescent labeled derivatives were injected into HPLC system passing through a silica gel column as described above. Table 2 shows the relative recoveries of bile acids at two different concentrations. The relative recoveries ranged from 96 to 107% at higher concentration and from 94 to 108% at lower concentration, respectively. It is noted that there were no interfering endogenous bile acids in an LM fraction except the trace amount of CA, which could be corrected by a blank test.

### *3.2. Application for the determination of $C_{27}$-bile acids formed with rat liver light mitochondrial fraction*

The determination of the stereochemistry of 24-hydroxylated bile acids (VA and DVA) and quantitative determination of the products in $\beta$-oxidation were carried out by the above method. A partially purified LM fraction was prepared as a peroxisome enriched fraction and used for the incubating experimental. Since the initial formation of CoA esters of $C_{27}$-bile acids required for proceeding a peroxisomal $\beta$-oxidation, CoA esters of THCA, DHCA 24*E*-THCA and 24*E*-DHCA were prepared by the modification of reported procedure [18]. And these CoA esters were used as the substrates for the incubation with LM fraction. The reaction conditions were set to convert about 10% of substrates into the total products. Fig. 3a shows the chromatogram of incubating mixture of 25*R*-THCA CoA ester as a substrate with LM fraction. The used substrate was stereochemically pure 25*R*-THCA, however, the formation of 25*S*-THCA was observed by the epimerization as a reported result [19]. This enzymatic epimerization was also confirmed by the incubation of 25*R*-THCA CoA ester with heat-treated LM fraction to give 25*R*-THCA as a sole component. In the chromatogram, 24*E*-THCA was determined as a major intermediate. The three isomers of VA (24*R*,25*S*-, 24*R*,25*R*-, 24*S*,25*S*-VA) could be quantified and the amount of 24*R*,25*R*-isomer was much higher than that of other isomers. The final side chain degradation product, CA was also determined. Fig. 3b showed the result of incubating 25*R*-DHCA CoA ester with LM fraction. It also showed the formation of epimeric 25*S*-DHCA, and 24*E*-DHCA was a major product similar to the above result. The four stereoisomers of DVA were detected, and the 24*R*,25*R*-isomer was predominant among them. In these case, it is considered that VA and DVA were produced from CoA esters of THCA and DHCA, respectively, by the two step enzymatic reactions (dehydrogenation with acyl CoA oxidase and hydration with acyl CoA hydratase). Moreover, the partially purified LM fraction used in the experiment may be contaminated with mitochondrial and microsomal fractions. So it is somewhat complicated to elucidate the stereochemistry of intermediates produced by peroxisomal $\beta$-oxidation. The direct formation of VA or DVA by hydration was, therefore, investigated using CoA esters of 24*E*-THCA and 24*E*-DHCA. The results of the incubation of 24*E*-THCA and 24*E*-DHCA CoA esters with LM fraction showed the formation of the isomers of VA and DVA similar to the above results.

Table 2
Relative recoveries of the $C_{27}$-bile acids and related compounds

| | Recovery (%, mean ± S.D., $n = 3$)[a] | |
|---|---|---|
| Bile acid | Added 0.1 μg[b] | Added 1 μg[b] |
| (24*R*,25*S*)-VA | 95.1 ± 3.2 | 96.2 ± 4.0 |
| (24*S*,25*R*)-VA | 101.3 ± 2.0 | 98.3 ± 5.5 |
| (24*R*,25*R*)-VA | 94.3 ± 3.4 | 101.2 ± 7.2 |
| (24*S*,25*S*)-VA | 107.6 ± 4.4 | 103.4 ± 5.3 |
| CA | 98.6 ± 4.9 | 97.8 ± 4.0 |
| (24*R*,25*S*)-DVA | 96.7 ± 8.5 | 96.1 ± 6.6 |
| (24*S*,25*R*)-DVA | 98.0 ± 9.2 | 99.0 ± 2.7 |
| (24*R*,25*R*)-DVA | 98.4 ± 5.8 | 102.1 ± 7.5 |
| (24*S*,25*S*)-DVA | 105.4 ± 6.3 | 100.8 ± 6.7 |
| (25*S*)-THCA | 101.3 ± 7.4 | 98.9 ± 3.9 |
| (25*R*)-THCA | 105.1 ± 8.1 | 102.3 ± 6.2 |
| CDCA | 104.3 ± 4.5 | 101.0 ± 3.3 |
| 24*E*-THCA | 106.7 ± 6.9 | 106.5 ± 7.4 |
| (25*S*)-DHCA | 104.6 ± 9.7 | 101.5 ± 6.1 |
| (25*R*)-DHCA | 102.3 ± 9.2 | 99.3 ± 7.6 |
| 24*E*-DHCA | 99.6 ± 8.9 | 98.6 ± 6.1 |

[a]Relative recovery to the 0.25 μg of the added internal standard.
[b]Added amounts of bile acids.

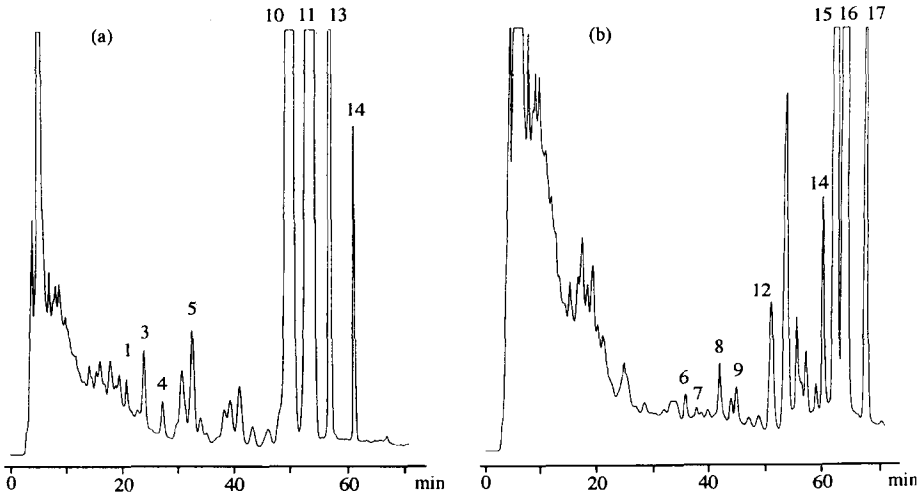


Fig. 3. Chromatograms of the incubating mixture of (25*R*)-3α, 7α, 12α-trihydroxy-5β-cholestan-26-oic acid CoA ester (a) and (25*R*)-3α, 7α-dihyroxy-5β-cholestan-26-oic acid CoA ester (b) with rat liver light mitochondrial fraction. (a) 1: (24*R*,25*S*)-VA, 3: (24*R*,25*R*)-VA, 4: (24*S*,25*S*)-VA, 5: CA, 10: (25*S*)-THCA, 11: (25*R*)-THCA, 13: 24*E*-THCA, 14: Internal standard. (b) 6: (24*R*,25*S*)-DVA, 7: (24*S*,25*R*)-DVA, 8: (24*R*,25*R*)-DVA, 9: (24*S*,25*S*)-DVA, 12: CDCA, 14: Internal standard, 15: (25*S*)-DHCA, 16: (25*R*)-DHCA. 17: 24*E*-DHCA.

The results of the quantitative determination of intermediates were summarized in Fig. 4. Both 25*R*- and 25*S*-isomers of THCA gave 24*E*-THCA at almost the same rate (Fig. 4c). This seemed to be non-stereoselectivity of the oxidase, however, in the present work it is uncertain that the enzyme can distinguish the stereochemistry of the substrate or not, because of the fast enzymatic epimerization (CoA ester) as described above. The similar results were obtained when the isomers of DHCA were incubated with LM fraction (Fig. 4c). As shown in Fig. 4a, the formation ratios of each isomeric VA from 25*R*-THCA were almost the same as those from 25*S*-THCA. This may also due to the initial epimerization of THCA CoA ester. The predominant isomer of VA was 24*R*,25*R*-isomer and the significant amount of 24*S*,25*S*-isomer was determined. In the case of DHCA (Fig. 4b), the same results were obtained. The order of the formation of epimeric VA was 24*R*,25*R* > 24*R*,25*S* and of epimeric DVA was 24*R*,25*R* > 24*S*,25*S* > 24*R*,25*S* > 24*R*,25*R*-isomer. Although the 24*S*,25*R*-VA could not be determined, these results indicated that both stereoisomeric THCA and DHCA were converted into VA and DVA through the same reaction pathway.

When 24*E*-THCA or 24*E*-DHCA CoA ester was used as a substrate, the same isomers of VA or DVA were produced as in the above case. However, the formation ratios of 24*R*,25*R*- and 24*S*,25*S*-isomers were increased (Fig. 4a, b). This seemed that the formation of these isomers were the results of the *cis*-addition of water to 24*E*-intermediates by the catalytic reaction with acyl CoA hydratase. The mechanism of the formation of other isomers (24*R*,25*S*- and 24*S*,25*R*-isomers) were not clear. The possibility of producing the above isomers is the epimerization of VA-CoA esters similar to THCA-CoA ester. However, the epimerization of VA CoA esters were not observed under the incubating conditions with or without LM fraction. Another possibility is the result of the contaminated other fractions which catalyze the *trans*-hydration of water to 24*E*-THCA.

In conclusion, the present method is very useful for the study of bile acid biosynthesis, especially, the study for the stereochemistry of intermediates in biosynthesis of bile acids. It is noted that the

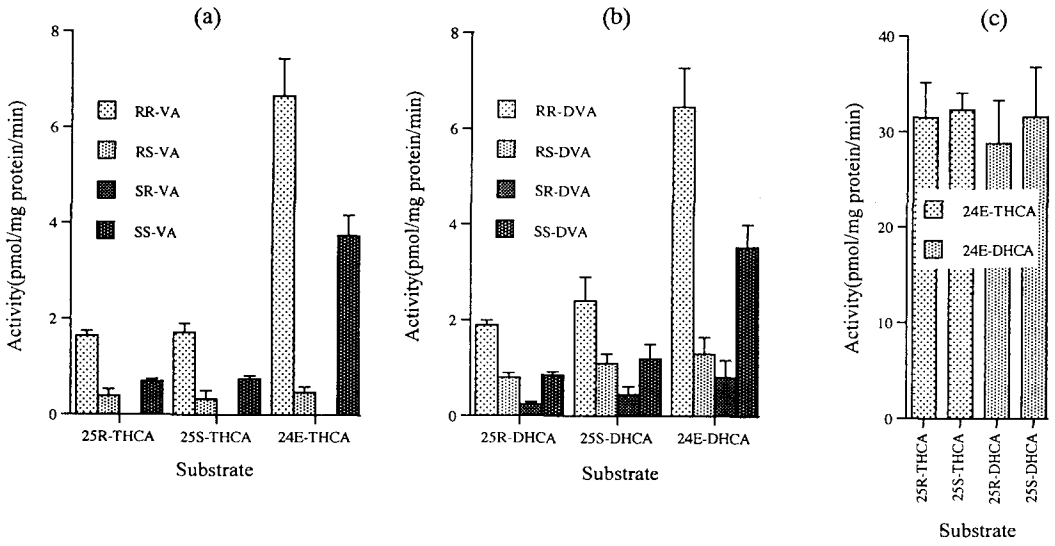


Fig. 4. Formation of stereoisomeric 3α,7α,12α,24-tetrahydroxy- and 3α, 7α,24-trihydroxy-5β-cholestan-26-oic acids (VA and DVA) in rat liver light mitochondrial fraction. (a) Formation of (24*R*,25*R*)-VA (▨). (24*R*,25*S*)-VA (▩), and (24*S*,25*S*)-VA (■). (b) Formation of (24*R*,25*R*)-DVA (▨), (24*R*,25*S*)-DVA (▩), (24*S*,25*R*)-DVA (■) and (24*S*,25*S*)-DVA (■). (c) Formation of 24*E*-THCA (▨) and 24*E*-DHCA (▩).

24-hydroxylated intermediates for the biosynthesis of CA and CDCA were predominantly produced by the *cis*-addition of water to 24*E*-intermediates to give 24*R*,25*R*- and 24*S*,25*S*-isomers. The differences of formation ratio of these two isomers may reflect the sensitivity of reaction of the enzyme, if these isomers were converted into CA at the same velocity.

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