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Sensitive analysis of serum 3α, 7α, 12α,24-tetrahydroxy-5β-cholestan-26-oic acid diastereomers using gas chromatography–mass spectrometry and its application in peroxisomal D-bifunctional protein deficiency

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Abstract **The final steps in bile acid biosynthesis take place in peroxisomes and involve oxidative cleavage of the side chain of C_{27}-5β-cholestanoic acids leading to the formation of the primary bile acids cholic acid and chenodeoxycholic acid. The enoyl-CoA hydratase and β-hydroxy acyl-CoA dehydrogenase reactions involved in the chain shortening of C_{27}-5β-cholestanoic acids are catalyzed by the recently identified peroxisomal D-bifunctional protein. Deficiencies of D-bifunctional protein lead, among others, to an accumulation of 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid (varanic acid). The ability to resolve the four C_{24}, C_{25} diastereomers of varanic acid has, so far, only been carried out on biliary bile acids using *p*-bromophenacyl derivatives. Here, we describe a sensitive gas chromatography–mass spectrometry (GC/MS) method that enables good separation of the four varanic acid diastereomers by use of 2R-butylester-trimethylsilylether derivatives. This method showed the specific accumulation of (24R,25R)-varanic acid in the serum of a patient with isolated deficiency of the D-3-hydroxy acyl-CoA dehydrogenase part of peroxisomal D-bifunctional protein, whereas this diastereomer was absent in a serum sample from a patient suffering from complete D-bifunctional protein deficiency. In samples from both patients an accumulation of (24S,25S)-varanic acid was observed, most likely due to the action of L-bifunctional protein on Δ24E-THCA-CoA. This GC/MS method is applicable to serum samples, obviating the use of bile fluid, and is a helpful tool in the subclassification of patients with peroxisomal D-bifunctional protein deficiency.—**Vreken, P., A. van Rooij, S. Denis, E. G. van Grunsven, D. A. Cuebas, and R. J. A. Wanders. **Sensitive analysis of serum 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid diastereomers using gas chromatography–mass spectrometry and its application in peroxisomal D-bifunctional protein deficiency. *J. Lipid Res.* 1998.** 39: **2452–2458.**

Supplementary key words peroxisome • bile acids • D-bifunctional protein • GC/MS

Peroxisomes play an important role in the biosynthesis of C_{24} bile acids from cholesterol. The process of chain shortening of C_{27} bile acids is believed to occur via β-oxidation of 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA (THCA-CoA) (1–4). As in mitochondrial β-oxidation, peroxisomal β-oxidation of bile acid precursors proceeds in four sequential steps. The first step is catalyzed by a distinct acyl-CoA oxidase (5–13) whereby THCA-CoA is converted into a α,β-enoyl CoA ester (24E-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA) which is subsequently hydrated in the second reaction forming a β-hydroxyacyl-CoA (3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA) (14–20). In the third reaction, the β-hydroxyacyl-CoA is dehydrogenated into a β-ketoacyl-CoA which is thiolytically cleaved in the fourth reaction (21, 22). The second and third reactions are catalyzed by a multifunctional protein, whereas the last step is carried out by a β-ketoacyl-CoA thiolase (21).

Recently, it has been demonstrated that there are at least two peroxisomal multifunctional proteins: L-bifunctional protein (MFE-I), which produces L-β-hydroxy-acyl-CoAs and is involved in the chain shortening of straight chain fatty acids, and D-bifunctional protein (MFE-II), which is involved in the degradation of branched chain fatty acids and bile acid intermediates (23). In the process of cholic acid biosynthesis, the hydration of 24E-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA by peroxisomal MFE-II yields specifically (24R,25R)-3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA which has been shown to accumulate in patients with D-bifunctional protein defi-

Abbreviations: THCA, 3α,7α,12α-trihydroxy-5β-cholest-26-oic acid; varanic acid, 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid; Δ24-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-ene-26-oic acid; MFE I, L-bifunctional protein, MFE II, D-bifunctional protein; MeSil, Methylester-trimethylsilylether; 2RButSil, 2R-butylester-trimethylsilylether; HPLC, high performance liquid chromatography; GC/MS, gas chromatography–mass spectrometry.

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ciency (24, 25). However, L-bifunctional protein also acts on 24E-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA forming the (24S,25S) isomer. The latter compound cannot, however, be dehydrogenated further by MFE-I, suggesting that MFE-I is most likely not involved in bile acid synthesis (26, 27).

The human MFE-II gene encodes a protein consisting of multiple domains: the N-terminal part of the protein harbors the β-hydroxyacyl-CoA dehydrogenase activity, the central part encodes the α,β-enoyl-CoA hydratase activity, and the C-terminal part contains a sequence showing high homology with sterol carrier protein-2 (28). Depending on the localization of the molecular defect, patients with D-bifunctional protein deficiency can be classified into three categories: patients with a complete deficiency of both the β-hydroxyacyl-CoA dehydrogenase and the α,β-enoyl CoA hydratase activity and patients with distinct deficiencies of either the β-hydroxyacyl-CoA dehydrogenase or α,β-enoyl CoA hydratase activity (29, 30). Recently, we identified a patient with isolated D-β-hydroxyacyl-CoA dehydrogenase deficiency due to a G16S missense mutation in the peroxisomal D-bifunctional protein (30).

In the present study we analyzed serum varanic acid profiles in this patient and in a patient with complete D-bifunctional protein deficiency to test whether these two defects could be discriminated based upon serum varanic acid analysis.

For this purpose we have developed a sensitive GC/MS method that enables the separation of the four C_{24}, C_{25} diastereomers of 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid in serum.

MATERIALS AND METHODS

Subjects

Patient V. suffered from an isolated deficiency of D-3-hydroxyacyl CoA dehydrogenase due to a G16S missense mutation and has been fully described elsewhere (30). Patient T. presented with craniofacial dysmorphism, slight hepatomegaly, and hypotonia. Biochemical studies in serum revealed highly abnormal very long chain fatty acids, an elevated pristanic acid concentration, abnormal serum bile acids, and normal erythrocyte plasmalogens. Studies in fibroblasts indicated that the patient suffered from a complete deficiency of peroxisomal D-bifunctional protein (Table 1) and showed an absence of immunoreactive D-bifunctional protein using the antibody prepared by Jiang et al. (15) (data not shown). Clinical details of this patient will be published elsewhere. Enzymatic studies were carried out as described previously (30).

Materials and reference compounds

A mixture of the C_{24}, C_{25} diastereomers of 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid (varanic acid) was synthesized as described previously (31), and their CoA esters were synthesized by the mixed anhydride method (27). Acetyl chloride was purchased from Merck, Darmstadt, Germany; R(−)-2-butanol was purchased from Fluka Chemie AG, Buchs, Switzerland; N-trimethylsilylimidazole (TMSI) was purchased from Pierce, Rockford, IL, and methanolic-HCl-3N was obtained from Supelco, Bellefonte, PA.

Fractionation of 3α,7α,12α,24-tetrahydroxy-5β-cholestanoyl-CoA diastereomers by HPLC

The CoA esters of diastereomeric 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid were separated by HPLC exactly as described in detail (16) to guarantee the correct elution order of the diastereomers. Briefly, samples were applied to a Mightysil 18 column (4 × 250 mm) with a flow rate of 1 ml/min using solvent A: 30 mM sodium acetate (pH 5)–methanol–acetonitrile 40:40:20 and solvent B: 30 mM sodium acetate (pH 5)–acetonitrile 50:50 in a linear gradient (from A/B = 90/10 to A/B = 5/95 over 40 min). Fractions containing the separated CoA esters of 3α,7α, 12α,24-tetrahydroxy-5β-cholest-26-oic acid diastereomers (elution order: 24S,25R-, 24R,25S-, 24S,25S-, and 24R,25R-isomer) were collected, evaporated, and subsequently incubated for 15 min at room temperature in 1 ml 0.3 N NaOH to hydrolyze the CoA esters. The free bile acids were isolated by acidification with 100 μL 25% HCl followed by two extractions with diethylether. GC/MS analysis of the diastereomers after derivatization with 2RbutSil yielded a distinct major peak for each diastereomer, thereby establishing that isomerization of the diastereomers did not take place during saponification and derivatization (see below). Recoveries were >95% (n = 3, tested with cholyl-CoA (data not shown)).

Serum sample preparation

Extraction of the unconjugated bile acids from serum was carried out essentially according to Setchell and Worthington (32) with slight modifications. Briefly, 100–300 μl serum was diluted 4 times with 0.1 N NaOH and heated to 64°C for 15 min before application to a prewashed octadecyl cartridge. After washing with 5 ml water, bile acids were eluted with 5 ml methanol and evaporated to dryness. The conjugated and unconjugated bile acids were redissolved in 0.1 N NaOH and extracted twice with hexane in order to remove most of the cholesterol. The resulting aqueous layer was acidified with 100 μl of 25% HCl. Free bile acids were extracted twice with 2 ml diethylether and subsequently converted to their MeSil or 2RbutSil derivatives (see below).

Derivatization

Methylester-trimethylsilylethers (MeSil). Isolated bile acids were allowed to react with 100 μl of 3 N methanolic HCl for 15 min at room temperature, evaporated, and subsequently allowed to react with TMSI for 15 min at room temperature.

2R-butylester-trimethylsilylethers (2RButSil). Isolated bile acids were allowed to react with 100 μL of a freshly prepared solution of 2R-butanolic-HCl (10% acetyl chloride in 2R-butanol) for 4 h at 50°C which resulted in complete derivatization. After the solvent was evaporated, the samples were reacted with TMSI for 15 min at room temperature. Aliquots of the derivatized bile acids were subjected to GC/MS procedures as described below.

Gas chromatograpy–mass spectrometry (GC/MS) procedures

A Hewlett-Packard model 5890A gas chromatograph equipped with a split/splitless injector was used for all separations. The chromatographic column consisted of a chemically bonded capillary fused silica CP-Sil 5CB-MS column (25 m × 0.25 mm ID × 0.25 μm DF, Chrompack the Netherlands). Helium was used as carrier gas and GC operating conditions were as follows. Injector temperature was set at 290°C, column inlet pressure was set at 155 Kpa. After splitless injection (splitless time 2 min), oven temperature was maintained at 160°C for 5 min, then increased at a rate of 20°C/min to a temperature of 300°C, kept there for 10 min and subsequently increased at a rate of 5°C/min to a final temperature of 320°C. MS (Hewlett-Packard 5970) interface temperature was set at 320°C. Mass spectrometry (electron impact,

TABLE 1. Analysis of the hydratase and dehydrogenase components of D-bifunctional protein in fibroblasts from patients V. and T.

Component	Enzyme Activity		
	Patient V.	Patient T.	Controls (n = 10)
	nmol/min/mg protein		
Enoyl-CoA hydratase	0.45	nd	0.28 ± 0.02
D-3-hydroxyacyl CoA dehydrogenase	nd	nd	0.11 ± 0.02

Control values represent mean ± 1 SD; nd, not detectable.

70 eV) was operated using both total ion current (range (60 m/z − 635 m/z) and selective ion monitoring mode (2.2 cycles/sec).

RESULTS

In two patients with D-bifunctional protein deficiency, detailed enzymatic and molecular analysis revealed that patient V. suffered from isolated D-3-hydroxyacyl-CoA dehydrogenase deficiency due to a G16S missense mutation in the MFE-II gene (30), whereas patient T. suffered from a combined deficiency of both the D-3-hydroxyacyl-CoA dehydrogenase and D-2-enoyl-CoA hydratase activities due to as yet unknown mutation(s) in the MFE-II gene (**Table 1**). Analysis of serum bile acids as their MeSil derivatives revealed an accumulation of C_{27}-bile acids in both patients. In both samples, a peak corresponding to 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid (isomer-I according to Clayton et al. (22)) could be clearly identified (data not shown). This GC/MS method, however, does not discriminate among the four diastereomers of 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid (**Fig. 1A**) and did not allow us to draw a conclusion about which diastereomers accumulated in each patient. As the commonly used HPLC analysis for separation of the four diastereomers of 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid as their *p*-bromophenacyl esters requires a substantial amount of serum or bile fluid (16, 33), we decided to modify the GC/MS procedure in order to achieve a good separation of the four diastereomers with the use of a minimum amount of serum.

It has been known that rigorous alkaline hydrolysis of samples causes loss of stereospecificity at the C_{24} and C_{25} chiral centers (34; A. van Rooij, unpublished data). In addition, varanic acid in serum is mainly present in its unconjugated form (>85%) as judged from analysis before and after enzymatic and/or alkaline deconjugation (22, 35; A. van Rooij, unpublished data). Therefore, we decided to use only the unconjugated bile acid fraction, to avoid rigorous alkaline hydrolysis. The newly developed method included a carboxyl derivatization with 2R-butanol (2RbutSil derivatives, see Materials and Methods) to produce an additional chiral center near the carboxyl end of the bile acid that would enhance the chromatographic selectivity of the C_{24},C_{25} diastereomers of 3α,7α,12α,24-tetrahydroxy-5β-cholest-26-oic acid. The results show that this alternative derivatization resulted in good separation among the four diastereomers (Fig. 1B). The mass spectra of the four diastereomers were identical and in good agreement with the mass spectra described in the literature for MeSil derivatives, taking into account the increase in side-chain mass fragments (+42) due to using 2RbutSil derivatives instead of MeSil derivatives (**Fig. 2**).

In order to establish the retention times and elution order for the four diastereomers, the HPLC fractions containing either (24R,25R-), (24R,25S-), (24S,25S-), or (24S, 25R-) CoA esters were hydrolyzed, derivatized (2RbutSil), and analyzed separately using the modified GC/MS procedure. We have used the same HPLC column and elution conditions as previously reported (16), where the elution order of the four diastereomeric CoA esters was independently determined and found to agree with the original assignments given by Xu and Cuebas (27). **Figure 3** shows that each of the four diastereomers yielded a distinct major peak allowing unequivocal assignment of the configuration in the GC/MS procedure.

Analysis of serum unconjugated bile acids as 2RbutSil derivatives (**Fig. 4**) showed that in patient V (isolated D-3-

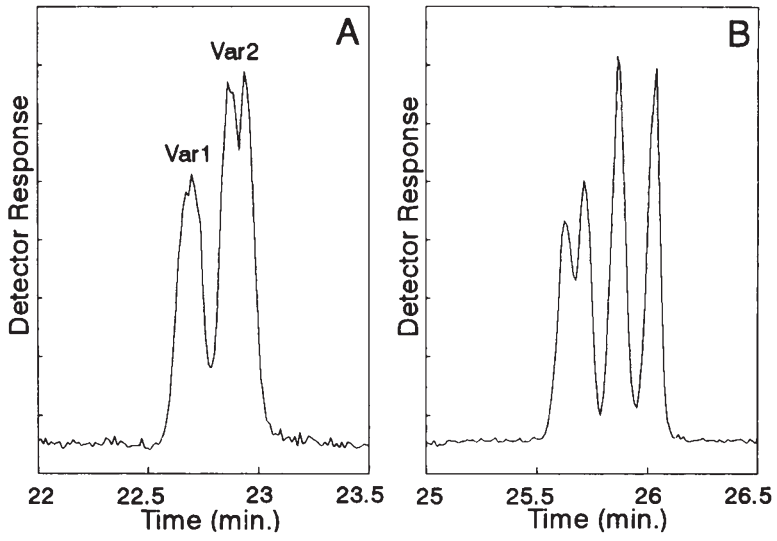

Fig. 1. Separation of varanic acid diastereomers. Diastereomeric varanic acid was analyzed after MeSil derivatization (A) or 2RbutSil derivatization (B) as described in Materials and Methods. Mass spectra of each peak were recorded (see Fig. 2). Panel A shows three peaks corresponding to Var I and Var II isomers according to Clayton et al. (22). Panel B shows four peaks representing the four varanic acid diastereomers.

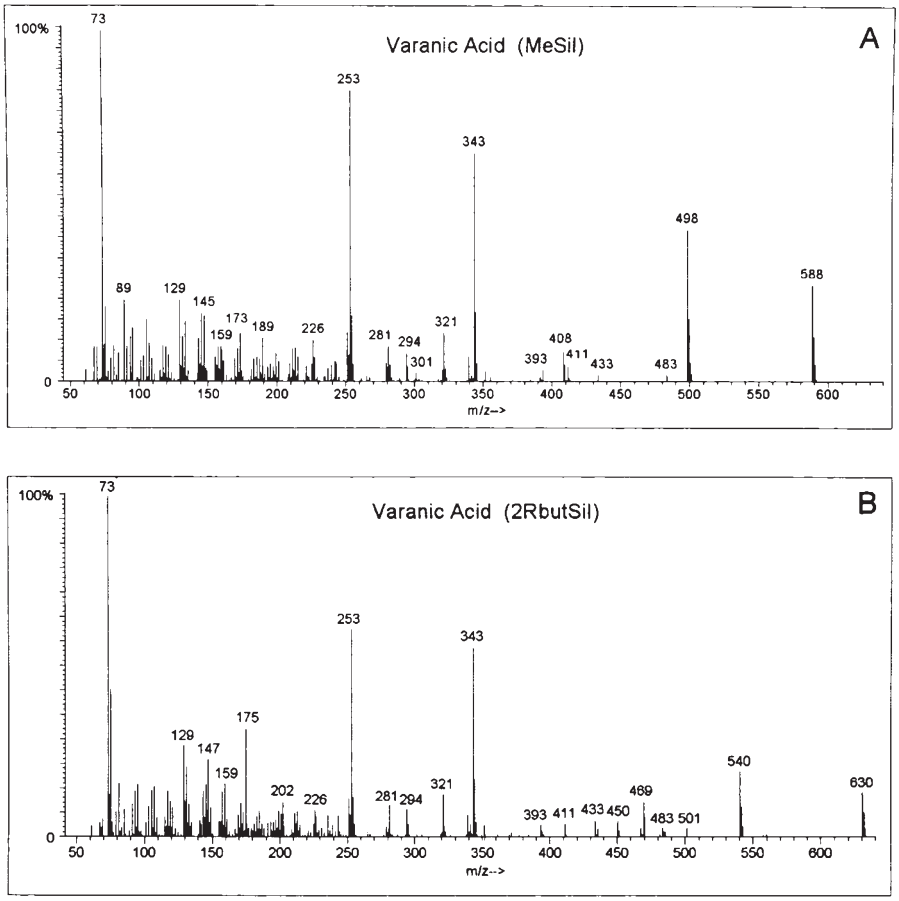

Fig. 2. Mass-spectrometric data of varanic acid diastereomers. Mass fragmentograms after MeSil derivatization (A) or 2RbutSil derivatization (B) of varanic acid diastereomers were recorded as described in Materials and Methods. Identical spectra for Var I and II were obtained in agreement with the literature (36) in panel A. Panel B represents the mass fragmentogram (identical for all four diastereomers) of 2RbutSil derivatized varanic acid. Note the +42 mass shift for fragments 408→450, 498→540 and 588→630 due the mass difference between 2RbutSil and MeSil derivatives in the side chain.

hydroxyacyl-CoA dehydrogenase deficiency) there was an accumulation of (24R,25R-) and, to a lesser extent, (24R,25S-), and (24S,25S-) varanic acid, whereas in patient T. (complete D-bifunctional protein deficiency) an almost exclusive accumulation of (24S,25S-) varanic acid was observed. Both patients accumulated small amounts of Δ24E-THCA, which elutes just before the 24R, 25R varanic acid diastereomer in this method (Fig. 4). The position and identity of this peak was verified by injecting chemically synthesized and purified Δ24E-THCA (27). Δ24Z-THCA could not be detected in the patient samples, whereas THCA and DHCA were present in substantial amounts in the patient samples. The latter compounds coelute at a retention time of 23.8 min and do not interfere in the region of interest of the chromatogram where the varanic acid acid diastereomers are located (data not shown). Two small peaks corresponding to 25-hydroxy-THCA and 26-hydroxy THCA were present at retention times 26.8 and 27.6 min, respectively (data not shown), indicating that analogous to previously described GC/MS methods using MeSil derivatives, these compounds do not interfere in the positions were the varanic acid diastereomers are located (22, 35). Moreover, as these compounds do not form the characteristic *m/z* 321 ion, they can be easily discriminated from the varanic acid diastereomers (36). Intermediates in the β-oxidation of DHCA like Δ24(E/Z)-DHCA or 24-hydroxy-DHCA cannot be detected using the multiple ion monitoring used (see legend of Fig. 4) as these compounds will produce different ions due to the presence of only two hydroxy groups at the steroid skeleton (36). Total ion current traces of the patient samples failed to detect peaks with mass spectra that could be derived from either Δ24(E/Z)-DHCA or 24-hydroxy-DHCA.

DISCUSSION

Peroxisomes play an important role in the oxidative cleavage of the side chains of C_{27} intermediates in the biosynthesis of bile acids. Hence, it is well known that these C_{27} bile-acids accumulate in several peroxisomal disorders, including defects in peroxisomal biogenesis and isolated defects in peroxisomal beta-oxidation (1–4, 33, 35,

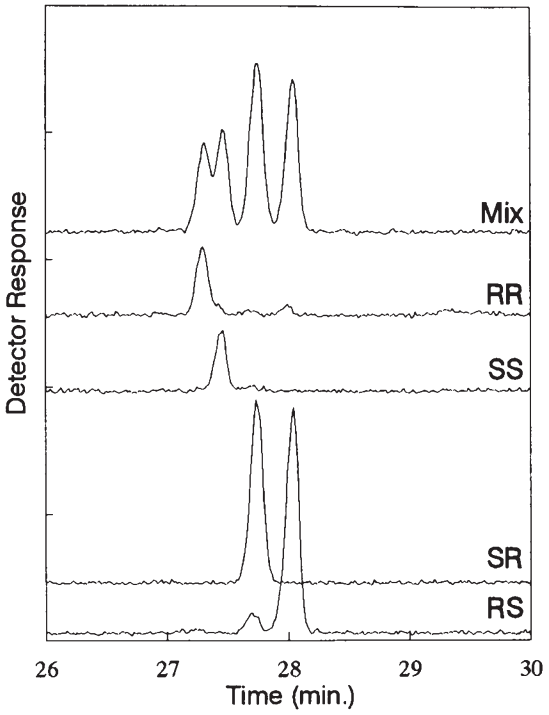

Fig. 3. Characterization of varanic acid diastereomers as 2RbutSil derivatives. Diastereomeric varanic acid (mix) and separated varanic acid diastereomers (24R, 25R), (24S, 25S), (24S, 25R) and (24R, 25S) were isolated from HPLC fractions and derivatized as described in Materials and Methods and subjected to GC/MS analysis.

37). However, a total absence of mature C_{24} bile acids like cholic acid and chenodeoxycholic acid has never been demonstrated in any of the peroxisomal disorders, suggesting that at least a small part of the C_{27} bile acid precursors can be metabolized in these disorders (26, 27, 33, 38).

Detailed analysis of serum bile acid profiles can provide a clue to the localization of the peroxisomal defect, as different profiles are observed in biogenesis defects and isolated defects in peroxisomal D-bifunctional protein (22, 33, 35, 37). In this paper we show that using a sensitive GC/MS analysis, a subclassification of defects in peroxisomal D-bifunctional protein is feasible. We show that a patient with an isolated defect in the 3-hydroxyacyl CoA dehydrogenase part of D-bifunctional protein mainly accumulates (24R,25R-) and (24R,25S-) varanic acid, whereas a patient with a complete D-bifunctional protein deficiency does not. The latter patient almost exclusively accumulates (24S,25S)-varanic acid. This observation agrees with the hypothesis that in addition to D-bifunctional protein, peroxisomal L-bifunctional protein exhibits affinity for the precursor Δ24E-THCA-CoA, specifically leading to the formation of (24S,25S)-varanic acid (26, 27). In patient V., the D-enoyl-CoA hydratase activity is normally functional leading to the formation of the (24R,25R)-varanic acid, which cannot be metabolized further as the D-3-hydroxyacyl CoA dehydrogenase activity is lacking. In patient T., the complete D-bifunctional protein is missing, thus leading to the accumulation of THCA-CoA and Δ24E-THCA-CoA. The latter compound is mainly converted to the (24S,25S) diastereomer catalyzed by the enoyl-CoA hydratase activity of L-bifunctional protein (27). Because the (24S,25S) diastereomers also accumulated to some extent in patient V., it is reasonable to assume that the latter reaction also occurs in the presence of a normally functional D-enoyl-CoA hydratase activity. The results reported here agree with a report by Clayton et al. (22) that also shows the accumulation of both (24R) and (24S) varanic acid isomers, although they considered these to be formed from the reduction of 24-oxo-THCA. Recently, a patient suspected to suffer from complete D-bifunctional protein deficiency, as concluded from the absence of immunoreactive D-bifunctional protein, was re-

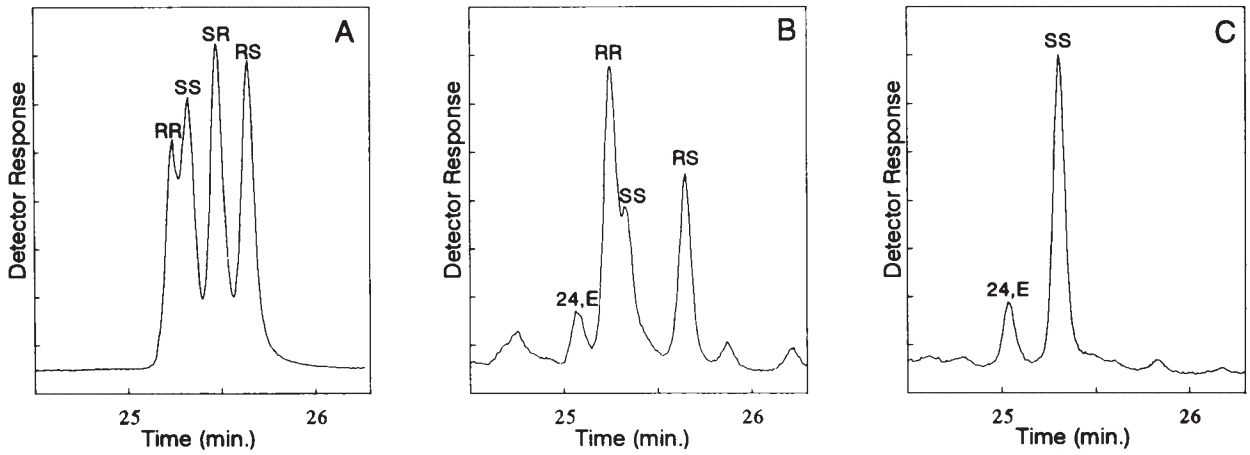

Fig. 4. Analysis of varanic acid diastereomers in patient serum samples. Serum samples were pretreated and derivatized as described in Materials and Methods. GC/MS procedures were carried out using multiple ion groups specific for the components of interest. A serum sample spiked with diastereomeric varanic acid was analyzed before in-between samples to allow accurate determination of retention times. Due to a unknown interference coeluting with (24S,25R)-varanic acid, only the low intensity but selective (see Fig. 2) ions for varanic acid diastereomers and Δ24-THCA (m/z = 321, 450, 540 and 630) were used. Panel A: varanic acid mix; Panel B: patient V. (isolated 3-hydroxyacyl CoA dehydrogenase deficiency); Panel C: patient T. (complete D-bifunctional protein deficiency). For explanation, see text.

ported (25, 33). This patient has previously been described as patient 2 in Suzuki et al. (39). Analysis of unconjugated biliary bile acids mainly showed accumulation of (24R,25R) and (24R,25S)-varanic acid diastereomers in this case (33). This accumulation was explained by assuming direct 24R hydroxylation of (R,S)-THCA, which has also been demonstrated in *Bombina orientalis* (40). It is clear that these observations are different from the observations in the serum sample of patient T.

Although it is difficult to explain these differences, one should realize that in the reported case biliary bile acid analysis was carried out, while in this report the analysis was carried out in serum samples that could differ in the relative composition of free versus conjugated bile acids. In both reports the analysis was performed on a single sample, which could suggest that the relative contribution of direct R-hydroxylation and action of L-bifunctional protein shows considerable intra-individual variation. It would be interesting to analyze more samples from different patients with either complete D-bifunctional protein deficiency or isolated D-3-hydroxy-acyl CoA dehydrogenase deficiency in order to test whether each group of patients shows a characteristic serum bile acid profile. The method described in this paper uses only a small amount of sample, which should allow this analysis to be performed in each patient suspected to suffer from either defect.

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