Pristanic acid and phytanic acid in plasma from patients with peroxisomal disorders: stable isotope dilution analysis with electron capture negative ion mass fragmentography

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Abstract A sensitive and selective stable isotope dilution method was developed for the accurate quantitation of pristanic acid and phytanic acid using electron capture negative ion mass fragmentography on pentafluorobenzyl derivatives. This technique allows detection of 1 pg of each compound and was applied to plasma from healthy controls and patients suffering from various peroxisomal disorders. The age-dependency of phytanic and pristanic acid levels in plasma from healthy controls was demonstrated. The involvement of peroxisomes in the β-oxidation of pristanic acid was concluded from its accumulation in plasma from patients with peroxisomal deficiencies. Pristanic acid/phytanic acid ratios were markedly increased in bifunctional protein and/or 3-oxoacyl-CoA thiolase deficiency, indicating their role in the (differential) diagnosis of disorders of peroxisomal β-oxidation. — **ten Brink, H.J., F. Stellaard, C.M.M. van den Heuvel, R.M. Kok, D.S.M. Schor, R.J.A. Wanders, and C. Jakobs.** Pristanic acid and phytanic acid in plasma from patients with peroxisomal disorders: stable isotope dilution analysis with electron capture negative ion mass fragmentography. *J. Lipid Res.* 1992. **33:** 41–47.

Supplementary key words phytanic acid α-oxidation • pristanic acid β-oxidation • pentafluorobenzyl esters

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a branched-chain fatty acid that can efficiently be degraded in healthy human beings. Its metabolic pathway is believed to involve an initial α-hydroxylation, followed by decarboxylation to yield pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) which can be degraded via subsequent cycles of β-oxidation (**Fig. 1**). Endogenous de novo biosynthesis of phytanic acid in humans has not been demonstrated. Its presence mainly originates from dietary intake of the substance itself, while conversion of dietary phytol as free alcohol rather than in a chlorophyll-bound form can account for some additional amounts of phytanic acid (1).

Phytanic acid was long thought to accumulate only in plasma and tissues from patients with classic Refsum's disease where the α-hydroxylation step is deficient (1). However, in recent years it has become clear that phytanic acid is also elevated in patients with a deficiency of morphologically distinguishable peroxisomes, i.e., Zellweger syndrome, infantile Refsum's disease (IRD), and neonatal adrenoleukodystrophy (NALD) (see (2) and (3) for reviews), and in patients with rhizomelic chondrodysplasia punctata (RCDP) (4). In all these cases a concomitant impairment of phytanic acid α-oxidation was measured.

Recently, Poulos et al. (5) reported that there is not only accumulation of phytanic acid but also of pristanic acid in plasma from patients with a generalized peroxisomal dysfunction. In Zellweger fibroblasts the oxidation of pristanic acid was found to be decreased (6). Furthermore, pristanoyl-CoA oxidase activity was identified in human liver and appeared deficient in the Zellweger syndrome (7). These findings suggest the involvement of peroxisomes in pristanic acid β-oxidation, raising the need for a reliable method to measure also pristanic acid concentrations in body fluids as a tool in the (differential) diagnosis of peroxisomal disorders.

Abbreviations: IRD, infantile Refsum's disease; NALD, neonatal adrenoleukodystrophy; RCDP, rhizomelic chondrodysplasia punctata; VLCFA, very long chain fatty acids; PFB, pentafluorobenzyl; GC–MS, gas chromatography–mass spectrometry; ECNCI, electron capture negative chemical ionization; SIM, selected ion monitoring; PrA, pristanic acid; PhA, phytanic acid.

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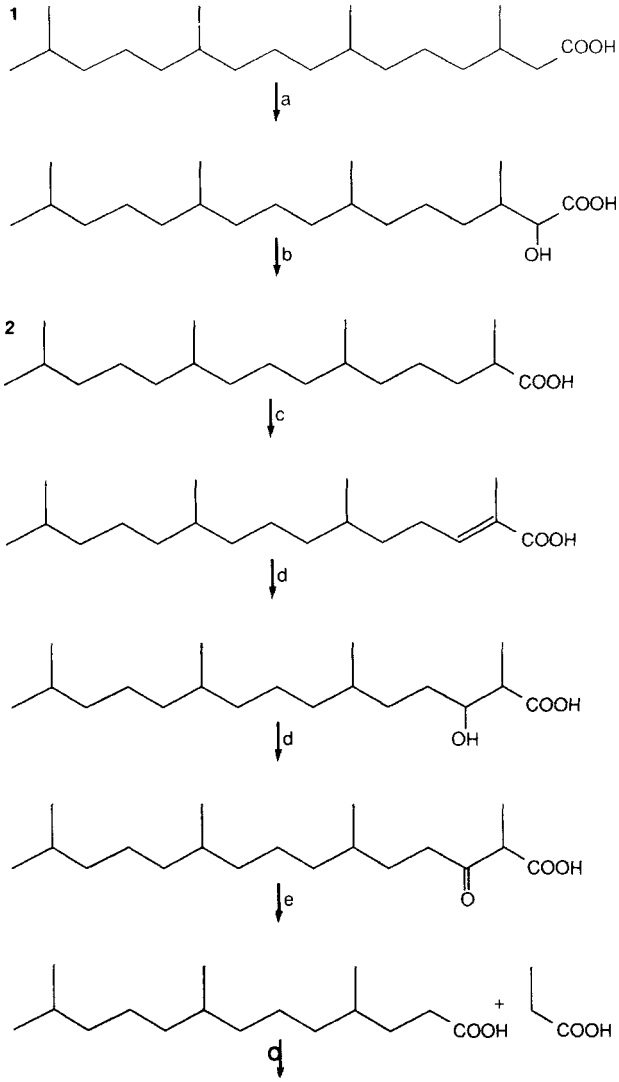

Fig. 1. Proposed pathway of phytanic acid (1) α-oxidation and pristanic acid (2) β-oxidation; a + b, phytanic acid α-oxidation; c, oxidase; d, bifunctional protein; e, thiolase.

Though application of gas chromatography—mass spectrometry has been reported for the quantitation of serum phytanic acid (8, 9), existing methods to determine plasma concentrations of pristanic acid and/or phytanic acid usually consist of a gas chromatographic assay with flame ionization detection of methyl esters, sometimes preceded by a thin-layer chromatographic purification step (10–14). Quantitation is done relative to an internal standard of which the extraction yield and detection efficiency inevitably differ from those of the analytes. These procedures suffice to detect elevated levels in plasma from patients who accumulate these substances. However, analysis in plasma from young infants, in whom dietary intake of these acids is still very limited, argues for a more sensitive quantitation technique. Moreover, calculation of pristanic acid / phytanic acid concentration ratios, which we recently introduced as an additional parameter for the diagnosis of defects in peroxisomal β-oxidation (15), demands determination of plasma concentration values for both acids with precision.

In this report we introduce a negative chemical ionization mass fragmentographic assay of pristanic acid and phytanic acid as their pentafluorobenzyl (PFB) esters. In combination with a stable isotopically labeled internal standard this provides a very sensitive, selective and accurate quantitation technique. The feasibility of the method is demonstrated by the analysis of pristanic acid and phytanic acid in plasma from patients suffering from different peroxisomal disorders and Refsum's disease, while the method enables us to obtain precise plasma values in healthy control infants as well.

PATIENTS

Plasma samples were analyzed from patients with various peroxisomal disorders which were divided into three groups according to Wanders et al. (3), depending upon whether a generalized, a multiple, or a single loss of peroxisomal functions was found. Diagnosis of peroxisomal disorders was based on clinical symptomatology and confirmed biochemically by multiple enzyme assays.

METHODS

Materials

Pristanic acid, phytanic acid, [2-methyl-$^2H_3$]pristanic acid (99 atom % $^2H_3$) and [3-methyl-$^2H_3$]phytanic acid (99 atom % $^2H_3$) were synthesized as described previously (16). Pentafluorobenzyl bromide (PFB-Br) was obtained from Pierce (Rockford, Il). Organic solvents and reagents were of analytical grade and were used without further purification.

Sample preparation

Analysis of pristanic acid and phytanic acid was performed on 100-μl samples of plasma. To the samples, 400 μl of an internal standard solution in toluene containing 0.2 nmol [2-methyl-$^2H_3$]pristanic acid and 2.0 nmol [3-methyl-$^2H_3$]phytanic acid were added. Hydrolysis was performed by adding 2.0 ml 1 N NaOH–methanol to the samples and heating for 45 min at 100°C. The cooled solutions were acidified with 6 N HCl to pH 1 and extracted with 3 × 3 ml hexane. The combined hexane extracts were evaporated to dryness at 40°C under a stream of nitrogen.

For derivatization, the residue was treated with 100 µl 7% PFB-Br–acetonitrile and 10 µl triethylamine for 15 min at room temperature. After adding 150 µl 0.5 N HCl, the derivatives were extracted into 1.0 ml hexane, and 2 µl of this hexane fraction was used for GC–MS analysis.

Gas chromatography–mass spectrometry

GC–MS was performed on a Kratos MS 80 mass spectrometer equipped with a Data General 120S/Eclipse data system and a Carlo Erba HRGC 5300 gas chromatograph. Chromatographic separation was achieved on a CP-Sil 19 CB capillary column (25 m × 0.22 mm, film thickness 0.21 µm, Chrompack, Middelburg, The Netherlands) which was inserted directly into the ion source. Samples (2 µl) were introduced onto the column using a glass falling needle injector operating at 320°C. The GC oven temperature was 270°C and GC–MS interface temperature was 280°C. Helium was used as carrier gas with a linear flow rate of 30 cm/sec.

The operating conditions for electron capture negative chemical ionization (ECNCI) detection were as follows: source temperature 280°C, moderating gas methane at optimized source pressure, electron energy 40 eV, filament emission current 1.5 mA. The resolving power was 1000 (10% valley). Mass spectra were recorded at a scan speed of 1 sec/decade. Mass fragmentography was performed by recording the ion intensities at – *m/z* 297.3, 300.3, 311.3, and 314.3, with operator-controlled determination of peak areas. Calibration curves were developed using a series of standard solutions in 1-butanol containing the labeled internal standards and variable amounts (0.1–5.0 nmol) of pristanic acid and phytanic acid carried through the entire procedure. Linear regression analysis was used to calculate pristanic acid and phytanic acid concentrations.

RESULTS

The ECNCI mass spectra of PFB ester derivatives of pristanic acid, phytanic acid and the deuterated analogues are given in **Fig.2**. No overlap is seen between the fragments of the analytes and those of the internal standards. They almost exclusively consist of the respective carboxylate anions [M–PFB]⁻ (with

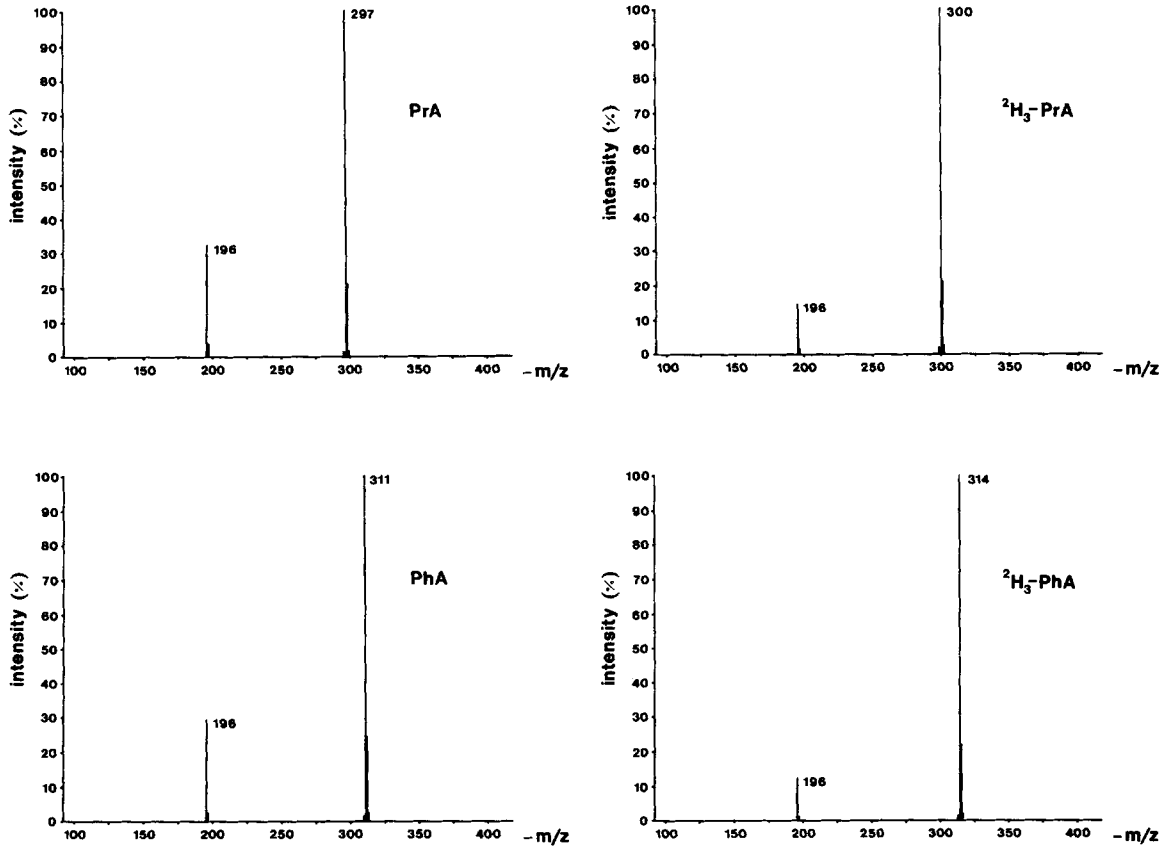

Fig. 2. ECNCI mass spectra of PFB ester derivatives of pristanic acid (PrA), [2-methyl-$^2H_3$]pristanic acid ($^2H_3$-PrA), phytanic acid (PhA), and [3-methyl-$^2H_3$]phytanic acid ($^2H_3$-PhA).

–m/z 297.3 and 300.3 for pristanic acid and 311.3 and 314.3 for phytanic acid), hence these ions were chosen for selected ion monitoring (SIM) measurement. A typical SIM chromatogram of a 0.1-ml plasma sample from a healthy control is shown in **Fig. 3.** Quantitation of compounds was not hampered by interference with co-eluting substances.

The detection limit of the method was found to be 1 pg for each acid (signal/noise 10), which corresponds to concentrations of 0.01 μmol/l in plasma. The sensitivity of the method allowed measurement of precise concentration values in all cases. Calibration curves for both pristanic and phytanic acid yielded straight lines (correlation coefficients 0.998–0.999). The inter-assay variation, determined over a 6-month period, and the intra-assay variation in measurements of pristanic acid and phytanic acid in control plasma are given in **Table 1.**

Pristanic acid and phytanic acid concentrations were measured in plasma from healthy subjects of various ages. The results depicted in **Table 2** show that both pristanic acid and phytanic acid concentrations, sometimes very low shortly after birth, may increase an order of magnitude within 2 years. For pristanic acid/phytanic acid ratios, however, no age-dependency was found.

Table 3 shows plasma pristanic acid and phytanic acid concentrations for controls and patients suffering from different peroxisomal disorders and Refsum's disease. Values are expressed not only in absolute terms but also relative to each other to serve as an additional discriminatory parameter.

DISCUSSION

In this report a specific, sensitive, and accurate method is presented for the quantitative analysis of pristanic acid and phytanic acid in plasma. The sample work-up procedure consists of hydrolysis of esterified fatty acids followed by solvent extraction and derivatization to PFB esters. The use of PFB ester derivatives is well established in the analysis of acidic compounds in biological samples (17–20). Loss of the electron capturing PFB group from acyl PFB esters results in the generation of a stable carboxylate anion, without formation of considerable amounts of other fragments. The highly efficient electron capture negative chemical ionization process combined with a low degree of fragmentation results in a very sensitive detection method. A high degree of specificity and accuracy is further obtained by the application of $^2H_3$-labeled analogues of the analytes as internal standards. If an additional acidic hydrolysis step is applied in the sample preparation procedure this method can conveniently be combined with the measurement of very long chain fatty acids (VLCFA) in plasma from patients suspect of a peroxisomal disease (20).

Several methods are practiced to measure phytanic acid levels in plasma, and a broad range of control values has been reported (**Table 4**); few data are available on pristanic acid in control plasma. We were able to demonstrate that in plasma from healthy infants both pristanic acid and phytanic acid levels are age-dependent. This finding confirms that the presence of phytanic acid results from dietary intake which may be very low at an early stage of life. Wanders et al. (21) have demonstrated that concentrations of phytanic acid in plasma from Zellweger patients are age-related: levels were found to be nor-

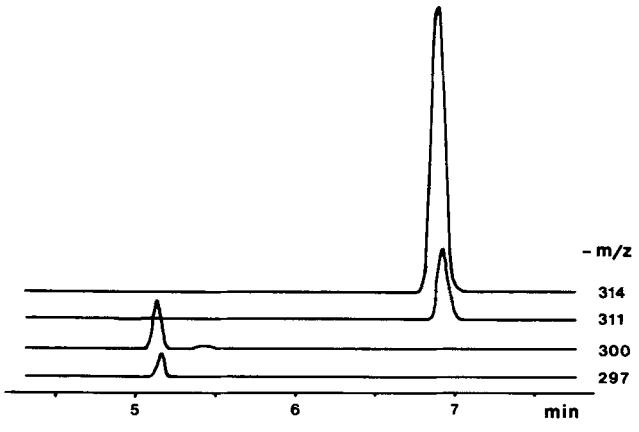

Fig. 3. Mass fragmentogram of PFB ester derivatives of PrA(– *m/z* 297), $^2H_3$-PrA (– *m/z* 300), PhA (– *m/z* 311), and $^2H_3$-PhA (– *m/z* 314), derived from 0.1 ml of control plasma.

TABLE 1. Intra-assay and inter-assay coefficients of variation (CV) in measurements of pristanic acid (PrA) and phytanic acid (PhA) and their ratios in control plasma

	Intra-assay (n = 10) CV	Inter-assay (n = 10) CV
	%	%
PrA	3.9	6.0
PhA	3.0	4.7
PrA/PhA	3.8	6.3

TABLE 2. Age-dependency of pristanic acid (PrA) and phytanic acid (PhA) concentrations and their ratios in control plasma

Age in Months (n)	PrA Mean (Range)	PhA Mean (Range)	PrA/PhA Mean (Range)
		μmol/l	
0–4 (39)	0.12 (0.01–0.60)	1.52 (0.04–5.28)	0.10 (0.03–0.35)
4–8 (16)	0.22 (0.01–0.84)	1.74 (0.21–5.70)	0.13 (0.04–0.28)
8–12 (8)	0.31 (0.02–0.77)	2.19 (0.38–4.40)	0.13 (0.05–0.23)
12–24 (12)	0.78 (0.12–1.47)	5.46 (1.34–8.62)	0.14 (0.06–0.24)
> 24 (43)	1.08 (0.12–2.98)	5.51 (0.49–9.88)	0.15 (0.03–0.39)

TABLE 3. Concentrations of pristanic acid (PrA) and phytanic acid (PhA) and their ratios in plasma from patients with various peroxisomal disorders and Refsum's disease

Patients (Age)	PrA	PhA	PrA/PhA
	μmol/l		
A. Generalized peroxisomal dysfunction			
Zellweger syndrome			
1 (3w)	0.28	1.70	0.16
2 (1m)	1.15	3.64	0.32
3 (1m)	4.29	12.2	0.35
4 (7m)	0.32	1.05	0.30
5 (15m)	0.69	2.95	0.23
6 (18m)	34.7	148	0.24
7 (3y)	16.7	54.5	0.31
8 (8y)	31.9	66.0	0.48
9 (10y)	10.8	28.8	0.38
Infantile Refsum's disease			
1 (16m)	38.6	177	0.22
2 (8y)	24.4	62.6	0.39
3 (8y)	8.44	35.0	0.24
4 (8y)	83.3	232	0.36
5 (11y)	7.99	27.5	0.29
6 (12y)	3.86	13.1	0.29
NALD			
1 (2y)	21.0	76.6	0.27
B. Multiple peroxisomal dysfunction			
RCDP			
1 (9m)	0.59	109	0.005
2 (5y)	0.08	85.7	0.001
3 (5y)	1.70	251	0.007
4 (6y)	0.26	51.6	0.005
5 (15y)	0.05	31.6	0.002
C. Single enzyme defects			
X-linked ALD			
1 (5y)	0.39	1.23	0.32
2 (6y)	0.62	4.15	0.15
3 (8y)	0.42	5.00	0.08
4 (30y)	2.32	12.5	0.19
5 (37y)	1.04	4.91	0.21
VLCFA acyl-CoA oxidase deficiency			
1 (4y)	0.74	3.72	0.20
Peroxisomal 3-oxoacyl-CoA thiolase/bifunctional protein deficiency			
1 (4m)	5.23	0.89	5.88
2 (11m)	33.1	5.45	6.08
3 (12m)	136	16.2	8.38
4 (15m)	59.0	27.3	2.16
5 (4y)	9.68	2.84	3.41
6 (4y)	26.8	20.6	1.30
7 (4y)	68.6	50.3	1.36
Refsum's disease			
1 (32y)	0.59	875	0.0007
2 (39y)	0.01	868	0.00001
3 (49y)	0.11	372	0.0003

Abbreviations: w, weeks; m, months; y, years.

TABLE 4. Control values (mean ± SD and/or ranges) of plasma pristanic acid (PrA) and phytanic acid (PhA) concentrations previously reported

Applied Method (Internal Standard)	PrA	PhA	Reference
	μmol/l		
GC (C19:0)	< 0.67	< 16.0	5
GCMS (C17:0)		< 16.0	8
GCMS (C17:0,[$^2H_4$]C16:0)		3.2 – 8.0	9
GC (C17:0)	1.48 ± 0.87	6.92 ± 2.18	13
GC (C27:0)		3.3 ± 1.4	14
GC (C17:0)		6.4 – 28.8	21
GC (C15:0)		12.80 ± 12.50 (1.90 – 39.90)	26

Values originally defined in other units are equally expressed in μmol/l.

mal in patients aged less than 17 weeks, and elevated in patients aged more than 40 weeks. A similar age-relation was reported by Lazarow and Moser (2). However, plasma phytanic acid levels in NALD, IRD, and RCDP reported by these authors showed marked differences among individual patients suffering from the same disease. Our results are in line with the former findings: the degree of accumulation of phytanic acid, and of pristanic acid, as a function of age varies among individual cases, possibly resulting from a combination of differences in gravity of the defects involved and amounts of dietary intake. As a consequence, for very young infants, phytanic acid and pristanic acid levels alone cannot serve as a diagnostic parameter; often, accumulation of these acids in plasma may not yet have started significantly. The additional finding of pristanic acid / phytanic acid ratios deviating from control values is indicative of a disturbance of the balance between dietary intake and a normal metabolic capacity.

Our results show that in patients with Zellweger syndrome, IRD, and NALD a common pattern is observed. Both phytanic acid and pristanic acid are elevated in plasma from patients although the concentration ratio of these substances is comparable to control values. The accumulation of phytanic acid as well as pristanic acid in these patients is probably due to the fact that both phytanic acid α-oxidation and pristanic acid β-oxidation are impaired. Accordingly, the absolute concentrations of both fatty acids measured in plasma from these patients would be determined by their dietary intake.

In RCDP phytanic acid is found to accumulate, whereas pristanic acid levels are low in these patients. Apparently, oxidation of pristanic acid can occur normally, like the oxidation of VLCFA. The same line of reasoning applies to Refsum's disease, where phytanic acid but not pristanic acid is found to be strongly elevated. The blocked formation of pristanic acid from phytanic acid leads to very low pristanic acid/phytanic acid ratios, reflecting the balance between dietary intake and efficient peroxisomal β-oxidation. In general, plasma pristanic acid/phytanic acid ratios in classic Refsum patients are lower than those in RCDP patients. It is unlikely that this difference reflects an age-dependency of phytanic acid accumulation (which is found more severe in the older

Refsum's disease patients). Most of the RCDP patients were old enough to accumulate phytanic acid at higher levels than actually observed. Rather, different pristanic acid / phytanic acid ratios in RCDP and Refsum's disease seem to reflect a difference in the metabolism of phytanic acid. Perhaps the metabolic defect is at a different location in the degradation pathway of phytanic acid. This hypothesis is strongly supported by complementary studies on phytanic acid α-oxidation that indicate that RCDP and Refsum's disease belong to different complementation groups (22).

In X-linked ALD, the β-oxidation of VLCFA is impaired due to a deficient peroxisomal VLCFA acyl-CoA synthase, resulting in accumulation of VLCFA (23). In plasma from one of the patients with this disease, only slightly elevated phytanic acid was measured; otherwise a normal phytanic acid–pristanic acid pattern was observed, indicating that VLCFA acyl-CoA synthase is not involved in pristanic acid β-oxidation.

Patients with pseudo-NALD show accumulation of VLCFA, caused by an isolated deficiency of peroxisomal VLCFA acyl-CoA oxidase (24). In plasma from one such patient pristanic acid and phytanic acid levels, as well as their ratio, were within the control range. Apparently, the pristanic acid β-oxidation is not disturbed by a deficient VLCFA acyl-CoA oxidase, indicating that this enzyme is not involved in the degradation of pristanic acid. It remains unclear whether the enzyme oxidizing pristanoyl-CoA is specific for this substrate or, for instance, is common for isoprenoid fatty acyl-CoA esters.

Our results further show that phytanic acid is elevated in plasma from a number of patients with a defect in peroxisomal fatty acid β-oxidation at the level of bifunctional protein and/or 3-oxoacyl-CoA thiolase (15). However, pristanic acid was found to accumulate much more strongly than phytanic acid, resulting in markedly increased pristanic acid/phytanic acid ratios. These results strongly suggest that peroxisomes are indeed the primary site of pristanic acid β-oxidation. Measurement of [1-^{14}C]phytanic acid oxidation in fibroblasts from patients with an isolated deficiency of bifunctional protein and/or peroxisomal thiolase revealed normal activities (R.J.A. Wanders, unpublished observations, see also (25)). This indicates that the accumulation of phytanic acid in these patients is secondary to the large accumulation of pristanic acid, probably causing product inhibition of phytanic acid α-oxidation. This is currently under investigation.

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