Analysis of pristanic acid β-oxidation intermediates in plasma from healthy controls and patients affected with peroxisomal disorders by stable isotope dilution gas chromatography mass spectrometry

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Abstract In this paper we report the development of highly sensitive, selective, and accurate stable isotope dilution gas chromatography negative chemical ionization mass spectrometry (GC-NCI-MS) methods for quantification of peroxisomal β-oxidation intermediates of pristanic acid in human plasma: 2,3-pristenic acid, 3-hydroxypristanic acid, and 3-ketopristanic acid. The carboxylic groups of the intermediates were converted into pentafluorobenzyl esters, whereas hydroxyl groups were acetylated and ketogroups were methoximized. Hereafter, the samples were subjected to clean-up by high performance liquid chromatography. Analyses were performed by selected monitoring of the carboxylate anions of the derivatives. Control values of all three metabolites were established (2,3-pristenic acid: 2–48 nm, 3-hydroxypristanic acid: 0.02–0.81 nm, 3-ketopristanic acid: 0.07–1.45 nm). A correlation between the concentrations of pristanic acid and its intermediates in plasma was found. The diagnostic value of the methods is illustrated by measurements of the intermediates in plasma from patients with peroxisomal disorders. It is shown that in generalized peroxisomal disorders, the absolute concentrations of 2,3-pristenic acid, 3-hydroxypristanic acid, and 3-ketopristanic acid were comparable to those in the controls, whereas relative to the pristanic acid concentrations these intermediates were significantly decreased. In bifunctional protein deficiency, elevated levels of 2,3-pristenic acid and 3-hydroxypristanic acid were found. 3-Ketopristanic acid, although within the normal range, was relatively low when compared to the high pristanic acid levels in these patients.—Verhoeven, N. M., D. S. M. Schor, E. A. Struys, E. E. W. Jansen, H. J. ten Brink, R. J. A. Wanders, and C. Jakobs. **Analysis of pristanic acid β-oxidation intermediates in plasma from healthy controls and patients affected with peroxisomal disorders by stable isotope dilution gas chromatography mass spectrometry.** *J. Lipid Res.* 1999. **40:** 260–266.

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In higher eukaryotes, including humans, oxidation of fatty acids takes place in both mitochondria and peroxisomes. Straight-chain fatty acids with short, medium, and long chains are mainly degraded in mitochondria, whereas very long chain fatty acids and branched-chain fatty acids, like pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) are oxidized in peroxisomes. In addition, the side chains of the bile acid intermediates di- and trihydroxycoprostanic acid (DHCA and THCA) are β-oxidized within the peroxisome (see refs. 1 and 2 for reviews). Mitochondrial and peroxisomal β-oxidation proceed via similar mechanisms, but the enzymes are different. In human peroxisomes, the first step of β-oxidation, which involves the FAD-dependent dehydrogenation of acyl-CoA esters to their corresponding *trans*-2-enoyl-CoA esters is catalyzed by two distinct acyl-CoA oxidases (3–5). One of these oxidases acts on saturated straight-chain acyl-CoA esters with different chain lengths. The other oxidase, branched-chain acyl-CoA oxidase, catalyses the dehydrogenation of pristanoyl-CoA to 2,3-pristenoyl-CoA and of di- and trihydroxycoprostanoyl-CoA to their unsaturated analogues (5).

The second and third steps of peroxisomal β-oxidation are catalyzed by multifunctional proteins. Recent work has shown that at least two multifunctional proteins are active in peroxisomes, multifunctional protein 1 and 2 (MFP1 and MFP2) (6–10). These enzymes differ in substrate specificity and in stereospecificity. The current concept is that MFP1 acts on straight-chain saturated fatty acids and is specific for the l-configuration of the 3-hydroxyacyl-CoA

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esters. MFP2, in contrast, forms and converts specifically d-3-hydroxyacyl-CoA esters and is involved in chain-shortening of the bile acid intermediates and of pristanic acid (8–10). Thiolytic cleavage of 3-ketoacyl-CoA esters is also catalyzed by two different enzymes within the peroxisome. A clofibrate-inducible 3-ketoacyl-CoA thiolase acts on straight-chain 3-ketoacyl-CoA esters (11), whereas branched-chain 3-ketoacyl-CoA esters are thiolytically cleaved by 58 kDa sterol carrier protein X, a non-clofibrate-stimulated enzyme (12).

It is generally assumed that the peroxisomal oxidation of fatty acids only involves chain-shortening and does not go to completion as the peroxisomal acyl-CoA oxidases do not accept short chain acyl-CoAs as substrates (13, 14). Recently, we demonstrated that pristanic acid undergoes three cycles of β-oxidation in peroxisomes, after which further oxidation occurs inside mitochondria (15).

Pristanic acid originates partly from phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) and partly from the diet. Different peroxisomal disorders in which pristanic acid accumulates have been described (see ref. 16 for overview). This accumulation of pristanic acid is never the sole biochemical abnormality in these disorders. In generalized peroxisomal disorders, in which no functionally active peroxisomes are present, pristanic acid accumulation is accompanied by accumulations of phytanic acid, very long chain fatty acids, DHCA and THCA, and pipecolic acid and decreased concentrations of other metabolites. In peroxisomal bifunctional protein deficiency and peroxisomal 3-ketoacyl-CoA thiolase deficiency, very long chain fatty acids, bile acid intermediates, and pristanic acid accumulate (17–19).

Although the known mechanism of β-oxidation of fatty acids implies that pristanic acid is converted into 2,3-pristenoyl-CoA, 3-hydroxypristanoyl-CoA, and 3-ketopristanoyl-CoA (**Fig. 1**), the existence of these intermediates has not been proven so far. Furthermore, it was not known whether or not these intermediates were detectable in plasma. Therefore, we developed sensitive methods to quantify these intermediates in plasma from healthy controls. In order to investigate the possible role of these methods in the differential diagnosis of peroxisomal disorders, we measured pristanic acid intermediates in plasma samples obtained from a large number of patients affected with peroxisomal disorders in which pristanic acid oxidation is disturbed.

MATERIALS AND METHODS

Biological samples

Human control plasma samples were obtained from subjects without a metabolic disorder and without liver and kidney pathology. Patient samples were obtained from patients suffering from peroxisomal disorders. The diagnosis was made by analysis of very long chain fatty acids, phytanic acid, pristanic acid, pipecolic acid, and bile acid intermediates in plasma. The diagnoses were confirmed by enzyme analysis, immunoblotting, and sometimes by complementation studies.

Synthesis of standards

The synthesis of pristanic acid and [2-methyl-^{2}H$_3$]pristanic acid has been described before (20). The key intermediate in the synthesis of unlabeled and deuterated standards not previously prepared was 4,8,12-trimethyltridecanal (**I**). It is readily accessible via oxidation of the corresponding alcohol 4,8,12-trimethyltridecan-1-ol (20). Coupling of this aldehyde **I** with suitable reagents forms a useful approach to build up the structure of pristanic acid derivatives at choice.
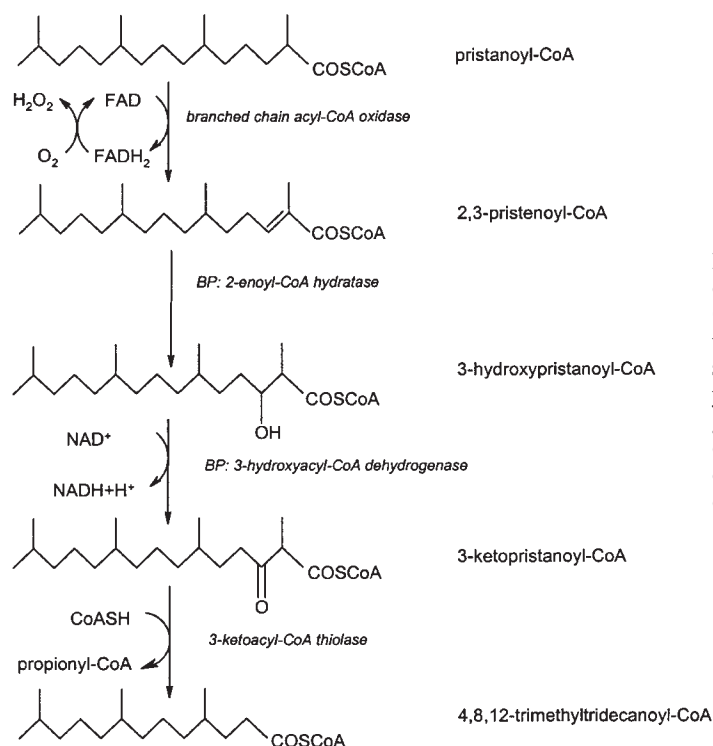

Fig. 1. Peroxisomal β-oxidation of pristanoyl-CoA. Branched-chain acyl-CoA oxidase converts pristanoyl-CoA into 2,3-pristenoyl-CoA. Bifunctional protein first forms 3-hydroxypristanoyl-CoA, which is further metabolized to 3-ketopristanoyl-CoA. The 58 kDa sterol carrier protein X thiolytically cleaves 3-ketopristanoyl-CoA, yielding propionyl-CoA and 4,8,12-trimethyltridecanoyl-CoA. In generalized peroxisomal disorders, all steps of peroxisomal β-oxidation are impaired. In bifunctional protein deficiency, one of the components or both components of the bifunctional enzyme are deficient; BP, bifunctional protein.

Reformatsky reaction of aldehyde **I** with ethyl 2-bromopropionate provided ethyl 3-hydroxypristanate, which was converted into free 3-hydroxypristanic acid and ethyl 3-ketopristanate. Condensation of aldehyde **I** with malonic acid via the Doebner modification of the Knoevenagel reaction was applied for the synthesis of 2,3-pristenic acid.

4,8,12-Trimethyltridecanal (I)

Pyridinium chlorochromate (1 g) was added to a solution of 4,8,12-trimethyltridecan-1-ol (1 g) in methylene chloride (30 ml). After stirring for 2 h at room temperature the solvent was evaporated, and the residue was extracted with petroleum ether (bp 40°–65°C). The extract was filtered over celite to remove some remaining solid, and concentrated to yield aldehyde **I** (0.88 g) as an almost colorless oil.

3-Hydroxypristanic acid ethyl ester (II)

A solution of d,l-2-bromopropionic acid ethyl ester (2 g) in benzene–ether 1:1 (5 ml) was slowly added to a suspension of Zn (1 g) in benzene–ether 1:1 (30 ml), and heated to reflux. Aldehyde **I** (0.88 g), dissolved in benzene–ether 1:1 (5 ml) was slowly added, and the reaction mixture was refluxed for 6 h. After cooling, the solution was filtered over celite, acidified with 2 N HCl, and extracted with petroleum ether (bp 40°–65°C) (2 × 25 ml). The combined extracts were washed with brine, dried with $MgSO_4$, filtered, and concentrated. Column chromatography over silica (50 g) with 1% ethyl acetate–petroleum ether (bp 40°–65°C) gave **II** (0.88 g) as an almost colorless oil (mixture of isomers).

3-Hydroxypristanic acid (III)

II (60 mg) was saponified by treating it with KOH (150 mg) in ethanol (3 ml) at reflux temperature for 2 h. After cooling and diluting with water (3 ml), the mixture was acidified and extracted with ethyl acetate (2 × 10 ml). The combined extracts were dried with $MgSO_4$, filtered, and concentrated to yield 3-hydroxypristanic acid (55 mg) as a slightly colored oil (mixture of isomers). The chemical purity of the sample was determined by comparing the flame ionization detector (FID) response of the sample (relative to a reference compound) with the theoretical FID response based on the effective carbon number. The purity of 3-hydroxypristanic acid was thus estimated at 23%.

3-Ketopristanic acid ethyl ester (IV)

Pyridinium chlorochromate (0.3 g) was added to a solution of **II** (0.2 g) in methylene chloride (20 ml), and the mixture was stirred for 4 h at room temperature. The solvent was evaporated, and the residue was extracted with petroleum ether (bp 40°–65°C) (2 × 15 ml). The combined extracts were filtered over celite and concentrated to yield **IV** as a slightly colored oil (0.2 g) with a purity of 69% as determined as described for 3-hydroxypristanic acid.

2,3-Pristenic acid (V)

Methylmalonic acid (0.2 g) was solved in pyridine (1 ml) and cooled with ice. A solution of **I** (0.4 g) in pyridine (1 ml) was slowly added, followed by 2 drops of piperidine. The solution was warmed at 50°C for 36 h in the dark. After cooling, the mixture was diluted with water (2 ml), acidified with HCl (2 N), and extracted with ethyl acetate (2 × 5 ml). The combined extracts were washed with brine, dried with $MgSO_4$, filtered, and concentrated. The crude product was purified by column chromatography over silica with 1% ethyl acetate–petroleum ether (bp 40°–65°C), giving 2,3-pristenic acid (0.2 g) as a colorless oil (mixture of isomers) with a purity of 69% as determined by FID response.

$^2H_3$-labeled standards

The [2-methyl-$^2H_3$]-labeled standards were synthesized from aldehyde **I** in an analogous manner. The Reformatsky reaction

was conducted with d,l-[3,3,3-$^2H_3$]2-bromopropionic acid ethyl ester, prepared from [3,3,3-$^2H_3$]propionic acid by α-bromination using bromine and red phosphorus and quenching of the intermediate acyl bromide with ethanol. The Doebner condensation was performed with [$^2H_3$]methylmalonic acid, prepared from diethyl malonate and [$^2H_3$]iodomethane followed by saponification of the resulting ester.

Sample preparation

2,3-Pristenic acid. An aliquot of 25–500 μl plasma was taken, depending on the availability of sample and the expected concentration of 2,3-pristenic acid. After addition of 0.015 nmol [2-methyl-$^2H_3$]pristenic acid as internal standard, 2 ml freshly prepared 10% 6 m HCl in acetonitrile was added, after which the sample was hydrolyzed for 45 min at 100°C. Hereafter, 2 ml 1 m NaOH in methanol was added and the sample was saponified again for 45 min at 100°C. These hydrolytic steps were performed to ensure complete release of the fatty acid moiety from CoA, from glycerol, from protein, etc. After acidification of the sample with 350 μl 6 m HCl it was extracted with 4 ml hexane. The hexane was evaporated and the residue was derivatized with 100 μl pentafluorobenzylbromide (PFB-Br) (7% in acetonitrile) and 10 μl triethylamine for 15 min at room temperature. After derivatization, the sample was acidified by addition of 200 μl 0.5 m HCl and extracted with 1 ml hexane. After evaporation of the hexane, the residue was dissolved in 45 μl acetonitrile and purified by high performance liquid chromatography (HPLC) as described below.

3-Hydroxypristanic acid. For analysis of 3-hydroxypristanic acid in plasma, an aliquot of 25–500 μl plasma was taken. [2-Methyl-$^2H_3$]3-hydroxypristanic acid (0.001 nmol) was added as internal standard. The sample was hydrolyzed and derivatized with PFB-Br as described for 2,3-pristenic acid. Hereafter, the sample was derivatized with 100 μl acetic anhydride and 100 μl pyridine for 1 h at 80°C. After evaporation of the reagents, the residue was dissolved in 45 μl acetonitrile and purified by HPLC as described below.

3-Ketopristanic acid. For analysis of 3-ketopristanic acid in plasma, to 100–500-μl aliquots of plasma, 0.55 pmol of [2-methyl-$^2H_3$]3-ketopristanic acid ethyl ester was added as internal standard. Methoxylamine·HCl (1 g/ml water) (200 μl) and a droplet of 6 m HCl were added, after which the sample was heated for 2 h at 80°C. After cooling, 500 μl 1 m NaOH in methanol was added and the samples were left overnight at room temperature to ensure hydrolysis under mild conditions. After acidification of the samples with 100 μl 6 m HCl, they were extracted with 2 ml hexane. The hexane fractions were evaporated under a nitrogen stream. PFB-Br (100 μl) (7% in acetonitrile) and 10 μl triethylamine were added, after which the samples were left at room temperature for 15 min. After acidification of the samples with 200 μl 0.5 m HCl, extraction with 1 ml hexane, and evaporation of the hexane, the residue was dissolved in 45 μl acetonitrile. Hereafter, the samples were purified by HPLC as described below.

Sample purification by reversed phase high performance liquid chromatography

A 20-μl aliquot of the prepared sample was applied onto a precolumn (LC18DB, length: 1 cm, internal diameter 4.6 mm, particle size 5 mm, Supelco, Bellefonte, PA) coupled to a C18 analytical RP-HPLC column (LC18S, length 25 cm, internal diameter 4.6 mm, particle size 5 μm, Supelco, Belafonte) using 100% acetonitrile as mobile phase with a flow rate of 1 ml/min. For 3-hydroxypristanic acid and 3-ketopristanic acid, a second analytical C18 column (LC18S, length 15 cm, internal diameter 4.6 mm, particle size 5 μm, Supelco) was coupled to the first analytical column. Chromatography was performed at 45°C (2,3-pristenic

acid), 35°C (3-hydroxypristanic acid), or 20°C (3-ketopristanic acid). Detection was accomplished with a UV detector at a wavelength of 225 nm. Derivatized standards (10 μm) were used to determine retention times of the analytes. Due to the constant performance of the HPLC set up, we were able to use a time-programmed fraction collector to collect 1-ml fractions, in spite of the fact that the metabolites in the plasma samples were usually not detectable by the UV detector due to their low concentrations.

Gas chromatography mass spectrometry

2,3-Pristenic acid. GC separation of the PFB ester of 2,3-pristenic acid was achieved on a very polar column (BPX70, 25 m × 0.32 mm, film thickness 0.25 μm, Scientific Glass Engineering, Ringwood, Australia) using helium as carrier gas. The injector temperature was 260°C, the injection volume was 1 μl. After 1 min at 80°C, the column temperature was raised with 5° per min to 250°C. The interface temperature was set at 280°C, the source temperature was 250°C. The mass spectrometer was used in the negative chemical ionization mode. NH_3 was used as ionization gas at an optimized ion source pressure. Selected ion monitoring (SIM) of the ions $-m/z$ 295 and $-m/z$ 298 was performed for 2,3-pristenic acid and its labeled internal standard, respectively. The two peaks that were observed in the SIM chromatogram were analyzed by high energy collision and constant B/E ratio linked scanning. This way, the daughter spectrum of the carboxylate anion of the fatty acid is produced, enabling confirmation of the positions of the methyl groups and the double bond in the molecule (Kratos Concept 1 H mass spectrometer).

3-Hydroxypristanic acid and 3-ketopristanic acid. For GC separation of the PFB-acetyl derivative of 3-hydroxypristanic acid and the PFB-methoxime derivative of 3-ketopristanic acid, a CPsil 19 CB capillary column (25m × 0.25 mm, film thickness: 0.2 μm Chrompack, Middelburg, The Netherlands) was used. The carrier gas was helium. The injector temperature was 300°C. One to 2 μl of sample was injected splitless. For 3-hydroxypristanic acid, the initial GC temperature of 160°C was kept for 1 min, after which the temperature was raised by 30°C per min to 320°C. For 3-ketopristanic acid, the GC was held for 1 min at 120°C, after which the temperature was increased by 35°C per min to 320°C. Hereafter, the GC was kept at 320°C for 5 min.

For both analyses, the interface temperature was 300°C, the source temperature was 250°C. The mass spectrometer was used in the negative chemical ionization mode with NH_3 as ionization gas. The ion source pressure was 9.10^{-5} Torr. For 3-hydroxypristanic acid and its labeled internal standard, the ions $-m/z$ 355 and $-m/z$ 358 were monitored. For 3-ketopristanic acid and its labeled internal standard, the ions $-m/z$ 340 and $-m/z$ 343 were monitored.

All GC-MS analyses were performed on a Hewlett-Packard system (Hewlett-Packard 5890 series II and Hewlett-Packard Engine 5989B). Interassay reproducibility was assessed by quantifying the analytes in two pooled plasma samples extracted and analyzed on different days. Concentrations were determined based on calibration curves processed on the same day.

RESULTS

The reproducibility of the quantifications of 2,3-pristenic acid, 3-hydroxypristanic acid and 3-ketopristanic acid are summarized in **Table 1**.

2,3-Pristenic acid

Chemical ionization mass spectrometry of 2,3-pristenic acid PFB ester generates a mass spectrum showing a prominent ion ($-m/z$ 295) corresponding to (M-PFB) (spectrum not shown). This ion was chosen for selected

TABLE 1. Reproducibility of the determination of 2,3-pristenic acid, 3-hydroxypristanic acid and 3-ketopristanic acid in 2 pooled plasma samples

	Pool 1	Pool 2
	nmol/l	
2,3-Pristenic acid	10 ± 1 (n = 14)	6 ± 1 (n = 10)
3-Hydroxypristanic acid	0.18 ± 0.09 (n = 8)	0.14 ± 0.12 (n = 8)
3-Ketopristanic acid	0.28 ± 0.04 (n = 12)	0.20 ± 0.04 (n = 7)

Measurements were performed on different days by GC-NCI-MS. Concentrations were calculated based on calibration curves processed on the same day. Values are given as mean ± SD.

ion monitoring (SIM) measurement. **Figure 2** represents the GC-NCI-MS chromatogram, recorded in the SIM mode, from 2,3-pristenic acid in control human plasma with added labeled internal standard. The internal standard shows two peaks with baseline separation. High energy collision and constant B/E ratio linked scanning showed that both peaks consist of 2,3-pristenic acid only. The second peak was the most prominent one, accounting for over 90% of the total amount of the compound. In the plasma samples, both peaks were present. In all plasma samples, the first peak accounted for ca. 3.5% of the total amount of 2,3-pristenic acid. Quantification was achieved by summation of both peaks. Control values of 2,3-pristenic acid were determined in 17 plasma samples with normal pristanic acid concentrations (**Table 2**). The ratios between 2,3-pristenic acid and pristanic acid in the samples are summarized in **Table 3**. In **Fig. 3**, the correlation between the pristanic acid and 2,3-pristenic acid concentrations in the samples is shown. Concentrations of 2,3-pristenic acid in plasma obtained from patients affected with generalized peroxisomal disorders and isolated bifunctional protein deficiency were measured (Table 2). In Fig. 3 it is shown that the correlation between pristanic acid and 2,3-pristenic acid in patients is different from those in controls.

3-Hydroxypristanic acid

The mass spectrum of the PFB-acetyl derivative of 3-hydroxypristanic acid showed a prominent ion corresponding to $-m/z$ 355 due to loss of PFB by chemical ionization. SIM measurements were performed by monitoring this ion. The results obtained with a control human plasma sample are shown in Fig. 2. Both the acetyl derivative of the biological 3-hydroxypristanate from the plasma and the chemically synthesized labeled internal standard show two peaks with baseline separation. This phenomenon may be caused by different gas chromatographic retention of different stereoisomers of the compound. As the exact nature of the two peaks was not known, quantification was achieved by summation of both peaks. Table 2 shows the concentrations of 3-hydroxypristanic acid in plasma from controls and patients affected with generalized peroxisomal disorders or bifunctional protein deficiency. Table 3 summarizes the ratios between the concentrations of 3-hydroxypristanic acid and pristanic acid in the samples. In Fig. 3, the correlations between the concentrations of 3-hydroxypristanic acid and
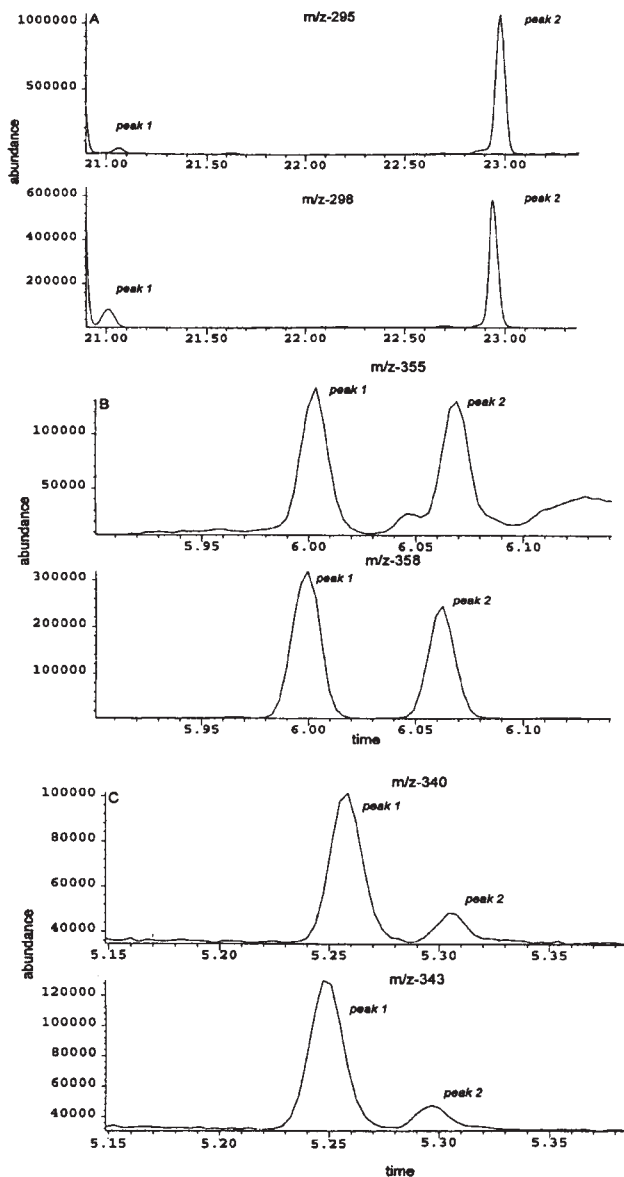
Fig. 2. GC-MS analysis of A: 2,3-pristenic acid in control human plasma, monitoring $-m/z$ 295 and $-m/z$ 298 for 2,3-pristenate in plasma and the internal standard respectively, B: 3-hydroxypristanic acid in control human plasma, monitoring $-m/z$ 355 and $-m/z$ 358 for 3-acetoxypristanate in pooled human plasma and the internal standard, respectively, and C: 3-ketopristanic acid in control human plasma, monitoring $-m/z$ 340 and $-m/z$ 343 for 3-ketopristanate-methoxime in plasma and the internal standard, respectively.

pristanic acid in the control samples are shown. In addition, the values from a patient affected with a generalized peroxisomal disorder are included in the graph, to illustrate the difference from the controls.

3-Ketopristanic acid

Loss of PFB from 3-ketopristanate-PFB-methoxime during chemical ionization resulted in the formation of one prominent peak with $-m/z$ 340 in the mass spectrum of this compound. This ion was chosen for SIM measurements. The SIM chromatogram of the 3-ketopristanic acid derivative showed two peaks due to formation of two isomers

TABLE 2. Concentrations of 2,3-pristenic acid, 3-hydroxypristanic acid, and 3-ketopristanic acid in plasma from controls and patients with generalized peroxisomal disorders or bifunctional protein deficiency

Subjects	Pristanic Acid	2,3-Pristenic Acid	3-OH-Pristanic Acid	3-Ketopristanic Acid
	μM	nM	nM	nM
Controls (n = 17)	0.20–4.4	2–48	0.02–0.81	0.07–1.45
Generalized peroxisomal disorders				
1	5.5	7	0.18	0.14
2	6.7	8	nd	0.07
3	7.1	6	0.28	0.35
4	9.7	26	0.32	0.14
5	16	5	nd	nd
6	17	21	0.21	0.28
7	24	26	0.53	0.41
8	32	57	1.22	1.31
9	43	35	0.83	0.28
10	47	46	0.78	0.97
Isolated peroxisomal β-oxidation defects				
1	0.22	13	nd	nd
2	0.95	43	nd	nd
3	4.9	262	nd	nd
4	5.4	952	3.91	0.07
5	7.4	221	2.07	n.d.
6	33	2898	11.0	1.31
7	37	1449	6.2	nd
8	68	4354	15.2	2.28
9	282	12972	76	nd

Not determined, nd.

during methoxime derivatization. As expected, these two peaks were always present in a fixed ratio, both in standard samples and in plasma samples. Figure 2 depicts the SIM chromatogram of a control plasma sample. Quantification of 3-ketopristanic acid in plasma was achieved by the use of calibration curves from the synthesized 3-ketopristanic acid ethyl ester. The concentrations of 3-ketopristanic acid in plasma from controls and patients affected with peroxisomal disorders are summarized in Table 2. In Table 3, the ratios between 3-ketopristanic acid and pristanic acid, both in controls and patients, are summarized. Figure 3 shows the correlation between the concentrations of 3-ketopristanic acid and pristanic acid in control samples. The patient with a generalized peroxisomal disorder is clearly abnormal.

DISCUSSION

In order to quantitate the low amounts of pristanic acid and its oxidative metabolites 2,3-pristenic acid, 3-hydroxypristanic acid, and 3-ketopristanic acid in plasma samples,

TABLE 3. Relative amounts of pristanic acid intermediates in plasma

Subjects	2,3-Pristenic/Pristanic × 10³	3-OH-Pristanic/Pristanic × 10³	3-Ketopristanic/Pristanic × 10³
Controls	6–18	0.07–0.60	0.08–1.7
GPD	0.31–2.68	0.01–0.04	0.01–0.05
BPD	30–176	0.17–0.72	0.01–0.04

GPD, generalized peroxisomal disorders; BPD, bifunctional protein deficiency.
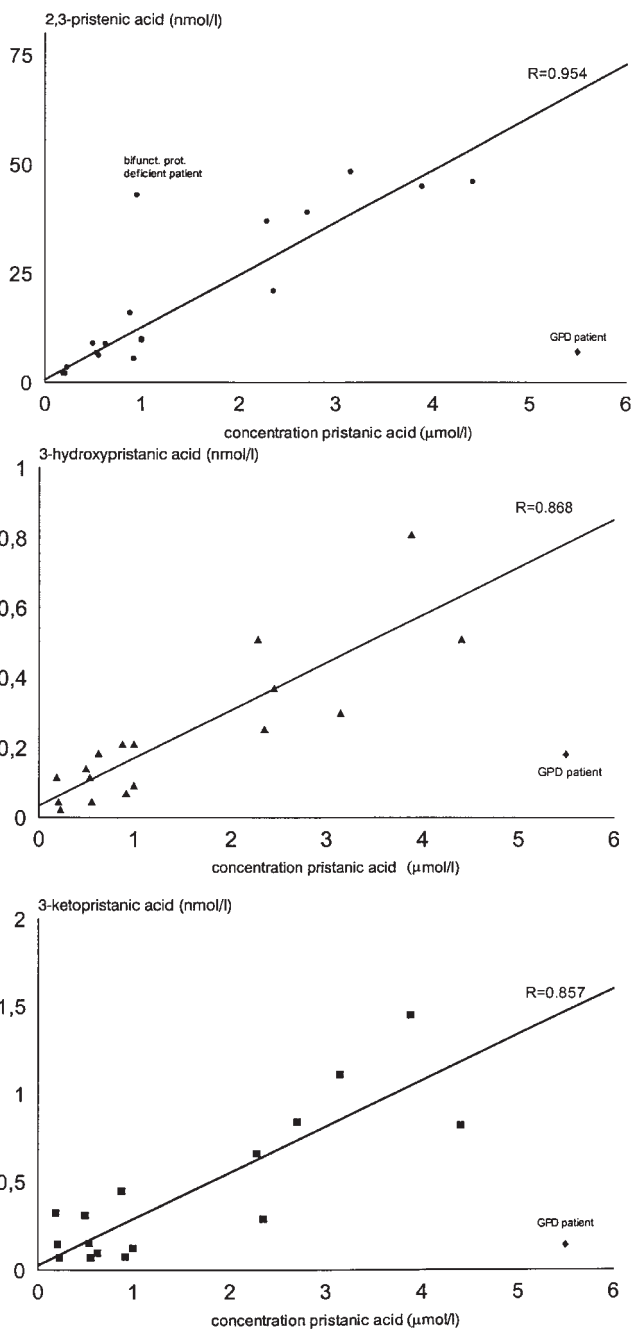
Fig. 3. Correlation between the concentrations of pristanic acid and its β-oxidation intermediates 2,3-pristenic acid, 3-hydroxypristanic acid, and 3-ketopristanic acid in control human plasma. The patients affected with a generalized peroxisomal disorder or bifunctional protein deficiency are clearly distinct; R, coefficient of correlation.

a highly selective and sensitive technique is required. Therefore, quantification was performed by stable isotope dilution GC-NCI-MS. The concentrations of 2,3-pristenic acid, 3-hydroxypristanic acid, and 3-ketopristanic acid in control plasma samples were in the nanomolar range. Early attempts to measure these low concentrations failed, as coeluting compounds interfered in the SIM chromatograms. Therefore, extensive clean-up of the samples by HPLC was introduced.

In controls, a clear correlation between the concentrations of pristanic acid and its intermediates was demonstrated.

In generalized peroxisomal disorders, both phytanic acid α-oxidation and pristanic acid β-oxidation are deficient. The concentrations of phytanic and pristanic acids, however, are not always elevated in plasma from affected patients. In very young patients, intake of phytanic acid and pristanic acid from the diet has been limited, which explains the lack of accumulation in these patients (19). We now demonstrate that in generalized peroxisomal disorders, 2,3-pristenic acid is, in all cases except one, within the control range. However, when comparing the concentrations of 2,3-pristenic acid with those of pristanic acid, it is clear that 2,3–pristenic acid in plasma from these patients is relatively low. In addition, 3-hydroxypristanic acid and 3-ketopristanic acid, although both within the control ranges or even slightly elevated, were low relative to the pristanic acid concentrations in these patients, as clearly reflected in the respective ratios.

The observation that pristanic acid intermediates are present in plasma from patients affected with generalized peroxisomal disorders may point to some residual activity of peroxisomal β-oxidation in these patients. However, it is also possible that small amounts of these intermediates are ingested with the diet and, due to defective β-oxidation, are not degraded.

In bifunctional protein deficiency, accumulation of pristanic acid occurs (18, 19). The accumulating pristanic acid is thought to inhibit phytanic acid α-oxidation, resulting in elevated phytanic acid concentrations. As pristanic acid accumulation is more pronounced than phytanic acid accumulation in these patients, the ratio pristanic acid/phytanic acid in plasma is increased (18). The present work shows that 7 out of 9 patients affected with bifunctional protein deficiency included in this study exhibited elevated levels of 2,3-pristenic acid. In addition, in all samples from bifunctional protein-deficient patients analyzed, 3-hydroxypristanic acid was elevated. In all 9 patients, 2,3-pristenic acid was elevated relative to the pristanic acid concentration, whereas the 3-hydroxypristanic acid/pristanic acid ratio was comparable to those in the controls. This suggests that, due to a presumed deficiency of the 3-hydroxyacyl-CoA dehydrogenase component of the bifunctional protein, 3-hydroxypristanic acid accumulated. This may then cause inhibition of the 2-enoyl-CoA hydratase component, resulting in 2,3-pristenic acid accumulation and, due to inhibition of branched-chain acyl-CoA oxidase, accumulation of pristanic acid. This suggests a phenomenon of feedback inhibition of enzymes by accumulating products.

In bifunctional protein deficiency, 3-ketopristanic acid is expected to be low, regardless of whether the hydratase or the dehydrogenase component of the bifunctional enzyme is deficient. Indeed, the 3-ketopristanic acid concentrations relative to the concentration of pristanic acid were decreased in bifunctional protein deficiency.

Measurement of pristanic acid and 2,3-pristenic acid in plasma provides a fast method to distinguish between gen-

eralized peroxisomal disorders and bifunctional protein deficiency, necessitating only one plasma sample. Although the ratio of pristanic acid/phytanic acid, being normal in generalized peroxisomal disorders and elevated in bifunctional protein deficiency, already hints at a diagnosis, this parameter is not always reliable. In our experience, the pristanic acid/phytanic acid ratio is sometimes elevated in patients suffering from atypical generalized peroxisomal disorders. Measurement of 2,3-pristenic acid in samples from these patients will give additional information for the correct diagnosis.

In addition, in isolated peroxisomal β-oxidation defects, the levels of pristanic acid intermediates may help to determine the exact defect. In bifunctional protein deficiency, elevated levels of 2,3-pristenic acid are always expected. 3-Hydroxypristanic acid is also expected to be elevated (in case of a 3-hydroxyacyl-CoA dehydrogenase deficiency) or low (in case of a hydratase deficiency). When 3-ketoacyl-CoA thiolase is deficient, accumulation of 3-ketopristanic acid is expected. This latter hypothesis could, unfortunately, not be verified as no plasma sample of the only known patient affected with peroxisomal 3-ketoacyl-CoA thiolase deficiency was available (19, 21).

In summary, we have demonstrated, for the first time, the existence of 2,3-pristenic acid, 3-hydroxypristanic acid, and 3-ketopristanic acid in human plasma. Furthermore, we have shown that in generalized peroxisomal disorders relatively low concentrations of these intermediates are found, whereas in the cases of bifunctional protein deficiency, accumulation of 2,3-pristenic acid and 3-hydroxypristanic acid occurs. The method enables distinction between generalized peroxisomal disorders and isolated peroxisomal β-oxidation defects. Future studies will focus on the investigation of plasma samples from patients with unknown peroxisomal fatty acid oxidation defects.

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