J. Inher. Metab. Dis. 18 Suppl. 1(1995) 76–83
© SSIEM and Kluwer Academic Publishers. Printed in the Netherlands

Measurement of very long-chain fatty acids, phytanic and pristanic acid in plasma and cultured fibroblasts by gas chromatography

G. DACREMONT*, G. COCQUYT and G. VINCENT
Department of Pediatrics, University Hospital Gent, Belgium

**Correspondence: Universitair Ziekenhuis, Kliniek voor Kinderziekten 'C. Hooft', De Pintelaan 185, 9000-Gent, Belgium*

Summary: Two methods are described, both currently used in our laboratory, for the quantitative analysis of very long-chain fatty acids, phytanic acid and pristanic acid in plasma and cultured fibroblasts by gas–liquid chromatography. The first method is based on the procedure developed by Moser and Moser (1991) and the second is based on the method of Onkenhout and colleagues (1989), which is an application of the original method of Lepage and Roy for plasma and fibroblasts. A survey is given of the concentrations of very long-chain fatty acids, pristanic and phytanic acid in plasma and fibroblasts from control subjects and all patients investigated so far in our laboratory.

Measurement of very long straight-chain fatty acids (VLCFA) and of pristanic and phytanic acid concentrations in plasma or serum is an established technique for the screening of peroxisomal disorders.

Initially VLCFA assays were only used for the diagnosis of X-linked adrenoleukodystrophy (McKusick 300100). However, the discovery by Singh et al (1984) that VLCFA β-oxidation is mainly a peroxisomal process led to the concept that VLCFA accumulation can be used as a marker for other peroxisomal disorders in which there is a disturbance in the β-oxidation of these acids. Indeed eight other peroxisomal disorders have been identified so far in which there is an impairment of the β-oxidation of VLCFA, resulting in their accumulation in body fluids and tissues. These disorders include:

(i) The generalized peroxisomal disorders or peroxisome assembly disorders: classical Zellweger syndrome (McKusick 214100), neonatal adrenoleukodystrophy (NALD; McKusick 20370), infantile Refsum disease (IRD; McKusick 266500) and pseudo-infantile Refsum disease.

(ii) The disorders caused by a deficiency of a single peroxisomal enzyme in the β-oxidation pathway: acyl-CoA oxidase deficiency (pseudo-NALD; McKusick 264470), bi- (tri-)functional enzyme deficiency and acyl-CoA thiolase deficiency (pseudo-Zellweger syndrome; McKusick 261510).

(iii) Zellweger-like syndrome, a disease with multiple enzyme deficiencies.

In man, very long straight-chain fatty acids and 2-methyl branched-chain fatty acids such as pristanic acid and the bile acid precursors di- and trihydroxycoprostanoic acid (2-methyl-substituted in their side-chains) are initially oxidized by two different acyl-CoA oxidases (Vanhove et al 1993). Hence, pristanic acid accumulation is a marker for a disturbance in the β-oxidation of branched-chain fatty acids. Pristanic acid accumulates in the peroxisome assembly disorders, in bifunctional enzyme deficiency and in acyl-CoA thiolase deficiency, two disorders that also have a single defect in the β-oxidation of VLCFA. In trihydroxycholestanoic acidaemia, only the branched-chain fatty acids accumulate, not VLCFA.

Phytanic acid, the precursor of pristanic acid, accumulates in the peroxisome deficiency disorders, in rhizomelic chondrodysplasia punctata and in Refsum disease. Additionally, it is also elevated in the two above-mentioned disorders with isolated β-oxidation defects where pristanic acid also accumulates. In these latter diseases the phytanic acid accumulation is probably due to the inhibition of phytanic acid α-oxidation by accumulating pristanic acid, while in the other diseases it is possibly due to a separate peroxisomal enzyme defect in the conversion of phytanic to pristanic acid. It is still not clear which step in the oxidation of phytanic acid is a peroxisomal process.

A variety of methods have been described for the measurement of VLCFA. Most laboratories use the original procedure developed by Moser and Moser (1991), which involves preparation of a total lipid extract, treatment of this extract with methanolic hydrochloric acid, which yields the methylesters, purification of the methylesters by thin-layer chromatography (TLC) and quantification by capillary gas–liquid chromatography (GLC). This procedure was in current use in our laboratory for 6 years and has been applied to about 4000 samples.

Two years ago we introduced the simple one-step procedure described by Onkenhout et al (1989). In this method total fatty acids are directly transesterified with acetyl chloride in the presence of methanol and benzene and the fatty acid methyl esters subsequently extracted and purified by TLC.

A detailed description of the two procedures is given below.

EQUIPMENT, CHEMICALS, MATERIALS AND SAMPLES

Equipment

- Gas chromatograph for capillary columns equipped with an oven with reproducible temperature control, a flame ionization detector, a splitless or on-column injection system and an accurate and sensitive integrator
- Capillary column (Ultra II Hewlett Packard) for VLCFA analysis
- Capillary column (OV 1701 Betron Scientific) for pristanic and phytanic acid analysis
- Vortex mixer
- Thermostated heating block or oven
- Laboratory centrifuge (preferably cooled)

Chemicals

- Solvents: chloroform, methanol, n-hexane, toluene and diethylether for *procedure 1*; benzene, methanol, n-hexane, toluene and diethylether for *procedure 2*. All solvents must be of the highest purity with the lowest amount of residue
- Fatty acid standards: pentadecanoic acid ($C_{15:0}$ FA) for pristanic and phytanic acid measurements; heptacosanoic acid ($C_{27:0}$ FA) for C_{22}, C_{24} and C_{26} measurements
- 2 mol/L Methanolic HCl (*procedure 1*)
- Iodine crystals
- Potassium carbonate (*procedure 2*)
- Potassium chloride (*procedure 1*)
- Acetyl chloride (*procedure 2*)

Solutions

- Pentadecanoic acid solution, 100 μg/ml in chloroform–methanol 2:1
- Heptacosanoic acid solution, 40 μg/ml in chloroform–methanol 2:1
- Mixture of methanol–benzene 4:1 (v/v) (*procedure 2*)
- 6% Potassium carbonate (*procedure 2*)
- Mixture of chloroform–methanol 1:1 (v/v) (*procedure 1*)
- 0.1 mol/L Potassium chloride (*procedure 1*)

Materials

- Precoated thin-layer chromatography (TLC) plates, silica 60 (20×20 cm, 0.25 mm thickness (e.g. Merck 5721))
- Borosilicate glass tubes (18×100 and 13×100 mm) with Teflon-lined screwcaps
- Thin-layer chromatography tanks
- Organic solvent-resistant dispensers
- Teflon-lined screwcapped vials, 2 ml

Biological samples

- *Plasma or serum:* Venous blood (with or without anticoagulant) 2–5 ml, is centrifuged for 10 min at 800*g*, preferably within 1 h of collection to avoid haemolysis, and the plasma or serum is separated. Normally the sample is stored at −4°C or lower temperature until analysis can be performed. However, since concentrations of saturated VLCFA, pristanic acid and phytanic acid remain unchanged by storage at room temperature for at least 10 days, samples can be mailed at ambient temperature.
- *Cultured fibroblasts:* Fibroblasts grown to confluency are harvested either by trypsinization or by 'scraping'. The culture medium is removed after centrifugation for 15 min at 800*g* and the fibroblast pellet is washed three times with buffered saline. After the last wash, the liquid is drained and the pellet is stored at −20°C or lower until analysis.
- For VLCFA analysis, at least 300–500 μg of fibroblast protein is required. In our experience, two culture flasks with a growth surface of 75 cm^2 are sufficient.

PROCEDURES

Preparation of fatty acid methylesters (FAME)

Procedure 1 (see Moser and Moser, 1991): preparation of the total lipid extract; acid methanolysis of the total lipid extract; purification of the fatty acid methylesters by TLC.

Plasma or serum

- Pipette 250 μl of plasma or serum into a 18×100 mm tube with Teflon-lined screwcap; add 50 μl (=5 μg) of $C_{15:0}$ and 50 μl (=2 μg) of $C_{27:0}$ solution as internal standard, and then 3.1 ml of chloroform–methanol 1 : 2 (v/v); cap and vortex vigorously for 5 min.
- Centrifuge the extracted plasma at 1000*g* for 15 min; transfer the supernatant to another screwcapped tube, taking care that the protein pellet is left. Add to the supernatant 1.55 ml of chloroform and 1 ml of 0.1 mol/L KCl; cap and vortex for 1 min.
- Centrifuge the vortexed sample for 5 min at 500*g*; remove the upper phase with a Pasteur pipette and dry the lower phase under N_2.
- To the dried lipid extract, add 2 ml of 2 mol/L methanolic HCl; cap the tube tightly with a Teflon-lined screwcap and heat the tube for at least 4 h at 80°C in a metal block thermostat or oven.
- After cooling to room temperature, add 2 ml of water and 4 ml of n-hexane; vortex for 3 min and centrifuge for 5 min. Transfer the upper n-hexane phase into another tube and repeat the extraction of the lower phase twice with 2 ml of hexane.
- Concentrate the combined hexane extracts to a smaller volume (~1 ml) under N_2; transfer the concentrated hexane extract to a 2 ml vial and evaporate further until dry.
- Dissolve the residue in 200 μl of n-hexane and apply 100 μl of the solution in a 1.5 cm band on a precoated TLC plate; a reference mixture and five samples can be run simultaneously. The plate is developed in 1 h in toluene–diethylether 97 : 3 (v/v).
- After air drying for 30 min, the five sample lanes are covered with a glass plate and the first lane containing the reference mixture is rendered visible with iodine vapour in a chromatography tank. Using the reference mixture, the positions of the FAME in the other lanes are outlined with a needle.
- The marked areas are scraped on to weighing paper, transferred to a centrifuge tube, and extracted three times with 1.5 ml of n-hexane. For each cycle, the tube is vortexed, sonicated and centrifuged for 3 min. The combined extracts are then evaporated in a 2 ml vial under N_2 until dry.
- The dried residue is finally taken up in 50 μl of n-hexane and approx. 1 μl is subjected to GLC for the analysis of VLCFA.
- For the measurement of phytanic and pristanic acids, the non-purified FAME solution is usually used. The 100 μl solution is concentrated to approx. 50 μl and 1 μl is subjected to GLC.

Cultured fibroblasts

- For VLCFA analysis of fibroblasts, suspend the fibroblast pellet (containing at least 500 μg of protein) in 400 μl of water and disrupt the cells by sonication to form a homogeneous suspension.

- Take two aliquots (10 and 20 μl) of this suspension for duplicate protein analysis and transfer 200 μl of the remaining suspension to a glass tube (18×100mm) with Teflon-lined screwcap.
- Add 25 μl (= 1 μg) of the heptacosanoic acid solution and proceed as described above for plasma.

Procedure 2

Based on the method described by Lepage and Roy (1986) and applied to serum by Onkenhout et al (1989), procedure 2 involves direct transesterification of the lipid in the plasma matrix and purification of the fatty acid methylesters by TLC.

Plasma or serum

- Into a 13×100mm glass tube with Teflon-lined screwcap, pipette 250 μl of serum or plasma; add 50 μl of the $C_{15:0}$ and 50 μl of the $C_{27:0}$ solution and then 2ml of methanol –benzene 4:1 (v/v) and mix.
- Add slowly, while stirring in an ice bath, 200 μl of acetyl chloride.
- Close the tube tightly and heat for 4h at 85°C in a heating block.
- Cool to room temperature and add, while cooling in an ice bath, 5ml of 6% aqueous potassium carbonate.
- Vortex briefly, close the tube and centrifuge for 10min at 750*g* at 4°C.
- With a Pasteur pipette, transfer the upper benzene layer into a 2ml vial.
- Evaporate under N_2 and dissolve the residue in 200 μl of hexane.
- Apply 100 μl of this solution to a precoated TLC plate and proceed with the purification of the FAME as described in *procedure 1*.

Fibroblasts

- Transfer 250 μl of homogeneous suspension (see *procedure 1*) to a 13×100mm screw-capped glass tube.
- Add 25 μl (1 μg) of the $C_{27:0}$ solution and 2ml of methanol–benzene 4:1 (v/v) and proceed as described for *plasma*.

Alternative to procedure 2

The direct-one step transesterification can also be performed by using methanolic HCl instead of methanol–benzene and acetyl chloride.

- To 250 μl of plasma or fibroblast suspension and internal standard is added 2ml of 2mol/L methanolic HCl.
- The tubes are heated for 4h at 85°C and the FAME is subsequently isolated and purified as described in *procedure 1*.

Capillary gas chromatography

For the assay of VLCFA in our laboratory we use a 25m×0.32mm ID cross-linked 5% phenylmethylsilicone (0.17 μm) capillary column (Hewlett Packard Ultra II). Helium is

used as the carrier gas, at a flow rate of 1 ml/min. The detector temperature is 320°C and the on-column injector temperature 60°C. The oven temperature is programmed as follows:

- The initial temperature of 60°C is immediately increased to 220°C at 50°C/min and held there for 15 min.
- Subsequently the temperature is increased to 250°C at 4°C/min and maintained there for 3 min.
- The final temperature of 285°C is reached by a temperature rise of 8°C/min and maintained for 15 min.

For the analysis of pristanic and phytanic acid an OV 1701 (14% cyanopropylphenyl) column (Betron) is used. The oven temperature is programmed as follows:

- The initial temperature of 60°C is maintained for 2 min and then increased to 200°C at a rate of 16°C/min and subsequently to 240°C at a rate of 4°C/min.
- The temperature of 240°C is maintained for 20 min and then increased to the final temperature of 280°C, which is maintained for 10 min.

RESULTS AND DISCUSSION

Table 1 summarizes the concentrations of VLCFA, phytanic acid and pristanic acid, in plasma and fibroblasts of controls and patients with different peroxisomal disorders as measured in our laboratory.

Note that our normal values are higher than those reported by Moser and Moser (1991) and by other laboratories that use essentially the same method (Schutgens et al 1993). The difference is most striking in fibroblasts. There is no clear explanation for this discrepancy unless our modes of extraction are more extensive, resulting in a higher recovery of the fatty acid methyl esters. Our control values are more in agreement with those reported by Onkenhout et al (1989), which were obtained by using *procedure 2*.

In our hands *procedure 1* and *procedure 2*, when they were compared in a number of duplicate plasma and fibroblast samples, gave similar results.

Our results obtained with X-linked adrenoleukodystrophy show that in virtually all affected patients the three parameters of $C_{26:0}$ concentration as well as $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ ratios are significantly elevated in both plasma and fibroblasts, although there is great variability among individuals.

In plasma of presumed heterozygotes we found abnormal values of the three parameters in only 60% of the cases. In the remaining cases sometimes only the $C_{26:0}$ or $C_{24:0}/C_{22:0}$ ratio was elevated. In 4 cases normal values of all three parameters were found. However, fibroblast analysis in these ambiguous cases revealed significantly elevated values for the three parameters, except in one case where the plasma values were normal. These findings are in agreement with the observation of Moser and Moser (1991) that 10–15% of heterozygotes were not detectable by VLCFA analysis in either plasma or cultured fibroblasts. The X-ALD heterozygote identification can be made more secure by DNA linkage analysis (Willems et al 1990).

Table 1 Concentrations of VLCFA, pristanic acid and phytanic acid in plasma and fibroblasts of controls and patients affected by different peroxisomal diseases

Sample	$C_{26:0}$	$C_{26:0}/C_{22:0}$ *ratio*	$C_{24:0}/C_{22:0}$ *ratio*	*Pristanic acid*	*Phytanic acid*
Plasma/serum (μg/ml)					
Controls (n=50)	0.30–0.80 (0.48)	0.016–0.035 (0.025)	0.65–1.00 (0.83)	n.d.–0.2 (0.1)	0.4–4.0 (0.8)
X-ALD-AMN (n=35)	0.70 –2.80 (1.42)	0.056–0.130 (0.072)	0.96–1.89 (1.39)	n.a.	n.a.
X-ALD heterozygotes (n=40)	0.55–1.75 (1.10)	0.018–0.108 (0.055)	0.68–1.44 (1.10)	n.a.	n.a.
Peroxisome deficiency disorders (n=9)	1.35–6.20 (3.30)	0.113–0.815 (0.345)	1.35–2.75 (1.90)	0.6–7.0 (3.0)	10.0–45.0 (25.0)
β-Oxidation deficiencies (n=4)	3.5–6.10 (4.40)	0.360–0.525 (0.410)	1.70–2.90 (2.20)	12.0–50.0 (31.0)	8.0–21.0 (12.0)
Refsum disease (n=3)	0.35–0.50 (0.45)	0.018–0.28 (0.022)	0.77–0.89 (0.83)	n.d.–0.20 (0.1)	800–1250 (925)
Fibroblasts (μg/mg protein)					
Controls (n=20)	0.15–0.35 (0.23)	0.08–0.20 (0.15)	1.70–2.30 (2.05)	n.a.	n.a.
XALD-AMN (n=12)	0.55–1.00 (0.65)	0.40–0.75 (0.51)	2.55–3.20 (2.70)	n.a.	n.a.
XALD heterozygotes (n=15)	0.45–0.95 (0.56)	0.30–0.65 (0.39)	2.45–2.95 (2.60)	n.a.	n.a.
Peroxisome deficiency disorders (n=6)	0.95–1.70 (1.45)	0.60–1.10 (0.85)	2.90–4.55 (3.50)	n.a.	n.a.
β-Oxidation deficiencies (n=3)	0.85–2.10 (1.70)	0.50–1.30 (0.75)	3.10–4.60 (3.70)	n.a.	n.a.

n.a.=not analysed; n.d.=not detectable; mean values in parentheses

In newborns, the diagnosis of ALD by measurement of VLCFA in plasma is hampered by the fact that sometimes (we estimate in about 20% of cases) elevated values of some or all of the three parameters are found that appear not to be related to a peroxisomal defect but are probably due to an immaturity of the peroxisomal system. These abnormal levels normalize after some weeks or even months. False positive results may also be observed in children who are receiving a ketogenic diet (Moser and Moser 1991) and in cases of severe liver malfunction (own observations).

False negative results in plasma from XALD patients appear also to be possible, as reported by Wanders et al (1993). In two cases of clinically suspected patients the plasma VLCFA levels were found normal, whereas they were clearly elevated in cultured fibroblasts.

Whenever there is clinical evidence for XALD and plasma VLCFA concentrations are difficult to interpret or normal, analysis of fibroblasts is recommended.

In the 7 patients diagnosed with a generalized peroxisomal disorder, all three parameters for VLCFA were strongly elevated in both plasma and fibroblasts. Plasma phytanic acid in blood samples taken at least 6 weeks after birth was also elevated. Except in one case, pristanic acid concentration was also elevated.

The VLCFA concentrations were also very high in plasma and fibroblasts of 4 patients in whom plasmalogen biosynthesis was normal and hence who were classified as disorders with an isolated β-oxidation defect. In all these patients, plasma pristanic acid concentration was very high and phytanic acid was also elevated, but to a lesser extent.

As already stated by ten Brink et al (1992), the pristanic acid/phytanic acid ratio is high in isolated β-oxidation defects while it is normal in disorders of peroxisome assembly. One of these patients was identified by immunoblotting and complementation analysis as a functional defect of the bifunctional enzyme. The defect in the other cases is still unidentified. They all have normal acyl-CoA oxidase activities (of both palmitoyl-CoA and pristanoyl-CoA oxidase) and the three β-oxidation enzymes are immunologically present. Significantly there is no accumulation of bile acid precursors in two cases.

REFERENCES

Lepage G, Roy CC (1986) Direct transesterification of all classes of lipids in a one-step reaction. *J Lipid Res* **27**: 114–120.

Moser HW, Moser AB (1991) Measurement of saturated very long chain fatty acid in plasma. In Hommes FA, ed. *Techniques in Diagnostic Human Biochemical Genetics*. New York: Wiley-Liss, 177–191.

Onkenhout W, Pael PFH vd, Heuvel MPH vd (1989) Improved determination of very long-chain fatty acids in plasma and cultured skin fibroblasts: application to the diagnosis of peroxisomal disorders. *J Chromatography* **494**: 31–41.

Schutgens RBH, Bouman IW, Nijenhuis AA, Wanders RJA, Frumau MEJ (1993) Profiles of very long chain fatty acids in plasma, fibroblasts and blood cells in Zellweger syndrome, X-linked adrenoleukodystrophy and rhizomelic chondrodysplasia punctata. *Clin Chem* **39**: 1632–1637.

Singh I, Moser AB, Goldfisher S, Moser HW (1984) Lignoceric acid is oxidized in the peroxisome: implications for the Zellweger cerebro-hepato-renal syndrome and adrenoleukodystrophy. *Proc Natl Acad Sci USA* **81**, 4203–4207.

ten Brink HJ, Stellaard F, Heuvel CMM vd et al (1992) Pristanic and phytanic acid in plasma from patients with peroxisomal disorders: stable isotope dilution analysis with electron capture negative ion mass fragmentography. *J Lipid Res* **33**: 31–37.

Vanhove GF, Veldhoven PP v, Fransen M et al (1993) The CoA esters of 2-methyl branched chain fatty acids and of the bile acid intermediates di- and trihydroxycoprostanic acids are oxidized by one single peroxisomal branched chain acyl CoA oxidase in human liver and kidney. *J Biol Chem* **286**: 10335–10344.

Wanders RJA, Schutgens RBH, Barth PG, Tager JM, Bosch H vd (1993) Postnatal diagnosis of peroxisomal disorders: a biochemical approach. *Biochemie* **75**: 269–279.

Willems P, Vits L, Wanders RJA et al (1990) Linkage of DNA markers at Xq 28 to adrenoleukodystrophy and adrenomyeloneuropathy present within the same family. *Arch Neurol* **47**: 665–669.