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Rapid stable isotope dilution analysis of very-long-chain fatty acids, pristanic acid and phytanic acid using gas chromatography–electron impact mass spectrometry

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Abstract

A common feature of most peroxisomal disorders is the accumulation of very-long-chain fatty acids (VLCFAs) and/or pristanic and phytanic acid in plasma. Previously described methods utilizing either gas chromatography alone or gas chromatography–mass spectrometry are, in general, time-consuming and unable to analyze VLCFAs, pristanic and phytanic acid within a single analysis. We describe a simple, reproducible and rapid method using gas chromatography/mass spectrometry with deuterated internal standards. The method was evaluated by analysing 30 control samples and samples from 35 patients with defined peroxisomal disorders and showed good discrimination between controls and patients. This method is suitable for routine screening for peroxisomal disorders.

Keywords: Very-long-chain fatty acids; Fatty acids; Pristanic acid; Phytanic acid

1. Introduction

Saturated very-long-chain fatty acids (VLCFAs) including C26:0 and C24:0 cannot be β-oxidized by mitochondria and have to undergo a few cycles of β-oxidation in peroxisomes before they can be handled by mitochondria. Therefore, in a number of peroxisomal disorders including defects of peroxisome biogenesis (e.g. Zellweger syndrome) and isolated defects in the pathway of peroxisomal β-oxidation (e.g. X-linked adrenoleukodystrophy, acyl-CoA oxidase deficiency, bifunctional protein deficiency and peroxisomal thiolase deficiency) [1–4], elevated concentrations of VLCFAs in plasma or cultured skin fibroblasts are a common feature. In addition, peroxisomes catalyze the degradation of branched-chain fatty acids like pristanic and phytanic acid [1,3]. Because these branched-chain fatty acids are not synthesized 'de novo' in the human body, newborns with defects in peroxisomal biogenesis often show normal levels of pristanic and phytanic acid despite (partially) defective phytanic acid α-oxidation. In time, however, these defects lead to

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elevated concentrations of both pristanic and phytanic acid in such patients resulting from dietary intake. Recently, the genetic and enzymatic defect in adult Refsum disease was elucidated and proven to be a defect in the peroxisomal enzyme phytanoyl-CoA hydroxylase explaining the accumulation of phytanic acid in the presence of normal concentrations of pristanic acid in these patients [5,6].

Although a number of methods have been published for either the analysis of VLCFAs or pristanic and phytanic acid [7–11], very few methods include the simultaneous analysis of these compounds. The advantage of such an analysis is obvious: it allows not only the detection of defects in peroxisomal biogenesis or β-oxidation but the same assay can be used in screening for adult Refsum disease and other peroxisomal defects [e.g. Rhizomelic chondriodysplasia punctata (RCDP)] in patients with sufficient dietary intake of branched-chain fatty acids causing plasma levels to be elevated.

In this paper, we describe a rapid and sensitive assay for the simultaneous analysis of VLCFAs and pristanic and phytanic acid using gas chromatography–electron impact mass spectrometry (GC–EI-MS). The method was applied to plasma samples of healthy controls and patients with various known peroxisomal disorders.

2. Experimental

2.1. Reagents and chemicals

3,7,11,15-Tetramethylhexadecanoic acid (phytanic acid), docosanoic acid (C22:0), tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0) were purchased from Sigma (St. Louis, MO, USA). 2,6,10,14-Tetramethylpentadecanoic acid (pristanic acid) was obtained from Prof. dr. G. Dacremont, Belgium (purity>95%).

3,3,5,5-$^{2}H_4$-C22:0 (isotopic purity:>98%), 3,3,5,5,-$^{2}H_4$-C24:0 (isotopic purity:>98%), 3,3,5,5-$^{2}H_4$-C26:0 (isotopic purity:>98%), $^{2}H_3$-phytanic acid and $^{2}H_3$-pristanic acid were obtained from Dr.H.J. ten Brink, Free University Hospital (Amsterdam, The Netherlands) [12]. *N*-Methyl-*N*-(*tert*.-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) was obtained from Pierce (Rockford, IL, USA). All organic solvents and other chemicals were of analytical grade and were used without further purification.

2.2. Samples

Plasma and serum specimens were obtained from 35 patients; 12 patients diagnosed with classical Zellweger syndrome, 16 (male) patients with proven X-ALD, 5 (female) patients diagnosed as heterozygotes X-ALD, one patient with adult Refsum disease, one patient with RCDP and 30 healthy controls. All diagnoses were verified using well-established enzymatic and immunological measurements in cultured skin fibroblasts (see Ref. [3] for details).

Blood was collected into EDTA or heparinized tubes and maintained at 4°C. The plasma was separated by centrifugation at 1500*g* for 10 min at 4°C and then stored at −20°C until analysis.

2.3. Analytical methods

2.3.1. Sample preparation

To 100 μl of plasma or serum, 100 μl of internal standard solution in toluene was added containing 0.1 nmol $^{2}H_3$-pristanic acid, 0.4 nmol $^{2}H_3$-phytanic acid, 5.0 nmol $^{2}H_4$-C22:0, 5.0 nmol $^{2}H_4$-C24:0 and 1.0 nmol $^{2}H_4$-C26:0. Acidic hydrolysis was performed by adding 2.0 ml of 0.5 mol/l HCl in acetonitrile, followed by a 45-min incubation at 110°C. Subsequently, alkaline hydrolysis was carried out by adding 2.0 ml of 1.0 mol/l NaOH in methanol followed by a 45-min incubation at 110°C. After cooling to room temperature, the pH was lowered by adding 0.5 ml of 10 *M* HCl. The fatty acids were then extracted into 4 ml of hexane. To remove most of the sterols, the organic layer was washed with 4 ml of 1 *M* KOH. The aqueous layer was acidified again with 0.5 ml of 10 *M* HCl and the fatty acids were re-extracted with 4 ml of hexane. To avoid interference of unsaturated fatty acids during the analysis, we added 25 μl of 1% bromine. The organic layer was evaporated to dryness under nitrogen at 45°C. The sample was then derivatized

Table 1
Single-ion monitoring

Compound	$[M-57]^+$
Pristanic acid	355.3
$[^2H_3]$-Pristanic acid	358.3
Phytanic acid	369.3
$[^2H_3]$-Phytanic acid	372.3
C22:0	397.4
$[^2H_4]$-C22:0	401.4
C24:0	425.4
$[^2H_4]$-C24:0	429.4
C26:0	453.4
$[^2H_4]$-C26:0	457.4

with MTBSTFA and pyridine (50 μl each) at 80°C for 30 min.

2.3.2. GC–MS

The fatty acids were analyzed on a Hewlett-Packard model 5890/5973 GC–MS system equipped with a CPsil 5 low-bleed capillary column (25 m× 0.25 mm I.D., film thickness 0.25 μm, Chrompack, Middelburg, The Netherlands). For separation the temperature programming started at 60°C (held for 1 min), increasing at 30°C/min to 160°C, then increasing to 230°C at 5°C/min and subsequently at 20°C/min increasing to 320°C (held for 10 min). The injection temperature and interface temperature were both 300°C. The injection volume was 1 μl at splitless mode with a splitless time of 1.5 min. Helium was used as carrier gas (50 kPa). Electron impact ionization was applied at 70 eV. MS acquisition was performed in the SIM (single ion monitoring) mode, monitoring the characteristic $[M-57]^+$ ions as listed in Table 1.

2.3.3. Calculation and statistics

The GC–MS data were analyzed quantitatively with HP-chemstation software which allowed a completely computerized analysis of the mass spectral data. The metabolites were quantified using calibration curves obtained for each individual fatty acid in the concentration range from 0–150 μmol/l (C22:0 and C24:0), 0–20 μmol/l (C26:0) and 0–100 μmol/l (pristanic and phytanic acid). Concentrations were calculated using a linear fit from calibration curves.

3. Results and discussion

3.1. Optimization of sample preparation procedure

Sample preparation for the analysis of VLCFAs and the branched-chain fatty acids pristanic and phytanic acid included both an alkaline hydrolysis and an acidic hydrolysis in order to optimize hydrolysis yields for both the saturated and the branched-chain compounds. Whereas acidic hydrolysis gave acceptable yields for the VLCFAs, the addition of an alkaline hydrolysis increased the yield of the branched-chain fatty acids by about 80%. Although alkaline hydrolysis alone gave good yields for C26:0, pristanic and phytanic acid, a combination of an alkaline and acidic hydrolyis was needed to yield optimal recoveries for C22:0 and C24:0 (Table 2). No significant difference in yield was observed when a Folch extraction was included in the sample preparation. Therefore, this laborious step was not included in the standard method for sample preparation. Because in normal plasma the concentration of the

Table 2
Effect of different types of hydrolysis on the recovery of various fatty acids

Compound[a]	Acidic hydrolysis (μmol/l)	Alkaline hydrolysis (μmol/l)	Acidic+alkaline hydrolysis (μmol/l)
C26:0	0.75±0.01	0.95±0.03	0.94±0.02
C24:0	59±1	45±1	66±1
C22:0	61±1	41±1	61±1
Phytanic acid	3.7±0.1	7.0±0.1	7.3±0.2
Pristanic acid	0.44±0.01	0.96±0.02	0.94±0.03

[a] A normal pool-plasma was analyzed in triplicate. Hydrolysis was carried out for 45 min at 110°C as indicated. Other procedures are described in the Section 2.

unsaturated fatty acid C18:2n-6 (linoleic acid) is about 1000 times higher than the concentration of phytanic acid and these peaks elute at approximately the same retention time under most chromatographic conditions, the peak shape for phytanic acid was initially not optimal. A bromine addition was included in the sample preparation in order to increase the retention time of C18:2n-6, thus decreasing the interference of C18:2n-6 with phytanic acid (data not shown). Altered retention times for other bromated fatty acids did not lead to interference in the analysis of other compounds of interest.

In order to remove most of the cholesterol present in the samples, an alkaline wash step was also included into the final protocol. Washing with 1 *M* KOH and re-extraction of the fatty acids with hexane removed >90% of the cholesterol present in the sample without significant reduction of the yield of both VLCFAs and branched-chain fatty acids (data not shown).

3.2. GC–EI-MS analysis

Derivatization of VLCFAs and branched-chain fatty acids using MTBSTFA yielded stable derivatives (Fig. 1) which characteristically loose the tertiary-butyl group ($m/z=57$). For all compounds the $[M-57]^+$ ion was the most abundant ion (100% base-peak). The recovery of authentic standards was linear for all components with correlation coefficients between 0.985 and 0.999 when calculated concentrations were plotted against the concentrations of the added authentic standards. Calculated recoveries were between 85–94% (Table 3). Fig. 2 shows representative mass chromatograms of a normal control, an adult Refsum patient and a Zellweger patient. Table 4 shows the coefficients of variation for the various compounds at both the normal and pathological level. The sensitivity of the method (detection limit (defined as a signal/noise ratio of 5:1) <50 nmol/l for C26:0) appeared to be sufficient to quantify the compounds at a clinically significant level of precision.

3.3. Analysis of control and patient samples

As expected, analysis of patients samples yielded abnormal values for C26:0 and the C26:0/C22:0 ratio for both Zellweger and X-ALD patient as compared to control values. The control values reported here are in good agreement with those mentioned in the literature [11,13]. In further agreement with literature data we found elevated values of phytanic acid for patients either diagnosed as having adult Refsum disease or RCDP, whereas the values for VLCFAs were normal in these patients (Table 5).

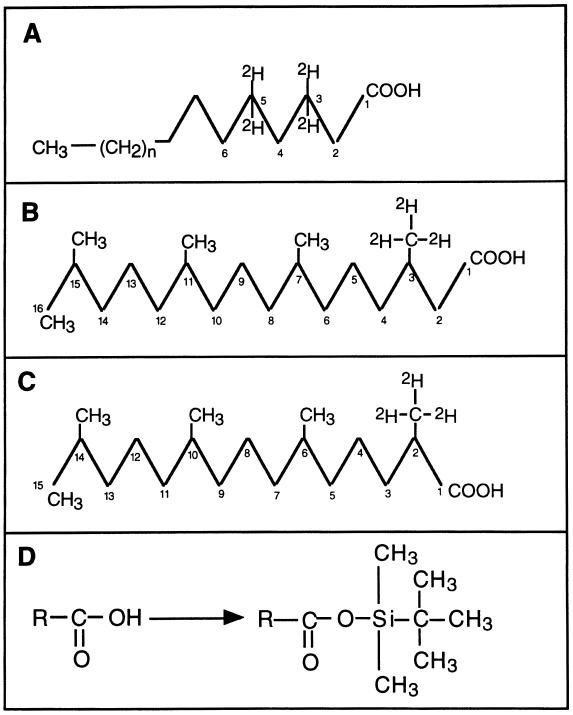

Fig. 1. Chemical structures of compounds. Schematic representation of the deuterated fatty acids [$^2H_4$]-C22:0 ($n=13$), [$^2H_4$]-C24:0 ($n=15$) and [$^2H_4$]-C26:0 ($n=17$) (panel A), [$^2H_3$]-phytanic acid (panel B) and [$^2H_3$]-pristanic acid (panel C). Panel D shows a representation of the MTBSTFA derivatization for the acid groups.

4. Conclusions

The results described in this paper show that we have developed a rapid and sensitive analytical method for the simultaneous measurement of very-long-chain fatty acids, pristanic and phytanic acid. Sample preparation does not include Folch extraction

Table 3
Recovery of VLCFAs, phytanic and pristanic acid

Compound	Curve ($y=ax+b$)[a]	Correlation coefficient (r^2)	Recovery (%)
C26:0	$y=0.86x+0.81$	0.985	86
C24:0	$y=0.93x+50.3$	0.998	93
C22:0	$y=0.94x+60.7$	0.998	94
Phytanic acid	$y=0.85x+3.4$	0.999	85
Pristanic acid	$y=0.96x+0.52$	0.998	96

[a] Recoveries were calculated by adding authentic standard solutions in five concentrations to a normal pool sample. The results are averages of three runs.

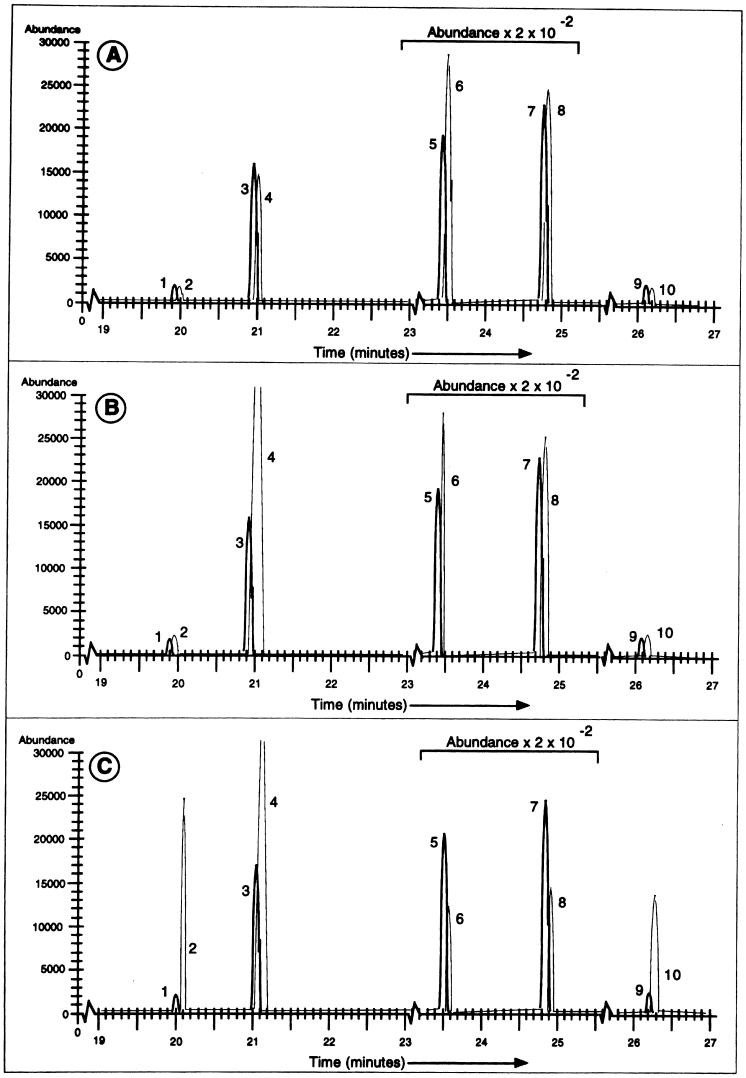

Fig. 2. Representative mass chromatograms (redrawn from original output) for a control (A), a patient with adult Refsum disease (B) and a Zellweger patient (C). Peaks: 1, [$^2H_3$]-pristanic acid; 2, pristanic acid; 3, [$^2H_3$]-phytanic acid; 4, phytanic acid; 5, [$^2H_4$]-C22:0; 6, C22:0; 7, [$^2H_4$]-C24:0; 8, C24:0; 9, [$^2H_4$]-C26:0; 10, C26:0.

Table 4
Reproducibility results

Normal pool ($n=20$)[a]				Abnormal pool ($n=20$)[a]			
Compound	Mean concentration (μmol/l)	C.V. (%) intra-assay	C.V. (%) inter-assay	Compound	Mean concentration (μmol/l)	C.V. (%) intra-assay	C.V. (%) inter-assay
C26:0	0.6	4.8	10.1	C26:0	1.7	1.7	8.2
C24:0	51.9	1.3	3.1	C24:0	62.8	1.7	4.3
C22:0	59.4	1.2	2.2	C22:0	58.4	1.4	3.4
Phytanic acid	3.4	0.9	4.8	Phytanic acid	20.8	1.7	3.0
Pristanic acid	0.5	1.1	4.8	Pristanic acid	2.2	1.7	4.4

[a] Normal and abnormal pools were obtained by mixing 50 plasma samples obtained from healthy controls (normal pool) or patients suffering from various peroxisomal disorders (abnormal pool).

or purification of samples by thin-layer chromatography as has been described for other methods [10,11,13] and is therefore less time consuming. The relative simplicity of the method and good reproducibility emphasize the usability of the method for routine screening of peroxisomal disorders and follow-up of X-ALD patients receiving dietary treatment.

Table 5
Results for controls and patients with various peroxisomal disorders

	C26:0 (μmol/l)	C24:0 (μmol/l)	C22:0 (μmol/l)	C26:0/C22:0 ratio	C24:0/C22:0 ratio	Phytanic acid (μmol/l)	Pristanic acid (μmol/l)
Controls ($n=30$)							
Median	0.9	52.4	62.5	0.014	0.841	4.4	0.5
(5–95%)[a]	(0.6–1.2)	(37.4–74.9)	(41.1–90.3)	(0.011–0.022)	(0.689–1.008)	(0.3–11.5)	(0.0–1.5)
Zellweger ($n=12$)							
Median	4.6	35.9	23.2	0.152	1.490	28.5	10.5
(5–95%)[a]	(1.8–8.1)	(21.7–73.4)	(12.5–52.4)	(0.069–0.453)	(0.919–2.527)	(1.6–115.7)	(0.5–30.3)
X-ALD ($n=7$)							
Median	2.3	75.6	49.4	0.053	1.307	8.4	0.8
(5–95%)[a]	(1.3–4.1)	(31.3–98.2)	(22.9–100.2)	(0.039–0.058)	(0.766–2.407)	(1.0–14.9)	(0.1–2.2)
X-ALD on glycerol trioleate/glycerol trierucate treatment [14] ($n=9$)							
Median	1.3	33.6	28.9	0.044	1.317	1.8	0.2
(5–95%)[a]	(0.9–2.5)	(22.4–72.2)	(18.5–48.6)	(0.014–0.178)	(1.019–1.657)	(0.6–7.6)	(0.0–1.7)
X-ALD-heterozygotes ($n=5$)							
Median	1.8	103.4	70.0	0.026	1.188	3.6	0.3
(5–95%)[a]	(1.2–2.3)	(65.2–107.3)	(57.7–89.0)	(0.015–0.039)	(0.922–1.677)	(1.4–13.0)	(0.2–1.9)
Class. Refsum	0.9	57.0	50.0	0.018	1.14	425.0	1.1
RCDP Type I	1.0	38.9	44.6	0.022	0.87	27.4	2.3

[a] 5%–95% interval.

References

[1] H. Van den Bosch, R.B.H. Schutgens, R.J.A. Wanders, J.M. Tager, Annu. Rev. Biochem. 61 (1992) 157.
[2] J.K. Reddy, G.P. Mannaerts, Annu. Rev. Nutr. 14 (1994) 343.
[3] R.J.A. Wanders, R.B.H. Schutgens, P.G. Barth, J. Neuropathol. Exp. Neurol. 54 (1995) 726.
[4] W.H. Kunau, V. Dommes, H. Schultz, Prog. Lipid. Res. 34 (1995) 267.
[5] G.A. Jansen, S.J. Mihalik, P.A. Watkins, H.W. Moser, C. Jakobs, H.S.A. Heijmans, R.J.A. Wanders, J. Inher. Metab. Dis. 20 (1997) 444.
[6] G.A. Jansen, S.J. Mihalik, P.A. Watkins, H.W. Moser, C. Jakobs, R.J.A. Wanders, Biochem. Biophys. Res. Commun. 229 (1996) 205.
[7] F. Stellaard, H.J. ten Brink, R.M. Kok, L. van den Heuvel, C. Jakobs, Clin. Chim. Acta 192 (1990) 133.
[8] H. Vallance, D. Applegarth, Clin. Biochem. 27 (1994) 183.
[9] U. Caruso, J. Chromatogr. 562 (1991) 147.
[10] W. Onkenhout, P.F.H. van der Poel, M.P.M. van den Heuvel, J. Chromatogr. 494 (1989) 31.
[11] R.B.H. Schutgens, I.W. Bouman, A.A. Nijenhuis, R.J.A. Wanders, M.E.J. Frumau, Clin. Chem. 39 (1993) 1632.
[12] H.J. Ten Brink, C. Jakobs, F. Bickelhaupt, in: T.A. Baillie, J.R. Jones (Eds.), Synthesis and Applications of Isotopically Labelled Compounds, Amsterdam, Elsevier, 1989, pp. 723–726.
[13] H.W. Moser, A.B. Moser, in: F.A. Hommes (Ed.), Techniques in Diagnostic Human Biochemical Genetics, New York, Wiley Liss Inc. 1991, pp. 177–191.
[14] P. Aubourg, C. Adamsbaum, M.C. Lavallard-Rousseau, N. Engl. J. Med. 329 (1993) 745.