Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 798 (2003) 159–162
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Short communication

Measurement of plasma pristanic, phytanic and very long chain fatty acids by liquid chromatography-electrospray tandem mass spectrometry for the diagnosis of peroxisomal disorders

David W. Johnson [a,*], Minh-Uyen Trinh [a], Tomoyuki Oe [b]

[a] *Department of Chemical Pathology, Women's and Children's Hospital, 72 King William Road, North Adelaide, SA 5006, Australia*
[b] *Center for Cancer Pharmacology, University of Pennsylvania, Philadelphia, PA, USA*

Received 11 June 2003; received in revised form 3 September 2003; accepted 5 September 2003

Abstract

High pressure liquid chromatography with a narrow bore C8 column has been used to separate pristanic, phytanic and very long chain fatty acids, important in the diagnosis of peroxisomal disorders, for their accurate isotope dilution quantification by tandem mass spectrometry. The fatty acids, isolated from plasma, were analysed as trimethylaminoethyl ester (quaternary ammonium) derivatives. Analysis time was 2.5 h and sample requirement was 10 μl of plasma. Good agreement with GC–MS methods for the levels of pristanic and phytanic acids, C26:0/C22:0 and C24:0/C22:0 ratios were obtained for 12 plasma samples from peroxisomal disorder patients and a set of controls.

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

1. Introduction

The single most useful biochemical test for the diagnosis, and further sub-classification, of a peroxisomal disorder is the measurement of total (de-esterified) branched chain and very long chain fatty acids (VLCFA) in plasma. In the majority of peroxisomal disorders tetracosanoic (C24:0) and hexacosanoic (C26:0) acids are increased relative to docosanoic (C22:0) acid with eight exceptions including Refsum's Disease and Rhizomelic Chondrodysplasia Punctata with elevated phytanic acid (C20:0 branched) (Fig. 1A). Pristanic acid (C19:0 branched) (Fig. 1B) is notably elevated in peroxisomal 3-oxoacyl-CoA thiolase deficiency and bifunctional protein deficiency.

Measurement of all these metabolites has been performed for many years by GC [1] using dual columns or by isotope dilution GC–MS [2]. An electrospray ionization tandem mass spectrometry (ESI–MS/MS) method for quantifying VLCFA, using dimethylaminoethyl ester derivatives [3], was recently developed for faster analysis with very small samples, including blood spots for neonatal screening. A 10-fold improvement in sensitivity was subsequently achieved using the trimethylaminoethyl (TMAE) ester iodide derivative [4] (Fig. 1C). Branched chain fatty acids could not be accurately measured by this method. Although structural isomers can often be differentiated by high energy MS/MS the product ion spectra of the TMAE derivatives of the branched and normal forms of C19:0 and C20:0 acids were indistinguishable.

A liquid chromatography separation step has been added to this ESI–MS/MS method to enable accurate quantification of pristanic and phytanic acids. A systematic trial of HPLC columns and solvent gradients was performed to determine the best combination for baseline separation of these fatty acid derivatives. Plasma samples from peroxisomal disorder patients, and a set of controls, were analysed to test the new method.

2. Experimental

2.1. Chemicals

Deuterium-labeled very long chain fatty acids were obtained from Larodan Fine Chemicals (Malmo, Sweden). Deuterium-labeled pristanic and phytanic acids were synthesised [5]. All other chemicals were obtained from Sigma/Aldrich (Castle Hill, NSW, Australia).

* Corresponding author. Fax: +61-8-81617100.
E-mail address: david.johnson@adelaide.edu.au (D.W. Johnson).

1570-0232/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2003.09.014

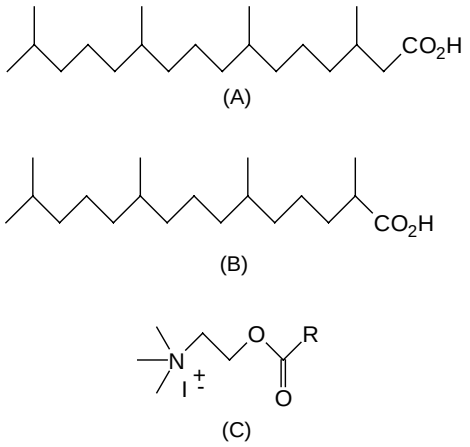

Fig. 1. Structures of (A) phytanic acid, (B) pristanic acid and (C) trimethylaminoethyl ester iodide derivatives.

2.2. Patient samples

Plasma samples from diagnosed peroxisomal disorder patients, used in this study, had been referred to our laboratory for very long chain fatty acid and bile acid analysis. Normal control plasma samples (from infants less than 2 years) were obtained from our metabolic screening laboratory.

2.3. Equipment

Mass spectrometric analysis was performed on an Applied Biosystems/MDS Sciex Model API365 (Concord, Ontario, Canada) tandem mass spectrometer. High pressure liquid chromatography was performed with an Agilent HP1100 binary pump (Waldbronn, Germany) system equipped with a Rheodyne injection valve (10 μl loop).

2.4. Sample preparation

To a 10 ml screw top glass tube was added 10 μl of a mixture of [$^2H_4$]-pristanic acid (20 μmol/l), [$^2H_4$]-phytanic acid (20 μmol/l), [$^2H_3$]-docosanoic acid (50 μmol/l), [$^2H_3$]-tetracosanoic acid (50 μmol/l), and [$^2H_3$]-hexacosaknoic acid (4 μmol/l) in ethanol. Plasma (10 μl), acetonitrile (360 μl) and hydrochloric acid (5 mol/l, 40 μl) were added. The tube was sealed and heated at 100 °C for 1 h. The cooled mixture was extracted with hexane (1 ml) and the solvent evaporated. Oxalyl chloride (2 mol/l in dichloromethane, 200 μl) was added to the residue and it was heated at 65 °C for 5 min with the tube sealed. The mixture was evaporated to dryness and dimethylaminoethanol (60 μl) was added. After 5 min at 25 °C, the mixture was evaporated to dryness. Methyl iodide (100 μl) was added to dissolve the residue. After 2 min at 25 °C, the mixture was evaporated and the residue was dissolved in ethanol (25 μl). Ethanol afforded narrower peaks than mobile phase. During the derivatization steps it is important to completely remove the excess of all derivatizing reagents. This can be aided by evaporating a small amount of dichloromethane after each step.

2.5. Column liquid chromatography

2.5.1. Column

A 150 mm × 2.1 mm, Alltech (Deerfield, Illinois, USA) Alltima C8 column, 5 μm particle size, operated at 23 °C.

2.5.2. Mobile phase

A solvent gradient of acetonitrile (with 0.1% trifluoroacetic acid) and water (with 0.1% trifluoroacetic acid) was employed. The gradient started at 80% acetonitrile, increased to 90% over 2.5 min, held at 90% for 5 min, increased to 100% acetonitrile over 2.5 min, held at 100% for 5 min, decreased to 80% over 5 min and held at 80% for 10 min. Flow rate was 150 μl/min. Injection volume was 10 μl.

2.6. Mass spectrometric analysis

2.6.1. Source

Ionspray operating at room temperature.

2.6.2. Mass analyser

Accelerating voltage 5000 V, positive ion, multiple reaction monitoring (MRM) mode with nitrogen collision gas.

2.6.3. Detection

The following 10 sets of ion pairs were monitored each for 100 ms: 384.4/325.4 (C19:0), 388.4/329.4 ([$^2H_4$]-C19:0),

Table 1
Fatty acid concentrations and ratios in the plasma of peroxisomal disease patients and normal controls measured by LC/ESI–MS/MS

	Pristanic (μmol/l)	Phytanic (μmol/l)	C24:0/C22:0	C26:0/C22:0
PBD patients ($n = 6$)				
Range	1.93–12.9	11.5–57.3	1.29–2.35	0.13–0.66
Mean ± S.D.	4.84 ± 4.14	23.9 ± 16.8	1.86 ± 0.41	0.37 ± 0.19
ALD/AMN patients ($n = 6$)				
Range	0.82–1.56	0.39–5.73	1.35–1.99	0.062–0.114
Mean ± S.D.	1.25 ± 0.32	3.69 ± 1.99	1.59 ± 0.25	0.091 ± 0.020
Normal controls ($n = 13$)				
Range	0.14–1.14	0.16–12.6	0.74–1.06	0.011–0.043
Mean ± S.D.	0.52 ± 0.34	3.34 ± 4.22	0.88 ± 0.09	0.025 ± 0.008

398.4/339.4 (C20:0), 402.4/343.4 ([$^2H_4$]-C20:0), 426.4/367.4 (C22:0), 429.4/370.4 ([$^2H_3$]-C22:0), 454.4/395.4 (C24:0), 457.4/398.4 ([$^2H_3$]-C24:0), 482.4/423.4 (C26:0) and 485.4/426.4 ([$^2H_3$]-C26:0).

3. Results and discussion

Good separation of the TMAE derivatives of pristanic and phytanic acids from their unbranched isomers was achieved with several C18 HPLC columns and a gradient of acetonitrile and water. Hexacosanoic acid, however, eluted too slowly. C8 HPLC columns afforded more rapid elution. The Alltech C8 column described in Experimental gave the best performance of several tried.

Mixtures of the five fatty acids were derivatized, separated by HPLC on the C8 HPLC column and analysed by ESI–MS/MS operated in multiple reaction monitoring mode. The intensities of 10 ion pairs, representing a neutral loss of 59 atomic mass units for each fatty acid and its

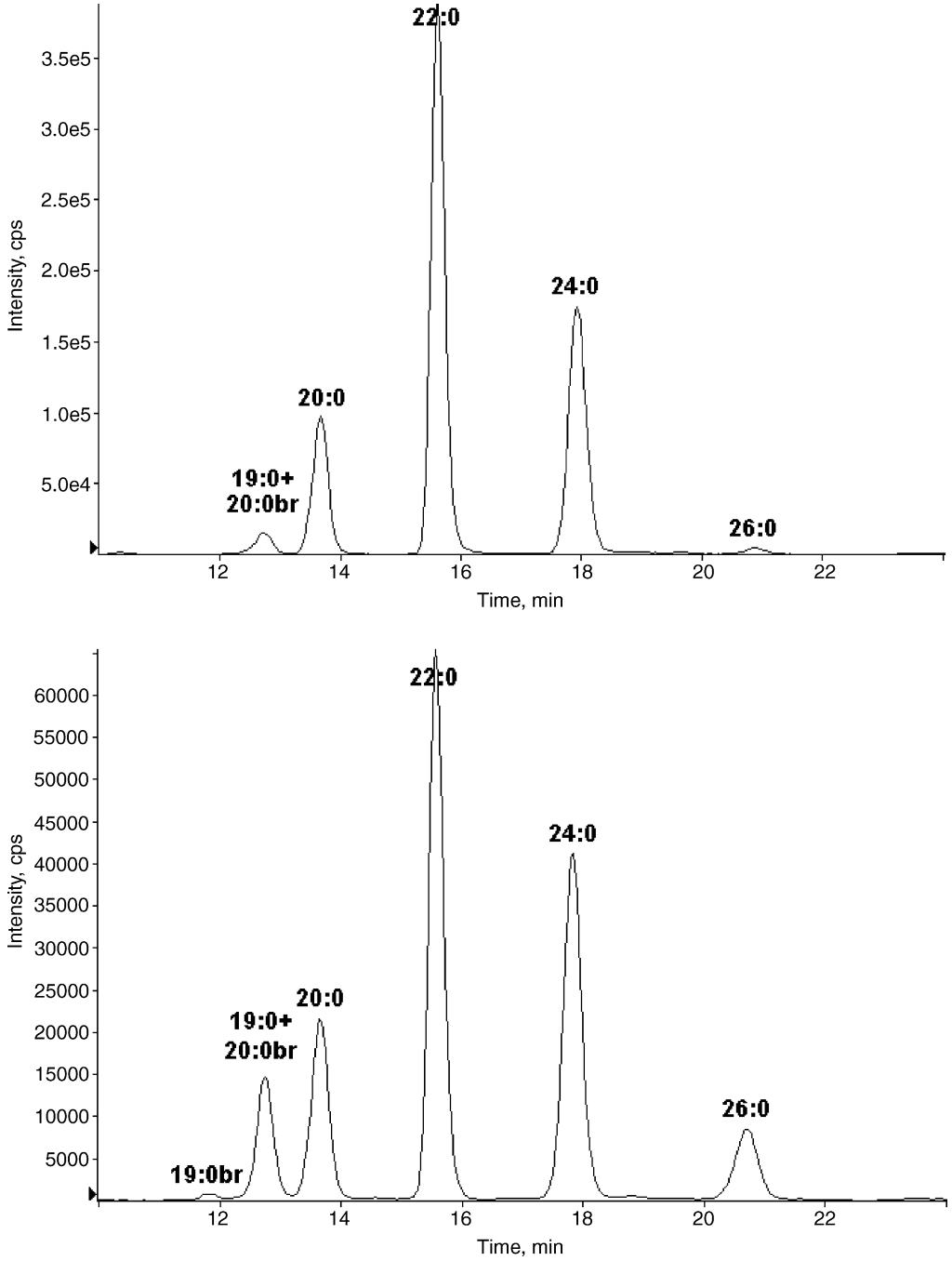

Fig. 2. Accumulated MRM ion chromatograms of unlabeled fatty acids from the LC/ESI–MS/MS analysis of plasma of (top) normal control and (bottom) peroxisome biogenesis defect patient. 19:0br is pristanic acid and 20:0br is phytanic acid.

corresponding labeled internal calibrator were measured for each a set of five standard mixtures. Calibration curves were constructed. Statistical analysis revealed response linearity for pristanic acid (0–40 μmol/l; $y = 5.23x + 0.02$ μmol/l; $R^2 = 0.993$), phytanic acid (0–100 μmol/l; $y = 1.77x + 0.09$ μmol/l; $R^2 = 0.998$), docosanoic acid (0–250 μmol/l; $y = 1.32x + 0.02$ μmol/l; $R^2 = 0.997$), tetracosanoic acid (0–250 μmol/l; $1.36x + 0.01$ μmol/l; $R^2 = 0.998$) and hexacosanoic acid (0–20 μmol/l; $y = 0.79x + 0.08$ μmol/l; $R^2 = 0.994$).

VLCFA and pristanic and phytanic acids were measured repeatedly (intraassay, $n = 10$; interassay, $n = 8$) in an adult plasma control. The imprecision in the measurements were pristanic acid (intraassay 19%, interassay 25%), phytanic acid (intraassay 4.3%, interassay 15%), docosanoic acid (intraassay 1.3%, interassay 6.8%), tetracosanoic acid (intraassay 2.4%, interssay 3.7%) and hexacosanoic acid (intraassay 23.8%, interassay 16.8%). The high variabilities in the measurement of pristanic and hexacosanoic acids reflect their low concentrations (<1 μmol/l) in normal plasma.

To rigorously test the LC/ESI–MS/MS method, plasma samples from six peroxisomal biogenesis defect (PBD) patients (including Zellweger, Infantile Refsum's and Neonatal ALD) were analysed. These patient samples were chosen because they contained small elevations in pristanic and phytanic acids as well as elevated VLCFA. Additionally, six samples from peroxisomal disorder patients diagnosed with X-linked adrenoleukodystrophy (ALD) and the related adrenomyelinoneuropathy (AMN) and from thirteen normal controls were analysed. Branched chain fatty acid concentrations and VLCFA ratios for each of these groups are summarized in Table 1. The branched chain fatty acid and VLCFA ratios are comparable with those obtained by GC–MS methods [2,6]. The only practical difficulty with the method arose from closely eluting peaks in the MRM (384.4/325.4) ion chromatogram in the quantification of pristanic acid, at low concentrations in several control samples. These peaks were resolved by starting at 70% acetonitrile and increasing the solvent program cycle time. The ion chromatograms from the LC/ESI–MS/MS analysis of a normal control and a PBD patient are shown in Fig. 2. Whilst phytanic (C20:0 branched) acid is seen to elute with identical retention time to nonadecanoic (C19:0) acid there is no contribution to the two MRM experiments for its quantification from any C19:0 present.

Baseline separation was achieved for both tetra methyl-branched pristanic and phytanic acids from their respective straight-chain isomers. Single methyl-branched isomers, such as iso- and anteiso-, which together comprise approximately 10% of a VLCFA, merged with the straight-chain isomers. For example, $[^2H_3]$-isolignoceric acid, with a peak width of 0.8 min, eluted 0.2 min faster than lignoceric acid (C24:0) during LC/ESI–MS/MS analysis. By contrast the methyl esters of $[^2H_3]$-isolignoceric and lignoceric acids are separable by capillary GC–MS [7]. Resolution of the iso-/anteiso- isomers from unbranched VLCFA is not necessary for it does not significantly affect the C26:0/C22:0 ratio for the diagnosis of peroxisomal disorders.

A reduction in the C26:0/C22:0 ratio for normal controls was observed after the addition of the LC separation step. The C26:0/C22:0 ratio mean of 0.038 for ESI–MS/MS analysis with dimethylaminoethyl ester derivatives [3] was reduced to a mean of 0.025 for the LC/ESI–MS/MS method. It is a common criticism of direct ESI–MS/MS analysis that it over-estimates low level metabolites (e.g. C26:0) in complex mixtures. A consequence of the more accurate measurement of the C26:0/C22:0 ratio is an improved diagnosis of ALD heterozygotes who have ratios intermediate between normal and peroxisomal disorder affected.

4. Conclusions

The method described combines the high sensitivity of a quaternary ammonium derivative for ESI–MS/MS analysis with HPLC separation for trace analysis of branched chain fatty acids in biological extracts. Whilst no faster than some GC–MS methods and an LC/API–MS method [8], with sample preparation time of 2 h and chromatographic separation cycle time of 30 min, it requires substantially less plasma (10 μl compared with typically 100–200 μl) which is an important consideration in pediatric testing. This can be further reduced, or precision improved, by the use of newer MS/MS instruments that are up to 100 times more sensitive than the one used in this study.

In a high throughput metabolic laboratory, plasma samples could be rapidly screened by the ESI–MS/MS method and the remainder of the sample subjected to LC/ESI–MS/MS analysis only if high C20:0/C22:0 and C26:0/C22:0 ratios were obtained.

References

[1] H.M. Harris, D.A. Applegarth, L.A. Clarke, J. Wong. Clin. Chem. 35 (1989) 703.
[2] N.M. Verhoeven, W. Kulik, C.M.M. van den Heuvel, C. Jakobs, J. Inher. Metab. Dis. 18 (Suppl. 1) (1995) 45.
[3] D.W. Johnson, J. Inher. Metab. Dis. 23 (2000) 475.
[4] D.W. Johnson, Rapid Commun. Mass Spectrom. 14 (2000) 2019.
[5] D.W. Johnson, A. Poulos, Chem. Phys. Lipids 48 (1988) 141.
[6] H.J. Ten Brink, F. Stellaard, C.M.M. van den Heuvel, R.M. Kok, D.S.M. Schor, R.J.A. Wanders, C. Jakobs, J. Lipid. Res. 33 (1992) 41.
[7] A. Poulos, P.C. Stockham, D.W. Johnson, B.C. Paton, K. Beckman, H. Singh, Lipids 34 (1999) 943.
[8] S. Schrade, M. Goggerle, H. Korall, J. Inher. Metab. Dis. 23 (Suppl. 1) (2000) 242.