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Determination of very-long-chain fatty acids in plasma by a simplified gas chromatographic–mass spectrometric procedure

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ABSTRACT

The concentration of very-long-chain fatty acids (VLCFA) (straight chain, more than 22 carbon atoms) in plasma or in cultured fibroblasts is one of the most important diagnostic criteria for the diagnosis of the peroxisomal disorders. A sensitive method for VLCFA assay in plasma, using small sample volume and a simplified procedure, is described. After adequate extraction and derivatization, methyl esters of VLCFA are separated, identificated and quantified by gas chromatography–mass spectrometry (GC–MS). The method is sensitive, reproducible, accurate and relatively simple. GC–MS equipment used for routine organic acid analysis can be used.

INTRODUCTION

The concentration of very-long-chain fatty acids (VLCFA) (straight chain, with 22 or more carbon atoms) in plasma or in cultured fibroblasts is one of the most important diagnostic criteria for peroxisomal disorders [1]. Previously reported methods for their determination are often complicated, time-consuming and require different equipment to that used for routine organic acid analysis [2,3].

In this paper, a simplified gas chromatographic–mass spectrometric (GC–MS) method for VLCFA assay in plasma using a small sample volume is described. The method is sensitive, reproducible and discriminates well affected patients from controls.

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EXPERIMENTAL

Chemicals were of analytical-reagent grade from Merck (Darmstadt, F.R.G.), unless indicated otherwise. VLCFA standards were obtained from Sigma (St. Louis, MO, U.S.A.).

Sample preparation

Plasma obtained from blood collected in heparin or EDTA can be stored at room temperature if analysed within one week, or at −20°C for longer periods.

A 40–200 nmol amount of internal standard (heptacosanoic acid; pentacosanoic or heneicosanoic acid can equally well be used) is added to 0.5 ml of plasma. Extraction is then performed as follows [4]. In a clear, screw-capped, glass tube, 5 ml of methanol–chloroform (1:1, v/v) are added to the sample. After shaking, the tube is allowed to stand for 1 h at room temperature. The supernatant obtained after centrifugation at 2000 *g* for 5 min is transferred into another tube and 2.5 ml of chloroform and 1.5 ml of water are added. After shaking (mechanical shaker, 2 min) and centrifugation at 2000 *g* for a few minutes to separate the two phases, the upper phase is removed and discarded. The lower phase is dried under nitrogen (the sample can be warmed to 37°C to facilitate the evaporation). The dried extract is rinsed from the sides of the tube with 0.5 ml of chloroform–methanol (2:1, v/v) and re-dried under nitrogen.

The sample is then derivatized with 1.5 ml of 1 *M* methanolic hydrochloric acid (Alltech, Deerfield, IL, U.S.A.) for a minimum of 16 h at 75°C in a well capped tube (or Silyl-vial). The tube is cooled to room temperature and extracted twice with 1 ml of hexane. The two hexane phases are mixed and dried under nitrogen.

The extract can be stored at −20°C for several weeks, and is reconstituted with 50 μl of hexane immediately before injection.

GC–MS analysis

The analyses were carried out in two laboratories with different equipment and analytical conditions.

Laboratory A (Genova) used a Hewlett-Packard Model 5988A GC–MS system equipped with an HP1 column (25 m × 0.2 mm I.D.; particle size 0.5 μm) with temperature programming from 180°C (held for 2 min) at 10°C/min to 290°C (held for 15 min). The injection volume was 1 μl, splitless mode, at 280°C. The transfer line temperature was 280°C. Electron-impact ionization was applied at 70 eV. The source temperature was 230°C.

Laboratory B (Manchester) used a Hewlett-Packard Model 5970 GC–MS system equipped with an HP1 column (12 m × 0.2 mm I.D.; particle size 0.33 μm) with temperature programming from 170°C (held for 2 min) at 5°C/min to 230°C, then at 12°C/min to 285°C (held for 5 min). The injection volume was 1 μl, split mode (1:20), at 280°C. The transfer line and source conditions were as for laboratory A.

TABLE I

MONITORED IONS IN MID MODE FOR VLCFA

Ions in italics are used for quantification, ratioed to the molecular ion of the internal standard (I.S.). M^+ and M − 32 ions are monitored at exact first decimal mass to increase the specific response ('mass defect' effect).

VLCFA	*m/z*
$C_{22:1}$	55, *320.4*, 352.4
$C_{22:0}$	74, *354.4*
$C_{24:1}$	55, *348.4*, 380.4
$C_{24:0}$	74, *382.4*
$C_{26:1}$	55, *376.4*, 408.4
$C_{26:0}$	74, *410.4*
$C_{27:0}$ (I.S.)	74, *424.4*

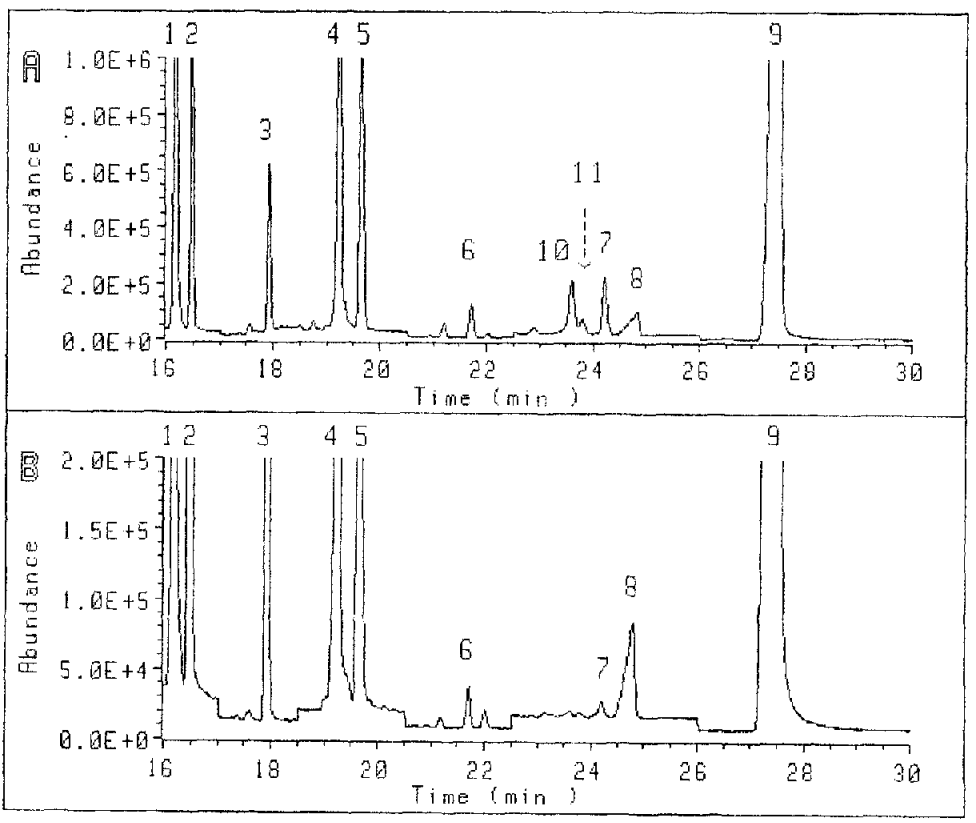

Fig. 1. Reconstituted chromatograms of plasma from (A) a patient affected with Zellweger syndrome and (B) a control. Chromatogram B is scaled up five times. Peak identification: 1 = $C_{22:1}$; 2 = $C_{22:0}$; 3 = $C_{23:0}$; 4 = $C_{24:1}$; 5 = $C_{24:0}$; 6 = $C_{25:0}$; 7 = $C_{26:0}$; 8 = cholersterol derivative; 9 = $C_{27:0}$ (I.S.); 10, 11 = $C_{26:1}$.

TABLE II

REPRODUCIBILITY TEST RESULTS

No.	Concentration (μM)			Molar ratio	
	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$	C_{24}/C_{22}	C_{26}/C_{22}
1	34.11	20.45	0.262	0.600	0.0077
2	34.5	18.15	0.308	0.526	0.0089
3	36.8	21.4	0.284	0.582	0.0077
4	37.1	21.95	0.321	0.592	0.0084
5	35.57	21.56	0.295	0.606	0.0083
6	33.7	19.95	0.261	0.592	0.0077
7	31.8	18.78	0.254	0.591	0.0080
Mean	34.80	20.32	0.282	0.584	0.0081
R.S.D.[a] (%)	4.93	6.60	7.77	4.223	5.270

[a] Relative standard deviation.

MS acquisition was performed in multiple ion detection (MID) mode, monitoring characteristic ions as listed in Table I.

After analysis, a 'macro' Pascal procedure allows integration (based on peak area), the print-out of the reconstituted chromatogram (Fig. 1) and a purity check of the $C_{26:0}$ peak, obtained overlapping the traces of the monitored ions.

A Pascal dedicated program ('Report', part of the main Hewlett-Packard software for GC–MS management) allows automated peak identification (based on retention times and relative ion intensities), referring to a standard mixture [$C_{22:0}$

TABLE III

RECOVERY TEST RESULTS

Averages of three runs.

Amount (μM)			Concentration measured (μM)		
$C_{22:0}$	$C_{24:0}$	$C_{26:0}$	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$
–	–	–	33.1	30.3	0.448
8.0	8.0	2.0	41.1	38.7	2.271
20.0	20.0	5.0	52.1	50.2	4.983
40.0	40.0	10.0	73.3	66.7	9.731
Curve type $y = a + bx$:					
Intercept (a)			−0.58	1.80	0.35
Slope (b)			1.01	0.87	0.88
Linear regression coefficient (r)			0.995	0.997	0.999

and $C_{24:0}$ (40 μM) and $C_{26:0}$ (10 μM)] of authentic acids. Peak identification is obviously checked by the operator using the same parameters.

The same program allows quantification based on the molecular ion (M − 32 for mono-unsaturated acids) response, ratioed to the internal standard peak.

RESULTS

The sensitivity of the method is sufficient to determine hexacosanoic acid in normal plasma (less than 50 nmol/l in plasma can be detected) at a clinically significant level of precision. The inter-assay reproducibility was tested (laboratory A), measuring VLCFA seven times on different days in the same normal plasma stored at −20°C; the results are given in Table II.

The recovery of authentic acids ($C_{22:0}$, $C_{24:0}$ and $C_{26:0}$) was linear (Table III) within the range of our results (controls and affected patients).

TABLE IV

RESULTS FOR AFFECTED PATIENTS, OBLIGATE CARRIERS AND CONTROLS

See text for abbreviations.

Case No.	Disease	Concentration (μM)			Molar ratio		DF^a
		$C_{22:0}$	$C_{24:0}$	$C_{26:0}$	C_{24}/C_{22}	C_{26}/C_{22}	
Laboratory A							
Patients:							
1	X-ALD	33.81	41.00	2.26	1.213	0.067	
2	X-ALD	16.53	25.97	1.09	1.571	0.062	
3	ZS	12.70	21.39	6.12	1.694	0.482	
Carriers:							
1	X-ALD	31.35	39.88	1.73	1.270	0.055	1.40
1[b]	X-ALD	44.24	40.20	2.87	0.908	0.065	1.77
Laboratory B							
1	ZS	40	61	15.5	1.52	0.39	
2	ZS	26	44	11	1.69	0.42	
3	P-ZS	14.5	10	2.8	0.74	0.23	
4	P-ZS	19	22	3.8	1.23	0.23	
5	P-NALD	61	67	6	1.1	0.098	
6	X-ALD	38	39	4.4	1.03	0.12	
Adult female controls (n = 9)							
Min.		19.9	13.1	0.21	0.66	0.008	0.38
Max.		39.3	35.1	0.52	0.89	0.013	0.59
Male controls (n = 11, pediatric age)							
Min.		18.1	13.6	0.22	0.54	0.008	
Max.		42.7	28.5	0.43	0.97	0.017	

[a] Discriminating function for X-ALD heterozygote [5].

[b] Second determination after one year.

Three cases of Zellweger syndrome (ZS), two of pseudo-Zellweger syndrome (P-ZS), three of X-linked adrenoleucodystrophy (X-ALD) and one of pseudo-neonatal adrenoleucodystrophy (P-NALD) were diagnosed in the two laboratories using this method (see results in Table IV). In addition, the obligate female carriers of X-ALD can be discriminated from controls using the discriminating function (*DF*) developed by Moser *et al.* [5], which takes into account the level of $C_{26:0}$ in plasma and also $C_{26:0}/C_{22:0}$ and $C_{24:0}/C_{22:0}$ ratios [$DF = 0.0279 \cdot C_{26}/C_{22} + 0.456 \cdot C_{24}/C_{22} + 0.473 \cdot C_{26}$ (μM).

CONCLUSIONS

The described method is sensitive and reproducible enough for the diagnosis of peroxisomal disorders and the follow-up of X-ALD patients in dietary treatment. We emphasize the relative simplicity of the method and that the analysis can be performed using the equipment usually required for organic acid analysis by GC–MS, changing only the analytical conditions. The method can also easily be modified to determine fatty acids with more than fourteen carbon atoms, either saturated or mono- or poly unsaturated.

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