Journal of Chromatography, 494 (1989) 31–41
Biomedical Applications
Elsevier Science Publishers B V , Amsterdam — Printed in The Netherlands

CHROMBIO 4847

IMPROVED DETERMINATION OF VERY-LONG-CHAIN FATTY ACIDS IN PLASMA AND CULTURED SKIN FIBROBLASTS APPLICATIONS TO THE DIAGNOSIS OF PEROXISOMAL DISORDERS

W ONKENHOUT*, P F H VAN DER POEL and M P M VAN DEN HEUVEL

Department of Pediatrics, University Hospital, P O Box 9600, 2300 RC Leiden (The Netherlands)

(First received February 28th 1989, revised manuscript received May 4th 1989)

SUMMARY

Current techniques for the determination of very-long-chain fatty acids from biological samples require laborious procedures including solvent extraction of lipids, purification hydrolysis derivatization purification of derivatized fatty acids by thin-layer chromatography and finally gas chromatographic analysis A comparison was made between such a procedure based on solvent extraction and a method based on a recently developed direct one-step transesterification reaction The latter method proved to be much faster and led to higher recoveries of all individual very-long-chain fatty acids from both plasma and skin fibroblasts The assay proved to be very convenient in the diagnosis of genetically determined disorders in which very-long-chain fatty acids accumulate in tissues and body fluids Because of its simplicity and speed and because it can be performed with as little as 100 μl of plasma, the method can be recommended as a valuable screening procedure for peroxisomal disorders

INTRODUCTION

In the group of peroxisomal disorders, several diseases are recognized which give rise to the accumulation of very-long-chain fatty acids (VLCFAs), e g , neonatal adrenoleukodystrophy (ALD) [1,2], Zellweger's syndrome [3,4], infantile Refsum's disease [5,6] and X-linked adrenoleukodystrophy [7,8] In the screening for peroxisomal disorders the determination in plasma of the concentration of 26 0 (hexacosanoic acid, cerotic acid) and the ratios of 26 0 and 24 0 (tetracosanoic acid, lignoceric acid) to 22 0 (docosanoic acid, be-

0378-4347/89/$03 50 © 1989 Elsevier Science Publishers B V

henic acid) is a widely applied biochemical assay [8] The procedures almost always include a lipid extraction step such as that described by Folch et al [9] and reference values of VLCFA levels and ratios in plasma and tissues are based on this isolation method [8] However, the procedure of Folch et al [9] is rather laborious and because of the need for several washing steps there is the risk of losses of lipids. For these reasons we applied a recently developed one-step direct procedure involving transmethylation of total long-chain fatty acids with acetyl chloride [10] to the determination of VLCFAs in plasma and human skin fibroblasts

EXPERIMENTAL

Reagents and materials

Acetyl chloride (98%) was obtained from Janssen Chimica (Beerse, Belgium) Boron trichloride (BCl_3) in methanol (10%, w/v) and 22 0, 23 0, 24 0, 25 0, 26 0 and 27 0 fatty acid methyl esters (FAMEs) were obtained from Alltech (Amstelveen The Netherlands) Cholesteryl behenate (CE), dibehenoyl-L-α-phosphatidylcholine (PC) and bovine erythrocyte sphingomyelin (SM), containing lignoceric acid, were bought from Sigma (St Louis, MO, U S A.) All solvents were of analytical-reagent grade and were used without purification

Borosilicate glass tubes (10 ml) with Teflon-lined screw caps (100×10 mm I D) were purchased from Tamson (Zoetermeer, The Netherlands) Thin-layer chromatographic (TLC) plates (20 cm×20 cm) were coated to a layer thickness of 0 25 mm by using a slurry of 25 g of silica 60 G (Merck, Darmstadt, F R G) in 50 ml of water for five plates Washing of the plates is critical [8], they were washed successively with chloroform–methanol–acetic acid (33 13 4, v/v) for 120 min and hexane–diethyl ether–acetic acid (45 5 0 5, v/v) for 50 min Finally, they were activated by heating for 1 h at 100 °C and stored in a desiccator until use

Biological specimens that were analysed included plasma samples ($n=30$) and cultured skin fibroblasts ($n=9$) from normal children and adults, and plasma from individuals with peroxisomal disorders Fibroblast cell lines were grown in Ham's medium supplemented with 10% fetal calf serum using standard procedures Cells were harvested by trypsinization one week after reaching confluence and collected by centrifugation Protein analysis was performed by the method of Lowry et al [11]

Instrumentation

Gas chromatography (GC) was performed on a Packard 430 instrument, equipped with a solid injection system (moving needle) (Chrompack, Middelburg, The Netherlands) and a flame ionization detector The column was a 25 m×0 25 mm I D CP Sil 8CB fused-silica (0 25 μm) capillary column

(Chrompack) Nitrogen was used as the carrier and make-up gas and the column flow-rate was about 1.2 ml/min. The injection port temperature was 275°C and the detector temperature 300°C. The oven temperature was programmed as follows: the initial temperature of 230°C was maintained for 6 min, then increased to 285°C at 3°C/min and held there for 2.5 min, the final temperature of 300°C was obtained by a temperature rise of 10°C/min and was maintained for 2 min. Peak integration was performed by using a Nelson 64 kbyte interface box and Nelson 2600 chromatography software (Chemical Laboratory Instruments, Schijndel, The Netherlands), running on an Olivetti M24 personal computer. FAME peaks were identified by comparison with retention times and electron impact mass spectra of authentic standards.

Mass spectrometry (MS) was performed on a VG Trio-2 quadrupole GC–MS system, equipped with an HP 5890 gas chromatograph and a Digital PDP 11/73 microcomputer. Injection was performed at 275°C using the same solid injection system as above. The column was a 50 m × 0.25 mm I.D. CP Sil 8CB fused-silica (0.12 μm) capillary column (Chrompack). Helium was used as the carrier gas at a flow-rate of 2.0 ml/min. The oven temperature was programmed as described above with the following exceptions: 285°C was held for 1 min and the final temperature of 300°C for 5 min. The interface temperature was 280°C and the ion source temperature 200°C. Mass spectra were continuously recorded from *m/z* 45 to 450 at a rate of 0.5 s per full scan, the ionization energy was 70 eV and the resolution, $M/\Delta M$ (10% valley), was about 500.

Methods

Preparation of FAMEs of total lipids extracted from plasma using a modified Folch extraction [9] (method I). To 250 μl of blood plasma in a 10-ml glass tube were added 5.0 μl of a 178 ng/μl solution of methyl heptacosanoate in *n*-hexane as an internal standard (IS). Protein precipitation and lipid extraction were performed by adding 2.5 ml of chloroform–methanol (1:1, v/v) and vortex-mixing for 2 min. The tube was shaken regularly for 1 h and the protein precipitate removed by centrifugation for 10 min at 1300 *g*. The solvents were collected in another tube. 1.25 ml of chloroform and 0.75 ml of water were added and, after vortex-mixing and centrifugation (5 min, 1300 *g*), the upper solvent layer was removed. The resulting chloroform layer, containing the several lipid fractions, was purified by adding twice 2 ml of chloroform–methanol–water (3:48:47, v/v), vortex-mixing (30 s), centrifuging (1 min, 1300 *g*) and removing the upper water layer each time. The crude lipid extract was dried at room temperature under a gentle stream of nitrogen and lipids were transmethylated by adding 1 ml of 10% BCl_3–methanol and heating for 2 h at 80°C in a Liebisch metal block thermostat. Subsequently, 1 ml of water and 3 ml of *n*-hexane were added and, after vortex-mixing and centrifugation, the supernatant was isolated. Extraction was repeated twice with 2 ml of *n*-hexane. The

combined *n*-hexane layers were dried under nitrogen and the residue was dissolved in 50 μl of *n*-hexane and placed as a single spot on a laboratory-made TLC plate, on which a maximum of six samples can be handled simultaneously The plate was developed for 45 min with toluene–diethyl ether (97 3, v/v) and FAMEs of a reference mixture in the first lane were rendered visible with iodine vapour to locate the position In the other lanes the corresponding areas were scraped off the plate, the silica was extracted three times with 1 ml of *n*-hexane, the solvent was evaporated under nitrogen and the residue was finally taken up in 25 μl of *n*-hexane and 1 μl was subjected to GC(-MS)

Preparation of FAMEs from plasma using a one-step transmethylation reaction [10] (method II) To 100 μl of blood plasma in a 10-ml glass tube were added 5 0 μl of a 178 ng/μl solution of methyl heptacosanoate in *n*-hexane as an IS and 2 0 ml of methanol–benzene (4 1, v/v) A 200-μl volume of acetyl chloride was added slowly during 1 min with magnetic stirring The tube was tightly closed and derivatization was performed for 1 h at 100°C in a heating block Subsequently, 5 ml of a 6% potassium carbonate solution in water was carefully added while cooling in a ice-bath, the tube was shaken and centrifuged and as much as possible of the benzene upper layer was isolated and dried under nitrogen The residue was dissolved in 50 μl of *n*-hexane and FAMEs were isolated by TLC as described above

Preparation of FAMEs from cultured skin fibroblasts Packed cells were suspended in 400 μl of water and disrupted by sonication to form a completely homogeneous suspension Two aliquots (5 and 10 μl) of this aqueous suspension were taken for duplicate protein analysis [11] From the resulting suspension 250 and 100 μl were transferred into 10-ml tubes and FAMEs were prepared according to methods I and II as described above

Recoveries of standards

CE, PC and SM standard lipid solutions were prepared in chloroform–methanol (1 1, v/v) at concentrations of 750 μg/ml (1 059 μmol/ml), 472 μg/ml (0 524 μmol/ml) and 887 μg/ml (1 090 μmol/ml) Volumes of 10 0 and 4 0 μl of the above solutions were placed in separate tubes, no water and 100 μl of water, respectively, were added, and 890 ng of 27 0 methyl ester were added to each tube FAMEs were prepared according to methods I (from the transmethylation step on) and II as described above The TLC purification step was not necessary and was therefore omitted

Quantification of VLCFAs

In the GC analyses, an identical detector response was assumed for all (saturated) VLCFA methyl esters Quantification was performed by determining the peak-area ratios of VLCFA methyl esters to the IS Concentrations are expressed as micromoles per litre of plasma or micrograms (of free VLCFA) per milligram of protein in fibroblast samples

RESULTS AND DISCUSSION

Fig 1 shows some representative gas chromatograms of VLCFA methyl esters from both plasma and fibroblasts, obtained after sample preparation by the two methods described above Because of the TLC purification step, which removed the cholesterol derivatives 3,5-cholestadiene and cholesterol methyl ether from the FAME mixture, no interfering peaks are present in any of the traces and quantification of the components is easy to perform

VLCFAs were quantified in plasma and fibroblast samples from normal control subjects and the results are shown in Tables I and II

The results show that the direct transmethylation procedure with acetyl chloride gives higher yields for all the VLCFAs concerned in both plasma and fibroblasts Lepage and Roy [10] found that this technique led to a 20% increase in total fatty acids from plasma compared with the method involving a Folch lipid extraction procedure In both plasma and fibroblasts the yields of 22 0, 23 0 and 24 0 were increased to the same extents, giving rise to about the same 24 0/22 0 peak-area ratios The yield of 26 0 is 1 75 and 2 4 times as high, respectively, and that of 25 0 is more than three times as high in both plasma and fibroblasts In plasma, the 26 0 concentration and 24 0/22 0 and 26 0/22 0 peak-area ratios are considered to be discriminatory parameters in distinguishing peroxisomal disorders with VLCFA oxidation deficiencies [8], whereas in fibroblasts in this respect only the 26 0/22 0 peak-area ratio is considered to be of particular importance [12] The normal values in plasma for 26 0 and 24 0/22 0 based on method I (Table I) are in close agreement with those found by Moser et al [8] However, the 26 0/22 0 ratios found in plasma and fibroblasts by method I (Tables I and II) are both about twice as high as those found by Moser and co-workers [8,12] On the other hand, Hall et al [13] determined the 26 0/22 0 ratio in plasma of controls by a high-performance liquid chromatographic (HPLC) method and found $0\ 026 \pm 0\ 013$ (means $\pm$ S D , $n = 25$), a value in agreement with our results of $0\ 027 \pm 0\ 0097$ (Table I)

In order to obtain more insight into differences in the analytical performances of the two methods, CE, PC and SM standard lipid solutions were treated according to these methods and the recoveries of 22 0, 22 0 and 24 0, respectively, were compared (Table III) The almost quantitative recoveries with method II are in agreement with the results obtained by Lepage and Roy [10] for fatty acids with up to twenty carbon atoms The great difference in the recovery of 24 0 from SM is probably caused by the more rigorous circumstances in the direct transesterification method which are needed to hydrolyse the relatively inert amide bond in SM 22 0 and 24 0 are especially prominent in the plasma SM fraction [14] The higher yields of 22 0 and 24 0 (and probably also of 23 0) by method II (Table I) can therefore be explained on the basis of the difference in recovery from SM Presumably 26 0 is predominantly

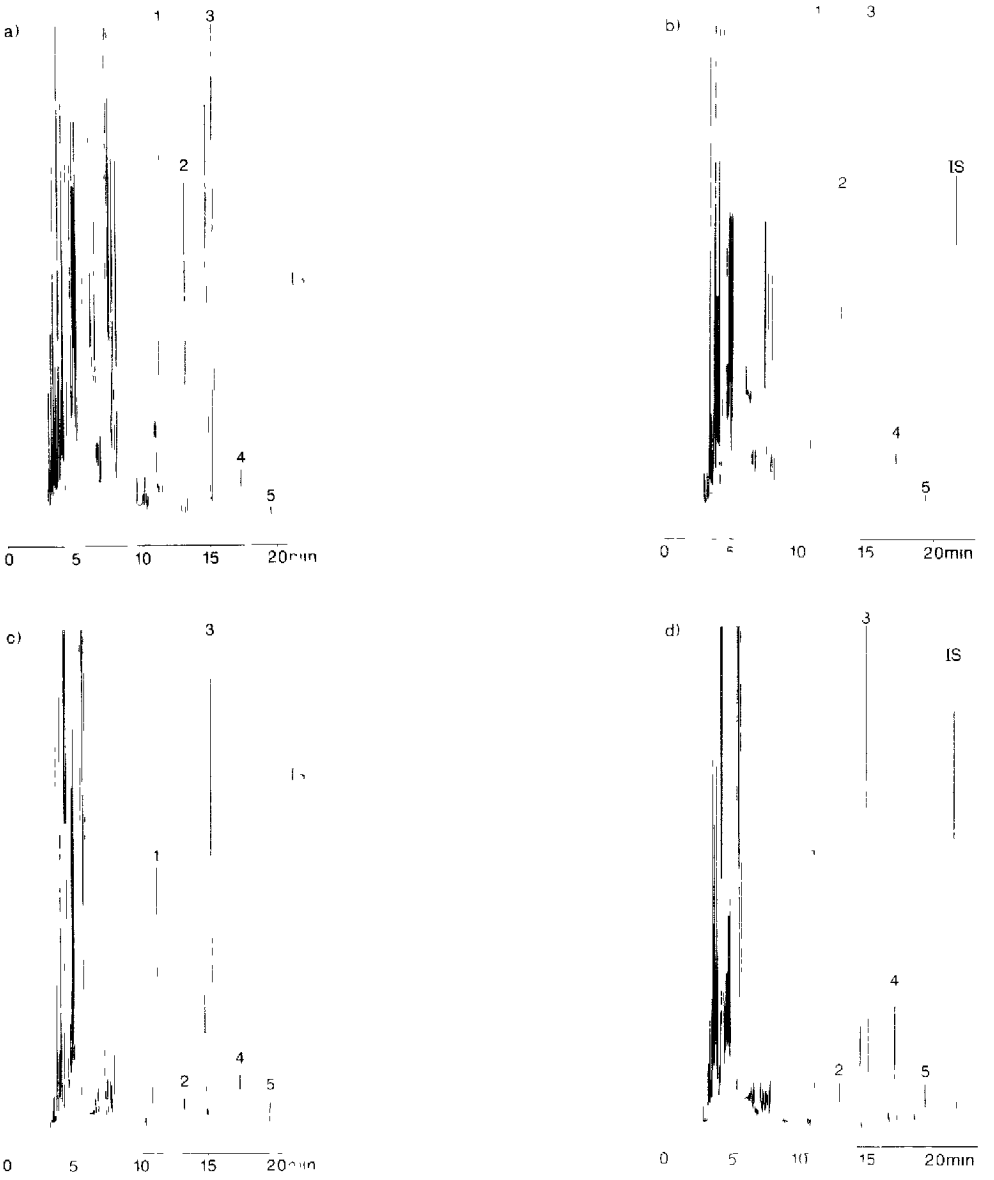

Fig 1 Gas chromatograms of VLCFA methyl esters prepared from (a) 250 μl of plasma from a healthy person by method I (b) 100 μl of the same plasma specimen by method II, (c) 250 μl of suspended skin fibroblasts from a healthy person by method I, (d) 100 μl of the same fibroblast suspension by method II Peaks 1-5 were identified as 22 0 23 0, 24 0, 25 0 and 26 0 methyl ester, respectively IS = internal standard (27 0 methyl ester)

TABLE I

VLCFA CONCENTRATIONS (μmol/l) AND RATIOS IN PLASMA SAMPLES FROM NORMAL CONTROLS ($n=30$) ACCORDING TO METHODS I AND II

Parameter	Method I		Method II		Difference in yield (%)
	Mean ± S.D.	Range	Mean ± S.D.	Range	
22:0	39.7 ± 10.8	23.8 - 71.4	48.5 ± 16.8	27.3 - 90.1	22
23:0	15.3 ± 4.02	8.6 - 22.9	18.6 ± 6.82	9.3 - 37.5	22
24:0	30.0 ± 8.05	13.8 - 50.4	36.9 ± 13.3	17.7 - 80.3	23
25:0	2.12 ± 0.78	0.82 - 4.31	6.71 ± 3.16	1.67 - 16.1	216
26:0	0.87 ± 0.26	0.44 - 1.40	1.52 ± 0.40	0.83 - 2.27	75
24:0/22:0	0.82 ± 0.15	0.55 - 1.10	0.83 ± 0.15	0.54 - 1.11	
26:0/22:0	0.027 ± 0.0097	0.012 - 0.048	0.039 ± 0.013	0.019 - 0.062	

TABLE II

VLCFA CONCENTRATIONS (μg/mg OF PROTEIN) AND RATIOS IN FIBROBLAST SAMPLES FROM NORMAL CONTROLS ($n=9$) ACCORDING TO METHODS I AND II

Parameter	Method I		Method II		Difference in yield (%)
	Mean ± S.D.	Range	Mean ± S.D.	Range	
22:0	0.81 ± 0.30	0.42 - 1.45	1.60 ± 0.59	0.84 - 2.97	98
23:0	0.19 ± 0.068	0.10 - 0.32	0.40 ± 0.13	0.23 - 0.68	111
24:0	2.06 ± 0.60	1.19 - 2.83	4.24 ± 1.04	2.55 - 5.95	106
25:0	0.37 ± 0.17	0.16 - 0.65	1.15 ± 0.53	0.44 - 2.06	211
26:0	0.15 ± 0.047	0.077 - 0.22	0.36 ± 0.099	0.17 - 0.53	140
24:0/22:0	2.60 ± 0.36	1.95 - 2.93	2.75 ± 0.37	1.99 - 3.23	
26:0/22:0	0.19 ± 0.041	0.15 - 0.27	0.24 ± 0.058	0.15 - 0.34	

present in the plasma CE fraction [14]. The slight difference in recovery between the two methods (Table III) cannot be the reason why method II yields 1.75 times as much of this particular VLCFA from plasma as method I (Table I).

In an additional experiment, the direct transmethylation procedure was applied to the protein precipitate obtained with method I. Only small amounts of 22:0, 23:0 and 24:0 were found (<5% of the amounts found in the lipid extract). 26:0, however, was prominently present and this could fully explain the large difference in yield. The amount of 25:0 found in the protein precipitate was even larger than that found in the lipid extract of plasma, explaining

TABLE III

RECOVERY OF VLCFAs FROM LIPID STANDARDS ACCORDING TO METHODS I AND II

Values represent the means of two separate determinations

Lipid	VLCFA	Recovery (%)	
		Method I	Method II
CE	22 0	4 3	99 2
PC	22 0	1 8	99 9
SM	24 0	2 8	97 9

TABLE IV

VLCFA CONCENTRATIONS AND RATIOS IN PLASMA SAMPLES FROM INDIVIDUALS WITH PEROXISOMAL DISORDERS AS DETERMINED BY METHOD II

Control values are expressed as means ± S D with the accompanying range in parentheses

Case	Sex	Age at diagnosis (years)	Concentration of 26 0 (μmol l of plasma)	24 0/22 0 ratio	26 0/22 0 ratio	Diagnosis
1	M	0 7	6 3	1 57	0 371	Infantile Refsum
2	F	0 2	3 7	2 46	0 639	Zellweger
3	M	1 0	3 5	1 57	0 277	Infantile Refsum
4	M	0 7	5 4	1 70	0 351	Infantile Refsum
5	M	24	5 1	1 68	0 105	ALD
6	M	32	2 5	1 30	0 061	AMN
7	M	42	2 9	1 76	0 101	AMN (index case)
8	M	56	3	1 39	0 091	AMN (maternal uncle of 7)
9	M	26	3 1	1 34	0 085	AMN (asymptomatic brother of 7)
10	M	30	2 9	1 66	0 097	AMN (asymptomatic brother of 7)
Controls			1 ± 0 40 (0 8 2 27)	0 83 ± 0 15 (0 54–1 11)	0 039 ± 0 013 (0 019 0 062)	

the three-fold greater yield of this specific VLCFA with method II The conclusion from this experiment is that a large part of the 25 0 but also of the 26 0 pool is tightly bound to plasma proteins, giving rise to incomplete recovery from plasma by solvent extraction methods such as the Folch et al procedure As the discrepancies found between the two methods are much higher for fibroblasts (Table II) than for plasma (Table I), except for 25 0, it seems reasonable to conclude that also in fibroblasts VLCFAs are bound very tightly to protein, leading to low yields in solvent extraction methods

The precision of the one-step transmethylation procedure was estimated by analysing aliquots of single plasma specimens on four different occasions The

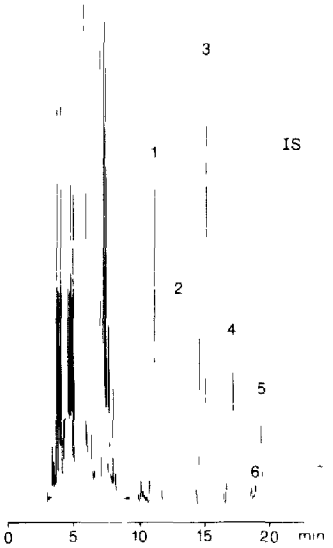

Fig 2 Gas chromatogram of VLCFA methyl esters prepared from 100 μl of plasma from patient 4 (Table IV) by method II Peaks 1–7 were identified as 22 0, 23 0 24 0 25 0 26 0, 26 1 and 28 0 methyl ester, respectively IS = internal standard (27 0 methyl ester)

analysis was performed on three control plasma samples and the coefficients of variation (C V s) for the 24 0/22 0 ratio ranged from 3 8 to 6 4%, for the 26 0/22 0 ratio from 24 3 to 28 6% and for the 26 0 concentration from 17.9 to 26 4% These values are satisfactory and, for instance, better than the C V s for the 24 0/22 0 ratio and the 26 0/22 0 ratio in the HPLC method of Hall et al [13], which ranged from 1 5 to 7 8% and from 23 1 to 44 4%, respectively

Table IV and Fig 2 show the practical applicability of the direct transmethylation procedure in diagnosing patients with peroxisomal diseases The method was applied to plasma samples from one patient with Zellweger syndrome, three with infantile Refsum's disease, one with ALD, one with adrenomyeloneuropathy (AMN) [15,16] and four individuals from a large kindred with AMN The diagnosis of patients 1–4 and 7 was confirmed enzymatically by the finding of deficient acyl-CoA d hydroxyacetone phosphate acyltransferase [17] and deficient VLCFA oxidation, respectively (assays performed by Drs R J A Wanders and R B H Schutgens, Department of Pediatrics, University of Amsterdam, The Netherlands)

Analysis of plasma samples from the above patients by method II revealed abnormal VLCFA concentrations and ratios (Table IV) All data except the 26 0/22 0 ratio for case 6, which just fell into the normal range, were significantly elevated It is generally known that in Zellweger syndrome and infantile Refsum's disease deviations from normal plasma values are much higher than in ALD [5,8] The data in Table IV are in agreement with this cases 1–4 show

especially strongly elevated 26 0/22 0 ratios in comparison with both controls and ALD/AMN patients In addition to elevated 26 0 levels, the concentrations of 22 0 were found to be significantly below normal in cases 1–4, ranging from 6 8 to 19 7 μmol/l plasma The concentrations of 24 0 were in the low normal range The other six patients exhibited normal 22 0 concentrations Hence the elevated 24 0/22 0 and 26 0/22 0 ratios found in plasma of Zellweger and infantile Refsum patients are at least partly caused by low 22 0 concentrations

In patients with peroxisomal disorders, abnormalities in plasma VLCFAs are not only confined to 22 0, 24 0 and 26 0 In each of the GC traces of VLCFA methyl esters of patients 1–4 a small peak (corresponding to about 0 5–1 5 μmol/l plasma) eluted just before 26 0 (Fig 2) On the basis of its retention time and electron impact mass spectrum [18], it was identified as 26 1 This peak was barely or not detectable in the ALD/AMN patients and in normal plasma These findings are in agreement with those of Moser et al [4] Another small, unusual but distinct peak was observed in eight of the ten GC traces (Fig 2) It eluted after the IS and, on basis of its electron-impact mass spectrum, which showed a highest mass fragment at *m/z* 438, it was identified as 28 0 The concentrations were estimated to range up to 1 2 μmol/l of plasma. In control samples the 28 0 peak was hardly or not detectable The above findings suggest that not only the levels of 26 1 [4,19] but also of 28 0 can be used as additional discriminatory parameters in the screening for peroxisomal diseases

The one-step derivatization procedure proved to be a simple, fast, precise, sensitive and reliable method for the determination of VLCFAs Very small amounts of material are needed and the analytical performance and the discriminatory power are very good Application of this method to samples from patients leads to findings comparable to those obtained by current methods based on solvent extraction procedures For these reasons we recommend the method to (bio)chemists who are screening patients for peroxisomal disorders

ACKNOWLEDGEMENT

The authors thank Dr B J H M Poorthuis for critically reading the manuscript

REFERENCES

1 P J Benke, P F Reves and J C Parker, Hum Genet , 58 (1981) 204
2 R Jaffe, P Crumrine Y Hashida and H W Moser, Am J Pathol 108 (1982) 100
3 R I Kelley, Am J Med Genet , 16 (1983) 503
4 A E Moser, I Singh F R Brown, G I Solish, R I Kelley P J Benke and H W Moser, N Engl J Med , 310 (1984) 1141
5 A Poulos, P Sharp and M J Whiting, Clin Genet , 26 (1984) 579

6 B.T. Poll-Thé, J.M. Saudubray, H. Ogier, R.B.H. Schutgens, R.J.A. Wanders, G. Schrakamp, H. van den Bosch, J.M.F. Trijbels, A. Poulos, H.W. Moser, J. van Eldere and H.J. Eyssen, J. Inher. Metab. Dis., 9 (1986) 169.
7 H.H. Schaumburg, J.H. Powers, C.S. Raine, K. Suzuki and E.P. Richardson, Arch. Neurol., 32 (1975) 577.
8 H.W. Moser, A.E. Moser, I. Singh and B.P. O'Neil, Ann. Neurol., 16 (1984) 628.
9 J. Folch, M. Lees and G. Sloane-Stanley, J. Biol. Chem., 226 (1957) 497.
10 G. Lepage and C.C. Roy, J. Lipid. Res., 27 (1986) 114.
11 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
12 H.W. Moser, A.E. Moser, J.E. Trojak and S.W. Supplee, J. Pediatr., 103 (1983) 54.
13 N.A. Hall, G.W. Lynes and N.M. Hjelm, Clin. Chem., 34 (1988) 1041.
14 H.W. Moser, A.B. Moser, K.K. Frayer, W. Chen, J.D. Schulman, B.P. O'Neil and Y. Kishimoto, Neurology, 31 (1981) 1241.
15 J.W. Griffin, E. Goren, H.H. Schaumburg, W.K. Engel and L. Loriaux, Neurology, 27 (1977) 1107.
16 S.F. Berkovic, A.J. Fellenberg, J.D. Zajac, A. Poulos, D.W. Warburton, A.C. Pollard and J.R. Merory, Aust. N.Z.J. Med., 13 (1983) 594.
17 R.B.H. Schutgens, R.J.A. Wanders, H.S.A. Heymans, A.W. Schram, J.M. Tager, G. Schrakamp and H. van den Bosch, J. Inher. Metab. Dis., 10 (Suppl. 1) (1987) 33.
18 B. Molzer, M. Korschinsky, H. Bernheimer, R. Schmid, C. Wolf and A. Roscher, Clin. Chim. Acta, 161 (1986) 81.
19 P. Aubourg, P.F. Bougnères and F. Rocchiccioli, J. Lipid. Res., 26 (1985) 263.