KAWASAKI MED. J. VOL. 17 : NO. 1～4, 29-36 (1991)

Very Long Chain Fatty Acid Contents in the Sera of Patients with Adrenoleukodystrophy and Adrenomyeloneuropathy Measured by Selected-Ion-Monitoring Gas Chromatography/Mass Spectrometry

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Accepted for publication on October 16, 1991

ABSTRACT. Saturated very long chain fatty acid (VLCFAs, C22 : 0-C28 : 0) contents in the sera of 9 adrenoleukodystrophy (ALD) patients, 4 adrenomyeloneuropathy (AMN) patients, and 11 age-matched normal controls were determined by gas chromatography (GC), GC/mass spectrometry (MS) and selected-ion-monitoring (SIM)-GC/MS. The following results were obtained. The C22 : 0 contents of the ALD and AMN groups were not significantly different from those of the control group. However, the C24 : 0 contents of the ALD and AMN groups were respectively, 1.9 and 1.5 times higher than control, and the C26 : 0 contents of these groups measured by SIM-GC/MS were 7.7 and 7.8 times higher than the control. These contents were about half of those measured by GC alone. The C28 : 0 content of human sera could be measured for the first time in this study using SIM-GC/MS. The order of the increasing rate of the individual VLCFAs of ALD and AMN as compared that with the control was as follows, C26 : 0 > C28 : 0 > C24 : 0.

Key words : saturated very long chain fatty acids — adrenoleukodystrophy — adrenomyeloneuropathy — selected-ion-monitoring gas chromatography/mass spectrometry

Adrenoleukodystrophy (ALD) is an X-linked hereditary disorder characterized by demyelination of the central and peripheral nerves and adrenocortical dysfunction.[1] Clinically, it usually develops in childhood and rapidly causes demyelination of the central and peripheral nerves. Adrenomyeloneuropathy (AMN), a subtype of ALD, develops in adult males, follows a more chronic course than ALD, and damages the spinal cord and peripheral nerves, rather than the cerebral cortex.[2,3] It has been revealed that very long chain fatty acids (VLCFAs) with a carbon chain length longer than 22 accumulate in the cholesterol ester fractions of the cerebral white matter and adrenal cortex of postmortem patients.[4,5] Similar accumulation of VLCFAs has also been found in peripheral nerves, cultured skin fibroblasts, cultured muscle, plasma and erythrocytes.[6-10] The VLCFA contents of these tissues, however, have usually been expressed as the ratios peak areas of VLCFAs to that of C22 : 0, as measured by gas chromatography (GC),[7,9] GC/mass spectrometry (MS),[8] or high-performance liquid chromatography.[11]

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In this study, measurement of the VLCFA content of the sera of ALD and AMN patients and normal controls was carried out as precisely as possible by GC and GC/MS for fatty acids from C22 : 0 to C26 : 0 and additionally by selected-ion-monitoring (SIM)-GC/MS for C26 : 0 and C28 : 0. The data obtained were compared with those reported previously by others.

MATERIALS AND METHODS

Sera

Nine specimens of sera from ALD patients, 4 from AMN patients and 11 from age-matched 2 - 10 year old healthy persons, were used. Among these specimens, eight sera of ALD and four of AMN were kindly provided by the Department of Neurology of the Brain Research Institute of Niigata University, the Division of Neurology in Chikugo National Hospital, and the Division of Neurology in Sumitomo Hospital.

Chemicals

Methyl esters (Me) of C13 : 0, C22 : 0, C24 : 0, C25 : 0, C26 : 0 and C28 : 0 were purchased from Sigma Chemical Co. (St. Louis, MO). [2,2-$^2H_2$] C28 : 0 (d_2-C28 : 0) was prepared according to the method of Noda *et al.*,[12] and its purity (>97%) was checked by proton NMR and SIM/GC-MS (data not shown).

Preparation of total fatty acid methyl esters in sera

Total fatty acids in each 0.1 ml of serum were transmethylated and extracted according to the method of Lepage and Roy.[13] As internal standards, 20 μg of C13 : 0 and 0.25 μg of C25 : 0 were added to each sample for GC and SIM-GC/MS, respectively.

Quantitative determination of methyl esters of C22 : 0, C24 : 0, C26 : 0, C28 : 0 in sera

The amounts of C22 : 0, C24 : 0, C26 : 0, were determined by GC, and those of C26 : 0 and C28 : 0 by SIM-GC/MS as described hereafter in detail. The GC [Model GC-8A, Shimadzu Co. (Kyoto, Japan)] installed with a silicone OV - 1 capillary column [Gaschrokogyo Co. (Osaka, Japan), 0.25 mm × 25 mm] was used under the following conditions: helium as the carrier gas; column temperature rising from 160 to 280°C at a rate of 4°C/min; injection port temperature of 290°C; detector temperature of 300°C. For quantitative determination of C22 : 0, C24 : 0 and C26 : 0, calibration curves were prepared for each fatty acid by plotting the peak area ratio against the weight ratios to the internal standard of C13 : 0 (data not shown). The operating conditions of GC for the GC/MS were the same as described for GC alone and the electron impact mode of MS [M - 80 B, Hitachi Co. (Tokyo, Japan)] was operated at an ionization voltage of 70 eV, an accelerating voltage of 3V, and an ion source temperature of 210°C. These GC/MS conditions were used occasionally to identify fatty acid peaks of GC. SIM-GC/MS was carried out by monitoring the selected ions of m/z 440 (molecular ion of Me d_2-C28 : 0),

m/z 438 (molecular ion of Me C28 : 0), m/z 395 (molecular ion of Me C28 : 0 -43), m/z 410 (molecular ion of Me C26 : 0) and m/z 396 (molecular ion of Me C25 : 0). The column temperature was programmed at an increasing rate of 30°C/min from 85 to 265°C, at 0.5°C/min from 265 to 272°C and at 10°C/min from 272 to 300°C. For quantitative determination of C26 : 0 and C28 : 0, calibration curves were prepared by plotting the peak area ratios of molecular ions against the weight ratios to an internal standard of C25 : 0. The calibration curve for Me C26 : 0/Me C25 : 0 is shown in Fig. 1. The examined

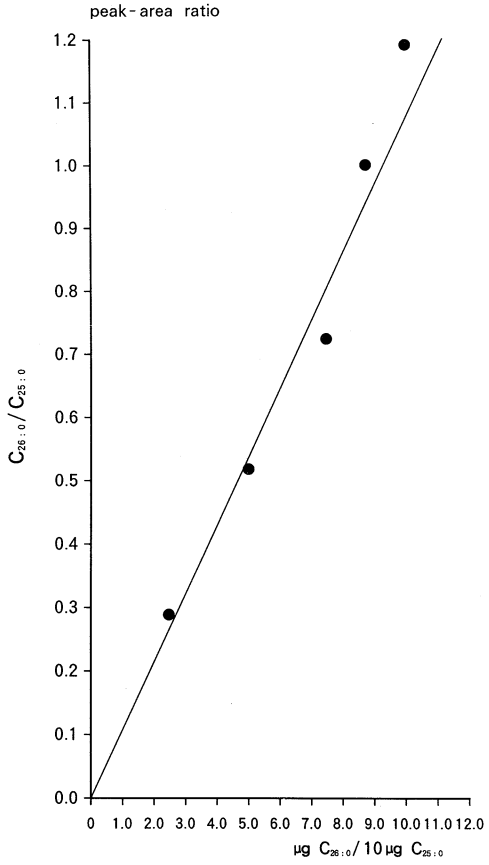

Fig. 1. Calibration curve for quantitative analysis of C26 : 0 using C25 : 0 as an internal standard by selected-ion monitoring gas chromatography/mass spectrometry. The peak area ratios of Me C26 : 0 (m/z 410) to Me C25 : 0 (m/z 396) were plotted against weight ratios.

sera sometimes contained endogenous C25 : 0. Therefore, the real peak area ratios of Me C26 : 0/Me C25 : 0 were calculated as follows :

$$\frac{ab}{b-a}$$

a : apparent peak area ratio with internal standard
b : apparent peak area ratio without internal standard

RESULTS

Identification of fatty acids and other substances in the total fatty acid methyl esters fraction using GC and GC/MS

Gas-chromatograms of the total fatty acid methylesters obtained from sera of the control and ALD and AMN patients are shown in Fig. 2 (A), (B) and

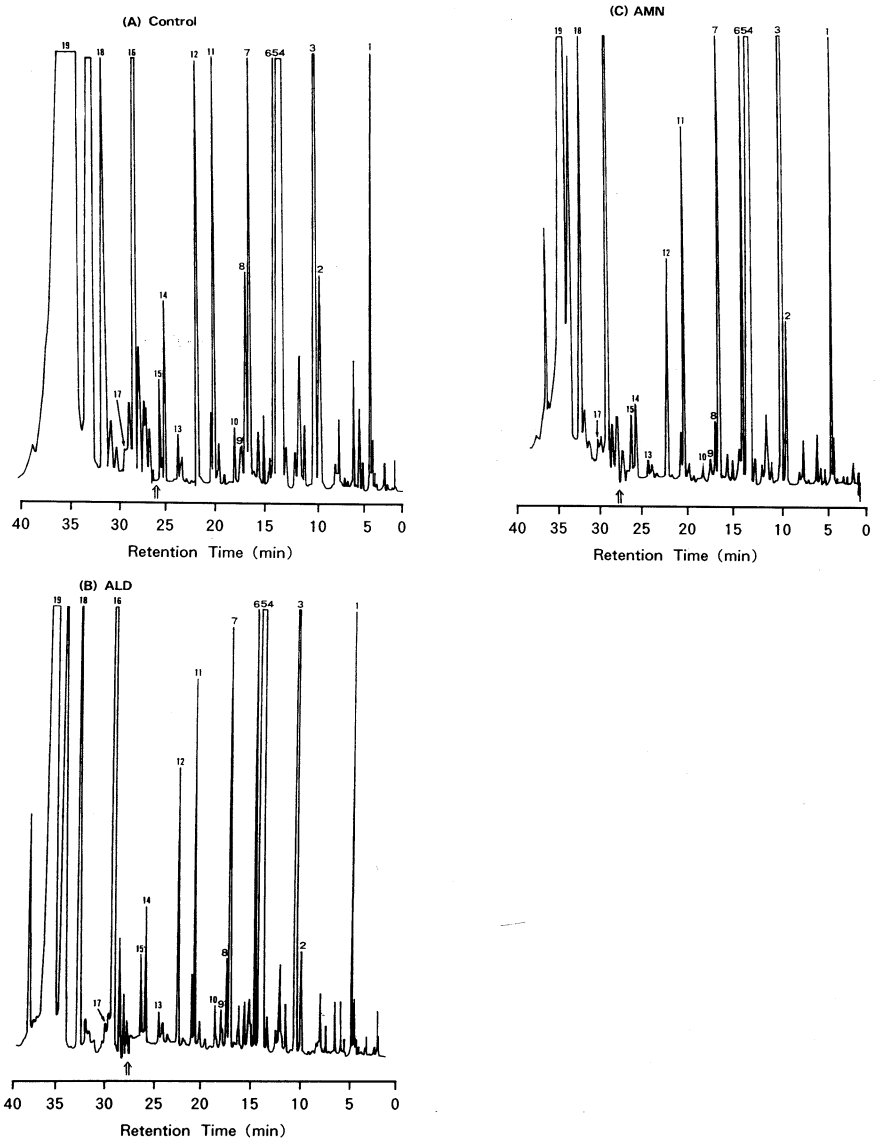

Fig. 2. Gas chromatograhic profiles of the methyl esters of fatty acids in the sera of controls (A), ALD (B), and AMN (C). Peak identifications: (1) C13 : 0 (internal standard); (2) C16 : 1; (3) C16 : 0; (4) C18 : 2; (5) C18 : 1; (6) C18 : 0; (7) C20 : 4; (8) C20 : 3; (9) C20 : 2; (10) C20 : 0; (11) C22 : 6; (12) C22 : 0; (13) C23 : 0; (14) C24 : 1; (15) C24 : 0; (16) cholesta-3,5-diene; (17) C26 : 0; (18) cholesterol methyl ether; (19) cholesterol. The arrows indicate 8-fold increased sensitivity.

(C), respectively. The numbered peaks were identified by GC/MS. Small peaks of C26 : 0 were found in samples from the sera of ALD and AMN, but not distinctly in those of the control. The peak corresponding to C28 : 0 was not detected in any sample by GC.

Quantitative analysis of VLCFA using GC

The C22 : 0, C24 : 0 and C26 : 0 contents of sera as determined by GC with the internal standard method are shown in the Table 1 (A), (B), (C). C26 : 0 in the control sera could be hardly detected by GC alone.

TABLE. 1. Very long chain fatty acid contents in sera

(A) Control

Case	C22 : 0	C24 : 0	C26 : 0		C28 : 0
	GC	GC	GC	SIM-GC/MS	SIM-GC/MS
1	118	27	ND[a]	0.13	0.0397
2	118	19	ND	0.13	0.0429
3	151	22	ND	0.13	0.0435
4	108	24	ND	0.13	nd[b]
5	119	17	ND	0.21	nd
6	148	20	ND	0.1	nd
7	116	20	ND	0.12	nd
8	103	16	ND	0.13	nd
9	135	15	ND	0.1	nd
10	127	20	ND	0.16	nd
11	117	12	ND	0.1	nd
Mean	123	19	—	0.13	0.042
±SD	15.3	4.2	—	0.032	0.002
n	11	11	—	11	3

(B) ALD

Case	C22 : 0	C24 : 0	C26 : 0		C28 : 0
	GC	GC	GC	SIM-GC/MS	SIM-GC/MS
1	119	32	1.03	1.79	0.288
2	143	17	1.53	0.67	0.15
3	132	55	2.7	1.23	0.057
4	103	25	ND	0.731	0.179
5	131	49	5.13	1.18	0.181
6	127	40	1.83	1.05	0.102
7	149	47	3.58	1.12	0.262
8	123	38	2.90	0.98	0.17
9	125	19	ND	0.27	0.055
Mean	128	36	2.08	1.0	0.161
±SD	13.4	13.0	1.69	0.42	0.081
n	9	9	9	9	9

(C) AMN

Case	C22 : 0	C24 : 0	C26 : 0		C28 : 0
	GC	GC	GC	SIM-GC/MS	SIM-GC/MS
1	102	28	1.99	1.01	0.203
2	97	32	4.2	1.33	0.292
3	115	28	ND	0.73	0.087
4	118	29	1.59	0.98	0.158
Mean	108	29	1.95	1.01	0.119
±SD	10	1.9	1.7	0.246	0.077
n	4	4	4	4	4

a. peak height too low to estimate accurately
b. not determined

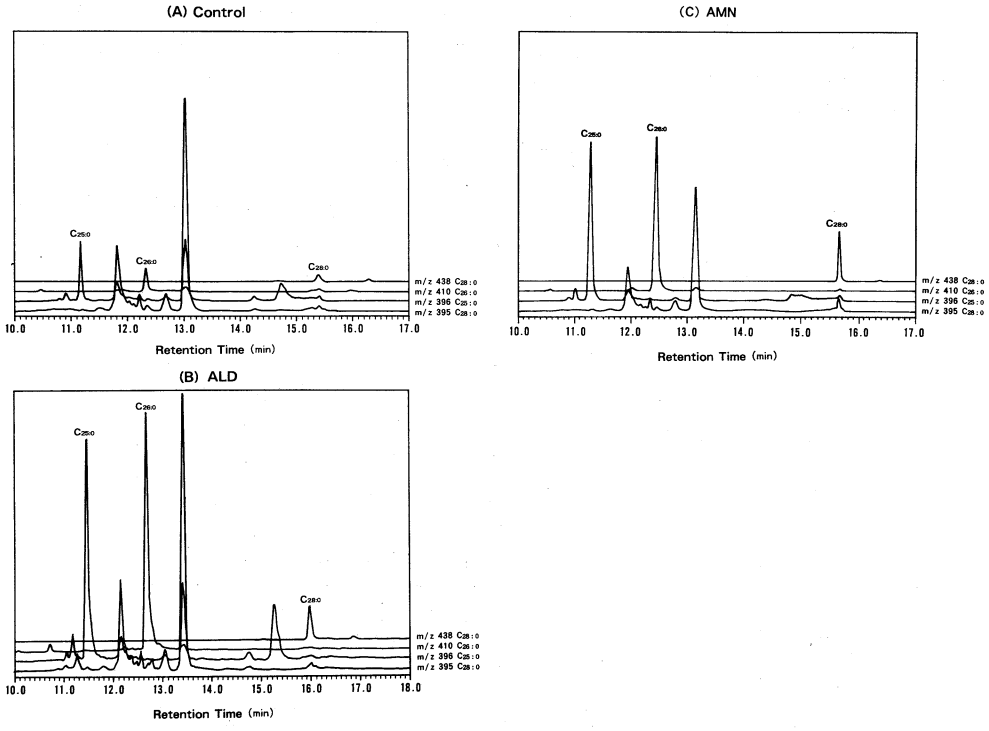

Fig. 3. Selected-ion monitoring gas chromatography/mass chromatograms of methyl esters of VLCFAs in the sera of conrols (A) ALD (B) and AMN (C) using methyl ester of C25 : 0 as an internal standard.

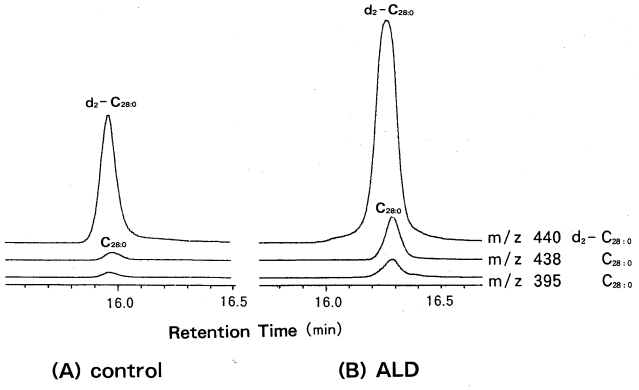

Fig. 4. Selected-ion-monitoring gas chromatography/mass chromatograms of methyl ester of C28 : 0 (m/z 438, m/z 395) in the sera of controls (A) and ALD (B) using methyl ester of d_2-28 : 0 (m/z 440) as an internal standard.

Quantitative analysis of C26 : 0 and C28 : 0 using SIM-GC/MS

As shown in Fig. 3 (A), (B), (C), C26 : 0 could be identified easily from the peak of m/z 410 (molecular ion). The relative retention time to Me C25 : 0 and the peak height increased after addition of authentic Me C26 : 0 to the

samples. But C28 : 0 was difficult to identify by the above methods because the peak at m/z 438 (molecular ion) was small. However, as shown in Fig. 4 (A), (B), C28 : 0 could be identified clearly by SIM-GC/MS after addition of d_2-C28 : 0 m/z 440 to the samples. The peaks of C28 : 0 m/z 438 and d_2-C28 : 0 m/z 440 had the same retention times.

All the measured serum fatty acid contents are listed in the Table 1 (A), (B), (C). The C22 : 0 content did not vary significantly among the three groups. The C24 : 0 contents of sera of ALD and AMN were respectively, 1.9 and 1.5 times higher than those of the normal controls ($p<0.01$, $p<0.01$). As also shown in Table 1 (B), (C), the mean values of the C26 : 0 contents in ALD and AMN measured by GC were much higher than those measured by SIM-GC/MS. The C26 : 0 contents in ALD and AMN measured by SIM-GC/MS were respectively, 7.7 and 7.8 times higher than those of the control ($p<0.05$, $p<0.05$). The C28 : 0 contents in ALD and AMN measured by SIM-GC/MS were respectively, 3.8 and 2.8 times higher than the control, ($p<0.05$, $p<0.05$).

DISCUSSION

An increase in the VLCFA content of several tissues of ALD or AMN patients was discovered several years ago. However, the reason for this increase is still unclear. Recently, it has been reported that VLCFAs content may increase as the result of a defect of β-oxidation due to the reduced lignoceroyl-CoA ligase activity in the peroxisome of ALD and AMN patients' tissues.[14] Moser *et al.* indicated that VLCFA content could increase transiently by immunological association with demyelination.[15]

To ascertain the increase in VLCFA content in ALD and AMN patients, we analyzed VLCFA in the sera of ALD and AMN patients and normal controls qualitatively and quantitatively using GC, GC/MS and SIM-GC/MS. As described above, the C22 : 0 contents of the sera did not vary greatly among the samples. Therefore, the GC peak area ratio of C24 : 0/C22 : 0 is thought to reflect approximately the content ratio of C24 : 0/C22 : 0 (data not shown). On the other hand, the C26 : 0 contents as measured by GC were usually higher than those measured by SIM-GC/MS, as shown in Table 1 (B), (C). Using SIM-GC/MS, we could qualitatively and quantitatively detect C28 : 0 in sera of ALD and AMN patients for the first time. The C24 : 0, C26 : 0 and C28 : 0 contents of the sera of ALD and AMN patients was found to be consistently higher than those of the controls with the order of increasing rate as C26 : 0> C28 : 0> C24 : 0. SIM-GC/MS seems to be essential for quantitative analysis of VLCFA with a carbon chain length longer than 24. For biochemical diagnosis of ALD and AMN patients and carriers, Moser *et al.* pointed out that the cultured skin fibroblasts should be screened because GC analysis gave too low a C26 : 0/C22 : 0 ratio in sera (1/10 of that in fibroblasts). Therefore, GC analysis alone was insufficient to exclude false negative or positive cases.[7] In this regard, the quantitative analysis of serum VLCFA by SIM-GC/MS, which is 10^2-10^3 times more sensitive than scanning mass chromatography by GC/MS, may displace the analysis of VLCFA using GC in cultured skin fibroblasts from ALD and AMN patients, and normal controls.

ACKNOWLEDGMENTS

The author wishes to thank Prof. T. Kusaka and Dr. M. Ikeda, Department of Biochemistry, Kawasaki Medical School and Prof. A. Terao, Department of Neurology, Kawasaki Medical School for useful advice on many aspects of this study. The author would also like to thank Dr. R. Koike, Department of Neuology of the Insitute of Brain Research at Niigata University, Dr. T. Sakai, Division of Neurology in Chikugo National Hospital and Dr. T. Mezaki, Division of Neurology in Sumitomo Hospital, for providing blood of the patients with ALD and AMN.

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