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Journal of Chromatography B, 760 (2001) 149–157

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Diagnostic patterns of very-long-chain fatty acids in plasma of patients with X-linked adrenoleukodystrophy

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Received 8 January 2001; received in revised form 1 April 2001; accepted 17 May 2001

Abstract

Pattern recognition analysis on the levels of the very-long-chain fatty acids (VLCFAs) in plasma is described for the visual discrimination of X-linked adrenoleukodystrophy (X-ALD) patients from normal healthy group. Plasma VLCFA compositions of 58 normal subjects and 16 X-ALD patients were examined by gas chromatography as their methyl esters to determine the area percentages of behenic acid (C22:0), lignoceric acid (C24:0) and hexacosanoic acid (C26:0) in the total fatty acids, and the concentration (μg/ml) of C26:0. When star symbol plotting was applied to the VLCFA values of C22:0 (%), C24:0 (%), C26:0 (%), C24:0/C22:0, C26:0/C22:0 and C26:0 (μg/ml) after normalization to the corresponding median values in normal group, the resulting deformed hexagonal star pattern was characteristic of each patient. Therefore, simple visual comparison with the equilateral hexagon of normal group average as the control pattern enabled one readily to discriminate X-ALD patients from the normal group. Additionally, canonical discriminant analysis performed on the six unnormalized VLCFA values correctly classified 74 plasma specimens into two separate clusters according to normal subject or X-ALD patient in the canonical plot.

Keywords: Very-long-chain fatty acids

1. Introduction

X-linked adrenoleukodystrophy (X-ALD) is a hereditary neurological disorder characterized by adrenal cortex insufficiency and progressive brain demyelination, which commonly appears in childhood. Its principal biochemical abnormality is the accumulation of saturated very-long-chain fatty acids (VLCFAs) with 22 or more carbons in tissues and body fluids, due to the impaired capacity of VLCFA coenzyme A to degrade these substances in peroxisome. Diagnosis of X-ALD is mainly based on clinical features combined with the absolute concentration of hexacosanoic acid (C26:0) as well as the ratios of C26:0 and lignoceric acid (C24:0) relative to behenic acid (C22:0) as biochemical diagnostic markers [1–8].

For the biochemical diagnosis of X-ALD with more certainty, it has been desired to transform the numerical values of these biochemical markers into visually discriminating patterns. In the literature, for the distinction of diabetic patient, a non-parametric

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00263-8

pattern recognition method was used [9]. SIMCA multivariate data analysis was employed for the classification of brain and pituitary tumor cells [10]. The differentiation of leprosy patients from normal controls was achieved by principal component analysis and partial least-square models [11]. However, attempts have been rarely made to apply pattern discrimination analysis to VLCFA composition of X-ALD patients to date. It seems, therefore, of interest to devise pattern recognition tools suitable for the visual distinction of X-ALD compared with a normal group.

In our previous works, star symbol and canonical plottings as the visual pattern recognition methods were found useful for the comparative analysis of urinary organic acid profiles among the groups of nonsmokers and smokers [12]. When these methods were applied to urinary organic acid profiles [13] and urinary nucleoside profiles [14], the uterine malignant tumor group was readily discriminated from the benign tumor group.

This work discusses our recent investigation on the effective pattern recognition of X-ALD. In this study, star symbol plotting of the six VLCFA levels such as C26:0 (μg/ml), C22:0 (%), C24:0 (%), and C26:0 (%), C24:0/C22:0 and C26:0/C22:0 after normalization to the corresponding median values in normal group was examined for the visual comparison between X-ALD and normal groups. The canonical discriminant analysis [12–14] on the unnormalized VLCFA values as the data vectors was examined for the classification of 74 plasma specimens into two groups according to X-ALD or normal.

2. Experimental

2.1. Chemicals

Fatty acids standards and potassium carbonate were purchased from Sigma (St. Louis, MO, USA). Acetyl chloride was purchased from Aldrich (Milwaukee, WI, USA). Acetonitrile, *n*-hexane, chloroform, methanol and all other chemicals were of analytical-reagent grade purchased from Merck (Darmstadt, Germany).

2.2. Plasma specimens

Plasma samples examined for this study were freshly collected (Seoul Clinical Laboratories, Seoul Medical Science Institute, Seoul, South Korea) from 58 normal individuals (36 males and 22 females) and 16 X-ALD patients under fasting conditions between October 1997 and June 1999. The ages of normal subjects diagnosed clinically healthy ranged from 0.01 to 61 years. The 16 male patients diagnosed as having X-ALD were aged from 5 to 19 years. The samples were immediately stored at −20°C until analyzed.

2.3. Sample preparation

Blood specimens were drawn into tubes containing EDTA or heparin, and centrifuged at 3000 rpm for 20 min at room temperature. Plasma was then separated and stored at −70°C for no longer than 3 days before analysis. VLCFAs were determined as their methyl esters according to the method of Moser and Moser described elsewhere [1]. Briefly, 100 μl of heptacosanoic acid (C27:0) (20 μg/ml) as the internal standard was added to 250 μl of plasma. Then 1 ml of 25% (v/v) methylene chloride in methanol and 200 μl of acetyl chloride were added and heated for 1 h at 75°C to form methyl esters. After cooling, 4 ml of 7.0 wt.% K_2CO_3 was added to quench the reaction by neutralization. The resulting fatty acid methyl esters were extracted with 5 ml of hexane, followed by extracting with 2.5 ml of acetonitrile to remove polar compounds. The hexane layer was taken and evaporated to dryness under a gentle stream of nitrogen. The dry residue was reconstituted in 100 μl of hexane to be analyzed by gas chromatography (GC).

2.4. Gas chromatography

GC analyses were performed with a Hewlett-Packard HP Model 6890 gas chromatograph, equipped with a split/splitless capillary inlet system and a flame ionization detection (FID) system and interfaced to a HP 3365A GC Chemstation (Hewlett-Packard, Avondale, PA, USA). The injector and detector temperatures were 260 and 280°C, respectively. Samples (ca. 2 μl) were injected into a HP-5

Table 1
Levels of saturated very-long-chain fatty acid variables measured in plasma samples of 58 normal subjects

Age (years), sex	Mean level[a] (range)					
	C22:0 (%)	C24:0 (%)	C26:0 (%)	C24:0/C22:0	C26:0/C22:0	C26:0 μg/ml
0.01–15						
M (n=16)	0.84 (0.52–1.14)	0.70 (0.44–1.06)	0.008 (0.004–0.015)	0.83 (0.68–1.01)	0.009 (0.004–0.018)	0.13 (0.08–0.24)
F (n=11)	0.86 (0.68–1.13)	0.70 (0.57–0.90)	0.006 (0.003–0.009)	0.82 (0.70–0.96)	0.008 (0.003–0.013)	0.13 (0.08–0.16)
16–30						
M (n=10)	0.81 (0.62–1.23)	0.75 (0.47–1.28)	0.007 (0.001–0.015)	0.91 (0.74–1.06)	0.008 (0.002–0.017)	0.12 (0.05–0.18)
F (n=8)	1.18 (0.98–1.41)	1.11 (0.89–1.31)	0.013 (0.007–0.017)	0.94 (0.90–0.98)	0.011 (0.007–0.016)	0.13 (0.10–0.18)
31–61						
M (n=10)	0.88 (0.64–1.30)	0.81 (0.53–1.31)	0.008 (0.003–0.014)	0.91 (0.79–1.04)	0.009 (0.004–0.012)	0.13 (0.10–0.16)
F (n=3)	0.86 (0.83–0.89)	0.68 (0.64–0.72)	0.007 (0.005–0.011)	0.80 (0.77–0.84)	0.009 (0.006–0.013)	0.14 (0.10–0.21)
Total						
M (n=36)	0.84 (0.52–1.30)	0.75 (0.44–1.31)	0.008 (0.001–0.015)	0.87 (0.68–1.06)	0.009 (0.002–0.018)	0.13 (0.05–0.24)
F (n=22)	0.97 (0.68–1.41)	0.85 (0.57–1.31)	0.009 (0.003–0.017)	0.86 (0.70–0.98)	0.009 (0.003–0.016)	0.13 (0.08–0.21)
Total mean[b]	0.89	0.79	0.008	0.87	0.009	0.13
Total median[c]	0.88	0.72	0.007	0.85	0.008	0.13

[a] Mean (range) values from triplicate runs of each plasma sample for each age range. Normal control samples were obtained from 22 female and 36 male subjects aged from 0.01 and 61 years in the fasting state.
[b] Mean values of total normal subjects.
[c] Median values of total normal subjects.

(SE-54 bonded phase) fused-silica capillary column (Hewlett-Packard; dimensions 30 m×0.25 mm I.D., 0.25 μm film thickness) in the splitless mode with a purge delay time of 0.7 min. The flow-rate of helium as the carrier gas was set to 1.2 ml/min in the constant flow mode. The oven temperature was held at 60°C for 2 min, then programmed at a rate of 20°C/min to 150°C and subsequently programmed to 280°C at a rate of 4°C/min.

2.5. Calculation of VLCFA values

Peak areas of 20 fatty acids (C14:0, C16:1, C16:0, C18:2, C18:1, C18:3, C18:0, C20:4, C20:5, C20:3, C20:2, C20:1, C20:0, C22:6, C22:5, C22:1, C22:0, C24:1, C24:0 and C26:0) were summed up and then the percentages of C22:0, C24:0 and C26:0 in the total fatty acids were calculated as area percents. The area percent ratios of C24:0 and C26:0 to C22:0 were computed and the concentration (μg/ml) of C26:0 in plasma was calculated according to the following equations [1]:

$$\mu g \text{ total fatty acid/ml plasma} = [(\text{total fatty acid area} - \text{area C27:0})/\text{area C27:0}] \times 8\ \mu g/ml$$

$$\mu g \text{ C26:0/ml plasma} = \mu g \text{ total fatty acid/ml plasma} \times \% \text{ C26:0} \times 0.01$$

2.6. Pattern recognition analysis

The VLCFA values measured for the 16 patients and 58 normal subjects were directly plotted as the data points except for the area percents of C22:0 and C24:0 to produce four scatter plots using MS Excel program. And horizontal lines corresponding to each mean and mean+3SD values of normal group were drawn in each scatter plot.

The VLCFA values of each patient were normalized to the corresponding median values in normal group. Subsequently, each normalized value was plotted as a line radiating from a common central point and the far ends of the six lines were joined together to produce a star pattern in the shape of

hexagon for each patient using MS Excel program as described earlier [12,14].

The unnormalized VLCFA values from 74 individual plasma samples as the data vectors were subjected to canonical discriminant analysis [12–14] by means of the statistical software package SAS. Canonical plots were then drawn on the basis of the first canonical discriminant function (CAN1) against the second canonical discriminant function (CAN2) of the variables for each plasma specimen.

3. Results and discussion

3.1. Plasma VLCFA composition in normal subjects

The 58 healthy subjects examined were grouped according to age ranges (0.01 to 62 years) and sex. And the levels of VLCFA variables were expressed as the mean (range) values of each group (Table 1). Large variation in each VLCFA level from subject to subject was observed. Among the three age groups, 18 controls (10 males and eight females) between ages 16 and 30 years had the highest values in all the variables except for μg/ml of C26:0. In 13 controls between 31 and 61 years, males had higher values in all the variables except for C26:0/C22:0 ratio and μg/ml of C26:0 as compared with females. The overall sex-related differences were significant only in the percentages of C22:0 ($P<0.001$) and C24:0 ($P<0.1$).

The numerical mean and median values of the total (Table 1) were used as the normal reference values for the diagnostic pattern analysis of X-ALD in this study. Among the normal mean values, μg/ml of C26:0 (0.13) was found to be lower than the corresponding typical American reference values (≥ 0.29) as reported elsewhere [1,5].

3.2. Plasma VLCFA composition in X-ALD patients

The levels of VLCFA variables in plasma samples from 16 X-ALD patients (P-1 through P-16) aged

Table 2
Levels of saturated very-long-chain fatty acid variables measured in plasma samples of 16 male patients with X-linked adrenoleukodystrophy

Patient (age in years)	Level[a]					
	C22:0 (%)	C24:0 (%)	C26:0 (%)	C24:0/C22:0	C26:0/C22:0	C26:0 (μg/ml)
P-1 (5)	0.79	1.24	0.054	1.57	0.068	0.35
P-2 (7)	0.52	0.89	0.051	1.71	0.098	0.66
P-3 (9)	0.78	1.19	0.062	1.52	0.079	0.45
P-4 (9)	0.59	0.71	0.029	1.21	0.049	0.39
P-5 (9)	0.39	0.78	0.049	2.02	0.127	1.41
P-6 (9)	0.69	1.15	0.039	1.68	0.057	0.68
P-7 (10)	0.65	1.42	0.100	2.16	0.153	0.74
P-8 (10)	0.68	1.51	0.078	2.24	0.116	1.19
P-9 (11)	0.31	0.47	0.027	1.50	0.086	1.19
P-10 (12)	0.57	1.00	0.062	1.74	0.108	0.84
P-11 (12)	0.51	0.74	0.046	1.45	0.090	0.73
P-12 (13)	0.61	1.15	0.070	1.89	0.115	1.47
P-13 (16)	0.64	1.23	0.130	1.92	0.203	1.23
P-14 (18)	0.65	1.16	0.051	1.77	0.078	0.63
P-15 (18)	0.47	0.62	0.029	1.34	0.062	0.57
P-16 (19)	0.66	1.59	0.112	2.41	0.169	0.83
Median	0.63	1.15	0.053	1.73	0.094	0.74

[a] Mean values from triplicate runs of each plasma sample. X-ALD patients were clinically diagnosed in all cases.

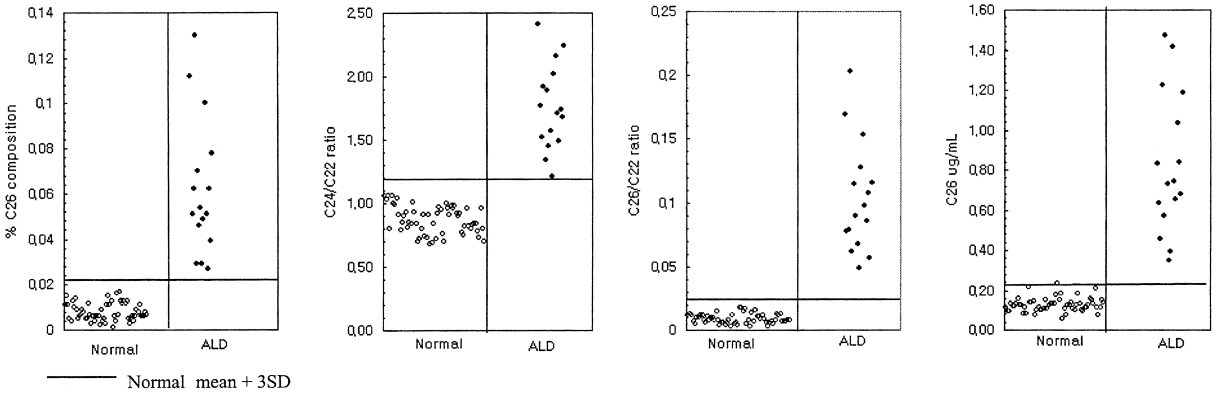

Fig. 1. Scatter plots of C26:0 (%), C24:0/C22:0 and C26:0/C22:0, and C26:0 (μg/ml) in plasma samples from 58 normal subjects (open circles) and 16 X-ALD patients (filled circles). Each variable of patients and normal subjects was directly plotted as the data points and horizontal lines corresponding to each mean + 3SD values of normal group were drawn in each scatter plot.

from 5 to 19 years were listed in the order of increasing age (Table 2). Like the normal subjects, large variations in the VLCFA levels from patient to patient were observed. As compared with the normal reference value (0.008) of C26:0 (%), its levels varied from 0.027 to 0.130 in all patients were significantly higher ($P < 0.002$). The differences in levels of C24:0/C22:0 (ranged from 1.21 to 2.41)

Table 3
Normalized values of saturated very-long-chain fatty acid variables measured in plasma samples of 16 male patients with X-linked adrenoleukodystrophy

Patient	Value[a]					
	C22:0 (%)	C24:0 (%)	C26:0 (%)	C24:0/C22:0	C26:0/C22:0	C26:0 (μg/ml)
P-1	0.9	1.7	7.7	1.8	8.5	2.7
P-2	0.6	1.2	7.3	2.0	12.3	5.1
P-3	0.9	1.7	8.9	1.8	9.9	3.5
P-4	0.7	1.0	4.1	1.4	6.1	3.0
P-5	0.4	1.1	7.0	2.4	15.9	10.9
P-6	0.8	1.6	5.6	2.0	7.1	5.2
P-7	0.7	2.0	14.3	2.5	19.1	5.7
P-8	0.8	2.1	11.1	2.6	14.5	9.1
P-9	0.4	0.7	3.9	1.8	10.8	9.2
P-10	0.7	1.4	8.9	2.0	13.5	6.4
P-11	0.6	1.0	6.6	1.7	11.3	5.6
P-12	0.7	1.6	10.0	2.2	14.4	11.3
P-13	0.7	1.7	18.6	2.3	25.4	9.4
P-14	0.7	1.6	7.3	2.1	9.8	4.9
P-15	0.5	0.9	4.1	1.6	7.8	4.4
P-16	0.8	2.2	16.0	2.8	21.1	6.4
Median	0.7	1.6	7.6	2.0	11.8	5.7

[a] Values normalized to corresponding normal median values.

and C26:0/C22:0 (varied from 0.049 to 0.203) of each patient from the respective reference values (0.87 and 0.009) were very significant ($P<0.001$). And the levels of C26:0 (μg/ml) in all patients were markedly increased (ranged from 0.35 to 1.47) compared to the normal mean value (0.13).

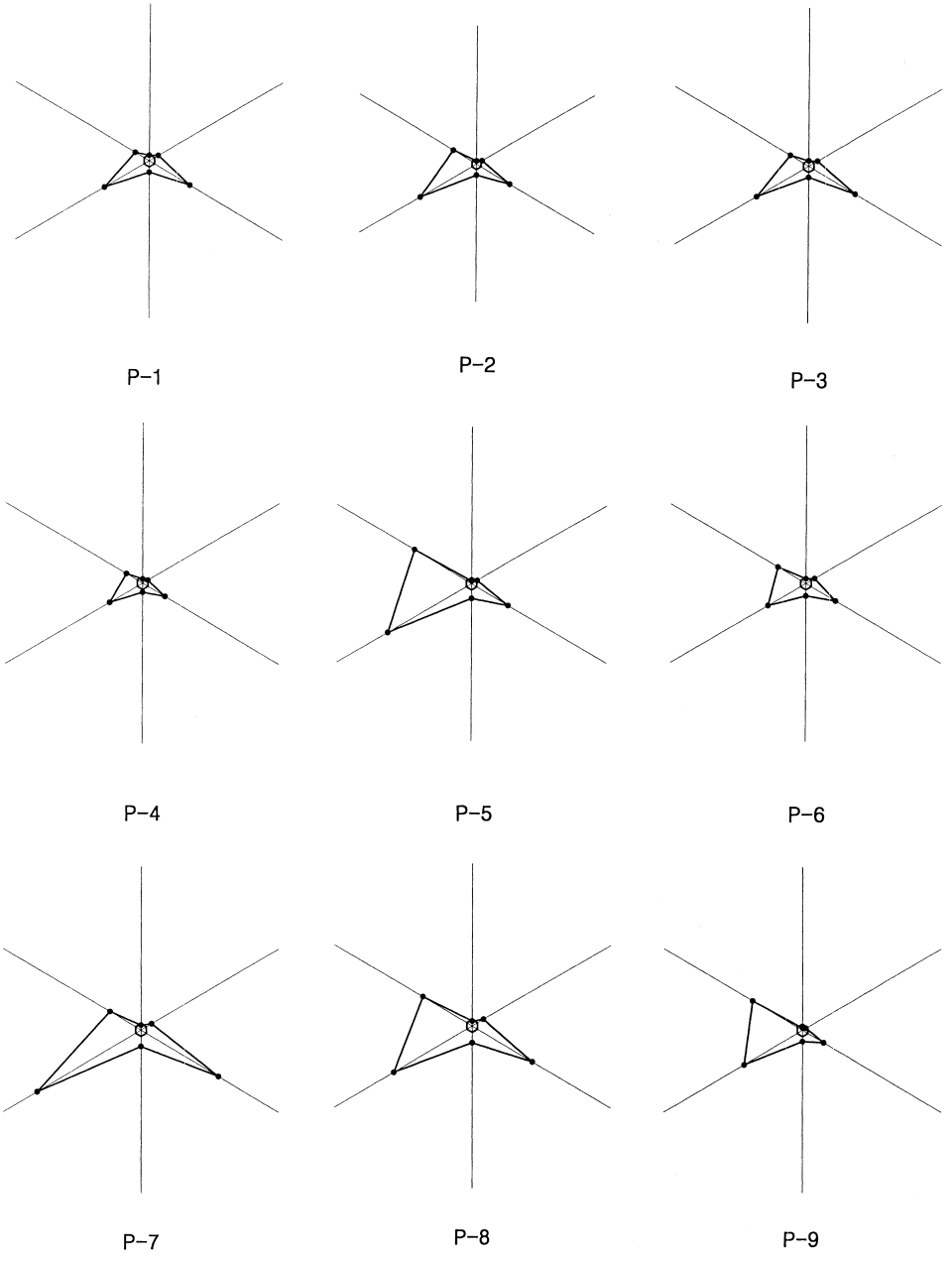

Fig. 2. Star symbol plots of individual X-ALD patients (P-1 though P-16), and patient and normal group averages drawn based on the levels of the very-long-chain fatty acid variables after normalization to the corresponding median values of the normal group. Rays: 1=C22:0 (%); 2=C24:0 (%); 3=C26:0 (%); 4=C24:0/C22:0; 5=C26:0/C22:0; 6=C26:0 (μg/ml).

3.3. Pattern recognition analysis

The scatter plots were drawn based on the VLCFA values except for the percents of C22:0 and C24:0 that were known insignificant. Each scatter plot showed large shifts in the distribution to higher values for the 16 patients (represented by filled circles) compared with 58 normal controls (represented by open circles) as demonstrated in Fig. 1. The data points of normal subjects in each scatter

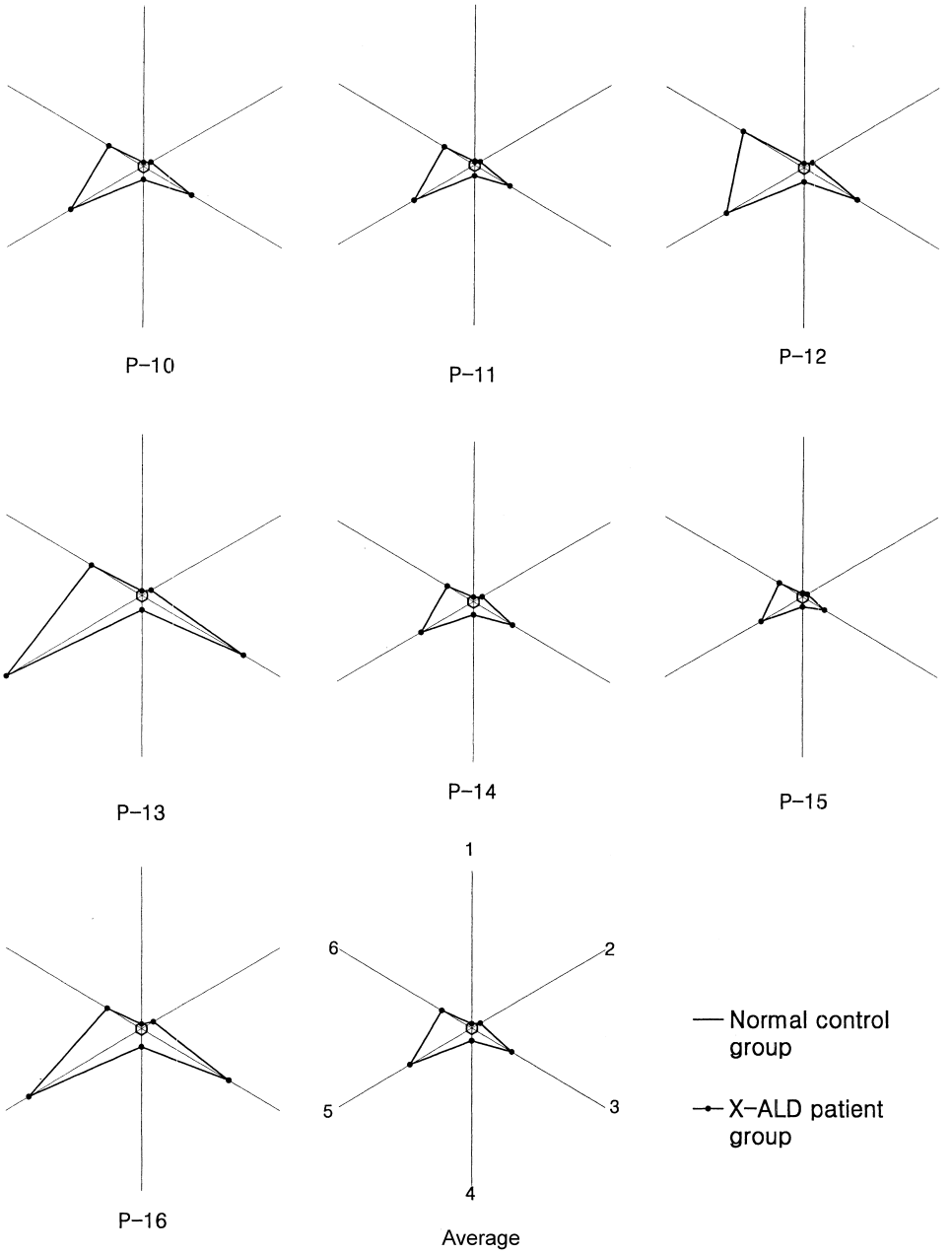

Fig. 2. (*continued*)

plot were clustered together compared with their wide distribution for the patients. The solid line corresponding to the upper + 3SD limit of the normal mean value in each plot imply that a subject with plasma VLCFA levels above the lines belongs to X-ALD patient group with 99.7% confidence. If each solid line were assumed to be the X-ALD marker line, the estimated diagnostic powers of C26:0 (%), C26:0/C22:0 and C26:0 (μg/ml) would be all 100%, but 94% for C24:0/C22:0.

Each VLCFA level in each patient was normalized to the corresponding median value in normal group to measure the magnitude of each elevation. The normalized values were surely more informative by expressing the elevation of the VLCFA levels in multiples (ranging from 0.4 to 25.4) of the normal

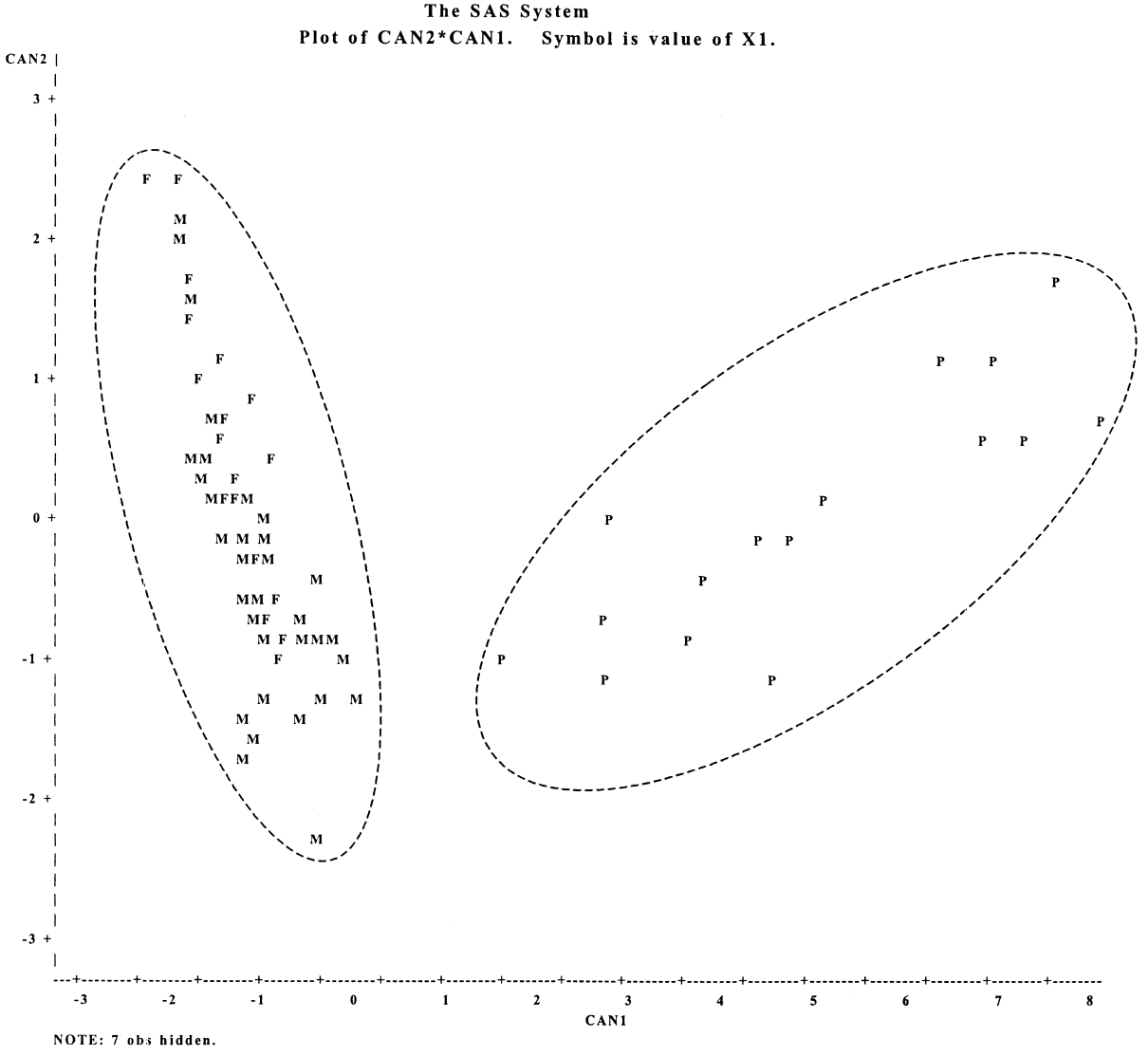

Fig. 3. Plot of the first canonical discriminant function (CAN1) against the second canonical discriminant function (CAN2) of the very-long-chain fatty acid variables for the 58 normal subjects (each represented by M for male and F for female) and 16 X-ALD patients (each represented by P). Variables: C22:0 (%), C24:0 (%), C26:0 (%), C24:0/C22:0, C26:0/C22:0 and C26:0 (μg/ml).

median values (Table 3). The low normalized values of C24:0/C22:0 (1.4 to 2.8) agreed well with its low diagnostic power as estimated in the scatter plot above.

However, the numbers themselves of the normalized values showed no readily apparent pattern of each patient. Therefore, our star symbol plotting method [12,14] was employed to display the six normalized values together in a visually discriminative hexagonal shape for each patient. The resulting star patterns (P-1 through P-16) of the 16 patients examined were all deformed hexagons as compared with the small equilateral hexagon of the normal group average placed in each center (Fig. 2). The normal hexagon served well as the control pattern for the X-ALD patients and thus the visual comparison between each patient and the normal group average was much easier than the conventional scatter patterns.

When canonical discriminant analysis was performed on the unnormalized VLCFA values, the 74 plasma specimens were well separated into two distinct clusters in the two-dimensional canonical function space (Fig. 3). The 16 X-ALD patients (represented by P) formed one cluster and the 58 normal subjects (M represented for male and F for female) another cluster. No sex discrimination was observed in the canonical plot.

4. Conclusions

The present star symbol plots based on the plasma levels of C26:0 (μg/ml), C22:0 (%), C24:0 (%), C26:0 (%), C24:0/C22:0 and C26:0/C22:0 after normalization to the corresponding median values in normal group were found effective for the visual recognition of X-ALD in deformed hexagonal patterns. The small equilateral hexagon of the normal group average served well as the control pattern for the X-ALD patients. Hence, the visual comparison between patients and the normal group average was much easier than the conventional scatter patterns. Canonical discriminant analysis applied to the unnormalized VLCFA levels correctly classified 74 plasma specimens into two separate clusters according to X-ALD or normal. The present results proved that star graphical and canonical plottings as the pattern recognition tools were complements each of the other for the visual diagnosis of X-ALD.

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