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Gas chromatographic–mass spectrometric determination of plasma saturated fatty acids using pentafluorophenyldimethylsilyl derivatization

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Abstract

An improved method for the detection of 11 saturated fatty acids (SFAs) including C12:0–C26:0 (even numbers only), C17:0, C19:0 and C23:0 in human plasma by gas chromatography–mass spectrometry (GC–MS) with a stable isotope internal standard as d_3-stearic acid is described. This procedure was based on acidic treatment, liquid–liquid extraction, and chemical derivatization prior to instrumental analysis. Eleven pentafluorophenyldimethylsilyl-SFA derivatives were well separated without any interfering peaks in plasma samples. The characteristic ions at M−15, constituting the base peaks in the electron impact mass spectra for 11 SFAs, permitted their sensitive detection by GC–MS in the selected ion monitoring (SIM) mode. The SIM responses were linear with correlation coefficients varying from 0.993 to 0.999 in the concentration range of 0.05~50 μg/ml for the 11 SFAs. The detection limits for SIM of the SFAs varied in the range of 0.05~10.0 pg. When applied to the plasma samples of normal subjects and patients with X-linked adenoleukodystrophy, which is one of the hereditary peroxisomal disorders, the present method enabled us to determine the SFAs with good sensitivity and good overall precision and accuracy within the concentration ranges of 0.14~82.35 μmol/l.

Keywords: Fatty acids; GC–MS; Flophemesyl; X-ALD; Plasma

1. Introduction

The common fatty acids (FAs) of plant and animal origin contain several carbon atoms in straight chains with a terminal carboxyl group. They also may be fully saturated and contain one, two, or more double bonds as saturated and unsaturated fatty acids (SFAs and USFAs), respectively. Especially, some of the SFAs are used as the qualitative biomarkers of fatty acid intake [1] and peroxisomal disorders in tissues or body fluids [2–5].

Gas chromatography–mass spectrometry (GC–MS) is a technique widely used for the identification of FAs in biological mixtures, usually as their

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methyl, benzyl and trimethylsilyl (TMS) esters [4,6–9]. These derivatives, however, are not considered satisfactory for unequivocal identification or sensitive detection purposes. The mass spectra obtained are typically dominated by intense ions at the lower mass, characteristic of the ester moiety rather than the structure of the acid itself, and the abundance of the high mass such as molecular ions, decreases with increasing the chain length of the acids. Also, the mass spectra of *tert*.-butyldimethylsilyl (TBDMS)-FA esters determinate the good mass fragment pattern, but as expected, do not have all FAs [10].

However, as well documented elsewhere [11,12], in the determination of the C26:0/C22:0 and C24:0/C22:0 ratio as the biomarkers of X-linked adrenoleucodystrophy (X-ALD), which is a peroxisomal disorders, the identification of C26:0 is very difficult. Since it is normally only 0.01% of the total lipid fatty acids, more sensitive methods of measuring very long chain fatty acids (VLCFAs) are, therefore, most desirable. A sensitive method with pentafluorobenzyl derivatization in electron-capture negative ion chemical ionization mass spectrometry (GC–EC-NCI-MS) was developed for the accurate quantification of VLCFAs [5].

For the facile and reproducible screening procedure, the best and most appropriate choice of derivatization in GC–MS with electron impact ionization is primary concern not only for GC properties, but also for the selection of efficient quantitative ions in order to trace quantification in the selected ion monitoring (SIM) mode. Therefore, we introduced our technique of pentafluorophenyldimethylsilyl (flophemesyl) derivatization, which had been confirmed as the stable and reproducible derivatization method in previous reports as hydroxyl-protecting groups in steroid analogues [13–16].

In the continuation of biomedical methodologies and clinical applications of endogenous compounds, the present method was undertaken to investigate the optimum conditions of GC–SIM-MS for SFAs as their flophemesyl ester derivatives. For reference, the present method was applied to determine C22:0, C24:0 and C26:0 in order to demonstrate the difference between the plasma concentrations of the normal controls and of the patients with X-ALD as diagnostic analysis. The structures of the derivatives that are new to the literature were confirmed by mass spectral patterns.

2. Experimental

2.1. Materials

All chemicals and solvents for GC–MS analyses were of analytical-reagent grade, and SFAs with a chain length between C12:0 and C26:0 were used in this study. Fatty acid standards, lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), margaric acid (C17:0), stearic acid (C18:0), nonadecanoic acid (C19:0), arachidic acid (C20:0), behenic acid (C22:0), tricosanoic acid (C23:0), lignoceric acid (C24:0) and hexacosanoic acid (C26:0) were purchased from Sigma (St. Louis, MO, USA). 18,18,18-$^{2}H_{3}$-Stearic acid as an internal standard (I.S.) was obtained from Cambridge Isotope Labs. (Andover, MA, USA). Flophemesyl chloride as silylating reagent was supplied by Acros Organics (Geel, Belgium).

2.2. Preparation of standard solution

Stock solutions of SFAs studied were prepared by diluting them with methanol at a concentration of 0.1 mg/ml, and the other SFAs with 20 more carbon atoms were prepared at the same concentration in a methanol–chloroform (1:2, v/v) mixture. These stock solutions were further diluted with the same organic solvent to prepare working solutions of varying concentrations from 0.1 to 10 μg/ml. The I.S. solution was dissolved at a concentration of 10 μg/ml in methanol.

2.3. Subjects and sample collection

Plasma samples were obtained from eight healthy volunteers (6~18 years of age) without remarkable medical histories and five patients (8~19 years of age) with X-ALD. All cases and controls in this study underwent the same diagnostic procedure in the same facilities. Patients groups received no treatment before plasma samples were obtained. The plasma specimens were collected in polyethylene

bottles and immediately stored at −20°C until analysis.

2.4. Sample preparation and derivatization

For the extraction of FAs in plasma samples, a previously reported method [12] was modified. Briefly, the procedure was as follows: to a 0.2-ml aliquot of plasma was added 0.2 μg of I.S., 1 ml of acetonitrile and 0.2 ml of 5 *M* HCl solution. The mixture was heated for 40 min at 80°C in order to release FAs from lipids. After cooling, 0.5 ml of water was added. Then FAs were extracted with 5 ml of *n*-hexane. The supernatant obtained by centrifugation at 2500 *g* for 10 min was evaporated to dryness in a vacuum rotary evaporator. The residue was derivatized with 50 μl of a flophemesyl chloride solution at room temperature for 15 min, and 2 μl of the sample was injected onto the GC column, using an autosampler. The reaction mixtures were directly examined by GC–MS. The standard samples containing 0.1–1.0 ng each of the 11 SFAs each was prepared to determine detection limits. Determination of the signal-to-noise ($S/N=3$) ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of water blank as their flophemesyl derivatives.

2.5. Gas chromatography–mass spectrometry

Analyses were carried out on a Hewlett-Packard (Avondiele, PA, USA) GC–MS system consisting of a Model 6890 gas chromatograph, a Model 5973 mass-selective detector, a HP G1701AA MSD Chemstation, and a Model 6890 series injector. For separation of FAs, a HP Ultra-2 (25 m×0.2 mm I.D., cross-linked 5% phenyl–methyl silicone, 0.33 μm film thickness) fused-silica capillary column was used. The column head pressure of helium as a carrier gas was set to 132 kPa, and split ratio was 1:12. The GC oven temperature was programmed from 230°C (3 min hold) to 290°C at a rate of 15°C/min and held there for 5 min. Then it was programmed to 320°C at a rate of 5°C/min and held for 3 min. The temperatures of detector and injector were 300°C and 280°C, respectively. The mass spectrometer was operated at 70 eV in the electron impact (EI) mode, and the SIM mode was used for quantifying 11 SFAs in human plasma. A dwell time of 100 ms and a relative EM voltage of 400 V higher than that in the scanning mode were chosen for each ion monitored.

2.6. Calibration

Calibration curves of each SFAs were set up over a concentration range of 0.05~50.0 μg/ml and are shown in Table 1. For the standard curve of C16:0 and C18:0, the working solution of 1.0~50.0 μg/ml was added in 0.2 ml of blank plasma. The concentrations of C12:0, C14:0, C22:0 and C24:0 ranged from 0.5 to 10.0 μg/ml and for the SFAs containing C17:0, C19:0, C20:0, C23:0 and C26:0, the concentration range was 0.05~5.0 μg/ml. After the addition of 0.2 μg of the internal standard, each sample was extracted and derivatized according to the method as described above for the plasma sample. For the quantitative analyses, relative peak-area ratios of SFAs standard over internal standard were calculated and plotted in the calibration range of each SFAs.

2.7. Calculation

Samples for intra- and inter-day assays as well as for recovery tests were prepared at three different concentrations; 0.5, 1.0 and 5.0 μg/ml or 1.0, 5.0 and 10.0 μg/ml depending on the kind of fatty acid, using plasma samples whose endogenous fatty acid levels were predetermined. They were analyzed in a day for intra-day assays, and on every other day for inter-day assays.

3. Results and discussion

3.1. Derivatization

In order to determine the low concentration of compounds accurately, it is necessary to use a technique that is both highly selective and sensitive. However, previous methods for their determination have often been unsatisfactory when used for sensitive identification purposes, e.g., when used to

Table 1
GC–SIM-MS data of 11 SFAs as their flophemesyl derivatives

No.	Fatty acid	M_r	RRT[a]	Quantitative ion (m/z)	Detection limit (pg)	Calibration range (μg/ml)	Linearity[b]
1	Lauric, C12:0	424	0.56	409	0.2	0.5–10.0	0.996
2	Myristic, C14:0	452	0.68	437	0.1	0.5–10.0	0.993
3	Palmitic, C16:0	480	0.83	465	0.05	1.0–50.0	0.995
4	Margaric, C17:0	494	0.91	479	0.4	0.05–5.0	0.998
5	Stearic, C18:0	508	1.00	493	0.05	1.0–50.0	0.996
6	Nonadecanoic, C19:0	522	1.11	507	1.0	0.05–5.0	0.998
7	Arachidic, C20:0	536	1.24	521	1.0	0.05–5.0	0.997
8	Behenic, C22:0	564	1.59	549	2.0	0.5–10.0	0.998
9	Tricosanoic, C23:0	578	1.68	563	4.0	0.05–5.0	0.999
10	Lignoceric, C24:0	592	1.83	577	4.0	0.5–10.0	0.994
11	Hexacosanoic, C26:0	620	2.13	605	10.0	0.05–5.0	0.999

[a] Retention time relative to that of d_3-stearic acid as its flophemesyl ester (9.36 min).
[b] Linearity was described with linear correlation coefficients for calibration curves.

determine the abundance of high mass, the formation of an intense molecular ion and a GC property. Moreover, the derivatization time was too long (methyl ester, 16 h [9]; TBDMS ester, 60 min [17]; and cyanomethylester, 40 min [12], etc.), and after derivatization, re-extraction of the derivatives was necessary [9,18].

The present derivatization with flophemesyl chloride was completed within 15 min at room temperature. All the carboxyl groups of 11 SFAs were readily converted to their corresponding flophemesyl esters. In preliminary work, flophemesyl-SFAs resulted in an amendable work-up process and, when stored in a derivatizing reagent, were more stable than TMS and TBDMS derivatives. The flophemesyl derivatives have longer retention times than other derivatives such as methyl, TMS and TBDMS, but demonstrate good GC properties.

3.2. Gas chromatography–mass spectrometry

Under the present GC conditions using a nonpolar dimethylpolysiloxane column, each flophemesyl derivative of the 11 SFAs showed a symmetrical peak shape within 21 min, and 11 flophemesyl-SFA derivatives were well separated without any interfering peaks in plasma samples (Fig. 1). Among the SFAs detected, C16:0 was most intense, followed by C14:0, C18:0, C17:0, C12:0, C19:0, and C20:0. The VLCFAs (C22:0~26:0) were relatively weak.

The present GC–MS assay was initially employed to verify the chemical nature of the flophemesyl-SFAs. It was observed that all of the SFA-esters that were prepared yielded characteristic mass spectra, including a significant proportion of $[M-15]^+$. The mass spectra of flophemesyl derivatives of 11 SFAs and d_3-stearic acid as I.S. obtained at 70 eV are shown in Fig. 2. The $[M-15]^+$ ion as base peaks, except d_3-stearic acid in the EI spectrum of all the flophemesyl esters studied which is postulated to have the resonance stabilized cyclic structure is formed by the fission of a methyl radical from the molecular ion. Some typical mass spectra for the flophemesyl esters of SFAs had a molecular peak and a large peak (above 80%) at m/z 121, which is produces by the elimination of the F_2 at the fluorohydrocarbon tropylium ion (m/z 159) [13]. The peaks at M−167 $[M\text{-}C_6F_5]^+$, 225 $[Si(CH_3)_2C_6F_5]^+$, 241 $[225+O]^+$, and 269 $[225+CO_2]^+$ also occurred to varying extents in all of the spectra as flophemesyl characteristic ions.

The base peak ion at M−15 permitted sensitive detection of most of the SFAs by GC–MS with SIM, it was used as the quantitative ions (Table 1). Their detection limits for SIM were femtogram levels (50~400 fg) except for the C19:0–26:0 whose detection limits were in the range of 1~10 pg. The GC–SIM-

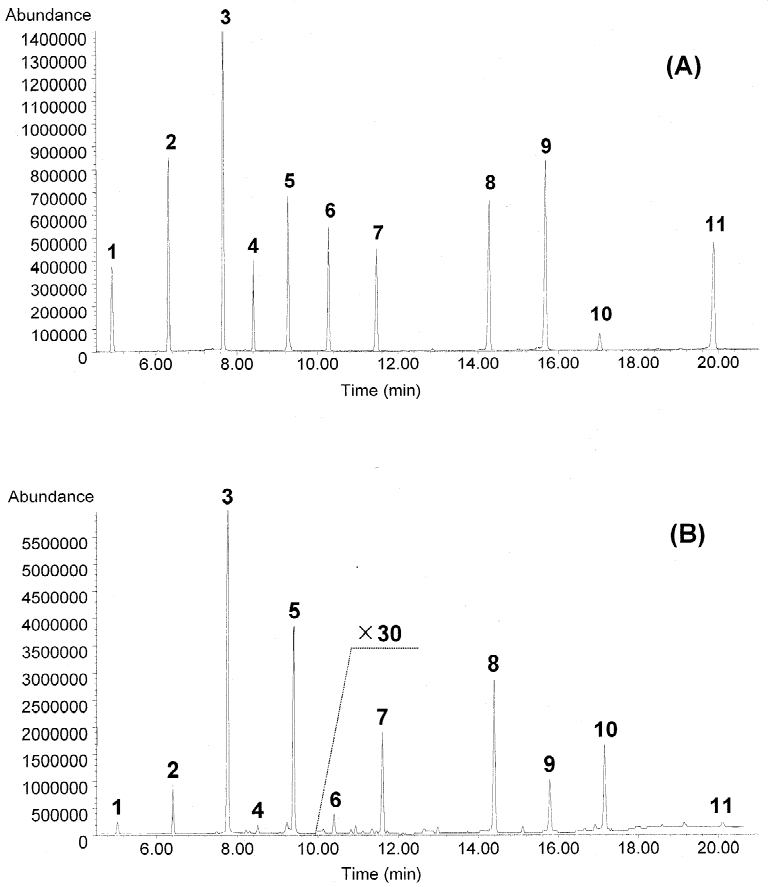

Fig. 1. Total ion chromatogram (TIC) of (A) the mixed standard solution of 11 saturated fatty acids (peaks 1~7, 50 ppm; peaks 8 and 9, 100 ppm; peaks 10 and 11, 200 ppm) in scan mode and (B) reconstructed ion chromatogram (RIC) of the normal subject plasma in SIM mode (for peak identities, see Table 1).

MS responses were, linear with correlation coefficients varying from 0.993 to 0.999 in the concentration range of 0.05~50.0 μg/ml for the SFAs studied.

3.3. Validation of analytical procedure

The precision of the present method was assessed by analyses of replicate aliquots of plasma specimens spiked with three different concentrations of each of fatty acids. The intra- and inter-day assay variances obtained by GC–MS analyses with SIM are given in Table 2. The recoveries ranged from 72.2~113.6% with good overall precision that appears to be satisfactory for the quantification of SFAs in unknown plasma samples.

3.4. Quantification of 11 SFAs in human plasma

Also, to evaluate the usefulness of this method for routine analysis, we estimated the concentration of SFAs, peroxisomal metabolites in plasma of patients with X-ALD that is one of hereditary peroxisomal disorders. When applied to plasma specimens from eight normal control subjects and five patients with X-ALD, the present method was quantitatively de-

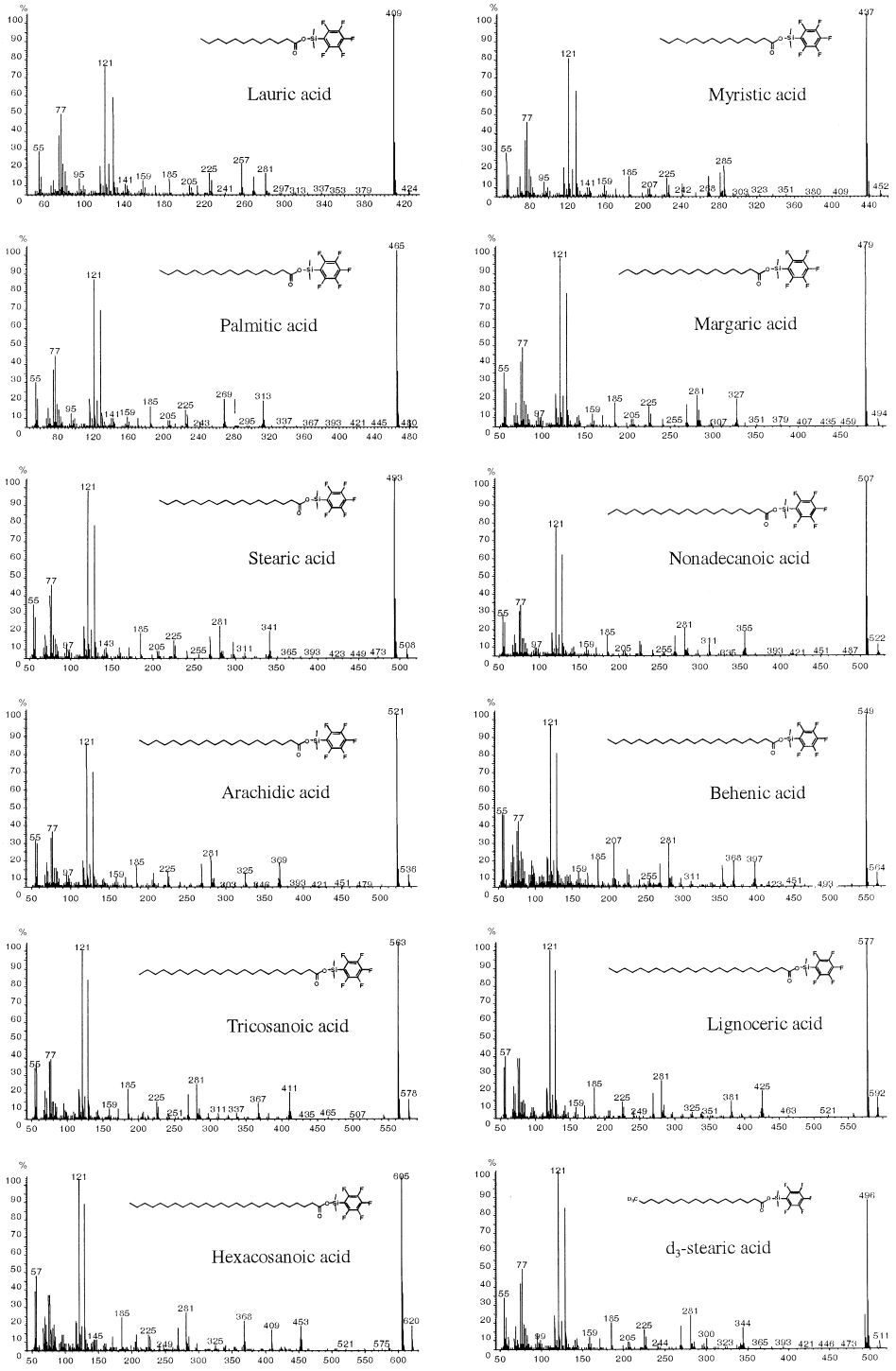

Fig. 2. Mass spectra of 11 saturated fatty acids and d_3-stearic acid as their flophemesyl esters. In scanning mode, mass range was m/z 50~650 at a rate of 0.45 scans/s. The EI spectrum of all the flophemesyl esters except a d_3-stearic acid ester is formed by the fission of a methyl radical from the molecular ion [M−15] as the base peaks.

Table 2
Recovery, precision and accuracy data for determination of 11 fatty acids

Fatty acid (Recovery, %, ±SD)	Amount added (μg/ml)	Intra-day (n=3)		Inter-day (n=3)	
		Mean±SD (μg/ml)	RSD (%)	Mean±SD (μg/ml)	RSD (%)
Lauric, C12:0 (86.9±3.4)	Blank	4.11±0.19	4.6	4.10±0.25	6.0
	0.5	4.42±0.17	3.9	4.62±0.09	1.9
	1.0	5.20±0.40	7.7	5.29±0.34	6.5
	5.0	10.90±0.26	2.4	10.69±1.23	11.5
Myristic, C14:0 (94.4±8.0)	Blank	1.57±0.06	3.6	1.73±0.07	4.1
	0.5	2.03±0.05	2.7	2.06±0.07	3.5
	1.0	2.52±0.33	12.9	2.33±0.17	7.2
	5.0	7.01±0.61	8.8	7.02±0.65	9.2
Palmitic, C16:0 (113.6±8.1)	Blank	9.58±0.34	3.6	9.72±0.40	4.1
	1.0	10.43±0.28	2.7	10.66±0.37	3.5
	5.0	14.52±1.88	12.9	15.34±1.10	7.2
	10.0	18.89±1.67	8.8	19.20±1.77	9.2
Margaric, C17:0 (76.0±6.78)	Blank	0.59±0.04	6.8	0.50±0.06	12.0
	0.5	1.03±0.03	3.0	0.92±0.03	3.3
	1.0	1.48±0.05	3.4	1.44±0.06	4.2
	5.0	5.38±0.28	5.2	5.39±0.31	5.8
Stearic, C18:0 (108.4±11.8)	Blank	22.48±1.09	4.8	22.16±2.19	9.9
	1.0	23.01±1.55	6.7	23.38±2.83	12.1
	5.0	27.63±2.68	9.7	25.98±1.91	7.4
	10.0	32.19±1.89	5.9	35.73±2.51	7.0
Nonadecanoic, C19:0 (74.3±6.6)	Blank	0.20±0.01	4.9	0.20±0.01	5.0
	0.5	0.68±0.03	4.4	0.71±0.04	5.4
	1.0	1.36±0.13	9.6	1.40±0.15	10.7
	5.0	6.93±0.46	6.6	7.25±0.51	7.0
Arachidic, C20:0 (72.2±8.6)	Blank	0.65±0.03	5.3	0.67±0.04	5.8
	0.5	1.08±0.05	4.6	1.13±0.05	4.4
	1.0	1.71±0.03	1.7	1.60±0.11	7.0
	5.0	5.44±0.22	4.0	5.74±0.25	4.4
Behenic, C22:0 (83.6±8.3)	Blank	2.30±0.11	4.9	2.00±0.16	8.0
	0.5	2.77±0.20	7.2	2.69±0.22	8.2
	1.0	3.27±0.22	6.7	3.16±0.26	8.3
	5.0	6.96±0.27	3.9	6.75±0.43	6.4
Tricosanoic, C23:0 (80.4±5.2)	Blank	0.88±0.03	3.6	0.83±0.05	5.7
	0.5	1.26±0.05	4.0	1.32±0.08	6.0
	1.0	1.99±0.07	3.6	1.80±0.10	5.8
	5.0	5.57±0.42	7.5	5.63±0.33	5.9
Lignoceric, C24:0 (84.9±6.3)	Blank	2.21±0.14	6.5	2.01±0.23	11.4
	0.5	2.69±0.24	9.3	2.55±0.20	7.8
	1.0	3.18±0.17	5.3	3.13±0.29	9.3
	5.0	7.03±0.69	9.8	7.17±0.45	6.3
Hexacosanoic, C26:0 (79.5±7.2)	Blank	0.10±0.01	8.0	0.10±0.01	9.0
	0.5	0.52±0.04	7.7	0.54±0.05	9.3
	1.0	1.23±0.08	6.5	1.19±0.07	5.9
	5.0	5.03±0.39	7.8	5.11±0.41	8.0

Table 3
Concentration and relative ratios of plasma saturated fatty acids in normal subjects and patients with X-ALD

Fatty acid	Normal subjects (n=8)			X-ALD patients (n=5)		
	Mean (μmol/l)	SD	Range (μmol/l)	Mean (μmol/l)	SD	Range (μmol/l)
Lauric, C12:0	10.17	9.04	3.79~27.82	13.86	6.15	6.74~22.87
Myristic, C14:0	6.32	0.94	4.98~7.83	19.70	5.28	12.16~24.73
Palmitic, C16:0	59.69	14.42	40.72~82.35	38.67	6.21	32.06~47.17
Margaric, C17:0	2.09	0.19	1.91~2.36	7.12	2.50	4.91~11.43
Stearic, C18:0	67.49	3.67	61.88~72.85	64.29	9.22	52.27~73.34
Nonadecanoic, C19:0	0.68	0.06	0.61~0.78	0.87	0.13	0.65~1.00
Arachidic, C20:0	3.43	0.81	2.46~4.77	2.68	1.05	1.56~4.42
Behenic, C22:0	13.02	3.51	7.38~18.23	12.69	4.67	7.06~19.26
Tricosanoic, C23:0	5.58	1.66	2.49~7.58	6.45	1.94	4.01~8.77
Lignoceric, C24:0	13.33	3.92	6.62~18.91	21.84	6.04	13.09~28.68
Hexacosanoic, C26:0	0.26	0.07	0.14~0.36	2.70	0.18	2.41~2.85
C24:0/C22:0 ratio	1.02	0.06	0.90~1.09	1.77	0.18	1.49~1.93
C26:0/C22:0 ratio	0.02	0.01	0.01~0.03	0.24	0.08	0.14~0.34

termined simultaneously with good GC properties within the overall concentration ranges of 0.14~82.35 μmol/l (Table 3). The results indicated that many of SFAs was equally abundant in both cases. Although the mean value and concentration range of some FAs, such as C14:0, C16:0 and C17:0 were different in two groups; however, the differences were not significant since the case of C26:0 was almost 10-times higher than that of the control group.

The relative ratios of C24:0/C22:0 and C26:0/C22:0 were compared between the control and patient groups. Especially in the patients with X-ALD, the ratio of C26:0/C22:0 (0.24±0.08) were significantly increased as compared with the normal values (0.02±0.01).

In this study, we found that the mean values of some SFAs were different from those of the controls, but the difference was not significant. Regarding VLCFAs, we found elevated C24:0 and C26:0 levels in the plasma of X-ALD patients, while the value of C22:0 was similar to that of the normal controls. Therefore, the ratios of C24:0/C22:0 and C26:0/C22:0 significantly increased in the patient groups and the C26:0 level and C26:0/C22:0 ratio were almost 10-times higher than those of normal subjects. These results match the previous results [11,12] reported in the literature. Fig. 3 shows the chromatogram of VLCFAs, including C22:0, C24:0 and C26:0 in the plasma of both the normal and the patients with X-ALD, respectively.

4. Conclusion

We present a new derivatization procedure for simultaneous GC–MS analysis of SFAs in human plasma. The uncatalyzed reactions by flophemesyl chloride rapidly react with carboxylic acid groups to produce the silyl-esters. The flophemesyl-SFAs derivatives show base peaks of M−15, not concerned with the chain-length and good separation, without any interfering peaks in SIM mode. Therefore, the flophemesyl esters of SFAs are very well suited to a derivatization technique for SIM quantification.

The advantages of using flophemesyl derivatization method include rapid reaction time, mild reaction conditions, good GC properties, and the formation of intense M−15 ions. By using this method, we could also more accurately determine characteristics of SFAs, including determining VLCFAs more sensitively and with a smaller sample volume than necessary in the previous methods.

Our study suggests that flophemesyl derivatization is selective and that it is a sensitive method for the

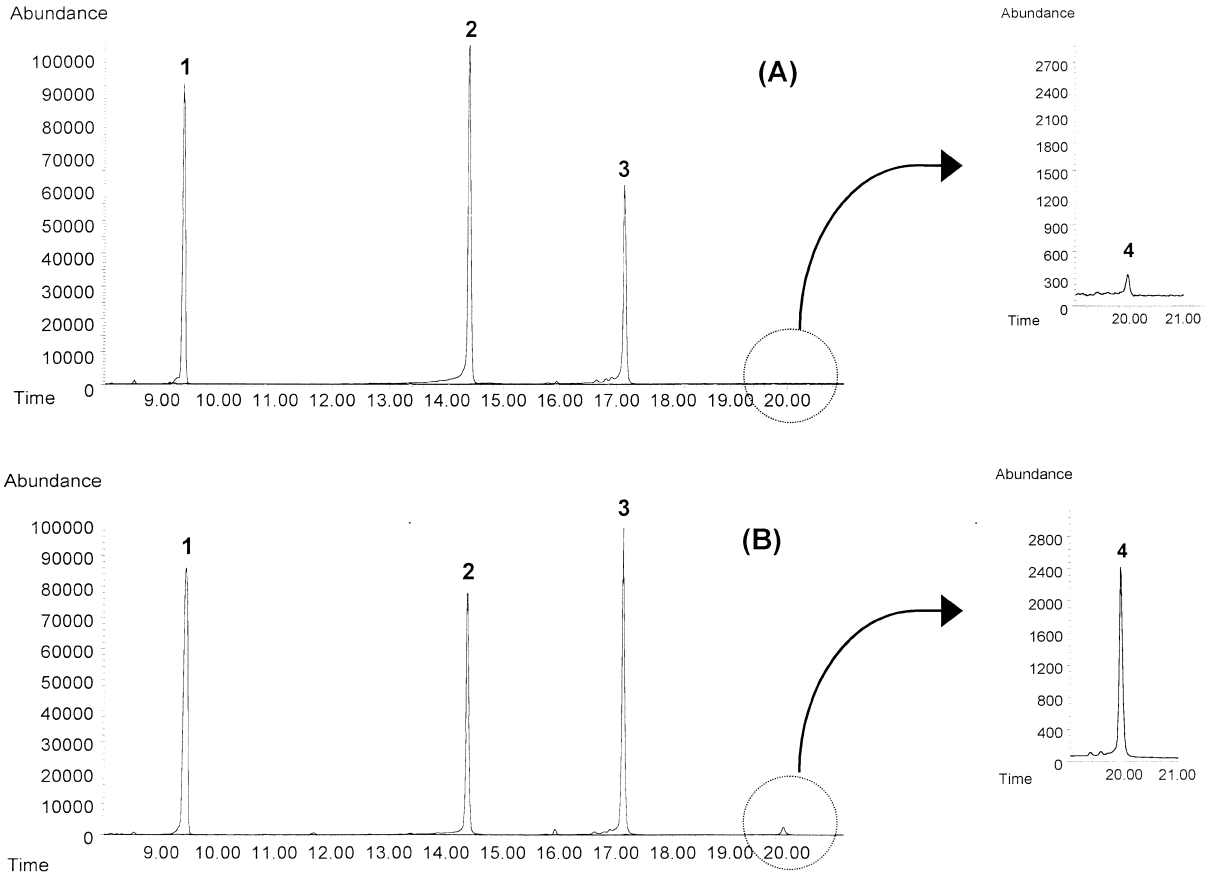

Fig. 3. GC–MS extracted ion chromatogram of plasma VLCFAs from (a) normal subjects and (b) patients with X-ALD. Peak 1, I.S. (m/z 496); peak 2, C22:0 (m/z 549); peak 3, C24:0 (m/z 577); peak 4, C26:0 (m/z 605).

analysis of SFAs in biological fluids. Also, this study suggests that it may be used effectively for the diagnosis and monitoring of diseases related to SFAs, such as X-ALD.

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