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Determination of very-long-chain fatty acids in serum by gas chromatography–nitrogen–phosphorus detection following cyanomethylation

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Abstract

A sensitive method for very-long-chain fatty acid analysis was developed by gas chromatography–nitrogen–phosphorus detection by using cyanomethyl derivatization. Bromoacetonitrile as alkylating reagent was used to improve nitrogen phosphorus detection detectability of compounds containing non-nitrogen. The carboxyl group of very-long-chain fatty acid was alkylated to cyanomethyl esters. Reaction conditions were 40 min at 60°C under potassium carbonate base. Heptacosanoic acid was used as an internal standard and hexane was used as a solvent of extraction. The extraction yield was 82.8% or more, relative standard deviation of the precision test was 8.3 % or more and the result of linearity test showed a good correlation coefficient of $r^2=0.999$ in the range of 0.1–50 μg/ml. The quantification limits were 10 ng/ml when 0.5 ml of serum was used. The present method proved simple, rapid, inexpensive and resistant to contaminants. When it was applied to serum samples taken from patients with X-linked adrenoleukodystrophy which is a hereditary X-linked disorder characterized by progressive demyelination and adrenal insufficiency during childhood, relative increase of the concentration of hexacosanoic acid and the concentration ratios of hexacosanoic, lignoceric to behenic acid was observed in comparison with control samples.

Keywords: Derivatization, GC; Fatty acids; Very-long-chain fatty acids

1. Introduction

The peroxisome, which is a cellular organelle with diverse metabolic function, usually contains one or more enzymes. A major reaction in peroxisome is break down of fatty acid molecules called β-oxidation.The deficiencies of the enzymes are related to the accumulation of very-long-chain fatty acids (VLCFA) (straight chain, with 22 or more carbon atoms) in tissues and body fluids [1–3]. The VLCFA accumulation can be used as a marker for most of the peroxisomal disorders, especially X-linked adrenoleukodystrophy (X-ALD). X-ALD is a hereditary neurological disorder characterized by adrenol insufficiency and progressive demyelination which commonly appears in childhood [4–7]. Particularly, absolute concentration of hexacosanoic acid and concentration ratios of hexacosanoic, lignoceric to

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behenic acid in patient tissues and body fluids are higher than those of a normal person [8–12]. Therefore, measurement of VLCFA for demonstration of an abnormality of peroxisome function is one of the most important diagnostic criteria [2,13].

Determination of fatty acids was reported by high-performance liquid chromatography (HPLC) [2,14,15] with various fluorescent derivatization. However, derivatization procedure is time-consuming and sensitivity is not enough for detecting fatty acid in normal plasma. As other methods for fatty acid analysis, gas chromatography–flame ionization detection (GC–FID) [1–3,9,16,17] after methylation and gas chromatography–mass spectrometry (GC–MS) [8,13] after methylation or trimethylsilylation were reported. In the quantification of VLCFA by GC–FID [3,18], the existence of an interference peak near the $C_{26:0}$ peak was reported. Difficulties in interpretation arise when there is an elevation in $C_{26:0}$ levels in body fluid (serum, plasma) without a change in the $C_{24:0}/C_{22:0}$ ratios [3]. In the case of GC–MS method [13], after extraction, the sample is derivatized as a methylester, but the derivatization time is for a minimum of 16h at 75 C. Therefore, in this study, we developed a gas chromatography–nitrogen–phosphorus detection (GC–NPD) method using bromacetonitrile as alkylating reagent [19]. In this method, the carboxyl group of VLCFA was derivatized as a cyanomethyl ester.

When this method is compared with others, derivatization time is short and the sample preparation process is fairly simple. A purification step is not necessary because NPD can exclude from detection non-nitrogen or phosphorus containing compounds. Finally, this method was applied to a patient with X-ALD for evaluating the usefulness for routine analysis in a clinical laboratory.

2. Experimental

2.1. Standards and reagents

VLCFA standards (behenic($C_{22:0}$), lignoceric ($C_{24:0}$), hexacosanoic($C_{26:0}$), heptacosanoic acids($C_{27:0}$) (internal standard)), and potassium carbonate were purchased from Sigma (St. Louis, MO, USA). Bromoacetonitrile ($CNCH_2Br$) was from Aldrich (Milwaukee, WI 53233, USA). Acetonitrile, *n*-hexane, chloroform, methanol, acetone, and all other chemicals as analytical-grade reagents were purchased from Merck (Darmstadt, Germany).

2.2. Preparation of stock and working solution

Stock solutions of behenic, lignoceric, hexacosanoic, and heptacosanoic acids (internal standard) were prepared by separately dissolving 10 mg of each compound in 10 ml of $CHCl_3$-CH_3OH (2:1, v/v). A working mixed solution was prepared by diluting to a final concentration of 0.1 mg/ml and 0.01 mg/ml. The working heptacosanoic acid (internal standard) was prepared by diluting to a final concentration of 0.1 mg/ml.

2.3. Sample preparation and derivatization

A 5, 25 and 50 μl (0.01 mg/ml), 25, 50 and 250 μl (0.1 mg/ml) of working mixed solution containing behenic, lignoceric and hexacosanoic acids, and a 40 μl of internal standard (heptacosanoic acid : 0.1 mg/ml) were added to 0.5 ml of serum. Then 2.3 ml of acetonitrile and 0. 3 ml of 5 *M* HCl solution were added, which was heated for 45 min at 100°C in order to release fatty acids from lipids. After cooling, 5 ml of hexane was added. Fatty acids were extracted into the hexane phase by shaking for 20 min and centrifugation for 10 min at 2500 rpm. The hexane phase was transferred into another glass vial, which was dried in a vacuum evaporator under a gentle stream of nitrogen. The dry residue was dissolved in 0.1 ml of acetone, then 10 mg of potassium carbonate and 20 μl of bromoacetonitrile were added. The solution was heated for 40 min at 60°C in a heating block. After cooling, 2 μl of sample were injected into the split mode (1:5) without concentration process.

2.4. Instrumentation

A Hewlett–Packard 6890 gas chromatograph (Avondale, USA) with a nitrogen– phosphorus detector, a Hewlett–Packard 3365 chemstation, and HP-5 (30 m × 0.32 mm I.D., 0.25 μm film thick-

ness) was used for the analysis of VLCFA. The injector temperature was 260°C and the oven temperature was programmed from 120°C (held for 2 min) at 10°C/min to 220°C, then at 4°C/min to 280°C (held for 13 min). The detector temperature was 300°C. Helium was used as the carrier gas (flow-rate 1.2 ml/min) and make-up gas (flow-rate 20 ml/min). The flow-rate of air and hydrogen were 60 ml/min and 3.5 ml/min, respectively.

All mass spectra for the identification of cyanomethyl derivatives were obtained with a HP 6890/5973 mass spectrometer. The ion source was operated in the electron impact (EI) ionization mode (70 eV, 230°C). Full scan mass spectra (m/z 40–550) were recorded for analyte identification. A capillary column was used for HP-5 MS (30 m × 0.25 mm I.D., 0.25 μm film thickness). The flow-rate of carrier gas (He) was 0.8 ml/min. The oven operating temperature and injector temperature were the same as described above. The temperature of transfer line was 300°C.

2.5. Matrix calibration

For the matrix calibration curve of VLCFA, the working standard solution of 0.01 mg/ml and 0.1 mg/ml were added in 0.5 ml of human serum against 8 μg/ml of internal standard. It was extracted and derivatized according to the method described above. Relative peak area ratios of VLCFA standard to internal standard was used for the plotting of matrix calibration curve in the range of 0.1–50 μg/ml (behenic and lignoceric acid) and 0.1–5 μg/ml (hexacosanoic acid).

2.6. Precision, recovery and quantification limit

VLCFA of 0.1–50 μg/ml and 8 μg/ml (I.S) are contained in serum by spiking standard solution. Then a precision test was performed by the present method. The recovery test was performed as follows; in the case of extraction from serum containing VLCFA of 0.1–50 μg/ml, the internal standard was added just before the derivatization reaction. The limit of quantification was calculated at a signal-to-noise ratio (S/N) of 3:1.

3. Results and discussion

3.1. Derivatization of VLCFAs by using bromoacetonitrile

The reaction scheme is shown in Fig. 1. It was reported previously by Shin et al. [19]. Bromoacetonitrile contains an alkyl moiety and a leaving group which is the general structure of alkyl reagents. The alkyl moiety characterizes the form of the derivatives, and the leaving group determines the reactivity of reagents. In this reaction, the cyano group was used as a chemical tag for the detection mechanism of NPD and the bromide was used as a leaving group. As a result of reaction, the carboxyl group of VLCFA was alkylated to a cyanomethyl ester.

3.2. Optimization of reaction time

To find the optimal reaction time of cyanomethyl derivatives, the reaction was performed for various times (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60 min) at 60°C. In this study, the reaction for 40 min at 60°C showed highest reaction efficiency and least interference peak.

3.3. Identification of VLCFA derivatives by GC–MS

The structures of VLCFA derivatives were confirmed by GC–MS. The total ion chromatogram (Fig. 2) and mass spectra are shown in Figs. 3–6. The molecular ions $[M^+]$ of behenic, lignoceric, hexacosanoic and heptacosanoic acids (I.S.) were m/z 379, 407, 435, and 449, respectively. The fragmentation patterns are shown to be characteristic ions of $[M-15]^+$, $[M-31]^+$, $[M-43]^+$ and $[M-99]^+$. Loss of CH_3 from the alkyl chain in molecular ions $[M-15]^+$ gives the ion m/z 364, 382, 420 and 434. The ions $[M-31]^+$ at m/z 348 [$CH{\equiv}CH$-$(CH_2)_{17}$ $COOCH_2CN$], 376 [$CH \equiv CH$-$(CH_2)_{19}$ $COOCH_2CN$], 404 [$CH \equiv CH$-$(CH_2)_{21}$

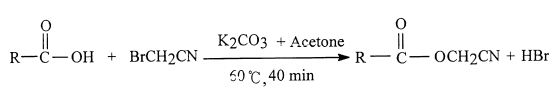

Fig. 1. Reaction scheme with bromoacetonitrile.

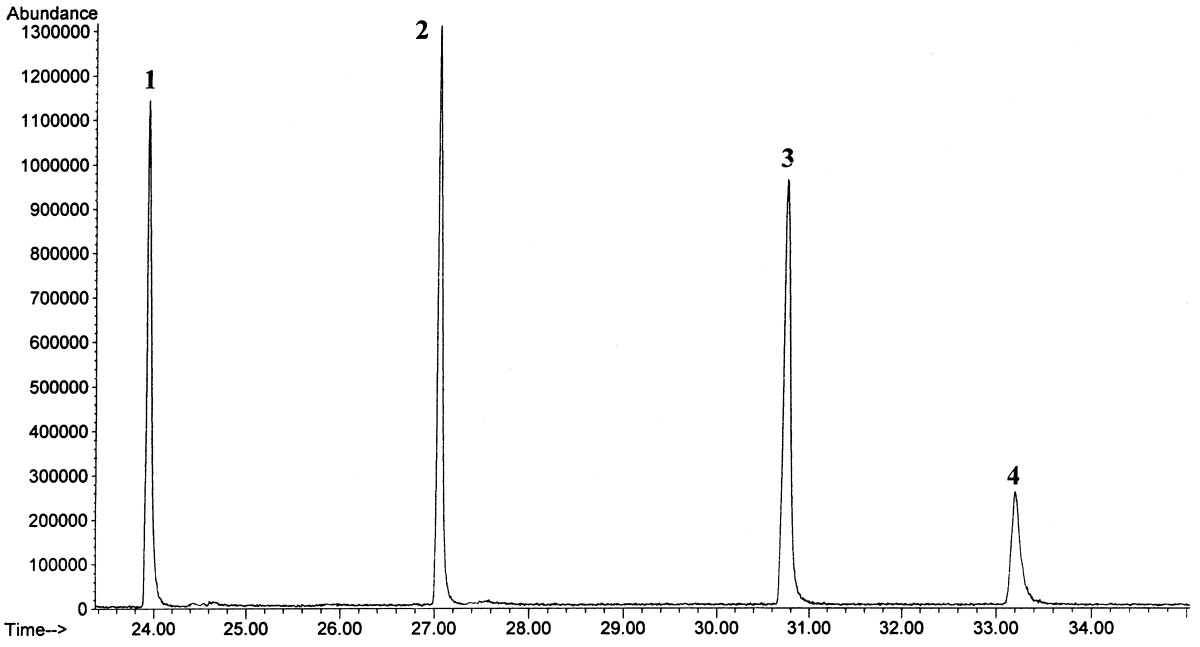

Fig. 2. Total ion chromatogram of VLCFA (1=behenic; 2= lignoceric; 3=hexacosanoic ; 4=heptacosanoic acid (I.S.).

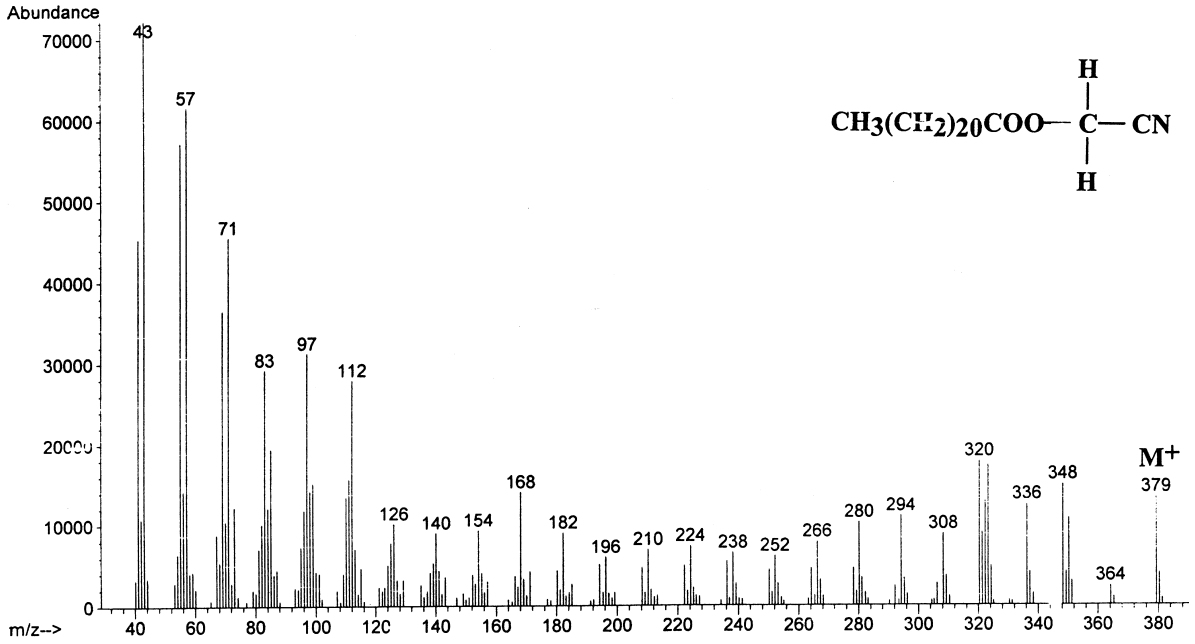

Fig. 3. Mass spectrum of behenic acid derivative.

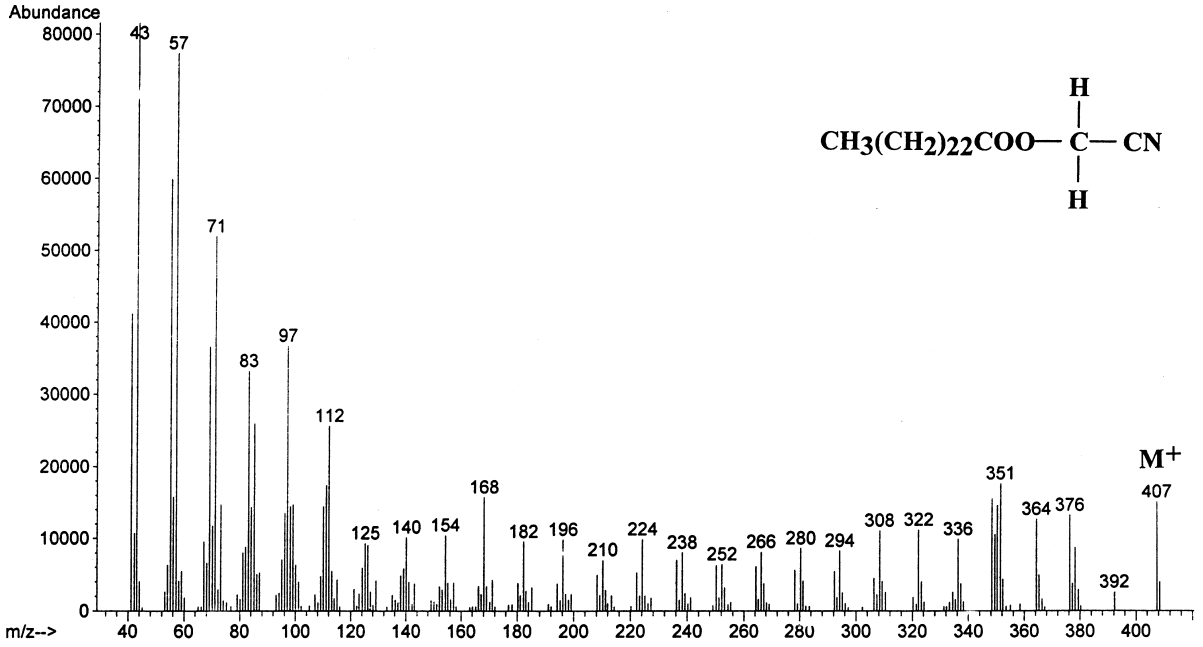

Fig. 4. Mass spectrum of lignoceric acid derivative.

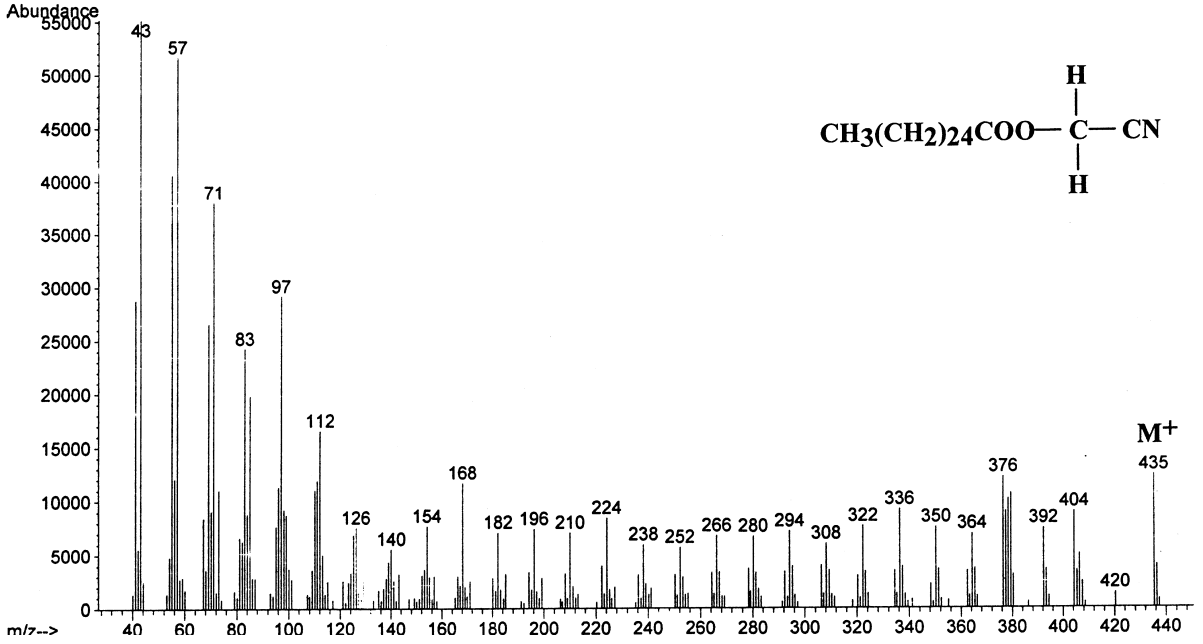

Fig. 5. Mass spectrum of hexacosanoic acid derivative.

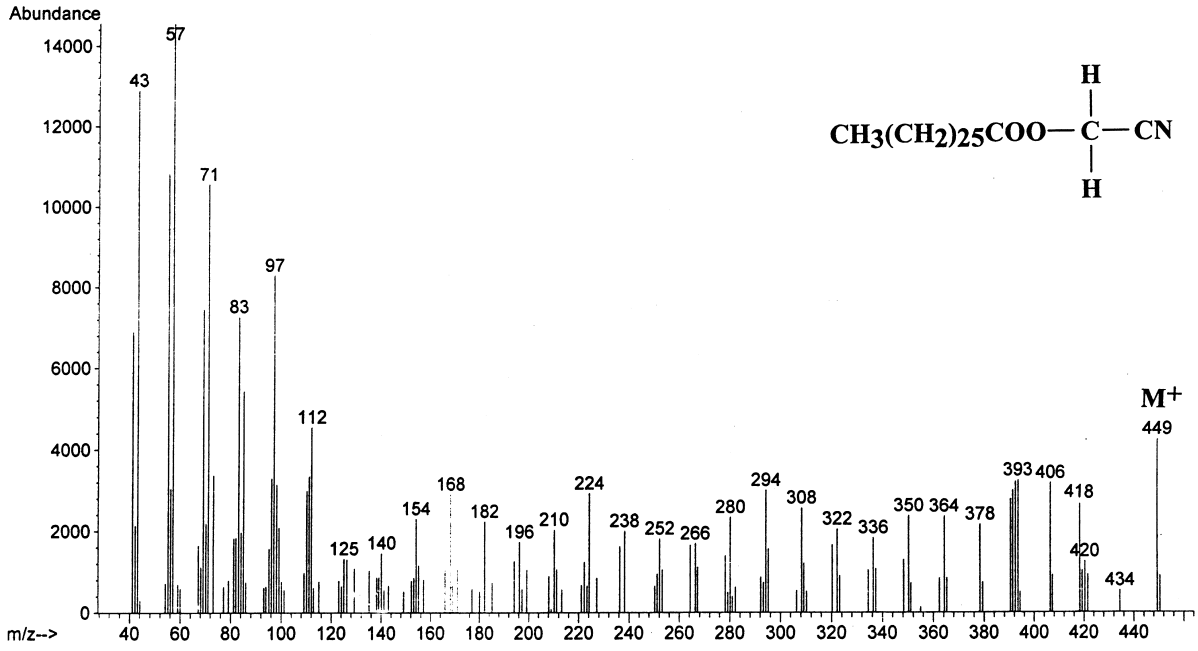

Fig. 6. Mass spectrum of heptacosanoic acid (I.S.) derivative.

$COOCH_2CN$], and 418 [CH ≡ CH-$(CH_2)_{22}$ $COOCH_2CN$] were produced by the loss of CH_3–CH_2 and two hydrogens from the alkyl chain. The ions $[M-43]^+$ at *m/z* 336, 364, 392, and 406 were produced by the loss of CH_3–CH_2–CH_2 from the alkylchain. The ions $[M-99]^+$ at *m/z* 280, 308, 336 and 350 were produced by a loss of CH_3–COO–CH_2CN. Intensive peaks, which stem from stepwise elimination of alkyl chains were observed at *m/z* 43, 57, 71, 83, 97 and 112 for the cyanomethyl esters of behenic, lignoceric, hexacosanoic and heptacosanoic acids (I.S.). The *m/z* 43 ($C_{22:0}$, $C_{24:0}$ and $C_{26:0}$) and 57 ($C_{27:0}$(I.S.)) were the base peaks of VLCFA cyano methyl esters. Mass fragmentation of VLCFA cyanomethyl esters showed patterns similar to that of VLCFA methyl esters.

3.4. Matrix calibration curve

The matrix calibration curves for behenic, lignoceric and hexacosanoic acids showed good linearity of $r^2=0.999$. The result of analysis were calculated by relative peak area ratios of each of the different concentrations of VLCFA standard [0.1, 0.5, 1.0, 5.0, 10.0 and 50.0 μg/ml ($C_{22:0}$ and $C_{24:0}$), 0.1, 0.5, 1.0 and 5.0 μg/ml ($C_{26:0}$)] to peak area of I.S.(8 μg/ml) in blank serum.

3.5. Precision, recovery and quantification limit

The result of the precision test was expressed as mean±S.D. (% R.S.D.) of concentration found. Concentration added zero (0.0) means blank serum without spiking standard solution. The relative standard deviation was less than 7.1% for behenic acid, 6.2% for lignoceric acid and 8.3% for hexacosanoic acid (Table 1). The precision, as shown by relative standard deviation, was less than 6% except for 0.1 ppm of behenic, lignoceric acid, and hexacosanoic acids. The result of the recovery test was expressed as mean values of concentration found. Percent recovery of behenic and lignoceric acids ranged from 85.1% to 91.5% in the range of 1–50 μg /ml, that of hexacosanoic acid was around 82.8% at 0.1 μg /ml and 84.4% in the range of 0.5–50 μg /ml, respectively (Table 2). The recovery was always higher than 82% using this method. The quantification limits ($S/N=3$) were 10 ng/ml when 0.5 ml of serum was used. It is possible to detect $C_{26:0}$

Table 1
Precision of VLCFA analysis in serum (n=3)

Concentration added (μg/ml)	Concentration found (μg/ml) mean±S.D (% R.S.D.)		
	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$
0.0[a]	12.87±0.83 (6.45)	11.66±0.64 (5.49)	0.15±0.01 (6.67)
0.1	12.96±0.92 (7.10)	11.75±0.73 (6.21)	0.24±0.02 (8.33)
0.5	13.32±0.76 (5.71)	12.07±0.51 (4.23)	0.53±0.02 (3.77)
1.0	13.85±0.61 (4.40)	12.52±0.53 (4.23)	0.98±0.05 (5.10)
5.0	17.83±0.62 (3.48)	16.61±0.44 (2.65)	5.06±0.07 (1.38)
10.0	22.70±1.14 (5.02)	21.26±1.07 (5.03)	9.30±0.20 (2.15)
50.0	61.06±1.76 (2.88)	58.86±1.69 (2.87)	42.54±1.38 (3.24)

[a] 0.0=Blank plasma was not spiked by VLCFA standard solution.

Table 2
% Recovery of VLCFA from serum (n=3)

Concentration added (μg/ml)	Concentration found[a] (μg/ml) (mean±S.D % recovery)[b]		
	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$
0.1	0.09 (85.4±4.3)	0.09 (85.2±3.5)	0.08 (82.8±2.8)
0.5	0.44 (87.2±3.9)	0.43 (85.5±3.4)	0.42 (84.2±3.6)
1.0	0.86 (86.1±2.1)	0.85 (85.1±2.9)	0.86 (85.7±3.4)
5.0	4.49 (89.7±2.8)	4.25 (85.0±2.5)	4.27 (85.4±2.1)
10.0	8.86 (88.6±3.6)	8.68 (86.8±1.9)	8.48 (84.8±2.6)
50.0	45.75 (91.5±2.3)	44.80 (89.6±3.1)	42.55 (85.1±1.8)

[a] Concentration found=(% mean recovery/100)×concentration added.
[b] % Recovery={extraction peak area ratio (peak area of $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$/peak area of internal standard)/neat peak area ratio (peak area of $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$/peak area of internal standard)}×100.

Table 3
Result of VLCFA analysis in patient with X-ALD and a normal person

Diagnostic category	Concentration (μg/ml)			Concentration ratio	
	$C_{22:0}$	$C_{24:0}$	$C_{26:0}$	$C_{24:0}/C_{22:0}$	$C_{26:0}/C_{22:0}$
X-ALD[a]					
1	7.60	16.57	1.14	2.18	0.15
2	2.94	4.94	0.50	1.68	0.17
3	12.00	20.76	1.32	1.73	0.11
Mean±SD				1.86±0.28	0.14±0.03
Normal controls[b]					
1	8.00	7.36	0.16	0.92	0.02
2	6.50	5.27	0.13	0.81	0.02
3	7.00	5.74	0.14	0.82	0.02
Mean±SD				0.85±0.06	0.02±0.00

[a] Heterozygotes for X-linked ALD.
[b] 1=Adult male; 2=Adult female; 3=Adult female.

concentration in serum of a normal person in existence as trace level and this method was optimum to carry out VLCFA analysis of normal persons and patients with X-ALD.

3.6. Application to the determination of VLCFA in serum of normal person and X-ALD patient

No interference peak was in the quantification of endogenous VLCFA. The result of quantification showed an apparent discrimination between normal person and patient with X-ALD. Fig. 7. showed chromatograms o f a blank serum, serum spiked with VLCFA standards (1.0 μg/ml of behenic, lignoceric and hexacosanoic acids against 8 μg/ml of heptacosanoic acid (internal standard)), and a serum of patient with X-ALD, respectively. Relative increasing the concentration of hexacosanoic acid and the ratios of hexacosanoic, lignoceric to behenic acid from serum of X-ALD patients was observed in comparison with control samples (Table 3). The absolute concentration of $C_{26:0}$ was 0.99±0.43 and it was almost sevenfold higher than that of normal person 0.14±0.03 ug/ml. The ratios of hexacosanoic and lignoceric to behenic acid in the case of X-ALD patients were 0.14±0.03 and 1.86±0.28, respectively. The ratio of lignoceric to behenic acid was double that of a normal person 0.85±0.06. Whereas, the ratio of hexacosanoic to behenic acid was seven times higher than that of a normal person 0.02±0.00.

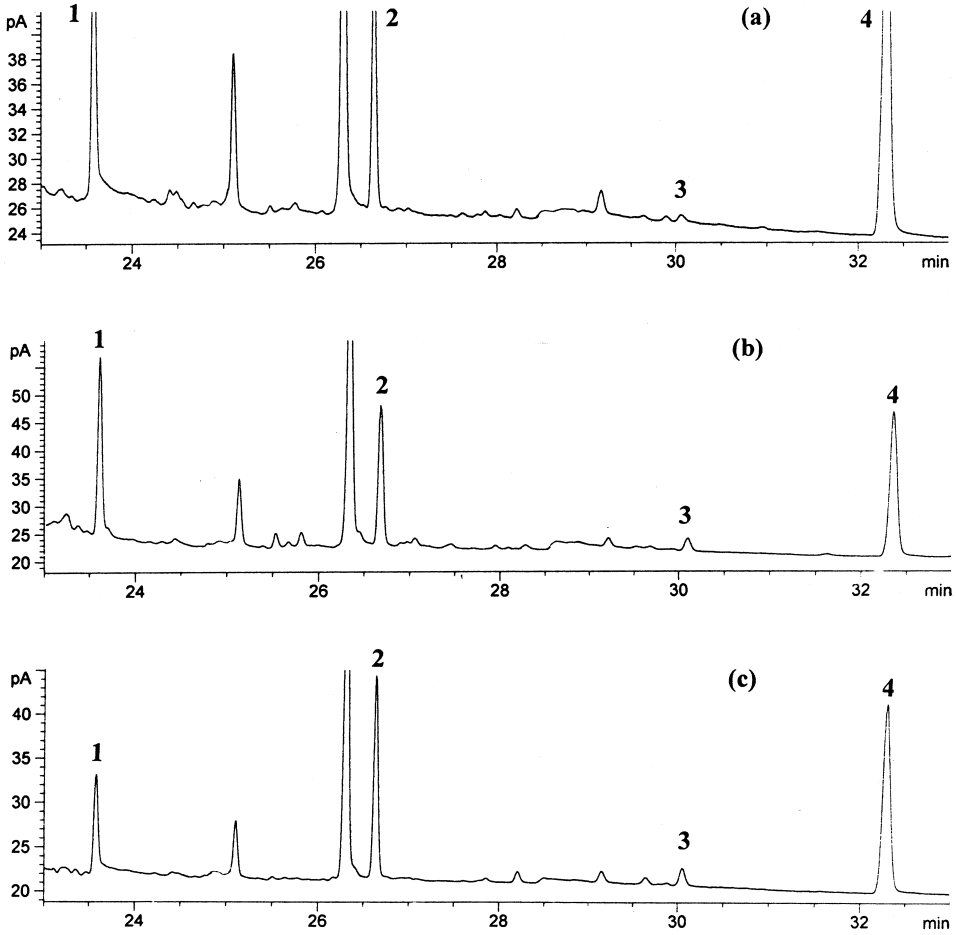

Fig. 7. Gas chromatograms of VLCFA in a blank serum (a), serum spiked with 1.0 μg/ml of VLCFA (b), and serum of X-ALD patient (c). (1=Behenic; 2= lignoceric; 3=hexacosanoic acid; 4=heptacosanoic (I.S.)).

4. Conclusion

A simple, rapid, resistant to contaminants and inexpensive method for the determination of VLCFA in serum was developed by GC–NPD. The carboxyl group of VLCFA was alkylated to cyanomethyl esters. In the present method, VLCFA analysis was satisfactorily performed without interfering with the substance and the NPD system proved appropriate for trace level determination of these compounds in serum. The absolute concentration of hexacosanoic acid and the ratio of hexacosanoic acid to behenic acid were seven times higher than those of a normal person, whereas, the ratio of lignoceric acid to behenic acid was twofold higher than that of normal person. Therefore, the described method can be used for routine analysis of VLCFA in the diagnosis of peroxisomal disorders in a clinical laboratory.

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