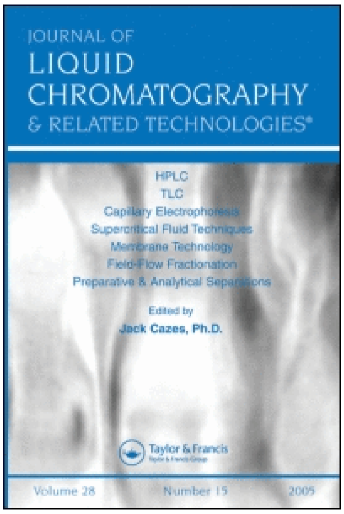

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# TRACE ANALYSIS OF VERY LONG CHAIN FREE FATTY ACIDS IN PLASMA BY FLUOROGENIC DERIVATIZATION AND LIQUID CHROMATOGRAPHY[†]

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## ABSTRACT

A simple and sensitive liquid chromatographic method is described for the simultaneous determination of docosanoic, tetracosanoic and hexacosanoic acids as fluorogenic derivatives. These acids, spiked in plasma, were extracted with n-heptane and the resulting extract was derivatized with 4-bromomethyl-7-methoxycoumarin (BrMmC) in dichloromethane using potassium carbonate and 18-crown-6 as catalysts. The derivatives obtained were chromatographed on a reversed-phase $C_8$ column with acetonitrile : water (91 : 9, v/v) as a mobile phase and 7-methoxy-4-(tricosanoyloxymethyl)-coumarin as an internal standard. The linear ranges for the determination of docosanoic, tetracosanoic, and hexacosanoic acids were over 31-500, 36-580 and 36-580 pmol, respectively. The limit of detection for each acid was about 0.3 pmol per 10 µL injection (S/N=3).

## INTRODUCTION

The abnormal accumulation of very long chain fatty acids (VLCFA) in tissues permits an approach to the identification of inherited neurodegenerative disorders such as the classical adrenoleukodystrophy and its chronic variant adrenoleukodystrophy.[1] Due to a defective peroxisomal beta-oxidation of VLCFA, the levels of fatty acids with chain length more than 22 carbons rise in plasma and other tissues.[2,3] The pathogenesis of the brain lesions in these disorders is unknown, but it has been suggested[4] that the excess of VLCFA in the myelin lipids could impair membrane stability and thus induce demyelination. The diagnosis of these diseases has generally involved measuring ratios of VLCFA in plasma as an indicator for the disorders.[5]

Several analytical methods have been reported for the assay of VLCFA in biological fluids. The GC-MS method[5,6] requires highly sophisticated equipment and is not amenable to routine clinical assay. Analytical derivatization coupled with liquid chromatography (LC)[7-13] is widely used for the determination of various fatty acids.

In this paper, a simple derivatization-LC was developed for the trace analysis of docosanoic, tetracosanoic, and hexacosanoic acids in plasma. The sample size used is small (0.1 mL of plasma). The VLCFA were simply extracted with n-heptane in acidic media. 7-Methoxy-4-(tricosanoyloxymethyl)coumarin, structurally similar to VLCFA derivative, was synthesized from tricosanoic and BrMmC and used as the internal standard.

## METHODS

### Chemicals and Solutions

Docosanoic acid (behenic acid, C22), tricosanoic acid (C23), tetracosanoic acid (lignoceric acid, C24), hexacosanoic acid (cerotic acid, C26), 18-crown-6 ether (18-crown-6), and anthracene were from TCI (Tokyo, Japan). Potassium carbonate (40-60 mesh) and phosphoric acid (85%) were from E. Merck (Darmstadt, Germany). Acetonitrile, 2-propanol, chloroform, dichloromethane, and n-heptane were from Fisher Scientific (Fair Lawn, NJ, USA). BrMmC was from Aldrich (Milwaukee, WI, USA). Distilled water purified with the Ultrapure R/O water system (Millipore, MA, USA) was used for all aqueous solutions.

The internal standard (I.S.), 7-methoxy-4-(tricosanoylxymethyl)coumarin was synthesized in our laboratory and its structure was confirmed by $H^1$-NMR, mass spectrometry, and elemental analysis.

Standard solutions of BrMmC and 18-crown-6 at various concentrations were prepared by dissolving the appropriate amounts of the respective compounds in dichloromethane and diluted if necessary. Solutions of I.S. (1.3 μM) and phosphoric acid (2 M) were prepared by dissolving the respective compounds in $CH_3CN$ and $H_2O$, respectively. VLCFA (C22, C24 and C26) spiked plasma at various concentrations was prepared by dissolving VLCFA in a mixed solvent of 2-propanol-phosphoric acid (2 M) (5:1, v/v) and diluted with plasma (1:1, v/v).

## Liquid Chromatography

A Waters LC system with a U6K injector, a Model 501 pump and a Model 470 fluorescence detector was used. A Nova-Pak $C_8$ column (150 x 3.9 mm I.D.; 4 μm) (Waters) and a mixed solvent of acetonitrile-water (91 : 9, v/v) at a flow rate of 1.3 mL/min were used. The column eluate was monitored at excitation wavelength 321 nm and emission wavelength 390 nm.

## Mass Spectrometry

A JEOL JMS-HX 100 mass spectrometer was used with fast atom bombardment (FAB) with an acceleration energy of 10 kV for the analysis of VLCFA derivatives.

## Extraction of VLCFA from Plasma

A 0.2-mL aliquot of VLCFA spiked plasma was transferred into a 10-mL test tube, then 1.0 mL of n-heptane was added. After being vortexed for 0.5 min, 0.6 mL of water and 2.0 mL of n-heptane were added successively and vortexed again for 2 min. The mixture was centrifuged at 1800 g for 5 min. A 2.0-mL amount of the supernatant was transferred into a 10-mL glass-stoppered test tube and evaporated to dryness in a cold-trap centrifugal evaporator (Tokyo Rikakikai, Eyela CVE-200D). The resulting residue was subjected to derivatization as described below.

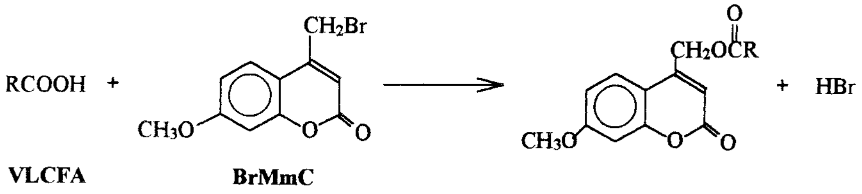

**Figure 1.** Reaction scheme for preparation of VLCFA derivatives and internal standard. R=C21, C23 and C25 for very long chain fatty acids (VLCFA) and R=C22 for internal standard.

### Derivatization Procedure

The residue was dissolved in 0.2 mL of dichloromethane and added successively with 0.1 mL of BrMmC solution (15 mM), 0.1 mL of 18-crown-6 solution (3 mM) and about 20 mg of potassium carbonate. The reaction mixture was shaken for 1.0 hr at 30°C in a thermostated water-bath. At the end of the reaction, 0.4 mL of I.S. (1.3 μM) was added to the derivatized solutions. A 10-μL aliquot of the solution was injected into the LC system.

## RESULTS AND DISCUSSION

For optimizing the conditions for derivatizing the VLCFA extracted, the spiked amounts of C22, C24, and C26 for study were 0.50, 0.58, and 0.58 nmol, respectively. Several parameters affecting the derivatization were investigated, including the amount of derivatizing agent, catalysts (18-crown-6 and potassium carbonate), and reaction time.

The effects of the tested parameters on the derivatization of VLCFA were evaluated by the peak area ratios of the resulting derivatives to the I.S. A reaction scheme for the preparation of VLCFA derivatives and the internal standard is shown in Figure 1.

### Stability of the VLCFA Derivatives

The stability of 7-methoxy-4-(tricosanoyloxymethyl)coumarin (I.S.) was studied in a medium of potassium carbonate (20 mg), 18-crown-6 (3 mM x 0.1 mL), and anthracene (4 μM x 0.2 mL) over a period of 6.0 h. The I.S. (an ester) was found stable at 30°C in 6.0 h in the presence of potassium carbonate in dichloromethane using anthracene (without functional group) as the second I.S. for evaluating the peak area ratio of the ester I.S. to anthracene. Consequently, 7-methoxy-4-(tricosanoyloxymethyl)coumarin was used as the I.S. for evaluating the stability of the VLCFA derivatives (C22 0.50 nmol, C24 0.58 nmol and C26 0.58 nmol) according to the present derivatization procedure.

No significant change of the peak area ratio of the derivatives to I.S. was found over a tested peroid of 6.0 h, indicating that the VLCFA derivatives are stable enough for the time required for LC analysis.

### Mass Spectral Analysis of the Derivatives

The VLCFA derivatives were synthesized by scaling up the amount of VLCFA with a similar method as indicated in the Derivatization procedure. The purified derivatives of VLCFA were identified by FAB-MS with nitrobenzyl alcohol as a matrix.

The quasi-molecular ions of the derivatives of C22, C24, and C26 were found at m/z = 529, 557, and 585, respectively, indicating the formation of esters from VLCFA and BrMmC. The retention times of the purified C22, C24, and C26 derivatives are identical to those from derivatization of standard solution of VLCFA under present LC condition.

### Effect of Amount of Derivatizing Agent

The effect of 0.1 mL of BrMmC at various concentrations (0-60 mM) on the derivatization of VLCFA extracted from plasma was studied. The plateau formation of the derivatives was found using BrMmC at concentration $\geq$ 15 mM as shown in Figure 2.

### Effect of Amount of Potassium Carbonate

The effect of potassium carbonate (30-50 mesh) at various amounts (0 - 100 mg) on the derivatization was studied. The results indicate that the use of potassium carbonate $\geq$ 10 mg is optimal for derivatization. In the absence of this base catalyst, no derivative was detected.

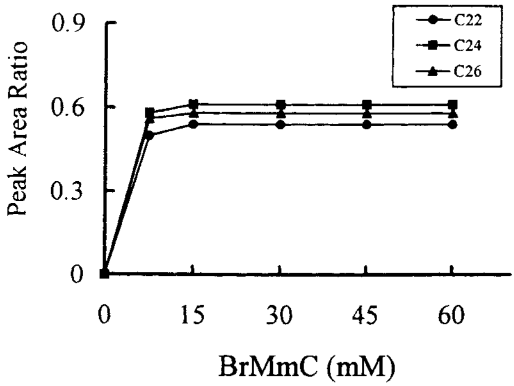

**Figure 2.** Effect of concentration of derivatizing agent on the derivatization of VLCFA extracted from plasma.

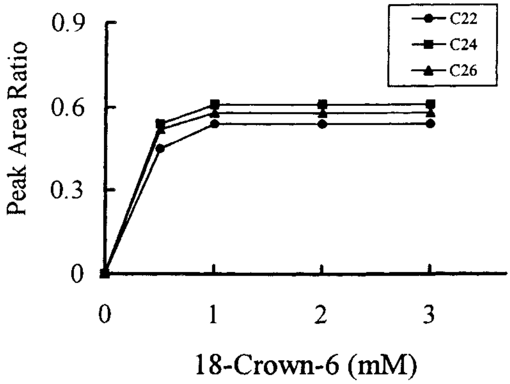

**Figure 3.** Effect of concentration of 18-crown-6 on the derivatization of VLCFA extracted from plasma.

## Effect of Amount of 18-Crown-6

The effect of 18-crown-6 solution (0.1 mL) at various concentrations (0-3 mM) on the derivatization was studied. The results indicate that in the absence of the catalyst in the reaction system, no derivative was detected. A concentration of 18-crown-6 solution $\geq$ 1 mM is needed for the optimal derivatization of the analytes as shown in Figure 3.

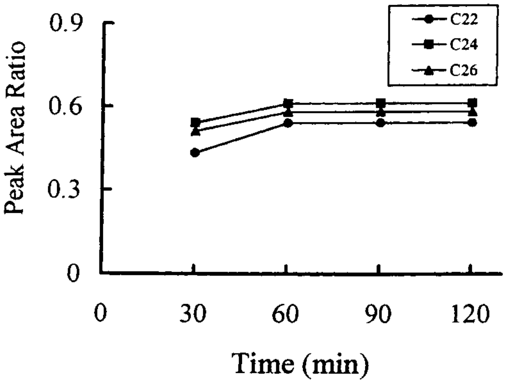

Figure 4. Effect of reaction time on the derivatization of VLCFA extracted from plasma.

### Effect of Reaction Time

The reaction time at 30°C required to reach an equilibrium for the derivatives were studied over a period of 2.0 h. The results indicate that 1.0 h is required for the derivatization of C22, C24, and C26 as shown in Figure 4.

### Analytical Calibration and Precision

Based on the optimum derivatization conditions, the Derivatization procedure for VLCFA spiked in plasma was established. To evaluate the quantitative applicability of the method, five different amounts of VLCFA in the range 0 ~ 0.500 nmol (0 ~ 0.170 μg) (C22), 0 ~ 0.580 nmol (0 ~ 0.213 μg) (C24), 0 ~ 0.580 nmol (0 ~ 0.230 μg) (C26) spiked in plasma were analyzed and the linearity between the peak-area ratios ($y$) and sample amount ($x$, μg) was examined. The linear regression equations for C22, C24, and C26 were $y = (0.085 \pm 0.003) + (2.678 \pm 0.022)x$, $r = 0.999$, $y = (0.056 \pm 0.002) + (2.699 \pm 0.080)x$, $r = 0.996$ and $y = (0.012 \pm 0.001) + (2.407 \pm 0.042)x$, $r = 0.996$, respectively. The precision based on known amount of VLCFA can not be evaluated, because the true values of endogenous C22, C24, and C26 in plasma are unavailable. Therefore, the precisions (relative standard deviation, RSD) based on the slope and intercept of the related regression equation for intra-day and inter-day analysis were studied and the results are summarized in Table 1.

### Table 1

### Precisions for the Slope and Intercept of the Regression Equations for VLCFA Analysis

| VLCFA | Slope±SD (% RSD) | Intercept±SD (% RSD) |
|---|---|---|
| Intra-day (n=7) | | |
| C22 | 2.678±0.022<br>(0.82) | 0.085±0.003<br>(3.53) |
| C24 | 2.699±0.080<br>(2.96) | 0.056±0.002<br>(3.57) |
| C26 | 2.407±0.042<br>(1.74) | 0.012±0.001<br>(8.33) |
| Inter-day (n=9) | | |
| C22 | 2.675±0.025<br>(0.93) | 0.084±0.004<br>(4.76) |
| C24 | 2.716±0.106<br>(3.90) | 0.053±0.003<br>(5.66) |
| C26 | 2.411±0.049<br>(2.03) | 0.013±0.001<br>(7.69) |

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* VLCFA, SD and RSD stand for very long chain fatty acids, standard deviation and relative standard deviation respectively.

**C22, C24, and C26 stand for docosanoic acid, tetracosanoic acid and hexacosanoic acid, respectively.

The RSDs for the slope and intercept are all below 4.0% and 8.4%, respectively. Typical chromatograms for the analysis of VLCFA are shown in Figure 5. This indicates that the plasma blank did not significantly interfere in the I.S.

The I.S. was synthesized from BrMmC and C23, an odd carbon number of VLCFA that is basically not an endogeneous substance. Small peaks 1, 2, and 3 with similar retentions to C22, C24, and C26, respectively in Figure 5 (b), assumed to be from endogeneous VLCFA.

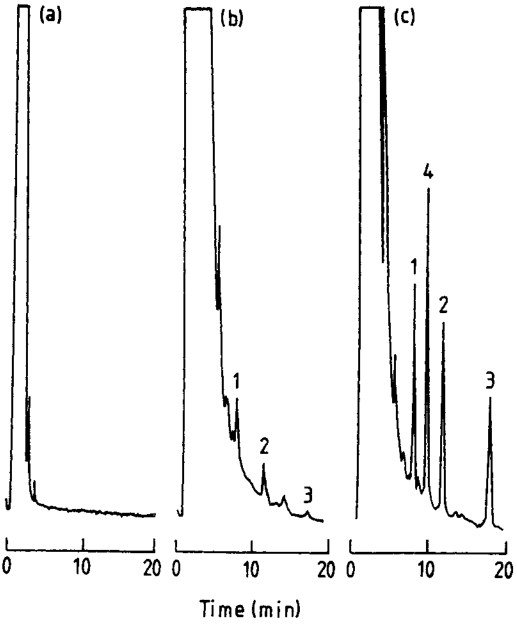

**Figure 5**. HPLC chromatograms for (a)reagent blank, (b)plasma blank and (c)plasma spiked with VLCFA (0.50 nmol C22, 0.58 nmol C24 and 0.58 nmol C26). Peaks: 1, C22 derivative; 2, C24 derivative; 3, C26 derivative; 4, internal standard. LC conditions: column, Nova-Pak C8 (150 x 3.9 mm, I.D.; 4 μm particle size); mobile phases, acetonitrile-water (91 : 9, v/v); flow rate, 1.3 mL/min; fluorescence detection : λex, 321 nm and λem, 390 nm.

The proposed method was tentatively applied to the determination of C22, C24, and C26 in normal plasma (n=15) by standard addition method[14] using 0.1 mL aliquot of plasma for analysis. The results indicate that average levels (mg/mL) of C22, C24, and C26 in plasma are 0.304 ± 0.021, 0.191 ± 0.027 and 0.055 ± 0.018, respectively. Application of the method to the diagnosis of patients with abnormal levels of VLCFA is being evaluated.

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