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A simple and sensitive liquid chromatographic method for the analysis of free docosanoic, tetracosanoic and hexacosanoic acids in human plasma as fluorescent derivatives

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ABSTRACT

Docosanoic (C22), tetracosanoic (C24) and hexacosanoic (C26) acids are saturated very-long-chain fatty acids (VLCFA) present at trace levels in biosamples. VLCFA can be used as potential biomarkers for the diagnosis of hereditary diseases such as X-linked adrenoleukodystrophy. Because the analytes to be detected are at trace levels, a sensitive fluorimetric liquid chromatographic method was developed to analyze VLCFA in plasma. The method is simple based on extracting VLCFA from plasma with toluene, and the obtained toluene extract was subject to the derivatization of VLCFA with a fluorescent reagent 2-(2-naphthoxy)ethyl-2-(piperidino)ethanesulfonate (NOEPES) without solvent evaporation/replacement. The resulting fluorescent derivatives were monitored by fluorimetric detection (excitation at 225 nm and emission at 360 nm), giving a high sensitivity with the limit of detection about 5.0 nM (S/N = 3, 10 μL injected) of the analytes. Application of the method to the analysis of VLCFA in the plasma of patients with adrenoleukodystrophy proved practical and effective.

1. Introduction

X-linked adrenoleukodystrophy (ALD) is a peroxisomal disorder caused by mutation in *ABCD 1* gene. The disease is biochemically characterized by the accumulation of VLCFA such as C24 and C26 in tissue and body fluids. Diagnosis of ALD is mainly based on clinical features combined with the concentrations of C22, C24 and C26 in biosamples [1–4].

VLCFA are saturated carboxylic acids without practical chromophore for being directly measured to give sufficient sensitivity for clinical monitoring. Therefore, detector-oriented derivatization coupled with various chromatography or mass spectrometry is frequently applied to address the problems. Among the methods that are available for the analysis of VLCFA in biosamples including capillary GC or LC with varied detector such as detector of flame-ionization (FID), ultra-violet (UV), fluorimetry (FD) or mass spectrometry (MS). The GC–FID and LC–UV methods analyzed VLCFA as methyl [5,6] and bromophenacyl [7] esters, respectively. They need lengthy analytical operation including solvent

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extraction/evaporation and solvent replacement for reconstituting a final sample suitable for analysis. The sensitive LC–FD methods analyzed VLCFA as coumarin [8] and anthryl derivatives [9], each leading to a chromatogram with serious tailing from excess reagent that may unfavorably affect the lower limit of quantitation. Moreover, the reagent (anthryl diazomethane) used for the derivatization of VLCFA is quite labile and needs careful operation. The GC–MS methods analyzed VLCFA as *tert*-butyldimethylsilyl [10], methyl [11,12] and pentafluorobenzyl derivatives [13] that also require lengthy solvent treatment. In addition, isotope mediated method is often used in quantitative mass spectrometry for better precision [10,11,13], including the LC–MS using isotope internal standard in the analysis of VLCFA [14].

The purpose of this study is to develop a simple and sensitive method for the analysis of VLCFA in human plasma, using 2-(2-naphthoxy)ethyl-2-(piperidino)ethanesulfonate (NOEPES) as a fluorescent reagent [15]. We found that the resulting VLCFA derivatives can be analyzed by simple isocratic LC with a sensitive fluorescence detector.

2. Experimental

2.1. Materials and reagents

C22 (99%), C24 (99%), C26 (>95%) and C23 (99%) (tricosanoic acid, as the internal standard, IS) were from Sigma (St. Louis, MO, USA). 18-Crown-6 ether (18-crown-6) was from TCI (Tokyo, Japan). 2-(2-Naphthoxy)ethyl-2-(piperidino)ethanesulfonate was prepared as reported [15]. Potassium carbonate, phosphoric acid and sulfuric acid were from E. Merk (Darmstadt, Germany), methanol, isopropanol, tetrahydrofuran and toluene were from Tedia (Fairfield, OH, USA). These chemicals were of analytical reagent grade. Distilled water purified with the Ultrapure R/O water system (Millipore, Bedford, MA, USA) was used for preparing water related solutions. Stock solutions of C22, C24, C26 and C23 (0.50 mM for each) were separately prepared in a mixed solvent of isopropanol–phosphoric acid (2 M) (5:1, v/v) and further diluted with the same solvent for subsequent use as working solution. Solutions of NOEPES and 18-crown-6 were prepared in toluene. Solutions of sulfuric and phosphoric acids were prepared in water.

2.2. HPLC conditions

A Hitachi (Tokyo, Japan) HPLC system with a Model L-2130 pump, a Model L-2200 autosampler and a Model L-2480 fluorescence detector was used. A Phenomenex Luna phenyl-hexyl analytical column (250 mm length × 4.6 mm I.D., 5 μm) with a Phenomenex (Torrance, CA, USA) phenyl-hexyl SecurityGuard cartridge (4 mm length × 3.0 mm I.D., 5 μm) and a mixed mobile phase of methanol–water–tetrahydrofuran (87:6:7, v/v) at a flow rate of 1.2 mL min^{-1} were used. The separated analytes were fluorimetrically detected by excitation at 225 nm and emission at 360 nm.

2.3. Preparation and extraction of plasma sample for standard addition analysis of VLCFA

A modified method [16] was used for the extraction of VLCFA in volunteer and patient plasma after spiked with known levels of VLCFA for standard addition analysis [17], i.e., aliquots (100 μL) of plasma sample in a series of microcentrifuge tubes (1.5 mL) were separately added with aliquot (100 μL) of VLCFA (C22, C24 and C26) at 5 levels of 0.0, 1.0, 1.5, 2.0 and 2.5 μM each with IS (C23; 5.0 μM), where the solutions of VLCFA and the IS for spiking were prepared in a mixed solvent of isopropanol–phosphoric acid (2 M) (5:1, v/v) (Section 2.1). After mixing the spiked plasma, aliquots (400 μL) of toluene were added to each tube and vortexed for 30 s. And then each was added with 400 μL of water and vortexed for additional 2 min. The resulting mixture was centrifuged at 1800 × *g* for 5 min. A

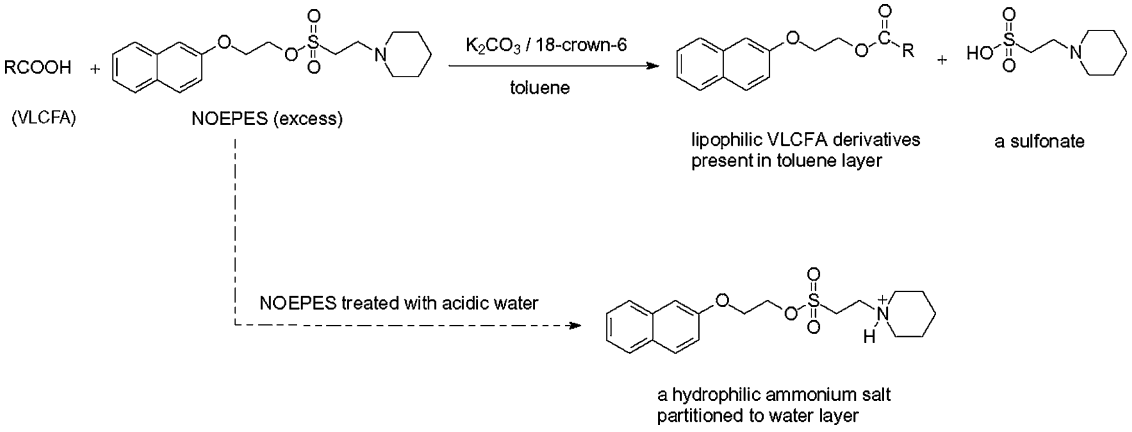

Fig. 1 – Simplified scheme for derivatizing docosanoic (C22), tetracosanoic (C24) and hexacosanoic (C26) acids based on nucleophilic substitution catalyzed by potassium carbonate and crown ether (solid arrow) and the removal of excess reagent (NOEPES) after derivatization as water soluble ammonium species (dashed arrow). RCOOH stands for the very-long-chain fatty acids (VLCFA) of C22, C24 and C26. The sulfonate obtained is a by-product of the nucleophilic substitution (as a leaving group). The molecular weights (MW) of the resulting VLCFA derivatives are 510, 538 and 566, respectively, for C22, C24 and C26.