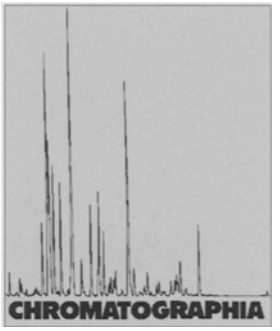
Highly Sensitive Determination of Long-Chain Free Fatty Acids by Fluorogenic Derivatization and Liquid Chromatography



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Key Words

Column liquid chromatography
Fluorometric detection
Long-chain free fatty acids
Naphthoxyethyl derivatives

Summary

A simple and sensitive method is described for the determination of biologically important fatty acids including dodecanoic, tetradecanoic, hexadecanoic, octadecanoic, hexadecenoic, octadecenoic and octadecadienoic acids. The method is based derivatization with 2-(2-naphthoxy)ethyl 2-(piperidino)ethanesulfonate (NOEPES) in toluene using potassium carbonate and 18-crown-6 as reaction activators. The resulting naphthoxyethyl derivatives were simply analyzed by isocratic HPLC with fluorometric detection (λex = 235 nm, λem = 350 nm). The excess fluorescence reagent (NOEPES) was readily removed after derivatization by a quick acid treatment. This minimizes the interference of excess NOEPES in fluorometric detection. The lower limit of quantitation 50 nM with a detection limit (S / N = 3) of about 7.5 nM (or 0.15 pmol per 20 µL injection). Application of the method to analysis of free fatty acids in plasma proved feasible (using only 10 µL of plasma sample).

Introduction

The analysis of free fatty acids in plasma or other biological samples is useful in the management of patients with various disease, including diabetes mellitus [1], thyremphraxis and hepatic dysfunction [2], heart disease and cancer [3–5] and spinal muscular atrophy [6]. Because free fatty acids do not contain a practical chromophore or fluorophore for direct detection by ultraviolet, visible or fluorescence spectroscopy; derivatization coupled with liquid chromatography is widely used in the analysis of free fatty acids at trace levels. In general, the reaction rate of fatty acids in aqueous solution is poor, due to the easy solvation of a carboxyl function by water, causing a fatty acid to be a less active nucleophile. Various reagents have been described for derivatization with fluorophores of acridine, anthracene, coumarin, fluorene, naphthalene, phenanthrene, pyrene or quinoxaline [7, 8]. In this work, the biologically important long-chain free fatty acids (LCFFA) were derivatized with a chemically removable reagent, 2-(2-naphthoxy)ethyl 2-(piperidino)ethanesulfonate (NOEPES). The resulting naphthoxyethyl derivatives give a high response with a fluorometric detector. Only a very small amount of human plasma (10 µL) was used to analyze LCFFA. The excess reagent (NOEPES) used for the derivatization can be readily removed from reacted toluene solution by an acidic aqueous solution thus minimizing interference from the excess of fluorescent NOEPES [9, 10].

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Experimental

Chemicals and Solutions

Dodecanoic acid (lauric acid, C12:0), tetradecanoic acid (myristic acid, C14:0), hexadecanoic acid (palmitic acid, C16:0), octadecanoic acid (stearic acid, C18:0), hexadecenoic acid (palmitoleic acid, C16:1), octadecenoic acid (oleic acid, C18:1), octadecadienoic acid (linoleic acid, C18:2) and heptadecanoic acid (margaric acid, C17:0, used as an internal standard, IS) were from Sigma (St. Louis, MO, USA). 18-Crown-6 ether (18-crown-6) was from TCI (Tokyo, Japan). Potassium carbonate, potassium bicarbonate, potassium fluoride , sulfuric acid and phosphoric acid (85%) were from E. Merck (Darmstadt, Germany). Toluene, 2-propanol and n-heptane were from Te-

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dia (Fairfield, OH, USA). Other chemicals were of analytical reagent grade. Distilled water purified with an Ultrapure R/O water system (Millipore, MA, USA) was used for all aqueous solutions. Standard solutions of NOEPES and 18-crown-6 at various concentrations were prepared by dissolving the appropriate amounts of the respective compounds in toluene and diluted if necessary. Solutions of sulfuric acid (1.0 M) and phosphoric acid (2.0 M) were prepared in water.

HPLC Conditions

A Waters LC system with a U6K injector, a Model 717 plus autosampler, a Model 510 pump, a Model 474 scanning fluorescence detector and a Model 746 integrator was used. A Symmetry C_8 column (150 × 3.9 mm I. D.; 5 μm) (Waters) and a mixed solvent of methanol-water (92 : 8, *v/v*) at a flow rate of 0.7 mL min^{-1} were used. The column eluate was monitored at 235 nm ex. and 350 nm em.

Derivatization Procedure

A 200 μL aliquot of LCFFA with IS in toluene, at various LCFFA concentrations was added to a 10 mL screw capped test tube containing 300 μL NOEPES (12 mM) in toluene, 100 μL of 18-crown-6 in toluene (20 mM) and about 5 mg potassium carbonate. The reactants were shaken at 95 °C for 30 min. After cooling, 300 μL of the solution were transferred to a test tube and washed with 1.0 mL of 1 M H_2SO_4 aqueous solution by vortex mixing for 30 s. An aliquot of the acid-washed toluene layer (100 μL) was mixed with an equal volume of methanol. The resulting solution was used for HPLC analysis with an autosampler (sample size, 10 μL).

Extraction of LCFFA from Plasma

A modified Dole and Meinertz [11] method was used for the extraction of LCFFA. A 10 μL aliquot of plasma in a 10 mL test tube was added with 190 μL of a reference solution of LCFFA with IS (15.0 μM) (LCFFA-IS). The LCFFA-IS (C12:0, C14:0, C16:0, C18:0, C16:1, C18:1, C18:2 and C17:0) was prepared by dissolving various amount of LCFFA and fixed amount of IS in a mixed solvent of 2-propanol-2 M phosphoric acid (5 : 1, *v/v*). The levels of various LCFFA spiked reflected that of LCFFA in normal plasma or serum. To the spiked mixture, 1.0 mL of n-heptane was added. After vortexing for 30 s, 0.6 mL of water and 2.0 mL of additional 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 aliquot of the supernatant was transferred into a 10 mL test tube and evaporated to dryness in a cold-trap centrifugal evaporator (Tokyo Rikakikai, Eyela CVE-200D). The resulting residue was subjected to the derivatization as described above.

Results and Discussion

For optimizing the conditions for derivatizing the standard mixture of LCFFA, 0.5 nmol of each LCFFA was used for the study (with IS 0.6 nmol) in 0.2 mL volume of toluene (i.e., LCFFA 2.5 μM and IS 3.0 μM). Several parameters affecting the derivatization were studied, including reaction temperature, reaction time, base catalyst and the amount of the derivatization reagent. Optimal conditions were evaluated by computing the peak areas of LCFFA derivatives.

Effects of Derivatizing Agent and 18-crown-6

In the derivatization procedure, various concentrations of NOEPES (0–14.0 mM × 0.3 mL)were studied for derivatization. Plateau formation of the derivatives was attained at concentrations ≥ 10.0 mM of NOEPES. Derivatization of LCFFA in the absence of 18-crown-6 results in a very poor yield of the derivatives. Therefore, the catalytic effect of 18-crown-6 (0–25 mM × 0.1 mL) on the derivatization was examined. The results indicate that 18-crown-6 at concentrations ≥ 15 mM is optimal for the derivatization. An excess of NOEPES was used to derivatize LCFFA at trace levels. Because the fluorescent reagent (NOEPES) is chemically removable, it can be easily removed as a water soluble ammonium species by protonation of the tertiary amino function of NOEPES with aqueous sulfuric acid as shown in Figure 1. This minimizes the reagent interference in separation and fluorometric detection.

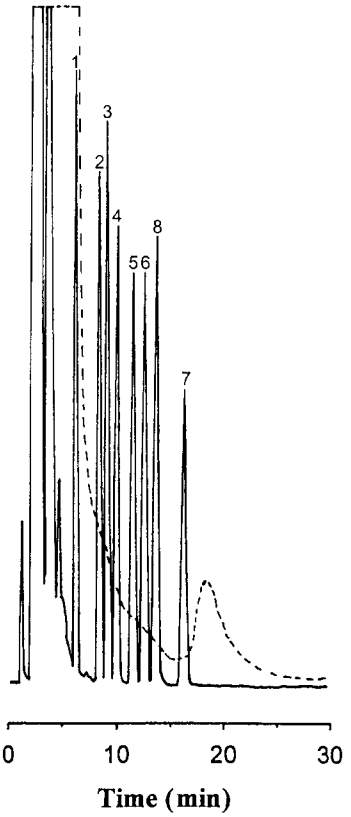

Figure 1. Composite chromatograms for a standard mixture of LCFFA (each at 2.5 μMwith IS at 3.0 μM). Peaks for LCFFA derivatives: **1** = C12:0; **2** = C14:0; **3** = C16:1; **4** = C18:2; **5** = C16:0; **6** = C18:1; **7** = C18:0 and **8** = C17:0 (IS), derivatized with NOEPES with (solid line) and without (dashed line) acid treatment. The reagent peak (dashed line) seriously interferes with the LCFFA derivatives in the derivatization without subsequent acid treatment. LC conditions: column, Symmetry C_8 (150 × 3.9 mm, I. D.; 5 μm particle size); mobile phase, methanol-water (92 : 8, *v/v*); flow rate, 0.7 mL min^{-1}; fluorescence detection : λex, 235 nm and λem, 350 nm.

Effects of Base Activator and Reaction Time

Several solid bases of potassium salt coupled with 18-crown-6 were studied as activators on the derivatization of LCFFA, including potassium carbonate, potassium bicarbonate and potassium fluoride. The results indicate that potassium carbonate is the best choice for the derivatization. The optimal amount of potassium carbonate (0–20 mg tested) on the derivatization found to be ≥ 2 mg.

About 1.5 hr at 95 °C is necessary to attain equilibrium, but a shorter reaction time of 0.5 h at 95 °C was used for the derivatization. Because the difference in yield between reacting for 0.5 and 1.5 h is small. The relative yields of the derivatives (based on peak areas) from reacting for

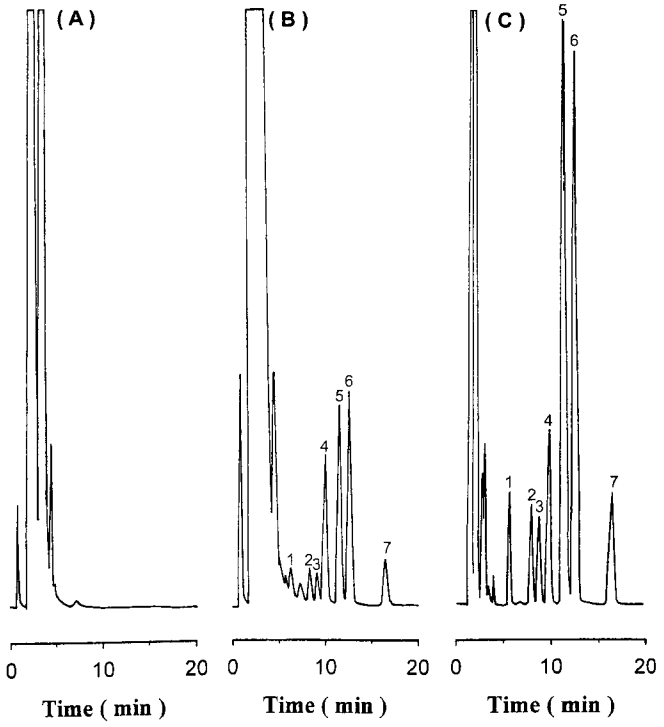

Figure 2. Chromatograms for (A) reagent blank, (B) plasma blank and (C) plasma spiked with LCFFA (C12:0, C14:0 and C16:1, each at 25.0 μM C18:0 and C18:2, each at 50.0 μM C16:0 and C18:1, each at 200.0 μM. Peaks for the LCFFA derivatives: **1** = C12:0; **2** = C14:0; **3** = C16:1; **4** = C18:2; **5** = C16:0; **6** = C18:1 and **7** = C18:0. LC conditions were the same as in Figure 1.

Table I. Analysis of long-chain free fatty acids (LCFFA) in normal human plasma.

Plasma sample	LCFFA concentration (μM)						
	C12:0	C14:0	C16:0	C18:0	C16:1	C18:1	C18:2
1	3.2	17.5	103.6	55.6	17.4	105.9	91.9
2	1.1	19.8	55.3	29.8	15.4	61.3	84.8
3	6.2	27.6	113.5	63.7	20.4	135.0	140.0
4	3.8	11.4	46.0	30.2	12.1	37.7	54.5
5	1.4	17.5	60.6	40.0	10.1	53.2	99.7
Mean	3.1	18.8	75.8	43.9	15.1	78.6	94.2
S.D.	2.1	5.8	30.6	15.3	4.1	40.4	30.8

0.5 h and 1.5 h are 82 and 100%, respectively. At the lower recovery figure the sensitivity of the method is high enough for the analysis of LCFFA levels in plasma.

Analytical Calibration and Precision

The stability of the LCFFA derivatives (2.5 μM) relative to IS at room temperature after derivatization was studied based on their peak area ratios. No significant change in peak area ratios of the derivatives to IS was found over 24 h, indicating that the derivatives are stable for the time required for HPLC analysis.

Based on the derivatization conditions optimized and formulated in the derivatization procedure, the quantitative applicability of the method to the determination of LCFFA was evaluated at five concentrations of each LCFFA over the range 0.05–2.5 μM with IS at 3.0 μM. The linearity was evaluated between the peak-area ratios of LCFFA derivatives to IS as ordinate (y) and the concentration of the LCFFA (μM) as abscissa (x). The results indicate that a good linearity between y and x over the range studied is obtained for the LCFFA, each giving a correlation coefficient of 0.999 for intra-day (n = 5) and inter-day (n = 6) analyses. The detection limit (as signal to noise ratio of 3) of various LCFFA was about 7.5 ~ 10.0 nM (or 0.15 ~ 0.2 pmol per 20 μL injection).

The precisions (relative standard deviation, RSD) and accuracy (relative error, RE) of the method were studied based on the peak-area ratios for the analysis of each LCFFA at 2.0, 0.8 and 0.2 μM. The results indicate that the% RSD and% RE for the intra-day (n = 5) and inter-day (n = 6) are all below 5.4 at the three levels.

Application to the Analysis of LCFFA in Plasma

The analysis of plasma spiked with and without LCFFA was studied following the extraction and derivatization protocols for LCFFA. Five different amounts of LCFFA spiked in varied amounts corresponding to their levels in biosample (C12:0, C14:0 and C16:1 at 0 ~ 25 μM; C18:2 and C18:0 at 0 ~ 50 μM; C16:0 and C18:1 at 0 ~ 200 μM) were prepared, and the linearity between the peak-area ratios (y) and sample amount (x, μM) was examined. A good correlation coefficient (r = 0.999) was obtained for each LCFFA over the range studied. The precision based on known amount of LCFFA cannot be evaluated, because the true values of endogenous LCFFA in plasma are unavailable. Therefore, the precisions (RSD) based on the slope and intercept of the related regression equations for intra-day (n = 5) and inter-day (n = 6) analysis were analyzed. The RSD for the slope and intercept are all below 3.6% and 6.3%. Figure 2 shows typical chromatograms for the analysis of LCFFA. This indicates that the plasma blank did not significantly interfere with the IS. Peaks 1, 2, 3, 4, 5, 6, and 7 in Figure 2 (B) from plasma blank with similar retention time to those peaks in Figure 2 (C) of spiked LCFFA could be assigned as endogeneous C12:0, C14:0, C16:1, C18:2, C16:0, C18:1 and C18:0, respectively. The IS (C17:0) is an odd carbon number LCFFA of non-endogeneous origin.

The method was applied to the analysis of LCFFA in normal plasma (n = 5) using standard addition and only 10 μL of plasma. The results are shown in Table I. The levels of various LCFFA analyzed are compatible with the reported values for plasma and serum [12–20]. Application of the method to the analysis of LCFFA in plasma of patients with diseases of lipid metabolic disorders should be feasible.

Conclusion

A simple and sensitive method has been developed for the analysis of LCFFA by fluorometric derivatization and isocratic LC. The amount of plasma required for the analysis of LCFFA is very small (10 μL), so that the method is suitable for biosample monitoring. NOEPES is chemically removable and highly fluorescent due partly to the structure with an alkoxy substituent (auxochrome) attached to the fluorophoric naphthalene skeleton. This sulfonate reagent is relatively stable as compared to those of diazoalkyl and haloacyl functions with various fluorophores. NOEPES is easy to store, but is generally less reactive than diazoalkyl or haloacyl reagents. Further application of the method to the analysis of fatty acids in clinical and agricultural samples is being developed.

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