Analysis of Fatty Acids in Mouse Cells Using Reversed-Phase High-Performance Liquid Chromatography

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Key Words

Column liquid chromatography
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Summary

A reversed-phase high-performance liquid chromatography (RP-HPLC) method was developed to analyze various fatty acids in recombinant mouse L cells. These fatty acids were the metabolites of oleic acid. A process was developed to extract fatty acids from the cell samples before RP-HPLC analysis. The samples were first saponified with 0.5 M NaOH in 96% ethanol then extracted with acidified ethyl acetate. After extraction, the sample was dried and dissolved in HPLC-grade methanol. After centrifugation to remove insoluble impurities, the sample was applied to a C_{18} RP-HPLC column using a gradient of acetonitrile (ACN)-H_2O. The eluted fatty acids were monitored by ultraviolet (UV) absorption at 195 nm and identified by retention time and adsorption spectrum comparison. This method successfully resolved various fatty acids and provided a tool for the elucidation of the fatty acid metabolic pathway in the cells.

Introduction

Fatty acids are the distinctive structural components of lipids and their compositions and biological functions can vary widely in tissues or cells [1]. As part of the strategy for the elucidation of cell metabolic pathway, it is necessary to analyze the fatty acids composition of the cell samples. The pathways are mostly still unknown.

Before the development of modern high-performance liquid chromatography (HPLC), thin layer chromatography and gas chromatography were extensively used for the separation, identification and quantification of fatty acids [2–7]. With the development of modern chromatographic technology, HPLC has become the preferred method for the isolation and analysis of fatty acids. Numerous applications of HPLC for the analysis of fatty acids have been reported [8–11]. HPLC offers a number of advantages over other techniques. A wide range of column packing materials is available for specific applications, and the columns can be used repeatedly. Since most separations can be achieved at ambient temperature under anaerobic conditions, HPLC is particularly well suited to compounds with reactive functional groups such as hydro-peroxide groups whereas unwanted rearrangements occur at the high temperatures required for gas chromatographic analysis. Most importantly, HPLC can resolve a wide range of components in a single analysis. High specificity and sensitivity are possible if a low range wavelength detector is used, and this is very important for fatty acids detection and quantification.

RP-HPLC is a very popular chromatography mode. The term reversed-phase implies that the stationary phase is a nonpolar solid and the mobile phase is a more polar solvent. RP-HPLC columns are efficient, stable, and reproducible. In RP-HPLC, good resolution basis of complex and thermolabile compounds can be obtained and it is simple to recover intact material for further analysis.

Although RP-HPLC is a very promising analytical method, very few studies have been devoted to the analysis of fatty acids, especially free fatty acids from animal cells. Although ethyl esters and other fatty acid derivatives have been used in the analysis of fatty acids, if free fatty acids are needed for further analysis or other purposes, it is not appropriate to convert fatty acids to their derivatives. In this case, it is desirable to establish a process to separate and analyze free fatty acids.

Free fatty acids only account for a very small part of the total fatty acids in animal cells [12]. Before RP-HPLC analysis, a procedure must be developed to release these fatty acids from the cells and remove some impurities such that the sample will not contaminate the HPLC column. This

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Table I. Fatty acid standards used for RP-HPLC analysis.

Name of fatty acids	Shorthand	Number of double bonds
Oleic acid (OA)	18:1ω9	1
γ-Linolenic acid (GLA)	18:3ω6	3
Linoleic acid (LA)	18:2ω6	2
Eicosadienoic acid (EDA)	20:2ω6	2
Di-homo-γ-linolenic acid (DGLA)	20:3ω6	3
Arachidonic acid (AA)	20:4ω6	4
Eicosapentaenoic acid (EPA)	20:5ω3	5
Adrenic acid	22:4ω6	4
Docosapentaenoic acid (DPA)	22:5ω3	5
Docosahexaenoic acid (DHA)	22:6ω3	6
Prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$)	–	1
Prostaglandin E_1 (PGE_1)	–	1
Prostaglandin E_2 (PGE_2)	–	2
Prostaglandin E_3 (PGE_3)	–	3

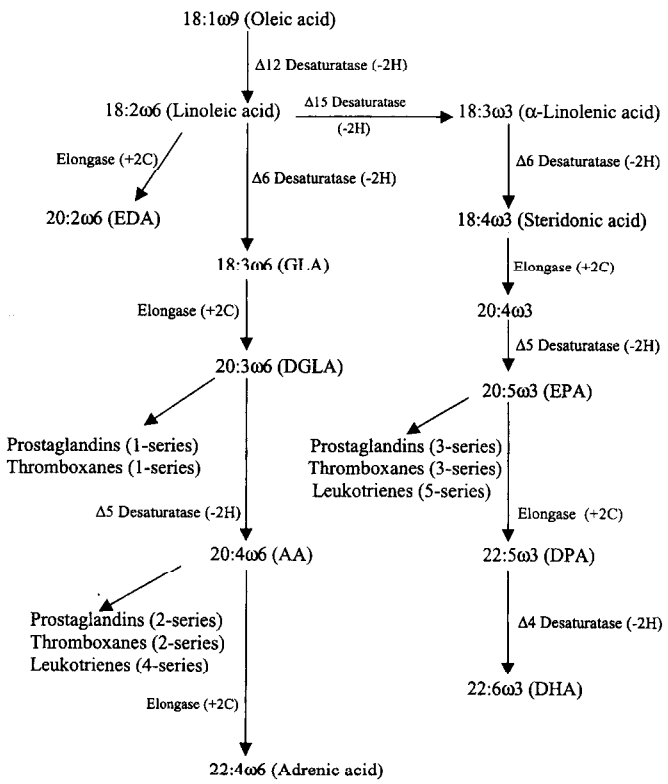

Figure 1. Metabolic pathway of oleic acid in recombinant mouse cells.

study describes methods to separate and analyze fatty acids in their free form to support the study on the metabolic pathway of oleic acid in mouse L cells. The objectives of this study were to develop an effective process to prepare fatty acids samples from cells for RP-HPLC analysis and establish an RP-HPLC method to analyze these fatty acid samples. Such a fatty acid isolation process together with the RP-HPLC method may also provide some useful information for the separation and analysis of fatty acids from other sources.

Experimental

Chromatographic System

The RP-HPLC analysis was performed on a Vydac C_{18} RP-HPLC column (Catalog # 218TP54, particle size 5 µm, pore size 300 Å, dimensions 0.46 cm × 25 cm) at ambient temperature using a Waters HPLC system (Millipore Corp., Bedford, MA) consisting of a dual-pump gradient system, an autosampler and a Photodiode Array detector. The mobile phase was the water solution of ACN. The wavelength range of the detector was set to 191.5 ~ 300 nm. The absorbance at 195nm was used to calibrate the concentrations of the fatty acids.

Materials

Control mouse L cells and L cells stably transformed with a *Mortierella Alpina* Δ12-desaturase cDNA were incubated for 24-hours in serum-free culture medium as described by Kelder et al. [13]. The Δ12 cells express an active Δ12-desaturase and demonstrate increased levels of linoleic acid compared to control cells [13]. Several fatty acids and eicosionids such as prostaglandins were chosen to serve as fatty acid standards for the RP-HPLC analysis (Table I) according to the polyunsaturated fatty acid (PUFA) metabolic pathway for omega-6 and omega-3 PUFA's [14, 15, 16] (Figure 1). All the fatty acid standards were reagent grade and were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA). HPLC grade ACN, methanol, ethyl acetate and analytical grade acetic acid were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Sample Preparation

Before the fatty acids from the cell samples can be analyzed using RP-HPLC, they must first be extracted and transferred into a solvent that is suitable for RP-HPLC analysis. However, free fatty acids only constitute a very small part of the total fatty acids present in the animal cells. Most fatty acids exist in the cells in the form of complex lipids [12]. In this work, a process was first developed to prepare free fatty acid samples from the mouse L-cells for RP-HPLC analysis. This method consists of four steps: saponification, solvent extraction, solvent change and RP-HPLC analysis.

The saponification step was used to release fatty acids from complex lipids. 0.5 N sodium hydroxide in a solution of 96% ethanol-4% water (*v/v*) was used to saponify the fatty acids. Ethanol was used to support the saponification reaction and dissolve the sodium salts of the fatty acids. First, 1 mL 0.5 N NaOH in 96% ethanol was added to the cell sample with around 10^6 cells. After cell disruption using an ultrasonic homogenizer, the sample was left at room temperature for at least 8 hours or overnight. The sample was then centrifuged at 7000 rpm for 5 minutes and the solid residue was discarded.

The purpose of the solvent extraction step was to separate fatty acids from salts and other unwanted water-soluble impurities. 1 mL 0.6 N HCl was added to the supernatant from the saponification step. This operation changed the fatty acids from their sodium salts to their free acid forms. 3 mL ethyl acetate was then added to the sample. After vortex mixing for 1 minute, the sample was left in room temperature 30 minutes for phase separation.

The solvent change step was used to transfer free fatty acids from the ethyl acetate-ethanol solution to pure methanol for the HPLC injection. Nitrogen was gently blown through the top phase from the extraction step under 30 °C to remove the organic solvents (ethyl acetate and ethanol). When most of the solvents were evaporated from the solution, the sample was lyophilized to remove the remaining water. The sample was then re-dissolved into 350 µL HPLC-grade methanol for RP-HPLC analysis.

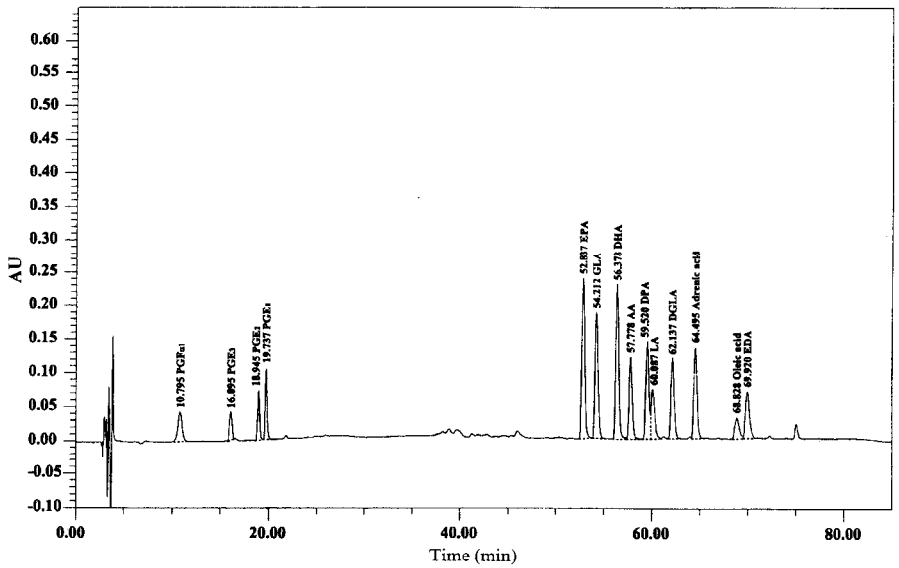

Figure 2. Chromatogram of fatty acid standards. The detection wavelength was 195 nm.

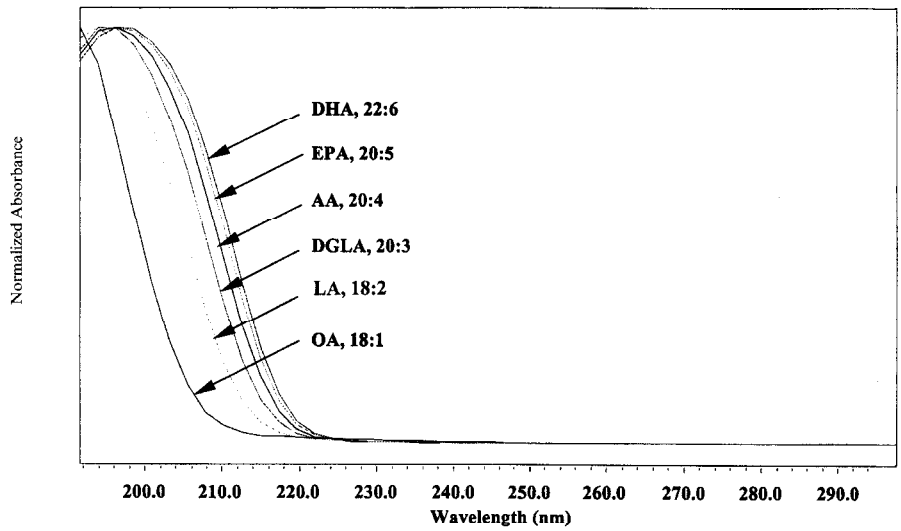

Figure 3. Comparison of the UV spectra (after normalization) of fatty acids with different numbers of double bonds and similar chain lengths.

Results

Separation of Fatty Acid Standards

A gradient elution method was used to resolve various fatty acids in a single HPLC run. Two mobile phases were used to generate the gradient: solution A: 25% ACN + 75% water; solution B: pure ACN. Acetic acid (0.12% *v/v*) was added to these two mobile phase solutions to adjust the pH and prevent the ion exchange side effect. The mobile phase changes from 100% A to 100% B linearly over 80 minutes. The flow rate was 1 mL min^{-1} from 0 to 80 minute and then was changed to 2 mL min^{-1} to clean the column. The run time for each analysis was 95 minutes. Figure 2 is the RP-HPLC profile of the free fatty acid standards.

Absorption Spectra of Fatty Acid Standards

Retention time alone cannot identify a fatty acid because different compounds may have the same retention time. An individual fatty acid can only be identified tentatively by retention time and it is necessary to confirm the identity of a particular component with a second method. In this study, the absorption spectrum over the wavelength range of 191.5–300 nm was used to identify the fatty acids. Figure 3 is the normalized UV absorption spectra of fatty acids containing different numbers of double-bonds. Each normalized spectrum was obtained by normalizing the actual spectrum with its peak absorbance and was thus independent of its actual concentration level. The UV absorption of fatty acids is mainly due to the double bond and the carboxylic group in the molecule. The contribution of the carbon chain length to the absorption spectra is negligible. Therefore, different fatty acids with the same number of double bonds have similar normalized UV spectra. The UV spectra of fatty acids with different numbers of double bonds are a little different. The normalized UV spectra could help to identify different fatty acids. Most importantly, spectrum comparison could be used to identify fatty acids from other impurities in the RP-HPLC profile.

Calibration Curves of Fatty Acids

In order to quantify fatty acid levels, the correlation between the peak area and the injection quantity for each fatty acid standard was obtained by a linear regression. Each fatty acid standard was dissolved into HPLC-grade methanol and then was diluted to different concentration levels for RP-HPLC analysis. The RP-HPLC conditions used to obtain the calibration lines were the same as those used to obtain the RP-HPLC profile of the fatty acid standards. The results including the low-

Table II. Calibration correlations of various fatty acids.

Fatty acid	Calibration correlation	Correlation coefficient (R^2)	Lowest limit of detectability (μg)
Oleic acid	y = 1.14E + 6x	0.992	0.0055
GLA	y = 5.15E + 6x	0.998	0.0012
LA	y = 2.35E + 6x	0.997	0.0027
EDA	y = 2.51E + 6x	0.994	0.0025
DGLA	y = 3.48E + 6x	0.999	0.0018
AA	y = 3.36E + 6x	0.999	0.0019
EPA	y = 6.25E + 6x	0.998	0.0010
Adrenic acid	y = 3.80E + 6x	0.998	0.0017
DPA	y = 3.81E + 6x	0.999	0.0017
DHA	y = 6.06E + 6x	0.998	0.0010
$PGF_{1\alpha}$	y = 2.79E + 6x	0.989	0.0023
PGE_1	y = 1.67E + 6x	0.999	0.0038
PGE_2	y = 1.38E + 6x	0.999	0.0046
PGE_3	y = 1.46E + 6x	0.995	0.0043

Table III. RP-HPLC analysis results for negative control sample and Δ12 sample.

Fatty acid	Negative control sample		Δ12 sample		$(M_{\Delta12} - M_{NC})/M_{\Delta12}$
	Peak area	Mass M_{NC} (μg)	Peak area	Mass $M_{\Delta12}$ (μg)	
DHA	357003.8	0.0589	594452.3	0.0981	0.67
AA	1306477	0.389	2277631	0.678	0.74
DPA	358849.8	0.094	613838.7	0.161	0.71
LA	2206754	0.939	12276646	5.224	4.56
DGLA	398114.9	0.114	611413.3	0.176	0.54
Adrenic acid	235970.3	0.062	612403.7	0.161	1.60
Oleic acid	16704087	14.653	6264032.6	5.495	−0.63
EDA	261340.3	0.104	2327435	0.927	7.91

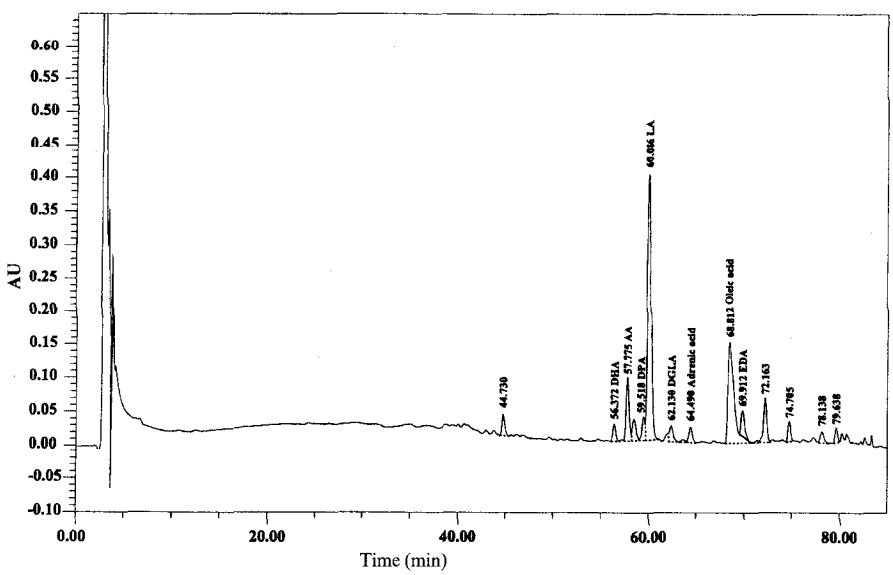

Figure 4. The RP-HPLC profile of the Δ12 cell sample. The detection wavelength was 195 nm.

est limits of detectability are listed in Table II [y: peak area; x: corresponding injection mass of fatty acid (μg)]. The linearity of the curves is very good as indicated by the correlation coefficient (R^2) values in Table II.

Chromatography of Control Samples Prepared from Mouse L Cells

Mouse L cells and Δ12-desaturase cells were compared in this study. RP-HPLC analysis was conducted under the same operational conditions as that for the fatty acid standards. After processing each cell sample for HPLC analysis following the sample preparation procedure described above, a final 350 μL methanol solution was obtained. For each RP-HPLC analysis, the injection volume is 100 μL. Figures 4 and 5 are the RP-HPLC profiles for a Δ12 cell sample and an L cell control cell sample, respectively. Eight fatty acids were identified in each of the two RP-HPLC profiles. The mass of these fatty acids was calculated according to the corresponding calibration correlations in Table II. The results are listed in Table III.

Discussion

For routine investigation, a simple and rapid sample preparation and also a RP-HPLC method were developed for the analysis of fatty acids in mouse cells. This RP-HPLC method successfully resolved various fatty acids with a wide range of hydrophobicity. Among the 14 fatty acids in the RP-HPLC profile of the fatty acid standards, 8 fatty acids were identified and quantified in both the Δ12 cell sample and the negative control cell sample. The remaining 6 fatty acids were not identified in these two profiles. This could be due to the low concentrations of these fatty acids in these samples. However, the decrease in the concentration of oleic acid and the increase in the concentrations of the remaining 7 fatty acids in the Δ12 cell sample clearly reflected the contribution of the Δ12 desaturatase to the metabolism of oleic acid in the recombinant mouse cells. The method described here can provide useful instructive information for the study on the oleic acid metabolic pathway in the recombinant mouse cells.

The purpose of this RP-HPLC method was to obtain the total fatty acid profile of the cell samples and was designed to analyze fatty acids with a wide range of hydrophobicity. A wide gradient range and relative long running time were used in this method. This method can also be adapted for other purposes. The mobile phase gradient range and the running time can be adjusted according to the types of the fatty acids to be analyzed.

In this work, acetic acid was used to adjust the mobile phase pH and prevent the ion exchange side effect. This makes it easier to further purify each fatty acid fraction following the RP-HPLC step when necessary since it is very easy to remove acetic acid by evaporation. The software used in the HPLC system supported the ability to subtract the effects of the

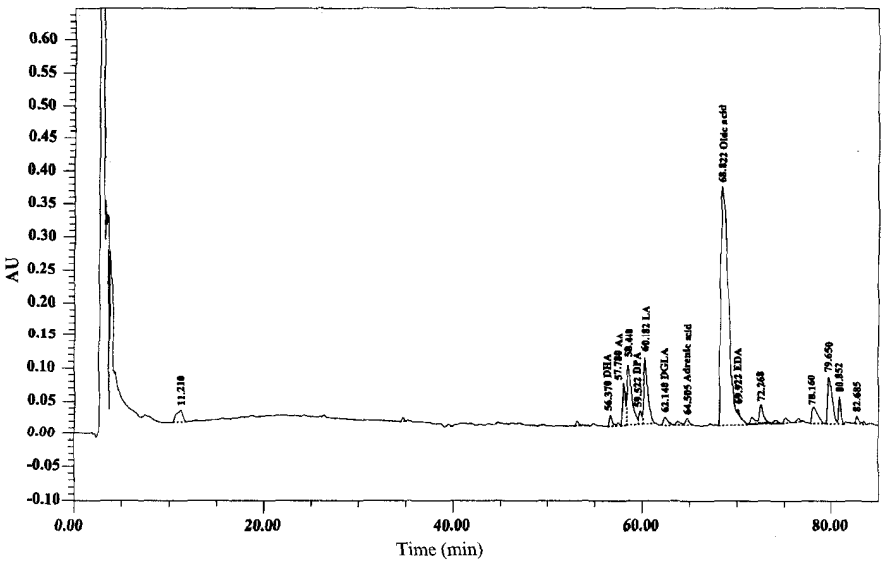

Figure 5. The RP-HPLC profile of the control mouse L cell. The detection wavelength was 195 nm.

mobile phase on a sample. The absorbance of acetic acid owing to the carboxylic group was not a problem. All the RP-HPLC profiles presented here are the results of baseline subtraction.

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