Long-Chain Free Fatty Acid Profiling Analysis by Liquid Chromatography–Mass Spectrometry in Mouse Treated with Peroxisome Proliferator-Activated Receptor α Agonist

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A change in the free fatty acid (FFA) profile reflects an alteration in the lipid metabolism of peripheral tissue. A high-throughput quantitative analysis method for individual FFAs therefore needs to be established. We report here an optimized LC-MS assay for a high-throughput and high-sensitivity analysis of the 10 major long-chain FFAs in mouse plasma and liver. This assay enables quantification of individual FFAs by using trace amounts of samples (2 μL of plasma and 10 mg of liver tissue). We apply this method to analyze the FFA profile of plasma and liver samples from an obese mouse model treated with bezafibrate, the peroxisome proliferator-activated receptor α (PPARα) agonist, and show a change in the FFA profile, particularly in the palmitoleic and oleic acid contents. This assay is useful for quantifying individual FFAs and helpful for monitoring the condition of lipid metabolism.

Key words: peroxisome proliferator-activated receptor α (PPARα); free fatty acid (FFA); ultra-high-performance liquid chromatography-mass spectrometry (UPLC-MS)

Free fatty acids (FFAs) have various physiological functions that depend on the FFA structure, i.e., the acyl chain length and saturation form. FFAs have recently been noted to play an important role as signaling factors in cells for both anti- and pro-inflammatory processes.1) Many previous studies have indicated that the signaling mechanism differed according to the acyl chain length and/or number of unsaturated bonds in FFA. For example, palmitoleic acid (POA) has improved hyperglycemia and hypertriglyceridemia by increasing the insulin sensitivity, due in part to suppressing the pro-inflammatory gene expression and improving the hepatic lipid metabolism in diabetic mice.2) Stearic acid (SA) and palmitic acid (PA) activate toll-like receptor 4 (TLR4) in macrophages and induce inflammation.3) Since FFAs have various functions, including those in anti- and pro-inflammatory processes, a change in the composition of FFAs has been observed in such disorders as obesity,5) insulin resistance,6) and diabetes.7) Although further research is still required to link the change in fatty acid profile to the metabolic syndrome, several fatty acids have been suggested as potential biomarkers of metabolic syndrome.8)

Peroxisome proliferator-activated receptor α (PPARα) is a ligand-activated transcription factor that regulates lipid metabolism.9–11) PPARα activation enhances fatty acid oxidation and decreases the levels of circulating and cellular lipids in obese diabetic patients.12,13) Regulating the PPARα activity is therefore one of the most important means of managing chronic diseases related to a dysfunction of the lipid metabolism in the liver. PPARα is expressed at a high level in the liver where it promotes β-oxidation, ketogenesis, and lipid transport.14,15) Fibrates are widely prescribed hypolipidemic drugs, these drugs exerting their effects through a PPARα-dependent mechanism. Bezafibrate has been identified as a PPARα agonist16,17) and improves dyslipidemia, lowers the plasma fibrinogen level, slows the progression of focal coronary atherosclerosis, and reduces the number of coronary events in young survivors of myocardial infarction.18) Many studies have also found functional food ingredients that activate PPARs. We have recently reported that tomato fruit contained linoleic acid (LIA) derivatives, i.e., 9-oxo-10,12-octadecadienoic acid (9-oxo-ODA) and 13-oxo-9,11-octadecadienoic acid (13-oxo-ODA), which could serve as PPARα agonists,19,20) Identifying PPARα agonists in food ingredients generates a valuable resource pool for preventing lifestyle-related diseases. Although PPARα is a major modulatory factor of FFA oxidation, it is unclear whether alterations in the long-
Liquid chromatography-mass spectrometry (LC-MS) has recently been used for an FFA analysis of samples in plasma or urine, and its potential for the sensitive and high-throughput analysis of individual FFAs by LC-MS and (b) to apply this method to the analysis of changes in the long-chain FFAs can be applied for monitoring the condition of lipid metabolism and identifying the markers for PPAR activation.

The aims of this present study were (a) to optimize a method for the sensitive and high-throughput analysis of individual long-chain FFAs by LC-MS and (b) to apply this method to the analysis of changes in the long-chain FFA profile in mouse plasma and liver after a treatment with bezafibrate, a PPARα agonist.

Materials and Methods

Chemicals. Analytical-grade cis-10-heptadecenoic acid (HDA), myristic acid (MA), palmitic acid (PA), palmitoleic acid (POA), linoleic acid (LNA), arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and ammonium formate were purchased from Sigma (St. Louis, MO, USA) or Wako (Osaka, Japan). 4-Hydroxy-2-methyl-5-flavone (HMF) was purchased from Extrasynthese (Lyon, France). Bezafibrate for the mouse experiments was purchased from Kissei Pharmaceutical Co. (Tokyo, Japan), and for the LC-MS analysis, from Sigma (St. Louis, MO, USA). All the solvents used in this study were purchased from Wako (Osaka, Japan). The LC-MS buffer (acetonitrile, ultrapure water, and formic acid) was of LC-MS grade.

Mice. Male KK-Ay mice, a useful model of obesity and diabetes, were purchased from Clea Japan (Tokyo, Japan). The mice were kept in individual cages in a temperature-controlled room at 23 ± 1°C and maintained under a constant 12-h light/dark cycle. All the animal experiments were approved by the Kyoto University Animal Care Committee.

The 4-week-old mice were maintained for 7 d on a standard diet and then assigned to two groups of similar average body weight. Each group was maintained on a 60% high-fat diet (HFD) for 4 weeks, with one group being fed HFD containing 0.2% (w/w) bezafibrate. The energy intake of all the mice was adjusted by pair feeding. At the end of the treatment period, anesthetized mice were sacrificed by cervical dislocation after 5 h of fasting, and tissue samples were collected as described next.

Extraction of the mouse plasma and liver samples. Mouse blood samples were centrifuged at 15,000 rpm for 10 min at 4°C, and resulting plasma samples (2 μL) were mixed with 98 μL of an FFA extraction solvent (99.5% ethanol containing 1 μg/mL of HDA as an internal standard) or a bezafibrate extraction solvent (80% methanol containing 0.1 μg/mL of HMF as an internal standard). The internal standard was used for calibration by plotting the ratio of the analyte signal to the internal standard signal. Mouse liver samples (10 mg) were homogenized with a Mill MM 300 mixer (Quimag, Hilden, Germany) at 25 Hz for 10 min in 1 mL of the FFA extraction solvent. The supernatant was collected as the tissue extract after centrifugation (15,000 rpm for 10 min at 4°C). The extract was filtered through a 0.2-μm-pore PVDF membrane (Whatman, Brentford, UK), and the resulting filtrate was used for LC-MS.

LC-MS for quantifying FFA and bezafibrate. LC-MS was performed with an Acquity UPLC system coupled to a Xevo Quadrupole Time-Of-Flight (qTOF)-MS system (Waters, Milford, MA), equipped with an electrospray source operated in the negative-ion mode with a lock-spray interface for accurate mass measurement. Leucine enkephalin was employed as the lock-mass compound. It was infused straight into the MS system at a flow rate of 20 μL/min at a concentration of 200 pg/μL in 50% acetonitrile and 0.1% formic acid. The data were acquired by using Mass Lynx software (Waters). External mass was calibrated by following the manufacturer’s protocol. The V mode was selected for a quantitative analysis, the dynamic range being set to “extended” to maintain the linearity of the mass signal within the dynamic range. An aliquot of the extracted sample (3 μL) was injected into an Acquity UPLC BEH-C18 reverse-phase column (2.1 × 100 mm column size, 1.7 μm particle size). The column temperature was set at 40°C. The amount of FFA or bezafibrate was estimated from calibration curves obtained by using analytical-grade standard compounds. The peak area of m/z [M – H] ± 0.05 Da was divided by the area of the internal standard, this value being used to generate the calibration curves.

The capillary, sampling cone, and extraction cone voltages were respectively set at 2300, 40, and 1.5 V for FFA detection. The respective source and desolvation temperatures were 120°C and 500°C, and the respective cone and desolvation gas flow rates were set at 50 and 1,000 L/h. Mobile phases A (90% acetonitrile, 10 mM ammonium formate, and 0.1% formic acid) and B (98% acetonitrile and 0.1% formic acid) were used for FFA separation. The buffer gradient consisted of 0.1% B for 0–5 min, 0.1 to 99.9% B for 5–6 min, 99.9% B for 6–11 min, 99.9% to 0.1% B for 11–12 min, and 0.1% B for 3 min before the next injection, at a flow rate of 400 μL/min.

The capillary, sampling cone, and extraction cone voltages for bezafibrate detection were respectively set at 2,600, 20, and 2.0 V. The respective source and desolvation temperatures were 120°C and 450°C, and the respective cone and desolvation gas flow rates were set at 50 and 800 L/h. Mobile phases A (H2O and 0.1% formic acid) and B (acetonitrile and 0.1% formic acid) were used for bezafibrate separation. The column temperature was set at 40°C. The buffer gradient consisted of 0.5% of 0.0% B for 0–10 min, 0.0% B for 10–15 min, 99.9% to 5.0% B for 15–15.5 min, and 5.0% B for 4.5 min before the next injection, at a flow rate of 300 μL/min.
Quantification of the mRNA expression levels. Total RNA was prepared from mouse liver by using Sepasol (Nacalai Tesque), according to the manufacturer’s protocol. M-MLV reverse transcriptase (Invitrogen, Corp.) was used to reverse-transcribe total RNA by a PCR SP thermal cycler (Takara Bio, Shiga, Japan). A real-time quantitative RT-PCR analysis was performed with a Light Cycler system (Roche Diagnostics), using SYBR green fluorescence signals to determine the mRNA expression levels. The oligonucleotide primer sets of mouse 36B4, the internal control encoding for the 36B4 ribosomal protein, and of the stearoyl-CoA desaturase-1-encoding gene (SCD-1) were as follows: mouse 36B4 (fwd: 5’-ttctcttcagctgttggg-3’; rev: 5’-gacacgctcagaagacgag-3’), mouse SCD-1 (fwd: 5’-ccgaaagacgccgagag-3’; rev: 5’-ccgaaagacgccggtagag-3’).

Statistical analysis. Data are presented as the mean ± SEM. Differences between groups were compared with Student’s t-test. Values of $p < 0.05$ were considered statistically significant.

Results and Discussion

Method optimization for the FFA analysis

Approximately 94% of FFAs in human plasma are composed of the 10 major FFA species, MA, PA, POA, SA, OA, LIA, LNA, AA, EPA, and DHA.27) We therefore analyzed individual FFAs, focusing on the major FFA species, by using an LC-MS-based method. We first investigated the optimum analytical conditions for LC-MS to improve the FFA peak sensitivity and high-throughput analysis, including optimizing the peak separation and quantification. The influence of ion suppression on the FFA peak detection and recovery rate was also investigated.

We used different LC elution buffer conditions and MS ionization parameters to improve the peak sensitivity of individual FFAs by LC. We initially analyzed FFAs based on acetonitrile/H$_2$O including 0.1% formic acid in the gradient mode (an acetonitrile rate from 30% to 90%). It proved difficult to detect the unfragmented deprotonated molecular ion ([M – H$^-$]) under this condition (data not shown). We next adopted 90% acetonitrile with 0.1% formic acid as the solvent under an isocratic condition and detected weak FFA-specific peaks (data not shown). To further improve the peak sensitivity, the LC elution buffer was supplemented with 10 mM ammonium formate as well as 0.1% formic acid. This condition enabled sensitive detection of FFAs as unfragmented deprotonated molecular ions ([M – H$^-$]) (Table 1 and Fig. 1). The resolution of the FFA peaks was significantly improved by adding ammonium formate to the LC buffer, indicating that ammonium formate would be useful for configuring FFA unfragmented deprotonated molecular ions.

We next optimized the MS ionization parameters, including the source voltage, temperature, and gas flow rate. Since FFAs containing a carboxyl group (R-COOH) are easy to deprotonate (R-COO$^-$), MS was operated in the negative-ionization condition without fragmentation, and monitored in the full-scan mode. We applied these LC-MS parameters to quantify FFAs, and the data indicate that these conditions provided a rapid, highly sensitive method for the FFA analysis.

Peak separation and quantification under the high-throughput analysis were achieved by optimizing the LC conditions. The FFA retention time (Rt) was dependent on the acyl chain length and unsaturation degree of the FFAs with our method. In respect of the LC method, FFAs with higher carbon content were eluted from the column at a slower rate than those with lower carbon content; for example, MA (C14:0) was eluted at 1.40 min, PA (C16:0) was eluted at 1.94 min, and SA (C18:0) was eluted at 2.94 min. In addition, the number of double bonds in FFA also affected its elution time. Our data indicate that LNA (C18:3, Rt = 1.18 min) was eluted from the column faster than LIA (C18:2, Rt = 1.47 min), OA (C18:1, Rt = 2.00 min), or SA (C18:0, Rt = 2.94 min) (Table 1 and Fig. 1). We validated this method to accurately quantify FFAs. Linearity

![Fig. 1. Elution Peaks of Standard FFAs.](image)

**Table 1.** Characteristics of Individual FFAs Determined by LC-MS

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular formula</th>
<th>Rt (min)</th>
<th>Monoisotopic mass</th>
<th>Detected mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>C$<em>{14}$H$</em>{29}$O$_2$</td>
<td>1.40</td>
<td>228.20893</td>
<td>227.20 [M – H$^-$]</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>C$<em>{16}$H$</em>{31}$O$_2$</td>
<td>1.94</td>
<td>256.24023</td>
<td>255.23 [M – H$^-$]</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>C$<em>{16}$H$</em>{31}$O$_2$</td>
<td>1.41</td>
<td>254.22458</td>
<td>253.21 [M – H$^-$]</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>C$<em>{18}$H$</em>{33}$O$_2$</td>
<td>2.94</td>
<td>284.27153</td>
<td>283.26 [M – H$^-$]</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>C$<em>{18}$H$</em>{31}$O$_2$</td>
<td>2.00</td>
<td>282.25588</td>
<td>281.25 [M – H$^-$]</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>C$<em>{18}$H$</em>{32}$O$_2$</td>
<td>1.47</td>
<td>280.24023</td>
<td>279.23 [M – H$^-$]</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>C$<em>{18}$H$</em>{32}$O$_2$</td>
<td>1.18</td>
<td>278.22458</td>
<td>277.22 [M – H$^-$]</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>C$<em>{20}$H$</em>{34}$O$_2$</td>
<td>1.35</td>
<td>304.24023</td>
<td>303.23 [M – H$^-$]</td>
</tr>
<tr>
<td>Eicosapentaenoic acid (EPA, C20:5)</td>
<td>C$<em>{20}$H$</em>{34}$O$_2$</td>
<td>1.11</td>
<td>302.22458</td>
<td>301.21 [M – H$^-$]</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA, C22:6)</td>
<td>C$<em>{22}$H$</em>{36}$O$_2$</td>
<td>1.25</td>
<td>328.24023</td>
<td>327.23 [M – H$^-$]</td>
</tr>
</tbody>
</table>
was achieved in the calibration curves for all FFAs (MA and liver PA: 200–5000 ng/mL; plasma PA: 200–2000 ng/mL; POA, SA, OA, LIA and AA: 20–5000 ng/mL; EPA: 2–2000 ng/mL; DHA: 20–10,000 ng/mL; Table 2), the lower limit of the linear range being between 2 and 200 ng/mL for all FFAs (Table 2). The validation of this method demonstrated linearity of the calibration curve for all FFAs at R² > 0.986 in their physiological range (Table 2). This linear range is appropriate for quantitatively analyzing FFAs in mouse plasma and liver.

The simple preparation procedure for FFA detection from plasma or tissue samples (ethanol extraction) did not exclude matrix components, except for proteins. We used a standard solution containing known amounts of FFAs (MA, PA, POA, SA, OA, LIA, LNA, AA, EPA, and DHA) for assessing the extraction efficiency. Aliquots of the plasma samples were spiked with the standard solution of FFAs. The rate of recovery for each FFA was not less than 60% in the mouse plasma sample, and in the range of 34–96% in the mouse liver sample (Table 2). The liver MA level showed a low recovery rate (approximately 34%), suggesting that the ionization of liver MA had been suppressed by other liver metabolites. These results indicate that all FFAs, except liver MA, could be detected with adequate sensitivity by our LC-MS method.

**Sensitive analysis of FFAs**

The purpose of this study was to develop a high-throughput and sensitive LC-MS method for quantifying FFAs in plasma and organs. We optimized the conditions for the quantification of ten FFAs in mouse plasma and liver by LC-MS. This analytical method is intended for major long-chain FFAs, including MA, PA, POA, SA, OA, LIA, LNA, AA, EPA, and DHA.

Previous studies have reported that 20 µL of plasma was required for FFA quantification by using a non-conversion-based method, and 5 or 10 µL of plasma was required for the dimethylaminoethyl or trimethylaminoethyl ester method. However, only 2 µL of plasma and 10 mg of liver were required to detect major FFAs by our method.

**Analysis of the FFA profile in mouse plasma and liver treated with bezafibrate**

To elucidate the effects of bezafibrate on the plasma and liver FFA profiles for mice, we investigated the levels of individual FFAs in plasma and liver samples from an obese mouse model, which had been treated or not with bezafibrate, by using the optimized analysis method. We first assessed whether bezafibrate was absorbed into the mouse plasma and liver, and detected bezafibrate as an unfragmented deprotonated molecular ion ([M − H]⁰) peak in the plasma and liver samples (Supplemental Figs. 1 and 2; see Biosci. Biotechnol. Biochem. Web site). The data show that the respective bezafibrate concentrations in mouse plasma and liver were 12.21 ± 0.97 µg/mL of plasma and 4.12 ± 0.14 µg/mg of liver.

The ten major FFAs, MA, PA, POA, SA, OA, LIA, LNA, AA, EPA, and DHA, could be detected in the plasma samples by this method. The concentrations of POA, OA, and LNA in the plasma were increased to 231.6%, 164.1%, and 123.5% of their respective contents in the control sample by the bezafibrate treatment, while those of AA and DHA were decreased to 72.5% and 38.7% of their respective contents in the control sample (Fig. 2). These data suggest that PPARα activation changed the plasma FFA profile.

We applied our assay system to the quantitative analysis of FFA in the mouse liver. The concentrations of POA, OA, and LIA in the liver were increased to 509.1%, 240.6%, and 133.1% of their respective contents in the control sample, but that of SA was decreased to 76.8% of the SA content in the control sample by the bezafibrate treatment (Fig. 3). Characteristically, the concentrations of monounsaturated FFAs, i.e., POA and OA, were increased in both the plasma and liver by the
bezafibrate treatment. POA and OA are respectively synthesized from PA and SA by stearoyl-CoA desaturase (SCD). We also found that the SCD-1 mRNA expression level in the liver was increased to 377.4% vs. the control by the bezafibrate treatment (Fig. 4). These data suggest that the increase of OA and POA by the bezafibrate treatment was involved in the enhancement of SCD expression. It has been reported that SCD was a target gene of PPARγ.29) It has recently been demonstrated that bezafibrate activated SCD in the rat liver, 30) consistent with our present data. Taken together, these results suggest that plasma POA and OA were markers of PPARγ activation.

The previous study confirmed that, in situations in which there was an increase of monounsaturated n-9 fatty acids in adipocytes, the lipolytic activity of the adipocytes was increased.31) Moreover, another study has demonstrated that supplementing POA to bovine adipocytes resulted in down-regulated lipogenesis and desaturation, and up-regulated β-oxidation.32) We confirmed in this present study that the bezafibrate treatment also decreased the adipose tissue weight (data not shown). These observations raise the possibility that the POA induced by bezafibrate contributed to the decreased adipose tissue weight. We found in this study that the plasma concentration of LNA was increased, but of AA and DHA were decreased by the bezafibrate treatment (Fig. 2). However, the concentrations of these FFAs in the liver were not changed by the bezafibrate treatment (Fig. 3). This raises the possibility that the change in concentrations of these FFAs reflects the FFA profiles of organs other than the liver. More physiological studies are needed to link these effects to PPARγ activation.

In summary, we report a high-throughput and highly sensitive LC-MS method for identifying and quantifying individual long-chain FFAs in mouse plasma and liver. This highly sensitive assay enables the quantification of individual FFAs by using trace amounts of samples (2 μL of plasma and 10 mg of liver tissue). We applied this method to analyze the long-chain FFA profiles of plasma and liver samples from an obese mouse model that had been treated with bezafibrate, and have shown a change in the long-chain FFA profile, particularly in respect of the POA and OA contents. This FFA assay can also be adapted for assessing the lipid metabolism in peripheral tissues.

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