LC/MS/MS Method for Quantitative Determination of Long-Chain Fatty Acyl-CoAs

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Long-chain acyl-CoA esters (LCACoAs) are activated lipid species that represent key substrates in lipid metabolism. The relationship between lipid metabolism disorders and type 2 diabetes has attracted much attention to this class of metabolites. This paper presents a highly sensitive and robust on-line LC/MS² procedure for quantitative determination of LCACoAs from rat liver. A fast SPE method has been developed without the need for time-consuming evaporation steps for sample preparation. LCACoAs were separated with high resolution using a C18 reversed-phase column at high pH (10.5) with an ammonium hydroxide and acetonitrile gradient. Five LCACoAs (C16:0, C16:1, C18:0 C18:1, C18:2) were quantified by selective multireaction monitoring using a triple quadrupole mass spectrometer in positive electrospray ionization mode. It is possible to perform a neutral loss scan of 507 for lipid profiling of complex LCACoA mixtures in tissue extracts. The method presented was validated according to ICH guidelines for quantitative determination of five LCACoAs for physiological concentrations in 100–200 mg of tissue with accuracies ranging from 94.8 to 110.8%, interrun precisions between 2.6 and 12.2%, and intrarun precisions between 1.2 and 4.4%. Due to the high sensitivity of the developed method, the amount of tissue biopsied for reliable quantification can be reduced. This may be advantageous in the quantification of LCACoAs in humans.

Long-chain acyl-CoA esters (LCACoA) are key substrates for lipid biosynthesis and oxidation and are effectors for many other important enzymatic reactions in reference to lipid metabolism. Additionally they play a role in the regulation of cell metabolism and cell signaling. In particular, the evident relationship between lipid metabolism disorders and type 2 diabetes has attracted much attention to these metabolites. Besides the established correlations between obesity and insulin resistance, which is one of the main characteristics of type 2 diabetes, it becomes apparent that the intensity of insulin resistance is especially affected by the distribution of triglycerides in different tissues. Recent investigations have focused on activated metabolites of triglycerides and free fatty acids, such as LCACoA, in cell metabolism. This subject has been investigated in many human and animal studies. In contrast to most long-chain fatty acids, long chain n-3 fatty acids have been shown to protect against insulin resistance. Thus, it seems that the fatty acyl-CoA profile is also a crucial point in the genesis of insulin resistance. A simple, efficient, and sensitive method for LCACoA quantification from biological tissues is crucial for investigating the role of LCACoAs and the LCACoA profile in type 2 diabetes.

There are a number of published methods employing different techniques, such as capillary electrophoresis with UV detection, HPLC/UV, HPLC with fluorometric detection, GC-FID, LC/MS/MS, and others.

References:


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The low sensitivity and selectivity of UV detection methods require chromatographic separation of interfering compounds and all LCACoA species, which results in very long analysis times, e.g., up to 120 min per run in the case of HPLC and 30 min per run for CE. In contrast, high selectivity and sensitivity can be achieved by using GC methods, especially GC/MS methods. The drawbacks of these methods include time-consuming derivatization steps, and the need for isotopically labeled internal standards for viable quantification. Recently published HPLC methods with fluorometric detection show impressive sensitivity with a linear detection range from 6 to 50 000 fmol. However, to achieve this high sensitivity, a derivatization step using chloroacetaldehyde is needed to introduce the fluorochrome (e.g., acyl etheno CoA). ELISA methods for LCACoA quantification have also been published, but they can neither reach the reported limit of detection (LOD) of fluorometric methods nor are they able to generate fatty acyl-CoA profiles. There are only a few papers reporting quantification of LCACoA using mass spectrometry. Kalderon et al. employed negative ESI-MS/MS in multireaction monitoring (MRM) mode without using any chromatographic separation technique. After tissue extraction, the samples are introduced into the ESI source by direct flow injection. In MRM mode, only interferences of isotopic distribution of saturated (M) and unsaturated (M-2) LCACoAs having the same chain length occur. The content of the respective LCACoAs must thus be corrected by subtracting the intensity contributed by the 13C isotope peaks of the corresponding M-2 LCACoA as shown by these authors. The less selective selected ion monitoring (SIM) quantification mode involves additional interferences, which must be separated by HPLC. Kasuya et al. described a method for medium-chain AcylCoA (MCACoA) quantification based on SIM with a positive ionization mode and an HPLC run time of 40 min. Here we report the first LC–MS2 method in positive ESI for the quantification of LCACoAs. By using positive ESI and ammonium hydroxide as mobile-phase additive, this new method provides excellent sensitivity with a LOD score in the femtomole range combined with the possibility of investigating fatty acyl-CoA profiles of complex LCACoA mixtures by neutral loss scans. Furthermore, HPLC was chosen to avoid ion suppression effects leading to nonlinear behavior and to eliminate interferences due to natural isotopic distribution. The LC–MS2 method presented is capable of baseline separating all interfering LCACoAs and thus constitutes a very fast and robust quantification method. Furthermore, the extraction procedure used for this method was accelerated by eliminating time-consuming evaporation steps within the extraction protocol.

**EXPERIMENTAL SECTION**

**Chemicals.** Water (HPLC grade), methanol (HPLC grade), acetonitrile (HPLC grade), ammonium hydroxide (25% aqueous solution), potassium phosphate monobasic (>99.5%), ammonium sulfate (>99%), palmitoyl CoA (95%), palmitoleoyl CoA (96%), heptadecanoyl CoA (98%), stearoyl CoA (98%), oleoyl CoA (95%), and linoleoyl CoA (94%) were purchased from Sigma-Aldrich Handels GmbH (Vienna, Austria). 2-Propanol (>99.7%) was obtained from Merck KGaA (Darmstadt, Germany).

**Animals.** After an overnight fast, male Wistar rats were anesthetized with pentobarbital, and the livers were freeze-clamped in situ, excised immediately, and placed in liquid nitrogen. Rats were then euthanized with a lethal dose of pentobarbital. Frozen tissues were powdered in liquid nitrogen using a mortar, and aliquots of 0.1–0.2 g were placed in dry ice-cooled sample vials and weighed immediately. Samples were stored at −70 °C until extraction.

**Extraction Method.** Sample preparation was performed analogously to the method reported by Deutsch et al. with significant changes to obtain a more practicable extraction procedure. All solutions and solvents used for extraction were precooled at 4 °C. A 1-mL aliquot of 0.1 M KH2PO4, 1 mL of 2-propanol, and 50 μL of 1.6 mM heptadecanoyl CoA internal standard were added to the frozen rat liver aliquots. The emulsion was homogenized using a Heidolph Diax 100 for 30–60 s. A 125-μL aliquot of saturated aqueous ammonium sulfate and 2 mL of acetonitrile were added to the homogenate and the resultant mixture was vortexed. After 10-min centrifugation at 2500g, the supernatant was diluted with 10 mL of 0.1 M KH2PO4, pH 4.9.

**Solid-Phase Extraction.** Solid-phase extraction was carried out on Oasis HLB 1 cm3 (30 mg) cartridges (Waters, Milford, MA). After washing with 3 mL of acetonitrile and conditioning with 2 mL of 25 mM KH2PO4, 4 mL of liver extract was loaded onto the cartridge and washed with 4 mL of water. Elution was done using 0.5 mL of 40:60 acetonitrile/water solution containing 15 mM ammonium hydroxide. The eluate was then further diluted with 2.5 mL of water.

**Preparation of Standard Solutions.** Stock solutions of LCACoA containing concentrations between 1 and 5 mM were prepared in 1:1 methanol/water and stored at −70 °C. Final concentrations of spike solutions and internal standard were prepared by diluting stock solutions with 10% aqueous acetonitrile. Calibration standard mixtures containing five LCACoAs were prepared by adding 200, 150, 100, and 50% of estimated average physiological amounts of each compound (palmitoyl-CoA 4 nmol, palmitoleoyl-CoA 2 nmol, stearoyl-CoA 2 nmol, oleoyl-CoA 6 nmol, and linoleoyl-CoA 6 nmol) to 200 μg of acyl-CoA-free tissue. LCACoA free tissue was obtained by storing liver aliquots for 1 h at room temperature. After 1 h, LCACoA concentrations are below the LOD. Extracts obtained from calibration standards were aliquoted and stored at −70 °C until used.

**HPLC/MS Conditions.** All experiments were carried out on an Ultimate-System ( Dionex, LCPackings) comprising a Famos autosampler with cooled tray coupled to a Quantum TSQ Ultra AM (ThermoFinnigan). The system was controlled by Xcalibur Software 1.3. Separation was performed on a Zorbax 300 Extend...
C18, 3.5-μm column (2.1 × 150 mm, Agilent). The flow rate was set to 200 μL/min. Elution solvent A consisted of 10:90 acetonitrile/water, and solvent B was pure acetonitrile, both containing 15 mM ammonium hydroxide. Elution solvent C, which consisted of 30:70:0.1 water/acetonitrile/formic acid was used to elute nonspecifically bound substances from the HPLC column to achieve long-term separation stability. The chromatographic conditions were as follows: 0% B to 45% B/55% A in 8 min; 8–10 min, isocratic 45% B/55% A; 45% B/55% A to 100% C in 2 min; 100% C for 5 min, and 100% C to 100% A in 2 min. Equilibration time with A was set to 8 min. Separation was performed at room temperature. Injection volume was 10 μL.

Positive ESI-MS/MS mass spectrometry was performed using the following parameters: spray voltage 5.5 kV, capillary temperature 300 °C, sheath gas pressure 30 AU, auxiliary gas 3 AU, optimized collision energy for neutral loss and MRM 40 eV. Neutral loss scans were obtained by scanning from 800 to 1100 m/z in 700 ms with a neutral loss mass of 507 Da. Quantitation was performed by MRM. Recorded reactions were as follows: 1004.33 → 497.305 m/z for palmitoyl-CoA, 1006.35 → 499.357 m/z for palmitoyl-CoA, 1030.35 → 523.355 m/z for stearoyl-CoA, 1032.36 → 525.365 m/z for oleoyl-CoA, 1034.38 → 527.345 m/z for stearoyl-CoA, and 1020.36 → 513.365 m/z for heptadecanoyl-CoA as internal standard.

Validation Procedure. Linearity was tested by three replicate analyses of five calibration standards (200, 150, 100, and 50% of expected physiological concentrations and LCACoA-free tissue). Accuracy was investigated by adding known amounts of LCACoA (180, 120, and 60% of estimated average physiological level of each LCACoA) to acylCoA-free liver homogenate. LCACoAs of five replicate samples of each concentration were extracted and measured twice. Intrarun precision was investigated by six independent measurements of one replicate of a high-level (180%) and low-level (60%) sample. Interrun precision was obtained by extracting and measuring twice five aliquots of a high-level (180%) and low-level (60%) sample.

RESULTS AND DISCUSSION

LC–MS Analysis. Only a few methods using mass spectrometry for quantification of LCACoAs have been published, the majority of them employing negative ionization mode.32,34 Negative ionization mode mass spectra show abundant [M–H]− and [M–2H]2− ions. In contrast, in positive ionization mode only one abundant ion, [M–H]+, is generated, thus leading to better sensitivity. In addition, when low contaminations of TFA are present in the LC–MS/MS-system, TFA causes a strong background noise and thus a significant loss of sensitivity in negative ESI. As TFA is often needed as a mobile-phase additive for achieving superior separation performance, negative ESI can be critical. No negative influences of TFA contaminations in the system were observed in the positive ESI mode. As HPLC eluents are very basic, positive charged ions of LCACoAs are not expected to exist in appreciable concentrations in solution. In fact, LCACoAs in HPLC eluents are present as anions due to their three phosphate esters. However, the high abundance of positive charged ions in ESI is probably due to gas-phase ion–molecule reactions or collision-induced dissociation of [M + NH4+] ions.35

![Figure 1. MS/MS fragmentation of protonated acyl-CoA C16:1 (parent ion 1006 m/z, collision energy 40 eV, direct flow injection, flow rate 0.2 mL/min, mobile phase acetonitrile/water 50:50 containing 15 mM ammonium hydroxide). Homologous fragmentations could be observed for all LCACoAs investigated.](image)

Scheme 1. Fragmentation Used for MRM and Neutral Loss Scans

The exact mechanism of positive ion formation under alkaline LC/MS/MS conditions ("wrong-way-round ionization") are not fully understood at this time.36 In the positive mode, the predominant ion in MS2 spectra is the fatty acyl–panthetheine fragment, which is derived from cleavage between the ADP and pantetheine residues with charge retention on the fatty acyl portion (Figure 1). We chose the fatty acyl–panthetheine for quantification because it is characteristic of CoA-activated substances, and the substance itself, and was very abundant. By choosing the fatty acyl–panthetheine of interest in MRM mode, we were able to develop a very sensitive and selective LC–MS2 method for quantification of LCACoAs. Furthermore, fatty acyl-CoA profiles of complex LCACoA mixtures can be obtained by using neutral loss scan of 507 Da. CoA-activated substances can be monitored by scanning for the neutral loss of phosphoadenosine diphosphate (Scheme 1). The total ion current of a neutral loss scan of a Wistar rat liver extract is shown in the upper panel of Figure 2. Chromatograms of m/z of CoA-activated fatty acids can be seen in the lower panels of this figure (m/z 1054 for C20:4 arachidonic acid, m/z 978 for C14:0, m/z 976 for C14:1, m/z 974 for C14:2, m/z 950 for C12:0, m/z 948 for C12:1, m/z 922 for C10:0).

Generally, for reliable quantification, all interfering peaks must be chromatographically separated. In our case, baseline separation

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of all interfering peaks is achieved in 10 min using the HPLC conditions described (Figure 3). Isotopic peaks (IP) of homologous LCACoAs are also separated. Figure 3 shows a MRM chromatogram of five different LCACoAs extracted from Wistar rat liver. For accurate quantification, it is necessary to separate all saturated and single and multiple unsaturated LCACoAs bearing the same number of carbon atoms, e.g., in this case, C18:0 acylCoA, C18:1 acylCoA, and C18:2 acylCoA. This is of utmost importance as in biological samples concentrations of saturated and single and multiple unsaturated LCACoAs bearing the same number of carbon atoms are not identical. For example, in the case of liver samples, concentrations of C18:1 acylCoA can be much higher than for C18:0 acylCoA. This leads to a contribution of the isotopic peak of C18:1 acylCoA (m/z +2) up to 40% of the C18:0 acylCoA signal. C18:2 acylCoA also shows a significant interference with C18:1 acylCoA as shown in Figure 3. Surprisingly this phenomenon was not reported by Mauriala et al.34

We observed a fast decrease in separation performance by using mobile phases with a pH of 10.5 (Figure 4). We believe that this phenomenon is due to nonspecific interactions of analytes present in the sample with the separation column. An additional column cleaning step with 30:70:0.1 water/acetonitrile/formic acid (pH 2.5) was thus used to regenerate the HPLC column after each run. By switching the pH from 10.5 to 2.5, nonspecifically bound analytes are removed as free silanol groups of the stationary phase are protonated and the charge distribution of LCACoAs is converted. By performing this cleaning step for 5 min after each run, no losses in separation performance could be observed. A chemically highly inert stationary phase is needed since this newly developed method uses mobile phases of pH varying from 2.5 to 10.5. By employing Zorbax 300 Extend C18 no changes in separation performance due to pH or nonspecific binding was observed.

Another objective in the development of this new method was to facilitate time-consuming sample workup. We thus focused on evaporation steps, as these are the most time-consuming parts of the LC–MS/MS methods described.28–31 Evaporation is required in all published LC–MS/MS methods to concentrate extracted analytes prior to analysis. Analytes are preconcentrated by a factor of 20–40 depending on the published method.34,35 Due to the superior sensitivity of this new method, which is due to extremely narrow HPLC peaks, comparable to GC peaks (Figure 32–34,38 Evaporation is required in all published LC–MS/MS methods to concentrate extracted analytes prior to analysis. Analytes are preconcentrated by a factor of 20–40 depending on the published method.34,35 Due to the superior sensitivity of this new method, which is due to extremely narrow HPLC peaks, comparable to GC peaks (Figure 2892 Analytical Chemistry, Vol. 77, No. 9, May 1, 2005

Figure 2. Neutral loss scan of liver extract (Wistar rat, neutral loss 507 Da, collision energy 40 eV). m/z of C10–C20 fatty acyl-CoAs are shown. HPLC conditions are described in the Experimental Section.

Figure 3. MRM chromatograms of an extract obtained from rat liver (Wistar rat) for quantification. IP, isotopic peaks of homologous LCACoAs. HPLC/MS conditions are described in the Experimental Section.

Figure 4. SRM chromatograms of C18:0 acyl-CoA (1034 − 527.345 m/z) derived from HPLC separation of an aqueous mixture of LCACoAs (70 pmol of C16:0 acyl-CoA, 35 pmol of C16:1 acyl-CoA, 35 pmol of C18:0 acyl-CoA, 105 pmol of C18:1 acyl-CoA, 105 pmol of C18:2 acyl-CoA) injected after 20 injections respectively. IP, isotopic peaks of C18:2 acyl-CoA (minor) and C18:1 acyl-CoA (major) (A) HPLC conditions as described in the Experimental Section. (B) HPLC conditions as in panel A, except that no column cleaning step with low-pH eluent C (30:70:0.1 water/acetonitrile/formic acid, pH 2.5) was performed.

3), and the excellent sensitivity of the TSQ Quantum Ultra mass spectrometer used, preconcentration steps are not needed. Instead, we were able to eliminate all evaporation steps by simply diluting the SPE extract with 2.5 mL of water. The acetonitrile solvent and was estimated at 60% by comparing intensities of standard deviation of blank; b, slope. c Percent of estimated average physiological concentration (amount per g of liver). d Values in parentheses are in picomoles.

Table 1. Linearity, Correlation Coefficients, Slopes, Intercepts, and LODs Obtained from Three Replicate Analyses

<table>
<thead>
<tr>
<th>compd</th>
<th>cor coeff (r²)</th>
<th>equation y = a (SD) + xb (SD)</th>
<th>LOD - 60% (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C16:0</td>
<td>0.9954</td>
<td>y = -0.026 (0.044) + x0.0162 (0.0003)</td>
<td>0.61 (120)</td>
</tr>
<tr>
<td>C16:1</td>
<td>0.9946</td>
<td>y = -0.026 (0.037) + x0.0176 (0.0004)</td>
<td>0.27 (27)</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.9956</td>
<td>y = -0.0027 (0.0008) + x0.00361 (0.00006)</td>
<td>2.2 (220)</td>
</tr>
<tr>
<td>C18:1</td>
<td>0.9955</td>
<td>y = -0.026 (0.037) + x0.0162 (0.0003)</td>
<td>0.25 (75)</td>
</tr>
<tr>
<td>C18:2</td>
<td>0.9951</td>
<td>y = -0.013 (0.054) + x0.0226 (0.0004)</td>
<td>0.35 (105)</td>
</tr>
</tbody>
</table>

a Mandel test passed for every compound. b LOD = 3.3σ/b; σ, standard deviation of blank; b, slope. c Percent of estimated average physiological concentration.

determined to be between 2.6 and 12% (Table 3). The long-term stability of the method was considered adequate as no significant alteration of calibration curves in terms of R² and slope was observed within the validation period of 10 days. The signal-to-noise ratio was between 140 and 400 for the lowest point (except blank) of all LCACoA standard curves, which is well above the criteria of LOQ. Limit of detection (LOD) estimated by the standard deviation of response of blank and the slope was 2.2% of estimated physiological level for stearoyl-CoA (220 pmol per g of liver) and below 0.7% for the rest of measured LCACoAs.

To the authors’ knowledge, validation data for the determination of LCACoAs are only available for the LC/MS method described by Mauriala et al.34 For the quantification of C16:0, these authors found an overall precision and accuracy of 12.5 and 94.1%, respectively. Validation data for other LCACoAs are not reported in this publication. Thus, regarding the determination of C16:0, the herein presented method shows significant improvements in precision and accuracy compared to this previously published method.

To demonstrate reproducibility, one liver sample from a Wistar rat was divided into six aliquots. All aliquots were worked-up independently, and LCACoAs concentrations were determined (Table 4). The amounts found in rat liver were 22.0 ± 3.1 nmol/g C16:0, 7.6 ± 0.8 nmol/g C16:1, 4.4 ± 0.7 nmol/g C18:0, 24.2 ± 2.5 nmol/g C18:1, and 41.0±3.0 nmol/g C18:2. The results reported here are in line with previously reported values.11

CONCLUSIONS

The LC−MS² method presented is a robust, sensitive, and easy to perform method for quantification of LCACoAs from biological samples.
tissue. The atypical HPLC conditions generate sharp peaks leading to high sensitivity, and by inclusion of a washing step, excellent long-term stability is achieved. In addition, all interferences generated by natural isotopic distribution of homologous LCACoAs are eliminated under the HPLC conditions used. Furthermore, by combining positive electrospray ionization and selective MRM scanning, LCACoAs can be quantitated under very robust and sensitive conditions. Finally, sample preparation is significantly accelerated by eliminating evaporation steps. Due to the high sensitivity of the developed method, the amount of tissue biopsied for reliable quantification can be reduced. This may be advantageous in the quantification of LCACoAs in humans.

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