A mass spectrometric approach to investigate cardiolipin metabolism in Barth syndrome
Valianpour, F.

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CHAPTER 4

Quantitative and Compositional Study of Cardiolipin in Platelets
by Electrospray Ionization Mass Spectrometry: Application for the
Identification of Barth Syndrome Patients

Fredoen Valianpour, Ronald J.A. Wanders, Peter G. Barth, Henk Overmars,
and Albert H. van Gennip

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Chapter 4

ABSTRACT Background: The concentration of cardiolipin (CL) in cultured skin fibroblasts is a useful indicator of Barth syndrome (BTHS; MIM 302060), but the sampling and culturing of fibroblasts are burdensome and time-consuming procedures. We investigated whether the analysis of CL in platelets might help to identify BTHS in patients suspected of having this condition.

Methods: We used HPLC and online electrospray ionization mass spectrometry (HPLC-ESI-MS) to quantify CL molecular species. The CL content of platelets was studied in blood samples of BTHS and non-BTHS patients. Control blood samples drawn from healthy adults were collected and analyzed within 24 h (n=10) and 48 h (n=10) to characterize any effect of sample shipping time on the CL content in platelets. Samples were collected from children 1–10 years of age who were not affected by BTHS (n=6) and from BTHS patients (n=4) and analyzed within 24 h. Results for all four groups were compared by a Student t-test for all individual analyses.

Results: We found different CL molecular species, e.g., (C18:2)4-CL. BTHS patients had a specific decrease of tetralinoleyl-CL concentrations in platelets (0.1–0.5 nmol/mg of protein; n=4) compared with all control groups (2.3–5.5 nmol/mg of protein; n=26). Only minor differences were observed among the different control groups.

Conclusions: Quantitative and compositional analyses of CL in platelets by the proposed method allow identification of BTHS patients more rapidly than gene analysis or analysis of CL in cultured skin fibroblasts. The abnormality of CL may explain the abnormal mitochondrial function observed in BTHS. The differences between the control groups did not cause any complication.

INTRODUCTION Cardiolipin (CL) is a unique phospholipid with a dimeric structure (diphosphatidylglycerol) that contains four acyl groups and carries two negative charges. CL is found almost exclusively in the mitochondrial inner membrane and plays a central role in mitochondrial oxidative phosphorylation because several respiratory chain complexes depend on CL (1, 2).

The trivial name “cardiolipin” was derived because this phospholipid was first found in animal hearts, where it is especially abundant. However, CL is found in the mitochondria of animal species, yeasts, and bacteria (3). The biosynthetic pathway of CL is similar to those of other phospholipids; it passes through the common intermediates, phosphatidic acid and phosphatidyl-CMP. However, the final step is a unique reaction that is different between prokaryotes and eukaryotes (4). In prokaryotes, diphosphatidylglycerol synthase catalyses the transfer of a phosphatidyl moiety from one phosphatidylglycerol (PG) to the free 3'-hydroxyl.
Quantitative and Compositional Study of Cardiolipin in Platelets

group of another PG (3–5), whereas eukaryotic diphasphatidylglycerol synthase links an activated phosphatidyl moiety (phosphatidyl-CMP) to PG (3,5–7). In animal tissues, CL contains almost exclusively 18-carbon fatty acids, and 80% of these are typically linoleic acid (C18:2n-6) (7).

This also appears to be true for the higher plants (7). Yeast PG differs by having more C16:1 and C18:1 fatty acids (4), whereas the bacterial lipid contains saturated and monoenoic fatty acids with 14–18 carbons (8,9). The precise function of CL is still a matter of debate. Variations in the concentrations of CL or its fatty acid composition affect the function of respiratory chain complexes (10). Recently, we showed defective incorporation of linoleic acid into CL in cultured skin fibroblasts of patients suffering from Barth syndrome (BTHS) (10).

BTHS (MIM 302060) is an X-linked recessive disorder characterized by infantile or childhood onset of dilated cardiomyopathy, neutropenia, skeletal myopathy (11), abnormal mitochondrial ultrastructure (12,13), and variable mitochondrial respiratory chain dysfunction. This dysfunction is evidently unrelatated to a single respiratory chain complex in skeletal muscle (11,14) and in cultured fibroblasts (15). Biochemical findings of BTHS include increased urinary excretion of 3-methylglutaconic acid, 3-methylglutaric acid and 2-ethylhydracrylic acid (16,17) and moderately decreased serum cholesterol concentrations (17). The gene involved in the disease is the G4.5 gene (18), or tafazzin (TAZ) gene (18), which is localized at Xq28 (19).

The TAZ gene has 11 exons and two ATG initiation sites (on exons 1 and 3). Diagnosis of BTHS has been by molecular analysis through the identification of mutations in the G4.5/TAZ gene. A biochemical method for BTHS was lacking until recently, when we presented a biochemical test based on the incorporation of linoleic acid into the phospholipids of cultured skin fibroblasts to distinguish between BTHS patients and controls (10). However, this method (10) and other conventional analytic methods for quantitative or compositional analysis of CL (20–23) are typically multistep procedures. Furthermore, they are time-consuming, and the analyses require a large amount of mitochondrial membrane.

The availability of a simple, rapid method for the microanalysis of CL is important for investigation of CL metabolism in various tissues. Our primary goal was to develop a rapid method to analyze CL isolated from platelets for research and diagnostic purposes. We used platelet samples from BTHS patients to show the usefulness of this method.

**MATERIALS AND METHODS**

All solutions were of analytical quality and were purchased from Merck. (C14:0)4-CL and (C14:0)2-PG [internal standard (IS)] were purchased from Avanti Lipids.
Commercially available \((C18:2)_4\)-CL was purchased from Fluka BioChemika (cat. no. 71238). The analytical HPLC LiChrospher Si 60 column (2 x 250 mm; 5 m particle size) was from Merck.

SAMPLES

To check the possible effect of sample shipping time, blood samples were collected (2 mL) with EDTA as an anticoagulant and kept at room temperature for 24h (24h blood samples; n=10) and 48h (48h blood samples; n=10). Blood samples (2 mL) from patients with BTHS were collected and analyzed within 24h (n=4; Table 3). Blood samples (2 mL) from children (age range, 2–10 years; n=6) without BTHS were collected and analyzed in the same manner as those from BTHS patients. All samples were collected according to institutional guidelines for blood sampling, including procurement of informed consent of the persons involved or their representatives.

PROCEDURES

ISOLATION OF PLATELETS

EDTA-anticoagulated whole blood was kept at room temperature and mixed continually. The blood (2 mL) was centrifuged at 600g for 10 min at room temperature, and the resultant platelet-rich plasma was then centrifuged at 11 000g (4°C). The pellets were washed twice with phosphate-buffered saline, centrifuged at 11 000g (4°C), and subsequently stored at 80°C.

EXTRACTION OF LIPIDS

Lipids were extracted using a modification of the method of Folch et al. (24). In brief, platelet pellets were suspended in 1 mL of water and sonicated twice for 10 s; 50 μL was then removed and used for protein measurement. We then added 50 μL of a 7.8 μmol/L solution of IS \([(C14:0)_4\)-CL] in chloroform–methanol (2:1 by volume) and 3 mL of a chloroform–methanol solution (2:1 by volume) to the remaining cell suspension. The mixture was then shaken vigorously and put on ice for 15 min.

Phase separation was achieved by centrifugation at 1000g for 5 min. The organic layer was removed, and the aqueous layer was extracted again with 3 mL of a chloroform–methanol solution (2:1 by volume). The organic layers were combined and washed with 1 mL of chloroform–methanol–water (1:46:47 by volume). The organic layer was evaporated under a stream of nitrogen gas. The residue was dissolved in 150 μL of chloroform-methanol-water (50:45:5 by volume) and designated the lipid extract.

HPLC

The HPLC system consisted of a HP1100 series binary-gradient pump, a vacuum degasser, a column temperature controller (all from Hewlett-Packard), and a Gilson 231 XL.
autosampler (Gilson). The column temperature was maintained at 22°C. The lipid extract was injected onto a 2.1 x 250-mm silica column (5μm particle diameter; Merck). The phospholipids were separated from interfering compounds by a linear gradient between solution B (chloroform) and solution A (methanol-water; 9:1 by volume). Both solutions contained 0.1 mL of 250 mL/L aqueous ammonia per liter of solution.

The gradient (0.3 mL/min) was as follows: 0–3 min, 20% A to 100% A; 3–8 min, 100% A; 8–8.1 min, 100% A to 0% A; and 8.1–14 min, equilibration with 0% A. All gradient steps were linear, and the total analysis time, including equilibration, was 14 min. We used a splitter between the HPLC column and the mass spectrometer, and 30 μL/min of eluant was introduced into the mass spectrometer. An electrically operated valve ensured that the eluant was introduced into the mass spectrometer from 4 to 8 min only.

**MASS SPECTROMETRY (MS)**

A Quattro II triple-quadrupole mass spectrometer (Micromass) was used in the negative electrospray ionization (ESI) mode. Nitrogen was used as the nebulizing gas, and argon was used as the collision gas at a pressure of 0.25 Pa. The capillary voltage was 3kV, the source temperature was set at 80°C, and the optimal cone-voltage energy was 30 V. Daughter fragments of m/z 723.6, 724.6, and 725.6 (CL molecular species) were obtained using optimal collision energy of 40 eV. High cone-voltage energy (100 V) was used to obtain the fatty acid composition in the CL fraction. Both tetralinoleyl-CL (C18:2)₄-CL and (C14:0)₄-CL (IS) were measured by single-ion monitoring of their double-charged ions in negative mode.

**VALIDATION**

The HPLC and mass spectrometer settings were optimized with a 10 μmol/L solution of commercially available (C14:0)₄-CL (synthetic) and (C18:2)₄-CL in chloroform-methanol-water (50:45:5 by volume) containing 0.1 mL/L of 250 mL/L aqueous ammonia. Of this calibrator solution, 5 μL was injected into the HPLC column, and one-tenth of the eluting solution was introduced into the mass spectrometer. The purity of the commercially available (C18:2)₄-CL was assessed by measuring all CL molecular species in the sample and calculating the purity of (C18:2)₄-CL as a percentage of the total. The linear response and detection limit for (C18:2)₄-CL were established by injection of calibration mixtures with different concentrations of this analyte and a constant concentration of (C14:0)₄-CL as the IS. The amount of (C18:2)₄-CL in the samples was calculated as follows: amount of (C18:2)₄-CL/mg of protein* (peak area analyte * IS concentration * response factor)/(peak area IS * mg of protein used in the assay).

The extraction efficiencies for both the IS and analyte were obtained by comparing their peak intensities in the extracted calibration mixtures with those in the unextracted
calibration mixtures with the same final concentrations without extraction. The intraassay (within-day) variation of the method was determined by measuring 10 times a nonenriched sample and a sample enriched with commercially available \(\text{(C18:2)}_4\)-CL at low (2 nmol/mg of protein), medium (10 nmol/mg of protein), and high (100 nmol/mg of protein) concentrations. The interassay (between-day) variation was determined by measuring blank samples and samples enriched with commercially available \(\text{(C18:2)}_4\)-CL (10 and 100 nmol/mg of protein) over 3 separate weeks. The recovery efficiency of the method was established by measuring 10 different samples before and after enrichment with a known amount of commercially available \(\text{(C18:2)}_4\)-CL. We investigated ion suppression from interfering substances by comparison of the peak intensities of both \(\text{(C18:2)}_4\)-CL and \(\text{(C14:0)}_4\)-CL in the enriched samples with those of calibrator solutions with similar concentrations.

In all experiments, no special precautions (e.g., cleaning of the high-voltage lens and sample cone) were taken to optimize the detection limit of the MS system. Platelets from different control groups and from BTHS patients were analyzed to establish the usefulness of this method. The results of these experiments were compared by a Student t-test for all individual analyses.

**RESULTS**

**GENERAL ASPECTS OF THE HPLC-ESI-MS METHOD**

CL was successfully separated from the nonpolar lipids and other phospholipids by HPLC, which increased the purity of the CL fraction. The solvent system formed a good matrix for the ionization of CL. After separation by HPLC, individual CL molecular species such as \(\text{(C18:2)}_4\)-CL were measured by scanning negatively charged ions. We detected doubly as well as singly charged ions of CL molecular species. Both ions had the same retention time and the same daughter ions, which indicated that both ions belonged to the same compound (data not shown). Under optimal experimental conditions, the amount of single-ion species was 0.5% of total ions (both singly and doubly charged). Thus, we used the doubly-charged ions to measure the CL species with high sensitivity by use of the single-ion monitoring application. Daughter-ion scanning and high cone-voltage MS were also used to determine the structure of CL molecular species.

Figure 1 shows the CL molecular species isolated from a platelet sample detected by tandem MS in the elution fraction (peak) of CL. The peak at m/z 619.6 belongs to \[\text{[(C14:0)}_4\text{-2H}]^2\text{-CL}\] (IS). The peaks at m/z 723.6, 724.6, and 725.6 belong to \[\text{[(C18:2)}_4\text{-2H}]^2\text{-CL}, \\text{[(C18:2)}_3\text{/(C18:1)} \text{1-2H}]^2\text{-CL}, \text{and [(C18:2)}_2\text{/(C18:1)}_2\text{-2H}]^2\text{-CL}\], respectively.

Figure 2 shows the daughter-fragment spectra of these molecular species. The m/z 723.6 peak has C18:2 (m/z 279.2; Figure 2) exclusively as a side chain, whereas the peaks at m/z
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Figure 1: Mass spectrum of CL molecular species from platelets. Phospholipids are extracted from platelets and separated by HPLC. The mass spectrum was obtained from the elution fraction of CL. The insets at the top show the relevant areas of the spectrum in more detail. Different molecular species are shown. The peak at m/z 619.6 belongs to [(C14:0)₄-2H]²⁻-CL (IS), whereas the peak at m/z 723.6 belongs to [(C18:2)₄-2H]²⁻-CL. The peak at m/z 724.6 corresponds to the second isotope peak of m/z 723.6 and to [(C18:2)₃/(C18:1)₁-2H]²⁻-CL. The peak at m/z 725.6 corresponds to the second isotope peak of m/z 724.6, to the fourth isotope peak of m/z 723.6, and probably to [(C18:2)₂/(C18:1)₂-2H]²⁻-CL. The peaks 0.5 Da from each peak correspond to the first isotope peaks. The peak clusters at m/z 711.6 and 736.6 also have their first isotope peaks at a 0.5-Da distance, which indicates that they are doubly-charged ions. Figure 3 shows these ions in more detail. Cone voltage was 30 V.

724.6 and 725.6 have both C18:2 and C18:1 (m/z 281.3; Figure 2) as side chains. However, the peak at m/z 724.6 corresponds to the second isotope peak of m/z 723.6, and the peak at m/z 725.6 corresponds to the second isotope peak of m/z 724.6 and to the fourth isotope peak of m/z 723.6. This is probably the reason that the ratio C18:2/C18:1 in Figure 2, panels B and C, is not, as one would expect, equal to 3 for [(C18:2)₃/(C18:1)₁-2H]²⁻-CL (m/z 724.6) or 1 for [(C18:2)₂/(C18:1)₂-2H]²⁻-CL. The peaks 0.5 Da from each peak corresponds to the first isotope peaks.

The peak clusters at m/z 711.6 and 736.6, shown in more detail in Figure 3, also have their first isotope peaks at a distance of 0.5 Da, which indicates that the corresponding ions carry a double charge. These ions may have the structures indicated in Table 1. Figure 4 shows high cone-voltage spectra (100 V) of the CL fractions from a control and a patient sample. The CL molecules were fragmented and the fragments detected.

The peaks at m/z 253.5, 255.2, 279.2, 281.3, 283.3, and 303.3 belong to the fatty acids palmitoleic (C16:1), palmitic (C16:0), linoleic (C18:2), oleic (C18:1), stearic (C18:0),
and arachidonic (C20:4) acid. Linoleic acid was the most abundant fatty acid in the control sample, whereas it was almost absent in the patient sample. The presence of these fatty acids confirms the existence of other CL molecular species that have fatty acids other than C18:2 and C18:1 as side chains, as indicated in Table 1 and Figures 1 and 2.

**Validation**

The response factor for \((C18:2)_4\)-CL was determined by using the slope and y-intercept of the calibration curve, which were obtained from a linear least-squares regression for analyte/IS peak-area ratio vs analyte concentration \((r^2 = 0.982)\). Because of the differences between the IS and the analyte and because detector response is dependent on these differences, we used this response factor to calculate the concentrations of \((C18:2)_4\)-CL in our samples.

The ratio between the analyte concentration and the protein concentration of the sample was linear up to at least 0.04 g/L protein \((r^2 = 0.966)\). When the protein concentrations of the samples exceeded 4 g/L, the samples were diluted before extraction. The detection limits were established and defined as the lowest signals with a signal-to-noise ratio of 3.

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![Figure 2: Mass spectra of daughter fragments of the three most abundant CL molecular species, m/z 723.6 (A), m/z 724.6 (B), and 725.6 (C), as indicated in the legend to Figure. 1. The mass spectra were obtained from the elution fractions of CL. The peak m/z 723.6 (A) has C18:2 (m/z 279.2) exclusively as a side chain, whereas the peaks at m/z 724.6 (B) and 725.6 (C) have both C18:2 as well as C18:1 (m/z 281.3) side chains. The ratio of C18:2 to C18:1 (B and C) is not 3 for \([(C18:2)_3/(C18:1)_2-2H)_2-CL\) (m/z 724.6) or 1 for \([(C18:2)_2/(C18:1)_2-2H)_2-CL\), as might be expected, probably because of interference from the isotope peaks. Cone voltage was 30 V, and collision energy was 40 eV.](image)
The detection limits obtained were 0.7 µmol/L for the IS and 0.4 µmol/L for the commercially available (C18:2)₄-CL. However, the lowest concentration with a measurable signal-to-noise ratio of 3 was µ10 nmol/g of protein, when a sample with at least 0.3 10⁻³ g/L protein was available.

Figure 3: Mass spectrum of CL molecular species from platelets, which shows the peak clusters around m/z 711.6 and 736.6 (from Figure 1) in more detail. First isotope peaks at a 0.5-Da distance indicate that these ions are doubly-charged ions. The fatty acid composition of these molecular species is given in Table 1. Note the existence of CL with arachidonic acid as a side chain (m/z 736.6 and 737.6).

Figure 4: Mass spectra of CL molecular species of platelets from a control and a BTHS patient sample. Phospholipids were extracted from platelets and separated by HPLC. A high cone-voltage (100 V) mass spectrum was obtained from the elution fraction of CL. CL molecules were fragmented, and the fragments were detected. Different fatty acids, such as linoleic (C18:2), palmitic (C16:0), oleic (C18:1), and arachidonic (C20:4), were detected and are indicated. Linoleic acid is the most abundant fatty acid in the control sample, whereas it is almost absent in the patient sample.
The extraction efficiency for (C18:2)\textsubscript{4}-CL was 82.6% +/- 3.9% for nonenriched samples, 89.3% +/- 2.6% for samples enriched with low concentrations, 96.7% +/- 4.2% for samples enriched with medium concentrations, and 94.5% +/- 5.4% for samples enriched with high concentrations of (C18:2)\textsubscript{4}-CL. The extraction efficiency for (C14:0)\textsubscript{4}-CL (IS) was in the same range. The variation in retention times for both components (analyte and IS) was +/- 0.2%. The intraassay variation was 6.8% for nonenriched samples, 3.7% for samples enriched with low concentrations, 5.9% for samples enriched with medium concentrations, and 6.1% for samples enriched with high concentrations of (C18:2)\textsubscript{4}-CL. The interassay CV was 11% for nonenriched samples and 14% for enriched samples.

The recovery efficiency of the method was 96.4% +/- 2.1%. The (C18:2)\textsubscript{4}-CL and (C14:0)\textsubscript{4}-CL ions were suppressed by 33.5% +/- 4.1% and 31.9% +/- 3.4%, respectively.

**APPLICATION TO SAMPLES FROM PATIENTS AND CONTROLS**

Using this method, we measured the concentrations of (C18:2)\textsubscript{4}-CL in platelets prepared from blood samples of healthy, adult volunteers after 24h (n=10) and 48h (n=10), from a group of children (ages 2–10 years; n=6), and from a group of BTHS patients (n=4). Table 2 summarizes the clinical, biochemical, and genetic findings for the BTHS patients.

Figure 5 shows chromatograms of doubly charged (C18:2)\textsubscript{4}-CL from a control sample and a BTHS patient sample. (C18:2)\textsubscript{4}-CL is markedly decreased in the patient sample.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>619.6</td>
<td>(IS) (C14:0)\textsubscript{4}</td>
</tr>
<tr>
<td>709.6</td>
<td>(C18:2)\textsubscript{2}/(C16:1)\textsubscript{2}</td>
</tr>
<tr>
<td>711.6</td>
<td>(C18:2)\textsubscript{3}/(C16:1)\textsubscript{1}</td>
</tr>
<tr>
<td>723.6</td>
<td>(C18:2)\textsubscript{4}</td>
</tr>
<tr>
<td>724.6</td>
<td>(C18:2)\textsubscript{3}/C18:1</td>
</tr>
<tr>
<td>725.6</td>
<td>(C18:2)\textsubscript{2}/(C18:1)\textsubscript{2}</td>
</tr>
<tr>
<td>735.6</td>
<td>(C18:2)\textsubscript{3}/(C20:4)\textsubscript{1}</td>
</tr>
<tr>
<td>735.6</td>
<td>(C18:2)\textsubscript{2}/(C18:3)\textsubscript{1}/(C20:3)\textsubscript{1}</td>
</tr>
<tr>
<td>736.6</td>
<td>(C18:2)\textsubscript{2}/(C18:1)\textsubscript{1}/(C20:4)\textsubscript{1}</td>
</tr>
<tr>
<td>737.6</td>
<td>(C18:2)\textsubscript{1}/(C18:1)\textsubscript{2}/(C20:4)\textsubscript{1}</td>
</tr>
</tbody>
</table>

**Table 1:** Possible fatty acid composition of different CL molecular species found in platelets.
Table 3 shows the quantification results for (C18:2)₄-CL in the various patient and control groups. We noted a significant difference between the BTHS patients and the control groups. We also observed a difference between results for blood samples from the 24-h adult group vs the 48-h adult controls and the pediatric group (P > 0.03).

**DISCUSSION** Phospholipids in biological membranes form extremely complex mixtures of closely related compounds. This complexity of structure makes complete separation of these phospholipids, including their molecular species, impossible by a single separation method. Therefore, different combinations of separation methods have been developed to identify and quantify the major phospholipids classes and their molecular species (25). In particular, the separation of CL from other nonpolar lipids requires special solvent composition (26, 27). These solvent systems are usually incompatible with ESI-MS.

Furthermore, the instrument response in ESI-MS depends on different factors, such as acyl chain length, acyl chain unsaturation, structure of the polar head group, total lipid concentration, solvent composition, and instrument settings (28, 29).

<table>
<thead>
<tr>
<th>Patientsₐ</th>
<th>Age at onset and presenting symptom</th>
<th>Age</th>
<th>Dilated cardiomyopathy</th>
<th>Mild peroximal muscle weakness</th>
<th>Neutropenia</th>
<th>Organic asiduria</th>
<th>TAZᵇ gene mutation and effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3 y muscle weakness</td>
<td>11.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3-mcga</td>
<td>C441G prem mature stopcodon</td>
</tr>
<tr>
<td>2</td>
<td>1st month non-specific illness, abnormal cardiac echogram</td>
<td>1.7</td>
<td>+</td>
<td>Ventricular noncompaction</td>
<td>+</td>
<td>3-mgcᵃ</td>
<td>Exon 2, AG&gt;AC Splice acceptor site</td>
</tr>
<tr>
<td>3</td>
<td>6 wk cardiac decompensation</td>
<td>10.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3-mgca</td>
<td>G877T G&gt;V Exon 8</td>
</tr>
<tr>
<td>4</td>
<td>Mild dilated cardiomyopaty and muscle weakness in adult</td>
<td>36+</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>3-mgca</td>
<td>G216R</td>
</tr>
</tbody>
</table>

*Table 2: Clinical, biochemical, and genetic findings for BTHS patients.*

ₐ Patients 1 and 4 are related to each other (30)

ᵇ Reference sequence GenBank accession no. NM_000116, Tafazzin, TAZ.

ᶜ + indicates the presence of condition

d 3-MGCA, 3-methylglutaconic acid; 3-MGRA, 3-methylglutaric acid; 2-EHT, 2-ethylhydracrylic acid.
Chapter 4

The major classes of phospholipids differ in the polar head group, which makes it possible to separate them on a normal-phase silica-based column. However, CL and other phospholipids were separated in a short time from interfering nonpolar components by a binary solvent system, without any complications in mixability of the gradient components. In addition, this method requires only a small amount of biological material. The solvent composition forms a very good matrix for the ionization of CL in ESI-MS. Using this method, we measured CL molecular species in different biological samples, such as platelets, fibroblasts, lymphoblasts, yeast cells, and several other tissues (data not shown). The method was not optimized for the measurement of other phospholipids.

We used platelets in this study because they are easily isolated from whole blood compared with other blood cells or fibroblasts, which must be cultured for several weeks before use. For the quantitative measurement of CL, we determined the detector response factor to correct the measured concentrations of \((C18:2)_4\)-CL before each set of analyses. We also defined and measured a pool sample of healthy controls before each set of analyses. Validation data show that our method can be used for the compositional analysis of CL and quantitative analysis of \((C18:2)_4\)-CL. Our data for the different CL molecular species in platelets are comparable with CL molecular species found in other human tissues (4).

However, because internal calibrators labeled with stable isotopes are lacking, precautions, such measuring the response factor and performing recovery experiments for both the IS and the analyte, are advised for the quantification of CL molecular species by ESI-MS.

![Figure S: Chromatograms of doubly charged \((C18:2)_4\)-CL from a control sample and a BTHS patient sample. Phospholipids were extracted from platelets and separated by HPLC. Single-ion recording was used to measure the ions. The y axes of both chromatograms are linked, and the chromatograms are overlaid.](image)

In yeast, different mitochondrial dys-functions occur when CL synthase or phosphatidylglycerophosphate synthase undergo mutation, which indicates that CL plays an important role in mitochondrial function.
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However, the exact function of CL is still unclear. Our newly developed method can be used to clarify the function of CL in relation to mitochondrial disorders. Because BTHS, an X-linked recessive disorder with several mitochondrial dysfunctions (12), has been associated with a possible remodeling defect of CL (10), we used our method to establish a procedure for the identification of BTHS patients. Although the diagnosis of BTHS is usually established by molecular studies, a screening method for BTHS is greatly needed because the clinical picture of this syndrome is nonspecific, because gene analysis is time-consuming, and because the number of patients is high. Recently, we introduced a biochemical test that incorporates radiolabeled linoleic acid into CL or the PG pool of fibroblasts (10).

Both CL and PG had normal de novo synthesis. A reduced incorporation of linoleic acid into both PG and CL, however, was observed in BTHS patients, whereas incorporation of the other fatty acids, such as C16:0, C18:1 and C20:4 were within the range of values obtained in a healthy population. The total CL pool was reduced, whereas the total PG pool was slightly increased. Because PG can be separated by onedimensional thin-layer chromatography more easily than CL from other phospholipids and natural lipids, we suggested the incorporation of linoleic acid into the PG pool as a biochemical test.

However, both of these methods are time-consuming (days to weeks) and are therefore not very attractive for use as a screening tool to pinpoint patients suspected of BTHS; our new method, in contrast, takes only 5 h for 10 samples. Because the clinical and biochemical abnormalities in BTHS patients are nonspecific, an easy and rapid method for BTHS detection is very attractive. As shown in Table 3, BTHS patients and controls showed a significant difference in CL values. The differences between individual samples within each control group show that the time between blood collection and analysis can cause minor

<table>
<thead>
<tr>
<th>Groups</th>
<th>Median</th>
<th>Range</th>
<th>P values versus BTHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h (n=10)</td>
<td>5.0</td>
<td>2.3-5.5</td>
<td>0.00001</td>
</tr>
<tr>
<td>48h (n=10)</td>
<td>3.0</td>
<td>2.5-3.6</td>
<td>0.000001</td>
</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n=6)</td>
<td>3.8</td>
<td>2.3-5.2</td>
<td>0.0002</td>
</tr>
<tr>
<td>BTHS (n=4)</td>
<td>0.14</td>
<td>0.1-0.5</td>
<td></td>
</tr>
</tbody>
</table>

*CL molecular species were separated as described in Materials and Methods, and (C18:2)4-CL was quantified. A significant difference between BTHS patients and control groups was obtained.
differences, which could be caused by platelet activation during sampling. We there-
fore suggest that the platelets be isolated immediately after blood collection. However, differences
between control groups did not cause any complications with respect to the identification of
patients with BHTS. Because CL is an exclusive mitochondrial inner-membrane phospholipid
involved in various mitochondrial functions (1–3), markedly decreased CL values can explain
the abnormal mitochondrial function observed in BTHS patients.

We conclude that the analysis of CL in platelets by HPLC-ESI-tandem MS is a rapid
and simple method to identify patients with BTHS. However, no evidence is yet available to
confirm that the decreased CL concentration is a primary effect of a mutation in the TAZ
gene. Thus, at this time, gene mutation analysis may be needed to complete the diagnosis.

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