Inborn Errors of Metabolism Diagnosed in Sudden Death Cases by Acylcarnitine Analysis of Postmortem Bile

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Fatty acid oxidation (FAO) disorders represent a frequently misdiagnosed group of inborn errors of metabolism. Some patients die at the first episode of fasting intolerance and, if appropriate investigations are not undertaken, often meet the criteria of sudden infant death syndrome (SIDS). To expand existing protocols for the postmortem diagnosis of FAO and other metabolic disorders, we tested the hypothesis that analysis for acylcarnitine in bile, a specimen readily available at autopsy, may be utilized for diagnostic purposes. Using electro-spray/tandem mass spectrometry, we analyzed for acylcarnitine postmortem bile specimens from two infants with long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency, one infant with glutaryl-CoA dehydrogenase deficiency, and 17 uninformative SIDS cases as controls. The affected cases, and none of the controls, showed marked accumulation of C12-C18 acylcarnitines or glutaryl carnitine (acyl/free carnitine ratio: 5.2, 2.7, and 1.9, respectively; controls 0.2 ± 0.1). In one patient, all other diagnostic methods were uninformative, suggesting that bile acylcarnitine profiling could lead to identification of previously overlooked cases.

Indexing Terms: pediatric chemistry/fatty acid oxidation/glutaryl carnitine/postmortem diagnosis/sudden infant death syndrome

Inborn errors of metabolism often present early in infancy with life-threatening episodes of metabolic decompensation. In view of the high mortality rate that is common among these disorders, sudden infant death syndrome (SIDS) has been sporadically associated with a number of inborn errors of amino acid and energy metabolism (1-3), including glutaryl-CoA dehydrogenase deficiency (4).5 These isolated cases probably result from a delayed diagnosis combined with atypical and (or) mild clinical phenotypes, and are unlikely to represent a significant cause of sudden infant death. On the other hand, the number of cases found to have been affected with a disorder of fatty acid oxidation (FAO), either postmortem or retrospectively (after the diagnosis of an affected sibling), has soared in the last 10 years (4-8). On the basis of these observations, we and others have postulated that FAO disorders may be responsible for as many as 5% of SIDS cases (6-9), possibly even more if children who die suddenly and unexpectedly in early life are considered.

After the diagnosis a decade ago of medium chain acyl-CoA dehydrogenase deficiency in a SIDS victim (10), anecdotal reports have associated at least 13 FAO disorders with sudden unexpected death in infancy (7). Among them, long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency has emerged as a common disorder and has been linked to acute fatty liver of pregnancy (11) and multiple cases of sudden infant death (12, 13).

Suspicion of a possible FAO disorder is usually raised by the finding at autopsy of diffuse microvesicular steatosis (4). A final diagnosis can be reached through various biochemical, enzymatic, and molecular methods (4, 6-8, 14), but only if fluid and tissue specimens are collected during autopsy for this purpose. In our experience, this is seldom done, which possibly contributes to the underestimation of metabolic disorders among sudden death cases. To increase the general awareness of these disorders, we have sought alternative biochemical methods that are based on the constant availability of a suitable sample other than blood or urine, require simple preparation procedures, and allow the diagnosis of as many disorders as possible on the basis of multiple independent criteria (15).

Recently, we proposed a new method based on the simultaneous quantitative determination of C6-C18 fatty acids and glucose from the methanol wash of a pellet obtained by ultracentrifugation of frozen liver homogenate (7). Our current postmortem protocol includes this method, liver histology, assay of liver carnitine, and organic acid analysis of urine when available. In our collective experience, we have thus far diagnosed postmortem >40 patients with FAO disorders (6-8, 16, 17; and unpublished observations); in addition, however, we encountered several sudden death cases that remained without a precise diagnosis despite one or more abnormal findings. We postulated that our current strategy could be improved by the analysis of bile, a specimen easily collected at autopsy, for the postmortem diagnosis of FAO and other metabolic disorders associated with the accumulation of characteristic acylcarnitines in blood and urine. We report here the preliminary results obtained in three confirmed cases, two of which were diagnosed at autopsy.

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5 Nonstandard abbreviations: ESI/MS-MS, electrospray tandem mass spectrometry; FAO, fatty acid oxidation; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; and SIDS, sudden infant death syndrome.

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Materials and Methods

Case Reports

Case 1. A Caucasian male presented shortly after birth with severe dilated cardiomyopathy and two life-threatening episodes of hypoglycemia. Pregnancy and delivery were unremarkable. Organic acid analysis showed massive amounts of C9-C14 saturated and unsaturated 3-hydroxydicarboxylic acids with minimal ketonuria, a pattern suggestive of LCHAD deficiency. The diagnosis of a defect in the α-subunit of the mitochondrial trifunctional protein, which contains LCHAD and long-chain enoyl-CoA hydratase activity, was later confirmed by assay of enzyme activities in cultured fibroblasts and molecular analysis (18). At age 18 months, the patient became lethargic and vomited. In spite of prompt hospitalization and close monitoring, several hours later he died suddenly and unexpectedly.

Case 2. A Caucasian male born to unrelated parents. There was no significant obstetric history, and his two older half-siblings are reported healthy. His clinical course consisted of poor feeding, frequent vomiting, and failure to thrive. He had multiple hospitalizations, but no metabolic investigations were undertaken. At age 3 months, he was unexpectedly found dead.

Case 3. An 8-year-old Hispanic male was diagnosed with cerebral palsy and seizure disorder. The clinical histories of this patient and of his three siblings, who reportedly have similar manifestations, are not available. The patient died suddenly and unexpectedly shortly after being admitted to a hospital for evaluation of a non-life-threatening condition (persistent abdominal distension).

Controls

As part of a large-scale retrospective study, >450 sudden death cases investigated by the Office of the Medical Examiner of the State of Maryland were tested for a possible FAO disorder (7, 15, and RG Boles et al., unpublished results). Seventeen SIDS cases who tested negative in all the investigations performed (review of liver histology, metabolite profile of liver homogenate, and assay of liver carnitine) were randomly chosen on the basis that a bile specimen was available. The analysis of postmortem liver, bile, and urine specimens for diagnostic purposes was approved by the Yale University human investigation committee (HIC no. 05125).

Acylcarnitine Analysis

Bile specimens (~50–100 μL) were spotted onto filter paper (no. 903; Schleicher and Schuell, Dassel, Germany), allowed to dry overnight, and stored at room temperature in polypropylene bags at room temperature until analysis. Two 3/16-in. (~5-mm)-diameter circles were punched out from the spot and extracted as previously described (19). Qualitative profiles of acylcarnitine methyl or butyl esters were obtained by electrospray tandem mass spectrometry (ESI/MS-MS) by monitoring precursor-ion scans of the common fragment at m/z 99 (methyl-) or m/z 85 (n-butyl-) of acylcarnitine derivatives with a VG Quattro ESI/MS-MS system (Fisons, Altrincham, UK) equipped with a Jasco PU-980 HPLC pump and a Jasco AS-950 autosampler (Jasco International, Tokyo, Japan) (20).

Other Methods

Total and free carnitine were measured in perfusate and supernatant of liver homogenate samples by a radioenzymatic assay (21), with some modifications. Bile specimens (50 μL) were incubated with 50 μL of cholesteryamine (50 g/L, 30 min at room temperature) and subsequently diluted 10-fold with deionized water to prevent bile acid inhibition of carnitine acetyltransferase (22). The extremely high values of esterified carnitine in patients’ bile samples were confirmed at several dilutions (up to 30-fold) and verified by a different method (23). C9-C18 fatty acids and glucose were determined in liver homogenate as previously described (7). Activities of LCHAD and three other mitochondrial enzymes measured as controls in liver homogenate were assayed by spectrophotometric methods (24). Urine organic acids were separated by solvent extraction and analyzed by gas chromatography/mass spectrometry as trimethylsilyl derivatives (25). Glutaric acid was determined by a gas chromatography/mass spectrometry stable isotope dilution method (26).

Results

In case 1, postmortem examination showed no fatty infiltration of the liver, kidneys, or heart; the modest inflammatory infiltration of the myocardium was consistent with myocarditis. The metabolite profile of liver homogenate was uninformative (Table 1), and total liver carnitine was 0.89 μmol/g wet weight (range of control values: 0.33–1.09, n = 18). A urine specimen revealed only trace amounts of the metabolites characteristic of this disorder (18).

![Table 1. Metabolite profile of liver homogenate in two cases with LCHAD deficiency.](https://example.com/table1.jpg)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanoic acid</td>
<td>0.2</td>
<td>nd</td>
<td>0.1–0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>cis-4-Decenoic acid</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Decenoic acid</td>
<td>1</td>
<td>nd</td>
<td>nd–1.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Dodecanoic acid</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Dodecanolic acid</td>
<td>1.8</td>
<td>3.2</td>
<td>nd–20.5</td>
<td>3.8</td>
</tr>
<tr>
<td>Tetradecanoic acid</td>
<td>nd</td>
<td>nd</td>
<td>nd–0.8</td>
<td>nd</td>
</tr>
<tr>
<td>Tetradecanolic acid</td>
<td>6.0</td>
<td>3.6</td>
<td>1.0–23.8</td>
<td>5.6</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>11.6</td>
<td>18.3</td>
<td>nd–13.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>176.9</td>
<td>56.8</td>
<td>20.6–142.5</td>
<td>54.9</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>80.4</td>
<td>112.3</td>
<td>13.5–242.0</td>
<td>66.2</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>89.6</td>
<td>114.5</td>
<td>8.6–142.7</td>
<td>34.5</td>
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<tr>
<td>Stearic acid</td>
<td>48.5</td>
<td>23.5</td>
<td>9.8–42.3</td>
<td>21.1</td>
</tr>
<tr>
<td>Glucose</td>
<td>204.9</td>
<td>nd</td>
<td>2–85.0</td>
<td>22.7</td>
</tr>
</tbody>
</table>

* n = 100. Source: Boles et al. (7).

nd, not detectable.
The autopsy of case 2 revealed a pale, lipid-filled liver. The metabolite profile of homogenate liver showed undetectable glucose and an increased concentration of palmitoleic acid, findings consistent with a possible FAO disorder (Table 1) (7,15). Total liver carnitine was 0.45 μmol/g wet weight. Urine organic acid analysis revealed hypoketic C₆-C₁₀ dicarboxylic aciduria and C₈-C₁₄ 3-hydroxydicarboxylic aciduria, suggesting possible LCHAD deficiency. This diagnosis was confirmed by assay of LCHAD activity in liver that had been collected at autopsy and stored without delay at −70 °C (Table 2). Three other mitochondrial enzymes (short-chain 3-hydroxyacyl-CoA dehydrogenase, short-chain 3-ketoacyl-CoA thiolase, and long-chain 3-ketoacyl-CoA thiolase) were tested as controls.

The organic acid analysis of postmortem urine from case 3 showed massive excretion of glutaric acid (1593 mmol/mol creatinine; controls: 0.5–13), 3-hydroxyglutaric acid, and glutaconic acid but no ketonuria or dicarboxylic aciduria, a pattern considered pathognomonic for glutaryl-CoA dehydrogenase deficiency (glutaric acidemia type I) (27). At autopsy, examination of the brain revealed bilateral widening of the operculum, spongy degeneration of the white matter, and localized lesions of the putamen and caudate nuclei, findings strongly supportive of the biochemical diagnosis. Two of the three siblings with a similar clinical history have subsequently been investigated, and their urine organic acid profiles have been pathognomonic for glutaric acidemia type I—massive excretion in urine of glutaric acid, 3-hydroxyglutaric acid, and glutaconic acid.

Figure 1 shows the acylcarnitine profiles (methyl ester derivatives) from case 1 and a control. The patient’s acylcarnitine profile was similar to those previously described in plasma in cases with LCHAD deficiency (28), i.e., strong signals for saturated and unsaturated carnitine esters of C₁₀-C₁₆ fatty acids and less intense signals for the esters of C₁₀-C₁₆ 3-hydroxy fatty acids. The acylcarnitine qualitative profile for case 2 was essentially identical. These findings were confirmed by analysis involving a different derivatization (n-butyl-, data not shown). The marked increase of

![Figure 1: Acylcarnitine profiles of postmortem bile obtained by precursor ion-scanning ESI/MS-MS: (top) case 1, LCHAD deficiency; (bottom) control.](image)

**Parents of 99ES+**

**4.77e6**

**Mass/Charge (m/z)**

Fig. 1. Acylcarnitine profiles of postmortem bile obtained by precursor ion-scanning ESI/MS-MS: (top) case 1, LCHAD deficiency; (bottom) control.

IS, internal standard (3-palmitoyl carnitine, 72 pmol); n=8, 10 . . . , chain length of saturated acylcarnitine species; n=1, chain length of monounsaturated acylcarnitine species; n=2, chain length of diunsaturated acylcarnitine species; *, fatty acylcarnitine methyl esters; **, fatty 3-hydroxyacylcarnitine methyl esters.

### Table 2. Mitochondrial enzyme activities in liver extract of one case with LCHAD deficiency diagnosed as SIDS.

<table>
<thead>
<tr>
<th>Enzyme measured</th>
<th>Activity, μmol/min per g protein</th>
<th>Controls, mean ± SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCHAD</td>
<td>132</td>
<td>268 ± 29 (8)</td>
</tr>
<tr>
<td>SCHAD</td>
<td>245</td>
<td>249 ± 26 (6)</td>
</tr>
<tr>
<td>LCHAD/SCHAD ratio</td>
<td>0.54</td>
<td>1.04 ± 0.05 (6)</td>
</tr>
<tr>
<td>SKAT</td>
<td>218</td>
<td>127 ± 6 (6)</td>
</tr>
<tr>
<td>LKAT</td>
<td>3.98</td>
<td>3.47, 4.10 (2)</td>
</tr>
</tbody>
</table>

SCHAD, short-chain 3-hydroxyacyl-CoA dehydrogenase; SKAT, short-chain ketothiolase; LKAT, long-chain 3-ketoacyl-CoA thiolase.
the acylcarnitine fraction in the postmortem bile specimens of these patients was confirmed by radioenzymatic determination of total, free, and acylcarnitine fractions (Table 3). The acylcarnitine profile of the case with glutaryl-CoA dehydrogenase deficiency (Fig. 2) was characterized by a strong signal at m/z 304, corresponding to glutaryl carnitine (20). When analyzed under identical conditions, control bile samples from SIDS cases that were uninformative by multiple analyses showed trace amounts of the C_{14}−C_{16} fatty acylcarnitines and no detectable accumulation of the other acylcarnitine species described above.

Discussion

Formation of carnitine esters is involved in four primary functions: (a) import of long-chain fatty acids into the mitochondria; (b) export of physiological short-chain acyl-CoA groups from mitochondria and peroxisomes; (c) buffering of the free CoA/esterified CoA ratio; and (d) enhancing urinary excretion of potentially toxic acyl-CoA groups accumulated as a consequence of a metabolic block (29). Because the latter function is limited when increasingly hydrophobic long-chain fatty acids accumulate, carnitine-mediated detoxification should theoretically not occur in long-chain FAO disorders (30). This conclusion, however, rests on the assumption that excretion in urine is the only relevant mechanism of carnitine-mediated detoxification in inherited metabolic diseases.

Gudjonsson et al. (31) reported that carnitine concentrations in bile of rats receiving L-[\(^{3}\)H]carnitine intraluminal injection approximated the concentrations found in the portal vein, suggesting passive rather than active transport into bile. Their conclusion was that only a relatively small fraction of a radioactive carnitine dose is excreted in the bile, predominantly in the esterified form. Several lines of experimental evidence otherwise suggest that the biliary excretion of long-chain and possibly other acylcarnitine species may have been significantly underestimated. For instance, carnitine esters represented as much as 83% of total carnitine in bile from rats fed a high-fat diet for 72 h (32). In the fasted state, similar results were obtained not only in rats but in humans as well (33). In another study, the release of carnitine and isovalerylcarmitine was investigated in perfused rat liver after recirculating perfusion with 50 µmol/L \(^{3}\)H carnitine and infusion of 5 mmol/L α-ketoisocaprate (34). α-Ketoisocaprate infusion produced accumulation of \(^{3}\)Hisovalerylcarmitine in the liver tissue.

![Fig. 2. Acylcarnitine profiles of postmortem bile from case 3 (glutaryl-CoA dehydrogenase deficiency).](image-url)

IS, internal standard (d_{9}-palmitoylcarnitine, 72 pmol). Other symbols as in Fig. 1.
within 15 min. Although no bile samples were examined in this study, a rapid decrease of radioactivity in the liver tissue and the disappearance of $^{3}He$ovalerylcarnitine in the perfusate at 60 min were explained by the authors as the consequence of rapid intrahepatic degradation of isovalerylcarnitine. However, this carnitine ester is an end product of isovaleryl-CoA catabolism that accumulates in body fluids of patients with isovaleryl-CoA dehydrogenase deficiency (35) and is not a substrate for the microsomal enzyme carnitine ester hydrolase (substrate specificity: CoA-C4) (36). A similar retardation is recognizable in a study of L-[CH3$^{3}H$]carnitine turnover in rats, in which unexplained carnitine loss was three times the amounts recovered in urine (37). Total carnitine excretion in rat bile has been estimated as 0.4–1.4 μmol 24 h$^{-1}$ 100 g body wt$^{-1}$ (31), which is in the same order of magnitude of urinary excretion (0.8–1.9 μmol 24 h$^{-1}$ 100 g body wt$^{-1}$) (37).

To our knowledge, however, our two patients with LCHAD deficiency and the patient with glutaric acidemia type I are the first cases of inborn errors of amino acid and fatty acid metabolism to have been assessed by metabolite profiling of bile. In all three cases, the qualitative profiles were convincingly suggestive of the correct diagnosis, as confirmed by a 10- to 25-fold increase of the respective acyl/free carnitine ratios. The detection of glutaryl-carnitine (C4), a compound readily excreted in urine, underlines the potential of bile analysis for the biochemical diagnosis of a broad spectrum of disorders not limited to those with accumulation of long-chain acylcarnitines, the increasing hydrophobicity of which precludes urinary excretion. In one LCHAD-deficient patient, all other diagnostic methods included in our current protocol (liver histology, metabolite profile and carnitine concentration of liver homogenate, and urine organic acids) were uninformative, suggesting that bile acylcarnitine profiling could lead to the identification of an even greater proportion of previously overlooked cases.

The finding of bile acylcarnitine concentrations in the mmol/L range was unexpected. Comparable concentrations of carnitine have been reported in seminal fluid (1–3 mmol/L), but >90% of that is represented by free carnitine excreted in the epididymal fluid under the regulation of androgen hormones (29, 38, 39). In bile, our results point instead to the existence of an active primary transport of carnitine esters, with a mechanism already demonstrated for the ATP-dependent glutathione S-conjugate carrier (40, 41). Localization (basolateral or canicular plasma membrane) (42), substrate specificity, and regulation of such a putative acylcarnitine carrier are unknown.

Comparison with postmortem plasma acylcarnitine profiles was not possible in these cases, for the specific reason that collection and proper storage of blood specimens for metabolic testing is not routinely performed. However, published data consistently show that plasma acylcarnitine concentrations in patients with FAO disorders at the time of diagnosis are low rather than high, being with few exceptions always <100 μmol/L (43)—at least 10- to 40-fold lower than the acylcarnitine concentrations observed in postmortem bile.

Fasting intolerance is considered the main factor causing life-threatening episodes in patients with FAO disorders (44). In view of the fact that bile accumulates in the gallbladder and is not released during fasting (45), acylcarnitines analysis of bile seems to be well suited for diagnostic purposes, especially in patients with long-chain FAO disorders (30). Although testing of additional cases is needed to establish the specificity of this application, we propose the use of bile acylcarnitine profiling for the postmortem diagnosis of metabolic disorders in infants and children who die suddenly and unexpectedly.

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References


