Diagnosing Inborn Errors of Lipid Metabolism with Proton Nuclear Magnetic Resonance Spectroscopy

Marlies Oostendorp,† Udo F.H. Engelke,† Michèl A.A.P. Willemsen, and Ron A. Wevers*

Background: Many severe diseases are caused by defects in lipid metabolism. As a result, patients often accumulate unusual lipids in their blood and tissues, and proper identification of these lipids is essential for correct diagnosis. In this study, we investigated the potential use of proton nuclear magnetic resonance (1H-NMR) spectroscopy to simultaneously identify and quantify unusual lipids present in the blood of patients with different inborn errors of lipid metabolism.

Methods: We extracted blood plasma or serum lipids in chloroform–methanol (2:1 by volume). After addition of the nonvolatile chemical shift and concentration reference compound octamethylcyclotetrasiloxane, we performed 1H-NMR measurements on a 500-MHz spectrometer. Assignments were based on the literature, computer simulations, and reference spectra of relevant authentic standards.

Results: Spectra of normal plasma samples allowed the identification of 9 lipid species. We found good correlation between conventional methods and 1H-NMR for cholesterol and triglyceride concentrations. We also investigated 4 inborn errors of lipid metabolism (3 in sterol metabolism and 1 in fatty acid metabolism). NMR analysis led to a correct diagnosis for all 4 diseases, whereas the concentration of the diagnostic metabolite could be determined for 3.

Conclusions: 1H-NMR spectroscopy of blood plasma or serum lipid extracts can be used to accurately identify and quantify lipids. The method can also identify unusual lipids in the blood of patients with inborn errors of lipid metabolism. This technique may therefore be applicable in clinical diagnosis and follow-up.

Proton nuclear magnetic resonance (1H-NMR) spectroscopy is a versatile technique that can be used in a wide range of disciplines. The best known medical applications are in vivo magnetic resonance imaging and spectroscopy, but in vitro 1H-NMR spectroscopy of body fluids has also been used to diagnose inborn errors of metabolism (1–3). In contrast to conventional techniques, NMR can detect the majority of all proton-containing metabolites in a single experiment lasting ~15 min. Furthermore, it is a nondestructive method and requires little or no sample preparation.

Currently, blood plasma or serum samples are measured either directly (4–6) or after deproteinization by ultrafiltration (3). The advantage of using the latter method is that broad, overlapping protein resonances are removed, thereby yielding a highly resolved spectrum in which only the water-soluble, low–molecular-mass metabolites are observed. Unfortunately, this also limits NMR as a diagnostic tool for inborn errors of metabolism to diseases involving accumulation or absence of these relatively small metabolites.

There are also many severe diseases caused by inherited defects in the metabolism or biosynthesis of different fatty acids and sterols. As a result, unusual lipids are often present in the blood and tissues of affected patients. Accurate identification and quantification of these metabolites are essential for correct diagnosis of the disease.

1H-NMR has several major advantages over gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry for analysis of lipids and sterols. First, authentic standards are usually not available for these metabolites. Accurate identification and quantification of the diagnostic metabolite is therefore not possible using GC-MS or LC-MS. In contrast, NMR can detect these metabolites and provide accurate quantification.

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Nonstandard abbreviations: 1H-NMR, proton nuclear magnetic resonance; GC-MS, gas chromatography–mass spectrometry; SLOS, Smith–Lemli–Opitz syndrome; CTX, cerebrotendinous xanthomatosis; 7DHC and 8DHC, 7- and 8-dehydrocholesterol, respectively; TMS, tetramethylsilane; and OMS, octamethylcyclotetrasiloxane.
required once the chemical shifts of the biologically relevant species are known [for the chemical shifts of many C_{27} sterols and their acetyl derivatives, see Ref. (7)]. Second, lipid identification is almost unequivocal if a few distinct resonances are resolved. Finally, sample preparation for \(^1\)H-NMR measurements can be fairly simple, whereas the conventional biochemical analysis of unusual lipids in body fluids may be complicated and time-consuming, sometimes involving derivatization steps and a combination of several types of chromatography.

One-dimensional \(^1\)H-NMR spectroscopy of intact blood plasma can detect several lipid signals (6). This technique can be used to determine the relative amounts of HDL\(_c\), LDL\(_c\), and VLDL-cholesterol by use of complex mathematical line fitting techniques (4). However, the diagnostic markers for inborn errors in lipid metabolism remain undetected because of their low concentrations, overlap with other metabolites, and protein-derived interferences. Here, we describe a simple procedure based on the Folch extraction (8) to isolate all lipids from blood plasma or serum samples by use of a chloroform–methanol extraction medium (2:1 by volume). Casu et al. (9) reported the NMR analysis of lipids extracted from erythrocytes and plasma of humans. Furthermore, \(^1\)H-NMR spectroscopy has been applied successfully in the diagnosis of the Smith–Lemli–Opitz syndrome (SLOS) (10, 11). This study demonstrates for the first time that several serum lipids can be quantified simultaneously by \(^1\)H-NMR spectroscopy. We show the clinical usefulness of the technique by successfully applying it to 4 inherited disorders in lipid metabolism: SLOS, cerebroretinodous xanthomatosis (CTX), sitosterolemia, and Refsum disease.

**Materials and Methods**

**AUTHENTIC STANDARDS**

All authentic standards of the metabolites accumulating in the investigated inborn errors of metabolism, except 8-dehydrocholesterol (8DHC) and 8-lathosterol, were commercially available and were purchased from Sigma. Authentic standards of cholesterol, 7-lathosterol, oleic acid, linoleic acid, palmitic acid, and stearic acid were also purchased from Sigma.

**SAMPLE PREPARATION**

We obtained blood plasma or serum samples from healthy controls and from patients diagnosed with an inborn error of lipid metabolism at the University Medical Centre, Nijmegen, The Netherlands. All samples were provided anonymously after routine diagnostic screening was performed and were kept frozen until NMR analysis. We extracted all lipid material from 1 mL of blood plasma or serum, using a Folch extraction (8) optimized for blood plasma (12). For each extraction we used 30 mL of a chloroform–methanol extraction medium (2:1 by volume). The extraction was performed in a 50-mL capped Teflon\(^{16}\) centrifuge tube (Nalgene). We discarded the water–methanol layer and denatured protein precipitate, and evaporated the chloroform layer to dryness in an AS290 automatic Speedvac concentrator (Savant Instruments). Subsequently, we redissolved the extract in ~650 \(\mu\)L of deuterated chloroform (CDCl\(_3\)) for NMR analysis. The use of fresh chloroform and methanol during sample preparation was important. Aged chloroform may contain phosgenes, which can react with the analytes and lead to incorrect results.

Unfortunately, the conventional chemical shift reference compound tetramethysilane (TMS) was not suitable as the concentration reference because of its high volatility. We therefore used octamethylycyclotetrasiloxane (OMS; Fluka), which has a boiling point of 448 K and a chemical shift of 0.094 ppm compared with TMS. We were not able to pipet an amount of OMS dissolved in CDCl\(_3\) into the sample because the use of plastic pipet tips with chloroform solutions led to sample contamination. Furthermore, chloroform leakage from the pipet tip could significantly increase the volume and, hence, concentration differences. We therefore determined OMS concentrations by carefully weighing the amount added to the sample. The final sample was placed in a calibrated 5-mm NMR tube (Wilmad Royal Imperial).

**NMR SPECTROSCOPY**

All high-resolution \(^1\)H-NMR spectra were obtained at 298 K on a Bruker DRX 500-MHz spectrometer with a triple-resonance inverse (TXI) \(^1\)H (\(^{15}\)N, \(^{13}\)C) probe head and equipped with \(x, y, z\) gradient coils. Shimming of the samples was performed automatically on the deuteron signal. The resonance line-width for OMS was <1 Hz in all spectra. For the 1-dimensional spectra, 64 transients were recorded into 32 000 data points with a spectral width of 6010 Hz and a 6-s recycle delay. A pulse width of 5 \(\mu\)s was used (corresponding to a 90-degree excitation pulse). An inversion recovery experiment revealed a T\(_1\) of 2.6 s for OMS.

Data were processed and analyzed with MestReC, Ver. 4.4.1 (www.mestrec.com). The free induction decay was apodized with a sine-square filter and subsequently Fourier-transformed after zero filling to 64 000 points. The phase was corrected manually, and metabolite signals were integrated (for peaks showing complex J-splitting) or fitted semiautomatically with a Lorentzian line shape (singlets only). The resulting areas were compared with the area of OMS to determine metabolite concentrations.

Two-dimensional \(^1\)H–\(^1\)H correlation (COSY) spectra were recorded with a spectral width of 6010 Hz in both dimensions, with 256 and 2000 data points in F1 and F2, respectively; 16 scans per increment; and a recycle delay of 6 s. Before Fourier transformation, both time domains were apodized with a sine-bell function and were zero-filled once. Resonance assignments were based on the literature (7, 13–15), 1- and 2-dimensional spectra of the authentic standards in chloroform, and computer simulations run on ACD/HNMR Predictor, Ver. 2.03 (ACD/labs). Furthermore, available authentic standards of the
accumulating metabolites were added to patient samples to confirm their assignment. Chemical shift values in this study are referenced to the chemical shift of OMS at 0.094 ppm.

GAS CHROMATOGRAPHY
For GC of sterols, we extracted samples in pentane and subsequently derivatized them using N,O-bis(trimethylsilyl)trifluoroacetamide (Fluka) and pyridine. GC analysis was performed with a CP-Sil-19 CB column (Chrompack) on a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector (16).

In the conventional analysis of plasma phytanic acid, all lipids were first extracted in chloroform–methanol (1:1 by volume). After evaporation of the organic solvent, the fatty acids were esterified by addition methanolic HCl. The resulting fatty acid methyl esters were subsequently extracted in hexane and analyzed by GC with the same equipment described above for sterol analysis.

CORRELATION STUDY
For the correlation study, we selected 15 patient plasma samples from routine samples of the clinical chemistry department to give a wide range of cholesterol and triglyceride values. They were selected anonymously, and the results were not available to the person doing the 1H-NMR analysis. Cholesterol and triglycerides had been measured enzymatically in 15 and 12 samples, respectively, with standard reagent assays on an AEROSET System (Abbott Laboratories), according to instructions of the manufacturer (17, 18).

For 1H-NMR analysis, samples were prepared as described above. Statistical analysis was performed by Passing–Bablok regression analysis (19). We determined the within-run CV of the NMR method by preparing 9 separate samples from the same blood serum and subsequently measuring their cholesterol and triglyceride content. Sample preparation and NMR measurements were carried out in 1 session.

INBORN ERRORS OF LIPID METABOLISM
The patient population consisted of the following: SLOS (OMIM 270400), 3 cases; CTX (OMIM 213700), 3 cases; sitosterolemia (OMIM 210250), 1 case; and Refsum disease (OMIM 266500), 1 case.

The patient materials from the SLOS, CTX, and sitosterolemia patients were obtained before treatment and therefore represent diagnostic samples. Six samples were obtained from the follow-up during therapy of a patient with Refsum disease. For every inborn error, previous detection of the accumulating metabolite as well as genetic analysis of the relevant gene had confirmed the diagnosis. The diseases are described briefly below.

SLOS. SLOS is caused by a deficiency of the enzyme 7-dehydrocholesterol reductase (EC 1.3.1.21), which catalyzes the conversion of 7-dehydrocholesterol (7DHC; see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/contentvol52/issue7) to cholesterol, the final step in cholesterol biosynthesis (20). As a result, 7DHC accumulation and low cholesterol concentrations are observed in SLOS patients. Furthermore, 8DHC (see the online Data Supplement) can be detected in the blood of affected patients, resulting from isomerization of 7DHC to 8DHC.

CTX. CTX is caused by a defect in the CYP27A1 gene, which encodes the mitochondrial enzyme sterol 27-hydroxylase (EC 1.14.13.15) (21). This leads to a block in bile acid synthesis, which in turn leads to the accumulation of unusual bile alcohols in urine and cholestanol in blood.

Sitosterolemia. Sitosterolemia is characterized by increased concentrations of the plant sterols β-sitosterol, campesterol, and stigmasterol (see the online Data Supplement) in blood and tissues. Sequence variations in the ABCG5 and ABCG8 genes, both of which encode for half-transporter proteins, have been identified in sitosterolemia patients (22). In healthy participants, ~5% of the 200–300 mg of plant sterols consumed daily is absorbed, and almost all plant sterols are rapidly excreted in the bile. Sitosterolemia patients absorb between 15% and 60% of the ingested plant sterols and excrete only very little (21–23). The mean β-sitosterol concentration in patients is increased ~100-fold (21).

Refsum disease. For patients with Refsum disease, phytanic acid α-oxidation can not take place because of a defect in the enzyme phytanoyl-CoA hydroxylase (EC 1.14.11.18) (24). Phytanic acid is totally exogenous in origin; it derives from the bacterial metabolism of chlorophyll in ruminants. Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid; see the online Data Supplement) accumulates in the blood and tissues of affected patients (25). Phytanic acid concentrations may reach 1300 µmol/L (typically <10 µmol/L) in plasma, where it is incorporated in triglycerides (26). Refsum patients are generally treated with a low–phytanic acid diet (26). Furthermore, plasmapheresis treatment can be used to rapidly lower plasma phytanic acid concentrations.

Results
NORMAL LIPID RESONANCES
The 1-dimensional 1H-NMR spectrum of the blood plasma lipid extract of a healthy volunteer is shown in Fig. 1. Resonance assignments were based on previously reported spectra (7, 13–15); 1-dimensional and 2-dimensional spectra of pure cholesterol, oleic acid, linoleic acid,
palmitic acid, and stearic acid dissolved in CDCl₃; and supporting computer simulations. In total, 25 lipid-related resonances could be assigned, leading to the identification of 9 molecular species: free cholesterol, esterified cholesterol, 7-lathosterol, triglycerides, spingomyelin, choline, glycerophospholipid, phosphatidylcholine, and fatty acids (esterified plus nonesterified; Table 1).

Although the spectrum in Fig. 1 shows many overlapping signals from fatty acyl chains, several distinct groups can be identified, leading to the identification of 9 molecular species: free cholesterol, esterified cholesterol, 7-lathosterol, triglycerides, spingomyelin, choline, glycerophospholipid, phosphatidylcholine, and fatty acids (esterified plus nonesterified; Table 1).

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On the other hand, the C-18 H₃ protons of cholesterol and its precursor 7-lathosterol are well resolved (see the online Data Supplement for molecular structures and atom numbering), and because the cholesterol C-19 H₃ group shows separate singlets for free and esterified cholesterol, it is possible to determine their relative amounts (~60%–70% of the total cholesterol is usually esterified). The C-3 H proton shows individual signals for esterified and nonesterified cholesterol as well, but unfortunately, the latter partially overlaps with the methanol contamination in Fig. 1. Additionally, the C-21 H₃ and C-26 H₃/C-27 H₃ proton resonances can be distinguished, although they overlap moderately with fatty acid methyl groups.

Finally, cross-peaks between the allylic, olefinic, and diallylic protons can be readily observed in a 2-dimensional ¹H-¹H correlation spectrum of a healthy control, as well as cross-peaks between the different protons of the glycerol backbone (data not shown). The crowded spectral region between 0.5 and 2.5 ppm could not be completely assigned because of overlap problems and complex J-splitting of the high number of cross peaks. However, changes in the observed cross-peak pattern can still contribute to the diagnosis of an inherited metabolic disease (see below).

**CORRELATION STUDY**

We measured cholesterol and triglyceride concentrations in 15 and 12 blood plasma samples, respectively, using conventional enzymatic analysis and ¹H-NMR spectroscopy. In the latter method, peaks 2 and 20 in Fig. 1 were used for cholesterol and triglyceride quantification, respectively. We compared the obtained results by Passing
The recycle time is not 5 times the T₁ relaxation time, OMS nation might be the effect of partial saturation of OMS. As of chloroform–methanol (2:1 by volume). Another explanation might be the effect of partial saturation of OMS. If the methyl singlets of cholesterol and sterols have a T₁ relaxation similar to that of OMS (~3 s). We estimate, therefore, that the error attributable to partial saturation cannot be significant.

We determined the within-run CV with 9 samples prepared from the same blood serum containing 7.6 mmol/L cholesterol and 1.4 mmol/L triglycerides (values determined by NMR). For cholesterol and triglyceride concentrations, the within-run CVs were 7.9% and 6.8%, respectively.

The detection limit can vary for different metabolites because it is dependent on the number of equivalent protons contributing to the NMR signal, the peak-splitting pattern, the number of scans, and the field strength of the spectrometer. The detection limit for the C-18 H₃ singlet of cholesterol at 0.68 ppm is estimated to be ~10 μmol/L, assuming that the peak can be distinguished when the signal-to-noise ratio is ≥3.

**INBORN ERRORS OF METABOLISM**

To assess the diagnostic ability of ¹H-NMR spectroscopy in lipid extracts, we investigated several samples from patients with a known inborn error of lipid metabolism. Diseases in 3 patients involved errors in sterol metabolism, whereas the disease in 1 individual was caused by a defect in the breakdown of an unusual branched chain fatty acid. The characteristic resonance frequencies of different metabolites are listed in Table 2 (also see the online Data Supplement).

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**Table 1. **¹H resonance assignments with chemical shifts, multiplicity, and J-coupling constants for signals identified in the lipid extract of blood plasma taken from healthy controls (n = 3).**

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>¹H shift, ppm</th>
<th>Assignment</th>
<th>Multiplicity, J (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.53</td>
<td>Total 7-lathosterol C-18 H₃</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>0.68</td>
<td>Total cholesterol C-18 H₃</td>
<td>S</td>
</tr>
<tr>
<td>3</td>
<td>0.86/0.87</td>
<td>Total cholesterol C-26 H₃/C-27 H₃</td>
<td>2×d (6.6)</td>
</tr>
<tr>
<td>4</td>
<td>0.88</td>
<td>Fatty acyl chain CH₃(CH₂)ₙ</td>
<td>t (6.9)</td>
</tr>
<tr>
<td>5</td>
<td>0.91</td>
<td>Total cholesterol C-21 H₃</td>
<td>d (6.5)</td>
</tr>
<tr>
<td>6</td>
<td>1.01</td>
<td>Free cholesterol C-19 H₃</td>
<td>s</td>
</tr>
<tr>
<td>7</td>
<td>1.02</td>
<td>Esterified cholesterol C-19 H₃</td>
<td>s</td>
</tr>
<tr>
<td>8</td>
<td>1.05–1.19</td>
<td>Multiple cholesterol protons</td>
<td>m</td>
</tr>
<tr>
<td>9</td>
<td>1.24–1.37</td>
<td>Fatty acyl chain (CH₂)ₙ</td>
<td>m</td>
</tr>
<tr>
<td>10</td>
<td>1.42–1.55</td>
<td>Multiple cholesterol protons</td>
<td>m</td>
</tr>
<tr>
<td>11</td>
<td>1.55–1.65</td>
<td>Fatty acyl chain –CH₂CH₂CO</td>
<td>m</td>
</tr>
<tr>
<td>12</td>
<td>1.79–1.88</td>
<td>Multiple cholesterol protons</td>
<td>m</td>
</tr>
<tr>
<td>13</td>
<td>1.98–2.09</td>
<td>Fatty acyl chain –CH₂CH=</td>
<td>m</td>
</tr>
<tr>
<td>14</td>
<td>2.24–2.35</td>
<td>Fatty acyl chain –CH₂CO</td>
<td>m</td>
</tr>
<tr>
<td>15</td>
<td>2.77–2.87</td>
<td>Fatty acyl chain –CH₂CH=</td>
<td>m</td>
</tr>
<tr>
<td>16</td>
<td>3.32/3.35</td>
<td>Sphingomyelin and choline N(CH₃)₃</td>
<td>2× s (largely overlapping)</td>
</tr>
<tr>
<td>17</td>
<td>3.48–3.57</td>
<td>Free cholesterol C-3 H</td>
<td>m</td>
</tr>
<tr>
<td>18</td>
<td>3.81</td>
<td>Phosphatidylcholine N=CH₂</td>
<td>s (broad)</td>
</tr>
<tr>
<td>19</td>
<td>3.96</td>
<td>Glycerocephospholipid backbone C-3 H₃</td>
<td>s (broad)</td>
</tr>
<tr>
<td>20</td>
<td>4.15/4.29</td>
<td>Glycerol backbone C-1 H₂/C-3 H₂</td>
<td>m (AB spin system)</td>
</tr>
<tr>
<td>21</td>
<td>4.32–4.43</td>
<td>Phosphatidylcholine PO=CH₂</td>
<td>m (broad)</td>
</tr>
<tr>
<td>22</td>
<td>4.57–4.65</td>
<td>Esterified cholesterol C-3 H₃</td>
<td>m</td>
</tr>
<tr>
<td>23</td>
<td>5.17–5.24</td>
<td>Glycerophospholipid backbone C-2 H₃</td>
<td>m (broad)</td>
</tr>
<tr>
<td>24</td>
<td>5.26</td>
<td>Glycerol backbone C-2 H₃</td>
<td>p (5.7)</td>
</tr>
<tr>
<td>25</td>
<td>5.29–5.43</td>
<td>Fatty acyl chain –CH=CH=</td>
<td>m</td>
</tr>
</tbody>
</table>

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* Peak numbers correspond with Fig. 1. Chemical shifts are referenced to OMS (δ = 0.094 ppm). All assignments were based on spectra of authentic standards, on the literature, or on computer simulations, unless specified differently.

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* s, singlet; d, doublet; t, triplet; p, pentet; m, multiplet.

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* Assignment based solely on the literature.

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* Assignment based on the literature and spectral simulation.
Table 2. Characteristic resonance assignments, including multiplicity and J-coupling constants used for identification of metabolites accumulating in several inborn errors of lipid metabolism.a

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Assignment</th>
<th>δH shift, ppm</th>
<th>Multiplicity, J (Hz)</th>
<th>Inborn error</th>
</tr>
</thead>
<tbody>
<tr>
<td>7DHHC</td>
<td>C-18 H₃</td>
<td>0.618</td>
<td>s</td>
<td>SLOS</td>
</tr>
<tr>
<td>8DHHC</td>
<td>C-18 H₃</td>
<td>0.651</td>
<td>s</td>
<td>SLOS</td>
</tr>
<tr>
<td>Cholestanol</td>
<td>C-18 H₃</td>
<td>0.645</td>
<td>s</td>
<td>CTX</td>
</tr>
<tr>
<td></td>
<td>C-19 H₃</td>
<td>0.817</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>7-Lathosterol</td>
<td>C-18 H₃</td>
<td>0.534</td>
<td>s</td>
<td>CTX lathosterosis⁢d</td>
</tr>
<tr>
<td></td>
<td>C-19 H₃</td>
<td>0.810</td>
<td>s</td>
<td></td>
</tr>
<tr>
<td>8-Lathosterol</td>
<td>C-18 H₃</td>
<td>0.606</td>
<td>s</td>
<td>CTX</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>C-18 H₃</td>
<td>0.680</td>
<td>s</td>
<td>Sitosterolemia</td>
</tr>
<tr>
<td></td>
<td>C-26 H₃/C-27 H₃/C-29 H₃</td>
<td>0.800–0.850</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-24 H</td>
<td>1.685</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>Phytanic acid (esterified to glycerol)</td>
<td>C-16 H₃/C-17 H₃</td>
<td>0.844</td>
<td>d (6.6)</td>
<td>Refsum disease</td>
</tr>
<tr>
<td></td>
<td>C-18 H₃/C-19 H₃</td>
<td>0.867</td>
<td>d (6.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C-20 H₃</td>
<td>0.927⁢c,e</td>
<td>d (5.6)</td>
<td></td>
</tr>
</tbody>
</table>

a Chemical shifts are referenced to OMS (δ = 0.094 ppm). Values were determined in patients and confirmed by use of authentic standards, unless stated otherwise.

b s, singlet; d, doublet; m, multiplet.

c Tentative assignment; no authentic standard available.

d No patient material available.

e Resonates at higher ppm value in pure compound (see text).

SLOS

The 0.55–0.75 ppm region of the 1-dimensional ¹H-NMR spectrum of a blood plasma lipid extract from a 5-month-old infant with SLOS is shown in Fig. 2B. It clearly differs from the spectrum of a healthy volunteer (Fig. 2A) and shows the diagnostic metabolites 7DHHC and 8DHHC. The characteristic C-18 H₃ resonances used for identification and quantification of cholesterol, 7DHHC, and 8DHHC are well resolved. The presence of 7DHHC was confirmed by addition of the pure compound to the sample and reanalysis by NMR spectroscopy. An additional experiment for 8DHHC could not be performed because this compound was not commercially available. More certain identification of both metabolites may be achieved when additional signals of these compounds are resolved. Unfortunately, the current experimental conditions did not provide the required higher resolution.

Samples from 2 other SLOS patients showed a similar NMR spectrum. Ruan et al. (10) and Xiong et al. (11) assigned 7DHHC and 8DHHC unequivocally in an ¹H-NMR spectrum. Their study quantifies both metabolites for the first time. The quantitative data for 7DHHC and 8DHHC show discrepancies between NMR and GC (Table 3A). These may relate to the long interval of time between the GC and NMR measurements (in some cases several years). It is known that the unsaturated sterols decompose easily during storage, processing, and/or analysis.

Accumulation of cholesta-5,7,9(11)-triene-3β-ol in the blood of SLOS patients has been reported (10). The C-18 H₃ protons of this compound have been reported to resonate at 0.566 ppm (7); however, we observed no resonance at this position in the NMR spectra of samples from SLOS patients. The concentration of this compound varies considerably and may be in the low micromolar range in SLOS patients. Ruan et al. (10) reported concentrations from 0.8 to 79.4 μmol/L. This may explain why we were unable to detect this compound in our patient samples.

CTX

The 1-dimensional lipid ¹H-NMR spectrum of a 24-year-old male CTX patient is given in Fig. 2C. It shows an abnormally high concentration of the diagnostic metabolite cholestanol (C-18 H₃ resonance at 0.645 ppm, close to the C-18 H₃ of cholesterol). The NMR method revealed a cholestanol concentration of 0.15 mmol/L, somewhat higher than the value determined by GC (0.11 mmol/L; Table 3B). This may be the result of a slight overlap with the tail of the cholesterol resonance. Furthermore, the spectrum shows 3 other peaks: the ¹³C satellite of the cholestanol C-18 H₃, the C-18 H₃ resonance of 7-lathosterol, and 1 unknown metabolite (peak X). Although 7-lathosterol is also observed in healthy controls (Fig. 1), increased concentrations were found in all investigated CTX patients (Table 3B), which corresponds with reference values determined by Woltbers et al. (27).

Interestingly, peak X at 0.61 ppm did not occur in the control spectra, but was present in the 2 other CTX patients as well [both female (16 and 46 years of age) and before the start of therapy], although it was much weaker in the 16-year-old female. GC and GC-MS measurements also displayed an unknown peak in CTX patients, which was tentatively identified as 8-lathosterol (see the online Data Supplement). On the basis of this finding and the following substantial supporting evidence, peak X was tentatively assigned as the C-18 H₃ resonance of 8-lathosterol: First, the relative peak area compared with cholesterol and 1 unknown metabolite (peak X). Although 7-lathosterol is also observed in healthy controls (Fig. 1), increased concentrations were found in all investigated CTX patients (Table 3B), which corresponds with reference values determined by Woltbers et al. (27).

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nual difference as 7DHC compared with 8DHC, i.e., the
position of the double bond (see the online Data Supple-
mment), and the frequency of peak X differed from 7-lathos-
terol in the same way as 8DHC from 7DHC (see Fig. 2B).
Although the chemical shift differences between 7- and
8-lathosterol and the chemical shift difference between
7DHC and 8DHC were not identical (0.073 and 0.031
ppm, respectively), this does provide some tentative evi-
dence. Third, our assignment was strongly supported by
chemical shift values found by Wilson et
al. (7) for 7- and 8-lathosterol. Finally, results described by
Wolthers et al. (27) confirm the presence of 8-lathosterol
in plasma of CTX patients, and the reported concentration
(60.0 μmol/L; n = 1) corresponded very well with the
mean concentration of 66.8 μmol/L determined by 1H-
NMR (Table 3B). Unfortunately, 8-lathosterol is not
commercially available for absolute confirmation of the
assignment. Furthermore, the current resolution does
not allow identification of additional 8-lathosterol sig-
nals.

Fig. 2. Upfield regions of the 1-dimensional 1H-NMR spectra of patient plasma lipid extracts, plasma from a healthy control, and the authentic
β-sitosterol and phytanic acid standards.
Methyl assignment for both authentic standards are shown (see the online Data Supplement for atom numbering). (A), control; (B), SLOS; (C), CTX; (D), control; (E),
sitosterolemia; (F), β-sitosterol; (G), Refsum disease; (J), phytanic acid. Peaks in panels A-C correspond to the C-18 H₃ resonance of the indicated metabolite. The peak
labeled 13C in panel C represents the cholesterol C-18 H₃ 13C satellite, whereas the peak with unknown assignment is labeled with an X. The peak numbering in panel
D is identical to the numbering in Fig. 1. Peaks labeled with an * in panels E and G correspond to β-sitosterol (sito) and phytanic acid signals, respectively. The C-20
H₃ resonance of esterified phytanic acid is indicated by # in panel G.
SITOSTOREMIA
The upfield region of the 1-dimensional $^1$H-NMR spectrum of the lipid extract of a sitosterolemic patient is shown in Fig. 2E. For comparison, the same spectral regions for a healthy control and for pure $^8$H$_{9252}$-sitosterol are shown in Fig. 2D and Fig. 2F, respectively. In the analysis, only $^8$H$_{9252}$-sitosterol was taken into account because this is the major plant sterol, constituting $65\%$ of all absorbed plant sterols (21). Campesterol and stigmasterol contribute $32\%$ and $3\%$, respectively. Unfortunately, $^8$H$_{9252}$-sitosterol quantification was impossible under the current experimental conditions because the C-18 H$_3$ and C-19 H$_3$ resonances of $^8$H$_{9252}$-sitosterol and cholesterol overlapped almost completely (Fig. 2E), and other $^8$H$_{9252}$-sitosterol resonances were not resolved into individual doublets and triplets to allow accurate quantification. Although the assignments of the C-26 H$_3$, C-27 H$_3$, and C-29 H$_3$ resonances ($0.80–0.85$ ppm) are therefore not exactly known, their presence in the sitosterolemia patient and absence in the healthy control is clear. Peak positions in the authentic standard corresponded exactly with the observed positions in the patient, and repeated NMR measurement after addition of pure $^8$H$_{9252}$-sitosterol confirmed the assignment. The weak resonances at $0.75$ to $0.80$ ppm (at right of the $\beta$-sitosterol signals) likely result from campesterol and dihydrobrassicasterol (24S stereoisomer of campesterol), because the corresponding peak positions were found in the authentic standard of campesterol (data not shown). Overlap with the campesterol signals increases the difficulty in quantifying $\beta$-sitosterol; therefore, higher resolution and precise knowledge of all assignments are necessary to allow quantification of both $\beta$-sitosterol and campesterol.

To support the diagnosis, we obtained a 2-dimensional $^1$H-$^1$H correlation spectrum. The resulting spectrum showed a characteristic cross-peak at $(1.68; 0.82)$ ppm, originating from the long-range spin–spin coupling between the C-24 H and C-26 H$_3$, C-27 H$_3$, or C-29 H$_3$ of $^8$H$_{9252}$-sitosterol (data not shown). Thus, the presence of $^8$H$_{9252}$-sitosterol can be measured by our NMR method, although quantification is still impossible.

REFSUM DISEASE
The $0.60–1.05$ ppm region of the 1-dimensional $^1$H-NMR lipid spectrum of a 29-year-old female patient with Refsum disease is shown in Fig. 2G. Assignment of the methyl resonances is given in the spectrum of pure phytanic acid (Fig. 2H). The doublet at $0.84/0.85$ ppm provides clear evidence for the presence of phytanic acid in the Refsum sample (see Fig. 2D). Remarkably, the C-20 H$_3$ protons, which resonate as a doublet at $0.97/0.98$ ppm.
in pure phytanic acid, appear absent in the patient spectrum. However, because of esterification with glycerol (26), this peak probably shifted to 0.92/0.93 ppm, where a clear difference can be seen between the Refsum case and a healthy control. After addition of pure phytanic acid to the patient sample, the doublet at 0.97/0.98 ppm was evident, confirming that phytanic acid was not present in its free form in the Refsum disease patient. Additional computer simulations confirmed a shift toward lower ppm values on esterification with glycerol.

Phytanic acid quantification by 1H-NMR (using one half of the C-16 H3/C-17 H3 doublet at 0.84 ppm) corresponded well with values determined by GC (Table 3C). We monitored the effect of plasmapheresis on the phytanic acid concentration by NMR analysis and observed a rapid decrease in phytanic acid concentration of 50%. The concentration return to its original value 3 days after the treatment (Fig. 3).

Discussion
Many inherited metabolic diseases are caused by defects in lipid metabolism or biosynthesis, often leading to accumulation of unusual lipids in the blood and tissues of affected patients. In this study, the successful use of 1H-NMR spectroscopy of blood plasma lipid extracts to accurately identify and quantify these lipids was demonstrated for 4 different inborn errors of lipid metabolism. Twenty-five lipid-derived resonances could be assigned in the 1-dimensional spectra of healthy controls, leading to the identification of 9 distinct molecular species. This number increases to 14 when one takes the investigated metabolic diseases into account.

Although 1H-NMR spectroscopy of lipid extracts was used previously by Kriat et al. (13) to study tumor-induced effects, its application in clinical diagnosis has hitherto been described only for the identification of unusual metabolites in the blood plasma of SLOS patients (10, 11). By using OMS instead of the highly volatile TMS as a concentration and chemical shift reference, we were able, for the first time, to accurately quantify metabolites in chloroform solutions by 1H-NMR. We obtained good correlations with conventional methods for total cholesterol and triglyceride concentrations. This does not exclude a possibly less efficient extraction and, hence, worse correlation for other metabolites. The results obtained for cholesterol indicate that a larger extraction volume needs to be used when high lipid concentrations are expected. Nevertheless, the currently applied protocol will likely be sufficient for the quantification of unusual lipids because their concentrations are typically low (<1 mmol/L) and because deviations are seen only at high concentrations (>6 mmol/L for cholesterol).

Because lipid 1H-NMR spectroscopy requires ~1 day of sample preparation and only a limited number of samples can be worked up, it cannot compete with the automated enzymatic analyses of cholesterol and triglycerides in blood. If an automated extraction procedure could be developed, it may shorten the sample preparation time. 1H-NMR spectroscopy does, however, provide an excellent alternative for the identification and quantification of unusual lipids. For example, separation of cholestanol and cholesterol can be tedious by conventional chromatographic methods, whereas both metabolites are readily identified with 1H-NMR spectroscopy based on their different C-18 H3 resonances. Similarly, the presence of 7DHC and 8DHC in the plasma of SLOS patients is immediately evident from a simple 1-dimensional 1H-NMR spectrum. Quantification of 8DHC may be more accurate with 1H-NMR spectroscopy because the lack of a commercially available authentic standard makes correct calibration of chromatographic measurements extremely difficult.

The diagnosis of SLOS, CTX, and sitosterolemia can be made with almost 100% certainty based on NMR analysis. However, for Refsum disease, the total clinical picture is required because phytanic acid also accumulates in persons with Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and rhizomelic chondrodysplasia punctata type 1 (26). The investigation of these diseases with lipid 1H-NMR spectroscopy is a topic for future research.

A database containing the resonances of many more authentic standards of molecules relevant to lipid metabolism needs to be established, which will be helpful for the assignment of unknown signals. For NMR spectra of urine samples, an analogous strategy has led to the identification of new inborn errors of metabolism (28–30), and it is likely that lipid NMR spectroscopy may also lead to the discovery of currently unknown inherited disorders.

In addition to resonance positions, which can provide clear information only when there is no overlap, peak ratios can be used to obtain additional important information. For example, the CH2/CH3 ratio (peaks 9 and 4, respectively, in Fig. 1) can provide evidence of the presence of branched chain fatty acids because this will lower

Fig. 3. Effect of plasmapheresis treatment on plasma phytanic acid concentrations in a female Refsum disease patient. The duration of the plasmapheresis is indicated in gray.
this ratio. For healthy individuals (n = 3), we found a CH3/CH2 peak ratio (SD) of 6.61 (0.07) (note that the CH3 integral has to be corrected for overlap with cholesterol resonances). This corresponds well with a ratio of 6.67 calculated for a theoretical 1:1 mixture of palmitic, oleic, and linoleic acid, which together constitute ~75% of all plasma fatty acids. As expected, the ratio for our Refsum disease patient was much lower (3.87), which is fully explained by the branching of the phytanic acid and its concentration compared with other fatty acids in the sample. Although the presence of phytanic acid can be more easily ascertained based on detection of the C-16 H3/C-17 H3 resonance (see the online Data Supplement), it is likely that peak ratios may be helpful for the detection of abnormalities in spectra without any clear unusual signals.

In conclusion, we have presented a new method for identification of unusual lipids in blood plasma or serum by means of 1H-NMR spectroscopy. Furthermore, metabolite concentrations can be accurately determined with the nonvolatile OMS as a chemical shift and concentration reference compound. The technique is well suited for the diagnosis and follow-up of inborn errors of lipid metabolism, as demonstrated in 4 different inherited diseases.

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