Simple diagnosis of the Zellweger syndrome by gas–liquid chromatography of dimethylacetals

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Abstract The absence of peroxisomes in patients with the cerebrohepatorenal syndrome of Zellweger leads to several biochemical abnormalities, including deficient synthesis of plasmalogens as well as accumulation of very long-chain fatty acids and intermediates in bile acid biosynthesis. Accumulation of very long-chain fatty acids in serum and fibroblasts has hitherto been used most extensively for diagnosis. Due to the relatively small amounts of the very long-chain fatty acids also in the Zellweger patients, and the risk for interfering impurities, such analyses are difficult. Direct assay of plasmalogens is also relatively difficult and time-consuming. In this report, we describe a relatively simple method for diagnosis, based on gas–liquid chromatography of a lipid extract of erythrocytes after methyl transesterification. The α,β-unsaturated ether in the plasmologen is converted to the dimethylacetal of the corresponding aldehyde, and the relative amount of plasmalogen is thus reflected by the ratio between 18:0 dimethylacetal and methyl stearate as well as by the ratio between 16:0 dimethylacetal and methyl palmitate. The ratio 18:0 dimethylacetal/methyl stearate was found to be 0.28 ± 0.03 (mean ± SD) after analyses of erythrocytes from healthy or non-Zellweger infants, but less than 0.02 in erythrocytes from three infants with the Zellweger syndrome. Preliminary work with amniotic fluid suggests that this analysis may be suitable also for prenatal diagnosis of the Zellweger syndrome. —**Björkhem, I., L. Sisfontes, B. Boström, B. F. Kase, and R. Blomstrand.** Simple diagnosis of the Zellweger syndrome by gas–liquid chromatography of dimethylacetals. *J. Lipid Res.* 1986. **27:** 786–791.

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The cerebrohepatorenal syndrome of Zellweger is an inborn error of metabolism clinically characterized by muscular hypotonia, liver enlargement, renal cysts, craniofacial malformations, and mental retardation (1). Usually the patients die before one year of age. Most probably the biochemical defects in this disease are related to the apparently complete lack of peroxisomes (2). Peroxisomes are important for two early steps in the biosynthesis of plasmalogens (3, 4), for the final step in the biosynthesis of bile acids (5, 6) as well as for oxidation of very long-chain fatty acids (7). Due to this, patients with the Zellweger syndrome have a markedly reduced formation of plasmalogens in various tissues (8) and fibroblasts have a reduced incorporation of labeled hexadecanol into plasmalogens (9). Furthermore, there is an accumulation of very long-chain fatty acids (10, 11) and bile acid intermediates (12, 13). Gas–liquid chromatographic determination of the ratio between hexacosanoic acid (26:0) and docosanoic acid (22:0) in serum (11), cultured skin fibroblasts (10), amniocytes (14), or amniotic fluid (15) has hitherto been used most extensively for natal and prenatal diagnosis of the disease. Direct assay of plasmalogens (8) or assay of incorporation of labeled hexadecanol into plasmalogens in fibroblasts (9) have also been used for diagnostic purposes. Assay of acyl-CoA dihydroxyacetone phosphate acyltransferase in cultured chorionic villi was also recently recommended for prenatal diagnosis (16). Since biosynthesis of bile acids occurs relatively late in the fetal development, assay of bile acid intermediates cannot be used as a prenatal test.

Due to the relatively small amounts of the very long-chain fatty acids in normal subjects as well as in Zellweger patients, such analyses are relatively difficult. According to our experiences there is also a definite risk for occurrence of interfering impurities, in particular phthalates (15). If the identity of the very long-chain fatty acids is not ascertained by combined gas–liquid chromatography–mass spectrometry (17), there may even be a risk for false positive results. Direct assays of plasmalogens together with other lipid classes (8, 18), as well as assay of enzymatic steps in the biosynthesis of plasmalogens (9, 16), are laborious and time-consuming. Assay of bile acid intermediates in serum is relatively easy (6, 13) but requires a mass spectrometer for a definitive analysis.

Abbreviations: GLC, gas–liquid chromatography; DMA, dimethylacetal; HPLC, high performance liquid chromatography.

We describe a simple method for diagnosis based on gas–liquid chromatography of a lipid extract of erythrocytes after methyl transesterification (19, 20). The α,β-unsaturated ether in the plasmalogen molecule is converted into a dimethyl acetal of the corresponding aldehyde during this procedure, and the relative amount of plasmalogen is thus reflected in the ratio between 18:0 aldehyde and stearic acid as well as by the ratio between 16:0 aldehyde and palmitic acid. As expected, this ratio was markedly reduced in lipid extracts of erythrocytes from patients with the Zellweger syndrome.

MATERIAL AND METHODS

Subjects and biological materials

Erythrocytes were obtained from seven healthy infants or infants with diseases unrelated to the Zellweger syndrome (age 3 months to 3 years) as well as from three infants with the Zellweger syndrome. Two of the latter patients (B. S. and I. B.) have been described in detail previously (13, 15, 21). Patient M. E. had the typical clinical features of the Zellweger syndrome (hypotonia, craniofacial malformation, hepatomegaly). The diagnosis was established by demonstration of accumulation of C_{27}-bile acid intermediates in serum and C_{26}-fatty acid in cultured fibroblasts. Samples of amniotic fluid were collected in the 16th pregnancy week from the mother giving birth to patient I. B. (15) and from six mothers who gave birth to healthy infants.

Materials

HPLC solvents were purchased from Rathburn Chemical Ltd. (Walkeburn, Scotland). Fatty acid standards were obtained from Nu-Chek-Prep (Elysian, MN). Methanolic sodium methoxide (0.5 M) was purchased from Applied Science Europe (Oud-Beijerland, The Netherlands). Methanolic hydrogen chloride (1 M) was prepared from 99% pure hydrogen chloride (AGA SpecialGas, Lidingö, Sweden). Bovine brain ethanolamine phospholipids (98%) containing 50% plasmalogen were obtained from Sigma Chemicals (St. Louis, MO). Dimethyl acetal standards, containing the dimethylacetal of the 16:0 fatty aldehyde as one dominating component, were purchased from Supelco Inc. (Bellafonte, PA). 2,6,Di-*tert*-butyl-cresol (BHT), was obtained from Fluka AB (Buchs, Switzerland). All other reagents and chemicals were from Merck (Darmstadt, GFR).

Chromatographic procedures

The capillary gas–liquid chromatographic system used, including a Hewlett-Packard 5710A gas chromatograph and a modified solventless injector (falling needle), has been described previously in detail (22). A home-made borosilicate glass capillary column (50 m × 0.3 mm i.d.) statically coated with Silar 5CP (Supelco) was used. Recognition and integration of the peaks were done with a Hewlett-Packard 3390 integrator coupled to an ABC 80 computer system (Luxor, Motala, Sweden).

High performance liquid chromatography was performed with a complete LDC-gradient Master System (model 1601) for solvent delivery. Detection of unsaturated ethanolamine phospholipids was achieved by a UV-Spectromonitor model 1204 at 205 nm. All the HPLC equipment was obtained from Milton Roy Co. (Riviera Beach, FL). The column used (25 cm × 4.6 mm i.d.) was packed with Nucleosil 50–5 μm (Machery-Nagel, GFR). The gradient profile used for separation of the phospholipids was essentially as described by Guichardont and Lugarde (23). Phosphorus was determined in some of the HPLC fractions according to the method of Rouser, Fleischer and Yamamoto (24). Combined gas–liquid chromatography–mass spectrometry was performed with an LKB 2091 instrument (LKB, Bromma, Sweden).

Lipid extractions and derivatizations

In most analyses, 0.2–0.5 ml of packed erythrocytes or 1–2 ml of amniotic fluid was used. All this material was extracted with chloroform–methanol 2:1 (v/v) according to the method of Folch, Lees, and Sloane Stanley (25). Fatty acid methyl esters were prepared by transesterification of 100 μg of the lipid extract with 1 M hydrogen chloride in methanol essentially as described by Carveau and Dubacq (26).

RESULTS

Analyses of lipid extracts of erythrocytes

Fig. 1 shows gas–liquid chromatograms obtained in analysis of derivatized lipid extracts of erythrocytes from a healthy infant and from an infant with the Zellweger syndrome. Peaks corresponding to dimethylacetals of 16:0 and 18:0 aldehydes were obtained in material from the healthy subject but not in material from the Zellweger infant. In addition to the 16:0 and 18:0 dimethylacetals, peaks with retention times expected for 14:0, 18:1, 20:0, and 20:1 dimethylacetals appeared in material from the healthy subjects. Due to lack of reference lipids and to the presence of contaminating compounds that sometimes occur near the latter peaks, only the 16:0 and 18:0 dimethylacetals were used in the following investigations.

The identity of the 16:0 dimethylacetal was established by combined gas–liquid chromatography–mass spectrometry. The mass spectrum was identical to that of the authentic compound and showed characteristic peaks at m/z 286 (M), m/z 254 (M–32), m/z 222 (M–64), m/z 124, m/z 110, m/z 96, m/z 82, m/z 68 (alkadiene fragments with varying chain lengths), m/z 71 ($CH_3(CH_2)$-C^+H_2) and m/z 75 ($C^+H(OCH_3)_2$). No authentic 18.0 dimethyl-

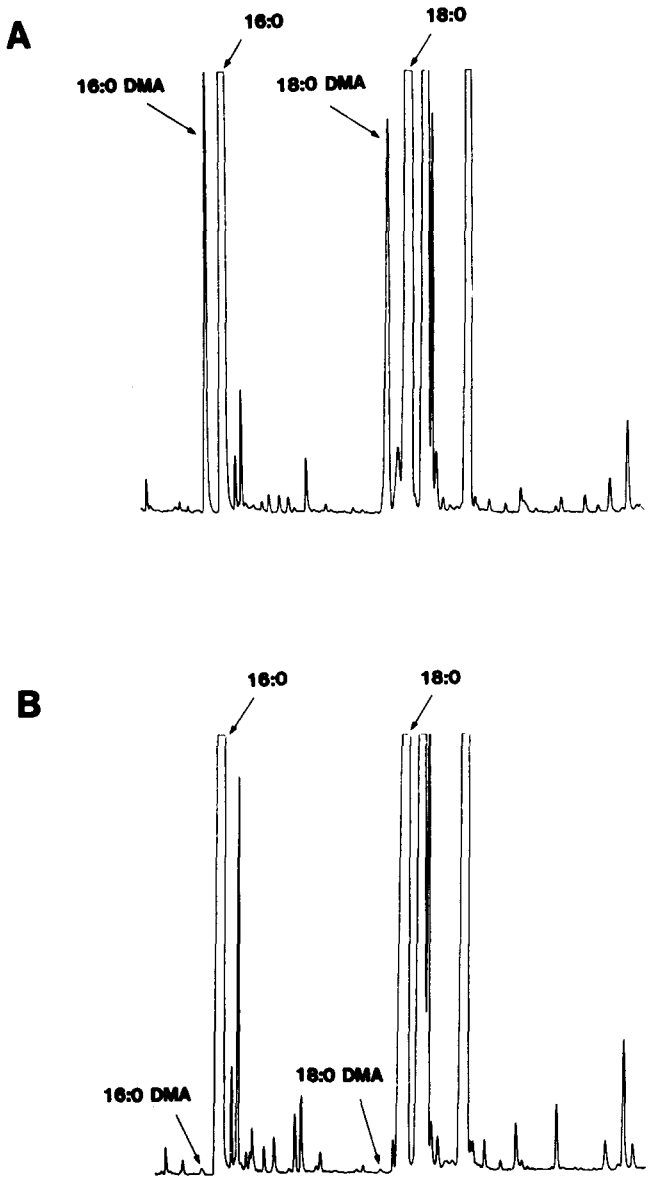

Fig. 1. Gas-liquid chromatography of a transmethylated lipid extract of erythrocytes from a healthy infant (A) and an infant with the Zellweger syndrome (B). For experimental details see Materials and Methods.

acetal was available, but the biological compound gave the expected mass spectrum with peaks at m/z 282 (M-32), m/z 250 (M-64), and, in addition, the above fragments at m/z 124, m/z 110, m/z 96, m/z 75, m/z 71, and m/z 68.

In order to ascertain that plasmalogens are the origin of the dimethylacetals (cf. refs. 19, 20), total lipid extracts from erythrocytes from healthy (non-Zellweger) infants were subjected to HPLC. Two peaks were obtained with retention times identical to those obtained in analysis of a reference ethanolamine phospholipid preparation containing 50% plasmalogens (cf. Material and Methods) (**Fig. 2A**). After transesterification and GLC, the first peak, denoted PE I, gave dimethylacetals in addition to the fatty acids. The material corresponding to the second peak, denoted PE II, gave only fatty acids. Transmethylation and GLC of the other lipid fractions obtained in the HPLC chromatography gave very little or no dimethylacetal.

It was concluded that the fraction denoted PE I in Fig. 2A contains mainly plasmalogens, and that transmethylation of this fraction gives 18:0 and 16:0 dimethylacetals together with small amounts of several other dimethylacetals whose identity was never ascertained.

In accordance with the reported lack of plasmalogens in patients with the Zellweger syndrome (8, 9), HPLC of a total lipid extract from erythrocytes of a subject with the Zellweger syndrome gave only the PE II peak, and the PE I peak was completely absent (Fig. 2B).

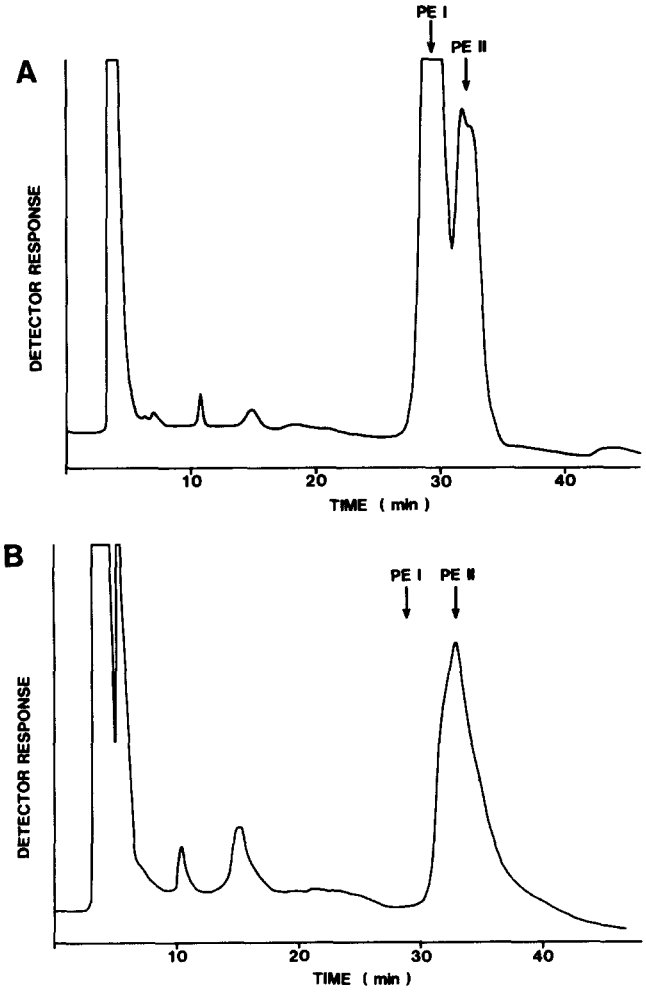

Fig. 2. HPLC-chromatogram of a crude lipid extract of erythrocytes of a healthy infant (A) and an infant with the Zellweger syndrome (B). For experimental details see Materials and Methods. PE I denotes the plasmalogen-containing phosphatidylethanolamine fraction and PE II denotes the plasmalogen-free phosphatidylethanolamine fraction.

Table 1 summarizes the results of direct analyses of total lipid extracts from erythrocytes of seven control subjects and three infants with the Zellweger syndrome. The ratio between 16:0 dimethylacetal and methyl palmitate was found to be 0.13 ± 0.02 (mean ± SD) in the control subjects but less than 0.02 in the three subjects with the Zellweger syndrome. The ratio between the 18:0 dimethyl acetal and methyl stearic acid was 0.28 ± 0.03 in the control subjects but less than 0.02 in the three Zellweger infants. It should be pointed out that the relative amounts of methyl palmitate and methyl stearate were similar in extracts from the control subjects as in the extracts from the Zellweger infants.

For reasons of comparison, and in order to get a more direct measurement of the relative content of plasmalogens in erythrocytes from the control subjects and the subjects with Zellweger syndrome, total lipid extracts of erythrocytes from all the subjects were also subjected to HPLC. The content of plasmalogens was estimated by measurement of phosphorus in the total lipid extract applied to the column and in the isolated PE I fraction.

In the analysis of material from the healthy subjects, the PE I fraction was found to contain 11.6 ± 2.0% of the total phosphorus. In the analysis of the material from the Zellweger infants, however, the PE I fraction was found to contain 0.4%, 0.5%, and 1.1%, respectively. Due to the absence of a UV-absorbing peak, the collection of the PE I fraction in the HPLC of the material from the Zellweger infants was difficult, and false high values may have been obtained.

For reasons of comparison, attempts were also made to quantitate very long-chain fatty acids in the crude lipid extracts from the erythrocytes. Only trace amounts of 26:0 and 26:1 fatty acids were detected in the material from the healthy and the non-Zellweger infant (corresponding to a ratio between 26:0 and 22:0 of less than 0.2).

Erythrocytes from two of the Zellweger infants were also found to contain only small amounts of 26:0 fatty acid, and the ratio between 26:0 and 22:0 was about 0.3. In the analysis of erythrocytes from one of the Zellweger patients (M. E.), however, there was a very prominent peak with a retention time corresponding to 26:0 fatty acid, and the ratio bewteen 26:0 and 22:0 was about 12.9 in this case.

TABLE 1. Relative content of aldehydes in erythrocytes from control subjects and subjects with Zellweger disease

Subject	16:0 Dimethylacetal/ Methyl Palmitate	18:0 Dimethylacetal/ Methyl Stearate
Control 1	0.113	0.253
Control 2	0.098	0.282
Control 3	0.129	0.289
Control 4	0.129	0.319
Control 5	0.140	0.297
Control 6	0.143	0.248
Control 7	0.132	0.260
Mean ± SD	0.126 ± 0.016	0.278 ± 0.026
Patient M. E.	0.003	0.001
Patient B. S.	0.012	0.013
Patient A. P.	0.002	0.000

Analyses of lipid extracts of amniotic fluid

In a previous study we analyzed amniotic fluid from normal pregnancies and a pregnancy resulting in a Zellweger infant. It was shown that the ratio between hexacosanoic acid and docosanoic acid was less than 0.12 in the control samples but 0.78 in the sample from the Zellweger pregnancy (15). **Table 2** summarizes the results of a reanalysis of the amniotic fluid from the Zellweger pregnancy and analyses of amniotic fluid from six pregnancies resulting in healthy infants. The ratio between 18:0 dimethylacetal and methyl stearate was 0.14 ± 0.02 in the analyses of the control samples and 0.03 in the analysis of the amniotic fluid from the Zellweger pregnancy. The mean ratio between methyl hexacosanoic acid and methyl docosanoic acid was 0.15 in the analyses of the control samples and 0.77 in the analysis of the samples from the Zellweger pregnancy. The ratio 26:0/22:0 was, however, relatively high in two of the control samples, 0.20 and 0.43, respectively. The possibility that these values were falsely high due to impurities contaminating the 26:0 peak cannot be completely excluded (cf. ref. 15).

DISCUSSION

As part of a general project to develop methods for diagnosis of some neurological disorders by gas–liquid chromatography of lipid extracts (27), we describe a simple method for diagnosis of the Zellweger disease based on analysis of plasmalogens. In the course of studies of the metabolism of the *sn*-1-monoethers of glycerol with hexadecyl, octadecyl, and 9-octadecenyl alcohol, Blomstrand and Gürtler (28) were the first to describe the separation of these compounds after acylation of the two hydroxyl groups with acetic acid anhydride. In the present study, however, conversion of the above unsaturated alcohols in the plasmalogens into dimethylacetals prior to GLC was thought to be more suitable. The C–O–C configuration in plasmalogens is relatively stable and is resistant to bases as well as to most oxidizing and reducing agents (19, 20). The bond may, however, be cleaved by acids, and Klenk (29) was the first to show that acid methanolysis of plasmalogens yields dimethyl acetal derivatives of aldehydes along with fatty acid methyl esters. It is evident that this method has several advantages compared to previous methods used for the diagnosis of the Zellweger disease. Due to the relatively high amounts of plasmalogens in

TABLE 2. Relative content of 18:0 aldehyde and 26:0 fatty acid in amniotic fluid from normal pregnancies and a pregnancy resulting in a Zellweger infant

Origin of Amniotic Fluid	18:0 Dimethylacetal/ Methyl Stearate	Methylhexacosanoic acid/ Methyl Docosanoic Acid
Control 1	0.138	< 0.05
Control 2	0.145	0.08
Control 3	0.149	0.07
Control 4	0.157	0.09
Control 5	0.130	0.20
Control 6	0.113	0.43
Mean ± SD	0.139 ± 0.016	0.15
Zellweger pregnancy	0.026	0.77

erythrocytes, the demand for sensitivity is much lower than is the case in analysis of very long-chain fatty acids. A very characteristic pattern was obtained in the GLC of lipid extracts from erythrocytes of Zellweger patients with an almost complete lack of dimethylacetals with a chain length from 14 to 20 carbon atoms. There was a good separation between 16:0 dimethylacetal and methyl palmitate as well as between 18:0 dimethylacetal and methyl stearate and, according to our experience, there is little or no risk for the presence of contaminating compounds in this region of the chromatogram. The ratio between 18:0 dimethyl acetal and methyl stearate in the analyses of erythrocytes from the Zellweger infants was less than 10% of that in the analyses of erythrocytes from control subjects.

In our determinations, analyses of erythrocytes were found to give more reproducible results than analyses of plasma or serum. Dietary factors may thus influence the results obtained with serum or plasma, and it is difficult to collect fasting samples from infants with the Zellweger syndrome.

In contrast to the results of our study, Heymans et al. (8) found a relatively small difference (about twofold) between the plasmalogen content in erythrocytes from healthy subjects and subjects with Zellweger syndrome. These authors used thin-layer chromatography to separate the different lipid classes, however, and their different results may have been due to incomplete separation.

Using the present transmethylation procedure, it was also shown in preliminary experiments that autopsy samples of liver and heart from patients with the Zellweger syndrome are lacking plasmalogens. Similar results have been reported by Heymans et al. (8).

In a previous study we showed that cell-free amniotic fluid from a pregnancy resulting in an infant with the Zellweger syndrome had about a sixfold increased ratio between 26:0 fatty acid and 22:0 fatty acid (15). As expected, the ratio between 18:0 dimethylacetal and methyl stearate was reduced in this sample of amniotic fluid, indicating that the present method may be used for prenatal diagnosis. The ratio 18:0 dimethylacetal/methyl stearate was at least fourfold higher in all the control samples as compared to the sample from the Zellweger pregnancy. Possibly due to contamination with impurities, analysis of one of the control samples gave a ratio between 26:0 fatty acid and 22:0 fatty acid that was more than 50% of that obtained in the analysis of the sample from the Zellweger pregnancy. It may be concluded that, under the specific conditions used here for prenatal diagnosis, use of dimethylacetals is more definitive than use of very long-chain fatty acids. It should be pointed out, however, that in contrast to most previous studies (14, 16), the present analyses were performed on crude centrifuged amniotic fluid and not on cultured amniocytes. Since it may take several weeks to cultivate amniocytes, analysis of cell-free amniotic fluid offers an advantage. Use of the present technique for prenatal diagnosis of the Zellweger syndrome is now under evaluation.

The Zellweger syndrome is the gravest form of the known peroxisomal diseases with a complete absence of peroxisomes. A variant form of Refsum's disease (30) and neonatal adrenoleukodystrophy (10) have several features in common with the Zellweger syndrome and are also regarded as peroxisomal diseases. There is an increasing need for biochemical tests for the diagnosis and classification of the different peroxisomal diseases. At the present state of knowledge, deficient synthesis of the *O*-alkyl bond in lipids and deficient synthesis of bile acids seem to be coexisting phenomena, while accumulation of very long-chain fatty acids is less specific and may be seen also in subjects with intact peroxisomes (14). The present method may be of value in futher attempts to subclassify the peroxisomal disorders.

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