Determining the Reference Intervals of Long-Chain Fatty Acids, Phytanic Acid and Pristanic Acid for Diagnostics of Peroxisome Disorders in Children

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ABSTRACT: In this paper we are given the following information about the biochemical properties of long-chain fatty acids (LCFA), phytanic acid and pristanic acids and their biological role. This article describes procedure of the analysis of these compounds by gas chromatography-mass spectrometry (GC-MS) from the sample preparation step to obtain a specific result. Presented reference values of long chain fatty acids and main biochemical markers of peroxisome disorders (phytanic acid and pristanic acid) in plasma and examples described of changes of these values in various pathologies, such as hereditary diseases in peroxisomes in children and adult. The complex GC-MS analysis of LCFA, pristanic acid and phytanic acid is an effective method to identify patients with peroxisome impairment, especially for diagnostics Zellweger syndrome spectrum, rhizomelic chondrodisplasia punctata type 1 and Refsume's disease. This article is intended for doctors clinical and laboratory diagnosis, specialists in the field of clinical genetics, pediatric neurologists, and scientists and audiences.

INTRODUCTION

The introduction of the achievements of molecular genetics, immunology, analytical biochemistry, morphology, and other sciences has led to undifferentiated state of a whole class of new diseases, related to change the structure and function of intracellular structures - lysosomes, mitochondria, peroxisomes, the so-called "diseases of cell organelles".

Due to the structure and function subcellular structures at different human pathology it was became possible isolate mitochondrial disease, lysosomal diseases, peroxisomal diseases. However, if the study of the first two classes of diseases is progressing significantly, the study peroxisomal diseases are not given enough attention [1, 2]. Peroxisomes play an important role in the catabolism of polyamines processes "peroxisome breathing», (β-oxidation of very long chain - 24-26 carbon atoms or more) and dicarboxylic fatty acids (C26 and above). These acids usually ingested in the diet and, because they are not part of the human lipid shall be destroyed. These related, in particular, phytanic acid, contained in plants. Oxidation of very long chain fatty acid with peroxisome enzyme acyl-CoA oxidase going in the liver, adipose tissue, kidney, intestines, lung, spleen, adrenals. In peroxisomes observed degradation some xenobiotics containing acetalphosphatides and catabolism of prostaglandins.

An important role is played by peroxisomes in the synthesis of certain vital components necessary for the body, for example, plasmalogens. These phospholipids in which the fatty acid is not combined with glycerin ester (enol) and aldehyde bond. They constitute from 5 to 20% of phospholipids and membranes necessary for formation of nervous tissue. Plasmalogen protect cells from oxygen free radicals. Peroxisomes are involved in transamination of glyoxylate, which are formed with the participation of glycolate oxidase and peroxisomal may further metabolized to oxalic acid. Alanine glyoxylate aminotransferase hereditary enzyme deficiency in liver peroxisomes leads to the development sof hyperoxaluria type I, since glyoxylate thus converted into oxalic acid.

Due to the variety of functions peroxisome becomes apparent that a violation of one or more metabolic functions may cause peroxisomal disorders [2]. Such disorders typically result in accumulation in tissues and

biological fluids of one, several or all of the respective metabolites, depending on the number of functional disorders. These savings are used for (differential) diagnosis of peroxisomal biochemical disorders accompanied by the absence or dysfunction of peroxisomes. Diagnosis is particularly important in identifying peroxisomal disorders in children, because, for example, with Zellweger syndrome, if the detection of the early stages does not occur, children die a few months after birth from severe hypotension, eating disorders, convulsions, seizures of liver and heart.

Peroxisomes or microbodies, are widely represented in human cells of all tissues except erythrocytes. They are a round or oval formations with diameter ranging from 0.2-1 mm (in liver and kidneys) to 0.1-0.2 micrometers (as amniocytes and fibroblasts). The peroxisomes, there are about 40 types of enzymes, takes an important part in the oxidative metabolism of cells, metabolism of bile acids, fatty acids, cholesterol, gluconeogenesis [3]. Peroxisomes play an important role in protecting cells from forming in their matrix of atomic oxygen (result of hydrogen peroxide decomposition) [2]. Part of peroxisome oxygen absorption is approximately 20% from summary oxygen consumption in the liver. Peroxisome enzymes use oxygen to oxidize various substrates, producing hydrogen peroxide. Excess hydrogen peroxide can be dangerous for the cells, however, thanks to the presence of enzyme - catalase, quickly decomposing hydrogen peroxide, prevented damage to cells, and the presence of superoxide dismutase witch protect cell from another toxic compound of oxygen - superoxide anion.

A recent study have shown that peroxisomes derived from a special subdomain of the endoplasmic reticulum and, therefore, do not have their own DNA, are semi-autonomous organelles that are able to grow and is divided into subsidiaries peroxisomes. It is now widely known that peroxisomes catalyze a number of important metabolic functions that can not be achieved by other organelles.

From the standpoint of human genetic diseases, of particular interest are the following processes: 1) beta-oxidation of fatty acids; 2) biosynthesis of lipid; 3) alpha-oxidation of fatty acids and; 4) glyoxylate detoxification. To accomplish this, a set of functions, peroxisomes have a unique set of enzymatic proteins that catalyze different reactions. Besides, the membranes have a system of selective peroxisome transport for transferring substrates from the cytosol into the organelles and outputting its end products of metabolism.

So far, there is no uniform classification of peroxisomal disorders. This is due to the small study the function of peroxisomes and the lack of a single criterion, which could form the basis of the classification. Attempts to use to justify the classification of morphological criteria (presence or absence of peroxisomes in the cells) were unsuccessful. In recent years there has been research to use as fundamental criteria peroxisomal disorders primary biochemical and genetic defects.

To date, the foundation of separation peroxisomal disorders based on two criteria - morphological (presence or absence of peroxisomes in the liver) and biochemical (violation of one or of several functions of peroxisomes), which must be assessed in each case at the same time. This allows you to identify three groups of peroxisomal disorders:

Group 1 - disorders associated with generalized violation of the biological functions of peroxisomes and the absence or significant decrease in the number of peroxisomes in the liver. This class includes syndrome Zellweger (SC), the infantile form of Refsume's disease (IRD), neonatal adrenoleukodystrophy (NALD), point osteochondrodystrophy, some forms of Leber's congenital amaurosis, rhizomielic chondrodysplasia punctate Type 1 (RCDP1), and others. For those diseases characterized by complete violation of the biogenesis of the peroxisomes, but to varying degrees. When RCDP first type biogenesis peroxisome broken partially and syndrome Zellweger mainly violated all peroxisome function, resulting in the accumulation of a number of peroxisomal metabolites in the plasma, whereas RCDP first type affected only biosynthesis lipids and alpha oxidation phytanic acid [4, 5].

Group 2 - disorders caused by violation of several biological functions of peroxisomes in the normal number of peroxisomes in the liver. These include the syndrome of pseudo Zellweger, D-bifunctional protein deficiency [6], Selvaganapathy syndrome etc.

Group 3 - includes disorders in which damaged the biological function of peroxisomes and there is a normal content of peroxisomes in the liver. This group is also divided into different subgroups, including disorders peroxisome beta-oxidation - X-meshed adrenoleukodystrophy (X-ALD), deficiency of acyl-CoA-oxidase 1 [7], failure 2-methyl-acyl-CoA reductase (MACoAR), deficiency of the protein transporting styrene (STB) [8], violations biosynthesis of lipids (failure dihydroxyacetone phosphate acyltransferase and alkyl dihydroxyacetone phosphate synthase) [9], violations of the alpha-oxidation of phytanic acid (Refsum's disease, adult type) [10] and, as the sole representative, violation of glyoxylate detoxification with hyperoxaluria first type, caused by lack of alanine aminotransferase glyoxylate [11].

Table 1 shows the various peroxisomal disorders and levels of LCFA, pristanic acid and phytanic acids for each of these disorders. The data indicate that the content of LCFA increased in the spectrum disorders Zellweger , and when X-ALD, deficiency of acyl-CoA oxidase and failure D-bifunctional protein, but normally in the case of other disorders including the deficit of the STB and the deficit MACoAR. However, if the last two violations accumulated pristanic acid and bile acids are intermediates di- and trihydroxychalcone acids. Pristanic acid level increases when the in the spectrum disorders Zellweger, at RCDP first type and Refsume's disease [10].

Table 1. Levels of long-chain fatty acids, pristanic acid and phytanic acid in various peroxisomal disorders.

Group 1	Long chain fatty acids	Pristanic acid	Phytanic acid
Spectrum of disorders Zellweger	↑	N-↑*	N-↑*
Rhizomielic chondrodysplasia punctate Type 1 (RCDP1)	N	↓-N	N-↑*
Group 2			
Peroxisome disorders of β-oxidation			
X-linked adrenoleukodystrophy (X-ALD)	↑	N	N
Deficiency of acyl-CoA-oxidase	↑	N	N
Deficiency of D-bifunctional protein	↑	N-↑*	N-↑*
Deficiency of the protein transporting styrene (STB)	N	N-↑*	N-↑*
Deficiency of 2-methyl-acyl-CoA reductase (MACoAR)	N	N-↑*	N-↑*
Disorders of the biosynthesis of lipids			
Rhizomielic chondrodysplasia punctate Type 2	N	N	N
Rhizomielic chondrodysplasia punctate Type 3	N	N	N
Violations of the alpha-oxidation of phytanic acid			
Refsum disease	N	N	N-↑*
Violation of detoxification of glyoxylate			
Hyperoxaluria First type	N	N	N

N= normal level; ↑ = higher level; * = Levels can range from normal to high, depending on the power and age

In order to use specific markers for the diagnosis of various metabolic disorders must be defined reference intervals in large healthy populations of different ages, nationalities and both genders. And though health care professionals understand the importance of reference intervals, many laboratories still do not have their own reference range, especially for the pediatric population.

The properties of the test substances

Long chain fatty acids as well as the fatty acid with a branched chain: pristanic acid and phytanic acid are extremely hydrophobic and practically insoluble in water. Inside the cell, they are in the form of esters of coenzyme A. These acids are generally present in lipid-tissues such as adipose tissue, but also, they can be components of various physiologically important lipids such as myelin. In this regard, LCFA and fatty acids with a branched structure are abundantly present in many tissues and organs. LCFA have a cyclic and branched structure exist in the form of esters such as triglycerides, phospholipids, cholesterol esters, or even in the form of carnitine esters. In the free form LCFA difficult to detect because, bind to plasma proteins, such as albumin. Consequently, LCFA not filtered by the kidneys and not be present in urine [13]. LCFA and saturated fatty acids with a branched structure are stable compounds, they are not destroyed in the presence of oxidants. In this regard, for the storage of samples is sufficient to freeze them. Although patients with abnormal peroxisome function will be observed an increased content LCFA with chain that longer than 26 carbon atoms [4], in the process of diagnosis can also use fatty acid: hexacosanoic acid C26:0, lignoceric acid C24:0, behenic acid C22:0, and their relations [12].

MATERIALS AND METHODS

The study included 168 healthy children aged 2 to 16 years who were passed routine medical inspection of Science research clinical institute of pediatrics. Pre from parents of patients was obtained written informed consent for the study. For analysis were collected from 2 to 5 ml of venous blood. As preferably used

anticoagulant EDTA, centrifuged for 10 minutes, then the plasma was collected by aspiration, and stored at – 200 C. If possible, blood samples should be done before breakfast, directly after sleep. However, since the level of LCFA shows only minimal daily variations are also suitable samples taken in the afternoon. Importantly, because the content long chain fatty acids, phytanic acid and pristanic acids does not change during storage of samples at room temperature for several days, the samples can be transported under these conditions, although we recommend them to freeze, especially if the transit time exceeds 48 hours. The level of content of the test compounds in frozen plasma is kept unchanged for 2 years. In our work we used the method of gas chromatography with mass detection (GC-MS) and electron impact ionization. Determination of content long chain fatty acids, phytanic and pristanic acids was performed by gas chromatography with mass spectrometric detection firm Shimadzu GCMS QP-5050A, AOC-equipped autoinjector -20i. The method involves the preliminary derivatization of N-methyl-N-(tert-butyldimethylsilyl)triflyuoroacetamide (MTBSTFA) in combination with the use of stable isotopes for the fatty acids: hexacosanoic acid C26:0, lignoceric acid C24:0, behenic acid C22:0, and pristanic acid (3, 7, 11, 15 tetramethylhexadecanoic acid) and phytanic acid (2, 6, 10, 14 tetramethylpentadecanoic acid) [14, 15]. In order to determine the total content of LCFA, pristanic acid and phytanic acid samples should be subjected to acid and alkaline hydrolysis followed by extraction with hexane.

A large number of plasma samples taken from different patients, were combined and thoroughly mixed. Of the total mixture aliquoted into eppendorf with 150 μl and stored at - 20 ° C. For each series of analysis used samples derived from a mixture of plasma sample one (pool).

RESULTS

Analysis chromatograms of the blood of patients, obtained by GC-MS from children with peroxisomal disorders and chromatograms of blood from children in the control group shows differences that can even evaluate visually, without quantifying processing the received data. A detailed analysis of chromatograms noteworthy increase in peak marker metabolites which are indicative of at respective pathologies. Thus, Figure 1 shows a chromatogram of patient with identified pathologies and patient from the control group.

The reference values were defined as confidence interval 2,5-97,5% spread in the control group. Reference values of unbranched fatty acids were obtained by analyzing the 168 control samples by GC/MS (Table 2).

Pathological values may differ for different inherited disorders peroxisomal functions. It is essential to link the cases with the maximum number peroxisome functions. Selective screening peroxisome violations in our lab can include an analysis of how LCFA, phytanic acid, pristanic acid, and pipecolinate bile acids in plasma and in erythrocytes plasmagene.
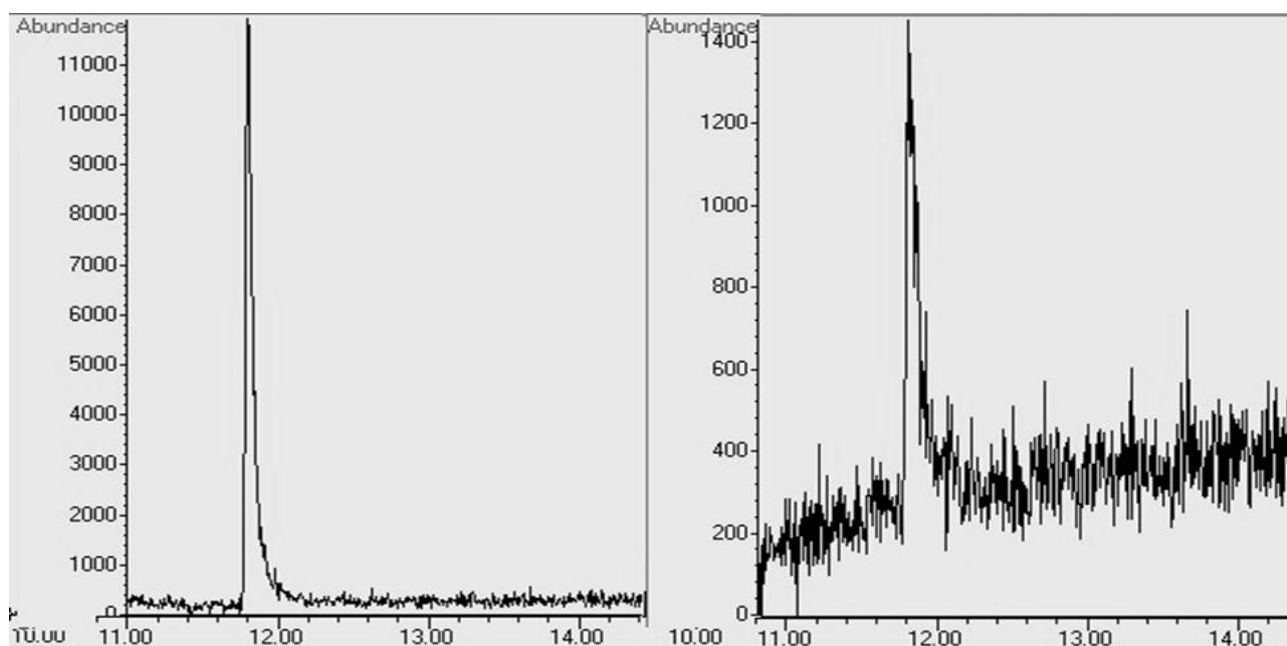

Figure 1. Comparison of typical chromatograms blood samples from a patient with Refsum's disease (marker - phytanic acid) and the patient from control group. The X-axis - time of chromatography (min), Y-axis- intensity of signal in absolute units.

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Table 2. Concentrations of long chain fatty acids plasma control samples (µmol /L).

Detection compound	Mean value	2,5-97,5% confidence interval
Behenic acid C22:0	77	42-121
Lignoceric acid C24:0	54	31-86
Hexacosanoic acid C26:0	0,78	0,45-1,34
C24/C22	0,76	0,56-0,94
C26/C22	0,01	0,01-0,02
Pristanic acid	1.52	0-3,4
Phytanic acid	3.26	0,92-8,5

DISCUSSION

At this moment in the literature is very little published research results with those obtained reference intervals of biochemical markers of peroxisome diseases both in children and in adult populations. The obtained data in this study are consistent with previously published [18, 19].

Long chain fatty acids synthesized in peroxisomes. These cell organelles not exhibit appreciable changes in protein activity during the day or with age. In this connection, reference values can be determined rather precisely. As it has already been described in earlier studies, long chain fatty acids concentration in control group is independent of age [15, 18]. Our findings can be applied to children with hypotonia and characteristic dysmorphic symptoms of the syndrome of Zellweger, but also adults (both men and women) with unexplained leukodystrophies. Probably, for adult patients would be limited to long chain fatty acids analysis aimed at the identification of X-linked adrenoleukodystrophy or adrenomyeloneuropathy (AMN), but recently published data on failure MACoAR and protein STB indicate the need for wider screening, because these patients equally possible flow and other metabolic processes [8].

With regard to pathological values, the most informative is a fatty acid C26:0. Most patients with the syndrome of Zellweger the content of fatty acid C26:0 is equal to 3-12 µmol/L, which exceeds the reference value of 3 to 10 times. For comparison, in men with the disease X-linked adrenomyeloneuropathy adrenoleukodystrophy and the levels of C26:0 in mostly 2-4 µmol/L. False-negative results for men with these diseases are extremely rare, in contrast to the levels of C26:0 in women, patients with X-linked adrenoleukodystrophy, which varies from 1.1 to 2.9 µmol/L and, thus, coincides with the normal. In the case of fatty acids C24:0, the situation is slightly different: there is a significant overlap between the levels of this acid in patients and normal control sample. However, the ratio of C24:C22 with the value for control sample < 0,92 is excessive for almost all patients and is equal to 1.06, but in women with X-ALD, the ratio of C24:C22 may be equal to up to 0.8. One analysis of long chain fatty acids not sufficient to completely exclude X-ALD; accurate test result in this disease can only be obtained if this analysis is accompanied by DNA analysis. In patients with hereditary disorders of peroxisomal function, induced by increased levels of fatty acids like C26:0 and the ratio of C24:C22. The increase in the levels of C26:0 rarely leads to the correct diagnosis. However, the constant deviation level of long chain fatty acids and/or their ratio should be checked when studying fibroblasts, in order to properly diagnose and recommend genetic testing of the family. False-positive level of long chain fatty acids is rare; the only well-known example is the ketogenic diet (a diet high in fat and low amount of carbohydrates), therefore, for the correct conclusion and the diagnosis must take into account the results of the analysis of the content at the same time of pristanic and phytanic acids. As a rule, in patients with impaired biogenesis of the peroxisome, or violations in the system peroxisomal β-oxidation show increased levels of both fatty acids with a branched structure in different ratios.

The exception, of course, are patients with ALD/AMN or deficiency of acyl-COA oxidase, having a normal level of branched fatty acids. Patients with Refsume's disease can have extremely high levels of phytanic acid, to 1500 µmol/L, and very low levels of pristanic acid (<1 µmol/L) due to a deficiency of phytanoil-CoA- hydrolase. A less pronounced increase in the level of phytanic acid observed in patients, patients with rhizomelic joints and connective tissue point of the first type and is observed both in the classic form of the disease, and in different variations. Values can vary from 200 to 900 µmol/L, to some extent depend on age. Now we discuss the cases of the appearance of excess phytanic acid in classical rhizomelic dot chondrodysplasia in newborns. In the laboratory of the authors involved in these cases was set to a normal level of phytanic acid in the plasma of patients under the age of one week (0.7 to 5.8 µmol/l). Patients aged from two to three weeks, have an increased level of phytanic acid from 9.1 to 13.2 µmol/L. As a rule, in patients at any age is impossible to determine the

level of plasmogen of red blood cells. In some cases there is a slight increase in the level of phytanic acid to a value of 15-35 μmol/L. Despite a detailed study of fibroblasts from several patients, explanation of this phenomenon still has not been found. The fact that the level of phytanic acid depends on the diet, can reduce the accumulation of phytanic acid due to diet. Patients with the Refsume's disease can achieve an almost normal level of phytanic acid in plasma by using a strict diet, accompanied by plasmapheresis, if necessary.

The authors of some articles have described cases of violation of the biogenesis of peroxisome, a deficit in D-bifunctional protein and acyl-CoA-oxidase in patients, analysis of plasma which had not revealed any deviations in the level of long chain fatty acids, phytanic, pristanic or bile acids. Obviously, peroxisome suspected violations should always be confirmed by the study of fibroblasts regardless of the results of the analysis plasma [6, 7].

CONCLUSION

Thus, the complex GC-MS analysis LCFA, pristanic acid and phytanic acid is an effective method to identify patients with peroxisome impairment, especially for diagnostics Zellweger syndrome spectrum, rhizomelic chondrodisplasia punctata type 1 and Refsume's disease. In disorders of biosynthesis of lipids, in particular with RCDP second and third types, and hyperoxaluria first type requires additional checking the content of other metabolites (level of plasmalogens in erythrocytes, glyoxylate, glycolate and oxalate levels in the urine). To diagnose diseases of peroxisome disorders recommended apply high-tech biochemical method GC-MS to determine the levels of very long chain fatty acids (VLCFA), phytanic acid and pristanic acid.

Therefore, for every laboratory is necessary to establish reference intervals for the performance of various markers of peroxisomal disorders of healthy children, that can't lead to significant errors in interpreting studies.

Competing interests
The authors declare that they have no competing interests.

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