Biochemical Diagnosis of Peroxisomal Disorders by GC/MS: Egyptian Patients with X-linked Adrenoleukodystrophy

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Abstract

Background: Peroxisomes are organelles responsible mainly for metabolism of lipids and peroxides. Lack of peroxisomes or dysfunction in any of their normal functions is the cellular basis for human peroxisomal disorders (PDs).

Aim of the Work: diagnosis of peroxisomal disorders among a high risk group of Egyptian patients using gas chromatography mass spectrometry**.**

Subjects and Methods: Forty six patients suspected to have peroxisomal disorders were included in this study. Their ages ranged from 2 to 20 years. They were referred to The Biochemical Genetics Department, National Research Centre from all over Egypt. Forty one (89%) were males while five were females (11%). Parental consanguinity was positive in 28 cases (61% out of 46). Very long chain fatty acids were quantified after extraction from plasma of all cases using gas chromatography/mass spectrometry (GC/MS) technique.

Results: The present study included 46 cases suspected clinically to have one of the peroxisomal disorders; four of them (8.7%) proved to have X-linked adrenoleukodystrophy by quantitative determination of the very long chain fatty acids after extraction from their plasma. The other 42 cases showed normal profile for very long chain fatty acids.

Conclusion:

This study showed that GC/MS analysis for VLCFA discriminates patients from controls, representing a non-invasive, reliable, specific and sensitive method for the diagnosis of peroxisomal disorders.

Introduction

Peroxisomes are organelles responsible for multiple metabolic pathways, mainly related to metabolism of lipids and peroxides. Lack of peroxisomes or dysfunction in any of their normal functions is the cellular basis for human peroxisomal disorders (PDs). PDs are clinically and genetically heterogeneous and can be classified mainly into two categories; peroxisome biogenesis disorders (PBDs) and single peroxisomal enzyme deficiencies (SEDs) **[1].** The PBDs include Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), infantile Refsum disease (IRD) and rhizomelic chondrodysplasia punctata (RCDP) type I. They are all caused by defects in PEX genes, which encode peroxins, proteins necessary for peroxisome biogenesis and the import of the peroxisomal matrix and membrane proteins. The second group includes disorders resulting from the deficiency of a single peroxisomal enzyme activity. About a dozen of such peroxisomal enzyme deficiencies have been identified [2].

Peroxisome biogenesis disorders (PBDs):

The PBD group comprises Zellweger spectrum disorders (ZSDs) and rhizomelic chondrodysplasia punctata (RCDP) type 1. The former group includes Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD). Patients with ZSDs usually presented with liver disease, variable neurodevelopmental delay, retinopathy, and perceptive deafness with onset in the first months of life. In addition, patients with ZS are severely hypotonic and weak from birth and have distinct facial features, periarticular calcifications, severe brain dysfunction associated with neuronal migration disorders and die before 1 year of

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age. The ZS; also known as cerebro-hepato-renal syndrome, is characterized by the absence of peroxisomes in the cells of the liver, kidney, and brain [3].

The biochemical anomalies correlated to these diseases present an accumulation of VLCFAs (C24:0, C25:0, C26:0), 3a, 7a, 12a-trihydroxy-5b-cholestanoic acid (THCA) and 3a, 7a-dihydroxy-5b-cholestanoic acid (DHCA) (from bile acids synthesis), branched-chain fatty acid (pristanic and phytanic acid) and a decrease in synthesis of plasmalogens and Docosahexanoic acid (DHA) [4].

Single peroxisomal enzyme deficiencies (SEDs):

SEDs are disorders in which the peroxisome is intact and functioning, but a defect in one enzyme process causes the primary biochemical abnormality. Although PEDs involve the loss of even a single peroxisome function, these diseases are severe and can closely mimic the PBDs [5]. SEDs may affect different peroxisomal pathways. The most known peroxisomal diseases involve etherphospholipid synthesis (Rhizomelic chondrodysplasia punctata Type 2 due to DHAPAT deficiency), peroxisomal β-oxidation (Rhizomelic chondrodysplasia punctata Type 3 due to alkyl- DHAP synthase deficiency), X-linked adrenoleukodystrophy, acyl CoA oxidase deficiency (AOX), D-bifunctional protein deficiency (BPD), 2-Methyl acyl CoA racemase deficiency, sterol carrier protein X deficiency, peroxisomal α-oxidation (Refsum disease is due to phytanoyl-CoA because of hydroxylase deficiency), glyoxylated toxification (determining hyperoxaluria Type 1) and H_2O_2 metabolism (Acatalasaemia) [5].

X-linked adrenoleukodystrophy (X-ALD) (MIM 300100):

X-ALD is the most common PD with impaired β-oxidation of saturated very-long-chain fatty acids (VLCFAs). Onset of symptoms is usually at approx. 7.2 years (ranges 2.75–10 years) with changes in behavior including emotional liability, withdrawal or hyperactive behavior. Focal or generalized seizures occur in the late stage of the disease. The EEGs may be normal or exhibit slowing of the background activity with a maximum in the posterior regions corresponding to the localization of the white matter lesions in the early stage. Slow wave would become progressively widespread and sometimes accompanied by paroxysmal discharges in correspondence with the deteriorated demyelinating process [6].

The principal biochemical abnormality is the elevated levels of saturated unbranched VLCFA particularly tetracosanoic (C24:0) and hexacosanoic acid (C26:0). The accumulation of VLCFA can be found in all tissues, body fluids and cultured cells [7]. The accumulation of VLCFA is the only biochemical alteration known to be present in all clinical variants of X-ALD including presymptomatic individuals.

Laboratory diagnosis of peroxisomal disorders:

There is no single laboratory test capable of identifying all these peroxisomal disorders. The selection of the correct laboratory analysis should be based on the clinical presentation of the patient involved.

Table (1): Biochemical abnormalities in peroxisomal disorders. [8]

	ZS	NALD	IRD	AOX	BPD	RCDP type I
Plasma						
VLFCA	↑	↑	↑	↑	↑	N
Di-and trihdroxycholestanoic	↑	↑	↑	N	N-↑	N
acid	↑*	↑*	↑*	N	↑*	N-↑*
Phytanic acid	↑**	↑**	↑**	N	↑**	N
Pristanic acid	↑	↑	↑	N	N	N
Piecolic acid						
Erythrocytes						
Plasmalogens	↓	N	N	N	N	↓
Liver						Present but
Peroxisomes	↓	↓	↓	N	N	abnormal
Fibroblasts						
De novo Plasmalogen synthesis	↓	↓	↓	N``	N	↓
C26:0 β-oxidation	↓	↓	↓	↓	↓	N
Pristanic acid β-oxidation	↓	↓	↓	N	↓	N
Phytanic acid α-oxidation	↓	↓	↓	N	N	↓
DHAPAT	↓	↓	↓	N	N	↓
Alkyl DHAP synthase	↓	↓	↓	N	N	↓↓
Acyl-CoA oxidase	↓	↓	↓	↓	N	N
D-Bifunctional protein	↓	↓	↓	N	↓	N
Catalase immunofluorescence	↓	↓	↓	reduced and enlarged	In no.	N

Patients and Methods

Patients: Forty six highly suspected patients to have peroxisomal disorders were included in this study. Their age ranged from 2 years to 20 years. They were referred from all over Egypt to the Biochemical Genetics department, National Research Centre. Forty one were males (while five were females. Positive history of parental consanguinity was present in 28 cases while it was negative in 18 cases.

Very long chain fatty acids were measured in plasma for all cases using gas chromatography/ mass spectrometry technique.

Sample preparation: Two ml venous blood is taken using EDTA as anticoagulant, centrifuged and plasma is collected and stored at -20 ^{0}C. Samples are preferred to be collected before breakfast after an overnight fast. One hundred µl of plasma and 1 mg of C17:0 as an internal standard were mixed with 2 ml of methanolic 5% hydrochloric acid and tightly capped. The mixture was shacked and incubated for 2 h at 100 ^{0}C. The methyl derivatives were cooled to room temperature and then extracted twice with 2 ml of n-hexane, dried by a nitrogen dryer, and finally dissolved in 1 ml of n-hexane supplemented with 0.05% butyrated hydroxytoluene for antioxidant. The extract was stored at - 20^o C until analysis.

Principle: In order to allow measurement of the total pool of VLCFA, samples need to be subjected to both acidic and alkaline hydrolysis, followed by extraction into hexane. After the hexane phase is washed once more, the sample is dried under nitrogen followed by addition of pyridine and N-methyl-N-(tert-butyldi-methylsilyl) trifluoroacetamide) MTBSTFA and heating

of the samples at 80°C. The sample is subsequently dried again under nitrogen and taken up in hexane, followed by GC-MS analysis [9].

Standards and Chemicals:

Internal standards:

1. C26:0-d**4** (3,3,5,5-**2**H**4**-hexacosanoic acid).
2. C24:0-d**4** (3,3,5,5-**2**H**4**-tetracosanoic acid).
3. C22:0-d**4** (3,3,5,5-**2**H**4**-docosanoic acid).

GC/MS analysis:

Measurements are performed on a bench-top GC-MS (Bruker Scion 456- Gas Chromatography) operating in the electron impact mode, and the acquired data are processed by the accompanying GC-MS software (Bruker MS Workstation ver. 8.). The GC is equipped with a CPSil19 capillary column (25 m×0.25 mm×0.20 µm; Varian); the injection system is used in the splitless mode and kept at 300°C. The interface to the mass selective detector is set at 290°C. GC separation of the analytes is achieved using the following column temperature program: initial temperature 60°C for 1 min; increase to 240°C at a rate of 30°C/min; further increase to 270°C at a rate of 10°C/ min; final increase to 300°C at 4°C/min, and 5 min isothermal at the latter level [9].

Procedure:

Plasma and internal standard mixtures were thawed on ice. 100 µl of plasma were transferred to 4-ml standard glass tubes equipped with screw caps and Teflon liner (Alltech). 100 µl internal standard solution plus 2 ml acid hydrolysis reagent were added and the tubes were tightly capped, mixed carefully and put the tubes in a heating block previously set at 110°C for 45 min. tubes are then left to cool down at room temperature. Two ml of alkaline hydrolysis reagent was added to the tubes, capped, mixed carefully and put in the heating block again for 45 min at 110°C. Tubes were allowed to cool to room temperature, then 0.5 ml of 25% hydrochloric acid and 4 ml hexane were added, tubes were tightly capped mixed for about 60 s by hand. After phase separation, the upper hexane layer was collected with the aid of a glass Pasteur pipette and transferred into a glass 10-ml tube equipped with a screw cap. 3.5 ml 1M KOH was added, tubes were tightly screwed and samples were mixed by hand. The upper hexane phase was removed by a glass pipette attached to a water pump. 0.6 ml of 25% hydrochloric acid (HCl) was added, 4 ml hexane again and the samples were mixed by hand. The upper hexane layer was transferred in a glass 4-ml tube with the aid of a glass Pasteur pipette. The samples were dried under nitrogen in a heating block set at 50°C. The tubes were rinsed with 50 µl pyridine and 100 µl MTBSTFA reagent was added, tubes were tightly capped and derivatisation were performed at 80°C for 30 min. Samples were dried again under nitrogen in a heating block set at 50°C. Finally tubes were rinsed with 200 µl hexane and the contents were transferred into a 1-ml injection vial for GC/MS analysis [9].

Quality Control: A pool of a large number of left-over plasma samples of patients is thoroughly mixed. Aliquots of 150ul are put into Eppendorf vials and stored at- 20^0C. These samples are stable for 1 year. Each series of analyses has one pool plasma sample. The concentrations of the three analytes are introduced into an electronic shewhart chart, which is renewed each year. Comparison of the results with the mean of all participating laboratories an indication of the accuracy.

Results

The present study included 46 cases suspected clinically to have peroxisomal disorders. They were referred to the Biochemical Genetics Department from all over Egypt. They included 41 (89%) males and 5 (11%) females while parental consanguinity was present in 18 (39%) cases. The distribution of sex and consanguinity rate is presented in figures (1) and (2) respectively.

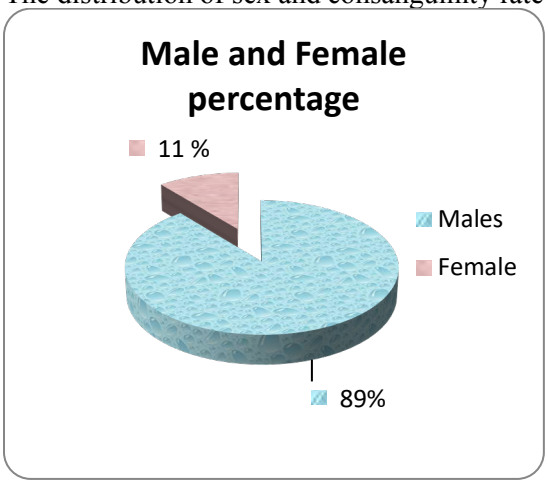

Figure (1): Male and female percentage

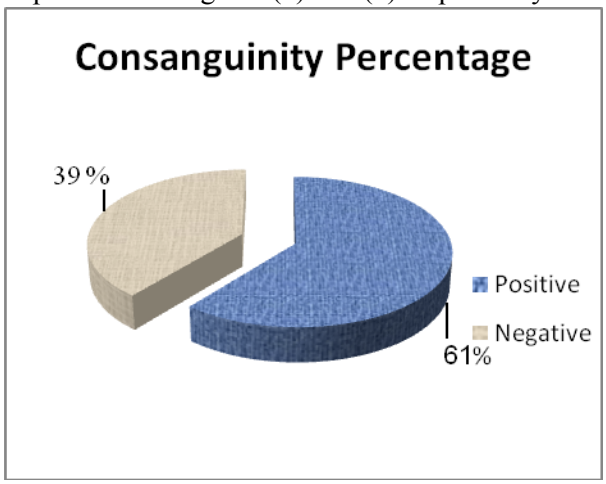

Figure (2) Parental consanguinity rate

Four cases of X-linked adrenoleukodystrophy (X-ALD) were diagnosed by their abnormal very long chain fatty acid profile, while the other 42 patients showed normal profile. They included two brothers (patients 1 and 2). The age, sex, parental consanguinity rate and biochemical results of the 4 diagnosed cases are shown in tables (2 and 3).

Table (2): age, sex and Consanguinity of the X-ALD diagnosed cases

Patient no	Age (year)	Sex	Consanguinity
1	3.75	♂	+ ve
2	5.5	♂	+ve
3	2.8	♂	- ve
4	6.7	♂	+ ve

Table (3): Biochemical results of the four diagnosed cases.

VLCFA	Patient 1	Patient 2	Patient 3	Patient 4	Reference Value
C 22:0	28.81	19.65	22.31	18.63	8.31-33.63 µg/ml
C 24:0	35.94	40.36	37.54	42.15	6.95-28.87 µg/ml
C 26:0	1.84	2.15	1.69	1.91	0.15-0.51 µg/ml
C 24:0/C22:0	1.248	2.053	1.682	2.262	0.76-0.92
C 26:0/C 22:0	0.041	0.109	0.075	0.102	0.004-0.022

Discussion

X-ALD is a metabolic disorder characterized by impaired peroxisomal beta-oxidation of very long-chain fatty acids (VLCFA; ≥ C22), which is reduced to about 30% of control levels [10]. Consequently, there is an accumulation of VLCFA in plasma and all tissues, including the white matter of the brain, the spinal cord and adrenal cortex [11]. There are at least six distinct types of X-ALD ranging in decreasing order of severity from the childhood cerebral form to asymptomatic persons including; childhood cerebral, adolescence and adult cerebral, Adrenomyeloneuropathy, Addison-only and asymptomatic and Phenotypes in female carriers [12].

In this study which included 46 cases clinically suspected to have peroxisomal disorders, four cases were diagnosed as having X-ALD. This agrees with previous studies on peroxisomal disorders that X-ALD is the most common type and it occurs in all regions of the world [13].

Since age range of the diagnosed cases is (from 2.8 to 6.7 years), they are all of the childhood cerebral type, which is the most severe phenotype. Patients with childhood cerebral type of X-ALD seem unaffected until the age of 2 to 10 years, when there is onset of adrenal insufficiency and progressive neurological dysfunction. Frequent initial symptoms include emotional liability, hyperactive behavior, school failure, impaired auditory discrimination and difficulties in vision. After onset of symptoms the course is rapidly progressive, leading to an apparently vegetative state within 2 to 4 years and to death at varying intervals thereafter [12].

Consanguineous marriages are frequent among Egyptians [14] it ranges from 29.5% to 75%. In many of previous studies on lysosomal disorders parental consanguinity exceeded 80% among the diagnosed cases [15]. This coincides with parental consanguinity found in this study where 61% of all studied cases were born to consanguineous parents, while the percentage was 50% among the positive cases; as patient 1 and 2 are brothers.

In the study group 41 cases were males (89%) and 5 cases were females (11%) while all X-ALD diagnosed cases were males which coincides with the mode of inheritance since it is caused by mutations in the ABCD1 gene located on the X-chromosome and accordingly all diagnosed cases were males [16].

As a result of the impaired VLCFA degradation due to the deficient import of VLCFA-CoA into peroxisome, the synthesis of VLCFA is enhanced in X-ALD and their accumulation occurs in different tissues in the body and their levels increases in plasma. Therefore their elevated levels in readily accessible materials like blood cells and plasma of X-ALD patients has been of crucial importance for their diagnosis [11]. Plasma VLCFA analysis is the best initial biomarker for the diagnosis of X-ALD. Three parameters are analyzed: the amount of hexacosanoic (C26:0), amount of tetracosanoic (C24:0) and the ratio of C24:0/C22:0 and C26:0/C22:0 [17]. In this study by analysis of these four plasma very long chain fatty acids parameters, four cases showed high concentrations of C 26:0 , C 24:0 and high ratio to behenic acid (C 24:0 / C 22:0 and C 26:0 / C22:0) which is diagnostic pattern for X-ALD as stated by Mosser et al., 2001[7]. The results of the patients are compared with that of the controls which showed normal pattern of VLCFA profile and using a pool of plasma sample as a quality control.

Every persistent abnormality of VLCFA levels and/or ratio should be checked by studies in fibroblast in order to arrive to an accurate diagnosis so that genetic counseling of the family can be arranged. A minority of patients with peroxisomal dysfunction cannot be diagnosed using plasma parameters and hence a strong clinical suspicion of proxisomal disease should always be verified by fibroblast

investigation, regardless of the outcome of plasma analysis [9].

In conclusion, VLCFA analysis in plasma are the best initial biomarker for the diagnosis of X-ALD using GC/MS as it is non-invasive, reliable, specific and sensitive method for the diagnosis. It requires a short-time analysis which could be applied in screening for peroxisomal disorders. Cases with strong clinical suspicion of peroxisomal disease should always be verified by fibroblast investigation.

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