BRIEF COMMUNICATION

SCREENING FOR ORGANIC ACID DISORDERS

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Summary: The detection of organic acidurias is a part of our screening programme for inherited metabolic diseases. Adapted procedure is differentiated and involves several steps: 1) thin-layer chromatography (TLC) in the case of an abnormal finding followed by 2) gas chromatography (GC). The next step of the investigation, using 3) gas chromatography mass-spectrometry (GS-MS) is reserved for more complicated and dubious analyses. In acutely sick patients and in the case of discrepancies between TLC results on the one hand, and clinical symptoms, supported by other laboratory findings on the other, the GC or GC-MS-analysis is performed immediately. Some examples of metabolic disorders, identified by this procedure, are presented.

Key words: *Organic acids; Screening; Inherited metabolic disorders*

Introduction

Profiling urinary organic acids is considered an invaluable tool for diagnosing the numerous metabolic diseases known as organic acidurias (2-5, 8). These are a group of genetic disorders in which an enzyme or cofactor defect in one of the metabolic pathways leads to accumulation and increased urinary excretion of specific acidic metabolites. Urinary organic acids (carboxylic acids and their amino acid conjugates) are water-soluble end products or intermediates of the metabolism of amino acids, sugars, lipids, steroids, biogenic amines and many other compounds. It should be pointed out that the products themselves are normal intermediates of the pathways involved, and it is the abnormal amounts present which is pathologic. Accumulated organic acids may be found in blood (organic acidemias), but in much lower concentration compared to urine (organic acidurias).

Organic acidurias manifest with an acute onset either early after birth or whenever later in life, often after provocation with a banal respiratory infect, vaccination or starvation. Otherwise, progression of neurological symptoms may be the only sign of disease.

Symptoms of the organic acidurias include vomiting, dehydration, tachypnoe, abnormal odour, seizures, hypotonia, hypertonia, ataxia, hepatomegalia, lethargy and death. Chronic manifestations are failure-to-thrive and mental retardation. Biochemical abnormalities, such as acidosis, hypoglycemia and hyperammonemia are due to increased production of ketoacids and to the accumulated organic acid intermediates, which then inhibit other pathways.

Prompt diagnosis of the specific enzyme abnormality and early treatment may prevent life threatening episodes and psychomotor retardation in some disorders.

The variety of clinical features and the similarity of symptoms for completely different disorders increase the importance of identifying excreted organic acids, direct assay for deficient enzyme usually being either time-consuming or not widely available.

Recently we have introduced a three-step procedure for detection of organic acid abnormalities, the first using TLC methodology, the second GC and the third GC-MS technique.

Experimental

Materials

Organic acid standards were supplied by Sigma-Aldrich and Serva, silylating agents (BSTFA:TMCS) were from Pierce, precoated micropulverised cellulose thin-layers (plastic sheets No. 5577) and all other chemicals were purchased from Merck.

A fasting plasma and fresh random or 24-hrs-urine samples should preferably be taken before the therapy is introduced. Cerebrospinal fluid is analysed in neurometabolic diseases and vitreous humour in post-mortem diagnoses.

Methods

High-performance thin layer chromatography (HPTLC)

We have adopted and slightly modified the method of Lee and Thurmon (6): Plasma or liquor are deproteinized with six times the volume of ethanol, after centrifugation the supernatant is concentrated to 1 ml. A volume of urine, equivalent to 2.2 μmol (250 μg) of creatinine is diluted with water (or concentrated at 30 °C in vacuum) to 1 ml. Whenever the nitroso-β-naphthol test for ketoacids in urine was positive, oxime derivatives of α-ketoacids were prepared by incubating urine at pH 14 (alkalised with NaOH, 8 $mol.l^{-1}$) with aqueous hydroxylamine-HCl (25 $g.l^{-1}$) at 60 °C for 30 min. After cooling, a volume equivalent to 250 μg creatinine was further processed. All samples, supplemented from the beginning with 60 μg phenylbutyric acid as an internal standard, were acidified to pH 1, saturated with NaCl, and vortex mixed for 5 min with 6 ml of ether-ethylacetate, 1:1. After 10 min the organic layer was removed, concentrated under nitrogen to the volume of 1 ml* and 20 μl were applied on the thin layer of cellulose. The plate was then stepwise (1 cm further with each development) developed in the solvent system n-propanol - ammonium hydroxide, 2 $mol.l^{-1}$ (7:3), using horizontal DS Chambers for TLC (Chromdes, Poland), with intermediate drying after each run and stained with aniline-xylose reagent (1 g xylose in 3 ml H_2O + 1 ml aniline in 96 ml methanol), diluted with methanol (2:1) before use.

Gas chromatography (GC)

As organic acids have low volatility, thermal instability and high polarity, derivatization is necessary prior to GC separation. The samples prepared for TLC were further processed for GC analysis, see footnote*. After spiking with an external standard (tetracosane) and evaporation to dryness under nitrogen, organic acids were derivatized with 100 μl of BSTFA-TMCS (99:1) at 60 °C for 30 min in a water bath. Trimethylsilyl derivatives in a volume of 1 μl (equivalent to 22 ng of creatinine) were analysed using capillary HP Ultra 2 column, 25 m x 0.33 mm ID, the Hewlett-Packard Model 5890 SE II with the oven temperature programmed from 50 to 300 °C, 5 °C/min and a flame ionisation detector (7) controlled by HP ChemStation and HP RetIndex.

Gas chromatography-mass spectroscopy (GC-MS)

When needed, the same sample as that used for GC is further analysed using the Varian 3300 gas chromatograph with a fused silica capillary column DB1701 (J&W), 30m x 0.25 mm ID, 0.25 μm coating, coupled to an Finnigan Magnum ion trap. The Lauber injector and transfer line temperature is kept at 250 °C and 260 °C, respectively, the oven temperature program is set from 60 to 300 °C with 5 °C/min increase. The parameters of ion trap are as follows: mass range from 50 to 550 amu, ionisation mode - electron impact, scan speed 4 μscans with auto ion control. The peaks are identified by reference to a mass spectral library.

Strategy of examination

1) Samples from acutely sick newborns with pronounced pathological laboratory results are immediately processed by GC (or GC-MS).
2) In all other cases the three-step examination is performed.

Results and discussion

HPTLC represents a prompt, inexpensive screening technique, used as the first approach in diagnostics of inherited metabolic diseases. The authors describing the procedure (6) claim quite reliable identification of samples with negative findings and those clearly pathological, provided that the child was examined promptly after the first symptoms had appeared and that it continuously received a normal amount of nutrients. This simple first-step procedure can be used in any general hospital laboratory, whenever an organic acid disorder is suspected.

The cost of GC discourages its use in a screening mode, therefore it is reserved for more advanced cases. GC provides high-efficiency resolution of numerous organic acids that are present in normal human urines. In a number of hereditary defects, organic acidurias are characterised mainly by quantitative changes of the normal acid profile, giving satisfactory results with GC alone. However, unknown compounds in some samples of the pathological urine, when detected by non-specific GC detectors such as the flame ionisation detector, could only be identified by the use of GC/MS.

Occasionally, we are simultaneously using both TLC and GC-MS, comparing the results. So far, our experience with the TLC is very good and several pathological conditions were detected, as illustrated in Figures 1 and 2.

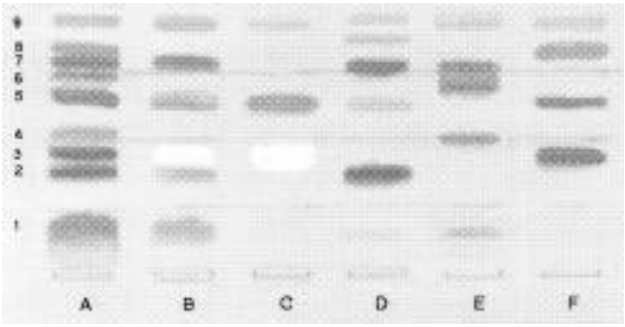

Figure 1. HPTLC of organic acids, multiple development, see text for details; A - standards: 1 - glutarate, 2 - methylmalonate, 3 - *3*-hydroxy-*3*-methylglutarate, 4 - ascorbate, 5 - lactate, 6 - homogentisate, 7 - hippurate, 8 - *3*-isovalerate, 9 - phenylbutyrate; B - normal newborn, C - lactic aciduria, D - methylmalonic aciduria, E - alcaptonuria, F - *3*-hydroxy-*3*-methylglutaric aciduria (B-F - urine samples).

* The same sample is further processed for gas chromatography, alternatively completed with mass spectrometry, if identification of abnormal or elevated organic acids is required.

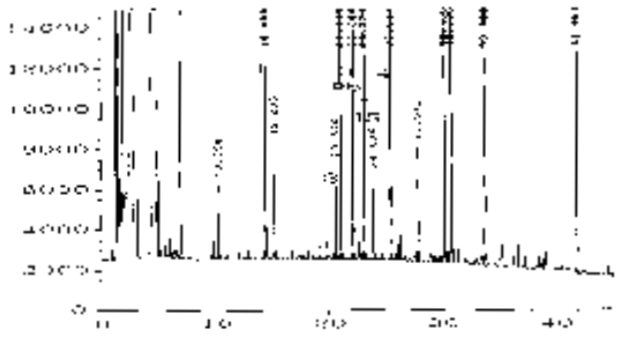

Figure 2. GC of urinary organic acids (trimethylsilyl derivatives) in a patient with 3-hydroxy-3-methylglutaryl-CoA lyase deficiency. 1 - *3*-hydroxyisovaleric acid, 2 - *3*-methylglutaric acid, 3 a,b - *3*-methylglutaconic acid, cis, trans, 4 - *3*-hydroxy-*3*-methylglutaric acid, IS - internal standard (phenylbutyric acid).

In general, analysis of organic acids is most reliable when the urine is collected during acute metabolic decompensation. Alternatively, the presence of an abnormality, which is associated with organic aciduria only intermittently, may be visible by provocative testing (by loading with metabolic intermediates or during starvation).

The determination of urinary organic acids contributes to the understanding of metabolic processes and serves as an important tool in the investigation of human diseases. In addition to organic acidurias, organic acid analysis may be an aid in the diagnosis of defects in other groups of metabolites. Examples are the presence of glycerol and glycerol-3-phosphate in patients with fructose-1,6-diphosphatase deficiency or with type I tyrosinaemia or type I glycogenosis. It may assist in the diagnosis not only inherited but also acquired disorders, e.g. catecholamines metabolites in neural tumours or oxalic, glyoxylic, glycolic and glyceric acids in the hyperoxaluric syndromes (1).

The advantage of the proposed three-step procedure is the possibility of eliminating the clearly normal samples from further, economically far exigent processing by GC or GC-MS at the beginning of an investigation.

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