High-Risk Screening and Diagnosis of Inborn Errors of Metabolism: A Practical Guide for Laboratories

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Abstract
Inborn errors of metabolism (IEM) are a large and heterogeneous group of genetic diseases. In most of these conditions, the presence of variants in specific genes leads to enzyme deficiencies that affect a particular metabolic step. The number of laboratories dedicated to the study of IEM is very limited worldwide, and its multiplication is urgently required for a more effective diagnosis. With the scarcity of specialized centers, the diagnosis of affected individuals comes too late or does not happen at all. Moreover, the biological samples have to travel long distances, compromising its quality and delaying still more the diagnosis. In this work, we suggest a practical guide for a basic biochemical laboratory to get involved in the study of IEM. This proposal was based on already described metabolic tests and involves the need of just a few, simple, and affordable instruments that can give an enormous quantity of information about the possible metabolic defect faced, such as a spectrophotofluorometer and a gas chromatography/mass spectrometry (GC/MS) instrument. The procedures proposed can be customized and adapted to particular needs and situations, which make it especially useful for developing countries.

Keywords
inborn errors of metabolism, biochemical diagnosis, gas chromatography/mass spectrometry, spectrophotofluorometry, screening

Introduction
Inborn errors of metabolism (IEM) are a large and heterogeneous group of genetic diseases. In most of these conditions, the presence of variants in specific genes leads to enzyme deficiencies that affect a particular metabolic step. Depending on the metabolic error involved, the clinical presentation can be severe or lethal with neurodegenerative process, skeletal abnormalities, and/or physiologic dysfunctions.1

The global incidence of each particular IEM is low, but considered all together they represent more than 1 affected individual each 1000 births. The occurrence of each disease can be significantly different in particular regions. Based on clinical suspicions, the biochemical study of IEM generally begins with qualitative or quantitative tests involving metabolites related to sugars, organic acids, amino acids, sterols, fatty acids, complex lipids, porphyrins, pterines, purines and pyrimidines, and so on. These studies can be definitive or presumptive to define a specific IEM, and they are complemented with enzyme assays and/or molecular genetic testing.2,3

The analytical technology used by sophisticated laboratories for the diagnosis of IEM mainly includes gas chromatography/mass spectrometry (GC/MS), tandem mass spectrometry (MS/MS), amino acid analyzer, high-pressure liquid chromatography (HPLC), thin-layer chromatography, electrophoresis, and specific spectrophotometric or fluorometric enzyme assays.

In the last few years, an important strategy for early identification of IEM was initiated with the study of acylcarnitines and...
Basic Biochemical Laboratory for IEM Study

Detailed biochemical procedures and its limitations can be found in the references cited in this section.

Screening Tests

Screening tests can be performed with a reactive strip or simple qualitative procedures as indicated subsequently. Despite being less used in the present days, these preliminary tests can be useful to guide the metabolic study, especially in emergency situations.2,3

Reactive Strips. Different reagent strips are commercially available for quick and qualitative analysis of several intermediates in urine, including protein, blood, leukocytes, nitrite, glucose, ketones (acetoacetic acid), pH, specific gravity, creatinine, bilirubin, and urobilinogen. Other strips allow the quick determination of reducing substances (glucose, lactose, fructose, galactose, pentoses, sulfite, etc). The interpretation of the results can be made by visual observation.

Qualitative tests. Complementing the reactive strip tests, there are simple qualitative assays that could bring useful information about the possible metabolic error present. The interpretation of the results can be made with simple sight, except the Ehrlich test and Bratton-Marshall reaction where a spectrophotometer is required.2,3 The most common tests are as follows:

- Benedict test for reducing substances,
- 2,4-Dinitrophenylhydrazine for α-keto acids,
- Nitroprusside test for cystine and homocystine,
- Nitrosonaphthol test for tyrosine metabolites,
- Ferric chloride test for PKU, Histidemia, MSUD, Tyrosinemia, Alkaptonuria, etc,
- Ehrlich test for porphyrins,
- Urine thiosulfate determination for defect of sulfate metabolism,
- Toluidine blue spot test for glycosaminoglycans (GAGs),
- Electrophoresis and thin-layer chromatography (TLC) of GAGs, oligosaccharides, sialyloligosaccharides, and
- Bratton-Marshall reaction.21

Spectrophotofluorometric Studies

These studies aim to determine total concentration of specific metabolites and to measure specific enzyme activities.2,3,7-18,22-24 Chitotriosidase activity can be used for screening, as it is increased in plasma and DBS in several LSDs. Creatinine and protein determinations are generally performed as parameters to normalize other biochemical assays that refer its results to creatinine or protein concentrations, such as organic acids, GAGs, sialic acid, enzyme assays in tissues or leukocytes, and so on. Creatinine concentration is also an important parameter for the investigation of defects in the creatine metabolism. A summary of the most common spectrophotofluorometric measurements is presented in Table 1.

Gas Chromatography and MS

Organic acid assay (total ion chromatogram, TIC). This is one of the most informative assays that can be easily performed to investigate many IEM. Through a simple organic acid profile, it is possible to identify particular products related to a specific enzyme deficiency or to visualize “flags” that would help guide new studies. It is important to note that the organic acid profiles can be different in clinically sick and asymptomatic patients. As the number of new pathologies is growing fast, new key metabolites are being identified. Thus, it is not unusual reviewing old profiles, even those obtained years before. The organic acid profile also offers interesting information about enriched diet, drug intake, bacterial or plastic contamination, and so on.2,3,25-28 A list of IEM that can be studied by organic assays is presented in Table 2.

Additional specific organic acid detection by GC and MS (simple ion monitoring, SIM). The extracted organic acids can also be analyzed by programming the mass spectrometer to detect only one or few isolated ions, allowing the identification and
quantification of metabolites at very low concentrations. The most important ions checked are usually succinylacetone (tyrosinemia type 1), orotic acid (urea cycle disorders), guanidinoacetoacetate (defects of creatine metabolism), methylmalonic acid (methylmalonic acidemia and disorder of cobalamin metabolism), mevalonic acid (mevalonic aciduria), and acetyl-N-aspartate (Canavan disease).29-34

Very long-chain fatty acids assay. Saturated and nonsaturated fatty acids and plasmalogens can be easily methylated and analyzed by GC/MS. The range of fatty acids to be screened include acids with medium-, long-, and very long-chain plasmalogens, along with pristanate and phytanate.2,3 The fatty acid profile can give important information about possible peroxisomal disorder as described in Table 2.

Sterol profile assay. Sterols can be separated and identified by GC-MS (TIC and SIM modes). The range of pathologies studied is detailed subsequently. Similar to the organic acid profile, many “unknown metabolites” could be the future markers for novel enzyme deficiencies identified in the future, in the pre- or post-squalene steps of cholesterol synthesis.2,3,35,36 A list of IEM that can be studied by the sterol profile is presented in Table 2.

Minimal Requirements for the Basic IEM Laboratory

Human Resources

Basically, the laboratory should count with qualified technicians for sample preparation, performance of the assays, and instrument operation. Inclusion of microtechniques with the use of multiwheel plate systems and/or microfluid-based enzyme assays22 is recommended. Autosamplers for GC/MS or other robotic implements are also helpful. This means an enormous saving of expensive artificial substrates, buffers, reading times, and so on. Well-organized laboratories are more efficient, saving time, human effort, and chemical products.
The technicians should be backed by professionals who have a background in inherited metabolic diseases to evaluate the results and to take the appropriate actions to confirm positive cases and direct the patients to quick management and therapy.

For the assessment of laboratory performance, it is very important to adhere to international schemes of quality control, such as ERNDIM, CDC, and others.

**Installations and Instruments**

The laboratory should have an appropriate size (around 100 m² is recommended), with adequate ventilation and temperature control. This last point is especially important due to the sensitive instrumentation required. Work benches, hermetic hood for manipulation of toxic gases, nitrogen and helium tanks, and a safe system for reagent storage and for discarding dangerous substances are also necessary.

A spectrophotofluorometer and a GC/MS are the major instruments required for the basic IEM laboratory. It is important to subscribe to maintenance programs. The first instrument is a single machine with UV-visible and fluorescence detectors. In the case that a spectrophotometer is already available, only a spectrofluorometer needs to be added.

Minor instruments also are required, and these are similar to the ones available in a basic biochemical laboratory, including vortex, pH meter, digital balance, water bath, plate incubator, TLC and electrophoresis systems, and so on.

**Final Considerations**

Figure 1 shows a flowchart for the biochemical study of IEM. It is clear that a vast number of IEM can be explored with this basic laboratory. Evidently, not all IEM will be covered, but the most important areas will be studied. The relatively low cost of the major equipment required to run this laboratory makes them quite affordable, especially when compared to the high cost of an MS/MS or other sophisticated equipment. This is essentially important in countries or regions where there is a lack of specialized centers to perform these studies. Once the basic laboratory is running, the range of the pathologies investigated can be expanded, incorporating new equipment, such as an amino acid analyzer, HPLC, MS/MS, and instruments for molecular genetic tests and for cell culture. Local or regional incidence of IEM and the availability of therapies should be considered for such expansion. If the resources are very limited, a GC/MS instrument should be the first option because of the large scope of IEM that can be studied through a simple chromatography profiling of organic acids, very long chain fatty acids, and sterols.

Finally, we should emphasize that the IEM are “rare diseases” when considered individually, but the entire group has a

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**Table 2. Details of GC/MS assays and the IEM studied.**

<table>
<thead>
<tr>
<th>GC/MS ASSAYS</th>
<th>IEM</th>
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<tbody>
<tr>
<td>Urine organic acid assay</td>
<td>Defects of aromatic amino acid metabolism</td>
</tr>
<tr>
<td>GC/MS, TIC counties 2,25-28</td>
<td>Phenylketonuria, tyrosinemia, hawkinsinuria, alkaptonuria</td>
</tr>
<tr>
<td>GC/MS, SIM</td>
<td>Defects of branched chain amino acids metabolism</td>
</tr>
<tr>
<td>Maple urine syrup disease, dihydrolipoyl dehydrogenase (E3) deficiency, isovaleric acidemia, 3-methylcrotonyl-CoA carboxylase deficiency, multiple carboxylase deficiency, 3-methylglutaconic aciduria, 3-OH-3-methylglutaric aciduria, 3-oxothiolase deficiency, propionic acidemia, methylmalonic acidemia, malonyl-CoA decarboxylase deficiency</td>
<td></td>
</tr>
<tr>
<td>Defects of dibasic amino acids metabolism</td>
<td></td>
</tr>
<tr>
<td>Glutaric aciduria type I, 2-Ketoacidic aciduria</td>
<td></td>
</tr>
<tr>
<td>Defects of fatty acid oxidation/pyruvate/Krebs cycle/ mitochondrial respiratory chain</td>
<td></td>
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<tr>
<td>Long-, medium-, and short-chain acyl-coA dehydrogenase deficiency, multiple acyl-CoA dehydrogenase deficiency</td>
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<tr>
<td>Lactic acidemia (pyruvate carboxylase deficiency, pyruvate dehydrogenase deficiency, multiple carboxylase deficiency, defects in oxidative phosphorylation), 2-ketoglutarate dehydrogenase deficiency, fumarase deficiency, Barth syndrome</td>
<td></td>
</tr>
<tr>
<td>Other organic acidurias:</td>
<td></td>
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<tr>
<td>5-Oxoo-prolinuria, D-2-OH-glutaric aciduria, 4-OH-butanic aciduria, Canavan disease, mevalonic aciduria, n-glyceric aciduria, glyceroluria, hyperoxaluria, ketosis, Reye’s syndrome, thymine and uracil acidurias. Urea cycle disorders and defects of creatine metabolism</td>
<td></td>
</tr>
<tr>
<td>Plasma very long-chain fatty acid assay</td>
<td>X-ALD, defects of phyticanic/pristanic acid metabolism, infantile Refsum disease, RCDP, Zellweger and Zellweger-like syndromes, dihydroxyacetone phosphate acyltransferase deficiency</td>
</tr>
<tr>
<td>GC/MS, TIC/SIM counties 2,3</td>
<td></td>
</tr>
<tr>
<td>Plasma Sterol profile assay</td>
<td>Mevalonate kinase deficiency, CHILD syndrome, CDPX2, Greenberg dysplasia, Smith-Lemli-Opitz syndrome, lathosterolosis, desmosterolosis, sitosterolemia, CTX.</td>
</tr>
<tr>
<td>GC/MS, TIC/SIM counties 2,3,35,36</td>
<td></td>
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</tbody>
</table>

Abbreviations: CDPX2, X linked dominant Conrad-Hunermann chondrodysplasia; CTX, cerebrotendineos xanthomatosis; GC/MS, gas chromatography/mass spectrometry system; IEM, inborn errors of metabolism; RCDP, rhizomelic chondrodysplasia punctata; SIM, simple ion chromatogram mode; TIC, total ion chromatogram mode; X-ALD, X linked adrenoleukodystrophy.
frequency higher than 1 per 1000 births. Moreover, the number of patients detected and the definition of new IEM are continually expanding worldwide.\textsuperscript{37-40} Many centers dedicated to the study of IEM are offering regular courses and training to physicians, biochemists, biologists, and related professionals, helping to extend the knowledge of these diseases and to encourage the health authorities and other international institutions to take appropriate actions to address these diseases.

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