Laboratory methods in the diagnosis of inherited metabolic disorders

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Laboratory investigation in classical inborn errors of metabolism

Can be at:

- Enzyme protein
- Metabolite levels
- At nucleotide
Laboratory techniques for the detection of inborn errors of metabolism

- Measurement of the enzymatic activity (spectrophotometric assays, analysers, fluorescence techniques)
- Measurement of the concentrations of metabolites (chromatography, analysers, spectrophotometric assays)
- Detection at protein level - western blot
- Sequencing of a gene - detection of mutations
Assays have developed

- Blood (plasma, white blood cells, red blood cells)
- Urine
- Cerebrospinal fluid
- Cultured cells
Enzymes

Enzymes are proteins that act as catalyst compounds, that increase the rate of chemical reactions. Enzyme catalysts bind the reactants, called substrates, convert them to products and release the products.

Factors affecting enzyme activity

- Temperature
- pH – Acidity and Basicity
- Enzyme and substrate concentration (affect the rate of real reaction)
- Presence of any inhibitors or activators
Spectrophotometer

- The spectrophotometer is an instrument which measures the amount of light of a specified wavelength which passes through a medium.
- The amount of light absorbed by a medium is proportional to the concentration of the absorbing material or solute present.
- The concentration of a colored solute in a solution may be determined in the lab by measuring the absorbance of light at a given wavelength ($\lambda$/nm).
- Absorbance is indicated with a capital A.
- In biochemistry it is used to determine enzyme-catalyzed reaction.
Spectrophotometer

PC / cuvettes / cuvettes carrier / temperature controller / solutions

UV/VIS lamp
Rate assay reactions

Rate assay reactions are reactions that are allowed to reach a stable rate in which the change in absorbance between readings is constant. The system performs several readings during this time, calculates absorbance change per minute (rate), and then uses the rate to calculate results.
End-point assay reactions

- End-point assay reactions are reactions that are allowed to react until all reactant is depleted and the absorbance is stable. When the reaction is complete, the system measures the absorbance readings.
Fluorescence techniques

- Analyses fluorescence from a sample
- Fluorometry is the measurement of fluorescence
- Fluorescence compounds have two characteristic spectra
  - An excitation spectrum (the wavelength and amount of light absorbed)
  - An emission spectrum (the wavelength and amount of light emitted)
- The instrument used to measure fluorescence is called a fluorometer
- A fluorometer generates the wavelength of light required to excite the analyte of interest; it selective transmits the wavelength of light emitted, then it measures the intensity of the emitted light
- The emitted light is proportional to the concentration of the analyte being measured
- For the enzyme analysis fluorescence substrates are being used
Fluorescence

- Basics of the technique
- Very simple
  - Light source excites sample
  - Emission is detected and recorded
Analysers

Analysers are fully automated clinical chemistry systems and can be configured to process samples using

- Photometric methods
- Immunoassay methods
  - An immunoassay is a biochemical test that measures the presence or concentration of a macromolecule in a solution through the use of an antibody
Analyser
Analyser (sample carrier)
Analysers
Principles of operation

Reagent pumps (loading of reagent)

Loading sample
Sample carrier

Incubation

A B A B A B............

Photometric or Immunoassay analysis

Reagent pumps (loading of reagent)
Chromatography

- Chromatography is a separation technique based on the different interactions of compounds with two phases a mobile phase and a stationary phase.
- Derived from the Greek word chroma meaning colour.
- Mikhail Tswett invented chromatography in 1901 during his research on plant pigments. He used the technique to separate various plant pigments such as chlorophylls, xanthophylls and carotenoids.
- Chromatography provides a way to identify unknown compounds and separate mixtures.
How does chromatography work

- In all chromatographic separations, the sample is transported in a **mobile phase**. The mobile phase can be a gas or a liquid.
- The mobile phase is then forced through a **stationary phase** held in a column or on a solid surface.
- The **sample** has the opportunity to interact with the stationary phase as it moves past it. Samples that interact greatly, then appear to move more slowly. Samples that interact weakly, then appear to move more quickly. Because of this difference in rates, the samples can then be **separated into their components**.
Chromatography methods classification

- Chromatography methods divided according to the type of mobile phase (liquid or gas), according to the type of stationary phase used in the system (e.g. column, paper, alumina, silica) or according to the principles of separation (e.g. adsorption, ion exchange, mass spectrometer)
Types of Chromatography

- Thin Layer Chromatography
- Paper Chromatography
- Gas-Chromatography Mass Spectrometry
- High Performance Liquid Chromatography
Thin Layer Chromatography

<table>
<thead>
<tr>
<th>Mobile phase</th>
<th>Stationary phase</th>
<th>Principle of separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>Sheet of glass coated with a thin layer of silica</td>
<td>Adsorption</td>
</tr>
</tbody>
</table>
Thin Layer Chromatography

As small amount of the mixture to be analysed is spotted near the bottom of this plate
Thin Layer Chromatography

- The TLC plate is then placed in a shallow pool of a solvent in a developing chamber so that only the very bottom of the plate is in the liquid.

- The mobile phase travels up the stationary phase carrying the samples with it. Components of the samples will separate according to how strongly they adsorb on the stationary phase versus how readily they dissolve in the mobile phase.
The $R_f$ value

- Each compound has its own $R_f$ value
- The retention factor, or $R_f$, is defined as the distance travelled by the compound divided by the distance travelled by the solvent

$$R_f = \frac{\text{distance travelled by the compound}}{\text{distance travelled by the solvent}}$$

- For example, if a compound travels 2.1 cm and the solvent front travels 2.8 cm, the $R_f$ is 0.75

```
\[ R_f = \frac{2.1}{2.8} = 0.75 \]
```
Thin Layer Chromatography

When the solvent has reached the top of the plate, the plate is removed from the developing chamber, dried, and the separated components of the mixture are visualized.

**Urine sugar chromatography**

- Xylose
- Fructose
- Galactose
- Lactose

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**Std**  **Std**  **Lactose**  **Gal**  **Lactose**  **Gal**  **Lactose**  **Gal**  **Normal**
### Paper Chromatography/Electrophoresis

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<tbody>
<tr>
<td>Liquid</td>
<td>Cellulose membrane</td>
<td>Adsorption</td>
</tr>
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</table>
Paper Chromatography/Electrophoresis

The mixture of compounds to be separated (e.g. mucopolysaccharides) is placed near one end of the strip of paper (cellulose membrane) and allowed to dry.
Paper Chromatography/Electrophoresis

- The membrane subjected on an electrophoresis apparatus which contains the buffer.
- Separation is based on the fact that charged molecules will migrate in an applied electrical field, according to molecular weight.
Paper Chromatography/Electrophoresis

The membrane is then staining with alcian blue ⇒ the spots are detected
Mucopolysaccharides electrophoresis

Glycosaminoglycans or mucopolysaccharides are large linear polysaccharides and are the major component of connective tissue.
High Performance Liquid Chromatography (HPLC)

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<th>Principle of separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid</td>
<td>Column</td>
<td>Reversed phase</td>
</tr>
</tbody>
</table>

Using this method for amino acids analysis
Parts of HPLC system

- **Pumps** (push the mobile phase through the column)
- The **column** (this is the mandatory HPLC part on which actual separation of compounds occur). Hydrophobic stationary phase – Polar (aqueous) mobile phase. The hydrophilic molecules are eluted first
- **Autosampler**
- **UV detector** to determine the chemicals as they exit the column
- **PC** – recording the results
Amino acids analysis by HPLC-reversed phase

- Hydrolysis of the protein or peptide sample to yield free amino acids
- Pre column derivatization of the sample
- Analysis by reversed phase HPLC

\[
\text{phenylisothiocyanate} + \text{R} = \text{S} \quad \text{NH}_2 - \text{CH} - \text{COO}^- \\
\downarrow \\
20 \mu l \text{ REAGENT} \\
\downarrow \\
\text{S} \quad \text{NH} - \text{C} - \text{NH} - \text{CH} - \text{COO}^- \\
\downarrow \text{DRY} \\
\text{DISSOLVE IN MOBILE PHASE AND INJECT}
\]
Amino acid Chromatogram
Gas chromatography-Mass spectrometry

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<tbody>
<tr>
<td>Gas (Helium)</td>
<td>Column</td>
<td>Mass analyser</td>
</tr>
</tbody>
</table>

The technique can be used to separate organic compounds in mixtures that are volatile (can be vaporized) and thermal stability.
Gas chromatography-Mass spectrometry

- The vaporized sample are transported into the column by the gas carrier
- The column is held in an oven that be programmed to increase temperature (TM) gradually and helps the separation
- As the TM increases, those compounds that have low boiling points elute from the column sooner
- The separated compounds elute from the column and enter a detector. The mass spectrometer (detector) breaks each molecule into ionized fragments and detecting these fragments using their mass to charge ratio (m/z)
Western Blotting

The western blot is an analytical technique used to detect specific proteins in a sample.

2. The separated molecules are transferred from the gel onto a nitrocellulose membrane.
3. Probing the membrane with a primary antibody that recognizes the target protein. In general the primary antibody which recognizes the target protein is not directly detectable. Therefore secondary antibodies are used.
4. Detection using variety of substrates colorimetric / chemiluminesces.
Polymerase Chain Reaction (PCR)/Sequencing

- **PCR** is a technique used to “amplify” – copy – small segments of DNA. The PCR reaction requires the following components: DNA template / DNA polymerase / primers / nucleotides

- **DNA sequencing** is a technique used to determine the exact sequence of bases (A, C, G and T) in a DNA molecule
Case presentation GM1 gangliosidosis

Male, D.O.B. 29/09/91

- Clinical features
  - Hypertonia of upper and lower limbs
  - Generalized oedema (face, eyes, lower limbs)
  - Phychomotor retardation
  - Coarse dysmorphic features

- Biochemical abnormalities
  - β-Galactosidase enzyme activity 5nmol/mg prot./hr
    (Normal Range: 198-458)

- Diagnosis
  - GM1 gangliosidosis (age of diagnosis 9 months old)
  - Lysosomal storage disease
Mutation analysis - GM1 gangliosidosis

Sequencing GLB1 gene c.1445G>A p.Arg482Hist
Thin Layer Chromatography Urinary Oligosaccharides (GM1 gangliosidosis)

- Oligosaccharides are low molecular weight carbohydrate chains composed of at least three monosaccharides subunit
- They may be covalently coupled to a protein moiety, in which case they are termed glycoproteins
- Lysosomes contains enzymes for glycoproteins degradation

![Thin Layer Chromatography Urinary Oligosaccharides](image)

- Glucose
- Lactose
- Stachyose

<table>
<thead>
<tr>
<th>Std</th>
<th>GM₁</th>
<th>GM₁</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
</table>
Western blot - GM1 gangliosidosis

62KDa ← marker
56KDa control
64KDa patient

β-gal-protein
Case presentation MSUD patient


- **Clinical features**
  - Hypotonia
  - Vomiting (feeding problems)
  - Lethargy
  - Seizures
  - Failure to thrive

- **Biochemical abnormalities**
  - Amino acid analysis, ↑Leu, ↑Isoleu, ↑Val
  - BCKD, branched chain α-ketoacid dehydrogenase, enzyme activity 0.009nmol/h/mg protein

- **Diagnosis**
  - Maple Syrup Urine Disease (age of diagnosis 13 days old)
HPLC/Maple syrup urine disease
Mutation analysis – MSUD patient

E1α (IVS5-1G>C)
Western blot - MSUD patient
Maple syrup urine disease
Branched chain amino acid pathway
GC/MS organic acids analysis – MSUD patient