Quantitation and Identification of Urine Mucopolysaccharides

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MetBioNet Workshop 2008
The Big Questions

- What are we measuring?
- Where does it come from?
- How do we measure it?
What are we measuring?
What are Mucopolysaccharides?
<table>
<thead>
<tr>
<th>Polysaccharide</th>
<th>Repeat Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin Sulphate</td>
<td>GlcUA-GalNAc</td>
</tr>
<tr>
<td>Dermatan Sulphate</td>
<td>GlcUA/IdUA-GalNAc</td>
</tr>
<tr>
<td>Heparan Sulphate</td>
<td>GlcUA/IdUA-GlcNAc</td>
</tr>
<tr>
<td>Keratan Sulphate</td>
<td>Gal-GlcNAc</td>
</tr>
<tr>
<td>Hyaluronin</td>
<td>GlcUA-GlcNAc</td>
</tr>
</tbody>
</table>

- All highly sulphated at 2, 4 or 6 positions

25-10000 Polymer Units per chain
Biosynthesis

- Protein cores made on the ER and transferred to the cell membrane.
- Sequential addition of the carbohydrate units.
- Completed chain expelled into matrix or integrated into the cell membrane.
Catabolism

- Matrix MPS in endocytosed by cells and transferred to the lysosome for breakdown
- Peptidases break down the core protein
- Exoglycosidases sequentially remove the carbohydrate chain (Sulphatases and Glycosidases)
- Endoglycosidases (hyaluronidase, heparanidase) can partially degrade bigger molecules.
Where does it come from?
<table>
<thead>
<tr>
<th>GAG</th>
<th>Localization</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronin</td>
<td>Synovial fluid, Vitreous Humour, ECM of loose connective tissue</td>
<td>Large polymers, shock absorbing</td>
</tr>
<tr>
<td>Chondroitin Sulphate</td>
<td>Cartilage, Bone, Heart Valves</td>
<td>Most abundant GAG</td>
</tr>
<tr>
<td>Heparan Sulphate</td>
<td>Basement membranes, Components of cell surfaces</td>
<td>Contains higher acetylated glucosamine than heparin</td>
</tr>
<tr>
<td>Heparin</td>
<td>Component of intracellular granules of mast cells</td>
<td>More sulphated than heparan sulphates</td>
</tr>
<tr>
<td>Dermatan Sulphate</td>
<td>Skin, Blood Vessels, Heart Valves</td>
<td></td>
</tr>
<tr>
<td>Keratan Sulphate</td>
<td>Cornea, Bone, Cartilage aggregated with chondroitin sulphates</td>
<td></td>
</tr>
</tbody>
</table>
Sources of Glycosaminoglycans – How does it get in the urine?

- Cell Death
- Leakage From Cells
- Extracellular Matrix
- Plasma
- Urine
What sort of Glycosaminoglycans are in Urine?

- Molecular weights are lower than in tissues and those in patients with MPS disorders are even lower.
- Most glycosaminoglycans are probably partially degraded glycosaminoglycans with the core protein removed.
- Wide spread of molecular weights particularly in MPS patients.
How do we measure them?
Spot Tests

- Toluidine Blue Spot Test
- Alcian Blue Spot Test
- Albumin Turbidimetric Spot Test
- Cetylpyridinium Chloride Precipitation Test
Quantitative Tests

- Uronic Acid Quantitation
  - Measures Glucuronic & Iduronic Acid using nasty chemicals! Does not measure keratan sulphate.

- Alcian Blue Quantitation

- 1,9 Dimethylmethylene Blue (DMB Quantitation)
Alcian Blue
Alcian Blue Quantitation

- Add urine to Alcian Blue in buffer pH5.8 + 10mmol/l MgCl₂
- Allow to stand to precipitate GAG
- Wash in ethanol
- Resuspend pellet in solvent which release GAG.
- Measure OD at 690nm with appropriate standard(S) calculate relative to standard.
- Take ratio to creatinine
1,9 Dimethylmethylene Blue (DMB)
DMB Quantitation

- Incubate urine sample with DMB in Tris-Formate Buffer pH?
- Measure end point OD at 510nm with appropriate standards and relate to standard curve.
- Take ratio to creatinine
- One step assay
- Can be adapted for Centrifugal Analyser
- Requires only 60ul urine
GAG concentrations vary with age
Separation Techniques

- Thin Layer Chromatography

- Cellulose Acetate Electrophoresis
  - 1 Dimensional
  - 2 Dimensional
GAGs are extracted from 25 ml urine by CPC or Alcian Blue precipitation with washes.
An aliquot of the redissolved GAG is spotted on to the plate and put through a series of six solvents of increasing ethanol concentrations containing calcium acetate.
It is the stained with Alcian Blue and destained in acetic acid solution.
Cellulose Acetate Electrophoresis
GAG Isolation

- GAGs are isolated from 2 ml urine by precipitation with Alcian Blue in 10 mmol/l MgCl$_2$
- Redissolve in NaCl/methanol mixture and add sodium carbonate solution to facilitate precipitation on non-GAG material.
- Centrifuge and precipitate the GAGs from the supernatant with ethanol.
- Resuspend the pellet in water
Cellulose Acetate Electrophoresis
1 D Electrophoresis

- Apply a small aliquot of dissolved isolated GAG (fixed amount of GAG) on to a rectangular membrane (8 samples including standards and a Morquio QA).
- Subject to electrophoresis in a barium acetate buffer pH 5.8 for 4-5 hours.
- Stain with Alcian Blue and destain in acetic acid solution.
Apply 1-2ul of GAG solution to the corner of a square membrane.

Subject to electrophoresis in pyridine:acetic acid:water 1-1.5 hours.

Turn through 90 degrees and subject to electrophoresis for 3 hours in barium acetate buffer.

Stain in Alcian Blue and destain in acetic acid.
One Dimensional Cellulose Acetate Electrophoresis

- Chondroitin Sulphate
- Heparan Sulphate
- Dermatan Sulphate
- Keratan Sulphate

Standard, MPS II Sanfilippo, MPS I Hunter/Hurler, Normal Heavy Pattern, MPS III Sanfilippo, Normal pattern, MPS IV Morquio, MPS IV Morquio
Two Dimensional Electrophoresis

- Chondroitin Sulphate
- Heparan Sulphate
- Keratan Sulphate (Trace)
Two Dimensional Electrophoresis
MPS2
Two Dimensional Electrophoresis
Electrophoresis MPS4
Anomalies

- If large loads are used traces of dermatan and keratan sulphate are occasionally seen.
- Hyaluronic Acid may very occasionally appear as a narrow band between Chondroitin and Heparan Sulphate. It has been reported to be excreted in certain renal tumours and is regularly seen in amniotic fluid.
Problems

- Infected urines – rarely a problem. Infection level needs to be high to cause problems.
- Slys (MPS VII) can be difficult!
- Heparin – be wary of apparently raised heparan sulphate in samples from patients on ITU or cardiac patients.
- Drugs - rarely a problem
- Funny spots and bands – lubricants used on the skin may contain mucopolysaccharides. It is more common in urines collected in bags,
- It is said that sometimes neonates may not be clearly abnormal in some disorders
- Adult patients sometimes do not have as clearly abnormal patterns
Amniotic Fluid GAGs

- GAGs can be isolated from the amniotic fluid supernatant collected at 16-18 week gestation.
- The position and pattern of individual GAGs can vary slightly from the ones in urine.
- Hyaluronic Acid is a major component.
- The test can reliably detect MPS I & II but MPS III & IV can sometimes be a problem.
- It is best to do both 1D and 2D electrophoresis.
Quality Assurance

- **Internal**
  - GAG Standards are from non-human sources!

- **External**
  - EQA Scheme run by Willink Unit
    - 3 urine samples 3-4 times per year
    - Reports on screening tests, quantitation and separation technique results.
  - ERNDIM analyte scheme
    - GAG Quantitation only