Quantitation and Identification of Urine Mucopolysaccharides

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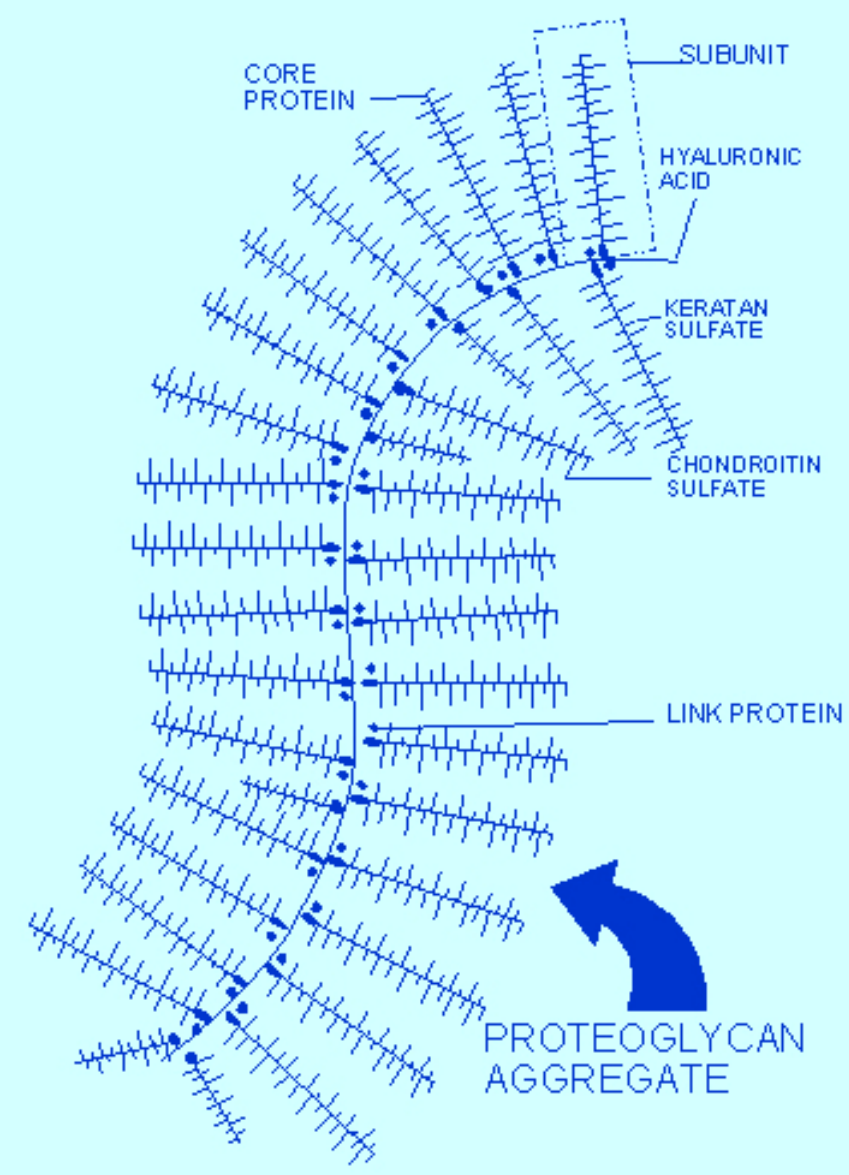
MetBioNet Workshop 2008

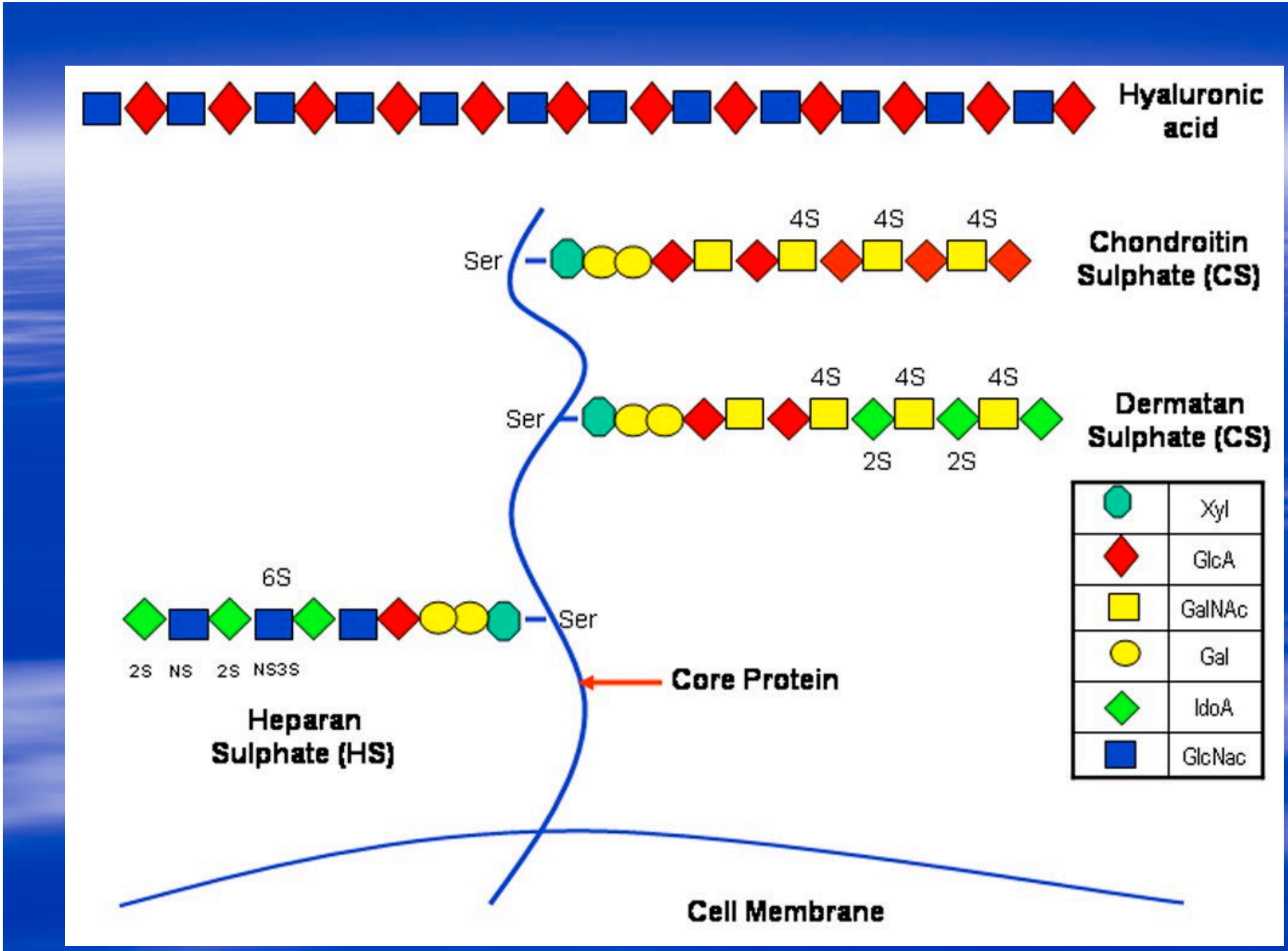
The Big Questions

- What are we measuring?
- Where does it come from?
- How do we measure it?

What are we measuring?

What are Mucopolysaccharides?




Hyaluronic acid
Ser
4S
4S
4S
Chondroitin Sulphate (CS)
Ser
4S
4S
4S
2S
2S
Dermatan Sulphate (CS)
Xyl
GlcA
GalNAc
Gal
IdoA
GlcNac
6S
Ser
2S
NS
2S
NS3S
Heparan Sulphate (HS)
Core Protein
Cell Membrane

Chondroitin Sulphate	GlcUA-GalNAc
Dermatan Sulphate	GlcUA/IdUA-GalNAc
Heparan Sulphate	GlcUA/IdUA-GlcNAc
Keratan Sulphate	Gal-GlcNAc
Hyaluronin	GlcUA-GlcNAc

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.

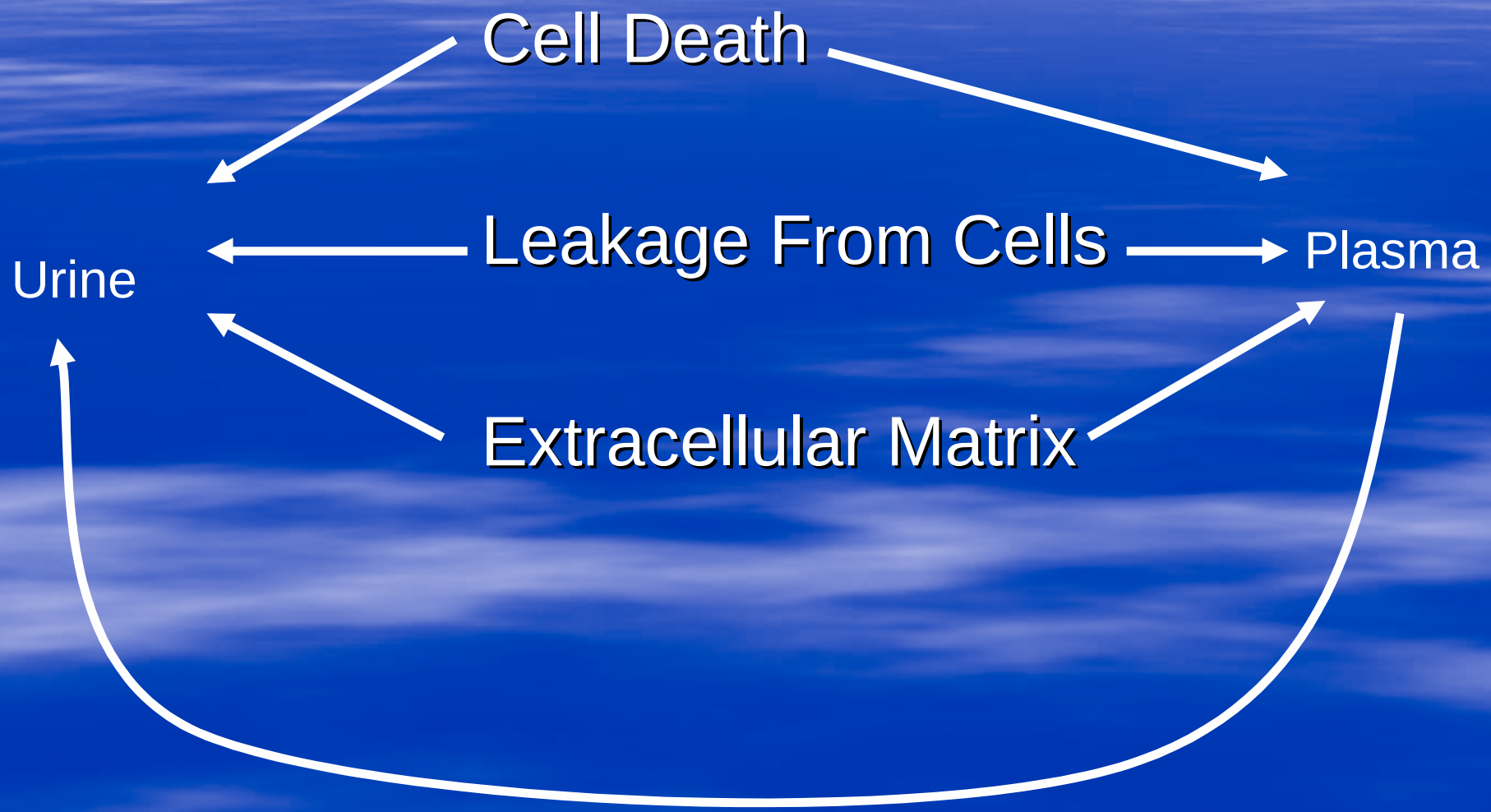
Catabolism

- 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?

GAG	Localization	Comments
Hyaluronin	Synovial fluid, Vitreous Humour, ECM of loose connective tissue	Large polymers, shock absorbing
Chondroitin Sulphate	Cartilage, Bone, Heart Valves	Most abundant GAG
Heparan Sulphate	Basement membranes, Components of cell surfaces	Contains higher acetylated glucosamine than heparin
Heparin	Component of intracellular granules of mast cells Lining the arteries of the lungs, liver and skin	More sulphated than heparan sulphates
Dermatan Sulphate	Skin, Blood Vessels, Heart Valves	
Keratan Sulphate	Cornea, Bone, Cartilage aggregated with chondroitin sulphates	

Sources of Glycosaminoglycans – How does it get in the 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?

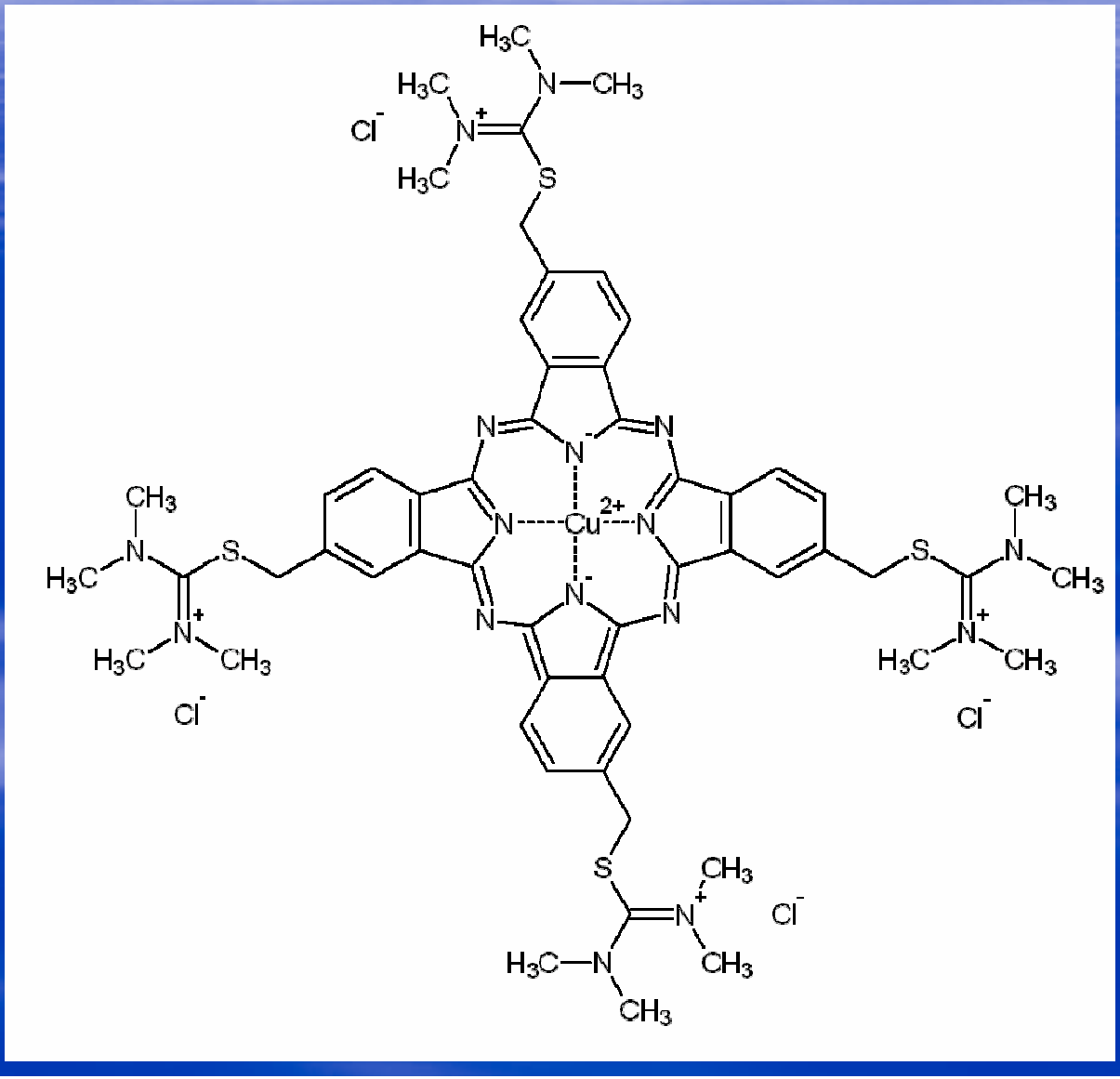
Spot Tests

- 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)

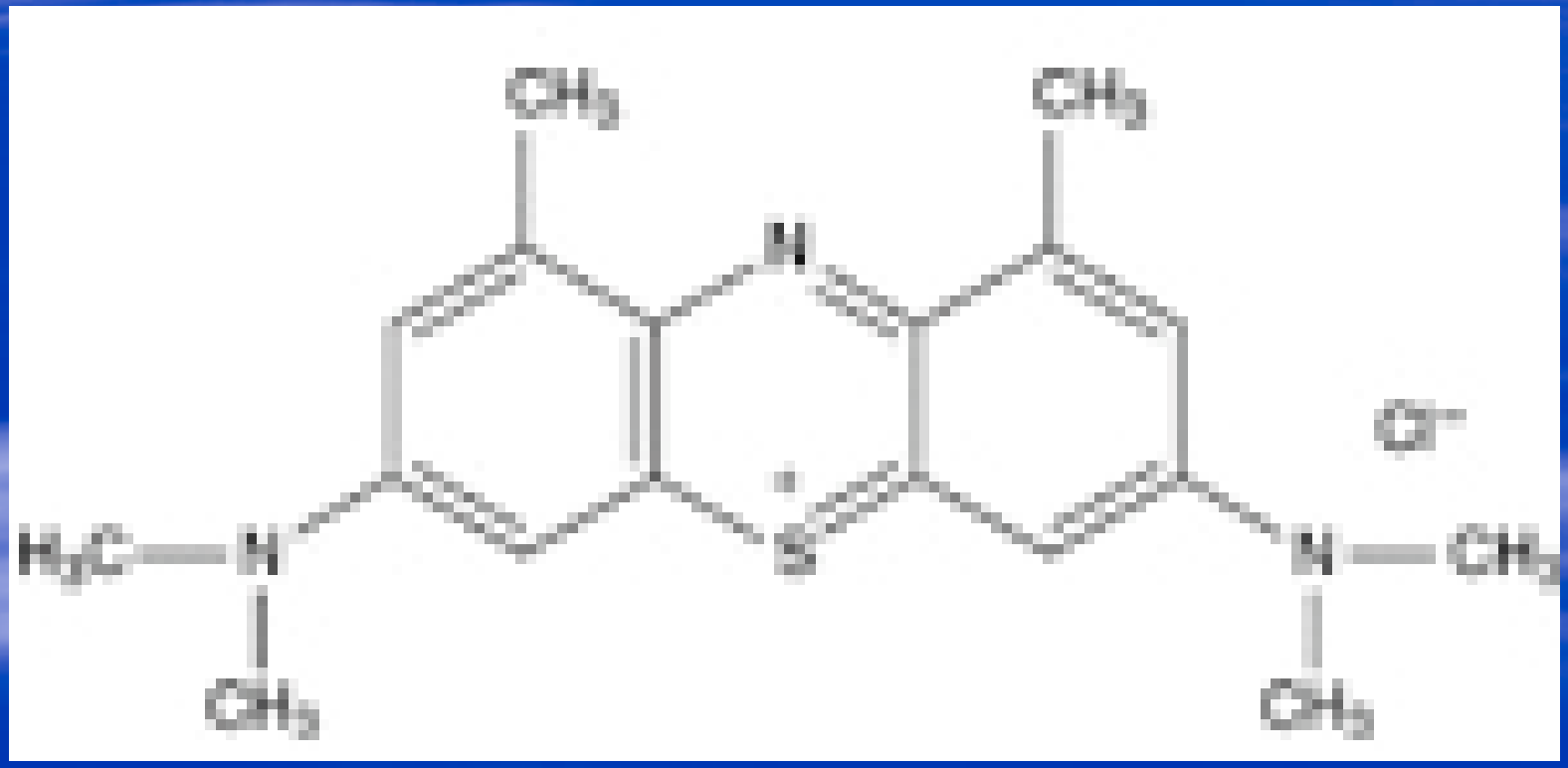
Alcian Blue


H3C
N
CH3
H3C
N+
Cl-
H3C
S
N
N
N-
N
N
N
Cu2+
N
N
N-
N
N
CH3
N
S
H3C
N+
CH3
Cl-
CH3
N
S
H3C
N+
CH3
Cl-
S
CH3
N+
Cl-
H3C
N
CH3
CH3

Alcian Blue Quantitation

- Add urine to Alcian Blue in buffer pH5.8 + 10mmol/l $MgCl_2$
- 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

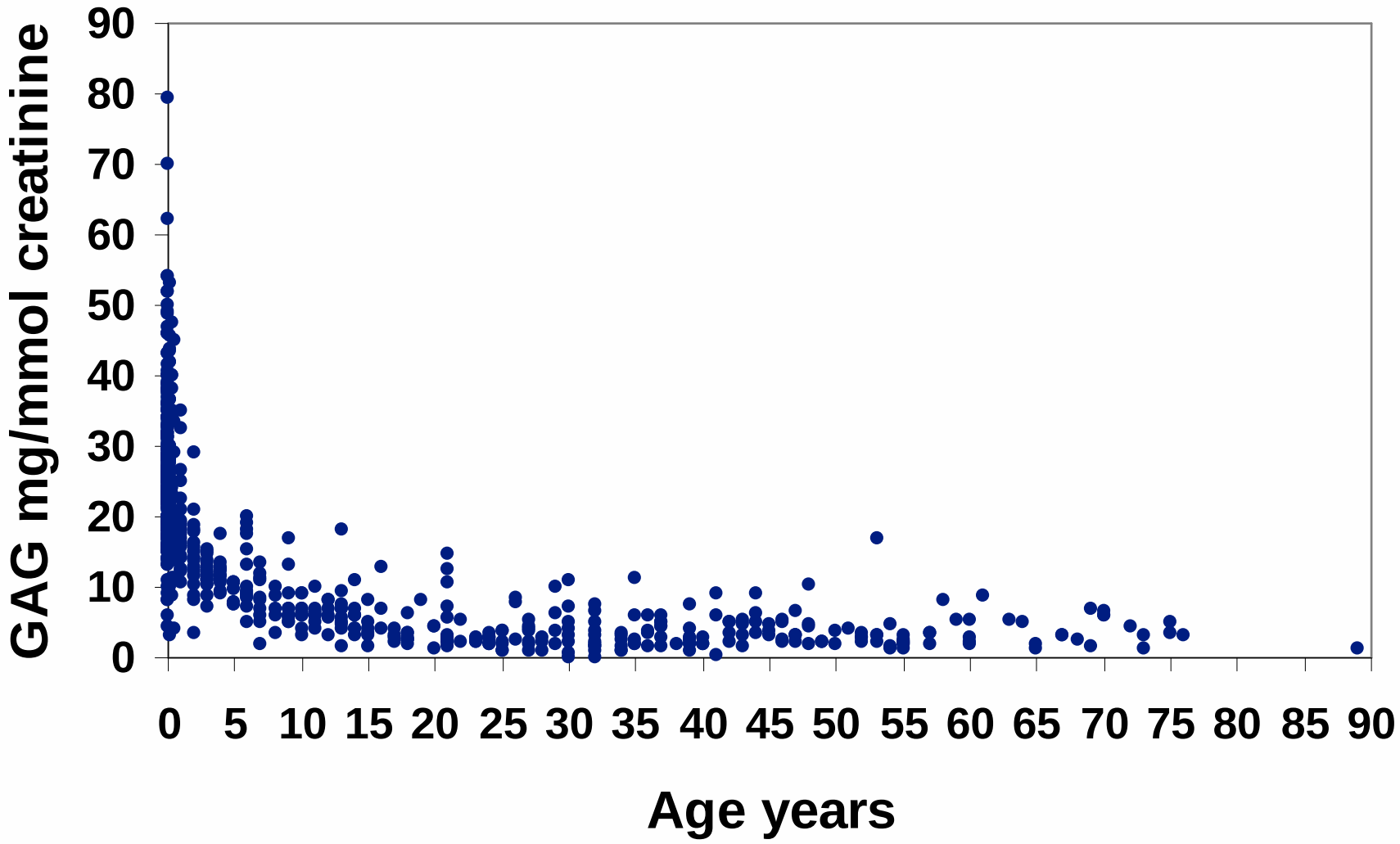
1,9 Dimethylmethylene Blue (DMB)



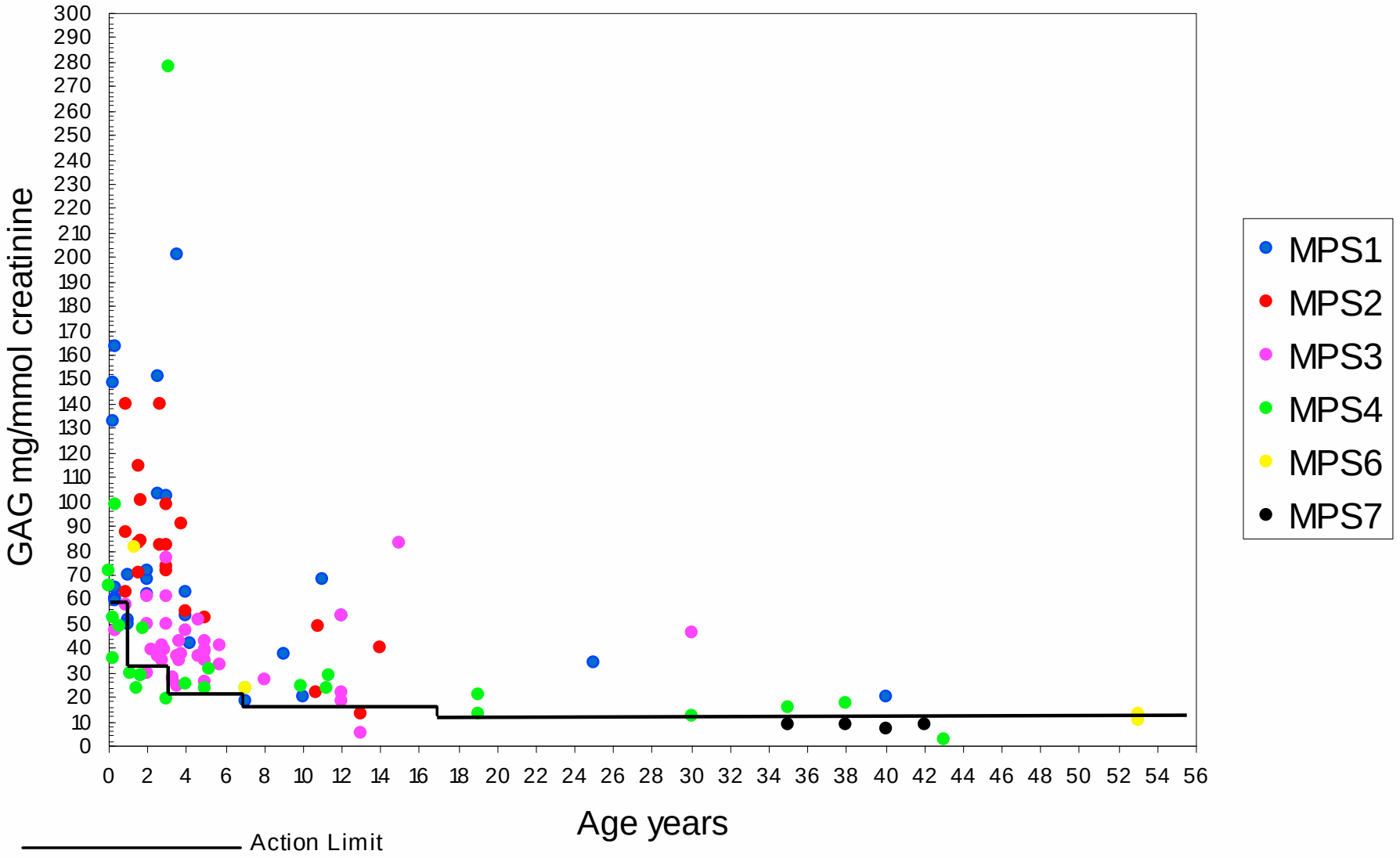
DMB Quantitation

- 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



DMB Quantitation - MPS Patients



Separation Techniques

- Thin Layer Chromatography
- Cellulose Acetate Electrophoresis
  - I Dimensional
  - 2 Dimensional

Thin Layer Chromatography

- 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

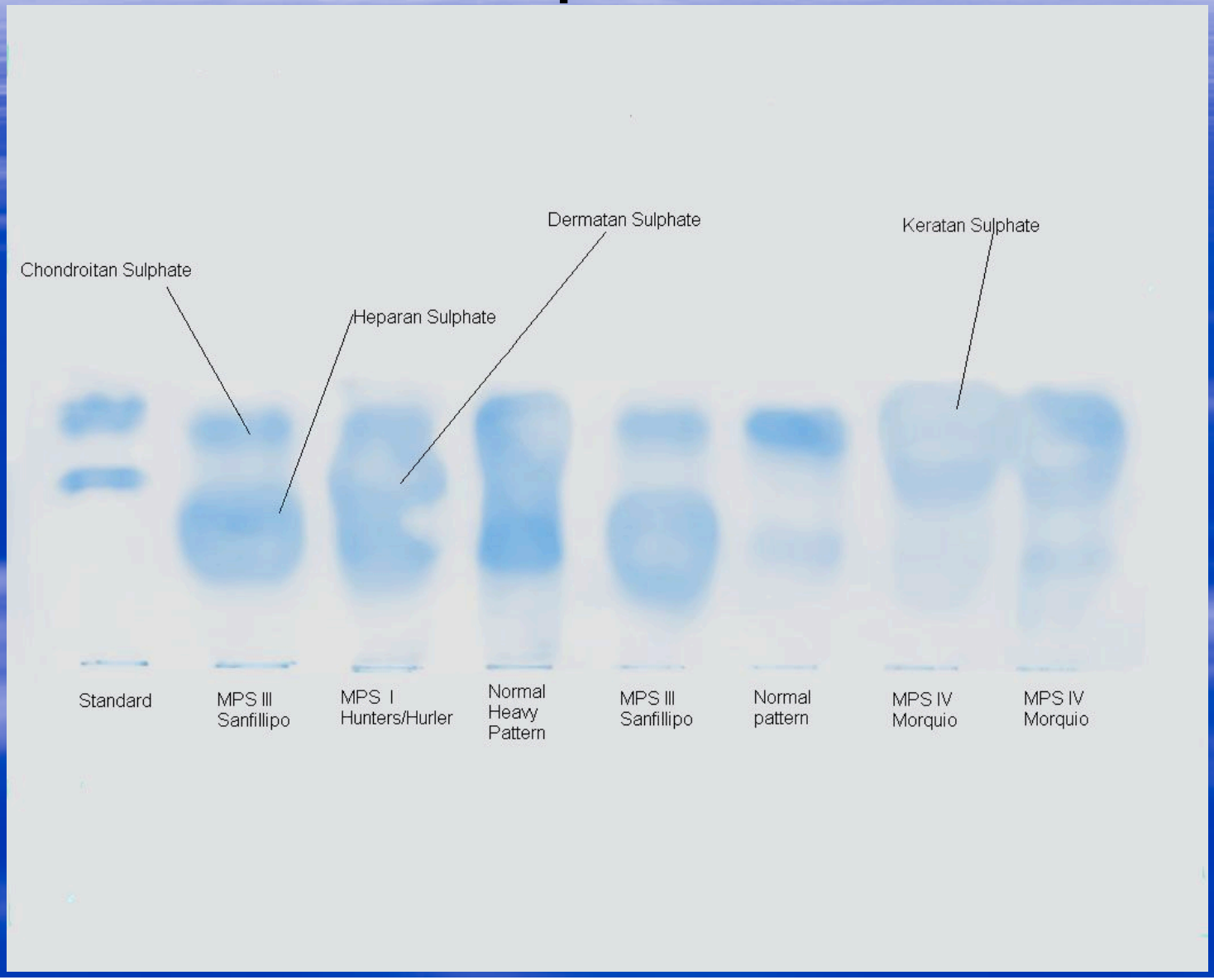
Cellulose Acetate Electrophoresis
1 D Electrophoresis

- 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.

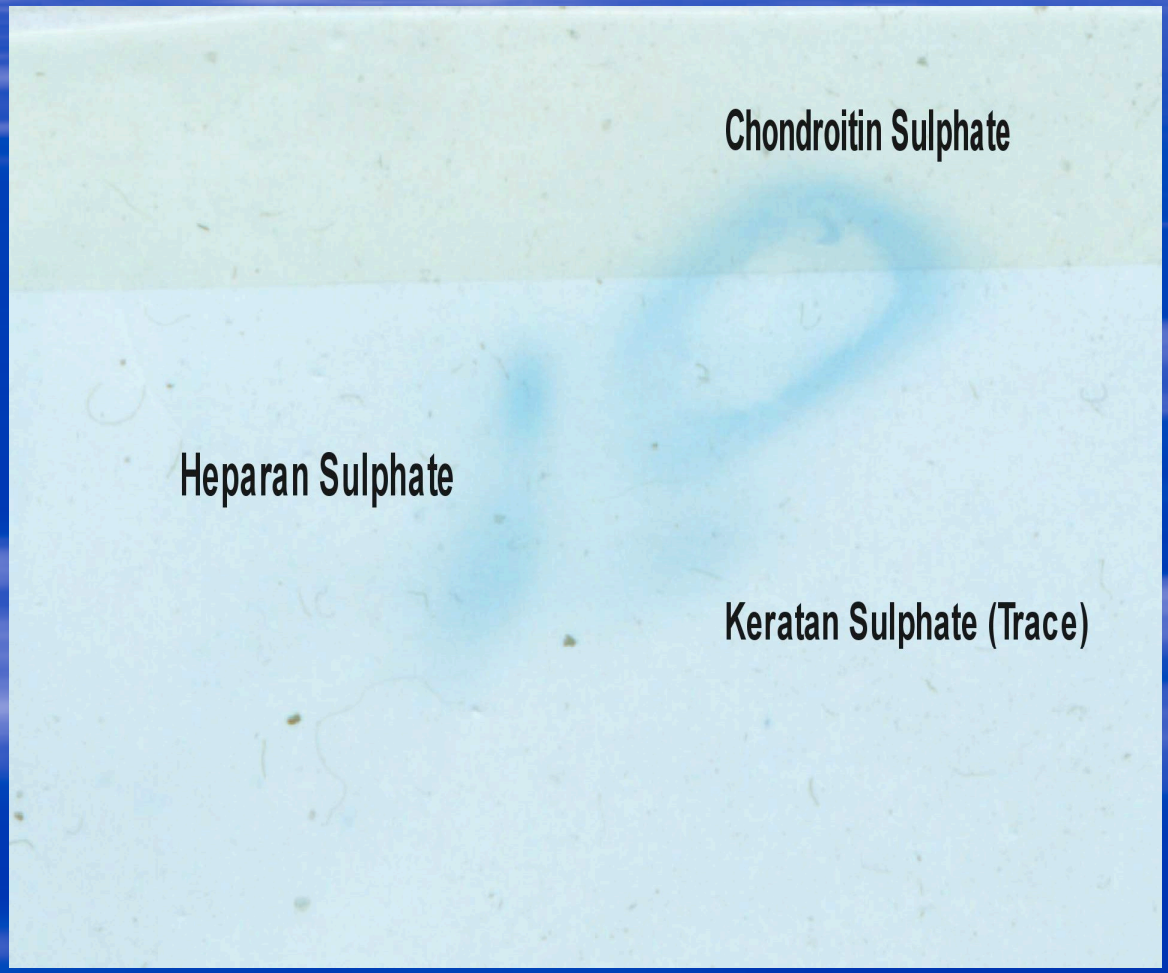
Cellulose Acetate Electrophoresis
2 D Electrophoresis

- 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

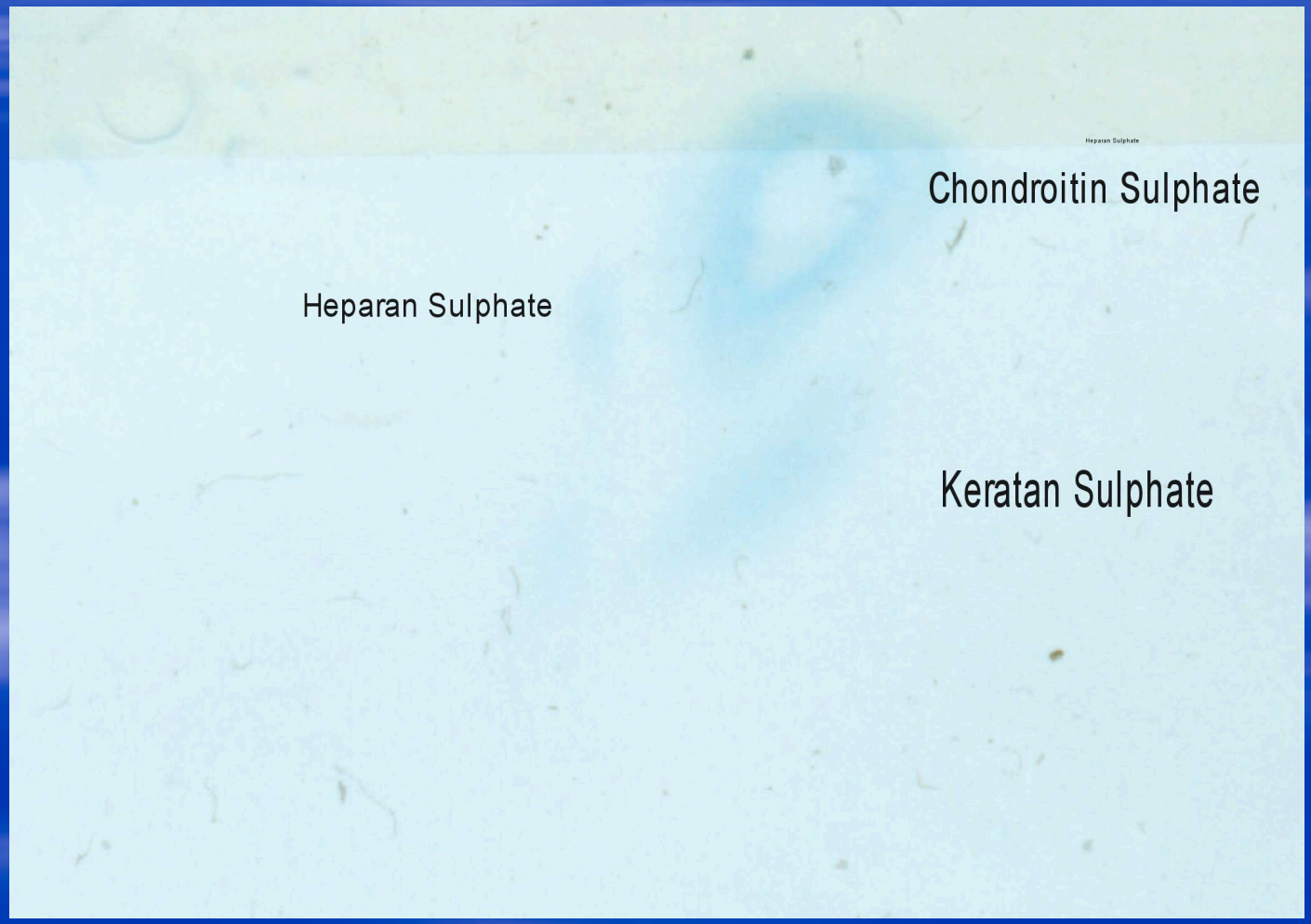


Two Dimensional Electrophoresis



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 form the ones in urine.
- Hyaluronic Acid is a major component.
- The test can reliably detect MPS1 & 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