HYALURONIDASE: A COLOURIMETRIC ASSAY

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In the B.P. assay for hyaluronidase (based on the work of Dorfman and Ott, 1947) the sample of enzyme is allowed to degrade a sample of hyaluronic acid and undegraded hyaluronic acid is estimated by turbidimetric measurement of the clot formed when serum is added to the digest. High precision in the assay is dependent on several factors including; accurately changing the pH from pH 5.0 to pH 3.75 and determining the absorbance (640nm) at exactly thirty minutes after formation of the unstable clot.

Whiteman's method (1973) of assaying hyaluronic acid with Alcian Blue 8GX has now been used for estimating hyaluronidase. When a solution of this dye is added to hyaluronic acid a precipitate is formed. This is washed and redissolved in 40% (w/v) di-isobutyl-sulphosuccinate, pH 5.8, and the absorbance of the resulting solution at 603nm is proportional to hyaluronate concentration. Hyaluronate partially degraded by hyaluronidase gives less precipitate with Alcian Blue than when intact. When the assay is carried out on an enzymic digest A_{603nm} is inversely proportional to enzyme concentration. The absorbance of the post-precipitation supernatant is however directly proportional to the hyaluronidase concentration. The binding of Alcian Blue to hyaluronic acid was greatest at pH 5.8 but was sufficiently strong at pH 5.0, the optimum pH for hyaluronidase, to ensure the linearity of the standard curve so obtained.

The assay, as developed, was carried out in capped, disposable, plastic, conical centrifuge tubes (1.9ml). All of the solutions used were prepared in sodium acetate buffer solution 0.05M, pH 5.0 containing 0.05M magnesium chloride. Hyaluronidase solution, 0.5ml, containing 2-20 units of enzyme activity (Sigma, Type IV or Fisons Batch 2230G) was added to 200µl of substrate solution, containing 60µg hyaluronic acid, (Sigma, Grade III-S) in each tube. The tubes were capped, shaken and incubated at 37°C for 25 minutes after which 1 ml of Alcian Blue solution (Gurr) (0.02% w/v) was added. The tubes were capped, shaken and immediately centrifuged at 11,600 rpm for 5 minutes in a Beckman Microfuge B centrifuge. The absorbance of the supernatant solution was determined at 603nm. A typical standard curve was linear over the range of 0.0-1.2 absorbance units (corrected for blank) against 2-16 hyaluronidase units.

The assay has about the same sensitivity as the B.P. assay but is more economical and has several other advantages:

i) it requires less hyaluronic acid (60µg instead of 125µg)
ii) it requires no serum
iii) no change of pH is necessary during the assay
iv) Alcian Blue acts as an inhibitor of the hyaluronidase, possibly because the complex excludes the enzyme from the inter-saccharide bonds
v) the overall assay time is reduced by about 40%
vi) the colour is very stable (for more than one week).

The assay could readily be semi-automated and may be suitable for complete automation using a dialysis/diffusion module in a sequential auto-analyser.

British Pharmacopoeia (1973) Her Majesty's Stationery Office.
Dorfman, A., Ott, M.L. (1947) J. Biol. Chem. 172: 367
Whiteman, P. (1973) Biochem. J. 131: 343

We acknowledge the gift of Hyaluronidase by Messrs. Fisons Ltd., Pharmaceuticals Division, Crewe, Cheshire.