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ASSAY / ANALYTICAL PROCEDURE
HYALURONIDASE

1. METHOD OF ASSAY: That of Dorfman in which enzymatic reduction in turbidity, resulting when hyaluronic acid is mixed with serum albumin at an acid pH, is measured spectrophotometrically at 600 nm and 37^{O}C.

2. UNIT DEFINITION: That amount of enzyme which causes a reduction in turbidity under specified conditions similar to that caused by one unit of an international standard.

3. REAGENTS:

3.1 0,02 M Sodium Phosphate Buffer pH 6,9 / 0,45% NaCl
Dissolve 1,40g $NaH_2PO_4.2H_2O$ [MM 156,01] or 1,24g $NaH_2PO_4.H_2O$ [MM 137,99], 1,56g Na_2HPO_4 [MM 142,0] and 4,5g NaCl [MM 58,44] in ± 800 ml distilled H_2O. Check pH6,8 - 7,0 and dilute to 1 000ml in a volumetric flask.

3.2 0,3 M Phosphate Buffer pH 5,3
Dissolve 36,77g KH_2PO_4 [MM 136,09] and {(0,873g Na_2HPO_4 [MM 142,0]) or (1,65g $Na_2HPO_4.7H_2O$ [MM 268,25]) or (2,2g $Na_2HPO_4.10H_2O$ [MM 358,50])} in ± 800ml distilled H_2O. Check pH5,3 and dilute to 1 000ml in a volumetric flask.

3.3 50% Hydrochloric Acid
To 50ml distilled water, carefully add 50ml conc. HCl [MM 36,46]. Mix well.

3.4 Acid Albumin Solution pH 3,72 - 3,78

3.4.1 Add 4,56ml glacial acetic acid [MM 60,05] to ± 900ml distilled H_2O. Mix well. Dissolve 3,26g CH_3COONa [MM 82,03] or 5,4g $CH_3COONa.3H_2O$ [MM 136,08] in the diluted acid. Adjust pH to 3,72 – 3,78 with HCl [3.3] and final volume to 1ℓ with distilled water.

3.4.2 Dissolve 1mg Bovine Serum Albumin Fraction 5 (BSA) / ml pH3,72 – 3,78 buffer solution. (PREPARE FRESH DAILY).

3.5 Enzyme Diluent
Dissolve 1mg BSA / ml pH6,9 buffer [3.1]. Store on ice. (PREPARE FRESH DAILY).

3.6 Substrate
Dissolve 4mg hyaluronic acid (Biozyme Laboratories Code HA2F)/ml phosphate buffer pH5,3 [3.2]. Do not stir solution but leave overnight in coldroom to dissolve. Add 0,1ml toluene [MM 92,14]/10ml substrate and stir before use. [This stock solution with toluene can be stored for ± 4 weeks at 5^{O}C.]. Immediately before assay, prepare a series of substrate dilutions in buffer [3.2] between $^{1}/_{18}$ and $^{1}/_{25}$. Mix 0,5ml of each dilution with 0,5ml buffer [3.1], incubate at 37^{O}C for 5 minutes and add 5ml acid albumin [3.4] at t_o (zero time). Read $A_{600\ nm}$ after exactly 10minutes. Use dilution yielding $0{,}38 \leq A_{600\ nm} \leq 0{,}40$ for preparation of stock solution. Store at 37^{O}C. (PREPARE FRESH DAILY)

3.7 Enzyme Sample
Dissolve 5mg enzyme/ml ice-cold diluent (3.5). Immediately before assay, dilute solution to yield expected 4 units/ml diluent (3.5).

3.8 Enzyme Standard
Dissolve International Standard (USP or BP) or Internal House Standard to yield exactly 4 units/ml diluent (3.5).

4. PROCEDURE: Into 125 x 16 mm test-tubes pipette the following:

	BLANK	STANDARD (If required)				SAMPLES				
TUBE No.	1	2	3	4	5	6	7	8	9	10
EnzymeDiluent (ml)[3.5]	0,5	0,2	0,3	0,4	0,5	0,2	0,2	0,2	0,2	0,2
Standard (ml) [3.8]	-	0,3	0,2	0,1	-	-	-	-	-	-
Sample (ml) [3.6]	-	-	-	-	-	0,3	0,3	0,3	0,3	0,3
Buffer pH 5.3 (ml) [3.2]	0,5	-	-	-	-	-	-	-	-	-
Activity u/Test	0	1,2	0,8	0,4	0	UNKNOWN				

Mix and equilibrate at 37^{O}C.

At 1 minute intervals, start reaction by adding 0.5ml substrate at 37^{O}C to tubes 2 to 10. Incubate for **exactly** 30 minutes. Kill reaction by adding 5ml acid albumin [3.4] at minute intervals. Incubate for 10 minutes at 37^{O}C, and measure $A_{600\ nm}$ at minute intervals. Subtract blank value from all enzyme values. Draw a standard curve by plotting absorbance versus enzyme activity for 0,0; 0,4; 0,8; 1,2 units respectively. Use the standard curve to determine the activity of the sample. Alternatively, the activity of the sample may be determined using a computerised regression model. (Currently, the method used at Faizyme).

5. CALCULATION: Units/mg material = $\frac{\text{sample units from standard curve x dilution}}{\text{volume enzyme x mg enzyme/ml original solution}}$

OR see computer printout for calculations

6. BIBLIOGRAPHY: Dorfman A.: (1955) Methods in Enzymology 1 166. Ed by Colowick S.P. and Kaplan N.O. Academic Press, New York.