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Optimization of the USP assay for hyaluronidase

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Abstract: The current USP XXII assay for hyaluronidase (EC 3.2.1.35, HAse) determines activity indirectly by measuring the amount of undegraded hyaluronic acid (HA) substrate remaining after the enzyme is allowed to react with the HA for 30 min at 37°C. To be acceptable as a substrate, the HA must pass a USP suitability test. In this study, seven HA samples, which differed in their anatomical origin, their commercial supplier, and their chondroitin sulphate content, were tested as substrates. One of these did not pass the USP suitability test and therefore would not be an officially acceptable substrate; however, it was carried through the investigation along with the others in order to demonstrate its effect on the analysis. All seven HAs were used as substrates to assay testicular hyaluronidases from three different suppliers. The standard by which the other hyaluronidase activities were measured was USP hyaluronidase reference standard. The activity values calculated for a particular hyaluronidase differed significantly depending on which HA was used as substrate in its assay. Optimal results, as judged on the bases of initial purity, suitability for the assay, linearity of the standard curve, and per cent relative standard deviation of the measured activity, were obtained with a HA substrate derived from vitreous humour.

Keywords: *Hyaluronidase; hyaluronic acid; USP XXII assay; optimization; substrate.*

Introduction

The group of substances called hyaluronidases consists of a number of similar enzymes that cleave the glycosidic bonds of certain glycosaminoglycans. The main substrate of the enzyme HAse is hyaluronic acid which is a high molecular weight polysaccharide consisting of repeating disaccharides of alternating D-glucuronic acid (GlcUA) and 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine, GlcNAc) molecules. The GlcUA and the GlcNAc are joined by a beta (1,3)-D linkage while the GlcNAc to GlcUA linkage is beta (1,4)-D (Fig. 1). Hyaluronic acid is found in the extracellular matrix, especially in soft connective tissue [1, 2]. Other sources are umbilical cord, embryonic pig skin, human serum, cock's comb, Rous chicken sarcoma, and rabbit ovum. *Streptococcus pyogenes*, *Pseudomonas aeruginosa* and some encapsulated strains of group A and C streptococci also synthesize HA [1].

In most tissues, HA occurs in association with proteins and other glycosaminoglycans [3], particularly chondroitin sulphates A and C, which contain the saccharide *N*-acetylgalactosamine (GalNAc) in place of the GlcNAc found in HA (Fig. 1). The methods of isolation of HA, viz., precipitation by salts, detergents, or alcohol, remove most of the proteinaceous material but leave a product which is contaminated with varying amounts of chondroitin sulphate which itself acts as a substrate for HAse.

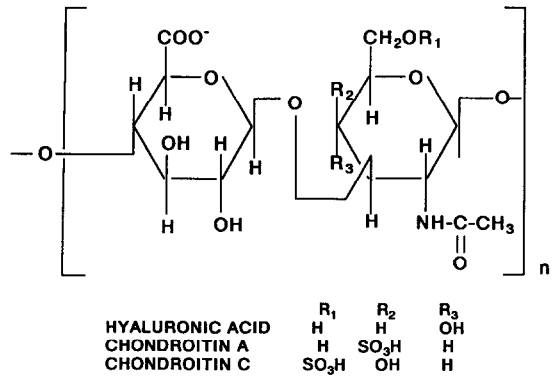

Figure 1
Structures of mucopolysaccharides.

Hyaluronic acid is a highly polymerized molecule whose molecular weight varies depending on its source, although it does not differ in elemental analysis, optical rotation, or the ratio of monosaccharides [1]. For example, synovial fluid HA has a molecular weight in the range $1.2–8.4 \times 10^6$ while human umbilical

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cord and vitreous humour have molecular weights of 3.6–4.5 × 10^6 and 0.3–1.3 × 10^6, respectively [1]. In solution HA is assumed to exist as an expanded coil which is stabilized by hydrogen bonds causing expansion and stiffening of the coil [2]. The viscosity of the solution also varies with the source due to the change in the molecular weights. The higher molecular weight HAs are more polymerized and give more viscous solutions [4].

Hyaluronidase degrades the glycosaminoglycan HA by either of two mechanisms depending on the source of the enzyme, e.g. snake venom, testicular, bacterial, and leech HAses [5]. The enzyme works either as an endo-glycanohydrolase which cleaves the 1,3 bond leaving a reducing glucuronic acid end group as with leech HAse, or cleaves the 1,4 bond leaving a reducing *N*-acetyl glucosamine end group as with testicular or snake venom HAse. Bacterial hyaluronidases, e.g. streptococci HAse, act by an elimination reaction producing in quantitative yields 4,5-unsaturated disaccharides, D-glucuronic acid and *N*-acetylglucosamine. Hyaluronidases, except for the leech variety, also break down substrates other than HA such as chondroitin sulphates A and C which are sulphated at the C-4 and C-6 positions of the GalNAc, respectively. The enzyme attacks chondroitin sulphate A or C at a rate that is approximately one-fifteenth that of HA [5].

The activity of HAse is determined by its effects on the substrate HA. Assays have been described which measure the change in the substrate in one of three different ways: physicochemically, biologically, or chemically. Physicochemical assays use as a measure of activity the reduction in viscosity of a HA solution caused by the depolymerizing action of HAse. These assays are difficult to perform and are inaccurate due to the non-Newtonian behaviour of HA solutions. Biological methods, which attempt to quantify by measuring the spreading of dyes in the skin after breakdown of the extracellular matrix by HAse, are extremely variable. Chemical assays [1, 4–6] which measure colorimetrically the increase in reducing groups produced by enzyme action are subject to error caused by the fact that the undegraded substrate as well as the products has reducing properties.

The current USP XXII assay for HAse is a turbidimetric method with HA as the substrate [7]. In order to be a suitable substrate for the official assay, the HA must pass preliminary tests for inhibitor content and turbidity production [8]. In the assay, first described by Kass and Seastone [9], the substrate, HA, forms a turbid suspension when an excess of acidified serum is added at a rigidly controlled hydrogen ion concentration. The HA and serum form a complex which disappears as the HA is depolymerized with HAse. Complex formation is measured as turbidity which has been shown to be proportional to HA concentration [10]. The turbidity unit is defined as the amount of enzyme which will reduce the turbidity obtained with 0.2 mg of substrate to that obtained with 0.1 mg of substrate in 30 min at 37°C [1, 4, 5]. A source of error in this assay derives from the fact that, in order for turbidity to be an accurate measure of undegraded substrate, the assumption must be made that each cleavage produces particles too small to be able to cause turbidity [9]. For a particle to be non-turbidity-producing its molecular weight must be less than 6000–8000 [11]. Given the high initial molecular weight of the substrate, the assumption cannot be true especially in the early stages of the reaction.

As was previously mentioned, commercially available preparations of HA can differ significantly in molecular weight and the amount of impurities depending on their anatomical source and method of isolation. Even though a particular product is able to pass the USP preliminary tests, different amounts of proteins and other glycosaminoglycans, which can adversely affect the assay for HAse, will be present. The purpose of this research has been to investigate a number of potential substrates, which differed in purity and source, in order to determine whether these variables have a significant effect on the apparent activities of some commercially available hyaluronidases. The wide variations observed indicate the need for agreement on a standardized substrate or more selective preliminary tests.

Experimental

Reagents and chemicals

Hyaluronic acid from human umbilical cord was obtained from Cooper Biomedical (Malvern, PA, USA), Fluka (Hauppauge, NY, USA), and Sigma (St Louis, MO, USA). HA from bovine vitreous humour was purchased from Sigma. Hyaluronidase from bovine testes

was used as received from Calbiochem (La Jolla, CA, USA) (2500 units mg^{-1}, cat. no. 385931), Fluka (Hauppauge, NY, USA) (lyophilized powder, 0.02 units mg^{-1}, cat. no. 53710) and Sigma (lyophilized powder, 750–1500 units mg^{-1}, cat. no. H3884). Horse serum was from Difco Laboratories (Detroit, MI, USA). Gelatin was from Ruger (Irvington, NJ, USA). Cetylpyridinium chloride (CPC), practical grade, was a prouct of J.T. Baker (Phillipsburg, NJ, USA). Alcian Blue 8GX and chondroitin sulphate, Grade III from whale or shark cartilage, were obtained from Sigma. Hyaluronidase USP was supplied by the USP Reference Standards (Rockville, MD, USA). All other chemicals were reagent grade and used as received.

Water was purified to a conductivity of 0.055 microsiemens by passing deionized water through a Gelman water purifier. Ethanol 95% v/v, was supplied by Publicker Distillation Products Inc. (Greenwich, CT, USA).

Apparatus

All spectrophotometric measurements were made using a Hewlett–Packard (Valley Forge, PA, USA) model 8451A Diode Array Spectrophotometer. Assay temperatures were maintained at 37 ± 0.2°C with a water bath from Precision Scientific Co. (Chicago, IL, USA).

In the purification of HA, a lyophilizer (Virtis Co., Gardiner, NY, USA), a Flash Evaporator, Model 1040, from Buchler Instruments (Fort Lee, NJ, USA) and GF/B glass microfibre filters (Whatman, Inc., Clifton, NJ, USA), were used.

Procedures

Preparation of buffer solutions. Acetate buffer (pH 4.3) was prepared by dissolving 14 g of potassium acetate and 20.5 ml of glacial acetic acid in water to make 1000 ml.

Acetate buffer (0.2 M, pH 6.0) was prepared by dissolving 16.4 g of anhydrous sodium acetate and 0.45 ml of glacial acid in each 1000 ml of water.

Acetate buffer (0.1 M, pH 6.0) was prepared by diluting 0.2 M acetate buffer with an equal volume of water, and adjusting the pH with glacial acetic acid.

Phosphate buffer (pH 6.3) was prepared by dissolving 2.5 g of anhydrous sodium biphosphate, 1.0 g of anhydrous sodium phosphate, and 8.2 g of sodium chloride in water to make 1000 ml.

Preparation of standard solutions. Hyaluronic acid stock solution was prepared by drying the material over magnesium perchlorate in a vacuum desiccator for 48 h. A solution was made at a concentration of 0.5 mg per ml of water and stored at a temperature not exceeding 5°C and was used within 30 days.

Diluent for hyaluronidase was prepared by dissolving 330 mg of hydrolysed gelatin in 250 ml of phosphate buffer and 250 ml of water.

An HAse stock solution of concentration 1.5 units per ml of diluent was prepared fresh daily.

Horse serum was reconstituted with water to its original volume according to the manufacturer's instructions and diluted with nine volumes of acetate buffer. The pH was adjusted to 3.1 with 4 N hydrochloric acid and the solution was allowed to stand at room temperature for 18–24 h. It was then stored at a temperature of 0–4°C and used within 30 days. For each day's use, one volume of the serum solution was diluted with three volumes of pH 4.3 acetate buffer to make a 1:40 solution.

Hydrolysed gelatin was prepared by dissolving 50 g gelatin in 1000 ml of water, heating in an autoclave at 121°C for 90 min, and freeze-drying the solution.

Purification of HA [7]. HA (200 mg) was dissolved in 50 ml of 0.4 M sodium chloride and placed in a 37°C water bath overnight. A 6 ml volume of 10% w/w CPC was added and the mixture stirred for 3 h. The cloudy solution was filtered through a Buchner funnel containing Whatman GF/B filter paper until the solution was clear. The filtrate was then dripped into 0.05% w/w CPC to obtain a concentration of 0.1 M in sodium chloride, so that an HA–CPC precipitate was formed. The precipitate was collected after centrifugation and dissolved overnight in 57 ml of 2 M sodium chloride–ethanol solution (100:15, v/v) in a 37°C water bath, which served to dissociate the HA–CPC complex. Hyaluronic acid was then precipitated by adding two parts ethanol to one part of the HA solution. Chondroitin sulphate content was determined as described below.

Determination of chondroitin sulphate content [12]. Alcian Blue dye (8GX) was dissolved in 15% phosphoric acid–2% sulphuric acid to

give a dye concentration of 1.0 mg ml^{-1}. A 100 µl volume of the HA solution (approximately 3 mg ml^{-1} in water) was mixed with 1.2 ml of the dye solution. After at least 15 min, the absorbance of each tube was determined at 480 nm using a blank of Alcian Blue and water. A standard curve was prepared by using a solution of chondroitin sulphate (1 mg ml^{-1}).

Tests for suitability of HA substrate. The inhibitor content and turbidity production tests were performed as directed in the USP XXII [8].

Turbidimetric assay. All measurements were made in triplicate using the method outlined in the USP XXII [7].

Results and Discussion

Substrate purification

The HA samples from the various sources were analysed for chondroitin sulphate content by the Alcian-Blue method. The results, shown in Table 1, indicated that the umbilical cord samples as received were relatively high in chondroitin, with values ranging from 10.4 to 19.2%. After purification using the cetylpyridinium chloride procedure, the sulphated glycan content was reduced by 86.5% in the case of the Sigma sample while the Cooper Biomedical and Fluka substrates had decreases of 61.5 and 94.3%, respectively. The Sigma vitreous humour substrate initially contained only 1.5% chondroitin, and was not purified further.

Substrate suitability

The USP XXII suitability test [8] for HA was performed on all substrates, purified and unpurified. The results are summarized in Table 2. To be considered suitable for use in the USP assay for HAse, the substrate must pass both an inhibitor content test and a turbidity production test. For the former, a solution of the substrate in an acetate buffer must give an absorbance value that is at least 75% of that observed with a phosphate-buffered solution. All the substrates passed this portion of the suitability test with most substrates giving higher absorbance values for the acetate–buffered substrate. Unpurified Sigma and purified Fluka umbilical cord HA gave lower absorbances for the acetate-buffered substrate but the reductions were small, 4% for Sigma and 7% for Fluka, and well within the prescribed limits.

The turbidity production test required that a phosphate-buffered solution of HA at a concentration of 250 mg ml^{-1}, when mixed with USP diluent for HAse in the ratio of 1:1, give an absorbance of at least 0.26 at 640 nm in a 1-cm cell. All substrates produced values above 0.26 except for the purified Fluka umbilical cord HA which gave a reading of 0.22. Therefore, this was the only sample tested that failed one of the tests for suitability and was not an acceptable substrate for the USP HAse assay. The fact that the absorbance for each of the three umbilical cord samples decreased after purification may be ascribed to the partial breakdown of the HA polymer during the

Table 1
Chondroitin sulphate content*

HA	%CH†	SD	RSD
Umbilical cord HA			
S	11.1	0.91	8.1
PS	1.5	0.17	11.0
CB	10.4	0.41	3.9
PCB	4.0	0.39	9.8
F	19.2	1.02	5.3
PF	1.1	0.11	10.2
Vitreous humour HA			
S	1.5	0.14	9.2

*Abbreviations used: HA, hyaluronic acid; %CH, per cent chondroitin sulphate; SD, standard deviation; RSD, per cent relative standard deviation; S, unpurified Sigma; PS, purified Sigma; CB, unpurified Cooper Biomedical; PCB, purified Cooper Biomedical; F, unpurified Fluka; PF, purified Fluka.
†Per cent chondoitin sulphate is the mean of three trials.

Table 2
USP suitability tests for HA substrate*

HA	HA + diluent	PBHA	ABHA
Umbilical cord HA			
S	0.3603†	0.3768	0.3620
PS	0.3087	0.2161	0.3351
CB	0.3851	0.4176	0.4641
PCB	0.2737	0.2814	0.3058
F	0.4658	0.4497	0.4557
PF	0.2146	0.1625	0.1511
Vitreous humour HA			
S	0.5332	0.3450	0.4865

*Abbreviations used: HA, hyaluronic acid; HA + diluent, hyaluronic acid and diluent for hyaluronidase solutions; PBHA, phosphate-buffered hyaluronic acid; ABHA, acetate-buffered hyaluronic acid; S, unpurified Sigma; PS, purified Sigma; CB, unpurified Cooper Biomedical; PCB, purified Cooper Biomedical; F, unpurified Fluka; PF, purified Fluka.
†Values are in terms of absorbance units.

Table 3
Hyaluronidase activities*

HA	Calbiochem			Fluka			Sigma		
	a†	SD	RSD	*a*†	SD	RSD	*a*†	SD	RSD
Umbilical cord HA									
S	1810.5	108.2	6.0	923.2	207.1	22.4	nd‡	nd	nd
PS	1847.6	558.7	30.2	517.7	41.2	8.0	230.5	30.7	13.1
CB	2046.6	484.4	23.7	987.6	107.4	10.9	205.4	18.8	9.2
PCB	2470.5	225.4	9.1	756.2	233.8	30.9	240.5	51.3	21.3
F	2925.0	779.4	26.6	1025.9	226.0	22.0	309.7	59.7	19.3
PF	2033.5	16.5	0.8	458.1	141.3	30.8	229.0	22.3	9.7
Vitreous humour HA									
S	2639.1	176.8	6.7	495.4	96.6	19.5	285.4	21.7	7.6
Overall average									
All	2253.3	428.0	14.7	737.7	150.5	20.6	250.1	34.1	13.4

*Abbreviations used: HA, hyaluronic acid; *a*, activity in units mg^{-1}; SD, standard deviation; RSD, per cent relative standard deviation; *S*, unpurified Sigma; PS, purified Sigma; CB, unpurified Cooper Biomedical; PCB, purified Cooper Biomedical; F, unpurified Fluka; PF, purified Fluka.

†Commercial claims of potency are: Calbiochem, 2277 units mg^{-1}; Fluka, 500 units mg^{-1}; Sigma, 300 units mg^{-1}. Hyaluronidase activities are the averages of three trials.

‡Not determined.

purification procedure to produce particles with molecular weights of less than 6000–8000, which will not produce turbidity. Hyaluronic acid is known to depolymerize under mechanical shear [7]. The higher absorbance for the unpurified samples may also be ascribed to their relatively high content of chondroitin. Despite the fact that the purified Fluka material would not be judged suitable for the USP assay, it was subjected to the remaining tests for comparison purposes.

Turbidimetric assay

The USP turbidimetric assay for HAse activity was performed with the three HAses using each of the seven substrates discussed above. The activities of these HAses, i.e. Calbiochem, Fluka and Sigma, were calculated using a standard curve determined with the USP reference standard HAse. The results of these assays, which are the averages of triplicate runs, are listed in Table 3 and depicted in Figs 2–5. When the activity plots for each of the HAses are compared, the position and shape of the curve for any individual HA is consistent, indicating that the particular enzyme preparation is not a variable in the assay. However, for any individual enzyme, the curves for each of the substrates are very different in slope and *y*-intercept, indicating that the substrate is a variable in the assay and that the measured activity of the enzyme is dependent on the proper choice of substrate.

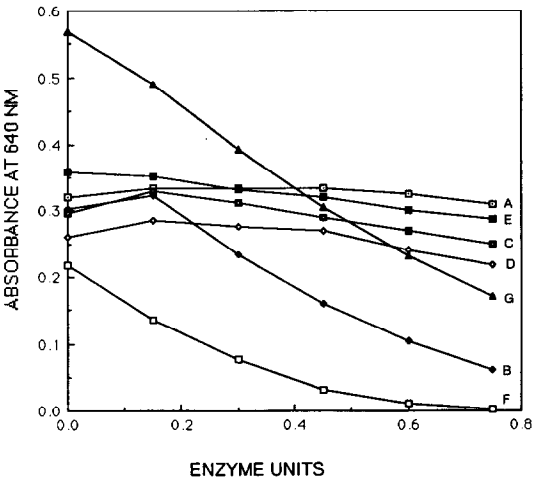

Figure 2
Results of turbidimetric assay for USP hyaluronidase. Hyaluronic acids: A, Sigma umbilical cord; B, purified Sigma umbilical cord; C, Cooper Biomedical umbilical cord; D, purified Cooper Biomedical umbilical cord; E, Fluka umbilical cord; F, purified Fluka umbilical cord; and G, Sigma vitreous humour.

In the USP XXII assay, turbidity is produced by the reaction of HA with serum and an assumption is made equating the turbidity-producing particles of HA with undegraded substrate. However, this assumption is valid only if each cleavage produces a non-turbidity producing unit, i.e. one with a molecular weight less than 6000–8000 [11]. The results in Figs 2–5 indicate that many combinations of substrate and enzyme yield initial increases in

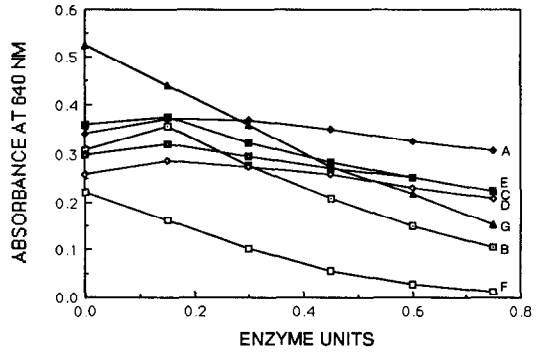

Figure 3
Results of turbidimetric assay for Calbiochem hyaluronidase. Key for hyaluronic acids as Fig. 2.

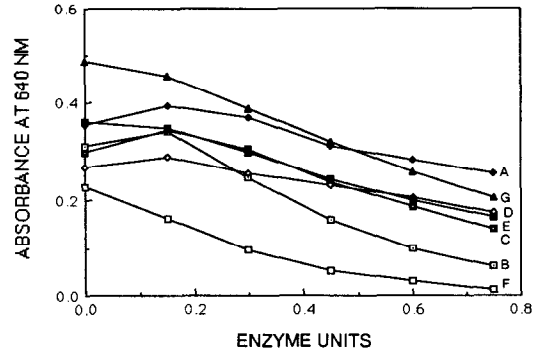

Figure 4
Results of turbidimetric assay for Fluka hyaluronidase. Key for hyaluronic acids as Fig. 2.

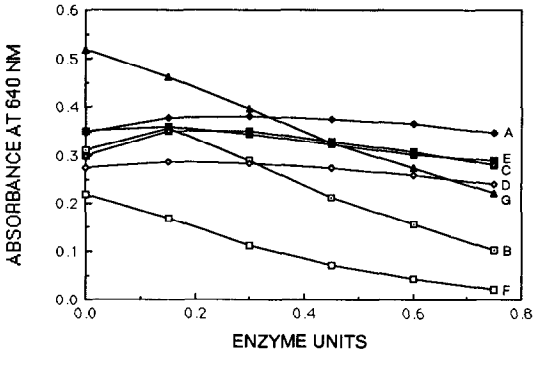

Figure 5
Results of turbidimetric assay for Sigma hyaluronidase. Key for hyaluronic acids as for Fig. 2.

turbidity which precede the declining straight portion of the line. This was due to the production of particles from the highly polymerized substrate which were still capable of reacting with the acidified serum to produce turbidity [5, 9]. Therefore, the assumption that each cleavage produces non-turbidity producing units was not valid, at least not in the region of low HAse concentration. It should be noted that the only substrates that did not produce an initial increase in turbidity were the purified Fluka HA, which was not suitable for the USP procedure because of its overall low turbidity-producing ability, and the vitreous humour substrate, which produced the highest turbidities and the most regular calibration curve.

The average values of the activities of the three HAse samples, determined using each of the seven substrates, are shown in Table 3. As is directed in the USP XXII, the analyses were performed using five concentrations of enzyme ranging from 0.15 to 0.75 units. However, in calculating the activity, the extreme values were eliminated and only the middle three used. This procedure was conducted in triplicate, using USP HAse as the reference standard, to obtain the values in Table 3. From an inspection of the range of activities obtained, it is apparent that, depending on the substrate used, very different activity values can be obtained for any enzyme preparation. The activities of the Calbiochem HAse ranged from 1810.5 to 2925.0 units mg^{-1} while the claim of potency was 2277 units. Fluka HAse had a range of 458.1–1025.9 units mg^{-1} for the seven substrates, while the claim of activity was 500 units per mg. The range for Sigma HAse was 205.4–309.7 units mg^{-1} and the claim was 300 units mg^{-1}. For the combination of Sigma HAse and unpurified Sigma umbilical cord HA, a reliable activity value could not be determined due to the consistent variability of the results. Fluka HAse had the most variation among the activity values calculated for the various substrates with mean RSD of 20.6%. Sigma HAse gave the least variation with mean RSD of 13.4%. Calbiochem HAse had a mean RSD of 14.7% (Table 3).

The differences in the results obtained with the various substrates for a particular enzyme can be attributed to factors such as the source of the substrate and the amounts of impurities present. With respect to anatomic source of the substrate, it has been reported that HA from vitreous humour has a lower molecular weight than the umbilical cord substrate [1, 2, 5]. Therefore, when reacted with the enzyme, there is a greater probability of each cleavage producing oligosaccharides whose size is less than that required to produce turbidity. Also,

because of the smaller size on average of each vitreous humour HA molecule, less enzyme is required to reduce the turbidity to the same level as would be needed for umbilical cord substrate. The result is a calibration curve with a more linear response, no initial rise in turbidity and no decrease in slope at higher enzyme concentrations. This allows a more accurate estimation of the activity of the enzyme as well as increased sensitivity [13].

Another factor which affects the activity results is the presence of chondroitin sulphate, a polysaccharide which is also hydrolysed by HAse but at a rate one-fifteenth as great as that of HA. According to Meyer *et al.* [13], the only mucopolysaccharide found in the vitreous humour is HA; however, HA from other sources is always contaminated with varying amounts of chondroitin sulphate. As can be seen from Table 1, the substrates have different amounts of chondroitin sulphate present depending on the source, the supplier, and whether the substrate was treated by the purification procedure described previously. Since chondroitin sulphate is structurally similar to HA, it competes with HA for the enzyme and, therefore, less enzyme is available to react with the HA. This gives a slower decrease in turbidity resulting in flatter calibration curves and, therefore, less sensitivity and discrimination in the analysis. The Sigma vitreous humour and purified Sigma and Fluka umbilical cord HA, which have considerably lower chondroitin sulphate content than unpurified Cooper Biomedical, Sigma, and Fluka, gave steeper slopes. Purification of the Cooper Biomedical umbilical cord HA did not change the response to the enzyme. This could be due to the relatively high level of chondroitin sulphate remaining, 4%, as compared to the purified Sigma and Fluka umbilical cord substrates and the Sigma vitreous humour HA which had chondroitin sulphate contents of 1.5, 1.1 and 1.5%, respectively. The purified Fluka substrate had the lowest chondroitin sulphate content but failed the USP suitability test for HA. The curve obtained with the latter was concave and lower in value than the other curves, a situation which could presumably be attributed to the breakdown of the substrate, as mentioned previously.

In order to determine whether the differences between the means, in this case the activities of the enzymes for the different substrates, were statistically significant, an analysis of variance, comparing differences in sample means to the variation within the sample means, was performed [14]. For each of the three HAses, statistically significant differences were found between the majority of the means of the various substrates. Therefore, in all three cases, the activity values of HAse differ depending on the substrate.

Examination of the results in Table 3 and Figs 2–5 indicates that passing the USP suitability tests is not sufficient to guarantee a suitable substrate. In fact, most of the substances which passed the suitability tests gave flat response curves. Based on the results obtained in this study, Sigma vitreous humour HA is the optimum substrate for this assay. Due to the initially lower chondroitin sulphate content, the substrate need not be purified in any way to remove substances that might interfere with the USP turbidimetric assay. In addition, its lower molecular weight allows greater accuracy and sensitivity from the turbidimetric assay since there is no rise in turbidity at lower enzyme concentrations, and the calibration curve is straight with a relatively steep slope. The turbidity, in this case, may be more accurately measuring undegraded HA due to the lower molecular weight of the substrate as opposed to the larger umbilical cord substrate where an initial cleavage gives turbidity-producing particles. The next best would be a substrate from umbilical cord origin, but one which had been treated to lower its chondroitin content to below 2%.

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