Title: PROCESS FOR ISOLATING AND PURIFYING OVINE HYALURONIDASE

Abstract: The disclosure relates to a process for preparing a hyaluronidase preparation suitable for pharmaceutical applications. In a preferred embodiment, the process includes the use of viral filtration steps to increase the purity of the final product. The process provides a method that enhances the purity of hyaluronidase preparations presently available in commerce. The methods are preferably used to purify hyaluronidase from mammalian sources. In an alternative embodiment, the methods disclosed can be used to purify recombinant hyaluronidase.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
PROCESS FOR ISOLATING AND PURIFYING OVINE HYALURONIDASE

Related Applications

This application claims the benefit of US Provisional Application No. 60/463,516, filed April 15, 2003, which is hereby incorporated by reference in its entirety.

Field of the Invention

The disclosure relates to a process for preparing a mammalian testicular hyaluronidase preparation suitable for pharmaceutical applications. In a preferred embodiment, the process is used to purify hyaluronidase from ovine testes and includes the use of viral filtration steps to increase the purity of the final product. The process also provides a method that enhances the purity of hyaluronidase preparations presently available in commerce. The methods are preferably used to purify hyaluronidase from mammalian sources. In an alternative embodiment, the methods disclosed can be used to purify recombinant hyaluronidase.

Background of the Invention

Hyaluronidase is a versatile class of enzymes that are expressed in vertebrates and invertebrates alike. The mammalian hyaluronidase catalyzes the random hydrolysis of 1,4-linkages between 2-acetamido-2-deoxy-b-D-glucose and D-glucose residues in hyaluronate.

The hyaluronidase from bovine testes has a reported molecular weight of 65,000 (Lathrop et al. 1990 J Cell Biol 111:2939). The bovine testicular hyaluronidase hydrolyzes the endo-N-acetylhexosaminic bonds of hyaluronic acid and chondroitin sulfuric acids A and C (but not B), primarily to tetrasaccharide residues (Ludovig et al. 1961 J Biol Chem 236:333).

Typical purification protocols call for the use of standard chromatographic techniques to produce a purified solution possessing hyaluronidase activity. Tolksdorf, et al. 1949 J Lab Clin Med 34:74; and Kass & Seastone, 1944 J Exp Med 79:319 have articulated a generally accepted assay protocol to determine hyaluronidase activity. These purification protocols were sufficient to provide relatively crude hyaluronidase preparations.

Brief Description of the Drawings

Figures 1-11 show flow charts of the described hyaluronidase preparation methodology.
Figure 12. Typical chromatograms: A. for Standard; B. for Sample.

**Detailed Description of the Preferred Embodiment**

Ovine hyaluronidase is an enzyme product purified from ovine testes and capable of hydrolyzing mucopolysaccharides of the type of hyaluronic acid.

**Amino Acid Sequence - α-form (SEQ ID NO: 1)**

The consensus sites for glycosylation are underlined. The site of cleavage that yields the β-form of hyaluronidase is assigned by homology with the bovine sequence and is indicated as bold and underlined.

Ovine 1 LDFRAPPLISNTSFLWAWNAPAERCVKIFKLPPDLRLFSVKGSPQKSATG 10
Ovine 51 QFITLFYADRLLGYYPHIDEKTGTNYVGGIPQLGNLKNHLEKAKKDIAYYI
Ovine 101 PNDSVGALIVIDWENWRPTWARNWPKDVYRDESLVQLKQNPQLSPEAS
Ovine 151 KIAKVDFTETAGKSFMQETLKLGLRPNHLWGYYLFPCYNHNYONQTYN
Ovine 201 GNCSDELKRRNDDLWLKESTALFSPVYLNIKLKSTPKAAYFVRNRVQE
Ovine 251 AIIRLSKLASVESPLPVYHROPVFDTDGSSTYLSQGDLVNSVGEIVALGAS 15
Ovine 301 GIIMWGSNLNLSTMQSCMNLOYLGYNLLTTLNPYINVTIALAMCSQVLCHE 20
Ovine 351 GVCTKQWNSSDYHLNPMNFAIQTGKGKTYVPGKVTLEDLQIFSDFKY
Ovine 401 CSCYANINCKKRVDIKNHHSVNVCMADICIEGPVKLQPSHDSSNOTNEAS
Ovine 451 TTTVSSISPSTTATTVSPTPEKQSPECLKVRCLEAIANVTQTGCQGVKW 25
Ovine 501 KNTSSQSQSSIQNIKNQTTY

**Molecular Weight**

The molecular weight based on mobility in 4-20% gradient reduced sodium dodecylsulfate (SDS) polyacrylamide gels is 70-74 kDa for the α-form and 60-63 for the β-form (see hyaluronidase content assay below).

**Identification test**

The identification of hyaluronidase in samples of the drug substance is based on demonstration of a hyaluronidase enzymatic activity.

**Activity ID Test:**

Demonstration of hyaluronidase enzymatic activity is a non-quantitative variation of the activity assay (see “Potency” below). A solution of approximately 0.2 mg/mL drug substance is prepared in the 20 mM sodium phosphate diluent buffer at pH 6.90. In a 16x125 mm glass test tube, 1 mL of the drug substance solution is reacted with 1 mL of 0.5 sodium hyaluronate substrate solution and incubated at 37°C for 10 minutes. A blank consisting of 1 mL of 20 mM sodium phosphate buffer with 1 mL of substrate solution is
run simultaneously. After 10 minutes, the absorbance of the blank and drug substance solutions are read in the spectrophotometer at 600 nm. The value obtained by subtracting the absorbance of the sample from the absorbance of the blank must be greater than 0.4 to prove the presence of a hyaluronidase activity in the drug substance. This demonstrates that the hyaluronidase present is testicular in origin because only this enzyme of the six known mammalian hyaluronidases has significant enzymatic activity above pH 5.0.

**Microbial Limits Test**

No *E. coli, S. aureus, P. aeruginosa,* or *Salmonella.* Total microbial contamination less than $10^3$ organisms per gram.

$\text{pH}$: between 5.2 and 7.2, in a solution of three mg in one mL of deionized water.

**Impurity Tests**

**Process-related Impurities**

**Annexin II** – The annexin II content of drug substance is determined by electrophoretic content assay, quantitated against an internal standard curve of in-house annexin II reference standard. Specification: between 0.29 and 0.57 mg annexin per mg protein. The annexin II reference standard is purified from drug substance by Protein G affinity chromatography and size-exclusion chromatography. Batches of reference standard are qualified for use by testing for purity (SDS PAGE), identity (western blot), protein concentration and amino acid content (amino acid analysis) and in the electrophoretic content assay described here.

**Assay Method:**

Annexin II content is assessed with 12-well 4-20% gradient Tris-glycine sodium dodecyl sulfate (SDS) polyacrylamide gels. Samples of drug substance are reduced and denatured in 2X Tris-glycine SDS sample preparation buffer containing 2-mercaptoethanol at 500 µL per 10 mL of buffer at a final concentration of 0.12 mg/mL. A standard curve of annexin II reference standard is prepared at 5 concentrations: 7.42, 14.84, 29.68, 44.52 and 74.2 µg/mL (Std. 1-5, respectively). These are diluted 1:1 in reducing 2X sample preparation buffer (see above). A designated lot of Vitrase® finished product is used as a system suitability standard, also prepared at 0.12 mg/mL. One sample lane is used by a sample of broad range reduced molecular weight standards. All samples and standards are loaded at a volume of 10 µL of sample per well in the following order:

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<td>Std. 1</td>
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<td>Std. 4</td>
<td>SSS</td>
<td>Std. 5</td>
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-3-
TS = DRUG SUBSTANCE test sample
SSS = system suitability standard lot
Std 1 thru Std 5 = standard curve of in-house annexin II reference standard
MWS = broad range molecular weight standard

Gels are electrophoresed at a constant voltage of 120 V for 120 +/- 5 minutes, or until the bromphenol blue tracking dye line reaches the bottom of the gel. When the run is complete, the gel is carefully removed from its plastic cassette and placed in 50 mL of colloidal Coomassie blue stain solution. Gels are left to stain 13-17 hours with constant mixing provided by a gel rocker platform.

Upon completion of the stain, the gel is destained over a period of not less than 7 hours in deionized water on the gel rocker platform, using multiple changes of water during the process.

Following destaining, the gel is quantitated on a scanning laser densitometer. The densitometer yields a band density histogram that is integrated to give an area under the "peaks" in the histogram.

Calculations:
1. The band density peak area data points for each annexin II concentration of the standard curve is plotted against their theoretical concentrations in Microsoft Excel, complete with correlation coefficient ($r^2$) value and curve equation.

2. The annexin II band mass (in µg) in all drug substance and system suitability samples is calculated from the quantitated band peak areas, and interpolated from the standard curve.

3. A mean annexin II mass for each set of three samples, (drug substance or system suitability) is calculated.

4. The mean annexin II mass for each sample is expressed as a percentage of total protein using the following equation:
   $\%$Annexin II = mean annexin II in sample (in µg) x 100/total protein loaded (in µg).
   The sample meets the requirement if the content is between 0.29 and 0.57 mg annexin per mg protein.

System Suitability Criteria:
Results from this assay are unacceptable if the following criteria are not met:
1. The densitometer must pass its internal calibration check
2. The annexin II band must migrate between the 31 and 36.5 kDa molecular weight standards in gel sample lane 12

3. The mean of system suitability samples tested in triplicate must be within 15% of historically determined values

Acceptance Criteria:

Data from this assay are unacceptable if the following criteria are not met.

1. The calculated $r^2$ value for the standard curve must be $\geq 0.98$.

2. The percentage coefficient of variance for triplicate data points (SSS and TS samples) must be $\leq 10\%$.

3. The mean calculated annexin II for samples must fall within the range of 0.076-0.39 $\mu$g for drug substance.

IgG fragment – The IgG heavy chain fragment ("IgG") content of drug substance is quantitated with a high performance liquid chromatography (HPLC) method using an affinity column and IgG reference standard curve. Specification: $\leq 0.23$ mg IgG per mg protein.

The IgG reference standard is prepared in a one-step affinity purification by Protein G chromatography. Batches of IgG reference standard are qualified for use by testing for purity (SDS PAGE), identity (Western blot), concentration (colorimetric protein assay) and content (HPLC method).

The method uses a standard HPLC system with a tunable UV detector set at 280 nm. The column is a 4.6 x 100 mm Poros OH pre-column, attached in sequence to a 4.6 x 100 mm Poros G affinity column. Samples of drug substance are loaded in a mobile phase of 0.05 M sodium phosphate, pH 7.2, supplemented with 0.15 M sodium chloride. All IgG is retained by the column, and removed by an elution buffer of 0.1 M glycine, 5% acetic acid at pH 2.5. All chromatography is performed at ambient temperature. The eluting gradient has a cycle time of 10 minutes and takes the following shape:

<table>
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<tr>
<th>Time</th>
<th>% Solvent A</th>
<th>% Solvent B</th>
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Solvent A = 0.05 M sodium phosphate, pH 7.2, 0.15 M sodium chloride
Solvent B = 0.1 M glycine, 5% acetic acid, pH 2.5

Preparation of standards, samples and check samples:
The IgG content is determined in comparison to an in-house purified ovine testicular IgG reference standard. The IgG standards are prepared at concentrations of 500, 250, 120, 60 and 30 µg/mL in Solvent A. The IgG standards are loaded onto the column at 100 µL per injection.

Prepare drug substance samples by dissolving 10 mg of drug substance in 3.5 mL of Solvent A in a 5-mL volumetric flask. The samples are mixed briefly and incubated for not less than 12 hours at 2-8°C to ensure complete dissolution. Bring the drug substance sample to a final volume of 5.0 mL with more Solvent A, and mix. Samples are filtered with a 0.45-µm syringe filter fitted to a 3-mL syringe. All drug substance samples are analyzed with 100-µL injections. The IgG content of a given drug substance is measured in 3 separate weighings, analyzed at n = 2 injections of each. Samples of drug substance may be stored at 2-8°C for up to 3 days.

Check samples of Vitrase® finished product are prepared by dissolving the contents of 2 vials in 0.5 mL (each) of Solvent A. Mix by vortexing briefly and then allow them to stand for not less than 30 minutes to ensure complete dissolution. Combine the vial contents and filter as described for drug substance. Each check sample preparation is analyzed with 2 injections of 100-µL volume. Check sample preparations can be stored at 2-8°C for up to 3 days.

**System Equilibration:**

The system is equilibrated with Solvent A for 20 minutes, first at a flow rate of 2 mL/minute for 5 minutes, and then at 4 mL/minute for 15 minutes. Four 100-µL injections of Solvent A are used to establish that the UV signal is stable.

**Assay Method:**

Once a stable baseline has been established, the standard curve of five IgG standard concentrations is run at one injection per standard concentration. This is followed by all drug substance sample injections and then the check sample injections. A final pair of injections of the 250-µg/mL (or 25 µg) IgG standard are used to verify that retention times and signal amplitudes have not changed during the chromatography run.

**Calculations:**

To determine the concentration of IgG in a sample, first perform a linear regression analysis of the standard curve peak areas versus the theoretical mass injected per standard to derive the correlation coefficient ($r^2$) value, the slope and the y-intercept.

\[
\text{IgG mass injected (µg)} = \frac{\text{(Sample Peak Area - Intercept)}}{\text{slope}}.
\]
IgG Concentration (µg/mL) = IgG mass injected (µg) / Injection Volume (mL)

The sample protein concentration is determined with a colorimetric protein assay and used to calculate the IgG content per mg of total protein in the sample.

\[
\text{IgG Concentration (µg/mL) / Protein Concentration (µg/mL)} = \frac{\text{µg IgG/µg protein}}{\text{µg total sample protein}}.
\]

It meets the requirement if the content is \( \leq 0.23 \text{ mg} \text{ IgG per mg protein} \).

The % relative error is calculated by (use absolute value of subtraction number):

\[
\%RE = 100 \times \frac{\text{Injection 1 (µg)} - \text{Injection 2 (µg)}}{2}
\]

The Relative Retention Time for IgG is calculated by:

\[
\text{RRT} = \frac{\text{RT}_{\text{sample}}}{\text{RT}_{250-µg/mL \text{ (or 25 µg) standard}}}
\]

**System Suitability:**

Results from this assay are unacceptable if the following chromatographic conditions are not met.

1. The theoretical plate count for the 25-µg IgG standard must be \( \geq 2300 \).
2. The peak retention time for IgG must be within 4.17 ±0.5 minutes and non-bound proteins must elute at 0.79±0.5 minutes.
3. The baseline shift upon switching from loading to elution buffer must not exceed 0.005 AU in the blank injections.
4. The % coefficient of variation for the 3 injections of the 250 µg/mL (or 25 µg) IgG standard must be \( \leq 10\% \).

**Acceptance Criteria:**

Data from this assay are unacceptable if the following criteria are not met.

1. The \( r^2 \) value for the standard curve must be \( \geq 0.99 \).
2. All sample concentrations must fall within the range of the IgG standard curve.
3. Each injection of the 25-µg standard must fall within the range of 22.5 – 27.5 µg (±10%)
4. The % Relative Error for any two injections of the same sample must be \( \leq 5\% \),
5. The Relative Retention Time of IgG must be within 0.9 – 1.1 minutes.

**Loss on drying** – Tare a glass-stoppered weighing vial that has been dried overnight at 110°C. Place 150 milligrams ±10 milligrams of the drug substance sample in the bottle and accurately weigh the bottle and the contents. Place the bottle in the drying chamber, evacuate the chamber to less than 10 inches of Hg, and dry two (2) hours ±1 minute at 60°C with the lid removed, but also in the chamber. Release the vacuum, open
the chamber door and immediately replace the lid. Weight the dried container and sample after it has cooled to ambient temperature and subtract the weight obtained from the tare weight. Calculate the percent of the original weight that was lost on drying. The loss on drying should not be more than 5% of the original tare weight.

5 Product-related Impurities

Quantitation

Hyaluronidase content - The hyaluronidase content of drug substance samples is quantitated by an electrophoretic method. Hyaluronidase is quantitated against a standard curve of in-house hyaluronidase reference standard with a system suitability sample provided by Vitrase® finished product. Specification: between 0.10 and 0.23 mg hylauronidase per mg protein.

The ovine hyaluronidase standard is purified from drug substance by Protein G affinity chromatography, followed by size-exclusion chromatography and Concanavalin A affinity chromatography. Batches of reference standard are qualified for use by testing for purity (SDS PAGE), identity (western blot), protein concentration and amino acid content (amino acid analysis) and in the electrophoretic content assay. Under reducing conditions, ovine testicular hyaluronidase migrates as a pair of discreet bands that differ in apparent mass by about 7 kDa. Both forms retain full enzymatic activity.

Assay Method:

Hyaluronidase content is assessed with 12-well 4-20% gradient Tris-glycine SDS polyacrylamide gels. Samples of drug substance are reduced and denatured in 2X Tris-glycine SDS sample preparation buffer containing 2-mercaptoethanol at 500 μL per 10 mL of buffer, a final concentration of 0.1 mg/mL. A standard curve of annexin II reference standard is prepared at 5 concentrations: 0.092, 0.0506, 0.0276, 0.0138 and 0.0092 μg/mL (Std's 1-5, respectively). These are diluted 1:1 in reducing 2x sample preparation buffer (see above). A designated lot of Vitrase® finished product is used as a system suitability standard, also prepared at 0.1 mg/mL. One sample lane is used by a sample of broad range reduced molecular weight standards. All samples and standards are loaded at a volume of 10 μL of sample per well.

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TS = DRUG SUBSTANCE test sample
SSS = system suitability standard lot
Std 1 thru Std 5 = standard curve of in-house annexin II reference standard
MWS = broad range molecular weight standard

Gels are electrophoresed at a constant voltage of 120 V for 120 +/- 5 minutes, or until the bromphenol blue tracking dye line reaches the bottom of the gel. When the run is complete, the gel is carefully removed from its plastic cassette and placed in 50 mL of colloidal Coomassie blue stain solution. Gels are left to stain 15-17 hours with constant mixing provided by a gel rocker platform.

Upon completion of the stain, the gel is destained over a period of not less than 7 hours in deionized water on the gel rocker platform, using multiple changes of water during the process.

Following destaining, the gel is quantitated densitometrically on a scanning laser densitometer.

**Calculations:**

Plot the band density total peak area against the theoretical mass values for each standard in the standard curve, to yield an \( r^2 \) value and equation for the line.

Calculate the total mass of the summed hyaluronidase bands in each sample using the band density peak area of the sample and the equation from the standard curve.

Calculate the mean hyaluronidase mass for each set of three hyaluronidase samples.

The mean hyaluronidase mass is expressed as a percentage of the total protein by:

\[
\%\text{Hyaluronidase} = 100 \times \frac{\text{mean sample hyaluronidase (\mu g)}}{\text{total protein (\mu g)}}
\]

The sample meets the requirement if the content is between 0.10 and 0.23 mg hyaluronidase per mg protein.

**System Suitability Criteria:**

Results from this assay are unacceptable if the following criteria are not met:

1. The densitometer must pass its internal calibration check.

2. The hyaluronidase bands must migrate between the phosphorylase b (97.4 kDa) and glutamic dehydrogenase (55.4 kDa) molecular weight standards in gel sample lane 1.

3. The mean of system suitability samples tested in triplicate must fall within 2 standard deviations the historically determined value.
Acceptance Criteria:

Data from this assay are unacceptable if the following criteria are not met.

1. Each standard curve must be comprised of at least 5 points.
2. The calculated r² value for each standard curve must be ≥0.98.
3. The % coefficient of variance for each set of triplicate data points must not exceed 15%.
4. The mean hyaluronidase content of drug substance samples must fall between 0.092 and 0.32 μg.

**Total Protein** - The total protein assay is a colorimetric method based on the binding of Coomassie Brilliant Blue G-250 to proteins in solution. Proteins are quantitated relative to a standard curve of bovine serum albumin. Specification: between 0.55 and 0.83 mg protein per mg drug substance.

Solutions:

The acidic dye concentrate, bovine serum albumin standard and 0.9% saline solutions are obtained from commercial sources. The dye concentrate is diluted 5:1 in deionized water and filtered through Whatman #1 filter paper prior to use. This 5x-diluted dye solution is stable for up to 14 days when stored at 2-8°C.

Samples of approximately 10 mg (+0.5 mg) of drug substance are weighed out and diluted to approximately 1 mg/mL in 0.9% saline. Such drug substance preparations are left to stand for not less than 12 hours to ensure full dissolution prior to use. The sample is then vortexed gently to mix, and filtered through a 0.2-μm cellulose acetate membrane. Reconstituted drug substance sample solutions may be used for up to 7 days when stored at 2-8°C.

Assay Method:

The bovine serum albumin stock solution is 2.0 mg/mL. Dilutions are prepared in 0.9% saline to final concentrations of 0.9, 0.7, 0.5, 0.3 and 0.1 mg/mL in volumes of 500 μL. These standards are prepared fresh daily. A blank consisting solely of 0.9% saline is included. All samples and standards are pipetted in triplicate into 13x100 mm glass tubes at 100 μL per tube.

The assay is initiated by pipetting 5.0 mL of dilute dye reagent into each tube at timed intervals of 20 seconds. After each addition, the tube is covered with a plastic cap and vortexed briefly to mix. The assays are then incubated at room temperature for 10 minutes. The saline blank is used to blank the spectrophotometer at 595 nm – the tubes are
read directly in the spectrophotometer cuvette holder. After 10 minutes incubation, the samples are read in the same order as initiated, at the same 20-second intervals.

**Calculations:**

Protein concentrations are interpolated from the regression line of standard curve absorbance at 595 nm (A_{595}) versus theoretical standard concentration. The assay reporting range is from 0.35 – 0.9 mg/mL – drug substance samples outside this range must be re-assayed with fresh dilutions to bring them into the standard curve range.

Because the drug substance contains proteins (in particular, the IgG fragment) that do not respond in the same way as BSA to the Coomassie Blue dye, a 1.2 correction factor is applied to all concentrations obtained by this assay.

Concentrations of drug substance samples are reported in units of mg protein per mg drug substance. This is calculated from the concentration in mg/mL by the following expression:

\[
\text{mg protein/mg drug substance} = \frac{(PC \times 10 \text{ mL})}{SW},
\]

where PC is the protein concentration in mg/mL and SW is the original drug substance sample weight in mg.

The sample meets requirement if the protein content is between 0.55 and 0.83 mg protein per mg drug substance.

Additionally, a coefficient of variance (\%CV, or \%RSD) is calculated for all drug substance samples run in triplicate.

**System Suitability:**

Results from this assay are unacceptable if the following criteria are not met:

1. The spectrophotometer calibration must be current.
2. The baseline reading on the blank must be stable at 0.00 ± 0.01 AU for at least 20 seconds.

**Acceptance Criteria:**

Data from this assay are unacceptable if the following criteria are not met.

1. The \( r^2 \) value for the standard curve must be \( \geq 0.98 \).
2. All samples must fall within the 0.35 – 0.9 mg/mL reporting range of the assay.
3. The \%CV value for all drug substance samples run in triplicate must be \( \leq 10\% \).

**Potency Assay**

Potency is measured with a hyaluronidase activity assay in which enzyme is incubated with hyaluronic acid substrate for a fixed time period and the non-degraded
substrate detected by the turbidity formed when reacted with an acidic albumin solution. Turbidity is measured spectrophotometrically at 600 nm. Hyaluronidase activity is quantitated relative to a USP hyaluronidase standard curve run simultaneously. Specific activity is calculated using the values from the activity and total protein assays. Potency specification: between $7.32 \times 10^3$ and $1.37 \times 10^4$ USP Units per mg drug. Specific activity specification: between $1.2 \times 10^4$ and $1.9 \times 10^4$ USP Units per mg protein.

**Solutions:**

The sodium hyaluronate substrate solution is prepared at 0.5 mg/mL in 0.3 M sodium phosphate at pH 5.30-5.35. All USP hyaluronidase standard, drug substance and check sample solutions are prepared in a diluent buffer of 20 mM sodium phosphate, pH 6.90±0.05 supplemented with 0.45% sodium chloride and 0.01% bovine serum albumin. The acidic albumin solution is 24 mM sodium acetate, pH 3.75 ±0.05, with 0.1% bovine serum albumin.

The USP hyaluronidase standard solution is prepared to yield a nominal activity of 15 USP U/mL. For the current lot of USP hyaluronidase, this means dissolving 45.3 mg of standard in a final volume of 25 mL of 20 mM sodium phosphate buffer.

Samples of drug substance are initially diluted to approximately 1 mg/mL in 0.9% saline in a 10-mL volume. This initial saline dilution is further diluted, based upon the manufacturer’s release activity value for the particular drug substance lot to be assayed, to yield a final concentration of about 9 USP U/mL. This secondary dilution is made in the 20 mM sodium phosphate buffer.

Check samples are made from lots of Vitrase® finished product in the following manner. A single vial of finished product is dissolved in 5.4 mL of 0.9% saline, delivered from a 10-mL syringe. This solution is filtered through a 5-µm filter needle and returned to its original vial. Dilute 125 µL of this solution to a final volume of 25 mL of the 20 mM sodium phosphate buffer to yield the check sample.

**Assay Method:**

All assays consist of a 10-point standard curve, a check sample, and the drug substance test samples. The standard curve contains USP hyaluronidase standard at concentrations of 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 10.5, 12.0, 13.5 and 15.0 USP U/mL in a final volume of 1 mL of the 20 mM sodium phosphate buffer in 16x125 mm glass test tubes. Check samples consist of 1 mL of the secondary dilution pipetted into the glass test tubes. Likewise, drug substance samples are of 1-mL volumes, pipetted into the test tubes.
Typically, drug substance samples are assayed multiple times on multiple preparations at \( n = 2 \) assays per preparation, allowing for the natural variability of the assay procedure. All standards, check samples and drug substance samples are vortexed briefly to mix, and the tubes capped with plastic stoppers prior to being pre-incubated for 5 minutes in a circulating water bath at 37-38°C.

Assays are initiated by uncapping the tube, pipetting 1 mL of the 0.5 mg/mL hyaluronate solution into the tube, recapping it and immediately vortexing before returning it to the water bath. Care should be taken to vortex at a relatively low speed to avoid foaming the solution. Assays are initiated every 30 seconds until all have received the substrate solution. The incubation time at 37-38°C is 45 minutes. At \( t = 45 \) minutes, the assays are quenched by the addition of 10 mL of acidic albumin solution: uncap the tube, add the acidic albumin, recap it, and mix by gently inverting. The assays are quenched in exactly the same order they were initiated, and left standing at room temperature for 20 minutes before being read at 600 nm in the spectrophotometer at \( t = 65 \) minutes (relative to their respective start times). To read at 600 nm, the tube is first re-mixed by gentle inversion, and then the sample decanted into a disposable 4.5-mL polystyrene cuvette. Samples are read every 30 seconds, maintaining the temporal lockstep of the process. The spectrophotometer is initially blanked with a cuvette full of deionized water.

**NOTE: the relative timing of these events throughout the assay is critical.** Turbidity development is non-linear with respect to time, and improper timing causes spurious or inaccurate activities from the mistimed samples.

**Calculations:**

The standard curve is plotted as a third-order polynomial function of USP U/mL versus the observed absorbance at 600 nm (A<sub>600</sub>). Check sample and drug substance sample activity values are then interpolated from this standard curve from their A<sub>600</sub> values. For the purpose of simplicity and reproducibility, analysts may find it most convenient to set up spreadsheet activity calculation tables whereby entering the raw A<sub>600</sub> data, the polynomial coefficient values from the standard curve equation, the mass value (drug substance samples only) and the sample dilution factors for drug substance and check samples yields calculated activity values for these samples from pre-entered equations in fixed cells of the spreadsheet.

The sample meets the potency requirement if the activity content is between \( 7.32 \times 10^3 \) and \( 1.37 \times 10^4 \) USP Units per mg drug substance.
System Suitability Criteria:

Results from this assay are unacceptable if the following criteria are not met:

1. Calibration of the spectrophotometer must be current.
2. The correlation coefficient ($r^2$ value) of the USP standard curve must be $\geq 0.999$.
3. The check sample activity value must fall within +/- 6% of the historically established value for that lot of finished product.
4. The % relative error between replicate assays (e.g. check samples) must be $\leq 6\%$.

Acceptance Criteria:

Data from this assay are unacceptable if the following criteria are not met.

1. The measured concentration of each sample assay must fall within the range of 3.0 – 13.5 USP U/mL.
2. The % relative error between replicate assays must be $\leq 6\%$.

Specific Activity - Specific activity is calculated as the quotient of the activity and total protein assay results. The sample meets the requirement if the specific activity is between $1.2 \times 10^4$ and $1.9 \times 10^4$ USP Units per mg protein.

Bacterial endotoxins – less than 60 eu per milligram drug substance.

Hyaluronidase Preparation Methodology

The disclosure relates to a process for preparing a hyaluronidase preparation suitable for pharmaceutical applications. In a preferred embodiment, the process includes the use of viral filtration steps to increase the safety level of the final product. The process provides a method that enhances the purity of hyaluronidase preparations presently available in commerce. The methods are preferably used to purify hyaluronidase from mammalian sources. In an alternative embodiment, the methods disclosed can be used to purify recombinant hyaluronidase.

Tissue Sources

Purified preparations of mammalian beta-glucuronidase enzymes are prepared preferably from mammalian testes. Examples of preferred of mammalian sources include ovine, bovine, porcine, and equine. However, any mammalian source can be used with the described methods. There are currently 4,629 currently recognized species of mammals. A taxonomic hierarchy that includes Order, Family, Subfamily, and Genus is found in Wilson, D. E., and D. M. Reeder (eds.) 1993 Mammal Species of the World, Smithsonian Institution Press, 1206 p. (Available from Smithsonian Institution Press), which is hereby incorporated by reference.
Extraction of hyaluronidase

Typically a preliminary step of the purification of hyaluronidase is to isolate testes from a preferred mammalian source. The testes can be processed immediately or preferably are frozen for later use. When frozen, the testes should be thawed at 2-8°C for 40-44 hours. The testes are then minced and filtered to extract the hyaluronidase. The isolated material is then precipitated for a first time, preferably at 15% saturated ammonium sulphate. The temperature of the buffers is preferably maintained at 2-8°C. The pH of the precipitation step is preferably maintained at a pH of 3.60 ± 0.1. The precipitate is filtered, preferably for less than 5 hours. Following filtration, the material is assayed for hyaluronidase activity and protein concentration, and the total units are calculated. The isolated material is then subjected to a second salt cut or precipitation. Ammonium sulphate is added to the level of approximately 85% saturation while maintaining the solution at pH 3.60 ± 0.1. These processes are described more completely in Figures 1 and 2.

Figures 3 and 4 illustrate the second step in the preparation of hyaluronidase from mammalian testes. The product of the purification Step 1 (15/85) purification is thawed and ammonium sulphate is added to 35% saturation. The solution is stirred and filtered. The pH of the filtrate is adjusted to 4.10 ± 0.20 and a sample is typically taken for quality control analysis. The solution is then brought to an 85% saturated concentration of ammonium sulphate and stirred. The solution is then filtered and the precipitate (35/85 precipitate) is collected and stored.

Figures 5 through 8 illustrate the third step of the purification protocol. The 35/85 precipitate is thawed and resuspended for further processing. The solution is dialyzed against 20mM potassium phosphate solution. The dialyzed solution is then filtered and concentrated. The concentrate is stored at 2-8°C overnight. Samples are typically taken as indicated in Figure 5 for quality control purposes. The concentrate is then subjected to DEAE sephadex fractionation. As described in Figure 6, fractions are collected and assayed for hyaluronidase activity. Selected fractions are combined and then precipitated with 85% saturated ammonium sulphate. This solution is stirred and then filtered. The precipitate (0/85) is collected, weighed, filtered, and clarified. The solution is precipitated with PEG6000 to a concentration of 20%. This suspension is centrifuged and the precipitate is collected. The precipitate is then resuspended and filtered. Quality control testing is typically performed. The product is dried and stored for further processing.
The fourth step of the procedure is illustrated in Figure 9 through 11. The product is dissolved for CM sephadex column fractionation. The sample is applied to the column and fractions are collected. Active fractions are pooled and precipitated with ammonium sulphate to 85%. The precipitate (0/85) is collected and weighed. The sample is dialyzed against a 20 mM potassium phosphate. As illustrated in Figure 10, the dialysate is then filtered and the pH is adjusted.

As depicted in Figure 11 the product is filtered to remove potential viral contaminants, freeze-dried and tested.

**Hyaluronidase activity**

Hyaluronidase activity was measured using a turbidity assay. This assay is used to determine the activity of hyaluronidase in the final product, the drug substance [active pharmaceutical ingredient (API)], and in-process intermediates during manufacture of the API or final product. Hyaluronidase activity is determined using a modification of the turbidity assay described in USP 26 for Hyaluronidase for Injection. Briefly, the dissolved hyaluronidase enzyme is allowed to react with the substrate, hyaluronic acid, for a set period of time followed by inactivation of the enzyme and precipitation of non-degraded hyaluronic acid by an acidic albumin solution. The degree of resultant turbidity is measured by absorbance determination at 600 nm using a UV-Visible Spectrophotometer. Enzyme activity is inversely proportional to the turbidity of the solution. Quantitation is based on comparison to turbidity data from a primary USP bovine hyaluronidase standard of known enzyme activity, run under the same conditions. The hyaluronidase preparation has $7.32 \times 10^3 - 1.37 \times 10^4$ USP Units/mg. Typically the hyaluronidase preparation has a pH from 5.2 to 7.2.

**Total protein**

Total protein of the hyaluronidase preparation is measured by generally accepted protein concentration assays. These assays are used to determine the protein concentration of in-process intermediates, active pharmaceutical ingredient (API), and final product. This assay is based on the method of Bradford for protein quantitation. It is a dye-binding assay in which a proportional color change of the dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of the dye, Coomassie Brilliant Blue G-250, shifts from 465 nm to 595 nm when binding to protein occurs. The Coomassie blue dye binds to primarily amine containing amino acid residues, especially arginine. The color yield for an individual protein may depend on its amino acid
composition. Total protein of the hyaluronidase preparation is 0.55 – 0.83 mg/mg protein. Specific activity of the preparation ranges from $1.2 \times 10^4$ – $1.9 \times 10^4$ USP Units per mg protein.

**Water Content**

The hyaluronidase preparation typically has a water content of $\leq 12\%$ by Karl Fischer analysis. This assay is used to determine the amount of water present within in-process intermediates, active pharmaceutical ingredient (API), and final product using the Karl Fischer Coulometric method as delineated in the U.S. Pharmacopoeia 25 (United States Pharmacopoeia), which is hereby incorporated by reference in its entirety. The Karl Fischer Coulometric assay is based on the titration of iodine against water and sulfur dioxide in the presence of a base and an alcohol. When all the water is used up excess iodine is generated and detected at the double platinum electrode. The Karl Fischer coulometer calculates and prints the percent (%) water of the injected sample based on weight. Alternatively, the water content of the hyaluronidase preparation typically is $\leq 5\%$ loss on drying.

**Concentration of Bacterial Endotoxins and Microbial Limits**

The hyaluronidase preparations disclosed possess a limited concentration of bacterial endotoxins. The *Limulus* amoebocyte lysate (LAL) test is used to determine the concentration of bacterial endotoxins in a given sample. The LAL test is based on the observation that bacterial endotoxins react with a lysate derived from circulating cells associated with the blood clotting mechanism of the horseshoe crab, *Limulus polyphemus*. Bacterial endotoxins are present in the hyaluronidase preparation at $\leq 60$ endotoxin units per mg.

Microbial limits are determined using the assay outlined in USP 61. This test is designed to demonstrate that the viable aerobic microorganisms present in the product are free of *E. coli*, *S. aureus*, *P. aeruginosa* or Salmonella. Total microbial contamination less than $10^3$ organisms per gram.

**Quantitation of Annexin II and IgG in the Final Product**

The hyaluronidase preparations for use in the disclosed method have a prescribed concentration of annexin II. To quantitate annexin II in final product, active pharmaceutical ingredient (API) and HY05A in-process lyophilized intermediate (HY05A) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A preferred embodiment of the purified solution typically contains four major protein components (alpha
hyaluronidase, beta hyaluronidase, annexin II, and an IgG fragment), as well as lactose and other buffer components. SDS-PAGE separates proteins based on their apparent molecular weight. This method describes the procedure for running SDS-PAGE gels, scanning gels by densitometry, and quantitating annexin II by ImageQuant® densitometry software. Annexin II content determined by SDS-PAGE analysis from 0.29 – 0.57 mg annexin per mg protein.

The hyaluronidase preparations for use with the disclosed methods contain a particular level of immunoglobulin. The amount of immunoglobulin (IgG) heavy chain fragment, referred to as “IgG,” in drug substance [active pharmaceutical ingredient – (API)], in-process intermediates and final product was measured by high performance liquid chromatography (HPLC). A preferred embodiment is a formulation containing several proteins, lactose, and phosphate buffer. The formulation contains hyaluronidase (the active ingredient) and two major impurities, annexin II and IgG. In order to characterize the drug product, set product specifications and provide process controls, the amounts of various components need to be determined.

The HPLC procedure used for quantitation of IgG is discussed below. The procedure uses an affinity column in which separation is achieved based on the binding affinity of the protein of interest to a ligand attached to the stationary phase. This procedure utilizes protein G as the ligand. During HPLC separation, the IgG is bound to the stationary phase using a buffer at neutral pH, and then is eluted in a step-wise manner with a buffer of low pH. The critical factor in the elution step is low pH. Salt is present to reduce nonspecific binding. IgG content determined by HPLC analysis is approximately ≤ 0.23 mg IgG per mg protein.

**Hyaluronidase content is determined by SDS-PAGE analysis**

To quantitate hyaluronidase protein content by running sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for final product, various process intermediates, and active pharmaceutical ingredient (API). A preferred embodiment typically contains four (4) major protein components [alpha hyaluronidase, beta hyaluronidase, annexin II, and an immunoglobulin (IgG) fragment], as well as lactose and other buffer components. SDS-PAGE separates proteins based on their apparent molecular weight. This method describes the procedure for running SDS-PAGE gels, scanning gels by densitometry, and quantitating hyaluronidase by ImageQuant® densitometry software.
Typically the hyaluronidase preparations contain 0.1-0.23 mg hyaluronidase per mg protein. EDTA content is 23 – 33 µg per mg.

**A Preferred Hyaluronidase Preparation**

The disclosed hyaluronidase preparations are highly purified preparations typically containing 0.10-0.23 mg hyaluronidase per mg protein determined by SDS-PAGE analysis. The preparation typically has a specific activity ranging from $1.2 \times 10^4 - 1.9 \times 10^4$ USP Units per mg protein and a total protein concentration of 0.55 – 0.83 mg/mg protein. As administered, $7.32 \times 10^3 - 1.37 \times 10^4$ USP Units/mg as measured by turbidity assay, a pH from 5.2 to 7.2, and a water content of $\leq 12\%$ by Karl Fischer analysis or $\leq 5\%$ loss on drying. The preparation will comprise bacterial endotoxins $\leq 60$ endotoxin units per mg drug substance. The composition will have an absence of *E. coli*, *S. aureus*, *P. aeruginosa* or *Salmonella*, and a total microbial contamination of less than $10^3$ organisms per gram protein. Other protein components of the preparation include an annexin content of about 0.29 - 0.57 mg annexin per mg protein as determined by SDS-PAGE analysis, and an IgG content of approximately $\leq 0.23$ mg IgG per mg protein as determined by HPLC analysis.

**EXAMPLE 1**

**Ophthalmic Toxicities of Thimerosal, Hyaluronidase (ACS) and Hyaluronidase (Wydase®) in Rabbits**

Certain types of enzymes, when contacted with the vitreous humor following hemorrhage thereinto, will accelerate the rate at which the hemorrhagic blood is cleared from the vitreous humor.

In this regard, a method is provided for accelerating clearance of hemorrhagic blood from the vitreous of the eye, said method generally comprising the step of contacting, with the vitreous humor, a quantity of hyaluronidase at a dose which is sufficient to accelerate the clearance of hemorrhagic blood from the vitreous without causing damage to the retina or other tissues of the eye. Preferably, the hyaluronidase is selected to have a molecular weight distribution which allows the hyaluronidase to be administered intravitreally at doses above 1 IU, and preferably above 15 IU, and advantageously above 75 IU, in the absence of thimerosal, without causing toxic damage to the retina or other tissues of the eye. This hemorrhage-clearing method may be performed without any vitrectomy or other surgical manipulation or removal of the vitreous humor, thereby avoiding the potential risks and complications associated with such vitrectomy procedures.
The preferred route of administration of these hemorrhage-clearing enzymes is by intraocular injection directly into the vitreous body. Alternatively, however, the hemorrhage-clearing enzyme(s) may be administered by any other suitable route of administration (e.g., topically) which results in sufficient distribution of the enzyme(s) to the vitreous body to cause the desired hemorrhage-clearing effect.

The preferred injectable solution may contain a hyaluronidase which has a molecular weight distribution which allows it to be administered intravitreally at doses above 1 IU, and preferably above 15 IU, and advantageously above 75 IU, without causing toxic damage to the eye, along with inactive ingredients which cause the solution to be substantially isotonic, and of a pH which is suitable for injection into the eye. This preferred hyaluronidase preparation is preferably devoid of thimerosal. Such solution for injection may be initially lyophilized to a dry state and, thereafter, may be reconstituted prior to use.

Under USP 6,610,292 and 6,551,590, which are hereby expressly incorporated by reference in their entireties, the term "hyaluronidase (ACS)" as used herein describes a hyaluronidase solution for intravitreal injection which is devoid of thimerosal and which is devoid of hyaluronidase molecular weight fractions above 100,000, between 50,000-60,000 and below 20,000, as determined by electrophoresis gel (4-20% gradient SDS-PAGE). Such hyaluronidase may be derived from ovine testicles and is available commercially from Biozyme Laboratories Limited, San Diego, California, which source may be a starting material for the disclosed process for isolating and purifying ovine hyaluronidase. This specific molecular weight distribution of the hyaluronidase (ACS) results in less ophthalmic toxicity than other hyaluronidase preparations, while exhibiting desirable therapeutic efficacy in a number of ophthalmic applications.

As described in the following examples, hyaluronidase (ACS) may be injected directly into the posterior chamber of the eye at dosage levels which bring about desirable therapeutic affects, including but not necessarily limited to the intravitreal hemorrhage clearing effect, without causing significant toxicity to the eye or associated anatomical structures.

Fifty-Two (52) healthy rabbits of the New Zealand Cross variety (26 male, 26 female) weighing 1.5 kg to 2.5 kg, were individually marked for identification and were housed individually in suspended cages. The animals received a commercially available pelleted rabbit feed on a daily basis, with tap water available ad libitum.
The animals were divided into thirteen groups of 4 animals each (2 male, 2 female). Two animals in each group (1 male, 1 female) were selected for pretreatment fundus photography and fluorescein angiography.

The fundus photography was performed by restraining the animals and visualizing the optic nerve, retinal arcades and fundas with a KOWA® RC-3 Fundus Camera loaded with Kodak Gold 200 ASA film.

The fluorescein angiography involved a 1.5 ml injection of 2% sterile fluorescein solution via the marginal ear vein. Approximately 30 seconds post-injection the fluorescein was visualized upon localization of the optic nerve, retinal vessels and fundas.

The following day, each animal was anesthetized by intravenous administration of a combination of 34 mg/kg of ketamine hydrochloride and 5 mg/kg xylazine. The eyelids were retracted using a lid speculum, and the eyes were disinfected with an iodine-providone wash.

Experimental treatments of either balanced salt solution (BSS), BSS+thimerosal, hyaluronidase (Wydase®) or hyaluronidase (ACS) were administered by injection using a 1 cc tuberculin syringe with a 30 gauge, 0.5 inch needle attached thereto. The hyaluronidase (ACS) solution utilized in this example was free of thimerosal and constituted the specific formulation set forth in Table 5.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronidase (ACS)</td>
<td>7,200 I.U.</td>
</tr>
<tr>
<td>Lactose USP</td>
<td>13.3 mg</td>
</tr>
<tr>
<td>Phosphate USP</td>
<td>5 mmole</td>
</tr>
</tbody>
</table>

Table 5. Specific Formulation

Table 6. The experimental treatments administered to each animal group were as follows:

<table>
<thead>
<tr>
<th>Group #</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BSS</td>
</tr>
<tr>
<td>2</td>
<td>BSS + 0.0075 mg Thimerosal</td>
</tr>
<tr>
<td>3</td>
<td>BSS + 0.025 mg Thimerosal</td>
</tr>
<tr>
<td>4</td>
<td>Hyaluronidase (Wydase®) 1 I.U.</td>
</tr>
<tr>
<td>5</td>
<td>Hyaluronidase (Wydase®) 15 I.U.</td>
</tr>
<tr>
<td>Group #</td>
<td>Treatment</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>6</td>
<td>Hyaluronidase (Wydase®) 30 I.U.</td>
</tr>
<tr>
<td>7</td>
<td>Hyaluronidase (Wydase®) 50 I.U.</td>
</tr>
<tr>
<td>8</td>
<td>Hyaluronidase (Wydase®) 150 I.U.</td>
</tr>
<tr>
<td>9</td>
<td>Hyaluronidase (ACS) 1 I.U.</td>
</tr>
<tr>
<td>10</td>
<td>Hyaluronidase (ACS) 15 I.U.</td>
</tr>
<tr>
<td>11</td>
<td>Hyaluronidase (ACS) 30 I.U.</td>
</tr>
<tr>
<td>12</td>
<td>Hyaluronidase (ACS) 50 I.U.</td>
</tr>
<tr>
<td>13</td>
<td>Hyaluronidase (ACS) 150 I.U.</td>
</tr>
</tbody>
</table>

The day following the injections (Day 1), the 26 animals which were subjected to the fundus photography and fluorescein angiography were observed using the same methods as for the pre-dose examination.

On Day 2 following the injections, the 13 male rabbits that had received the fundus photography and fluorescein angiography at pre-dose and Day 1, as well as the 13 female rabbits that were not selected for photography were euthanized with a sodium pentobarbital based drug. The eyes were then surgically removed and placed in a fixture solution of 2.5% glutaraldehyde with 0.1 M phosphate buffered saline at pH 7.37.

Alternatively, one randomly selected rabbit was euthanized by pentobarbital injection but then fixed by intracardiac injection of the of the glutaraldehyde solution into the left ventricle to determine the effect of the fixation procedure on the histology findings within the enucleated eyes.

On Day 7, the 13 female rabbits that had been previously photographed and angiography performed were subjected to the same observations following the methods previously described.

The remaining 26 animals were euthanized as described above 7 days after dosing. The eyes were fixed in the same manner as those which had been fixed on day 2. Also, one randomly selected rabbit was subjected to the same intracardiac glutaraldehyde fixation procedure described hereabove for the previously randomly selected animal.

The eyes of the animals treated in this example were examined grossly and microscopically for evidence of treatment-related toxicities. A table setting forth a summary of the histological evidence of toxicity or non-toxicity in each treatment group, is set forth in Table 1.
In summary, the eyes of the BSS-treated control group were free of toxicity at 2 and 7 days post dose.

The eyes of the Group No. 2 animals treated with BSS+thimerosal (0.0075 mg) were free of toxicity at day 2, but exhibited evidence that there was a breakdown of the blood-retinal barrier at day 7.

The Group No. 3 animals treated with BSS+thimerosal (0.025 mg) exhibited severe treatment-related toxic effects, at days 2 and 7 post dose.

The Group No. 4 animals treated with Wydase® at the 1 I.U. dose were free of toxicity at days 2 and 7, however, the eyes of the animals in Group Nos. 5-8 treated with Wydase® at dosages ranging from 15 I.U.-150 I.U. exhibited generally dose-related toxic effects at days 2 and 7 post dose.

The eyes of animals in treatment Groups Nos. 9-13 treated with the hyaluronidase (ACS) at dosages ranging from 1 I.U. through 150 I.U., were free of evidence of toxic effects at days 2 and 7 post dose.

Accordingly, it is concluded that thimerosal and the thimerosal-containing Wydase® formulation do cause toxic effects in the eyes of rabbits at the dosages tested, however, the hyaluronidase (ACS) caused no toxic effects in these animals at the dosages tested.

The results of the examinations conducted on day 7 are summarized in Table 1. As shown, in Table 1, significant toxic effects were observed on day 7 in the eyes of rabbits treated with BSS plus thimerosal (0.0075 mg.) and hyaluronidase (Wydase®) at all doses between 1 I.U.-150 I.U. In contrast, no toxic effects were observed in the eyes of animals treated with the hyaluronidase (ACS) at doses between 1 and 150 I.U.

**EXAMPLE 2**

**Safety and Efficacy of the Hyaluronidase (ACS) Injected Intravitreally in Rabbit Eyes**

In this example, 12 healthy rabbits of the New Zealand Cross variety were marked for identification and individually housed in suspended cages. The animals received commercially pelleted rabbit feed on a daily basis and tap water was available *ad libitum*.

The animals were randomly divided into four (4) treatment groups of three (3) animals each.

Initially, the eyes of each animal were examined by dilation with 1-2 drops of 10% Tropicamide followed by gross examination, indirect opthalmoscopy using a 20 diopter lens, and slit lamp examination of the anterior anatomy of the eye.
Following the initial examination of the animals eyes, 100 µl or 10 µl of blood was injected intravitreally into each eye of each animal.

On day 2, the animals of each treatment group received a single intravitreal injection of either BSS or the hyaluronidase (ACS) into the right eye, in accordance with the following treatment schedule:

<table>
<thead>
<tr>
<th>Group #</th>
<th>Left Eye</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>BSS (30 µl) x 1</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>25 I.U. Hyaluronidase (ACS) in 30 µl x 1</td>
</tr>
<tr>
<td>C</td>
<td>None</td>
<td>50 I.U. Hyaluronidase (ACS) in 30 µl x 1</td>
</tr>
<tr>
<td>D</td>
<td>None</td>
<td>75 I.U. Hyaluronidase (ACS) in 30 µl x 1</td>
</tr>
</tbody>
</table>

The hyaluronidase (ACS) preparation used in this experiment was the preferred formulation described hereabove and shown in Table 5.

On days 3, 5, 7, 14 and 21 the eyes of each animal were again examined by slit-lamp to evaluate the cornea, anterior chamber and iris. In addition, the eyes of each animal were dilated with 10% tropicamide solution and the retina was examined by indirect ophthalmoscopy with a 20 diopter lens.

The observed hemorrhage-clearing efficacy of the hyaluronidase (ACS) is summarized in Table 2. In general, the left eye (untreated) of each animal in each treatment group contained hazy vitreous and some blood clots, due to the quantity of blood which had been injected therein. The right eyes of the BSS treated (control) animals of Group A also contained hazy vitreous and some blood clots, while the right eyes of all hyaluronidase-treated animals in Treatment Groups B-D contained vitreous which was clear and through which transvitreal visualization of the retina was possible. Furthermore, the retinas of the rights eyes of all animals in Treatment Groups B-D appeared normal and free of treatment-related toxicity.

The results of this experiment indicate that intravitreally administered hyaluronidase (ACS) was effective at single doses of 25-75 I.U. to accelerate the rate at which blood was cleared from the eyes of the treated animals and further that such single doses of hyaluronidase (ACS) administered in this experiment did not cause observable toxic effects in the eyes of the rabbits treated in this experiment.

The observations following each dose were consistent and are summarized in Table 3. In general, the left eye (untreated) of each animal in each treatment group, contained hazy vitreous humor and some blood clots, due to the quantity of blood which had been
injected therein. The right eyes of the BSS treated (control) animals of Group A also contained hazy vitreous and some blood clots, while the right eyes of all animals in treatment Groups B-E (i.e., the animals treated with hyaluronidase (ACS)) contained clear vitreous through which transvitreal visualization of the retina was possible. Furthermore, the retinas of the right eyes of all animals in treatment Groups B-D appeared to be normal and free of treatment-related toxicity, even after multiple doses of the hyaluronidase (ACS).

The results of this experiment indicate that intravitreally administered hyaluronidase (ACS) was effective, at single doses of 25-75 I.U. x 4, to accelerate the rate at which blood was cleared from the eyes of rabbits and that such doses of the hyaluronidase (ACS) did not cause observable toxic effects in the eyes of the treated rabbits, even after four (4) consecutive doses of the hyaluronidase (ACS) administered at 2 week intervals.

**EXAMPLE 3**

**Safety and Efficacy of the Hyaluronidase (ACS) Injected Intravitreally in Human Eyes**

The primary objective of this study was to determine if a balanced salt solution containing a highly purified hyaluronidase extract from ovine testicular tissue could be injected into the vitreous of visually impaired eyes without eliciting any serious ocular adverse effects.

**Materials and Methods**

Balanced Salt Solution (BSS) was employed as the placebo control, and was obtained from Allergan Pharmaceuticals (Irvine, Calif.). The BSS contained 0.64% sodium chloride, 0.075% potassium chloride, 0.048% calcium chloride dihydrate, 0.03% magnesium chloride hexahydrate, 0.39% sodium acetate trihydrate, 0.17% sodium citrate dihydrate, sufficient sodium hydroxide/hydrochloric acid for adjustment of pH to 7.1-7.2, and water for injection (q.s. 100%). Thirty microliter aliquots of BSS or hyaluronidase specific formulation X (Table 7) were loaded into a 300 µl microsyringe fitted with a 29 gauge needle 0.5 inches in length. The loaded microsyringes were then used to inject the material into the vitreous of the patient's eye.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyaluronidase (ACS)</td>
<td>6,500 I.U.</td>
</tr>
<tr>
<td>Lactose USP</td>
<td>5.0 mg</td>
</tr>
<tr>
<td>Phosphate USP</td>
<td>0.02 mmoles</td>
</tr>
</tbody>
</table>
Initially, eight human subjects with at least one visually impaired eye were randomly assigned to receive intravitreally either 50 µl of 50 I.U. of the hyaluronidase (ACS) in BSS or BSS alone (3:1 ratio). After one month of follow-up to assure the 50 I.U. dosage was well-tolerated, a second group of six visually impaired subjects were enrolled in the study and randomly assigned to a higher hyaluronidase (ACS) dosage group (100 I.U.) or the BSS control in a 2:1 ratio.

Procedures used to evaluate the safety of the test articles were completed at various intervals throughout the study, and included indirect ophthalmoscopy, fundus photography, fluorescein angiography, electroretinography, external eye examination, slit lamp biomicroscopy, applanation tonometry, pachymetry, and autorefraction.

A concurrent placebo control group was included in the study so that adverse events peculiarly related to hyaluronidase (ACS) could be distinguished from those attributable to the vehicle (BSS)/injection procedure. Only visually impaired eyes were treated, moreover, since the test articles were injected proximate to the retina and any untoward responses of a serious nature could have been sight threatening. Patients were assigned to treatment using a computer generated randomization scheme beginning with the number 601 for the first phase of the study, and 701 for the second. Neither the patients nor investigators were aware of whether it was the BSS vehicle or hyaluronidase (ACS)/BSS solution that was being injected intravitreally.

Following establishment of a baseline for each patient, the subjects were injected with either the enzyme or the placebo control. Patients were placed in a sitting position on a comfortable chair. One or two drops of a local anesthetic were topically instilled into the eye that was to be treated, after which the patient was asked to look down and a sterile cotton swab soaked in Proparacaine Hydrochloride Ophthalmic solution was applied for 10 seconds to an area on the sclera approximately 4-5 mm above the cornea (superior position/12:00 meridian). The test article was then injected into the vitreous through a 29 gauge needle attached to a 200 µl microsyringe that was inserted up to the full length of the needle at the site of application of the second anesthetic.

Results

Although only infrequently attaining statistical significance, the slit lamp biomicroscopy data suggested that a substantially higher proportion of patients treated with the hyaluronidase (ACS)/BSS preparations as opposed to BSS alone exhibited anterior segment pathologic changes, the most prominent being the presence of cells and flare in the
anterior chamber. After the sixth (one month post treatment) visit, however, no intergroup differences were observed for any of the slit lamp assessed variables.

Retinal/cortical responses, as measured by electroretinography/visual evoked potential, deteriorated over time in one patient treated with BSS and two who were given 50 I.U. of hyaluronidase (ACS)/BSS. However, alterations in electroretinographic patterns were always bilateral and did not occur in either the treated or untreated eyes of the patients assigned to high dose (100 I.U.) hyaluronidase (ACS)/BSS, nor did fluorescein angiographic test results indicate that retinal ischemia was present in any eye irrespective of treatment.

The indirect ophthalmoscopic exams revealed liquefaction and the establishment of posterior vitreal detachment (PVD) in the eyes of the test subjects. The vitreous was characterized as exhibiting a high degree of motility and/or liquefaction soon after injecting the test articles, which was expected for the hyaluronidase (ACS)-containing preparations. Certain test eyes injected with BSS control showed liquefaction and PVD, which was likely present before treatment, since the latter did not possess any enzymatic activity and was given in very small volume (30 µl).

Concerning PVD, in the first group of patients, four of the six patients to be treated with hyaluronidase (ACS) displayed the absence of PVD by slit lamp biomicroscopy (i.e., 601, 602, 604, and 606) (See Table 8 below). After treatment, each of these subjects showed the presence of PVD. The results from the second group of patients were less clear, due to difficulties in imaging the vitreous using slit lamp microscopy.

Table 8. Human Safety Study with 50 µl and 100 µl Hyaluronidase (ACS) Injection Intravitreally

<table>
<thead>
<tr>
<th>Number</th>
<th>Enzyme Dose</th>
<th>Treated Eye</th>
<th>Split Lamp Biomicroscopy of Presence of PVD</th>
<th>Day for PVD</th>
<th>Vitreous Motility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Baseline</td>
<td>Treated</td>
<td></td>
</tr>
<tr>
<td>601</td>
<td>50</td>
<td>OD</td>
<td>NO</td>
<td>YES</td>
<td>2 Days</td>
</tr>
<tr>
<td>602</td>
<td>50</td>
<td>OD</td>
<td>NO</td>
<td>YES</td>
<td>1 Day</td>
</tr>
<tr>
<td>603</td>
<td>BSS</td>
<td>OD</td>
<td>YES</td>
<td>YES</td>
<td>--</td>
</tr>
<tr>
<td>604</td>
<td>50</td>
<td>OD</td>
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<td>1 Day</td>
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<tr>
<td>605</td>
<td>BSS</td>
<td>OS</td>
<td>YES</td>
<td>YES</td>
<td>--</td>
</tr>
<tr>
<td>606</td>
<td>50</td>
<td>OD</td>
<td>NO</td>
<td>YES</td>
<td>14 Days</td>
</tr>
<tr>
<td>607</td>
<td>50</td>
<td>OD</td>
<td>YES</td>
<td>YES</td>
<td>--</td>
</tr>
<tr>
<td>608</td>
<td>50</td>
<td>OD</td>
<td>YES</td>
<td>YES</td>
<td>--</td>
</tr>
<tr>
<td>701</td>
<td>100</td>
<td>OS</td>
<td>NO</td>
<td>?</td>
<td>--</td>
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<tr>
<td>702</td>
<td>100</td>
<td>OD</td>
<td>NO</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>
Given the results from Example 2 where injection of hyaluronidase (ACS) into the vitreous of rabbits at various doses up to 150 I.U. did not result in any significant histopathologic changes in an earlier preclinical study, it was expected that doses below 150 I.U. would be well-tolerated in humans. Consistent with this expectation, the intravitreal administration of hyaluronidase (ACS)/BSS into visually impaired eyes in the current trial elicited few symptoms, all of which were believed attributable to the injection procedure itself as they occurred with comparable frequency in each of the study groups, and treatment-related adverse sequelae were relatively mild and of short duration.

Furthermore, treatment of human eyes with hyaluronidase (ACS) was observed to increase the incidence of observed posterior vitreal detachment. The observed increase in PVD in patients injected intravitreally with hyaluronidase (ACS) shows that the methods described herein are effective in inducing liquefaction and detachment of the vitreous humor. Thus, the results of the present study indicate that hyaluronidase (ACS) can be injected into the vitreous of humans without eliciting any serious or long-term ocular complications.

EXAMPLE 4

Use of Hyaluronidase to Accelerate the Clearance of Hemorrhagic Blood from the Vitreous of the Eye

The Example set forth herebelow describes cases in which intravitreal hyaluronidase (ACS) was used to accelerate the clearance of hemorrhagic blood from the vitreous of the eye. The hyaluronidase used was the thimerosal-free hyaluronidase (ACS) formulation described above and shown in Table 9.

In this experiment, six (6) human patients (5 female, 1 male) who presented with vitreous hemorrhage were treated with single intravitreal injections of hyaluronidase (ACS) at dosages of 50-200 I.U.

The hyaluronidase (ACS) administered in this experiment was prepared by the formulation, described hereabove and shown in Table 9.

<table>
<thead>
<tr>
<th>Table 9. Specific Formulation Z</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ingredient</strong></td>
</tr>
<tr>
<td>Hyaluronidase (ACS)</td>
</tr>
<tr>
<td>Lactose USP</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>Phosphate USP</td>
</tr>
</tbody>
</table>

All of the patients treated in this experiment had a history of diabetic retinopathy, and were found to have vitreous hemorrhages of varying duration. In each patient, the amount of blood present in the vitreous was sufficient to prevent viewing of the retina by standard funduscopic means.

Each patient received a single intravitreal injection of hyaluronidase (ACS). Four (4) patients received a dose of 50 I.U., one (1) patient received a dose of 70 I.U., and one (1) patient received a dose of 200 I.U.

The observed results of this experiment are summarized in Table 4.

In the six (6) patients treated in this example, the hemorrhagic vitreous became sufficiently clear to permit trans-vitreal viewing of the retina within 6-16 days of the single intravitreal injection of the hyaluronidase (ACS). Such clearing of the vitreous was subjectively determined to have occurred significantly faster than that which would have been expected to occur in these patients without hyaluronidase treatment.

It should be noted that unlike the fluorescein leakage observed at higher doses of hyaluronidase (ACS) in rabbits, no toxicity was observed in the present human based study.

**EXAMPLE 5**

**Use of Hyaluronidase to Treat Other Ophthalmological Disorders**

Even a single intravitreal administration of hyaluronidase (ACS), at an experimental dose, is efficacious in treating certain ophthalmological disorders. Patients suffering from previously diagnosed disorders of the eye, including proliferative diabetic retinopathy, age-related macular degeneration, amblyopia, retinitis pigmentosa, macular holes, macular exudates and cystoid macular edema, have exhibited improvement in the clinical symptoms of these disorders upon treatment with hyaluronidase (ACS).

Hyaluronidase (ACS) is capable of being administered intravitreally at doses of or in excess of 1 I.U. without causing toxic damage to the eye and thus is useable to effect prompt liquefaction of the vitreous body and concomitantly the disconnection or detachment of the vitreous body from the retina and other tissues (e.g., epiretinal membranes, macula). As a result of this vitreal liquefaction and detachment, the physical pulling forces of the vitreous on the retina and other tissues are minimized and the rate of natural turnover of fluids within the vitreous is accelerated. Accordingly, hyaluronidase (ACS) is particularly suitable for the treatment of many disorders (e.g., proliferative
diabetic retinopathy, age-related macular degeneration, amblyopia, retinitis pigmentosa, macular holes, macular exudates and cystoid macular edema) which benefit from liquefaction/disconnection of the vitreous and/or accelerated clearance of toxins or other deleterious substances (e.g., angiogenic factors, edema fluids, etc.) from the posterior chamber of the eye and/or from tissues adjacent the posterior chamber (e.g., the retina or macula). Moreover, liquefaction of the vitreous is also believed to remove the matrix, in the form of the polymerized vitreous, necessary to support neovascularization. Thus, the present method is useful in preventing or reducing the incidence of retinal neovascularization.

Furthermore, many ophthalmic disorders have as a causative component, a destabilization of the blood-retina membrane. This destabilization permits various components (e.g., serum components, lipids, proteins) of the choriocapillaries to enter the vitreal chamber and damage the retinal surface. This destabilization is also a precursor to vascular infiltration of the vitreal chamber, known as neovascularization.

Accordingly, embodiments of the present method are directed toward the prevention and treatment of various disorders of the mammalian eye which result from damage or pathology to the vascularization of the eye or which result in damage to the blood-retinal barrier. Examples of such diseases include but are not limited to proliferative diabetic retinopathy, age-related macular degeneration, amblyopia, retinitis pigmentosa, macular holes, macular exudates, and cystoid macular edema, and others in which the clinical symptoms of these disorders respond to the hyaluronidase (ACS) treatment.

**EXAMPLE 6**

**Hyaluronidase Treatment of Proliferative Diabetic Retinopathy (PDR)**

Diabetic retinopathy is the leading cause of blindness in working age Americans. The incidence of retinopathy increases with the time of the disease state, from a level of about 50% manifestation in diabetics with the disease for 7 years to approximately 90% of those with the disease for more than 20 years. It is estimated that PDR affects an estimated 700,000 Americans.

The retinovascular consequences of diabetes essentially consist, in part, of microvascular leakage and capillary nonperfusion resulting from chronic hyperglycemia. Microvascular leakage may in turn result in retinal edema, lipid exudates and intraretinal hemorrhages. Capillary nonperfusion results in the formation of intraretinal microvascular
abnormalities (IRMA). These abnormalities are arteriovenous shunts formed to perfuse retinal regions deprived of vascularization by diabetes-mediated arteriole degeneration.

Expression of vascular endothelial growth factor from a hypoxic retina in areas of capillary nonperfusion is thought to result in the development of extraretinal neovascularization. Such neovascularization and its associated fibrous components may spontaneously involute or be complicated by vitreous hemorrhage or traction retinal detachment. Neovascularization may be easily seen on fluorescein angiogram by the profuse leakage of dye from these new vessels since they lack the tight endothelial junctions of the retinal vasculature. Impaired axoplasmic flow in areas of retinal hypoxia result in cotton wool spots.

Proliferative diabetic retinopathy (PDR) requires careful screening of diabetics for early identification and treatment since PDR remains largely asymptomatic in the early stages. Proliferative diabetic retinopathy can be classified into three subgroups: (1) nonproliferative retinopathy; (2) preproliferative retinopathy; (3) proliferative retinopathy. Each classification has certain morphological characteristics. Features of nonproliferative retinopathy include capillary microangiopathy (microvascular obstructions and permeability changes, nonperfusion of capillaries, retinal capillary microaneurysms, basement membrane thickening, and internal microvascular abnormalities (IRMA)); intraretinal hemorrhages; exudates; and macular changes. Preproliferative retinopathy is indicated by any or all of the changes described for nonproliferative retinopathy and the following: significant venous beading, cotton-wool exudates, extensive IRMA and extensive retinal ischemia. Proliferative retinopathy is indicated by extraretinal neovascularization and fibrous tissue proliferation, vitreous alterations and hemorrhage, macular disease, and retinal detachment.

The creation of fibrovacular tissue is an especially important complication of PDR since it often will lead to retinal damage mediated by the vitreous. The fibrovacular tissue may form preretinal membranes that create dense adhesions with the posterior hyaloid membrane. These adhesions are responsible for transmitting the forces of vitreous traction to the retina, which may result in retinal detachments.

The vitreous base is normally firmly attached to the adjacent retina and to the outer circumference of the optic nerve head, known as the ring of Martegiani. The attachment of the vitreous to the retina in all other sites between the ring of Martegiani and the vitreous base is much less firm. Neovascularization from the retina leads to the formation of
vascular strands extending into the vitreous from the nerve head or elsewhere in the fundus. Contraction of these strands may cause partial or complete retinal detachment.

Retinal detachment at the macula is a major complication of PDR. Most retinal detachments resulting from PDR begin as tractional detachments without holes, but they may become rhegmatogenous by the formation of retinal holes at some later point in the disease. The tractional detachments are caused by abnormal vitreoretinal adhesions or vitreal traction with subsequent shrinkage of the fibrous bands and elevation of the retina.

The present method contemplates treatment of PDR in the preproliferative and proliferative states using hyaluronidase (ACS) intravitreal injections. Without being limited to a particular mechanism, it is believed that the effect of intravitreal hyaluronidase (ACS) injection is to promote the clearance of the liquid phase of the vitreous. The rate of transfer of intravitreally injected tritiated water from the mid vitreous to the choroid was significantly increased after depolymerization of vitreous hyaluronic acid by injected hyaluronidase (ACS). The present method capitalizes upon this observation to liquefy the vitreous, for example, in order to promote the clearance of various growth inducing factors and other serum products leaked into the vitreous due to the presence of PDR. It is further contemplated that the hyaluronidase (ACS) treatment of the present method may be performed alone or in combination with other treatments of PDR.

EXAMPLE 7

Treatment of Non-Proliferative Diabetic Retinopathy

Purpose: To determine the effect of hyaluronidase (ACS) on progression of moderately severe to severe non-proliferative diabetic retinopathy (NPDR in the presence or absence of an induced posterior vitreous detachment (PVD). Methods: sixty patients evaluated by ultrasonography and masked fundus photography were randomly assigned to: saline (0.05 ml), hyaluronidase (ACS) (75 I.U., 0.05 ml), SF6 gas (0.3 ml) or hyaluronidase (ACS) plus SF6 gas 4 weeks later. PVD was assessed through week 16; seven-field fundus photographs were obtained as surrogate baseline and 12 months later. Results: Of all eyes treated, without regard to PVD, those with stable ETDRS scores were: saline 38% (6/16), hyaluronidase (ACS) 67% (10/15), SF6 40% (6/15) and hyaluronidase (ACS) plus SF6 43% (6/14). Worsening of ETDRS scores were: saline 38% (6/16), hyaluronidase (ACS) 13% (2/15), SF6 20% (3/15) and hyaluronidase (ACS) plus SF6 21% (3/14). Percent of eyes with a complete PVD on or prior to 16 weeks and stable ETDRS scores were: saline 0% (0/14), hyaluronidase (ACS) 50% (6/12), SF6 30% (3/10) and hyaluronidase (ACS)
plus SF6 40% (4/10). Reduced visual acuity across all groups was the most frequently reported ocular adverse event. Conclusions: Eyes treated with hyaluronidase (ACS) had stable ETDRS retinopathy scores compared to saline. The same number of saline-treated eyes had stable ETDRS scores as worsened scores. The percent of eyes with a complete PVD and stable ETDRS scores was highest in hyaluronidase (ACS) group. These data indicate that induction of a PVD and/or enzymatic liquefaction of the vitreous has the ability to affect the progression of diabetic retinopathy.

EXAMPLE 8

Treatment of Preproliferative Diabetic Retinopathy

In this Example, a diabetic patient manifesting preproliferative diabetic retinopathy is treated for this complication of diabetes mellitus through the intravitreal injection of hyaluronidase (ACS). The purpose of this treatment is to reduce or prevent the development of proliferative diabetic retinopathy manifested by extraretinal neovascularization and fibrous tissue proliferation, vitreous alterations and hemorrhage, macular disease, and retinal detachment.

Once a patient has been diagnosed with diabetes, increased ophthalmic surveillance is performed, given the high percentage of individuals suffering from this disease later developing proliferative diabetic retinopathy (PDR). This increased surveillance should include periodic retinal examinations and fluorescein angiograms to monitor the extent of venous beading, IRMA, and retinal ischemia.

When preproliferative diabetic retinopathy begins reaching the proliferative stage, the hyaluronidase (ACS) treatment is commenced. This stage is defined as the presence of venous beading in 2 or more quadrants, IRMA in one or more quadrants, and/or microaneurysm and dot hemorrhages in all quadrants. Once these indicia are present, hyaluronidase (ACS) method of treatment is initiated.

The patient is to receive a full ophthalmic examination to establish a baseline of ocular health. The ophthalmic examination includes indirect ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected) symptomatology, fundus photography, fluorescein angiography, electroretinography and A-scan measurements.

Following the preliminary examination, an intravitreal injection of hyaluronidase (ACS) is given to the patient's affected eye. If both eyes are affected, they may be treated separately. The eye to be treated is injected with 50 μl of 50 L.U. of the hyaluronidase
(ACS) ophthalmic solution described above intravitreally to promote the depolymerization of vitreous hyaluronic acid, resulting in the liquefaction of the vitreous.

After treatment, the patients' eyes are to be examined on days one (1), two (2), seven (7), fifteen (15), thirty (30) and sixty (60). On each examination day the patient is monitored for vitreous liquefaction. Additionally, the patient is monitored for posterior vitreous detachments using indirect ophthalmoscopy with scleral depression. Finally, the extent of PDR presented by the patient is continuously monitored through periodic retinal examinations and fluorescein angiograms to monitor the extent of venous beading, IRMA, and retinal ischemia.

**EXAMPLE 9**

**Treatment of Proliferative Retinopathy**

In this Example, a diabetic patient manifesting proliferative diabetic retinopathy is treated by the intravitreal injection of hyaluronidase (ACS). The purpose of this treatment is to reduce the extent of proliferative diabetic retinopathy, to prevent further manifestations of the disease after removal of any extraretinal neovascularized tissue, and to reduce the likelihood of retinal detachment.

A patient presenting proliferative diabetic retinopathy is to receive the hyaluronidase (ACS) method of treatment in combination with surgical treatment of the neovascularized tissue. The proliferation usually begins with the formation of new vessels with very little fibrous tissue component. They arise from primitive mesenchymal elements that differentiate into vascular endothelial cells. The newly formed vascular channels then undergo fibrous metaplasia; that is, the angioblastic buds are transformed into fibrous tissue.

The new vessels leak fluorescein, so the presence of proliferation is especially noticeable during angiography. The new vessels and fibrous tissue break through the internal limiting membrane and arborize at the interface between the internal limiting membrane and the posterior hyaloid membrane. The fibrovascular tissue may form preretinal membranes that create dense adhesions with the posterior hyaloid membrane. These adhesions are extremely important because they are responsible for transmitting the forces of vitreous traction to the retina during the later stage of vitreous shrinkage.

The proliferative stage of PDR is defined as the presence of three or more of the following characteristics: new vessels, new vessels on or within one disc diameter of the optic nerve, severe new vessels (as defined by one-third disc area neovascularization at the
optic nerve or one-half disc area neovascularization at the optic nerve or one-half disc area neovascularization elsewhere), and preretinal or vitreous hemorrhage.

Once diagnosed as entering the proliferative stage, the patient is to receive a full ophthalmic examination to establish a baseline of ocular health. The ophthalmic examination includes indirect ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected visual acuity) symptomatology, fundus photography, fluorescein angiography, electoretinography and A-scan measurements.

Following the preliminary examination, an intravitreal injection of hyaluronidase (ACS) is given to patient's affected eyes. If both eyes are affected, they may be treated separately. The eye is injected with 50 µl of 50 l.u. of the hyaluronidase (ACS) ophthalmic solution intravitreally to promote the depolymerization of vitreous hyaluronic acid, resulting in the liquefaction of the vitreous. In addition to depolymerization of the vitreous, the neovascularized tissue is also treated directly to minimize subsequent damage to the retina using panretinal photocoagulation.

Panretinal photocoagulation (PRP) may be used to treat patients presenting PDR in conjunction with the hyaluronidase (ACS) method of treatment. Panretinal photocoagulation is a form of laser photocoagulation. Currently lasers such as the argon green (614 nm), argon blue-green (488 and 514 nm), krypton red (647 nm), tunable dye, diode and xenon arc lasers, are used for retinal surgery. Laser energy is absorbed predominantly by tissues containing pigment (melanin, xanthophyll, or hemoglobin) producing thermal effects on adjacent structures. Krypton red lasers are the preferred method of treatment, as they are better able to penetrate nuclear sclerotic cataracts and vitreous hemorrhage than the argon lasers, which require more energy to produce equal levels of penetration.

The parameters used during laser retinal surgery may be modified depending on the goal of the photocoagulation. At lower power setting, using longer durations of treatment and producing larger spot sizes, the laser has a coagulative effect on small vessels. Focal laser photocoagulation is used in diabetes to stop leakage of microaneurysms. The laser spot is place directly over the microaneurysm to achieve a slight whitening and closure of the aneurysm. When applied as a grid over an edematous area of retina, the laser may reduce microvascular leakage. At higher energy levels, laser ablation of tissue is possible. Panretinal photocoagulation is thought to be effective by destroying tissue, reducing the
amount of ischemic tissue in the eye. Confluent laser spots may be used over a neovascular membrane to obliterate the abnormal vessels.

It should be understood that the present method does not require a particular order of treatment. In one embodiment, the patient is first treated with hyaluronidase (ACS) and then laser treatment. In another embodiment the patient is first undergoes laser treatment followed by the hyaluronidase (ACS) treatment.

After treatment, the patients' eyes are to be examined on days one (1), two (2), seven (7), fifteen (15), thirty (30) and sixty (60). On each examination day the patient is monitored for vitreous liquefaction. Additionally, the patient is monitored for posterior vitreous detachments using indirect ophthalmoscopy with scleral depression. Finally, the extent of PDR presented by the patient is continuously monitored through periodic retinal examinations and fluorescein angiograms to monitor the extent of venous beading, IRMA, retinal ischemia, neovascularization, and vitreal hemorrhage. Evidence of new neopolymerization or incomplete depolymerization of the vitreous would warrant a repeat treatment of the patient as described above.

EXAMPLE 10

Hyaluronidase Treatment of Age-Related Macular Degeneration

The present methodology also contemplates utility in the treatment of age-related macular degeneration (AMD). Age-related macular degeneration consists of a gradual, often bilateral decrease of vision. It is the most common cause of legal blindness in adults. It is probably caused by aging and vascular disease in the choriocapillaries or the afferent retinal vessels. There are basically two morphologic types of AMD: "dry" and "wet".

The underlying abnormality of AMD is the development of involutional changes at the level of Bruch's membrane and the retinal pigment epithelium (RPE). The hallmark lesion of such changes is the druse. Clinically, drusen (the plural form of druse) appear as small, yellow-white deposits at the level of the RPE. Drusen may be categorized as hard, soft or basal laminar drusen.

The present method is directed both to the treatment and prevention of wet and dry forms of AMD. In the wet form the disease, the condition is thought to affect the choriocapillaries. The choriocapillaries are a component of the choroid which serves to vascularize the globe. The choriocapillaries consists of a rich capillary network that supply most of the nutrition for the pigment epithelium and outer layers of the retina. Damage to
the choriocapillaries is thought to result ultimately in neovascular complications, a cause of macular degeneration.

In the dry form, nondisciform macular degeneration results from a partial or total obliteration of the underlying choriocapillaries. Ophthalmoscopically, degeneration of the retinal pigment epithelium and hole formation may be observed. Also, subpigment epithelial deposits of material such as calcium chelates or proteinaceous material and others may be observed. In dry AMD, secondary retinal changes generally occur gradually, resulting in the gradual loss of visual acuity. Nevertheless, in some percentage of patients, a severe loss of vision results.

The present method contemplates utility in treating dry AMD and preventing macular degeneration through liquefaction of the vitreous. It is contemplated that the liquefaction of the vitreous would result in an increase in the rate of clearance from the retina of deposited material that later results in macular degeneration.

Wet AMD most frequently results from choriocapillary insufficiency, leading to subsequent subpigment epithelial neovascularization. Neovascularization also is thought to occur as an adaptation of retinal vascularization to inadequate oxygenation as a result of vesicular damage. Neovascularization may also cause several other disorders such as detachment of the pigment epithelium and sensory retina. Typically the disease usually begins after 60 years of age, manifesting in both sexes equally and in patients presenting the disease, bilaterally.

Perhaps the most important complication of age-related macular degeneration is the development of defects in Bruch's membranes of the globe through which new vessels grow. This epithelial neovascularization may result in the production of exudative deposits in and under the retina. The neovascularization may also lead to hemorrhage into the vitreous, which may lead to degeneration of the retina's rods and cones, and cystoid macular edema (discussed below). A macular hole may form which results in irreversible visual loss.

Although affecting only 10% of patients with AMD, neovascular complications of AMD account for the overwhelming majority of cases of severe visual loss. Risk factors include increasing age, soft drusen, nongeographic atrophy, family history, hyperopia, and retinal pigment epithelial detachments. Symptoms of choroidal neovascularization in AMD include metamorphopsia, paracentral scotomas or diminished central vision. Ophthalmoscopic findings include subretinal fluid, blood, exudates, RPE detachment,
cystic retinal changes, or the presence of grayish green subretinal neovascular membrane. Fluorescein angiography is often an effective method of diagnosis. During this diagnostic procedure, progressive pooling of the dye in the subretinal space, seen as blurring of the boundaries of the lesion or leakage from undetermined sources are indicators of the disease. Other components of choroidal neovascular membranes as delineated by fluorescein angiography include elevated blocked fluorescence, flat blocked fluorescence, blood, and disciform scar.

The present understanding of neovascular AMD suggests that classic choroidal neovascularization is the lesion component most strongly associated with rapid visual deterioration. Accordingly, treatment of AMD must encompass all neovascular and fibrovascular components of the lesion. At present, treatment is only indicated when classic neovascularization has boundaries that are well demarcated, and photocoagulation has been shown to be beneficial.

In eyes with extrafoveal choroidal neovascularization (>200 microns from the foveal center), argon laser photocoagulation diminished the incidence of severe visual loss, ($6 lines) at 5 years from 64% to 46%. Recurrent neovascularization developed in one-half of laser-treated eyes, usually in the first year after treatment. Recurrent neovascularization was invariably associated with the development of severe visual loss.

In eyes with juxtafoveal choroidal neovascularization (1 to 199 microns from the foveal center), krypton laser photocoagulation diminished the incidence of severe visual loss from 45% to 31% at 1 year, although the difference between untreated and treated groups was less marked at 5 years.

Laser treatment remains an essential therapeutic method for the treatment of AMD, however, the present method would augment this approach by reducing the reoccurrence of neovascularization.

**Treatment of Age-Related Macular Degeneration**

In this Example, a patient manifesting age-related macular degeneration is treated with an intravitreal injection of hyaluronidase (ACS). The purpose of this treatment is to reduce or prevent the development of neovascularization, macular disease, and retinal damage.

Once a patient reaches the age of 60, increased ophthalmic surveillance is performed to detect the presence of AMD. This increased surveillance should include periodic retinal examinations and fluorescein angiograms to monitor for the presence of
subretinal fluid, blood, exudates, RPE detachment, cystic retinal changes, or the presence of grayish green subretinal neovascular membrane.

When AMD is diagnosed, a regime of hyaluronidase (ACS) treatment is commenced coupled with or without other treatments such as photocoagulation. As the first step of treatment, the patient is to receive a full ophthamlic examination to establish a baseline of ocular health. The ophthamlic examination includes indirect ophthammoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected) symptomatology, fundus photography, fluorescein angiography, electroretinography and A-scan measurements.

Following the preliminary examination, an intravitreal injection of hyaluronidase (ACS) is given to the patient's affected eye manifesting AMD. If both eyes are affected, they may be treated separately. The eye to be treated is injected with 50 μl of 50 I.U. of the hyaluronidase (ACS) ophthamlic solution (described above) intravitrealy to promote the depolymerization of vitreous hyaluronic acid, resulting in the liquefaction of the vitreous.

Laser photocoagulation treatment of the hyaluronidase (ACS) injected eyes may be required. The laser treatment protocol described in Examples 8 and 9 should be followed when treating AMD. In an alternative embodiment, photocoagulation treatment occurs before the enzyme treatment of the present method.

After treatment, the patients' eyes are to be examined on days one (1), two (2), seven (7), fifteen (15), thirty (30) and sixty (60). Because of the possibility of reoccurrence, the patient should return for periodic examinations on a monthly basis thereafter. On each examination day the patient is monitored for vitreous liquefaction. Additionally, the patient is monitored for posterior vitreous detachments using indirect ophthammoscopy with scleral depression. Finally, the extent of AMD presented by the patient is continuously monitored through periodic retinal examinations and fluorescein angiograms to monitor for the presence of subretinal fluid, blood, exudates, RPE detachment, cystic retinal changes, or the presence of grayish green subretinal neovascular membrane. Additional hyaluronidase (ACS) and/or laser treatments may be required if indicia of reoccurring neovascularization are observed.

The following Example demonstrates the efficacy of the present method, even without the use of photocoagulation.
Improvement in Symptoms of Macular Degeneration Following Intravitreal Hyaluronidase Infection

A greater than seventy-nine (79) year old male human being presented with a history of age-related macular degeneration, and uncorrected vision of 20:400 in his right eye. A single dose of 100 I.U. of the hyaluronidase (ACS) was injected intravitreally into his right eye. The other eye remained untreated.

The patient was repeatedly examined post-dose and the vision in his left (untreated) eye remained unchanged, while the vision in his right (treated) eye was observed to improve as follows:

<table>
<thead>
<tr>
<th>Time (Post-Dose)</th>
<th>Vision (uncorrected)</th>
<th>Vision (corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>20:400</td>
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</tr>
<tr>
<td>3 days</td>
<td>cf @ 1 ft.</td>
<td>none</td>
</tr>
<tr>
<td>1 wk</td>
<td>20:400</td>
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</tr>
<tr>
<td>2 wk</td>
<td>20:400</td>
<td>none</td>
</tr>
<tr>
<td>4 wk</td>
<td>20:300</td>
<td>none</td>
</tr>
</tbody>
</table>

*cf denotes finger counting

No adverse effects of the hyaluronidase (ACS) were observed in this experiment.

EXAMPLE 11

Hyaluronidase Treatment of Amblyopia

The term amblyopia is derived from Greek and means dull vision (amblys--dull, ops--eye). Poor vision is caused by abnormal development in visual areas of the brain, which is in turn caused by abnormal visual stimulation during early visual development. The pathology associated with amblyopia is not specific to the eye, rather, it is located in the visual areas of the brain including the lateral geniculate nucleus and the striate cortex. This abnormal development is caused by three mechanisms: (1) blurred retinal image called pattern distortion; (2) cortical suppression, or (3) both cortical suppression plus pattern distortion. The present method is primarily concerned with pattern distortions caused by media opacity. More specifically, the present method addresses issues of vitreous opacity.

Amblyopic vision is usually defined as a difference of at least two Snellen lines of visual acuity. Critical to the treatment of amblyopia is early detection and early intervention. The strategy for treating amblyopia caused by vitreous opacity is to provide a clear retinal image by altering the opacity of the vitreous so that clear vision results.

In this Example, a patient manifesting amblyopia resulting from vitreal opacity was treated with an intravitreal injection of hyaluronidase (ACS). The purpose of this treatment
was to reduce the opacity of the vitreous by increasing the exchange rate of the liquid in the vitreous.

A forty (40) year old female human being having a history of amblyopia presented with uncorrected vision of 20:400 in her right eye and corrected vision in that eye of 20:200. A single 100 I.U. dose of the hyaluronidase (ACS) was injected intravitreally into her right eye. The other eye remained untreated.

The patient was examined repeatedly post-dose and the vision in her left (untreated) eye remained unchanged while the vision in her right (treated) eye was observed to improve as follows:

<table>
<thead>
<tr>
<th>Vision (Post-Dose)</th>
<th>Time (uncorrected)</th>
<th>Vision (corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 wk.</td>
<td>20:200</td>
<td>20:70(-1)</td>
</tr>
<tr>
<td>8 wk.</td>
<td>20:200</td>
<td>20:60(-2)</td>
</tr>
<tr>
<td>12 wk.</td>
<td>20:200</td>
<td>20:60(-1)</td>
</tr>
<tr>
<td>52 wk.</td>
<td>20:200</td>
<td>20:60(-1)</td>
</tr>
</tbody>
</table>

No adverse effects of the hyaluronidase (ACS) were observed in this patient.

EXAMPLE 12

Hyaluronidase Treatment of Retinitis Pigmentosa

Retinitis pigmentosa (RP) is the name given to a group of heritable disorders of progressive retinal degeneration characterized by bilateral nyctalopia constricted visual fields and abnormality of the electroretinogram. Early symptoms include difficulty with dark adaptation and midperipheral visual field loss. As the disease progresses, visual field loss advances, typically leaving a small central field of vision until eventually even central vision is affected. Central acuity may also be affected earlier in the course of disease either by cystoid macular edema, macular atrophy, or development of a posterior subcapsular cataract. RP represents a varied group of diseases whose common thread is the abnormal production of at least one protein in photoreceptor outer segments critical to light transduction.

One clinical result of RP is the destabilization of the blood-retinal barrier of the perifoveal capillaries and the optic nerve head. This destabilization results in leakage of fluorescein dye observed by angiography. In addition to leakage, accumulation of fluid as microcysts in the outer plexiform layer may occur and be observed. These fluid filled cysts may eventually burst, resulting in damage to the retinal layer. The present method contemplates treating RP related damage to the retina by promoting the accelerated clearance of the tissue fluid accumulating in the microcysts.
A fifty-nine (59) year old male human being presented with a history of retinitis pigmentosa. The uncorrected vision in his left eye was 20:400 and with correction was also 20:400. A single intravitreal injection of 100 I.U. of the hyaluronidase (ACS) was administered to the left eye of the patient. The other eye remained untreated.

The patient was examined repeatedly following the dose of hyaluronidase (ACS) and the vision in the patient's right (untreated) eye remained unchanged, while the vision in the patient's left (treated) eye was observed to improve as follows:

<table>
<thead>
<tr>
<th>Time</th>
<th>Intraocular Pressure</th>
<th>Unaided Vision Acuity</th>
<th>Best Corrected Vision Acuity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>17 mm</td>
<td>20/400</td>
<td>20/400</td>
</tr>
<tr>
<td>1 Day Post Treatment</td>
<td>26 mm</td>
<td>HM*/1 ft</td>
<td>HM/1 ft</td>
</tr>
<tr>
<td>1 wk.</td>
<td>--</td>
<td>HM/1 ft</td>
<td>HM/1 ft</td>
</tr>
<tr>
<td>2 wk.</td>
<td>14 mm</td>
<td>cf** @ 3 ft.</td>
<td>cf @ 3 ft.</td>
</tr>
<tr>
<td>3 wk.</td>
<td>14 mm</td>
<td>20:300</td>
<td>20:300</td>
</tr>
<tr>
<td>4 wk.</td>
<td>12 mm</td>
<td>20:200</td>
<td>20:80</td>
</tr>
<tr>
<td>8 wk.</td>
<td>15 mm</td>
<td>20:80</td>
<td>20:40</td>
</tr>
<tr>
<td>9 wk.</td>
<td>15 mm</td>
<td>20:60</td>
<td>20:40</td>
</tr>
<tr>
<td>10 wk.</td>
<td>18 mm</td>
<td>20:80</td>
<td>20:40</td>
</tr>
</tbody>
</table>

HM* denotes "hand movement"

cf** denotes "finger counting"

The study results demonstrate that there were significant improvements in the visual performance of the subject, both with respect to Unaided Visual Acuity (improving from 20:400 to 20:80) and Best Corrected Visual Acuity (improving from 20:400 to 20:40). Also, while changes in the intraocular pressure of the subject during the treatment period were observed, the intraocular pressure appeared to return to baseline levels approximately two weeks after the time of injection. Although these results are from a single patient, they appear sufficiently promising to warrant further studies.

EXAMPLE 13

Hyaluronidase Treatment of Macular Holes

A rupture or bursting open of the macula is known as a macular hole. Interestingly, this condition usually occurs in women in their sixth through eighth decades, or after trauma such as lightening injury, solar injury, scleral buckling, or in staphylomatous eyes. Symptoms include metamorphopsia and diminished visual acuity.

Macular hole formation is thought to result from tangential traction across the retinal surface induced by the posterior cortical vitreous with involvement of fluid movement within a posterior vitreous synergetic cavity. The posterior vitreous syneresis
cavity is present in the vast majority of patients presenting macular holes. It is thought that as the posterior vitreal gel retreats from the retinal surface, the resulting gap between the two surfaces creates an area wherein movement of the vitreous humor may negatively interact with the retinal surface. The tangential movement of the vitreous humor within the space of the posterior vitreous synerysis cavity is thought to promote tears of the retinal membrane, resulting in the creation of macular holes.

The present method contemplates the use of hyaluronidase (ACS) to depolymerize the vitreous so as to eliminate the conditions which result in macular hole formation. Upon depolymerization of the vitreous, the posterior vitreous synerysis cavity is eliminated as a result of hyaluronidase (ACS)-mediated reorganization of the vitreous. The elimination of this cavity permits the fluid between the vitreous and the retina to move freely about the vitreal chamber, dispersing any harmful forces that would have otherwise have been directed against the retinal surface.

A patient presenting the early signs of macular hole formation is treated with an intravitreal injection of hyaluronidase (ACS). The patient to be treated presents the various signs of premacular hole formation. These include loss of the foveal depression associated with a yellow foveal spot or ring. The fovea has begun to thin in the region of hole formation and the lesion may obtain a reddish appearance. Fluorescein angiography at this stage may appear normal or show faint hyperfluorescence. The appearance of an eccentric full thickness dehiscence denotes an advanced early stage of the disease. Upon observance of these symptoms hyaluronidase (ACS) treatment is commenced.

The hyaluronidase (ACS) treatment of the present method is commenced when the formation of a macular hole is diagnosed. The patient is to receive a full ophthalamic examination to establish a baseline of ocular health. The ophthalamic examination included indirect ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected) symptomatology, fundus photography, fluorescein angiography, electroretinography and A-scan measurements.

Following the preliminary examination, an intravitreal injection of hyaluronidase (ACS) is given to the patient's affected eye. If both eyes are affected, they may be treated separately. The eye to be treated is injected with 50 µl of 50 I.U. of the hyaluronidase (ACS) ophthalmic solution described above intravitreally to promote the depolymerization of vitreous hyaluronic acid, resulting in the liquefaction of the vitreous.
After treatment, the patients’ eyes are to be examined on days one (1), two (2), seven (7), fifteen (15), thirty (30) and sixty (60). On each examination day the patient is monitored for vitreous liquefaction. Fluorescein angiography, considered a particularly effect method of monitoring the course of the treatment, is also performed. Additionally, the patient is monitored for posterior vitreous detachments using indirect ophthalmoscopy with scleral depression.

EXAMPLE 14

Hyaluronidase Treatment of Macular Exudates

Macular exudates are material that penetrates the blood-retina barrier and seeps through the macula into the vitreal chamber. There are two kinds, soft exudates and hard exudates. The soft exudates are actually not exudates but clusters of ganglion cell axons in the nerve fiber layer that have undergone a bulbous dilation at a site of ischemic damage or infarction. Hard exudates are commonly exuded as a result of microvascular changes in background retinopathy. Hard exudates appear yellow and waxy are often deposited in a circular fashion about the macula. They consist of lipid and proteinaceous material derived from the exudation of serum components from leaking vessels or from the lipid products of degenerating neural elements within the retina. Adsorption of hard exudates is primarily mediated by macrophagic resorption, however, the rate of this process may be slow since exudation often occurs in the outer plexiform layer within the avascular zone of the retina.

The present method is particularly useful in reducing the extent of exudative accumulation resulting from the destabilization of the retinal membrane since hyaluronidase (ACS) depolymerization of the vitreous promotes an increased turn-over rate of the aqueous components of the vitreous.

A patient presenting macular exudates is treated with hyaluronidase (ACS) injection method of treatment. The patient is to receive a full ophthalmic examination to establish a baseline of ocular health. The ophthalmic examination included indirect ophthalmoscopy, slit-lamp biomicroscopy, peripheral retinal examination, intraocular pressure measurements, visual acuity (unaided and best corrected) symptomatology, fundus photography, fluorescein angiography, electroretinography and A-scan measurements.

Following the preliminary examination, an intravitreal injection of hyaluronidase (ACS) is given to the patient’s affected eye. If both eyes are affected, they may be treated separately. The eye to be treated is injected with 50 µl of 50 I.U. of the hyaluronidase
(ACS) ophthalmic solution described above intravitreally to promote the depolymerization of vitreous hyaluronic acid, resulting in the liquefaction of the vitreous.

After treatment, the patients' eyes are to be examined on days one (1), two (2), seven (7), fifteen (15), thirty (30) and sixty (60). On each examination day the patient is monitored for vitreous liquefaction. Fluorescein angiography, considered a particularly effect method of monitoring the course of the treatment, is also performed. Additionally, the patient is monitored for posterior vitreous detachments using indirect ophthalmoscopy with scleral depression.

EXAMPLE 15

Treatment of Cystoid Macular Edema

Cystoid macular edema is a common ocular abnormality resulting form a diverse group of etiologies. Most the causes of this condition stem from a disturbance of the blood-retinal barrier of the perifoveal capillaries and the optic nerve head that result in fluid leakage which accumulates in microcysts of the outer plexiform layer. This region is a relatively thin and under vascularized area of the retina. Clinically, a cystoid macular edema produces a honey-comb appearance when examined with fluorescein angiography. As the edema progresses, the outer plexiform layer may rupture, producing a lamellar hole. The hole may be confined to the inner layer of the retina or it may eventually progress to a complete macular hole.

The present method contemplates the treatment of cystoid macular edema and the prevention of macular hole formation through the hyaluronidase (ACS)-mediated depolymerization of the vitreous.

A patient presenting the indicia of cystoid macular edema is treated with an intravitreal hyaluronidase (ACS) injection as described in Examples 13 and 14.

EXAMPLE 16

Other Pharmacological Uses of Hyaluronidase

Hyaluronidase has been used therapeutically for many years now. Its proven and diverse effects, which occur mainly in intercellular connective tissue, are primarily attributable to the breakdown of hyaluronic acid in the tissue. The therapeutically useful consequences of this action, i.e. reduced viscosity of intercellular cement and increased membrane and vascular permeability, are due to its "spreading effect". The permeability-enhancing effect of hyaluronidase after administration of fluids and/or radiopaque media is of therapeutic significance.
Hyaluronidase - The Spreading Effect:

- Reduces the viscosity of intercellular cement.
- Increases membrane and vascular permeability.

=> Enhances spatial processes and leads to temporal acceleration when injected via intracutaneous, subcutaneous, intradermal, intravenous or intramuscular route.

**Results:** Improves diffusion and penetration of solutions, suspensions and emulsions into the surrounding tissue.

Hyaluronidase accelerates and enhances the absorption of injected drugs (antibiotics, cytostatic agents, local anesthesia, chemotherapeutic agents, antivirals, etc.) by the tissue, even when large volumes of the medications are administered in solution, suspension or emulsion form.

Hyaluronidase has been successfully used in orthopedics, surgery, ophthalmology, internal medicine, oncology, gynecology, dermatology, etc. for many years.

The experimental use of hyaluronidase was tested in numerous areas of medicine. The substance has been administered clinically in various indications and therapies.


Hyaluronic acid is often applied during ophthalmic surgery (e.g., cataract surgery), for example, to keep the anterior chamber of the eye intact or to protect the corneal endothelium during lens implantation. This results in an increase in intraocular pressure.

Measurements have shown that introduction of hyaluronidase in the anterior chamber of the eye can effectively decrease the intraocular pressure postoperatively.

Hyaluronidase was also found to be effective in reducing the intraocular pressure in patients who underwent trabeculectomy for treatment of wide-angle glaucoma (doses of 300 IU were administered as a subconjunctival injection). The authors concluded that hyaluronidase reduced the number of complications and improved the prognosis of trabeculectomy. Hyaluronidase can also be helpful in retro- and peribulbar anesthesia for cataract surgery when used in combination with local anesthetics such as lidocaine and bupivacaine (with or without adrenaline). The effects of hyaluronidase in local anesthesia of the eye were reported back in 1949. Later research studied the effects of the combined use of procaine + adrenaline + hyaluronidase for retrobulbar anesthesia in patients.
Improvement of akinesia of the eye muscles was observed in subjective assessments. The hyaluronidase-induced improvement of the action of anesthesia may be due to the fact that the orbita is an optimal injection site.

In a double-blind comparison of bupivacaine versus lidocaine with and without hyaluronidase in groups of 50 patients each, investigators observed a significantly larger number of patients with complete anesthesia in the hyaluronidase group. It was also found that none of these patients required additional anesthetics, because the induced muscular blockade persisted throughout the entire operation. These findings were unequivocally confirmed in a later study.

The onset of effect of local anesthesia in conjunction with hyaluronidase was investigated in 1990. Investigators found that, in all patients in whom the enzyme supplement was injected together with an anesthetic, the anesthetic effect required for surgery occurred within 5 minutes after the injection. In the control groups, on the other hand, an additional dose of anesthesia was required, and the onset of the anesthetic effect occurred later.

The addition of hyaluronidase to local anesthesia improved the conditions for surgery. Hyaluronidase was found to be highly effective when added to lidocaine and noradrenaline in retrobulbar anesthesia in senile cataract operations.

In one clinical study, the addition of 75 IU of hyaluronidase to 0.75% bupivacaine and 2% mepivacaine lead to improved motor blockade, analgesia and hypotonus of the eyeball.

Studies on the use of hyaluronidase in strabotomies demonstrated that a sufficient anesthetic effect of mepivacaine and lidocaine can be achieved if supplemented with hyaluronidase. Even small volumes of hyaluronidase displayed the desired spreading effect.

The findings for retrobulbar anesthesia generally apply for peribulbar and subconjunctival applications as well. The combination of local anesthetics with hyaluronidase leads to a reliable blockade of the ocular muscles and, thus, to an improvement of operation conditions.

The effect of hyaluronidase on local anesthesia was again studied in another prospective, randomized, controlled, double-blind study. This study also showed that the addition of hyaluronidase can lead to additive effects.
These results were confirmed when 80 patients with senile cataracts were administered 150 IU of hyaluronidase as a supplement to anesthesia. Their results were later confirmed in another group of 70 patients who received 250 IU of hyaluronidase.

Investigators demonstrated that ocular circulatory changes occur during the combined use of lidocaine / bupivacaine and hyaluronidase. They detected significant reduction in the ocular pulsation volume and a significant reduction in intraocular pressure.

Local anesthesia with the addition of 150 IU of hyaluronidase proved to be a reliable alternative for cataract operations.

Regarding peribulbar anesthesia, investigators recommended that a mixture of 2% lidocaine, 0.5% bupivacaine, and 1550 IU hyaluronidase be warmed to body temperature to eliminate symptoms of pain in the target areas. In a separate study, two concentrations of hyaluronidase were used as a supplement to peribulbar anesthesia. Three groups received either 0, 50 or 150 IU of the hyaluronidase supplement, and the onset of effect, analgesia and akinesia were assessed. No statistically significant differences between the 50 IU and 150 IU hyaluronidase groups could be detected. The authors concluded that the addition of hyaluronidase did not lead to any complications.

After comparing the results of peribulbar anesthesia with retrobulbar anesthesia, other authors concluded that the addition of hyaluronidase is useful in both cases, that hyaluronidase is extremely effective in preventing the vitreous body from bulging into the posterior chamber of the eye, and that it significantly reduces the occurrence of vis a tergo.

Hyaluronidase makes is possible for lidocaine and bupivacaine to spread more rapidly within the peribulbar space.

The injection pressure of local anesthesia administered prior to cataract operations was investigated in 50 patients in a double-blind study. The study concluded that significant (sufficient) akinesia of the extraocular muscles can be achieved by administration of 1% etidocaine, 0.5% bupivacaine, and 50 IU hyaluronidase.

**Glaucoma:** Hyaluronidase is useful for treatment of glaucoma or to alleviate intraocular pressure.

**Combination with local anesthetics:** When hyaluronidase was combined with a local anesthetic, e.g., procaine or lidocaine, the onset of effect was quicker, the analgesic region was enlarged, and postoperative pain was significantly reduced.

One study showed that the anesthetized area after subcutaneous injection of a combination of procaine and hyaluronidase was almost twice as large as that of procaine
alone. This study also demonstrated that the duration of anesthesia after administration of combined preparations of hyaluronidase and procaine / adrenaline led to a 6-fold extension of the anesthetic effect.

The effects of bupivacaine plus adrenaline with or without the addition of hyaluronidase on blockade of the axillary plexus brachialis were compared in a double-blind study. The enzyme neither influenced the time of occurrence of anesthesia, nor the occurrence of inadequate blockade of the plexus, nor the level of the plasma bupivacaine level. The study demonstrated that the duration of anesthesia was reduced.

**Orthopedics, diseases of the supportive and locomotive apparatus:** For many years now, hyaluronidase has been successfully used for treatment of various diseases of the supportive and locomotive apparatus, e.g., acute conditions of the synovial sheath, surrounding connective tissue and varied inflammations in these areas (paratendinitis crepitans, humeroscapular periarthritis, humeral epicondylitis, tibial condylitis, radial styloiditis, etc.). Good treatment results can usually be achieved (especially in combination with exercise or physiotherapy) if hyaluronidase therapy is started as early as possible, even if the affected limb cannot be immobilized. Investigators successfully treated patients with acute tendovaginitis crepitans with hyaluronidase in 1968.

Combination therapy with hyaluronidase (300 IU) and Vitamin B12 injections for treatment of peritendinitis and periarthritis humeroscapularis has been reported. The efficacy of hyaluronidase in the treatment of epicondylitis and tendovaginitis was studied in a total of 53 patients. Daily intravenous doses of 1500 to 3000 units of hyaluronidase led to a decrease in symptoms or clear relief from complaints.

The findings from other studies show the same treatment success in paratendinitis, humeral epicondylitis, humeroscapular periarthritis, and radial and ulnar styloiditis; a dose of 3000 IU of hyaluronidase every two days was used in most cases.

Hyaluronidase was successfully used in combination with mephenesin for treatment of arthrosis, primarily in patients with gonarthrosis. The success rate in chronic cases was particularly impressive.

The findings that i.v. administration of hyaluronidase in patients with Bechterew’s disease produced long-lasting effects is of great therapeutic significance. Administration of an average 1500 to 9000 IU of hyaluronidase i.v., every 3 days increased the mobility of patients studied.
Regarding its mode of action, investigators assume that hyaluronidase depolymerizes mucopolysaccharides deposited in the connective tissue matrix during extrasosseous calcification processes that occur due to increased mesenchymal metabolism. If calcium salts are present, they can be depolymerized as long as calcium does not occur as tertiary calcium phosphate. This can restrict the formation of extrasosseous calcification. The improvement of flexibility (e.g., in the affected spinal segment) could be attributed to the presumed depolymerization resulting from the loosening of previously formed connective structures. It is assumed that hyaluronidase is able to influence the composition and structure of the dermis, and that it can promote the re-synthesis of the proteoglycans.

In the treatment of joint stiffness, which often occurs as a complication of supracondylar fractures, prior or simultaneous administration of hyaluronidase can provide the patient quicker relief from symptoms.

**Treatment of malignant diseases:** When used as a supplement to chemotherapy of malignant tumors, hyaluronidase can dissolve hyaluronic acid-containing areas around tumor cells and tumor cell conglomerates, thereby enabling a higher concentration of the cytostatic agent to take effect in the desired target area. Moreover, hyaluronidase may induce a related enhancement of immunological defensive processes, e.g., by creating direct contact between immunocompetent cells ("natural killer cells") and antigens on the tumor surface.

The usefulness of hyaluronidase (i.v., i.m. and intravesical) for treatment of malignant diseases (hematological systemic diseases, carcinomas of the breast, cerebral metastases, glioma, squamous cell carcinomas in the ENT region, adenocarcinomas of the lung and colon, and carcinomas of the bladder) is the subject of various clinical studies. In isolated cases, hyaluronidase was usually found to increase the response rate to cytostatic agents if high doses of the enzyme are administered prior to administration of the cytostatic agent. Hyaluronidase supplements can improve the patient's response to chemotherapy when used in therapy-resistant patients with malignant hematological diseases.

In oncology, hyaluronidase has proven to be particularly useful when administered as a supplement to cytostatic agents like doxorubicin and adriamycin. The enzyme improves the penetration of doxorubicin in the cells and increases the activity of adriamycin in breast cancer.

The combined administration of cisplatin, vindesin, hyaluronidase and radiation therapy for treatment of advanced squamous cell carcinoma in the head and neck region
was found to be highly effective: a high rate of remissions and improved tolerance of cytostatic therapy was observed in carcinoma patients.

Hyaluronidase led to a decrease in adhesion-related multicellular drug resistance in carcinomas of the breast. This mechanism of action is based on the reduction of cell-contact-dependent inhibition of growth and on the sensitization of cells for the cytostatic agents.

**Hyaluronidase in tumor treatment:**

- Hyaluronidase increases the effects of cytostatic agents used for treatment of such malignant diseases as hematological systemic diseases, carcinomas of the breast, cerebral metastases, glioma, squamous cell carcinomas in the ear, nose and throat region, adenocarcinomas of the lung and colon, and carcinomas of the bladder.
- In clinical studies, hyaluronidase was found to induce cessation of growth (remission) of various tumors.
- Therapy-resistant patients respond better to cytostatic agents if an intravesical dose of the enzyme is instilled prior to the cytostatic drug.
- Hyaluronidase can improve the subjective well-being and the quality of life of tumor patients.

**Dermatology:** Investigators have concluded that hyaluronidase is useful in certain dermatological diseases, such as, for example, progressive scleroderma, which is a systemic disorder of the entire vascular connective tissue system, with its most important characteristic being the displacement of collagen fractions. Of pathomechanistic significance is the fact that the histomorphological skin changes that occur in scleroderma begin with a dermal edema rich in acidic mucopolysaccharides (hyaluronic acid, chondroitin sulphate). Histopathological and chemical tests have shown that part of the ground substance occurs as cement in the collagen fibers. It would therefore appear that acidic mucopolysaccharides, soluble collagen, and polymeric collagen are responsible for the sclerosis.

Investigators published further findings on high-dose i.v. hyaluronidase therapy in patients with primary diseases such as keloids and localized scleroderma. The substance achieved good results in both diseases. The same applies for progressive scleroderma, but the increase in active flexibility of the joint was not permanent. High-dose i.v.
administration of hyaluronidase for treatment of progressive scleroderma was therefore recommended only as a supplementary medication, as no influence on older areas of sclerosis could be demonstrated. Assessment of the selected routes of administration showed that local administration of the substance to the affected areas of the skin did not lead to treatment success. Only intravenous injection or infusion achieved, at minimum, improvement of symptoms in most patients.

**Treatment of myocardial infarction:** The use of hyaluronidase for treatment of acute myocardial infarction was first described in 1959. Studies have shown that the administration of hyaluronidase in the acute stage, i.e., in the early stage of fresh myocardial infarction (2 to 4 hours after the onset of infarction) can reduce the size of the necrotic area in the heart.

Investigators studied medications that lead to a reduction in infarction size, e.g., beta blockers, nitrates, calcium antagonists, etc. Hyaluronidase was found to have a favorable effect on concomitantly administered thrombolytic agents such as streptokinase. This appears to be attributable to the ability of hyaluronidase to entrap oxygen radicals.

Statisticians performed meta-analyses in which they studied the role of hyaluronidase and other cardiovascular drugs with a potential for reducing the size of myocardial infarction.

They found that hyaluronidase supplements reduced the mortality rate by 15 to 20 percent.

Investigators also recommended the use of hyaluronidase, in addition to conventional agents for treatment of acute myocardial infarction (nitrates, beta receptor blockers, calcium antagonists).

Despite differences in the data and, in some case, contradictory findings, it appears that the use of hyaluronidase for treatment of myocardial infarction has been definitively proven and confirmed.

**Miscellaneous indications:** Another indication is for treatment of submucosal fibrosis. Experience with 150 patients over a 10-year period has shown that the combination of hyaluronidase and dexamethasone is able to reduce symptoms over a long period of time in most cases.

Local injection of chymotrypsin, hyaluronidase and dexamethasone was also reported to induce good treatment results. Surgical excision was, however, performed in therapy-resistant patients.
326 patients with oral submucosal fibrosis were randomized into 2 groups and treated with either steroids and hyaluronidase or with topical vitamin A steroid and oral iron preparations. Treatment with steroids and hyaluronidase was found to be much less problematic.

Hyaluronidase has also been used in hair transplants and in therapeutic and cosmetic surgery of the scalp. It was found effective in improving the diffusion of local anesthetics.

The use of hyaluronidase for prevention of postoperative adhesions following surgery has also been reported.

In cerebral abscess, hyaluronidase was alternatively combined with dexamethasone or antibiotics, primarily to eliminate edema in high-risk patients.

Hyaluronidase was found to improve the absorption of locally administered drugs and to reduce the risk of progression of skin necrosis in patients treated with intravenously administered Vinca alkaloids.

The use of hyaluronidase as an antidote for the extravasation of chemotherapeutic agents has also been described. Hyaluronidase is one of the few antidotes that can be used as an antidote for Vinca alkaloids or epipodophyllotoxins such as etoposide.

Gynecology is another area of application for hyaluronidase. When injected in the perineal region prior to the expulsive stage of labor, hyaluronidase was found to soften the consistency of the birth canal of first-time mothers, which often eliminated the need for episiotomy.

Hyaluronidase is also useful for facilitation of partial and complete aspiration of viscous joint effusions and pleural effusions, i.e., it liquefies the effusions. The enzyme is also used for treatment of edema of various origins and for treatment of arthritic joint changes.

Hyaluronidase is a treatment for corneaplasty, corneal scars, opacification, and haze, and cornea in need of delamination.

Hyaluronidase can be used as an alternative or adjunct to conventional mechanical vitrectomy.

Hyaluronidase is also useful for the induction of retinal detachments.

Hyaluronidase is indicated as an adjuvant to increase the absorption and dispersion of other injected drugs; for hypodermoclysis; and as an adjunct in subcutaneous urography for improving resorption of radiopaque agents.
Summary: The proven and diverse activity of hyaluronidase occurs mainly in the intercellular connective tissue. This action is clearly attributable to the breakdown of hyaluronic acid in the tissue. The therapeutically useful consequences of this action (reduced viscosity of intercellular cement, increased the permeability of membranes and vessels) occur due to a “spreading effect.”

The hyaluronidase-related enhancement of diffusion and increase in permeability that occurs after administration of liquids and/or radiopaque media is of therapeutic significance. The substance is able to accelerate and increase the absorption of drugs (antibiotics, cytostatics, local anesthetics, etc.) by the tissue, even when large volumes of the medication are injected in solution, suspension or emulsion form. If it is not possible to administer a certain drug intravenously in cases where a rapid onset of effect is necessary, a “pre-injection” of hyaluronidase can accelerate (by 200 to 300%) the absorption of subcutaneous or intramuscular doses of the drug in the bloodstream, which is of particular significance for internal medicine.

The efficacy of hyaluronidase in treatment of disorders of the supportive and locomotive system can be attributed to the so-called “softening effect” of the enzyme.

When administered early and, especially, when coupled with additional exercise and/or physiotherapy measures, the “antiphlogistic” effect of hyaluronidase makes it possible to control acute symptoms involving the synovial sheaths and the surrounding connective tissue (peritendinitis crepitans, humeroscapular periarthritis, humeral epicondylitis, tibial condylitis, radial styloiditis, etc.)

Joint stiffness (e.g., due to supracondylar fracture) can also be treated successfully.

Due to its diffusion potential, hyaluronidase can also be used for treatment of posttraumatic hematomas or edemas of any origin, and for liquefaction of joint and pleural effusions in orthopedics.

Hyaluronidase is indicated to be useful as an anti-edema and anti-inflammatory agent in the prevention of transplant rejection. It has been shown in pre-clinical experiments to lend itself to this role, because it breaks down hyaluronan in damaged tissues. Hyaluronan, a glucosaminoglycan with unique water-binding capacity, draws water into some transplanted organs causing edema. This in turn impairs organ function which may lead to the transplanted organ failure and being rejected. In addition to attracting water, hyaluronan attracts certain cells of the immune system and therefore may be instrumental in initiating inflammatory reactions. Studies have confirmed that
hyaluronidase treatment can be used to reduce edema and inflammation after organ transplantation.

When hyaluronidase is administered prior to administration of a local anesthetic such as procaine ("pre-injection"), the onset of effect of the anesthetic is quicker, the anesthetic region is larger, and the pain after completion of the procedure is significantly lower.

Optimal and efficient combinations of hyaluronidase and local anesthetics are now widely used, particularly in ophthalmology and especially in cataract surgery. The preoperative administration of hyaluronidase with certain local anesthetics (procaine, lidocaine, bupivacaine, etc.) for retro- and peribulbar anesthesia is useful in various ophthalmologic operations. The enzyme accelerates the onset of effect of the anesthetic agent and causes reliable blockade of the eye muscles which, in turn, creates excellent conditions for surgery. In combination with vasopressors such as adrenaline, hyaluronidase increases the duration of anesthesia in the treated area and prevents the rapid diminishment of local anesthesia. Hyaluronidase is also effective for treatment of postoperatively increased internal eye pressure due to the administration of viscoelastic substances such as sodium hyaluronate during ophthalmic surgery.

Hyaluronidase is also widely used in dermatology, i.e., in selected skin disorders involving the connective tissue system and characterized by degeneration of it (scleroderma, keloid formation, psoriasis, chronic varicose ulcer, etc.).

Hyaluronidase is also useful in gynecology, i.e., for prevention of episiotomy.

The usefulness of hyaluronidase has been validated for treatment of myocardial infarction.

The most recent clinical studies have shown that hyaluronidase is helpful as a supplement to chemotherapy in patients with cancer (myeloma, Hodgkin's disease, non-Hodgkin's lymphoma, breast cancer [also with concomitant cerebral metastasis], cerebral lymphomas, gliomas, squamous cell carcinomas in the ear, nose and throat region, and carcinomas of the bladder). The enzyme not only increases the patient's response to the cytostatic agents, but also drastically improves the patient's overall subjective feeling of well-being and the remission rate.

Preliminary evidence of the usefulness of this therapy principal has also been found in colon carcinoma, adenocarcinoma of the lung, bronchial carcinoma, hypernephroma, carcinomas of the stomach, pancreas and ovaries, myelosarcoma, and neurinoma. In
different tumor types, the administration of hyaluronidase was found to induce temporary
cessation of tumor growth, and it improved the response of therapy-resistant patients to the
cytostatic agent. Furthermore, it is assumed that hyaluronidase stimulates the immune
system. Positive findings after administration of the enzyme as a supplement to
chemotherapy of malignant tumors give one reason to expect that hyaluronidase may one
day be able to expand the potentials of anti-tumor chemotherapy and improve the results of
therapy.

From a toxicological point of view and in light of the wide range of available data
on the various applications of hyaluronidase, there does not seem to be any reason to
prohibit the use of the enzyme in humans.

No serious pathological organ changes were detected in an acute toxicity test with
administration of a single dose of hyaluronidase, nor after long-term administration of the
substance.

However, one should always remember that the enzyme is antigenic in nature, when
used alone or mixed with other substances. These effects can never be completely excluded
due to the complicated nature of the process used to isolate the enzyme. Thus, there is a
need for a hyaluronidase preparation suitable for pharmaceutical applications, which need is
met by the disclosed process for isolating and purifying ovine hyaluronidase.

In light of the positive clinical findings on hyaluronidase, one may conclude that
hyaluronidase is a therapeutically versatile enzyme that promises to be a therapeutically
useful agent in new areas of medical practice now and in the future.

**EXAMPLE 17**

**Hyaluronidase for Injection**

In this Example for use of hyaluronidase as a spreading agent, hyaluronidase for
injection dehydrated in the solid state under high vacuum with the inactive ingredients
listed below, is supplied as a sterile, nonpreserved, white, odorless, amorphous solid. The
product is to be reconstituted with Sodium Chloride Injection, USP, before use.

Each vial of 6200 USP units contains 5 mg lactose, 1.92 mg potassium phosphate
dibasic, and 1.22 mg potassium phosphate monobasic.

The USP/NF hyaluronidase unit is equivalent to the turbidity-reducing (TR) unit
and equal to 0.81 International Units (IU).

The reconstituted solution is clear and colorless, with an approximate pH of 6.7 and
osmolality of 290 to 310 mOsm.
Hyaluronidase for injection is to be reconstituted in a vial to a concentration of 1000 Units/mL of Sodium Chloride Injection, USP by adding 6.2 mL of solution to the vial. Prior to administration, the reconstituted solution should be further diluted to the desired concentration, commonly 150 Units/mL, see table below. The resulting solution should be used immediately after preparation.

A 1mL syringe and a 5-micron filter needle are supplied in a hyaluronidase for injection kit. Following reconstitution of hyaluronidase for injection, as described above, apply the 5-micron filter needle to the 1mL syringe. Draw the desired amount of hyaluronidase for injection into the syringe, and dilute according to the table below.

<table>
<thead>
<tr>
<th>Desired Concentration</th>
<th>Amount of hyaluronidase reconstituted solution (1000 Units/mL)</th>
<th>Additional Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 Units/mL</td>
<td>0.05 mL</td>
<td>0.95 mL</td>
</tr>
<tr>
<td>75 Units/mL</td>
<td>0.075 mL</td>
<td>0.925 mL</td>
</tr>
<tr>
<td>150 Units/mL</td>
<td>0.15 mL</td>
<td>0.85 mL</td>
</tr>
<tr>
<td>300 Units/mL</td>
<td>0.3 mL</td>
<td>0.7 mL</td>
</tr>
<tr>
<td>Group</td>
<td>Treatment and Dosage</td>
<td>Results by day 7</td>
</tr>
<tr>
<td>-------</td>
<td>--------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1</td>
<td>BSS</td>
<td>Fundus Photography: Normal; Fluorescein Angiography: Normal; Histology: Normal</td>
</tr>
<tr>
<td>2</td>
<td>BSS + Thimerosal (0.0075 mg)</td>
<td>Fundus Photography: Breakdown of blood-retinal barrier; Fluorescein Angiography:</td>
</tr>
<tr>
<td>3</td>
<td>BSS + Thimerosal (0.025 mg)</td>
<td>Severe retinal effects and intraretinal hemorrhage; Histology: Severe retinal</td>
</tr>
<tr>
<td>4</td>
<td>Hyaluronidase (Wydase®) - 1 I.U.</td>
<td>necrosis; Fluorescein Angiography: Slight fluorescein leakage; Histology: No</td>
</tr>
<tr>
<td>5</td>
<td>Hyaluronidase (Wydase®) - 15 I.U.</td>
<td>significant change; Fluorescein Angiography: Compromised blood-retinal barrier;</td>
</tr>
<tr>
<td>6</td>
<td>Hyaluronidase (Wydase®) - 30 I.U.</td>
<td>Histology: Severe retinal damage</td>
</tr>
<tr>
<td>7</td>
<td>Hyaluronidase (Wydase®) - 50 I.U.</td>
<td>Fundus Photography: Normal; Fluorescein Angiography: Normal; Histology: Normal</td>
</tr>
<tr>
<td>8</td>
<td>Hyaluronidase (ACS) - 1 I.U.</td>
<td>Fundus Photography: Normal; Fluorescein Angiography: Normal; Histology: Normal</td>
</tr>
<tr>
<td>9</td>
<td>Hyaluronidase (ACS) - 15 I.U.</td>
<td>Fundus Photography: Normal; Fluorescein Angiography: Normal; Histology: Normal</td>
</tr>
<tr>
<td>10</td>
<td>Hyaluronidase (ACS) - 30 I.U.</td>
<td>Fundus Photography: Normal; Fluorescein Angiography: Normal; Histology: Normal</td>
</tr>
<tr>
<td>11</td>
<td>Hyaluronidase (ACS) - 50 I.U.</td>
<td>Fundus Photography: Normal; Fluorescein Angiography: Normal; Histology: Normal</td>
</tr>
<tr>
<td>12</td>
<td>BSS - Balanced Salt Solution</td>
<td>Fundus Photography: Normal; Fluorescein Angiography: Normal; Histology: Normal</td>
</tr>
<tr>
<td>13</td>
<td>BSS - Balanced Salt Solution</td>
<td>Fundus Photography: Normal; Fluorescein Angiography: Normal; Histology: Normal</td>
</tr>
</tbody>
</table>
Table 2. Hemorrhage clearing Efficacy of Single-Dose Intravitreal Hyaluronidase (ACS) in the Rabbits

(12 New Zealand rabbits are injected with 10 µl or 100 µl of blood in both eyes intravitreally)

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Rabbits</th>
<th>Eye Treatments</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left Eye</td>
<td>Right Eye</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>BSS</td>
<td>3</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>25 I.U. of Hyaluronidase (ACS)</td>
<td>3</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>50 I.U. of Hyaluronidase (ACS)</td>
<td>3</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>75 I.U. of Hyaluronidase (ACS)</td>
<td>3</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*BSS - Balanced Salt Solution*
<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Rabbits</th>
<th>Eye Treatments</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Left Eye</td>
<td>Right Eye</td>
</tr>
<tr>
<td>A</td>
<td>BSS</td>
<td>None</td>
<td>30 μl of BSS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>25 I.U. of</td>
<td>None</td>
<td>25 I.U. of Hyaluronidase (ACS) in 30 μl</td>
</tr>
<tr>
<td></td>
<td>Hyaluronidase (ACS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>50 I.U. of</td>
<td>None</td>
<td>50 I.U. of Hyaluronidase (ACS) in 30 μl</td>
</tr>
<tr>
<td></td>
<td>Hyaluronidase (ACS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>75 I.U. of</td>
<td>None</td>
<td>75 I.U. of Hyaluronidase (ACS) in 30 μl</td>
</tr>
<tr>
<td></td>
<td>Hyaluronidase (ACS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>Dose of Hyaluronidase (ACS) Injected</td>
<td>Time to Hemorrhage Clearance (Days post Dose)</td>
<td>Subjective Visual Acuity</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
<td>---------------------------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>Female 54 years old</td>
<td>50 L.U.</td>
<td>6 days</td>
<td>Clear vitreous</td>
</tr>
<tr>
<td>Female 65 years old</td>
<td>50 L.U.</td>
<td>16 days</td>
<td>Clear vitreous</td>
</tr>
<tr>
<td>Female 68 years old</td>
<td>50 L.U.</td>
<td>11 days</td>
<td>Clear vitreous</td>
</tr>
<tr>
<td>Female 85 years old</td>
<td>200 L.U.</td>
<td>8 days</td>
<td>Clear vitreous</td>
</tr>
<tr>
<td>Female 60 years old</td>
<td>70 L.U.</td>
<td>7 days</td>
<td>Clear vitreous</td>
</tr>
<tr>
<td>Male 25 years old</td>
<td>50 L.U.</td>
<td>14 days</td>
<td>Clear vitreous</td>
</tr>
</tbody>
</table>

Table 4. Hemorrhage Clearing Efficacy of Single Intravitreal Injection of Hyaluronidase (ACS) in Human Patients with Diabetic Retinopathy.
It will be appreciated by those skilled in the art that the invention has been described hereabove with reference to certain presently preferred embodiments and examples only, and no effort has been made to exhaustively describe all embodiments in which the invention may take physical form or be practiced. Indeed, various modifications may be made to the specific embodiments and examples described here above, without departing from the intended spirit and scope of the present invention. Accordingly, it is intended that all such reasonable modifications to the above be included within the scope of the following claims.
WHAT IS CLAIMED IS:

1. A composition of matter comprising a hyaluronidase purified from ovine testes and capable of hydrolyzing mucopolysaccharides of the type hyaluronic acid; and having a molecular weight based on mobility in 4-20% gradient SDS polyacrylamide gel of 70-74 kDa for the α-form and 60-63 kDa for the β-form; and having a specific activity ranging from $1.2 \times 10^4$ and $1.9 \times 10^4$ USP Units per mg protein; and no thimerosal.

2. A method for preparing a purified mammalian hyaluronidase comprising:
   a) providing a testicular source;
   b) extracting the testicular source, whereby a crude testicular extract is prepared;
   c) precipitating the crude testicular extract to form a precipitate that is suspended to form a crude testicular suspension;
   d) dialyzing the crude testicular suspension using food-grade dialysis reagents, whereby a crude dialysate is prepared;
   e) submitting the crude dialysate to ion exchange chromatography, whereby a crude eluate is isolated;
   f) lyophilizing the crude eluate and forming a crude lyophilizate;
   g) suspending the crude lyophilizate, whereby a crude preparation is produced;
   h) submitting the crude preparation to ion exchange chromatography, whereby a purified eluate is isolated;
   i) dialyzing the purified eluate with food-grade dialysis reagents, whereby a purified dialysate is prepared; and
   j) subjecting the purified dialysate to viral filtration, whereby a purified mammalian hyaluronidase is prepared.

3. A product made by the process of Claim 2.

4. A method for accelerating the clearance of hemorrhagic blood from the vitreous humor of a mammalian eye, said method comprising contacting with the vitreous humor an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to accelerate the clearance of hemorrhagic blood from said vitreous humor.

5. A method for inducing liquefaction of a vitreous humor to treat a disorder of a mammalian eye, said method comprising contacting with said vitreous humor of said
mammalian eye an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to liquefy said vitreous humor whereby said disorder is treated.

6. The method of Claim 5, wherein said method is carried out for the purpose of treating nonproliferative diabetic retinopathy.

7. The method of Claim 5, wherein said method is carried out for the purpose of treating preproliferative diabetic retinopathy.

8. The method of Claim 5, wherein said method is carried out for the purpose of treating proliferative diabetic retinopathy.

9. The method of Claim 5, where in said method is carried out for the purpose of treating age-related macular degeneration.

10. The method of Claim 5, wherein said method is carried out for the purpose of treating amblyopia.

11. The method of Claim 5, wherein said method is carried out for the purpose of treating retinitis pigmentosa.

12. The method of Claim 5, wherein said method is carried out for the purpose of treating macular holes.

13. The method of Claim 5, wherein said method is carried out for the purpose of treating macular exudates.

14. The method of Claim 5, wherein said method is carried out for the purpose of treating cystoid macular edema.

15. The method of Claim 5, wherein said liquefaction achieves posterior vitreal detachment (PVD).

16. A method for treating glaucoma comprising administering an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to treat glaucoma.

17. A method for treating malignant disease comprising administering an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to treat malignant disease.

18. A method for treating myocardial infarction comprising administering an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to treat myocardial infarction.
19. A method for reducing edema or inflammation after organ transplantation comprising administering an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to reduce edema or inflammation after organ transplantation.

20. A method for treating corneal scar, opacification, or haze comprising administering an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to treat said corneal scar, opacification, or haze.

21. A method for enhancing diffusion of a radiopaque media comprising administering an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to enhance the diffusion of a radiopaque media.

22. A method for increasing the absorption and dispersion of an injected drug comprising administering an amount of the hyaluronidase of Claim 1 to provide a dose of at least 1 International Unit effective to increase the absorption and dispersion of said injected drug.

23. The composition of matter of Claim 1 in a solution form further comprising lactose and phosphate.

24. The composition of matter of Claim 1 in a lyophilized form.
MBR1300 Hyaluronidase 15/85 Precipitate (Step 1)

Prepare 2 x 340L 170mM Acetic Acid/11mM HCL/1mM EDTA (Vessel A & B)

Prepare 300L 170mM Acetic Acid/11mM HCL/EDTA/15%AS
(5 Days Expiry at 2–8°C)

Thaw 6 x 25Kg (150Kg) Ovine Testes at 2–8°C for 40–44hr

Ovine Testes Thaw 40–44hr at 2–8°C

Mince 3x25Kg Ovine Testes into Vessel A

Buffer Temp at 2–8°C

Mince 3x25Kg Ovine Testes into Vessel B

Stir with Lightnin Mixer for 3–4mins at 40Hz

Add 11.4Kg Celite 545AW to each Vessel and Stir in with Paddle

Stir at Room Temp with Lightnin Mixer for 3–4mins at 40Hz

Sieve Extracted Suspensions in Vessel A & B through 2 Mono Nylon Filter Cloth Nets (1600µm) in Half Coffin Vacuum Filter. Collect into 2 x 500L Polypropylene Vessels by Pump

Label as (Sieved Extract) (Vessel A & B)

Rinse Filter with 10L Purified Water and Add to Vessels

Add Celite 545AW (90g/L) to each Vessel and Stir in with Paddle

Stir at Room Temp with Lightnin Mixer for 2–10mins at 40Hz

Add Ammonium Sulphate (85g/L) over 1–5mins to Precipitate 0/15 each Vessel

15% Precipitation pH = 3.60 ±0.10

Stir with Lightnin Mixer for 35–45mins (Speed Hz determined by volume)

FIG. 1

Measure pH 3.60 ±0.10
MBR1300 Hyaluronidase 15/85 Precipitate (Step 1)

1. Prepare Cellite 545AW
2. Prepare 2 Large Coffin Vacuum Filters with Cellite 545AW Beds
3. Filter on 2 Large Coffin Vacuum Filters
4. Pump contents into 500L Polypropylene Vessels and Hold at 2-8°C
5. Filtration Time <5hr Temp<15°C
6. Wash each Filter Bed with 2x75L of 170mM Acetic Acid/11mM HCL/1mM EDTA/15%AS and Add to Vessels (Total Filtration Time<5hr)
7. Buffer Temp at 2-8°C
8. Measure and adjust pH 3.60 ±0.10
9. IPC1 Clarified 15% Vessel A & B Hyaluronidase Activity A280, Specific Activity Total Units (MU)
10. Clarified 15% Temp <15°C
11. Label as Clarified 15% (Vessel C)
12. Pump Approx One Third of Vessel A & B into Vessel C
13. Add Ammonium Sulphate (510g/L) over 5-10mins to Precipitate 15/85 each Vessel
14. Stir with Lightnin Mixer for 35-45mins (Speed Hz determined by volume)
15. Prepare Celpure P1000
16. Prepare 1 Large Coffin Vacuum Filter with Celpure P1000 Bed
17. Label as 15/85 Suspension (Vessel A, B, C)
18. Hold at 2-8°C for 17-24hrs
19. Filter 15/85 Suspension on Large Coffin Vacuum Filter at 2-8°C for 22-26hr
20. 15/85% Precipitation pH = 3.60 ±0.10
21. Label as 15/85 Precipitate
22. Collect 15/85 Precipitate from Filter into Polyethylene Bags and Weigh
23. Store at -10°C to -25°C

FIG. 2
Prepare 50mM Sodium Acetate/pH3.9/2mM EDTA (10 x Precipitate wt)

Prepare 150L 50mM Sodium Acetate/pH3.9/2mM EDTA/35%AS

(5 Days Expiry at 2–8°C)

Thaw 3 x 15/85 Precipitates for 20–24hr at 2–8°C

Add Precipitates to 50mM Sodium Acetate/pH3.9/2mM EDTA (10 x wt)

Buffer Temp at 2–8°C

Stir at Room Temp with Lightnin Mixer for 45–55mins
(Speed Hz determined by volume)

Label as Dissolved 15/85 Precipitate

Add Ammonium Sulphate over 1–5mins to precipitate to 35% (209g/L)

35% Suspension pH = 4.10 ± 0.20

Label as 35% Suspension

Stir with Lightnin Mixer for 35–45mins
(Speed Hz determined by volume)

Measure pH 4.10 ± 0.20

Add Celpure P1000 (29g/L) and Stir in With Paddle

Prepare Celpure P1000

Stir with Lightnin Mixer for 2–10mins at 40 Hz

Prepare 1 Large Coffin Vacuum Filter with Celpure P1000 Bed at 2–8°C

Filter on 1 Large Coffin Vacuum Filter at 2–8°C

Wash Bed with 2 x 80L of Prepare 150L 50mM Sodium Acetate/pH3.9/2mM EDTA/35%AS

Buffer Temp at 2–8°C

FIG. 3
MBR1308 Hyaluronidase 35/85 Precipitate (Step 2)

- Label as Clarified 35% (Vessel A & B)
- Pump Filtrate into Two 500L Polypropylene Vessels
- Measure and adjust pH 4.10 ±0.20
- Clarified 35% Vessels A & B
  Hyaluronidase Activity A280, Specific Activity
- Add Ammonium Sulphate over 1–5min to 85% Concentration (370g/L)
- Stir with Lightnin Mixer for 35–45mins
  (Speed Hz determined by volume)
- Label as 35/85 Suspension (Vessel A & B)
- Prepare Celpure P1000
- Prepare 1 Large Coffin Vacuum Filter with Celpure P1000 Bed at 2–8°C
- Filter on 1 Large Coffin Vacuum Filter at 2–8°C
  Re-filter the first 100L
  (Total Filtration Time 16–20hr)
- Label as 35/85 Precipitate
- Collect Precipitate into Polyethylene Bags and Weigh
- Store at −10°C to −25°C

FIG. 4
**MBR1301 Hyaluronidase HY05A (Step 3)**

1. **MBR1316**
   - Prepare 20mM Potassium Phosphate/1mM EDTA/pH6.0
   - (7 Days Expiry at 2–8°C)

2. Measure pH
   - 6.00 ±0.10

3. Thaw 35/65 Hyaluronidase Precipitate Overnight at 2–8°C for 12–24hr

4. Suspend Precipitate in Minimum Vol Buffer (3–8L)

5. Buffer Temp at 2–8°C

6. Stir with Lightnin Mixer for 30–45mins at 2–8°C
   - (Speed Hz determined by volume)

7. Prepare C110 Dialysis Tubing, 7m lengths
   - (Maximum 8L Slurry per Tube)

8. Dialyse in C110 Dialysis Tubing against 10 x 125L 20mM Potassium Phosphate/1mM EDTA/pH6.0 at 2–8°C over 3 days

9. Buffer Temp at 2–8°C

10. Measure & Adjust pH
    - 6.00 ±0.10

11. On Day 4 Remove from Dialysis Tubing. Rinse Each Tube with 1000ml Buffer

12. Buffer Temp at 2–8°C

13. Measure & Adjust pH
    - 6.00 ±0.10

14. Prepare Celpure P1000

15. Prepare 50cm Vacuum Filter with Celpure P1000 Bed

16. Label as HY05A Ex Dialysis

17. At 2–8°C Filter on 50cm Vacuum Filter. Wash Bed with 2 x 5L Buffer. Collect Filtrate into Polypropylene Vessel

18. Label as HY05A Clarified Ex Dialysis

19. Measure & Adjust pH
    - 4.40 – 4.80

20. IPC3
    - Clarified Ex Dialysis Hyaluronidase Activity A280, Specific Activity
MBR1301 Hyaluronidase HY05A (Step 3)

- **Label as HY05A 40L Fraction**
- **Collect 40L Fraction at 2-8°C**
- **Collect 3 x 10L Fractions at 2-8°C Clamp Column Overnight at 2-8°C**
- **Collect 12 x 10L Fractions at 2-8°C**

**IPC6**
- 40L Fraction Hyaluronidase Activity
- Total Units (MU)
- ≤10% of Charge

**IPC7**
- Fraction 1, 2, 3 etc.
- Hyaluronidase Activity
- Total Units (MU)
- A280
- USP U/A280
- U/ml

**MBR1317**
- Elute column with 100L of 20mM Potassium Phosphate/1mM EDTA/ pH 6.0/2M NaCl at 2-8°C
- Regenerate Resin with 150L of 0.1M NaOH and 500L 0.01M NaOH

**Select Fraction to Give Calculated USP U/A280 of 1980.**
- (First Frx USP U/A280 > 1860)
- (Last Frx USP U/A280 > 750)

**Label as HY05A Combined "A" Fraction**
**Combine Selected Fractions**

**IPC8**
- Combined A Fraction
- Hyaluronidase Activity
- Total Units (MU)
- % of Charge
- USP U/A280 > 1960

**Measure pH**
Add Ammonium Sulphate over 1–5mins to a Concentration of 85% (610g/L)

Stir with Lightnin Mixer Until Dissolution and then for 35–45mins (Speed Hz determined by volume)

Label as HY05A Fraction "A" 0/85

Prepare Celpure P300

Prepare 2 x 50cm Vacuum Filters with Celpure P300 Bed

Filter on 2 x 50cm Vacuum Filters and Dry 16–20 hours at 2–8°C

Label as HY05A 0/85 Precipitate

Collect the 0/85 Precipitate and Weigh

Store Overnight at 2–8°C

Add Buffer (7 x w/v)

Buffer Temp at 2–8°C

Measure and Adjust pH 6.00 ±0.10

Stir with Heidolph Bench Stirrer for 45–55mins at 2–8°C (Speed Hz determined by volume)

Label as HY05A Dissolved 0/85 Precipitate

FIG. 6B
MBR1301 Hyaluronidase HY05A (Step3)

- Prepare Celpure P300
- Prepare 24cm Vacuum Filter with Celpure P300 Bed
- Filter on 24cm Vacuum Filter at 2-8°C and Collected Material
- Wash Filter with Calculated Volume of Buffer (to give AS Entrainment of 5%) and Combine with Filtrate
- Measure pH 4.50 - 5.50 Adjust if Necessary
- Add PEG6000 over 1-5min to Precipitate to 20% PEG at 2-8°C
- Label as HY05A 20% PEG Suspension
- Stir with Heldolph Bench Stirrer for 40-60 mins at 2-8°C (Speed Hz determined by volume)
- Transfer Vessel to Secondary Vessel. Pack Ice Packs Around Primary Vessel. Store at 2-8°C for 12-24hr
- Decant 20% Suspension into Polypropylene Bottles and Weigh

FIG. 7A
Label as HY05A
20% PEG Precipitate

Leave 20% Precipitate in Vessel

Centrifuge 20% Suspension at 10,000rpm for 25±1min at 0°C

Discard Supernatant
Store Precipitates for 12–24hr at 2–8°C

Combine Precipitates and Add Buffer to Give a Hyaluronidase Activity of 250,000 USP U/ml

Stir with Heidolph Bench Stirrer until visually dissolved. Stir for a Further 20–25mins at 2–8°C (Speed Hz determined by volume)

Add 12.2g of Celpure P300

Stir with Heidolph Bench Stirrer for 2–10mins at 2–8°C (Speed Hz determined by volume)

Buffer Temp at 2–8°C

FIG. 7B
**MBR1301 Hyaluronidase HY05A (Step 3)**

- Prepare Celpure P300
- Prepare 11 cm Vacuum Filter with Celpure P300 Bed
- Label as HY05A Clarified Dissolved 20%Pprecipitate
- Filter on 11 cm Vacuum Filter at 2-8°C
- Wash Filter with 300 ml Buffer and add to Filtered Material
- Buffer at 2-8°C
- Measure pH
- IPC11
  - Clarified Dissolved 20% Precipitate
  - Hyaluronidase Activity, A280, Specific Activity
- Store Material for 12-24 hr at 2-8°C
- MBR1316 Prepare 1M K$_2$HPO$_4$
- Measure and Adjust pH 6.00 ± 0.10
- Filter Through a 0.2μm Gelman Filter. Wash Filter with Buffer to Give an Hyaluronidase Activity of 186,000 USP U/ml
- Label as HY05A Pre Freeze Dried
- Buffer at 2-8°C
IPC12

Pre-Freeze Dried Hyaluronidase Activity, A280, Specific Activity

Pre-Freeze Dried pH = 6.00 ± 0.10

Final Product Testing
Hyaluronidase Activity
A280, Specific Activity
Appearance
Solubility
pH

Pool and Weight Freeze Dried Material into 3L Sterile Bag in Humidity Control Chamber

Transfer to Second Bag and Seal, Transfer to Secondary Keg with Desiccant Packs and Seal

Store at -10°C to -25°C

Label as Hyaluronidase HUGA

Label as Hyaluronidase HUGA (Sampled for QC)

Divide Material equally into 2L Flasks and Shell Freeze

Freeze Dry for 72–90 hr Vacuum ≤250 x 10^-3 mbar Temperature ≤ -36°C

Remove Flasks Under Nitrogen and Transfer to Humidity Control Chamber, ≤5% RH
at 2–8°C, Wash Column with Maximum of Column Wash Buffer at 3±0.3L/hr by Pump (Max 35L or A280 <0.2 after 25L)

Buffer at 2–8°C

Measure and Adjust pH 6.00 ±0.10

Label as HY06A 250nM Wash Fractions 5L etc

At 2–8°C, Collect 5 X 5L Fractions and 5 X 2L Fractions at 3±0.3L/hr by Pump

IPC14

250mM Wash Fractions Hyaluronidase Activity A280

Clamp Column Overnight at 2–8°C

Elute Column with 30L of Column Elute Buffer at 3±0.3L/hr by Pump

Buffer at 2–8°C

Measure and Adjust pH 6.00 ±0.10

FIG. 9B
MBR1315 Hyaluronidase HY06A (Stage 4)

1. Add Ammonium Sulphate to a Concentration of 85% (610g/L) over 1-5 mins
2. Label as HY06A Fraction "A" 85% A8
3. Stir with Lightnin Mixer for 35-45 mins (Speed Hz determined by volume)
4. Prepare Colpure 300
5. Prepare 24cm Vacuum Filter with Colpure 300 Bed
6. Filter on 24cm Vacuum Filter at 2-8°C Re-filter the First 5L Twice
7. Label as HY06A 0/85 Precipitate
8. Collect the 0/85 Precipitate and Weigh
9. Process Same Day or Store Overnight at 2-8°C
10. Suspend in 0.3-0.5L Buffer
11. Stir with Heidolph Bench Stirrer for 30-40 mins at 2-8°C (Speed Hz determined by volume)
12. Buffer Temp at 2-8°C
13. Measure and Adjust pH 6.00 ±0.10

FIG. 10A
Prepare C40 Dialysis Tubing

Dialyse in C40 Dialysis Tubing against 5 x 125L 20mM Potassium Phosphate/1mM EDTA/pH 6.0 at 2-8°C over 3 days

Test for Presence of Ammonium ions (Nassar test)

On Day 4, remove from Dialysis. Rinse each tube with 2x25mL Buffer and combine with bulk material. Stir with stirring rod.

Dialyse to remove Ammonium ion

Measure and Adjust pH 6.00 ±0.10

Buffer Temp at 2-8°C

PC17A

FIG. 10B
MBR1315 Hyaluronidase HY06A (Stage 4)

- Filter Material Through 0.2μm Sartolab P Filter via Pump at 50 - 100 ml/min
- Label as HY06A HY06A Pre Freeze Dried
- Wash Filter with Buffer (Volume determined by Hyaluronidase Activity U/ml)
- Filter Material Through Asahi 20N Planova Virus Removal Filter
- Post Use Leakage/Integrity Test
- Pre-Use Leakage Test

Buffer Temp at 2-8°C
- Measure and Adjust pH 6.00 ±0.10
- pH of Pre Freeze-Dried 6.00 ±0.10

IPC19
- Pre Freeze-Dried Hyaluronidase Activity A280, Specific Activity

FIG. 11A
FIG. 11B

Divide Material equally into 1.2L Flasks and Freeze Dry

Freeze Dry for 72–90hr
Vacuum ≤250 x 10⁻³ mBar
Temperature ≤−36°C

Remove Flasks Under Nitrogen and Transfer to Humidity Control Chamber at ≤5% RH

Label as Hyaluronidase HY06A

Pool and Weigh Freeze Dried Material into Sterile Polypropylene Bottle in Humidity Control Chamber

Transfer to Secondary Keg with Desiccant Packs and Seal

Store at −15°C to −25°C

Final Product QC Testing

Hyaluronidase Activity
Appearance
pH
Solubility
Microbial Limit
Endotoxin
ISTA Pharmaceuticals
Biozyme Laboratories, Ltd.
Craig, William S.
Chesham, John

PROcedures for isolating and purifying ovine hyaluronidase

US 60/463,516
2003-04-15
1

FastSEQ for Windows Version 4.0

Leu Asp Phe Arg Ala Pro Pro Leu Ile Ser Asn Thr Ser Phe Leu Trp
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Ala Trp Asn Ala Pro Ala Glu Arg Cys Val Lys Ile Phe Lys Leu Pro
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Pro His Ile Asp Glu Lys Thr Gly Asn Thr Val Tyr Gly Ile Gly Ile Pro
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Ala Tyr Tyr Ile Pro Asn Asp Ser Val Gly Leu Ala Val Ile Asp Trp
100 105 110
Glu Asn Trp Arg Pro Thr Trp Ala Arg Asn Trp Lys Pro Lys Asp Val
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Tyr Arg Asp Glu Ser Val Glu Leu Val Leu Glu Asp Pro Gln Leu
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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/26 C12N15/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, Sequence Search, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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X Further documents are listed in the continuation of box C.

| X | Patent family members are listed in annex. |

* Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search
23 August 2004

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Paletlaan 2 NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040, Tx: 31 651 epo nl, Fax: (+31-70) 340-3016

Date of mailing of the international search report
06/09/2004

Authorized officer
Young, C

Form PCT/ISA/210 (second sheet) (January 2004)
## INTERNATIONAL SEARCH REPORT

### C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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INTERNATIONAL SEARCH REPORT

Box No. 1  Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material
      □  a sequence listing
      □  table(s) related to the sequence listing

   b. format of material
      □  in written format
      □  in computer readable form

   c. time of filing/furnishing
      □  contained in the international application as filed
      □  filed together with the international application in computer readable form
      □  furnished subsequently to this Authority for the purpose of search

2. □ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: