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**Description**

This is a continuation-in-part of U.S. Serial No. 10/290,908 filed November 7, 2002 .

**FIELD OF THE INVENTION**

The present invention is in the field of clinical medicine, biochemistry and molecular biology. The present invention features therapeutics and methods for treating mucopolysaccharidosis VI as well as production and purification procedures for producing such therapeutics.

**BACKGROUND OF THE INVENTION**

MPS VI (Maroteaux-Lamy syndrome) is a lysosomal storage disease in which the affected patients lack the enzyme *N*-acetylgalactosamine-4-sulfatase (ASB). The enzyme metabolizes the sulfate moiety of glycosaminoglycan (GAG) dermatan sulfate (Neufeld, et al., "The mucopolysaccharidoses" The Metabolic Basis of Inherited Disease, eds. Scriver et al., New York:McGraw-Hill, 1989, p. 1565-1587). In the absence of the enzyme, the stepwise degradation of dermatan sulfate is blocked and the substrate accumulates intracellularly in the lysosome in a wide range of tissues. The accumulation causes a progressive disorder with multiple organ and tissue involvement in which the infant appears normal at birth, but usually dies before puberty. The diagnosis of MPS VI is usually made at 6-24 months of age when children show progressive deceleration of growth, enlarged liver and spleen, skeletal deformities, coarse facial features, upper airway obstruction, and joint deformities. Progressive clouding of the cornea, communicating hydrocephalus, or heart disease may develop in MPS VI children. Death usually results from respiratory infection or cardiac disease. Distinct from MPS I, MPS VI is not typically associated with progressive impairment of mental status, although physical limitations may impact learning and development. Although most MPS VI patients have the severe form of the disease that is usually fatal by the teenage years, affected patients with a less severe form of the disease have been described which may survive for decades.

Several publications provide estimates of MPS VI incidence. A 1990 British Columbia survey of all births between 1952 and 1986 published by Lowry *et al* (Lowry, et al., Human Genet 85:389-390 (1990)) estimates an incidence of just 1:1,300,000. An Australian survey (Meikle et al., JAMA 281(3):249-54) of births between 1980-1996 found 18 patients for an incidence of 1:248,000. A survey in Northern Ireland (Nelson, et al., Hum. Genet. 101:355-358 (1997)) estimated an incidence of 1:840,000. Finally, a survey from The Netherlands from 1970-1996 calculated a birth prevalence of 0.24 per 100,000 (Poorthuis, et al., Hum. Genet. 105:151-156 (1999)). Based on these surveys, it is estimated that there are between 50 and 300 patients in the U.S. who are diagnosed with all forms of this syndrome.

There is no satisfactory treatment for MPS VI although a few patients have benefited from bone marrow transplantation (BMT) (Krivit, et al., N. Engl. J. Med. 311(25):1606-11 (1984); Krivit, et al., Int. Pediat. 7:47-52 (1992)). BMT is not universally available for lack of a suitable donor and is associated with substantial morbidity and mortality. The European Group for Bone Marrow Transplantation reported transplant-related mortality of 10% (HLA identical) to 20-25% (HLA mismatched) for 63 transplantation cases of lysosomal disorders (Hoogerbrugge, et al., Lancet 345: 1398-1402 (1995)). Other than BMT, most patients receive symptomatic treatment for specific problems as their only form of care. It is an object of the present invention to provide enzyme replacement therapy with recombinant human *N*-acetylgalactosamine-4-sulfatase (rhASB). No attempts to treat humans with rhASB have been made. Likewise, no acceptable clinical dosages or medical formulations have been provided. Several enzyme replacement trials in the feline MPS VI model have been conducted.

**SUMMARY OF THE INVENTION**

The present invention encompasses the production, purification, and the use of a composition comprising a highly purified *N*-acetylgalactosamine-4-sulfatase in the precursor form. The DNA and encoded amino acid sequence of the precursor form is set forth in SEQ ID NOS: 1 and 2, respectively. The signal sequence is predicted to be amino acids 1-38 of SEQ ID NO: 2, and recombinant production has resulted in product commencing at either amino acid residue 39 or 40 of SEQ ID NO: 2.

In a first aspect, the present invention features novel compositions for use in treating diseases caused all or in part by a deficiency in *N*-acetylgalactosamine-4-sulfatase (ASB). A use comprises administering an effective amount of a pharmaceutical composition to a subject in need of such treatment. In the preferred embodiment, the pharmaceutical composition comprises highly purified *N*-acetylgalactosamine-4-sulfatase in the precursor form, or a biologically active fragment, mutant or analog thereof alone or in combination with a pharmaceutically suitable carrier. The subject suffers from a disease caused all or in part by a deficiency of *N*-acetylgalactosamine-4-sulfatase. In other embodiments, this method features transferring a nucleic acid encoding all or a part of an *N*-acetylgalactosamine-4-sulfatase (ASB) or a biologically active mutant or analog thereof into one or more host cells *in vivo*. Preferred embodiments include optimizing the dosage to the needs of the organism to be treated, preferably mammals or humans, to effectively ameliorate the disease symptoms. In preferred embodiments the disease is mucopolysaccharidosis VI (MPS VI) or Maroteaux-Lamy syndrome.

This first aspect of the invention specifically provides a composition comprising precursor human *N*-acetylgalactosamine-4-sulfatase, wherein said precursor human *N*-acetylgalactosamine-4-sulfatase has a purity of at least equal to or greater than 99% based on total protein, wherein said purity is measured using the reverse-phase HPLC (RP-HPLC) method, and wherein the composition is free of detectable bands of about 47-48 kDa upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie stain, optionally wherein said precursor human *N*-acetylgalactosamine-4-sulfatase is a recombinant precursor *N*-acetylgalactosamine-4-sulfatase. Thus, the invention contemplates use of a composition of the present invention in preparation of a medicament for the treatment of a deficiency in ASB activity, as well as a pharmaceutical composition containing a composition of the present invention for use in treating a deficiency in ASB activity. The deficiency in ASB activity can be observed, e.g., as activity levels of 50% or less, 25% or less, or 10% or less compared to normal levels of ASB activity and can manifest as a mucopolysaccharidosis, for example mucopolysaccharidosis VI (MPS VI) or Maroteaux-Lamy syndrome. The therapeutically effective amount is an amount sufficient to provide a beneficial effect in the human patient and preferably provides improvements in any one of the following: joint mobility, pain, or stiffness, either subjectively or objectively; exercise tolerance or endurance, for example, as measured by walking or climbing ability; pulmonary function, for example, as measured by FVC, FEV<sub>1</sub> or FET; visual acuity; or activities of daily living, for example, as measured by ability to stand up from sitting, pull clothes on or off, or pick up small objects. The human ASB is preferably administered as a highly purified recombinant preparation as described herein. Preferred preparations contain rhASB with a purity greater than 99%, 99.2%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9%, as measured by reverse-phase HPLC. Preferred preparations also contain the precursor form of ASB at high purity, so that processed forms of ASB are not detected on Coomassie-stained SDS-PAGE. Most preferably the purity of the precursor form of ASB is greater than 99%, 99.2%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9%, as measured by size exclusion chromatography (SEC)-HPLC. The human ASB is also preferably formulated with a

surfactant or non-ionic detergent as described herein, optionally excluding a formulation with polyoxyethylene sorbitan 20 or 80 at 0.005%.

Administration of rhASB at doses below 1 mg/kg per week, e.g. at 0.2 mg/kg per week, has been found to have a beneficial effect. The invention contemplates doses of at least 0.1 mg/kg, 0.2 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, or 1.5 mg/kg per week, and may range up to 2 mg/kg, 4 mg/kg, 5 mg/kg or higher per week. The preferred dose is 1 mg/kg/week. Such doses are preferably delivered once weekly but optionally may be divided in equal amounts over more frequent time periods such as biweekly or daily. A variety of parenteral or nonparenteral routes of administration, including oral, transdermal, transmucosal, intrapulmonary (including aerosolized), intramuscular, subcutaneous, or intravenous that deliver equivalent dosages are contemplated. Administration by bolus injection or infusion directly into the joints or CSF is also specifically contemplated, such as intrathecal, intracerebral, intraventricular, via lumbar puncture, or via the cisterna magna. A variety of means are known in the art for achieving such intrathecal administration, including pumps, reservoirs, shunts or implants. Preferably the doses are delivered via intravenous infusions lasting 1, 2 or 4 hours, most preferably 4 hours, but may also be delivered by an intravenous bolus.

Other means of increasing ASB activity in the human subjects are also described, including gene therapy that causes the patient to transiently or permanently increase expression of exogenous or endogenous ASB. Transfer of an exogenous hASB gene or a promoter that increases expression of the endogenous hASB gene is possible through a variety of means known in the art, including viral vectors, homologous recombination, or direct DNA injection.

In a second aspect, the present invention features novel pharmaceutical compositions comprising a composition comprising precursor human N-acetylgalactosamine-4-sulfatase, wherein said precursor human N-acetylgalactosamine-4-sulfatase has a purity of at least equal to or greater than 99% based on total protein, wherein said purity is measured using the reverse-phase HPLC (RP-HPLC) method, and wherein the composition is free of detectable bands of about 47-48 kDa upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie stain, optionally wherein said precursor human N-acetylgalactosamine-4-sulfatase is a recombinant precursor N-acetylgalactosamine-4-sulfatase for treating a disease caused all or in part by a deficiency in N-acetylgalactosamine-4-sulfatase (ASB). Such compositions may be suitable for administration in a number of ways such as parenteral, topical, intranasal, inhalation or oral administration. Within the scope of this aspect are embodiments featuring nucleic acid sequences encoding all or a part of an N-acetylgalactosamine-4-sulfatase (ASB) which may be administered *in vivo* into cells affected with an N-acetylgalactosamine-4-sulfatase (ASB) deficiency.

A method to produce an N-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof in amounts which enable using the enzyme therapeutically is taught herein. The method may comprise the step of transfecting a cDNA encoding for all or a part of a N-acetylgalactosamine-4-sulfatase (ASB) or a biologically active mutant or analog thereof into a cell suitable for the expression thereof. Preferably, the cells are grown in a constant or continuous culture or in perfusion. Alternatively, the cells are grown in a medium that lacks G418. In some embodiments, a cDNA encoding for a complete N-acetylgalactosamine-4-sulfatase (ASB) is used, preferably a human N-acetylgalactosamine-4-sulfatase (ASB). However, in other embodiments, a cDNA encoding for a biologically active fragment or mutant thereof may be used. Specifically, one or more amino acid substitutions may be made while preserving or enhancing the biological activity of the enzyme. In other preferred embodiments, an expression vector is used to transfer the cDNA into a suitable cell or cell line for expression thereof. In one particularly preferred embodiment, the cDNA is transfected into a Chinese Hamster Ovary (CHO) cell, such as the CHO-K1 cell line. In yet other preferred embodiments, the production procedure comprises the following steps: (a) growing cells transfected with a DNA encoding all or a biologically active fragment or mutant of a human N-acetylgalactosamine-4-sulfatase in a suitable growth medium to an appropriate density, (b) introducing the transfected cells into a bioreactor, (c) supplying a suitable growth medium to the bioreactor, and (d) separating the transfected cells from the media containing the enzyme.

A transfected cell line which features the ability to produce N-acetylgalactosamine-4-sulfatase (ASB) in amounts which enable using the enzyme therapeutically is taught herein. In a preferred embodiment, the N-acetylgalactosamine-4-sulfatase is precursor N-acetylgalactosamine-4-sulfatase. Preferably, the recombinant CHO cell line such as the CHO K1 cell line stably and reliably produces amounts of an N-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof which enable using the enzyme therapeutically. Especially preferred is the transgenic CHO-K1 cell line designated CSL4S-342. In some preferred teachings, the transgenic cell line contains one or more copies of an expression construct. Preferably, the transgenic cell line contains about 10 or more copies of the expression construct. In even more preferred embodiments, the cell line expresses the recombinant N-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof in amounts of at least about 20-40 micrograms per 10<sup>7</sup> cells per day.

Novel vectors suitable to produce N-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof in amounts which enable using the enzyme therapeutically are also taught herein.

Novel N-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof produced in accordance with the methods taught herein and thereby present in amounts which enable using the enzyme therapeutically are taught herein. The specific activity of the N-acetylgalactosamine-4-sulfatase (ASB) according to the present invention is preferably in the range of 20-90 units, and more preferably greater than about 50 units per mg protein. In the preferred embodiment, the N-acetylgalactosamine-4-sulfatase is highly purified precursor N-acetylgalactosamine-4-sulfatase.

A novel method to purify N-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof is taught herein. According to a first embodiment, a transfected cell mass is grown and removed leaving recombinant enzyme. Exogenous materials should normally be separated from the crude bulk to prevent fouling of the columns. Preferably, the growth medium containing the recombinant enzyme is passed through an ultrafiltration step. Preferably, the method to purify the precursor N-acetylgalactosamine-4-sulfatase comprises: (a) obtaining a fluid containing precursor N-acetylgalactosamine-4-sulfatase; (b) reducing the proteolytic activity of a protease in said fluid able to cleave the precursor N-acetylgalactosamine-4-sulfatase, wherein said reducing does not harm said precursor N-acetylgalactosamine-4-sulfatase; (c) contacting the fluid with a Cibacron blue dye interaction chromatography resin; (d) contacting the fluid with a copper chelation chromatography resin; (e) contacting the fluid with a phenyl hydrophobic interaction chromatography resin; (f) recovering said precursor N-acetylgalactosamine-4-sulfatase. Preferably, steps (c), (d) and (e) can be performed sequentially. Those skilled in the art readily appreciate that one or more of the chromatography steps may be omitted or substituted, or that the order of the chromatography steps may be changed within the scope of the present invention. In other preferred embodiments, the eluent from the final chromatography column is ultrafiltered/diafiltered, and an appropriate step is performed to remove any remaining viruses. Finally, appropriate sterilizing steps may be performed as desired.

**DESCRIPTION OF THE FIGURES**

Figure 1 provides a flow diagram of the method for producing a human *N*-acetylgalactosamine-4-sulfatase (ASB) according to the present invention.

Figure 2 provides a flow diagram of the method for purifying a human *N*-acetylgalactosamine-4-sulfatase (ASB) according to the batch process method (Table 14).

Figure 3 provides the flow diagram of the method for purifying a human *N*-acetylgalactosamine-4-sulfatase (ASB) according to the perfusion process method (Tables 14 and 15).

Figures 4A-4C depicts results obtained for the chromatograms of the Blue Sepharose Column (Figure 4A), Copper Chelating Sepharose Column (Figure 4B) and Phenyl Sepharose Column (Figure 4C).

Figure 5 depicts a 4-20% polyacrylamide gradient gel showing the result of a silver-stained SDS-PAGE of the perfusion process purification method (Tables 14 and 15).

Figures 6A-6F depicts the results on 4-20% polyacrylamide gradient SDS gels of the following samples: lane 1, NEB broad range prestained molecule weight standards (MW in kDa); lane 2, 5 µg ASB from lot AS60001 (old batch process); lane 3, 5 µg ASB from lot AP60109 UF4 (perfusion process); lane 4, 5 µg ASB from lot AP60109 UF10 (perfusion process); lane 5, 5 µg ASB from lot AP60109 UF15 (perfusion process); lane 6, 5 µg ASB from lot AP60109 AUF18 (perfusion process); lane 7, 5 µg ASB from lot AP60109 AUF22 (perfusion process); lane 8, 5 µg ASB from lot AP60109 AUF25 (perfusion process); and, lane 9, 5 µg ASB from lot AP60109 AUF27 (perfusion process). The gels are stained either with Coomassie R-250 or silver-stained.

Figure 7A depicts the results on silver-stained 4-20% polyacrylamide gradient SDS gels. Figure 7B depicts the results on Coomassie stained 4-20% polyacrylamide gradient SDS gels. Lane 1, lot AP60202 UF4; lane 2, lot AP60202 UF10; lane 3, lot AP60202 UF18; lane 4, lot AP60202 (BMK); lane 5, lot 102PD0139x B3; lane 6, lot 102PD0139x B5; lane 7, perfusion reference standard rhASB-202-002; lane 8, lot 102PD0139 P1; lane 9, lot 102PD0139 P2; and, lane 10, Mark 12 standard (MW in kDa).

Figures 8A-8C depicts profiles obtained for the Blue Sepharose Column (Figure 8A), Copper Chelating Sepharose Column (Figure 8B) and Phenyl Sepharose Column (Figure 8C). In Figure 8B, cathepsin activity is indicated by the red line.

Figure 9A depicts the results of a silver-stained 4-20% polyacrylamide gradient SDS gels. Figure 9B depicts the results of the proteins transferred from the gel of Figure 9A transferred onto nitrocellulose and probed by anti-rhASB antibodies. Figure 9C depicts the results of the proteins transferred from the gel of Figure 9A transferred onto nitrocellulose and probed by anti-cathepsin antibodies. BioLabPreStain indicates molecular weight standards (MW in kDa).

Figure 10 shows serum anti-ASB antibody levels over 96 weeks of treatment with rhASB in a Phase 1/2 clinical study in humans.

Figure 11 shows the reduction in total urinary GAG levels over 96 weeks of treatment with rhASB in a Phase 1/2 clinical study in humans.

Figure 12 shows a comparison of urinary GAG levels at week 96 in humans treated with rhASB to age-appropriate normal levels.

Figure 13 shows the improvement in results of the 6-minute walk test over 96 weeks of treatment with rhASB in a Phase 1/2 clinical study in humans.

Figure 14 shows serum anti-ASB antibody levels over 48 weeks of treatment in a Phase 2 clinical study in humans.

Figure 15 shows the reduction in total urinary GAG levels over 48 weeks of treatment in a Phase 2 clinical study in humans.

Figure 16 shows a comparison of levels at week 48 in humans treated with rhASB to age-appropriate normal levels.

Figure 17 shows the improvement in the 12-minute walk test results over 48 weeks of treatment in a Phase 2 clinical study in humans.

Figure 18 shows the improvement in the 3-minute stair climb results over 48 weeks of treatment in a Phase 2 clinical study in humans.

Figure 19 shows results of the Expanded Timed Get Up and Go Test over 48 weeks of treatment in a Phase 2 clinical study in humans.

Figures 20 and 21, respectively, show results of joint pain and joint stiffness questionnaires over 48 weeks of treatment in a Phase 2 clinical study in humans.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention encompasses a composition comprising precursor human *N*-acetylgalactosamine-4-sulfatase, wherein said precursor human *N*-acetylgalactosamine-4-sulfatase has a purity of at least equal to or greater than 99% based on total protein, wherein said purity is measured using the reverse-phase HPLC (RP-HPLC) method, and wherein the composition is free of detectable bands of about 47-48 kDa upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie stain, optionally wherein said precursor human *N*-acetylgalactosamine-4-sulfatase is a recombinant precursor *N*-acetylgalactosamine-4-sulfatase and the use thereof. The purity of *N*-acetylgalactosamine-4-sulfatase in the precursor form is at least equal to or greater than 99% by total protein as determined by the reverse-phase HPLC method. Preferably, the purity is at least equal to or greater than 99.1, 99.2, 99.3 or 99.4%. Even more preferably, the purity is at least equal to, or greater than 99.5, 99.6, 99.7 or 99.8%. Even much more preferably, the purity is at least equal to or greater than 99.9%. The purity of precursor *N*-acetylgalactosamine-4-sulfatase is measured using the reverse-phase HPLC method (see Example 9). All percent purity is based on total protein as determined by the reverse-phase HPLC method. The consistently repeatable high purity obtainable using the purification process disclosed herein makes it possible to treat these patients that require long-term, chronic treatment with high purity rhASB preparations administered at every treatment, e.g., weekly. Thus, the invention contemplates administering precursor rhASB of such high purity over a long period of time, e.g. 12 weeks, 24 weeks, 48 weeks, 96 weeks or more.

In a first aspect, the present invention features a composition comprising precursor human *N*-acetylgalactosamine-4-sulfatase, wherein said precursor human *N*-acetylgalactosamine-4-sulfatase has a purity of at least equal to or greater than 99% based on total protein, wherein said purity is measured using the reverse-phase HPLC (RP-HPLC) method, and wherein the composition is free of detectable bands of about 47-48 kDa upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie stain, optionally wherein said precursor human *N*-acetylgalactosamine-4-sulfatase is a recombinant precursor *N*-acetylgalactosamine-4-sulfatase and use thereof for treating diseases caused all or in part by a deficiency in *N*-acetylgalactosamine-4-sulfatase (ASB). In one embodiment, this use features administering a composition of the present invention alone or in combination with a pharmaceutically suitable carrier. Transferring a nucleic acid encoding, all or a part of an *N*-acetylgalactosamine-4-sulfatase (ASB) or a biologically active mutant thereof into one or more host cells *in vivo* is taught herein. Preferred embodiments include optimizing the dosage to the needs of the organism to be treated, preferably mammals or humans, to effectively ameliorate the disease symptoms. In preferred embodiments the disease is mucopolysaccharidosis VI (MPS VI), Maroteaux-Lamy syndrome.

The purity of precursor *N*-acetylgalactosamine-4-sulfatase is that whereby the composition is essentially free of any contaminating cell proteins which can cause an immunological or allergic reaction by the subject who is administered precursor *N*-acetylgalactosamine-4-sulfatase. A composition is essentially free of such contaminating host cell proteins if the composition, when administered to a subject, does not cause any immunological or allergic reaction. The high purity of the precursor *N*-acetylgalactosamine-4-sulfatase is important for avoiding an immunological or allergic reaction by the subject to the impurities present in the pharmaceutical composition. This is especially

true of proteins of the cells from which the precursor *N*-acetylgalactosamine-4-sulfatase is purified. When recombinant precursor *N*-acetylgalactosamine-4-sulfatase is expressed and purified from Chinese Hamster Ovary cells, the Chinese Hamster Ovary proteins can cause immunological or allergic reactions (e.g. hives) in the subject. The only means to avoid this type of reaction is to ensure that the precursor *N*-acetylgalactosamine-4-sulfatase is sufficiently pure so that the contaminating Chinese Hamster Ovary proteins are not of sufficient amount to cause such reaction(s). The purity of the pharmaceutical composition is especially important as subjects include patients suffering from MPS VI and are thus already immunologically compromised.

Also preferably the purity of the precursor *N*-acetylgalactosamine-4-sulfatase (ASB) is such that the composition has only trace amounts of processed or degraded forms. Proteases present in the host cells cleave the *N*-acetylgalactosamine-4-sulfatase into lower molecular weight forms. While some of these forms may also be enzymatically active, the precursor form is preferable for cellular uptake and lysosomal targeting and thus a higher amount of non-processed precursor ASB in the final preparation is desirable. Moreover, degraded forms of ASB in the drug product may also engender higher incidence or amounts of antibodies to ASB itself, which is highly undesirable in cases such as this where long-term therapy of patients is required. The perfusion purification process described herein results in a highly pure preparation that is essentially free of contaminating host cell proteins or processed/aggregated forms of ASB as assayed by the combination of SDS-PAGE, RPHPLC and SEC-HPLC. When administered to humans, the product produced by this process appears to have a longer half-life.

The indication for recombinant human *N*-acetylgalactosamine-4-sulfatase (rhASB) is for the treatment of MPS VI, also known as Maroteaux-Lamy Syndrome. According to preferred embodiments, an initial dose of 1 mg/kg (~50 U/kg) is provided to patients suffering from a deficiency in *N*-acetylgalactosamine-4-sulfatase. Preferably, the *N*-acetylgalactosamine-4-sulfatase is administered weekly by injection. According to other preferred embodiments, patients who do not demonstrate a reduction in urinary glycosaminoglycan excretions of at least fifty percent are changed to a dosage of 2 mg/kg (~100 U/kg) within about three months of initial dosage. Preferably, the *N*-acetylgalactosamine-4-sulfatase (rhASB) or a biologically active fragment, mutant or analog thereof is administered intravenously over approximately a four-hour period once weekly preferably for as long as significant clinical symptoms of disease persist. Also, preferably, the *N*-acetylgalactosamine-4-sulfatase (rhASB) is administered by an intravenous catheter placed in the cephalic or other appropriate vein with an infusion of saline begun at about 30 cc/hr. Further, preferably the *N*-acetylgalactosamine-4-sulfatase (rhASB) is diluted into about 250 cc of normal saline.

In a second aspect, the present invention features novel pharmaceutical compositions comprising precursor human *N*-acetylgalactosamine-4-sulfatase, wherein said precursor human *N*-acetylgalactosamine-4-sulfatase has a purity of at least equal to or greater than 99% based on total protein, wherein said purity is measured using the reverse-phase HPLC (RP-HPLC) method, and wherein the composition is free of detectable bands of about 47-48 kDa upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie stain, optionally wherein said precursor human *N*-acetylgalactosamine-4-sulfatase is a recombinant precursor *N*-acetylgalactosamine-4-sulfatase for treating a deficiency in *N*-acetylgalactosamine-4-sulfatase. The recombinant enzyme may be administered in a number of ways in addition to the preferred embodiments described above, such as parenteral, topical, intranasal, inhalation or oral administration. Another aspect of the invention is to provide for the administration of the enzyme by formulating it with a pharmaceutically-acceptable carrier which may be solid, semi-solid or liquid or an ingestible capsule. Examples of pharmaceutical compositions include tablets, drops such as nasal drops, compositions for topical application such as ointments, jellies, creams and suspensions, aerosols for inhalation, nasal spray, liposomes. Usually the recombinant enzyme comprises between 0.05 and 99% or between 0.5 and 99% by weight of the composition, for example between 0.5 and 20% for compositions intended for injection and between 0.1 and 50% for compositions intended for oral administration.

To produce pharmaceutical compositions in this form of dosage units for oral application containing a therapeutic enzyme, the enzyme may be mixed with a solid, pulverulent carrier, for example lactose, saccharose, sorbitol, mannitol, a starch such as potato starch, corn starch, amylopectin, laminaria powder or citrus pulp powder, a cellulose derivative or gelatine and also may include lubricants such as magnesium or calcium stearate or a Carbowax or other polyethylene glycol waxes and compressed to form tablets or cores for dragees. If dragees are required, the cores may be coated for example with concentrated sugar solutions which may contain gum arabic, talc and/or titanium dioxide, or alternatively with a film forming agent dissolved in easily volatile organic solvents or mixtures of organic solvents. Dyestuffs can be added to these coatings, for example, to distinguish between different contents of active substance. For the composition of soft gelatine capsules consisting of gelatine and, for example, glycerol as a plasticizer, or similar closed capsules, the active substance may be admixed with a Carbowax or a suitable oil as e.g., sesame oil, olive oil, or arachis oil. Hard gelatine capsules may contain granulates of the active substance with solid, pulverulent carriers such as lactose, saccharose, sorbitol, mannitol, starches such as potato starch, corn starch or amylopectin, cellulose derivatives or gelatine, and may also include magnesium stearate or stearic acid as lubricants.

Therapeutic enzymes of the present invention may also be administered parenterally such as by subcutaneous, intramuscular or intravenous injection either by single injection or pump infusion or by sustained release subcutaneous implant, and therapeutic enzymes may be administered by inhalation. In subcutaneous, intramuscular and intravenous injection the therapeutic enzyme (the active ingredient) may be dissolved or dispersed in a liquid carrier vehicle. For parenteral administration the active material may be suitably admixed with an acceptable vehicle, preferably of the vegetable oil variety such as peanut oil, cottonseed oil and the like. Other parenteral vehicles such as organic compositions using solketal, glycerol, formal, and aqueous parenteral formulations may also be used.

For parenteral application by injection, compositions may comprise an aqueous solution of a water soluble pharmaceutically acceptable salt of the active acids according to the invention, desirably in a concentration of 0.5-10%, and optionally also a stabilizing agent and/or buffer substances in aqueous solution. Dosage units of the solution may advantageously be enclosed in ampoules.

When therapeutic enzymes are administered in the form of a subcutaneous implant, the compound is suspended or dissolved in a slowly dispersed material known to those skilled in the art, or administered in a device which slowly releases the active material through the use of a constant driving- force such as an osmotic pump. In such cases administration over an extended period of time is possible.

For topical application, the pharmaceutical compositions are suitably in the form of an ointment, cell, suspension, cream or the like. The amount of active substance may vary, for example between 0.05- 20% by weight of the active substance. Such pharmaceutical compositions for topical application may be prepared in known manner by mixing, the active substance with known carrier materials such as isopropanol, glycerol, paraffin, stearyl alcohol, polyethylene glycol, etc. The pharmaceutically acceptable carrier may also include a known chemical absorption promoter. Examples of absorption promoters are, e.g., dimethylacetamide (U.S. Patent No. 3,472,931), trichloro ethanol or trifluoroethanol (U.S. Patent No. 3,891,757), certain alcohols and mixtures thereof (British Patent No. 1,001,949). A carrier material for topical application to unbroken skin is also described in the British patent specification No. 1,464,975, which discloses a carrier material

consisting of a solvent comprising 40-70% (v/v) isopropanol and 0-60% (v/v) glycerol, the balance, if any, being an inert constituent of a diluent not exceeding 40% of the total volume of solvent.

The dosage at which the therapeutic enzyme containing pharmaceutical compositions are administered may vary within a wide range and will depend on various factors such as for example the severity of the disease, the age of the patient, etc., and may have to be individually adjusted. As a possible range for the amount of therapeutic enzyme which may be administered per day be mentioned from about 0.1 mg- to about 2000 mg or from about 1 mg to about 2000 mg.

The pharmaceutical compositions containing the therapeutic enzyme may suitably be formulated so that they provide doses within these ranges either as single dosage units or as multiple dosage units. In addition to containing a therapeutic enzyme (or therapeutic enzymes), the subject formulations may contain one or more substrates or cofactors for the reaction catalyzed by the therapeutic enzyme in the compositions. Therapeutic enzyme containing, compositions may also contain more than one therapeutic enzyme. Likewise, the therapeutic enzyme may be in conjugate form being bound to another moiety, for instance PEG. Additionally, the therapeutic enzyme may contain one or more targeting moieties or transit peptides to assist delivery to a tissue, organ or organelle of interest.

The recombinant enzyme employed in the subject methods and compositions may also be administered by means of transforming patient cells with nucleic acids encoding the *N*-acetylgalactosamine-4-sulfatase or a biologically active fragment, mutant or analog thereof. The nucleic acid sequence so encoding may be incorporated into a vector for transformation into cells of the patient to be treated. Preferred embodiments of such vectors are described herein. The vector may be designed so as to integrate into the chromosomes of the subject, e.g., retroviral vectors, or to replicate autonomously in the host cells. Vectors containing encoding *N*-acetylgalactosamine-4-sulfatase nucleotide sequences may be designed so as to provide for continuous or regulated expression of the enzyme. Additionally, the genetic vector encoding the enzyme may be designed so as to stably integrate into the cell genome or to only be present transiently. The general methodology of conventional genetic therapy may be applied to polynucleotide sequences encoding *N*-acetylgalactosamine-4-sulfatase. Reviews of conventional genetic therapy techniques can be found in Friedman, Science 244:1275-1281 (1989); Ledley, J. Inherit. Aletab. Dis. 13:587-616 (1990); and, Tososhev, et al., Curr. Opinions Biotech. 1:55-61 (1990).

A particularly preferred method of administering the recombinant enzyme is intravenously. A particularly preferred composition comprises recombinant *N*-acetylgalactosamine-4-sulfatase, normal saline, phosphate buffer to maintain the pH at about 5-7, and human albumin. The composition may additionally include polyoxyethylenesorbitan, such as polysorbate 20 or 80 (Tween -20 or Tween -80) to improve the stability and prolong shelf life. Alternatively, the composition may include any surfactant or non-ionic detergent known in the art, including but not limited to polyoxyethylene sorbitan 40 or 60; polyoxyethylene fatty acid esters; polyoxyethylene sorbitan monoisostearates; poloxamers, such as poloxamer 188 or poloxamer 407; octoxynol-9 or octoxynol 40.

Preferably the surfactant or non-ionic detergent is present at a concentration of at least 0.0001%, or at least 0.0005%, or at least 0.001%, 0.002%, 0.003%, 0.004% or 0.005% (w/v). Preferably the concentration is the lowest necessary to achieve the desired stability, but may be up to 0.005%, 0.006%, 0.007%, 0.008%, 0.009%, 0.01%, or 0.02% (w/v). Most preferably the composition comprises a polysorbate at a concentration of 0.005%  $\pm$  0.003% (e.g. 0.002% to 0.008%). These composition ingredients may preferably be provided in the following amounts:

<i>N</i> -acetylgalactosamine-4-sulfatase	1-5 mg/ml or 50-250 units/ml
Sodium chloride solution	150 mM in an IV bag, 50-250 cc total volume
Sodium phosphate buffer	10-100 mM, pH 5.8, preferably 10mM
Human albumin, optional	1 mg/mL
Tween -20 or Tween -80	0.001%-0.005% (w/v)

In a preferred embodiment, the ASB is formulated as 1 mg/mL in 150 mM NaCl, 10mM NaPO<sub>4</sub>, pH 5.8, 0.005% polysorbate 80.

A method to produce *N*-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof in amounts which enable using the enzyme therapeutically is taught herein. The method may comprise the step of transfecting a cDNA encoding for all or a part of a *N*-acetylgalactosamine-4-sulfatase (ASB) or a biologically active mutant or analog thereof into a cell suitable for the expression thereof. In some embodiments, a cDNA encoding for a complete *N*-acetylgalactosamine-4-sulfatase (ASB) is used, preferably a human *N*-acetylgalactosamine-4-sulfatase (ASB). However, in other embodiments, a cDNA encoding for a biologically active fragment or mutant thereof may be used. Specifically, one or more amino acid substitutions may be made while preserving or enhancing the biological activity of the enzyme.

An expression vector may be used to transfer the cDNA into a suitable cell or cell line for expression thereof. In one particularly preferred embodiment, the cDNA is transfected into a Chinese hamster ovary cell, such as the CHO-K1 cell line. In yet other preferred embodiments, the production procedure comprises the following steps: (a) growing cells transfected with a DNA encoding all or a biologically active fragment or mutant of a human *N*-acetylgalactosamine-4-sulfatase a suitable growth medium to an appropriate density, (b) introducing the transfected cells into a bioreactor, (c) supplying a suitable growth medium to the bioreactor, (d) harvesting said medium containing the recombinant enzyme, and (e) substantially removing the transfected cells from the harvest medium.

A suitable medium for growing the transfected cells is a JRH Excell 302 medium supplemented with L-glutamine, glucose and hypoxanthine/thymidine, optionally with or without G418. In a preferred medium, the JRH Excell 302 medium is further supplemented with folic acid, serine, and asparagine, and there is no G418 present in the medium. Using this preferred medium to culture cells provides a higher purity precursor rhASB compared to using the medium supplemented with G418 but not with folic acid, serine, and asparagine (see lane 2 of Figure 6). It is preferred to grow the cells in such a medium to achieve a cell density of about  $1 \times 10^7$  cells/ml resulting in 10-40 mg/ml of active enzyme. Moreover, it is preferable to grow the transfected cells in a bioreactor for about 5 to 15 days. More preferably, it is about 9 days. Preferably, the transfected cells are grown in a bioreactor using a perfusion-based process with collections continuing up to 35 days. More preferably, the transfected cells are grown in a bioreactor using a perfusion-based process with collections continuing up to 45 days. Even more preferably, the transfected cells are grown in a bioreactor using a perfusion-based process with collections continuing up to 60 days. Even much more preferably, the transfected cells are grown in a bioreactor using a perfusion-based process with collections continuing up to 90 days.

The transfected cells may be substantially removed from the bioreactor supernatant by filtering them through successive membranes such as a 10 µm membrane followed by a 1 µm membrane followed by a 0.2 µm. Any remaining harvest medium may be discarded prior to filtration.

Recombinant human *N*-acetylgalactosamine-4-sulfatase may be produced in Chinese hamster ovary cells (Peters, et al. J. Biol. Chem. 265:3374-3381). Its uptake is mediated by a high affinity mannose-6-phosphate receptor expressed on most, if not all, cells (Neufeld et al., "The mucopolysaccharidoses " The Metabolic Basis of Inherited Disease, eds. Scriver, et al. New York:McGraw-Hill (1989) p. 1565-1587). Once bound to the mannose-6-phosphate receptor, the enzyme is endocytosed through coated pits and transported to the lysosomes. At the pH of lysosomes, the enzyme is active and begins removing sulfate residues from accumulated dermatan sulfate. In MPS VI fibroblasts, the clearance of storage is rapid and easily demonstrated within 92 hours of enzyme exposure (Anson, et al., J. Clin. Invest. 99:651-662 (1997)). The recombinant enzyme may be produced at a 110-L (approximately 90 L working volume) fermentation scale according to a process according to the flow diagram outlined in Figure 1.

The recombinant enzyme can be produced using the following method as set forth in Table 1A-C.

**Table 1A: Cell Culture Process by the Fed Batch Process**

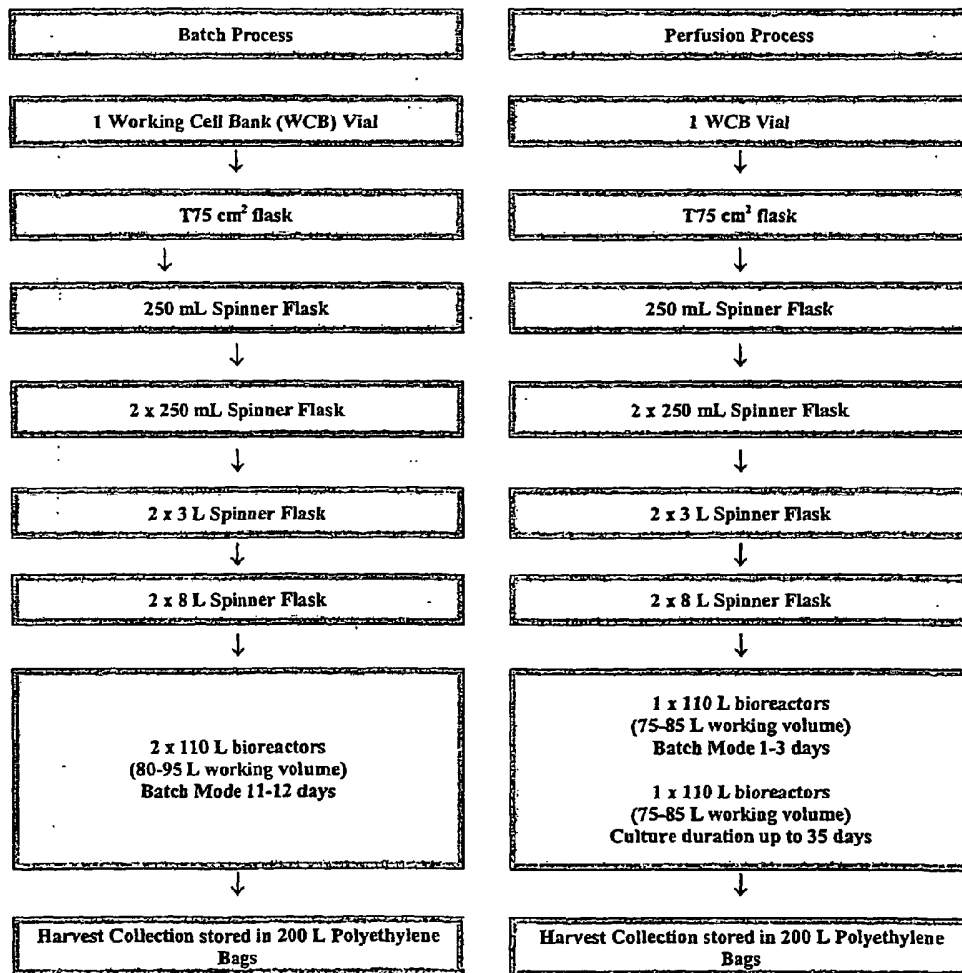
Step	Process	In-Process Testing
1. Thawing of the Working Cell Bank (WCB)	<ul style="list-style-type: none"> <li>Inoculate the thawed cells into one T-75 flask with 25mL of JRH Exell 302 medium supplemented with 4 mM L-glutamine, 4.5 g/L glucose and 10mg/L hypoxanthine/thymidine; further supplemented with folic acid, serine and asparagine (no G418)</li> <li>Culture for 3 days to achieve <math>1 \times 10^{10}</math> cell density</li> </ul>	<ul style="list-style-type: none"> <li>Cell count</li> <li>Cell viability</li> </ul>
	↓	
3. 250mL Spinner Flask	<ul style="list-style-type: none"> <li>Add cells to 175 mL of supplemented medium (no G418)</li> <li>Culture for 3 days</li> </ul>	<ul style="list-style-type: none"> <li>Cell count</li> <li>Cell viability</li> </ul>
	↓	
4. 1L Spinner Flask	<ul style="list-style-type: none"> <li>Add cells to 800mL of supplemented medium (no G418)</li> <li>Culture for 1-2 days</li> </ul>	<ul style="list-style-type: none"> <li>Cell count</li> <li>Cell viability</li> </ul>
	↓	
5. 8L Spinner Flask	<ul style="list-style-type: none"> <li>Add cells to 4L of supplemented medium (no G418)</li> <li>Culture for 1-2 days</li> </ul>	<ul style="list-style-type: none"> <li>Cell count</li> <li>Cell viability</li> </ul>
	↓	
6. 2x 8L Spinner Flask	<ul style="list-style-type: none"> <li>Split working volume into 2 8L Spinner Flasks</li> <li>Add cells to 5.5L of supplemented medium (no G418) to each 8L Spinner Flask</li> <li>Culture for 1-2 days</li> </ul>	<ul style="list-style-type: none"> <li>Cell count</li> <li>Cell viability</li> </ul>
	↓	
7. Inoculation of 110L Bioreactor	<ul style="list-style-type: none"> <li>Add cells to 7 mL of supplemented medium</li> <li>Culture 9 days</li> </ul>	<ul style="list-style-type: none"> <li>Cell count</li> <li>Cell viability</li> </ul>
	↓	
8. Production	<ul style="list-style-type: none"> <li>Approximately 9 days of growth in bioreactor</li> </ul>	<ul style="list-style-type: none"> <li>Cell Count</li> <li>Cell viability</li> <li>Activity</li> </ul>
	↓	
9. Harvest Supernatant	<ul style="list-style-type: none"> <li>Harvest is pumped into 100L bag, refrigerated overnight</li> </ul>	
	↓	
10. Cell Removal	<ul style="list-style-type: none"> <li>Cells are removed from the harvest medium by filtration through a 10 µm membrane cartridge followed by 1 µm and 0.2 µm cartridges. Since the cells have been allowed to</li> </ul>	QC Release Point



Step	Process	In-Process Testing
	settle overnight the final 5 to 10% of the harvest medium is discarded prior to filtration.	<ul style="list-style-type: none"> <li>• Activity</li> <li>• Bioburden</li> <li>• Endotoxin</li> <li>• Mycoplasma</li> <li>• In vitro advent. Agents</li> </ul>

In one embodiment, the transfected cells are grown in a cell culture process that is a perfusion-based process with collections continuing for up to 35 or more days with a collection rate of approximately 400 L per day from one 110 L bioreactor. Preferably, the collection rate is approximately 800 L per day from one 110 L bioreactor. A process flow diagram comparing the perfusion cell culture process with the batch cell culture process is shown in Table 1B. Comparisons to the fed batch process as well as details of the specific changes implemented for the perfusion-based cell culture process are summarized in Table 1C.

**Table 1B: Cell Culture Process Comparison Between Fed Batch and Perfusion Processes**



**Table 1C: Summary Description of the Differences between the Fed Batch and Perfusion Processes**

Production Step	Description (Batch)	Description (Perfusion)
CELL CULTURE	One batch is the result of one production run with two 110 L bioreactors.	Change: One batch is the result of one production run with one 110 L bioreactor

Production Step	Description (Batch)	Description (Perfusion)
Thawing of WCB vial	One vial for each production batch. Tests: Expected cell viability > 95% Expected $\geq 1 \times 10^6$ cells recovered	Change: Cell Viability: > 90% Viability of > 90% on thaw has proven to yield successful runs and product that meets specifications
↓		
T75 cm <sup>2</sup> flasks	Approximately $5 \times 10^6$ cells plated into 1 flask. Length of step: ~3 days Tests: Expected cell viability > 90% Expected $\geq 2 \times 10^7$ cells recovered	No Change
↓		
250 mL Spinner Flask	Cells from T75 is split onto a 250 mL spinner flask. Length of step: ~2 days Tests: Expected cell viability > 90% Expected $\geq 2 \times 10^8$ cells recovered	No Change
↓		
2 x 250 mL Spinner Flask	Cells from 1 x 250 mL spinner flask are split into two 250 mL spinner flask. Length of step: ~3 days Tests: Expected cell viability > 90% Expected $\geq 4 \times 10^8$ cells recovered	No Change
↓		
2 x 3 L Spinner Flask	Cells from 2 x 250 mL spinner flasks are split into two 3 L spinner flask Length of step: ~4 days Tests: Expected cell viability > 90% Expected $\geq 4.8 \times 10^9$ cells recovered	No Change
↓		
2 x 8 L Spinner Flask	Cells from 2 x 3 L spinner flasks are split into two 8 L spinner flask. Length of step: ~2 days Tests: Expected cell viability > 90% Expected $\geq 1.6 \times 10^{10}$ cells recovered	No Change
↓		
Inoculation of culture flask	Inoculum of $\geq 0.8 \times 10^{10}$ cells are added of $\geq 0.8 \times 10^{10}$ cells to each of two bioreactors containing 32 L culture medium each. Culture monitored with PC-interfaced control system.	Change: Inoculum of $\geq 1.6 \times 10^{10}$ cells added to one bioreactor containing 64 L culture medium. (Reflects the use of one bioreactor versus two.)
↓		
Production		Changes:
	Growth: 3 days of growth until culture reaches density of $> 3.2 \times 10^{10}$ cells. Vertical Split Culture media added to final volume of 95 L. Harvesting: Supernatant was harvested at day 11 or when cell viability fell below 70%. Supernatant is filtered and stored.	Growth/Transition/Harvesting: When cell density reaches $> 8 \times 10^{10}$ cells/mL, perfusion is started. The perfusion rate is gradually increased to 5 vessel volumes per day based on glucose levels. When the cell density is $> 1.28 \times 10^{12}$ the pH set point is adjusted to 7.35. Once increases in perfusion rates cease, glucose level is maintained by reducing cell density with a cell bleed. Harvested supernatant is collected and filtered.
Termination	Run is terminated at harvest	Change: Run is terminated after 35 or more days is reached, or activity falls below 2 mg/L for three consecutive days or after adequate harvested supernatant is collected.

The inoculum preparation for scale-up process is the same for the fed batch and perfusion processes. In one embodiment, the rhASB cell culture is initiated by thawing a single vial from the Working Cell Bank and transferring its contents (approximately 1 mL) to approximately 25 mL of EX-CELL 302 Medium (Modified w/ L-Glutamine, No Phenol Red) in a T75 cm<sup>2</sup> cell culture flask. In each expansion step, the cell culture is incubated until a viable cell count of approximately  $0.8 \times 10^6$  cells/mL is achieved. Each cell expansion step is monitored for cell growth (cell density) and viability (via trypan blue exclusion). All additions of EX-CELL 302 Medium (Modified w/ L-Glutamine, No Phenol Red) medium and cell transfers are performed aseptically in a laminar flow hood. The cell culture is expanded sequentially from the T75 cm<sup>2</sup> flask to a 250 mL spinner flask, to two 250 mL spinner flasks, to two 3 L spinner flasks, and finally to two 8 L spinner flasks. The entire scale-up process lasts approximately 14 days. When the two 8 L spinner flasks are at a density of at least  $1.0 \times 10^6$  cells/mL, the flasks are used to seed one 110 L bioreactor.

It is preferred that bioreactor operations for rhASB expression or production or manufacture utilize a perfusion based cell culture process. Preferably, the bioreactor, using the perfusion process, can control cell densities up to as high as 37 million cells per mL; compared to 4-5 million cells per mL using the fed batch process.

The perfusion-based process runs longer (35 days) than the fed batch process (11-12 days) and produces a greater volume of harvested cell culture fluid (approximately 400 L/day at a perfusion rate of 5 vessel volumes per day) compared to the fed batch process (190 L/run). Preferably, harvesting is performed up to 35 days for a total collection of approximately 8400 L of supernatant.

End of Production Cells (EPC) are evaluated for genetic stability, identity, sterility and adventitious agent contamination per ICH guideline. Preferably, EPC results, obtained from a 35-day long bioreactor run, AC60108, produced under cGMP conditions, show no growth or negative results or no detection of the presence of bacteria and fungi, mycoplasma, adventitious viral contaminants, murine viruses, or like contaminants or particles.

A transgenic cell line which features the ability to produce *N*-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof in amounts which enable using the enzyme therapeutically is taught herein. A recombinant Chinese hamster ovary cell line such as the CHO K1 cell line that stably and reliably produces amounts of *N*-acetylgalactosamine-4-sulfatase (ASB) which enable using the enzyme therapeutically is also taught herein. Especially preferred is the CHO-K1 cell line designated CSL4S-342. In some preferred embodiments, the cell line contains one or more of an expression construct. More preferably, the cell line contains about 10 or more copies of the expression construct. In even more preferred embodiments, the cell line expresses recombinant *N*-acetylgalactosamine-4-sulfatase (ASB) in amounts of at least about 20-80 or 40-80 micrograms per  $10^7$  cells per day.

Recombinant human *N*-acetylgalactosamine-4-sulfatase (rhASB) may be produced from a stable transfected CHO-K1 (Chinese hamster ovary) cell line designated CSL4S-342. The cell line is described in the literature (Crawley, J.Clin.Invest. 99:651-662 (1997)). Master Cell Bank (MCB) and Working Cell Bank (WCB) were prepared at Tektogen Inc. (Malvern, PA). The cell banks have been characterized per ICH recommended guidelines for a recombinant mammalian cell line.

Novel vectors suitable to produce *N*-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof in amounts which enable using the enzyme therapeutically are taught herein.

Novel *N*-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof produced in accordance with the methods taught herein and thereby present in amounts which enable using the enzyme therapeutically are described herein. The preferred specific activity of the *N*-acetylgalactosamine-4-sulfatase (ASB) according to the present invention is about 20-90 Unit, and more preferably greater than 50 units per milligram protein. Preferably, the enzyme has a deglycosylated weight of about 55 to 56 kDa, most preferably about 55.7 kDa. Preferably, the enzyme has a glycosylated weight of about 63 to 68 kDa, most preferably about 64 to 66 kDa. The present invention also includes biologically active fragments including truncated molecules, analogs and mutants of the naturally-occurring human *N*-acetylgalactosamine-4-sulfatase.

The human cDNA for *N*-acetylgalactosamine-4-sulfatase predicts a protein of 533 amino acids with a signal peptide of 41 amino acids (Peters, et al. J. Biol. Chem. 265:3374-3381). The predicted molecular weight is about 55.9 kDa after signal peptide cleavage. The recombinant enzyme has an apparent molecular weight of 64 kDa on SDS-PAGE due to carbohydrate modifications. The predicted protein sequence contains six potential N-linked oligosaccharide modification sites of which four may be used based on a 2,000 kDa average mass and 8,000 kDa difference between predicted and apparent mass. A mature form of the intracellular protein has three peptides attached by cystine bonds. The largest peptide has a molecular weight of 47 kDa; the other two has a molecular weight of 6 and 7 kDa respectively.

A description of a drug product produced and purified according to the methods of the present invention is provided in Table 2.

**Table 2: Drug Product Preliminary Specifications**

Test	Procedure	Specification
Activity	Fluorescence assay	20,000 - 120,000mUnits
Adventitious Viruses*	<i>In Vitro</i> Assay	Pass
Appearance	Visual	Clear, colorless to pale yellow solution
Bacterial Endotoxin	LAL	$\leq 2$ EU/mL
Chloride	Atomic Absorption	Report Value
ASB fibroblast Uptake Assay	TBD	$\leq 40$ nmol
Mycoplasma*	Points to Consider 1993	Pass
Particulates	USP	$\leq 600$ /vial at 25 $\mu$ m & $\leq 6000$ /vial at 10 $\mu$ m
PH	USP	5.5-6.8
Phosphate	Atomic Absorption	Report Value
Protein Concentration	UV 280	0.8-1.2 mg/ml
Purity	SDS PAGE	1 major band between 65 - 70 kDa
	RP-HPLC	> 95%
Residual Blue Dye	TBD	Report Value
Residual Copper	TBD	Report Value
Sodium	Atomic Absorption	Report Value

Test	Procedure	Specification
Specific Activity	Calculation	40,000 - 80,000 mUnits/mg
Sterility	21 CFR 610	Pass
* Tested on harvested supernatant from bioreactor (after cell removal by filtration).		

In a seventh aspect, the present invention features a novel method to purify *N*-acetylgalactosamine-4-sulfatase (ASB) or a biologically active fragment, mutant or analog thereof. According to a first embodiment, a transfected cell mass is grown and removed leaving recombinant enzyme. Exogenous materials should normally be separated from the crude bulk to prevent fouling of the columns. Preferably, the growth medium containing the recombinant enzyme is passed through an ultrafiltration and diafiltration step. In one method, the filtered solution is passed through a DEAE Sepharose chromatography column, then a Blue Sepharose chromatography column, then a Cu++ Chelating Sepharose chromatography column, and then a Phenyl Sepharose chromatography column. Such a four step column chromatography including using a DEAE Sepharose, a Blue Sepharose, a Cu++ Chelating Sepharose and a Phenyl Sepharose chromatography column sequentially results in especially highly purified recombinant enzyme. Those of skill in the art appreciate that one or more chromatography steps may be omitted or substituted or the order of the steps altered within the scope of the present invention. In other preferred embodiments, the eluent from the final chromatography column is ultrafiltered/diafiltered, and an appropriate step is performed to remove any remaining viruses. Finally, appropriate sterilizing steps may be performed as desired. The recombinant enzyme may be purified according to a process outlined in Figure 2. The quality of the recombinant enzyme is key to patients. The rhASB produced by this method is substantially pure (> 95%).

In a preferred embodiment, the ultrafiltration/diafiltration step is performed with a sodium phosphate solution of about 10 mM and with a sodium chloride solution of about 100 mM at a pH of about 7.3. In another embodiment, the DEAE Sepharose chromatography step is performed at a pH of about 7.3 wherein the elute solution is adjusted with an appropriate buffer, preferably a sodium chloride and sodium phosphate buffer. In additional preferred embodiments, the Blue Sepharose chromatography step is performed at a pH of about 5.5 wherein the elute solution is adjusted with an appropriate buffer, preferably a sodium chloride and sodium acetate buffer. Also, in preferred embodiments, the Cu++ Chelating Sepharose chromatography step is performed with an elution buffer including sodium chloride and sodium acetate. In especially preferred embodiments, a second ultrafiltration/diafiltration step is performed on the eluate from the chromatography runs wherein the recombinant enzyme is concentrated to a concentration of about 1 mg/ml in a formulation buffer such as a sodium chloride and sodium phosphate buffer to a pH of about 5.5 to 6.0, most preferably to a pH of 5.8. Phosphate buffer is a preferred buffer used in the process because phosphate buffer prevents critical degradation and improves the stability of the enzyme.

A more detailed description of particularly preferred purification methods within the scope of the present invention is set forth in Table 3.

**Table 3: Purification Process Overview**

Step	Process
1. UF/DF	Filtered harvest fluid (HF) is concentrated ten fold and then diafiltered with 5 volumes of 10 mM Sodium Phosphate, 100 mM NaCl, pH 7.3 using a tangential flow filtration (TFF) system.
	↓
2. DEAE Sepharose FF (flow through)	<ul style="list-style-type: none"> <li>• Pre-wash 1 buffer: 0.1 N NaOH</li> <li>• Pre-wash 2 buffer: 100 mM NaPO<sub>4</sub> pH 7.3</li> <li>• Equilibration buffer: 100 mM NaCl, 10 mM NaPO<sub>4</sub>, pH 7.3</li> <li>• Load: Product from Step 1</li> <li>• Wash buffer: 100 mM NaCl, 10mM NaPO<sub>4</sub>, pH 7.3</li> <li>• Strip buffer: 1 M NaCl, 10 mM NaPO<sub>4</sub>, pH 7.3</li> <li>• Sanitization buffer: 0.5 N NaOH</li> <li>• Storage buffer: 0.1 N NaOH</li> </ul>
	↓
3. Blue Sepharose FF	<ul style="list-style-type: none"> <li>• Pre-wash 1: 0.1 N NaOH</li> <li>• Pre-wash 2: H<sub>2</sub>O</li> <li>• Pre-wash 3: 1 M NaAc, pH 5.5</li> <li>• Equilibration buffer: 150 mM NaCl, 20 mM NaAc, pH 5.5</li> <li>• Load: DEAE flow through</li> <li>• Wash buffer: 150 mM NaCl, 20 mM NaAc, pH 5.5</li> <li>• Elution buffer: 500 mM NaCl, 20 mM NaAc, pH 5.5</li> <li>• Regeneration buffer: 1 M NaCl, 20 mM NaAc, pH 5.5</li> <li>• Sanitization buffer: 0.1 N NaOH, 0.5-2 hours</li> <li>• Storage buffer: 500 mM NaCl, 20 mM NaAc, pH 5.5,</li> </ul>
	20% ETOH

Step	Process
	↓
4. Cu <sup>++</sup> Chelating Sepharose FF	<ul style="list-style-type: none"> <li>• Sanitization buffer: 0.1 N NaOH</li> <li>• Wash buffer: H<sub>2</sub>O</li> <li>• Charge Buffer: 0.1 M Copper Sulfate</li> <li>• Equilibration buffer: 20 mM NaAc, 0.5 M NaCl, 10% Glycerol, pH 6.0</li> <li>• Load: Blue Sepharose Eluate</li> <li>• Wash Buffer 1: 20 mM NaAc, 0.5 M NaCl, 10% Glycerol, pH 6.0</li> <li>• Wash Buffer 2: 20 mM NaAc, 1 M NaCl, 10% Glycerol, pH 4.0</li> <li>• Wash Buffer 3: 20 mM NaAc, 1 M NaCl, 10% Glycerol, pH 3.8</li> <li>• Elution Buffer: 20 mM NaAc, 1 M NaCl, 10% Glycerol, pH 3.6</li> <li>• Strip Buffer: 50 mM EDTA, 1 M NaCl</li> <li>• Sanitization Buffer: 0.5 N NaOH, 0.5-2 hours</li> <li>• Storage Buffer: 0.1 N NaOH</li> </ul>
	↓
5. Phenyl Sepharose HP	<ul style="list-style-type: none"> <li>• Pre-wash 1 Buffer: 0.1 N NaOH</li> <li>• Pre-wash 2 Buffer: H<sub>2</sub>O</li> <li>• Equilibration buffer: 3 M NaCl, 20 mM NaAc, pH 4.5</li> <li>• Load: Cu<sup>++</sup> Chelating Sepharose Eluate</li> <li>• Wash Buffer 1: 3.0 M NaCl, 20 mM NaAc, pH 4.5</li> <li>• Wash Buffer 2: 1.5 M NaCl, 20 mM NaAc, pH 4.5</li> <li>• Elution buffer 1: 1.0 M NaCl, 20 mM NaAc, pH 4.5</li> <li>• Strip Buffer: 0 M NaCl, 20 mM NaAc, pH 4.5</li> <li>• Sanitization Buffer: 0.5 N NaOH</li> <li>• Storage Buffer: 0.1 N NaOH</li> </ul>
	↓
6. UF/DF	The purified rhASB is concentrated and diafiltered to a final concentration of 1.5 mg/ml in formulation buffer (150 mM NaCl, 10 mM NaPO <sub>4</sub> , pH 5.8) using a TFF system.
	↓
7. Formulation (if necessary)	• Dilute with additional formulation buffer to 1.0mg/ml
	↓
8. Viral Reduction/ Sterile filtration	• 0.02 µm filtration into sterile container
	↓
9. Vialing	• Product filled into 5cc Type 1 glass vials, manually stoppered, crimped and labeled.

The formulated bulk drug substance can be sterilized through a 0.04 micron or preferably a 2 micron filter in a class 100 laminar flow hood into Type 1 glass vials. The vials may be filled to a final volume of about 5mL using a semi-automatic liquid filling machine. The vials may then be manually stoppered, sealed and labeled.

A more preferred method to purify a precursor *N*-acetylgalactosamine-4-sulfatase comprises: (a) obtaining a fluid containing precursor *N*-acetylgalactosamine-4-sulfatase; (b) reducing the proteolytic activity of a protease in said fluid able to cleave the precursor *N*-acetylgalactosamine-4-sulfatase, wherein said reducing does not harm said precursor *N*-acetylgalactosamine-4-sulfatase; (c) contacting the fluid with a Cibacron blue dye interaction chromatography resin; (d) contacting the fluid with a copper chelation chromatography resin; (e) contacting the fluid with a phenyl hydrophobic interaction chromatography resin; and (f) recovering said precursor *N*-acetylgalactosamine-4-sulfatase. Preferable, steps (c), (d) and (e) are performed sequentially. This method requires no more than three chromatography steps or columns. In order to obtain highly purified precursor *N*-acetylgalactosamine-4-sulfatase, no further chromatography steps or columns are required. This method does not comprise the fluid contacting a DEAE Sepharose resin. The recovered precursor *N*-acetylgalactosamine-4-sulfatase has a purity of at least equal to or greater than 99%. The overall recovery yield can be at least about 40-60%.

Preferably, obtaining the fluid containing the precursor *N*-acetylgalactosamine-4-sulfatase comprises growing a culture of cells transformed with a gene encoding *N*-acetylgalactosamine-4-sulfatase; preferably, the gene encodes human *N*-acetylgalactosamine-4-sulfatase. Preferably, the cells are mammalian cells. More preferably, the mammalian cells are Chinese Hamster Ovary cells. The obtaining step can

further comprise harvesting the fluid from said culture of cells. The obtaining step can further comprise concentrating said fluid to about 20x.

A feature of this method is an early separation of protease activity and the precursor *N*-acetylgalactosamine-4-sulfatase. This separation can comprise either (1) the reduction, inhibition, or inactivation of the protease activity, or (2) the physical separation of the protease(s) from the precursor *N*-acetylgalactosamine-4-sulfatase. Preferably, this separation occurs as early as possible during the purification process. The purpose is to keep to a minimum the number of molecules of precursor *N*-acetylgalactosamine-4-sulfatase being cleaved into the mature or processed form and/or other degraded form(s). The precursor form of *N*-acetylgalactosamine-4-sulfatase is the preferred form, as opposed to the mature or processed form, because it is more readily taken up into the target tissue and for subsequent targeting to the lysosome. The earlier or sooner the protease activity is separated from the precursor *N*-acetylgalactosamine-4-sulfatase; the fewer the number of molecules of the precursor form would be cleaved into the mature or processed form.

The activity of the protease is reduced or inhibited by adjusting the fluid to a pH value between about 4.8 to 8.0. Preferably, the pH value is between about 4.8 to 5.5. More preferably, the pH value is between about 4.8 and 5.2. The specific protease activity that is desired to be reduced is protease activity that specifically cleaves precursor form of *N*-acetylgalactosamine-4-sulfatase into the mature or processed forms. The protease activity is found in one or more cysteine protease. A cysteine protease that specifically cleaves precursor *N*-acetylgalactosamine-4-sulfatase is cathepsin L. This cathepsin L has a molecular weight of about 36 kDa in its inactive form that is converted to its active forms of 21-29 kDa in size upon exposure to pH of less than 5.0 (see Figure 9C). The pH can be adjusted into any value whereby the protease(s) is not converted from its inactive form to its active form and the desired precursor *N*-acetylgalactosamine-4-sulfatase, or biological activity thereof, is not harmed or not irreversibly harmed.

Preferably, step (c) comprises passing the fluid through a Cibracon blue dye interaction chromatography column. More preferably, the Cibracon blue dye interaction chromatography column is a Blue Sepharose 6 Fast Flow column. Preferably, step (d) comprises passing the fluid through a copper chelation chromatography column. More preferably, the copper chelation chromatography column is a Chelating Sepharose Fast Flow column. Preferably, step (e) comprises passing the fluid through a phenyl hydrophobic interaction chromatography column. More preferably, the phenyl hydrophobic interaction chromatography column is a Phenyl Sepharose 6 Fast Flow High Sub column. Preferably, the temporal sequence of steps (c), (d) and (e) is step (c), step (d) and step (e).

The recovering step can comprise ultrafiltration and/or diafiltration of the fluid. The recovering can comprise filtering the fluid to remove DNA and/or filtering the fluid to remove virus. The filtering, for removing virus, can comprise passing said fluid through a 0.02  $\mu$ m filter.

This method can also be used to purify a *N*-acetylgalactosamine-4-sulfatase or biologically active fragment, analog or mutant thereof.

The purity of rhASB is measured or determined using reverse-phase high performance liquid chromatography ("RP-HPLC"), which separates proteins based on differences in hydrophobicity. This assay uses a C4 column (Phenomenex Jupiter) as the stationary phase and a gradient of water:acetonitrile as the mobile phase. The protein samples are initially injected onto the column in water; under these conditions, all proteins will bind to the column. A gradually increasing concentration of acetonitrile is then infused through the column. This acetonitrile gradient increases the hydrophobicity of the mobile phase, to the point where individual proteins become soluble in the mobile phase and elute from the column. These elution times are accurately reproducible for each individual protein in a mixture. Proteins are detected as peaks on a chromatogram by ultraviolet absorbance at 210 nm. The areas of each peak are calculated, and the sample purity can be calculated as the ratio of the rhASB peak area to the total area of all peaks in the chromatogram. RP-HPLC is a proven high-resolution, reproducible method of determining the purity of rhASB.

Studies prior to this application indicate that the purity of ASB was determined by performing an impurity protein ELISA. This method of using impurity protein ELISA (the details of which are not disclosed) probably used antibodies raised against a mixture of potential host-cell impurity proteins. The ELISA would likely be performed using a standard curve of the same mixture of potential impurity proteins used to generate the antibodies. Test samples would likely be quantitated for impurity levels, relative to this standard mixture. These assays are valuable tools in protein purification, but are less accurate than RP-HPLC for determining the product purity for the following reasons:

- (1) In the RP-HPLC assay, the proportions of rhASB and impurities are both determined by the same measurement (UV absorbance). In the ELISA, the impurity concentration is determined by antibody binding whereas the target protein content is determined by another assay method (usually UV absorbance or Bradford). The "percent purity" value should be calculated as the ratio of two quantities with the same units, experimentally determined by the same method.
- (2) For the ELISA to work well, the sample detected by the antibody should have the same protein composition as the standard. This is very unlikely to be the case in an impurity protein ELISA. The assay standard for this type of assay would be a mixture of many individual proteins, against which the antibodies were generated. However, only a small subset of impurity proteins should be present in purified rhASB product. Therefore, the antibody reagent will now be detecting a different mixture of proteins, and the response versus the standard will probably be quite nonlinear. When this occurs, the assay yields a different net value for each sample dilution, so one does not know which dilution (if any) is giving the correct value.
- (3) In addition, not all potential impurity proteins are immunogenic or immunogenic to the same degree in animals such as rabbits, used to generate the antibodies. Therefore, the impurity level determined by ELISA may only reflect a subset of the impurities present in the purified products. It is entirely possible to have one or more major impurities in the product that are totally undetectable. In contrast, RP-HPLC detects all proteins because UV absorbance is a universal property of protein molecules.
- (4) Finally, there are two types of impurities in a purified drug product: product-unrelated impurities (host cell proteins as discussed above) and product-related impurities (degradation products including processed forms and aggregates). The latter cannot be detected by the impurity ELISA but can be readily detected by RP-HPLC.

Therefore, actual numbers obtained from the impurity protein ELISA are open to question, and RP-HPLC numbers are based on a firmer foundation.

In addition, SDS-PAGE analysis permits detection of both host cell impurities and processed or degraded forms of the desired drug protein product. When used in conjunction with Western blot, product-unrelated impurities from host cell contaminants can be differentiated from product-related impurities. Finally, SEC-HPLC permits a level of quantification of product-related impurities because it can detect impurities of different molecular weights, including lower molecular weight processed or degraded forms of the protein as well as monomers, dimers and other multimers.

An embodiment of this method of purification is depicted in Table 4.

**Table 4: Method of Purification**

Step	Process
Harvest Filtration	<ul style="list-style-type: none"> <li>Filtration through Clarification filters, 0.45µm filters and finally 0.2µm filter.</li> <li>Filtered pooled harvests are stored in polypropylene bags</li> </ul>
UF Concentration	<ul style="list-style-type: none"> <li>Equilibration and flush: 100 mM sodium phosphate, pH 7.3</li> <li>Load: filtered harvest fluid</li> <li>Concentration: Concentration to 20X</li> <li>Filtration: Filter the diluted product through a 0.2 µm filter into storage container</li> </ul>
pH adjustment and filtration	<ul style="list-style-type: none"> <li>pH adjustment: Add 10% glacial acetic acid to pooled 20X concentrates to a final pH of equal to or less than about 7.3; preferably, the pH is about 4.0 to 7.3; more preferably, the pH is about 4.5 to 5.5; even more preferably, the pH is about 5.0</li> <li>Load: pooled 20X concentrates</li> <li>Rinse: Water-for Injection (WFI)</li> <li>Filtration through Clarification filters and 0.2µm filter.</li> <li>Flush: 20 mM sodium acetate, 120 mM sodium chloride, pH 5.0</li> <li>The recovery yield can be at least about 83%</li> </ul>
Cibracon blue dye interaction chromatography column (Blue Sepharose 6 FF)	<ul style="list-style-type: none"> <li>Pre-Wash: 0.1N sodium hydroxide</li> <li>Wash: Water-for-Injection (WFI)</li> <li>Equilibration: 10mM sodium phosphate, pH is less than about 6.5; preferably, pH is about 5.0 to 6.5; more preferably, pH is about 6.45</li> <li>Load: pH adjusted and filtered pooled 20X concentrates</li> <li>Wash: 10mM sodium phosphate, pH 6.45</li> <li>Elution: 10mM sodium phosphate, 125mM sodium chloride, pH 6.45</li> </ul>
(Blue, Blue Sepharose)	<ul style="list-style-type: none"> <li>Regeneration: 10mM sodium phosphate, 1.0 M sodium chloride, pH 6.45</li> <li>Sanitization: 0.1 N sodium hydroxide</li> <li>Wash 1: Water-for-Injection (WFI)</li> <li>Wash 2: 10mM sodium phosphate, 1.0 M sodium chloride, pH 6.45</li> <li>Storage: 20% Ethanol</li> <li>The recovery yield can be at least about 84%</li> </ul>
Copper chelation chromatography column (Chelating Sepharose FF)	<ul style="list-style-type: none"> <li>Pre-Wash: 0.1N sodium hydroxide</li> <li>Wash: Water-for-Injection (WFI)</li> <li>Charge Buffer: 0.1M cupric sulfate</li> <li>Equilibration: 20mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH is less than about 6.0; preferably, pH is about 3.6 to 5.5; more preferably, pH is about 5.5</li> </ul>
(Copper, CC, Copper-Chelating)	<ul style="list-style-type: none"> <li>Load: Adjust glycerol content of pooled Blue Eluates to 10%, by adding 100mM sodium acetate, 2.0M sodium chloride, 50% glycerol, pH 5.2</li> <li>Wash 1: 20mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 5.5</li> <li>Wash 2: 20mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 3.9</li> <li>Elution: 20mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 3.6</li> <li>Eluate hold for 30-120 minutes prior to adjustment to pH 4.5 with 0.5M NaOH</li> <li>Regeneration: 50mM EDTA, 1.0M sodium chloride, pH 8.0</li> <li>Sanitization: 0.5M sodium hydroxide</li> <li>Storage: 0.1M sodium hydroxide</li> <li>The recovery yield can be at least about 86%</li> </ul>
Phenyl hydrophobic interaction chromatography column (Phenyl)	<ul style="list-style-type: none"> <li>Pre-Wash: 0.1 N sodium hydroxide</li> <li>Wash: Water-for-Injection (WFI)</li> </ul>

Step	Process
Sepharose 6 FF High Sub)	▪ Equilibration: 20mM sodium acetate, 2.0 M sodium chloride, pH is about 4.5 to 7.1; preferably, pH is about 4.5
	▪ Load: Adjust sodium chloride content of Copper Eluate to 2 M, by adding 20mM sodium acetate, 5 M sodium chloride, pH 4.5
	▪ Wash 1: 10 mM sodium phosphate, 2.0 M sodium chloride, pH 7.1
(Phenyl, Phenyl High Sub)	▪ Wash 2: 20mM sodium acetate, 2.0M sodium chloride, pH 4.5
	▪ Elution: 20mM sodium acetate, 250mM sodium chloride, pH 4.5
	▪ Regeneration: 20mM sodium acetate, pH 4.5
	▪ Sanitization: 0.5 N sodium hydroxide
	▪ Storage: 0.1 N sodium hydroxide
	▪ The recovery yield can be at least about 88%
UF/DF, DNA Filtration, Viral Filtration, Formulation	▪ Equilibration: 10mM sodium phosphate, 150mM sodium chloride, pH 5.8
	▪ Concentration: Concentration to NMT 1.5 mg/mL
	▪ Diafiltration: 10 mM sodium phosphate, 150 mM sodium chloride, pH 5.8
	▪ DNA Filtration: Product filtered through a DNA filter
	▪ Viral Filtration/Dilution: Product filtered through a 0.02 $\mu$ m filter and diluted to 1.0 mg/ml
	▪ Formulation: Polysorbate 80 at a concentration of 50 $\mu$ g/mL is added
	▪ Filtration: Filter the diluted product through a 0.2 $\mu$ m filter into storage container

The components of the drug product thus obtained are set forth in Table 5. The components of the drug product composition within the scope of the present invention are set forth in Table 6.

**Table 5: Drug Product Component**

Component	Description
Active Ingredient	Recombinant human <i>N</i> -acetylgalactosamine-4-sulfatase
Excipients	Sodium Phosphate, Monobasic, 1 H <sub>2</sub> O
	Sodium Phosphate, Dibasic, 7 H <sub>2</sub> O
	Sodium Chloride
Container	Kimble Glass, Type I 5 ml clear glass vial, Borosilicate West pharmaceuticals, S-127 4432150 Grey stopper

**Table 6: Drug Product Composition**

Component	Amount
RhASB	1 mg/mL
Sodium Phosphate, Monobasic, 1 H <sub>2</sub> O	9 mM
Sodium Phosphate, Dibasic, 7 H <sub>2</sub> O	1 mM
Sodium Chloride	150 mM

The invention having been described, the following- examples are offered to illustrate the subject invention by way of illustration, not by way of limitation.

#### EXAMPLE 1

##### Clinical Evaluation with recombinant human *N*-acetylgalactosamine-4-sulfatase

##### Summary

The indication for recombinant human *N*-acetylgalactosamine-4-sulfatase (rhASB) is the treatment of MPS VI, also known as Maroteaux-Lamy Syndrome. We propose a clinical development program for rhASB consisting of an initial open-label clinical trial that will provide an assessment of weekly infusions of the enzyme for safety, pharmacokinetics, and initial response of both surrogate and defined clinical endpoints. The trial will be conducted for a minimum of three months to collect sufficient safety information for 5 evaluable patients. At this time, should the initial dose of 1 mg/kg not produce a reasonable reduction in excess urinary glycosaminoglycans or produce a significant direct clinical benefit, the dose will be doubled and maintained for an additional three months to establish safety and to evaluate further efficacy.

##### Objectives



Our primary objective is to demonstrate safety of a weekly infusion of rhASB in patients with MPS VI for a minimum of a three-month period. Measurements of safety will include adverse events, immune response and allergic reactions (complement activation, antibody formation to recombinant enzyme), complete clinical chemistry panel (kidney and liver function), urinalysis, and CBC with differential.

One secondary objective is to evaluate efficacy by monitoring changes in several parameters known to be affected in MPS VI. These include a six-minute walk test (as a measure of exercise tolerance), full pulmonary function (PFT) evaluation, reduction in levels of urinary glycosaminoglycans and hepatomegaly (as measures of kidney and liver GAG storage), growth velocity, joint range of motion, Children's Health Assessment Questionnaire (CHAQ), visual acuity, cardiac function, sleeping studies, and two different global assessments; one performed by the investigator, one performed by the patient/caregiver. A second secondary objective is to determine pharmacokinetic parameters of infused drug in the circulation, and general distribution and half-life of intracellular enzyme using leukocytes and buccal tissue as sources of tissue. It is anticipated that these measures will help relate dose to clinical response based on the levels of enzyme delivered to the lysosomes of cells.

## Methods

We will conduct a single center, open-labeled study to demonstrate safety and to evaluate clinical parameters of treatment with rhASB in patients with MPS VI. Patients will be admitted for a two week baseline evaluation that will include a medical history and physical exam, psychological testing, endurance testing (treadmill), a standard set of clinical laboratory tests (CBC, Parcel 20, CH50, UA), a MRI or CAT scan of the body (liver and spleen volumetric determination, bone and bone marrow evaluation, and lymph node and tonsillar size), a cardiology evaluation (echocardiogram, EKG, CXR), an airway evaluation (pulmonary function tests), a sleep study to evaluate for obstructive events during sleep, a joint restriction analysis (range of motion will be measured at the elbows and interphalangeal joints), a LP with CNS pressure, and biochemical studies (buccal *N*-acetylgalactosamine-4-sulfatase activity on two occasions, leukocyte *N*-acetylgalactosamine-4-sulfatase activity on two occasions, urinary GAG on three occasions, serum generation for ELISA of anti-rhASB antibodies and 24 hour urine for creatinine clearance). In addition to the above evaluations, each patient will be photographed and videotaped performing some physical movements such as attempting to raise their hands over their heads and walking. Patients will be titrated with antihistamines such that pretreatment with these agents could be effectively employed prior to infusion of enzyme. The proposed human dose of 1 mg/kg (50 U/kg) will be administered weekly by i.v. infusion over 4 hours. The patient will remain in the hospital for the first two weeks, followed by short stays for the next four weeks. Treatment for the final six weeks will be conducted at a facility close to the patient's home. Patients will return to the hospital for a complete evaluation at three months. Should dose escalation to 2 mg/kg be required, the patients will follow the same schedule outlined above for the first twelve weeks. Under either scenario, a complete evaluation will also occur at 6 months from the time of entering the trial. Safety will be monitored throughout the trial. Patients completing the trial will be continued on therapy following an extended protocol for as long as safety and efficacy conditions warrant it until BLA approval.

## Patient Number and Enrollment Rate

A single patient will be enrolled at the onset of the trial, with two additional patients one month later, and two more patients two weeks later barring any unforeseen complications related to treatment. Additional patients will be admitted should any of the enrolled patients become critically ill, or if a child is in need of an acute clinical procedure for life threatening or harmful conditions.

## Diagnosis and Inclusion/Exclusion Criteria

The patient may be male or female, aged five years or older with a documented diagnosis of MPS VI confirmed by measurable clinical signs and symptoms of MPS VI, and supported by a diminished fibroblast or leukocyte ASB enzyme activity level. Female patients of childbearing potential must have a negative pregnancy test (urine  $\beta$ -hCG) just prior to each dosing and must be advised to use a medically accepted method of contraception throughout the study. A patient will be excluded from this study if the patient has previously undergone bone marrow transplantation; is pregnant or lactating; has received an investigational drug within 30 days prior to study enrollment; or has a medical condition, serious intercurrent illness, or other extenuating circumstance that may significantly decrease study compliance.

## Dose, Route and Regimen

Patients will receive rhASB at a dose of 1 mg/kg (~50 U/kg) for the first 3 months of the study. In the event that excess urine GAGs are not decreased by a reasonable amount and no clinical benefit is observed, the dose will be doubled. Dose escalation will occur only after all 5 patients have undergone 3 months of therapy. This rhASB dosage form will be administered intravenously over approximately a four-hour period once weekly for a minimum of 12 consecutive weeks. A peripheral intravenous catheter will be placed in the cephalic or other appropriate vein and an infusion of saline begun at 30 cc/hr. The patient will be premedicated with up to 1.25 mg/kg of diphenylhydramine i.v. based on titration experiments completed prior to the trial. rhASB will be diluted into 100 cc of normal saline supplemented with 1 mg/ml human albumin. The diluted enzyme will be infused at 1 mg/kg (about 50 units per kg) over a 4 hour period with cardiorespiratory and pulse oximeter monitoring. The patients will be monitored clinically as well as for any adverse reaction to the infusion. If any unusual symptoms are observed, including but not limited to malaise, shortness of breath, hypoxemia, hypotension, tachycardia, nausea, chills, fever, and abdominal pain, the infusion will be stopped immediately. Based on clinical symptoms and signs, an additional dose of diphenylhydramine, oxygen by mask, a bolus of i.v. fluids or other appropriate clinical interventions such as steroid treatment may be administered. If an acute reaction does occur, an assessment for the consumption of complement in the serum will be tested. A second i.v. site will be used for the sampling required for pharmacokinetic analysis.

## Evaluable patients

The data from any given patient will be considered evaluable as long as no more than two non-sequential infusions are missed during the 12 weeks of therapy. The initial, midpoint and final evaluations must be completed.

## Safety

The enzyme therapy will be determined to be safe if no significant acute reactions occur that cannot be prevented by altering the rate of administration of the enzyme, or acute antihistamine or steroid use. The longer-term administration of the enzyme will be determined to be safe if no significant abnormalities are observed in the clinical examinations, clinical labs, or other appropriate studies. The presence of

antibodies or complement activation will not by themselves be considered unsafe, but such antibodies will require monitoring by ELISA, and by clinical assessments of possible immune complex disease.

### Efficacy

One purpose of this study is to evaluate potential endpoints for the design of a pivotal trial. Improvements in the surrogate and clinical endpoints are expected as a result of delivery of enzyme and removal of glycosaminoglycan storage from the body. Dose escalation will be performed if mean excess urinary glycosaminoglycan levels are not reduced by a reasonable amount over three months and no significant clinical benefit is observed at 3 months. Improvements are expected to be comparable to those observed in the recently completed MPS I clinical trial and should include improved airway index or resolution of sleep apnea, improved joint mobility, and increased endurance.

### EXAMPLE 2

A comprehensive review of the available information for the MPS VI cat and relevant pharmacology and toxicology studies is presented below: Enzyme replacement therapy has been established as a promising treatment for a variety of inherited metabolic disorders such as Gaucher Disease, Fabry Disease and Mucopolysaccharidosis I. In some of these disorders a natural animal model offers the ability to predict the clinical efficacy of human treatment during pre-clinical studies. This was found to be true in MPS I (canine model). With this in mind, studies have been performed with the MPS VI cat prior to the commencement of human studies for this disease. Sufficient safety and efficacy data exist to proceed with a clinical trial in human MPS VI patients.

Studies of rhASB MPS VI cats indicate that no cat has died as a result of drug administration. As predicted, experiments in MPS VI cats also indicate that rhASB uptake is dependent on the presence of mannose 6-phosphate modified carbohydrate sidechains. RhASB in MPS VI cats has also been shown to clear storage from a variety of major organs and moderately alters bone density. Long-term dose-ranging efficacy studies suggest that a dose of 1 mg/kg/week is the lowest concentration to see significant clinical benefits. Studies has also been performed to compare enzyme distribution, clearance of tissue glycosaminoglycan storage, and decrease of urinary glycosaminoglycan levels after bolus and slow (2 hour) infusion. Studies in progress continue to evaluate the safety of weekly infusions of the projected clinical dose of 1 mg/kg of rhASB in cats suffering from MPS VI.

A spontaneous form of MPS VI in several families of Siamese cats was identified in the 1970's (Jezyk, Science 198:834-36 (1977)), and detailed reports of the pathological changes in these animals have been published (Haskins, et al., Am. J. Pathol. 101:657-674 (1980); Haskins, et al., J. Am. Vet. Med Assoc. 182:983-985 (1983); Konde, et al., Vet. Radiol. 28:223-228 (1987)). Although the clinical presentation of these cats is somewhat variable, they all exhibit general changes that have been reported in the literature (Jezyk, et al., Science 198:834-36 (1977); Konde, et al., Vet. Radiol. 28:223-228 (1987); Crawley, "Enzyme replacement therapy in a feline model of mucopolysaccharidosis type VI" PhD thesis, University of Adelaide, Adelaide, S. Australia, (1998)). Table 6 has been constructed from these sources to provide the "average" changes one would expect to observe in an untreated MPS VI cat:

**Table 7: MPS VI Cat Model**

Clinical Observation	Timing of Onset	Changes Relative to Human disease (independent of time)
• Facial dysmorphism:	2 months	• Similar to human disease
• Small head,		
• Broad maxilla,		
• Small ears		
• Diffuse corneal clouding	2 months	• Similar to human disease
• Bone abnormalities:	First signs at 2 months - progressive	• Similar to human disease - alterations in enchondral calcification
• Epiphyseal dysplasia,		
• Subluxations,		
• Pectus excavatum		
• Reduced body weight	3 months	• Similar to human disease
• Reduced cervical spine flexibility	Normal cat value is 180° at all ages.	• Similar to human disease
	In MPS VI:	
	3 months: 130-170°	
	5 months: 45-130°	
	6 months: 30-100°	
	11 months: 20-80°	
• Osteoporosis/Degenerative Joint Disease	1 year or more	• Similar to human disease
• Hind limb gait defects	See table below	• Carpal tunnel syndrome
• Hind limb paresis or paralysis		• C <sub>1</sub> -C <sub>2</sub> subluxation,
(thoracolumbar cord compressions)		• Cervical cord compression secondary to thickened dura more typical

Clinical Observation	Timing of Onset	Changes Relative to Human disease (independent of time)
• Grossly normal liver and spleen		• Liver and spleen enlarged in humans
• Thickened cardiac valves		• Similar to human disease
• No CNS lesions - mild lateral ventricle enlargement		• May be comparable to hydrocephalus in human disease

Other biochemical/morphological determinations indicate that by 35 days, organs of untreated cats have maximal storage of glycosaminoglycans in tissues (Crawley, et al., J. Clin. Invest. 99:651-662 (1997)). Urinary glycosaminoglycan levels are elevated at birth in both normal and MPS VI cats but after approximately 40 days, normal cats have decreased levels. MPS VI cats urinary glycosaminoglycans remain elevated or continue to increase until reaching steady state after approximately 5 months.

Variability in clinical presentation is seen in affected littermates. In addition to some variability in the timing of onset of particular abnormalities, the time course of progression for some of the clinical and pathological changes is also variable. In general, the bone lesions are typically progressive (Konde et al., Vet. Radiol. 28:223-228 (1987)), while the corneal clouding is not. In addition, some paralyzed cats have been noted to improve to severe paresis with time. Studies detailing disease progression in individual cats are limited to clinical (or radiographic) observations. Some of these have distinct pathological correlations, such as neurological deficit and cord compression secondary to proliferation of bony tissue in the thoracolumbar region (Haskins, et al., J. Am. Vet. Med. Assoc. 182:983-985 (1983)).

A six-month efficacy study enzyme replacement therapy using recombinant feline ASB in newborn MPS VI cats was conducted. This was prompted by the observation that several treated MPS VI cats developed antibodies to the human enzyme (refer to section 6.5). These antibodies may alter uptake and stability of the enzyme (Brooks, et al., Biochim. Biophys. Acta 1361:203-216 (1997)). Feline enzyme was infused at 1 mg/kg weekly. The major conclusions of the study were that urinary GAG, body weight/growth, bone morphometry and clearance of stored material from several tissues was improved relative to the same dose of human recombinant enzyme used in the previous study, that antibodies were not detected beyond the range observed in normal cats, and that the feline enzyme dose at 1 mg/kg was comparable in reversing disease as the human enzyme dose at 5 mg/kg in a head-to-head comparison (Bielicki, et al., J. Biol. Chem., 274:36335-43 (1999)). These studies indicate that an incremental improvement in endpoints and immunogenicity is possible when the cat-derived enzyme is given to cats. This provides additional support to dosing human patients with the human enzyme at 1 mg/kg/week. The results of this study are set forth in Table 7.

**Table 8: Efficacy of Weekly Bolus Injections of CHO-derived Recombinant Feline ASB in Newborn MPS VI Cats**

	Results	
<b>Dose</b>	1 mg/kg	
<b>Duration</b>	6 months (n=2)	3 months (n=3)
<b>Urinary GAGS</b>	Decreased to 2x normal	Decreased to 2x normal
<b>Antibody titers</b>	• Within range observed in normal cats	• To be completed
<b>Clinical</b>		
<b>Appearance</b>	• Persistent corneal clouding	• Persistent corneal clouding
	• Some resolution of facial dysmorphism	• Some resolution of facial dysmorphism;
	• Improved body shape	• Improved body shape
<b>Weight</b>	• Heavier than normal	• Slightly lighter than normal
<b>Spine Flexibility (normal = 180°)</b>	160°-180°	Not examined
<b>Neurological</b>	• Normal	• Normal
<b>Radiology</b>	• Improved quality	• Not examined
	• Density and dimensions of bone (similar to 1 mg/kg rh4S in ref. 10)	
<b>Gross</b>		
<b>Bone/Cartilage Thickness</b>	• Variable; decreased cartilage thickness	• Not examined
	• more uniform subchondral bone (similar to 1 mg/kg rh4S")	
<b>Spinal Cord</b>	• No compressions present	• Not examined
<b>Cellular Level</b>		
<b>Liver (Kupffer)</b>	• Complete lysosomal storage clearing	• Complete lysosomal storage clearing
<b>Skin</b>	• Almost complete reduction in storage	• Mild reduction
<b>Cornea/Cartilage (ear, articular)</b>	• No clearance of lysosomal storage compared with untreated MPS VI controls	• No clearance of lysosomal storage

<b>Gross</b>		
<b>Heart Valves</b>	• Significant reduction in lysosomal storage	• To be completed
<b>Aorta</b>	• Almost complete reduction in	• Mild reduction in
<b>Results</b>		
<b>Dose</b>	1 mg/kg	
<b>Duration</b>	6 months (n=2)	3 months (n=3)
	lysosomal storage	lysosomal storage

Table 9 provides a summary of all studies performed using recombinant human ASB in the MPS VI cat model.

**Table 9: RhASB Study Results**

No. Cat	Dose	Duration (Mo.)	Route of Administration	Urinary GAGS	Histopathology
1	0/8 mg/kg/14 d	7-22	Bolus i.v.	Decreased 50% compared to untreated cat	• Normalization of vacuolization in liver
	1.5 mg/kg/7d	22-27	Bolus i.v.		• Significant reduction in kidney and skin
1	0.5 mg/kg/14 d	12-23	Bolus i.v.	Decreased to near normal	• No correction in cornea and chondrocytes
	1.4 mg/kg/7 d	23-27	Bolus i.v.		• No kidney immune complex deposition
1	0.8 mb/kg/14 d	2-15	Bolus i.v.		
1	0.2 mg/kg/8 d	6	Bolus i.v.	Marginal reduction compared to untreated	N/A
4		5/6	Bolus i.v.	Decreased and maintained at 3x normal compared to untreated at 10x normal	• Complete lysosomal storage clearing in liver cells
					• No evidence of renal impairment or glomerular immune complex deposition
	1 mg/kg/7 d				• Significant reduction of lysosomal storage in heart valves
1		11	Bolus i.v.		• Gradient storage content from media to adventia in aorta
					• Mild reduction of lysosomal storage of skin (hip joint, dura, kidney)
					• No evidence of renal impairment or glomerular deposition
2		5/6	Bolus i.v.	Decreased and maintained at 2x normal compared to untreated at 10x normal	• No significant changes in lysosomal storage of cornea/cartilage
					• Complete lysosomal storage in clearing in liver and skin (hip joint, dura, kidney)
	5 mg/kg/7 d				• No evidence of renal impairment or glomerular deposition Near complete reduction in lysosomal storage in heart valves Thin band of vacuolated cells in outer tuncia media
1		11	Bolus i.v.		• No evidence of renal impairment or glomerular deposition
					• Near complete reduction of lysosomal storage in heart valves
					• Thin band of vacuolated cells in outer tuncia media
2	0.5 mg/kg 2x weekly				• Complete lysosomal clearing in liver
		6	Bolus i.v.	Decreased to 3x normal	• Mild to moderate reduction in skin
					• Variable reduction of lysosomal storage of heart valves
2					• Mild reduction of lysosomal storage in aorta
			Long infusion (2	Reduced after first or	• Reduction of lysosomal storage in

No. Cat	Dose	Duration (Mo.)	Route of Administration	Urinary GAGS	Histopathology
			hr)	second infusion to below untreated MPS VI cats	reticuloendothelial cells and very mild in heart valve and aorta after 5 infusion
2	1 mg/kg/7 d	1	Short infusion (10 min)		
5	1 mg/kg/7 d	6	Long infusion (2 hr)		

**EXAMPLE 3****Distribution and Feasibility**

An initial study was performed to document enzyme uptake and distribution, and to serve as a pilot study of potential endpoints for future efficacy studies (Crawley, et al., J.Clin.Invest. 97:1864-1873 (1996)). Recombinant human ASB was administered by bolus injection to affected cats once per week or once every two weeks at 0.5 up to 1.5 mg/kg. Evaluation of one untreated MPS VI cat (Cat D), and one normal cat provided the values from which comparisons were drawn. The data from the one untreated cat was further supported by historical assessment of 38 additional untreated cats. The acute uptake and distribution studies were conducted in normal cats using an immune assay technique that allowed the detection of human ASB in the presence of normal cat enzyme.

The major conclusions of these studies demonstrated wide uptake of enzyme with the expected predominance of liver and spleen uptake as observed in other enzyme replacement studies in MPS animal models. The uptake efficiency was dependent on the presence of mannose 6-phosphate modified carbohydrate side-chains on the enzyme. The half-life of the enzyme was determined to be 2-4 days. Therapeutically, the enzyme did clear storage from a variety of major organs and did moderately alter bone density. The cornea, bone morphology and cartilage defects were not effectively treated in older MPS VI cats. The study results are summarized in Table 10.

**Table 10: Summary: Distribution/Feasibility MPS VI Cat Study**

Table 10: Summary: Distribution/Toxicology MPS VI Cat Study					
Parameter	Findings				
Cat	A		B		C
	Treated MPS VI		Treated MPS VI		Treated MPS VI
Dose	0.8 mg/kg per 14 d	1.5 mg/kg per 7 d	0.5 mg/kg per 14 d	1.4 mg/kg per 7 d	0.8 mg/kg per 14 d
Age at dose (mo.)	7*-22	22-27	12*-33	23-27	2*-15
Infusion Parameters	2-10 ml (PB S) via cephalic v. for 5-20 minutes				
Plasma t <sub>1/2</sub> (i.v. bolus)	<ul style="list-style-type: none"><li>• 13.7±3.2 min @ 1 mg/kg</li><li>• 45 min @ 7.5 mg/kg</li></ul>				
	<i>All values relative to endogenous feline ASB enzyme four hours after infusion of 1 mg/kg rhASB in normal cats</i>				
	<ul style="list-style-type: none"><li>• Liver: 495x</li><li>• Spleen: 6x</li><li>• Lung: 22.3x</li><li>• Heart: 4.3x</li><li>• Aorta: 4x</li><li>• Skin: 31x</li><li>• Cartilage: 0x</li><li>• Cornea 0x</li></ul>				
Tissue t <sub>1/2</sub>	2-4 days @ 1 mg/kg in most organs (detectable enzyme in most tissues of cat B, but only in liver of A after 7 days)				
Neurological	<ul style="list-style-type: none"><li>• Ambulation fluctuated, but improved on higher dose</li></ul>	N/A		<ul style="list-style-type: none"><li>• Marginal progression to paretic gate by end of study</li></ul>	
Corneal Opacity	<ul style="list-style-type: none"><li>• Did not change with therapy (slit lamp exam 3x late in rx)</li></ul>				
Skeletal (x-rays) 4 views every 3 mo.	<ul style="list-style-type: none"><li>• Lesions progressed (no radiographic improvement)</li><li>• Increased bone volume/trabecular # in cat C (received earlier rx)</li><li>• Vertebral compression in cat C</li></ul>				
Anaphylaxis	<ul style="list-style-type: none"><li>• No anaphylaxis, minimal distress on infusion;</li></ul>				
Antibody response (Ig titers)	1 x 10 <sup>6</sup> (plasma could inhibit	64,000		64,000	

Parameter	Findings		
Cat	A	B	C
Untreated MPS VI = 4,000-32,000	enzyme activity <i>in vitro</i> )		
Urinary GAGS (at - 400 days)	• Decreased 50% compared to untreated cat	• Decreased to near normal	
Urinary dermatan sulfate (~ 400 days)	• Midway for all 3 cats (relative to untreated control D and normal)		
Body Weight	• 2.5-3.0 kg vs. normal 4-7 kg		
Liver/Spleen	• Grossly normal		
Heart Valves	• Grossly normal		
Cartilage	• Abnormal thickness and formation		
Microscopy (vacuolization)	• Normalization of vacuolization in liver,		
	• Significant reduction in kidney and skin,		
	• No correction in cornea and chondrocytes		
Kidney immune complex deposition	• Absent		

## EXAMPLE 4

## Efficacy in MPS VI Cats Treated from Birth

A long term dose-ranging efficacy study was performed in MPS VI cats starting at birth (Crawley, et al., J. Clin. Invest. 99:651-662 (1997)), and is summarized in Table 10. MPS VI cats were treated weekly with bolus i.v. injections of 0.2, 1 and 5 mg/kg of rhASB beginning at birth. A total of 9 cats were treated for 5, 6 or 11 months. In addition, 12 MPS VI and 9 normal cats were included as untreated controls. The major conclusions are that 0.2 mg/kg dose did not alter disease progression in the one cat studied, and the only documented clinical benefit was a reduction in the storage in liver Kupffer cells. Urinary GAG levels decreased to near normal during the trial in the higher dose groups. In addition to improvements in the major organs, the higher doses of therapy from birth were able to prevent or ameliorate the bony deformity of the spine and the abnormal form of many bones. There was a dose-dependent effect on improvement in L-5 vertebral bone mineral volume, bone trabecular thickness, and bone surface density between the 1 and 5 mg/kg doses, although both were equivalent in improving bone formation rate at 5 to 6 months of ERT (Byers, et al., Bone 21:425-431 (1997)). The mitral valve and aorta was dependent on dose and was less complete at 1 mg/kg but nearly complete at 5 mg/kg. No improvement of storage in cartilage and cornea was observed at any dose. The study suggests that the 1 mg/kg/week dose is the lowest concentration to see significant clinical benefit. The study results are summarized in Table 11.

**Table 11: Efficacy of Weekly Bolus Injections of CHO-derived Recombinant Human ASB in Newborn MPS VI Cats (Study PC-BM102-002)**

	Results			
Dose	1 mg/kg		5 mg/kg	
Duration	5/6 mo	11 mo	5/6 mo	11 mo
N	4	1	2	1
Biochemical				
Urinary GAGs	• Decreased and maintained at 3x normal compared to untreated at 10x normal		• Decreased and maintained at 2x normal compared to untreated at 10x normal	
Clinical				
Appearance	• Variable changes;		• Variable changes;	
	• Persistent corneal clouding by slit lamp		• Persistent corneal clouding by slit lamp	
Weight	• Intermediate (no rx vs. normal)		• Intermediate (no rx vs. normal)	
Spine Flexibility (normal=180) (untreated MPS VI=90°)	130-160°	90°	180°	160°
Neurological	mild hindlimb paralysis	• No deficits	• No deficits	• No deficits
Radiology	• Improved bone quality, density and dimensions		• Improved bone quality, density and dimensions	
			• Possibly superior to 1 mg/kg	
Gross				
Bone/Cartilage Thickness	• Variability, but improved	• Degenerative joint disease present	• Variability, but improved	• Degenerative joint disease present

	Results			
Dose	1 mg/kg		5 mg/kg	
Duration	5/6 mo	11 mo	5/6 mo	11 mo
Spinal Cord	• 1 of 4 with several mild compressions	• No compressions	• No cord compressions	
Cellular Level				
Liver (Kupffer)	• Complete lysosomal storage clearing	• Maintained	• Complete lysosomal storage clearing	• Maintained
Skin (hip Joint, Dura, Kidney)	• No evidence of renal impairment or glomerular immune complex deposition	• Mild reduction in lysosomal storage	• Complete lysosomal storage clearing	• Maintained
		• No evidence of renal impairment or glomerular deposition	• No evidence of renal impairment or glomerular deposition	• No evidence of renal impairment or glomerular deposition
Cornea/Cartilage (ear, articular)	NA	• No significant changes in lysosomal storage	NA	• No significant changes in lysosomal storage
Heart Valves	• Significant (Variable) reduction in lysosomal storage	• Significant (variable) reduction in lysosomal storage near complete	• Near complete reduction in lysosomal storage	
Aorta	• Gradient of storage content from media to adventitia		• Thin band of vacuolated cells in outer tunica media	

**EXAMPLE 5****Efficacy of Twice Weekly Infusions of Recombinant Human ASB in Newborn MPS VI Cats**

A six-month study was performed in newborn cats to evaluate a 0.5 mg/kg infusion given twice weekly. In addition, the enzyme used in this study was derived exclusively from the CSL-4S-342 cell line. The major conclusions of the study include that compared with the previously reported 1 mg/kg weekly dose, this study produced similar improvements in physical, biochemical, neurological and radiographic parameters. The most notable differences were slightly worsened cervical spine flexibility, and less clearance of lysosomal storage in the denser connective tissues such as the heart valves and aorta. The results are summarized in Table 12.

**Table 12: Efficacy of Twice Weekly Bolus Injections of CHO-derived Recombinant Human ASB in Newborn MPS VI Cats**

Parameter	Results
<b>Dose</b>	0.5 mg/kg
<b>Duration</b>	2x weekly; 6 months (n=2; cats 225f, 226m)
<b>Urinary GAGs</b>	• Decreased to 3x normal
<b>Antibody titres</b>	• Within range observed in normal cats
<b>Clinical</b>	
<b>Appearance</b>	• Persistent corneal clouding
	• Some resolution of facial dysmorphism
	• Improved body shape
<b>Weight</b>	• Intermediate (between no treatment and normal)
<b>Spine Flexibility (normal = 180°)</b>	90° - 150°
<b>Neurological</b>	• No hind limb paralysis
<b>Radiology</b>	• Improved quality, density and dimensions of bone (similar to 1 mg/kg rh4S in ref. 110)
<b>Gross</b>	
<b>Bone/Cartilage Thickness</b>	• Variable; decreased cartilage thickness and more uniform subchondral bone (similar to 1 mg/kg rh4S°)
<b>Spinal Cord</b>	• No compressions present
<b>Cellular Level</b>	
<b>Liver (Kupffer)</b>	• Complete lysosomal clearing
<b>Skin</b>	• Mild to moderate reduction in storage

Parameter	Results
Cornea/Cartilage (ear, articular)	• No clearance of lysosomal storage compared with untreated MPS VI controls
Heart Valves	• Variable reduction in lysosomal storage (complete in 225f; no change from untreated in 226m)
Aorta	• Mild reduction in lysosomal storage

**EXAMPLE 6****Evaluation of Enzyme Uptake and Distribution as a Function of the Rate of Enzyme Infusion in MPS VI Cats**

The primary goal of this study was to compare enzyme distribution, clearance of tissue GAG storage, and decrease of urinary GAG levels after bolus infusion and after slow (2 hour) infusion of an identical 1 mg/kg dose. The slow administration proposal is based on experience from preclinical and clinical studies of  $\alpha$ -L-iduronidase for the treatment of MPS I. In addition, the study provided the first data that enzyme produced at BioMarin from cell line CSL-4S-342 is biologically active and safe. Major conclusions of the study include that all four cats (two per group) treated in this study showed no acute adverse reaction to either the slow or fast infusion, and no detrimental effects of repeated enzyme infusions. However, bolus infusion results in high liver uptake which is not preferred. Slow infusion provides better distribution into tissues and therefore is a preferred method for clinical trial.

The tissue distribution of rhASB obtained in the study suggested that 2-hour infusions might increase enzyme levels in other organs apart from the liver, including increased activity in the brain. Reduction in urinary GAG was observed immediately after the first or second infusion to levels below the range observed in untreated MPS VI cats. Correction of lysosomal storage was observed in reticuloendothelial cells and very mild in some fibroblasts (heart valve) and smooth muscle cells (aorta) after 5 infusions. No other significant clinical response to infusions was observed in either group, however this was not unexpected due to the short duration of the study, and due to therapy starting after significant disease changes had already developed. The extended 2-hour infusion was safe and well tolerated relative to the shorter protocols used in previous studies. The 2-hour infusion may provide improvement in enzyme distribution based on the one cat that was evaluable for enzyme tissue distribution.

**EXAMPLE 7****6 Month Safety Evaluation of Recombinant Human N-acetylgalactosamine-4-sulfatase in MPS VI Affected Cats**

Two 6 month studies in MPS VI cats have been initiated using the enzyme produced by the manufacturing process according to the present invention. The purpose of these studies is to evaluate the safety and efficacy of weekly infusions of the projected human clinical dose of rhASB in cats suffering from MPS VI. Study 6 involves kittens dosed initially at 3 to 5 months of age. Study 7 involves kittens treated from birth with weekly infusions of the projected human clinical dose of rhASB. The studies are intended to assess potential toxicology. Cats will be observed for changes in behavior during infusion of the recombinant enzyme to assess possible immune responses. Serum will be monitored for complement depletion and for the formation of antibody directed against the recombinant enzyme. General organ function will be monitored by complete clinical chemistry panels (kidney and liver function), urinalysis, and complete blood counts (CBC) with differential. Urinary glycosaminoglycan levels will be monitored on a weekly basis at a set time points relative to enzyme infusion. Evidence of clinical improvements in disease will be documented. These data will provide additional assessment of the potential efficacy of the treatment and will validate the activity and uptake of the enzyme *in vivo*. The studies have and will be conducted in a manner consistent with the principles and practices of GLP regulations as much as possible.

Preliminary results of the first study indicate that administration of rhASB has not had any detrimental effects on any of the animals, with bodyweights and clinical chemistries generally maintained within reference ranges. However, both of the cats with significantly elevated antibody titers developed abnormal clinical signs during infusions, however both animals behaved normally once enzyme infusions ceased and did not appear to suffer any longer term ill effects. Extended infusion times (4 hours) and increased premedication antihistamines have allowed continued therapy in the cats without any abnormal clinical signs. Mild reduction in urinary GAG levels suggest some efficacy of therapy in reducing stored glycosaminoglycans in tissues or circulation, however fluctuations in these levels were observed over time making interpretation difficult. None of the 5 cats have shown obvious clinical improvements in response to ERT, but this will require at least 6 month treatment based on previous studies<sup>23</sup>. Antibody titers have developed in four out of the five cats, with noticeable increases in titers observed after 2 months of ERT. Two of these cats have developed significantly elevated titers after 2 or 3 months.

**EXAMPLE 8****Safety Profile for MPS VI Cats Treated with rhASB**

A study has commenced enrolling affected cats that were treated within 24 hours of birth. Forty-one MPS VI cats have been treated using rhASB. Administration of enzyme to normal cats has been restricted to one to two cats to confirm acute safety of new batches prior to exposure of the valuable affected animals to therapy. In summary, no MPS VI cat has died as a result of drug administration, although four cats have died as a result of viral infection or an underlying congenital abnormality. Enzyme for the studies was produced according to the production methods of the present invention. The preliminary data are set forth in Table 13.

**Table 13: MPS VI Cat Efficacy Study Summary from Hopwood Laboratory**

Study #	# of Cats	Dose/wk (mg/kg)	Rx Length (mos.)	Mortality
1	2	Variable	13-21	None
	1			
2	1	0.2	5	None
2	1	0.2	1	Died: congenital heart defect



Study #	# of Cats	Dose/wk (mg/kg)	Rx Length (mos.)	Mortality
-	2	0.5	3-5	1 died parvovirus
2	4	1	3-11	1 died parvovirus
	2			
-	1	1	6 (s.c.)	None
-	4	1	6	None
2	2	5	3-11	1 died parvovirus
	2			
4	2	0.5 (twice)	5	None
-	3	0.5 (twice)	5	None
5	4	1	1	None
6	5	1	Started	None
7	5	1	Started	None

**EXAMPLE 8A****Tissue Distribution with Slow Infusion**

A separate study was undertaken to compare the rate of uptake and tissue distribution of rhASB in young MPS VI cats (<10 weeks old at initiation) using a long- (two hours) or a short-infusion (10 -15 minutes) protocol (ASB-PC-004). Seven normal cats (12-24 months old) were sedated and had femoral artery cannulae implanted to allow blood sampling. rhASB at a dose of 1.0 mg/kg or 7.3 mg/kg was infused into the cephalic vein in a volume of 7 mL over 40 seconds. Serial heparinized arterial blood samples were obtained at approximately 2, 4, 6, 8, 10, 20, 30, 40, 60 and 120 minutes post-dose. Plasma was collected by centrifugation and frozen until analysis. After four hours, animals were euthanized and tissues were collected, weighed and frozen until analysis.

In a separate study, three normal cats (8-66 months old) received a 1.0 mg/kg IV bolus dose of rhASB in a 7 mL dose volume. One cat per time point was euthanized at 2, 4 or 7 days post-dose. Selected tissues were collected, weighed and frozen until analysis. One additional normal cat, not exposed to rhASB, was euthanized to provide feline ASB levels in tissues for analytical comparisons. rhASB or feline ASB were quantitated in plasma and tissues was performed using an ELISA assay technique. rhASB in samples was adsorbed to an immobilized anti-rhASB monoclonal antibody and feline ASB was adsorbed to a polyclonal anti-rhASB antibody that cross-reacts with feline ASB. The adsorbed ASB was then quantitated using a fluorescent substrate.

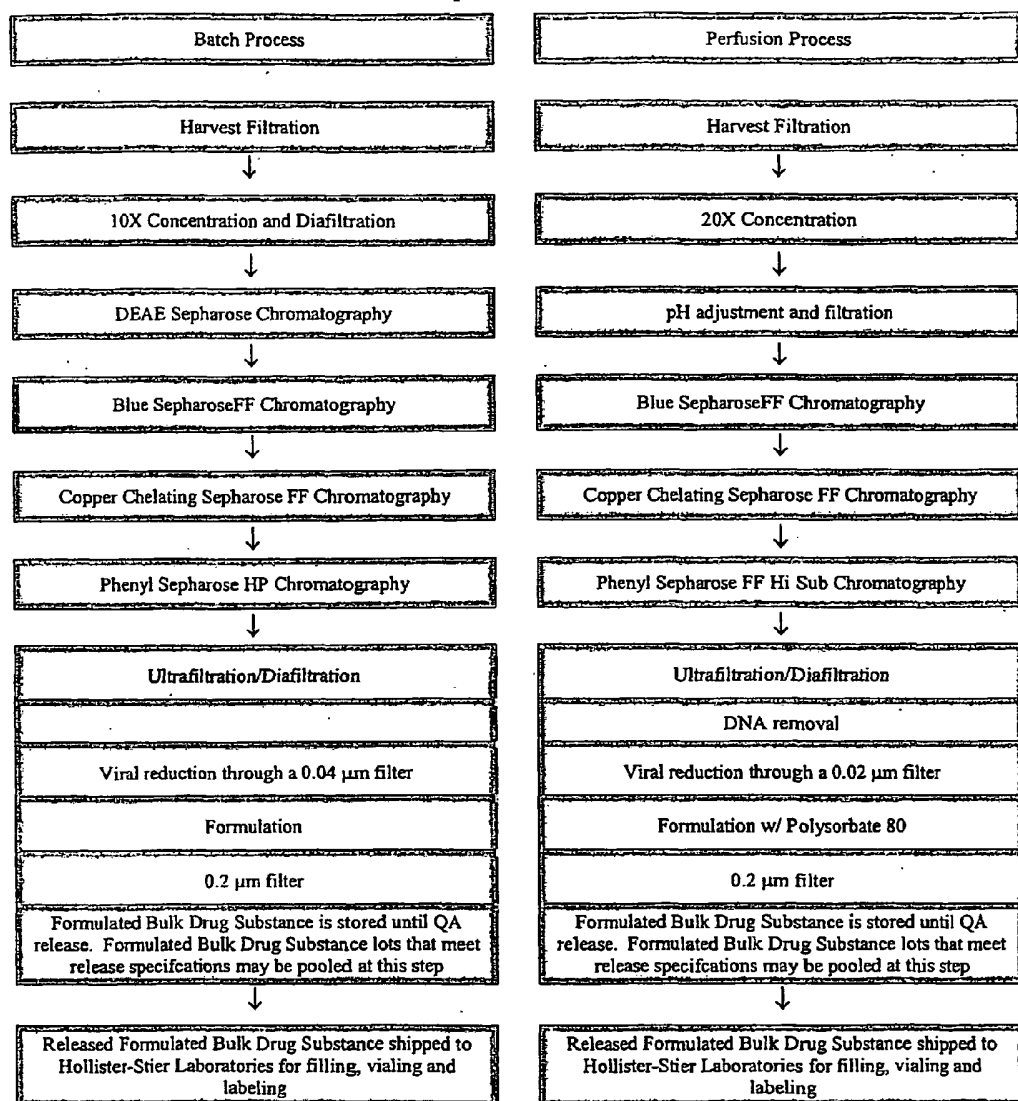
Plasma concentration analyses showed that the t<sub>1/2</sub> of rhASB at the 1.0 mg/kg dose was 13.7 ± 3.2 minutes and was ≈45 minutes for the 7.3 mg/kg dose. Tissue t<sub>1/2</sub> following the 1.0 mg/kg dose ranged from 2.4 -4.2 days in the liver, spleen, lung, kidney and heart. Low, but detectable levels were seen in these tissues (except heart) up to seven days following dosing. Four hours after a dose of 1.0 mg/kg ASB, the majority of the dose delivered to the liver, was detected in all tissues measured except cartilage and cornea, with the majority in the liver. With the exception of the cerebrum and cerebellum, levels were 4- (aorta) to 495- (liver) fold higher than those of feline ASB in the untreated control cat. The tissue half-lives determined in this study (2.4 -4.2 days at 1.0 mg/kg) support the weekly clinical dosing frequency.

The primary objective of this study was to evaluate the effects of infusion rate on enzyme distribution, clearance of tissue GAG storage, and decrease of urinary GAG levels. Groups (n =2/group) of 10-week-old MPS VI cats were administered short (10-15 minutes) or long (two hours) IV infusions of rhASB, 1.0 mg/kg once weekly for five weeks. Animals were euthanized two days after the last infusion and selected tissues (liver, spleen, heart, lung, kidney, skin, aorta, cerebrum, cerebellum, cartilage, cornea and lymph nodes) were collected for determination of ASB activity and for histopathological evaluation. Tissues were stored frozen until analysis. The study employed the same immune assay to determine the tissue ASB levels as described above (ASB-PC-001). One cat in the two-hour infusion group had reduced tissue enzyme levels compared to the other treated cats. High levels of enzyme were detected around the catheterization site following the last dose while low levels were seen in the contralateral limb. These findings suggest that the catheter had dislodged prematurely and had contributed to the low tissue levels of ASB seen in the tissues from this animal. Comparison of enzyme activities in the remaining three cats showed increased enzyme activity in the liver, lung, kidney, cerebrum and cerebellum and reduced activity in the mesenteric lymph nodes of the two-hour infusion cat relative to the 10-minute infusion animals. No enzyme was found in the skin, cartilage and corneas. Although the data from this study is limited, the findings from this study suggest that two-hour infusions may result in increased enzyme levels in the tissues (apart from the liver) relative to 10-minute infusions, indicating that the extended infusions provide better tissue distribution.

**EXAMPLE 9****Method of Manufacture and Purification (Perfusion Process)**

A process flow diagram comparing the purification processes for the fed batch and perfusion-based cell culture processes is provided in Table 14. Comparisons to the fed batch purification process as well as details of the specific changes implemented for the purification of the perfusion process material are summarized in Table 15. Table 16 depicts the purification method used for the perfusion-based cell cultures.

**Table 14: Purification Process Comparison between Batch and Perfusion Processes**



**Table 15: Summary Description of Purification Changes**

Production Step	Description (Batch)	Description (Perfusion)
PURIFICATION PROCESS		
		Changes:
	Tangential Flow Filtration: 2.8 m <sup>2</sup> Albumin retentive MWCO filters Operation:	Tangential Flow Filtration: 5.6 m <sup>2</sup> Albumin retentive MWCO filters Operation:
Concentration/ Diafiltration	<ul style="list-style-type: none"> <li>Concentration to 10X</li> <li>Diafiltered against 5 volumes 10 mM sodium phosphate, 100 mM sodium chloride, pH 7.3</li> </ul>	<ul style="list-style-type: none"> <li>Concentration to 20X</li> <li>No diafiltration, system rinsed with 100 mM sodium phosphate, pH 7.3 Rationale:</li> <li>Reduction of volume for storage at 4°C.</li> </ul>
↓		
	Column:	Step deleted
	2 x 30 cm diameter x 33 cm height	
DEAE Sepharose FF Flow-Through Chromatography	<ul style="list-style-type: none"> <li>Pre-Wash: 0.1 N sodium hydroxide</li> <li>Wash: Water-for-Injection (WFI)</li> <li>Neutralize: 100 mM sodium phosphate, pH 7.3</li> <li>Equilibration: 10 mM sodium phosphate, 100 mM sodium</li> </ul>	

Production Step	Description (Batch)	Description (Perfusion)
	chloride, pH 7.3	
	▪ Load: concentrated/diafiltered harvest	
	▪ Wash: 10 mM sodium phosphate, 100 mM sodium chloride, pH 7.3	
	▪ Strip: 10 mM sodium phosphate, 1.5 M sodium chloride, pH 7.3	
	▪ Regeneration: 10% glacial acetic acid	
	▪ Wash: Water-for-Injection (WFI)	
	▪ Sanitization: 0.5 N sodium hydroxide	
	▪ Storage: 0.1 N sodium hydroxide	
↓		
pH adjustment and filtration		▪ pH adjustment: addition of 10% glacial acetic acid to pooled 20X concentrates to a final pH 5.0
		▪ Load: pooled 20X concentrates
		▪ Filtration through clarification filters and 0.2 µm filter
		▪ Flush: 20 mM sodium acetate, 120 mM sodium chloride, pH 5.0 Rationale:
		▪ Increases rhASB binding capacity of the subsequent Blue Sepharose resin column.
↓		
Blue Sepharose FF Chromatography	Column:	Change:
	20 cm diameter x 11 cm height	Column:
	▪ Pre-Wash: 0.1 N sodium hydroxide	45 cm diameter x 16 cm height
	▪ Wash: Water-for-Injection (WFI)	
	▪ Neutralization: 1 M sodium acetate, pH 5.5	▪ Neutralization: omitted
	▪ Equilibration: 20 mM sodium acetate, 150 mM sodium chloride, pH 5.5	▪ Equilibration: 10 mM sodium phosphate, pH 6.45
	▪ Load: DEAE flow through adjusted to 20 mM sodium acetate, 150 mM sodium chloride, pH 5.5	▪ Load: pooled 20X concentrates, pH adjusted to 5.0 and filtered.
		▪ Wash: 10 mM sodium phosphate, pH 6.45
	▪ Wash: 20 mM sodium acetate, 150 mM sodium chloride, pH 5.5	Elution: 10 mM sodium phosphate, 125 mM sodium chloride, pH 6.45
	▪ Elution: 20 mM sodium acetate, 500 mM sodium chloride, pH 5.5	▪ Regeneration: 10 mM sodium phosphate, 1.0 M sodium chloride, pH 6.45
	▪ Regeneration: 20 mM sodium acetate, 1.0 M sodium chloride, pH 5.5	▪ Wash 2: 10 mM sodium phosphate, 1.0 M sodium chloride, pH 6.45
	▪ Sanitization: 0.1 N sodium hydroxide	Rationale:
	▪ Wash 1: Water-for-Injection	▪ Conditions allow improved removal of potential CHO impurities allowing for the deletion of DEAE Sepharose Chromatography.
	▪ Wash 2: 1 M sodium acetate, pH 5.5	
	▪ Storage: 20% Ethanol	
↓		
Copper Chelating Sepharose FF Chromatography		Changes:
	Column:	Column:
	14 cm diameter x 9 cm height	40 cm diameter x 16 cm height
	▪ Pre-Wash: 0.1 N sodium hydroxide	

Production Step	Description (Batch)	Description (Perfusion)
	▪ Wash: Water-for-Injection (WFI)	
	▪ Charge Buffer: 0.1 M cupric sulfate	
	▪ Equilibration: 20 mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 6.0	▪ Equilibration: 20 mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 5.5
	▪ Load: Adjust glycerol content of Blue Eluate to 10%, by adding 20 mM sodium acetate, 500 mM sodium chloride, 50% glycerol, pH 6.0	▪ Load: Adjust glycerol content of pooled Blue Eluate to 10%, by adding 100 mM sodium acetate, 2.0 M sodium chloride, 50% glycerol, pH 5.2
	▪ Wash 1: 20 mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 6.0	▪ Wash 1: 20 mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 5.5
	▪ Wash 2: 20 mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 4.0	▪ Wash 2: 20 mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 3.9
	▪ Wash 3: 20 mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 3.8	▪ Wash 3: omitted
	▪ Elution: 20 mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 3.6	Rationale:
	▪ Regeneration: 50 mM EDTA, 1.0 M sodium chloride, pH 8.0	▪ Consistent and reproducible product purity obtained with modified step
	▪ Sanitization: 0.5 M sodium hydroxide	
	▪ Storage: 0.1 M sodium hydroxide	
	↓	
Viral Inactivation	Pooled Eluate fractions held for 30-120 minutes prior to adjustment to pH 4.5 with 0.5 M NaOH	No Change
↓		
Phenyl Sepharose Chromatography		Change:
	Column:	Column:
	10 cm diameter x 16 cm height	40 cm diameter x 16 cm height
	Resin: Phenyl Sepharose High Performance	Resin: Phenyl Sepharose High Sub Fast Flow
	▪ Pre-Wash: 0.1 N sodium hydroxide	
	▪ Wash: Water-for-Injection (WFI)	
	▪ Equilibration: 20 mM sodium acetate, 3.0 M sodium chloride, pH 4.5	▪ Equilibration: 20 mM sodium acetate, 2.0 M sodium chloride, pH 4.5
	▪ Load: Adjust sodium chloride content of Copper Eluate to 3 M, by adding 20 mM sodium acetate, 5 M sodium chloride, pH 4.5	▪ Load: Adjust sodium chloride content of Copper Eluate to 2 M, by adding 20 mM sodium acetate, 5 M sodium chloride, pH 4.5
	▪ Wash 1: 20 mM sodium acetate, 3.0 M sodium chloride, pH 4.5	▪ Wash 1: 10 mM sodium phosphate, 2.0 M sodium chloride, pH 7.1
	▪ Wash 2: 20 mM sodium acetate, 1.6 M sodium chloride, pH 4.5	▪ Wash 2: 20 mM sodium acetate, 2.0 M sodium chloride, pH 4.5
	▪ Elution: 20 mM sodium acetate, 1 M	▪ Elution: 20 mM sodium acetate, 250 mM
		sodium chloride, pH 4.5
	▪ Regeneration: 20 mM sodium acetate, pH 4.5	
	▪ Sanitization: 0.5 N sodium hydroxide	Rationale:
	▪ Storage: 0.1 N sodium hydroxide	▪ Alternate resin has more favorable flow characteristics and capacity for rASB.
		▪ Wash 1 allows more robust clearance of potential protein impurities.
	↓	

Production Step	Description (Batch)	Description (Perfusion)
UF/DF Final		Change:
	Tangential Flow Filtration: < 0.4 m² MWCO 10 kDa Filters.	Tangential Flow Filtration: 2.8 m² MWCO 10 kDa Filters.
	▪ Equilibration: 10 mM sodium phosphate, 150 mM sodium chloride, pH 5.8	
	▪ Concentration: Concentration to NMT 1.5 mg/mL	
	▪ Diafiltration: 10 mM sodium phosphate, 150 mM sodium chloride, pH 5.8	
↓		
DNA Removal	Not Done	New Step:
		▪ DNA Filtration: Product filtered through an ion exchange-based DNA filter.
		Rationale:
▪ Additional DNA clearance to compensate for deletion of DEAE Flow-Through Chromatography step.		
↓		
Formulation	▪ Diluted to 1.0 mg/ml with 10 mM sodium phosphate, 150 mM sodium chloride, pH 5.8	
↓		
Viral filtration		Change:
	▪ Viral Filtration Product filtered through a 0.04 µm filter	▪ Viral Filtration: Product filtered through a 0.02 µm filter Rationale:
		▪ Use of smaller pore size to enhance viral clearance.
↓		
Formulation	Step occurs earlier in process (Prior to viral filtration)	Change:
		▪ Diluted to 1.0 mg/ml with 10 mM sodium phosphate, 150 mM sodium chloride, pH 5.8
		▪ Formulation: Polysorbate 80 is added to a concentration of 50 µg/mL.
↓		
Filtration	▪ Filtration: Filter the diluted product through a 0.2 µm filter into storage container	No Change

Table 16: rhASB Purification Method (Perfusion Process)

Step	Process
Harvest Filtration	<ul style="list-style-type: none"> <li>Filtration through Clarification filters, 0.45µm filters and finally 0.2µm filter.</li> <li>Filtered pooled harvests are stored in polypropylene bags</li> </ul>
UF Concentration	<ul style="list-style-type: none"> <li>Equilibration and flush: 100 mM sodium phosphate, pH 7.3</li> <li>Load: filtered harvest fluid</li> <li>Concentration: Concentration to 20X</li> <li>Filtration: Filter the diluted product through a 0.2 µm filter into storage container</li> </ul>
pH adjustment and filtration	<ul style="list-style-type: none"> <li>pH adjustment: Add 10% glacial acetic acid to pooled 20X concentrates to a final pH of 5.0</li> <li>Load: pooled 20X concentrates</li> <li>Rinse: Water-for Injection (WFI)</li> <li>Filtration through Clarification filters and 0.2µm filter.</li> <li>Flush: 20 mM sodium acetate, 120 mM sodium chloride, pH 5.0</li> </ul>
	<ul style="list-style-type: none"> <li>Pre-Wash: 0.1N sodium hydroxide</li> </ul>

Step	Process
Blue Sepharose 6 FF	<ul style="list-style-type: none"> <li>Wash: Water-for-Injection (WFI)</li> <li>Equilibration: 10mM sodium phosphate, pH 6.45</li> </ul>
(Blue, Blue Sepharose)	<ul style="list-style-type: none"> <li>Load: pH adjusted and filtered pooled 20X concentrates</li> <li>Wash: 10mM sodium phosphate, pH 6.45</li> <li>Elution: 10mM sodium phosphate, 125mM sodium chloride, pH 6.45</li> <li>Regeneration: 10mM sodium phosphate, 1.0 M sodium chloride, pH 6.45</li> <li>Sanitization: 0.1 N sodium hydroxide</li> <li>Wash 1: Water-for-Injection (WFI)</li> <li>Wash 2: 10mM sodium phosphate, 1.0 M sodium chloride, pH 6.45</li> <li>Storage: 20% Ethanol</li> </ul>
Chelating Sepharose FF	<ul style="list-style-type: none"> <li>Pre-Wash: 0.1N sodium hydroxide</li> <li>Wash: Water-for-Injection (WFI)</li> <li>Charge Buffer: 0.1M cupric sulfate</li> </ul>
(Copper, CC, Copper-Chelating)	<ul style="list-style-type: none"> <li>Equilibration: 20mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 5.5</li> <li>Load: Adjust glycerol content of pooled Blue Eluates to 10%, by adding 100mM sodium acetate, 2.0M sodium chloride, 50% glycerol, pH 5.2</li> <li>Wash 1: 20mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 5.5</li> <li>Wash 2: 20mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 3.9</li> <li>Elution: 20mM sodium acetate, 0.5 M sodium chloride, 10% glycerol, pH 3.6</li> <li>Eluate hold for 30-120 minutes prior to adjustment to pH 4.5 with 0.5M NaOH</li> <li>Regeneration: 50mM EDTA, 1.0M sodium chloride, pH 8.0</li> <li>Sanitization: 0.5M sodium hydroxide</li> <li>Storage: 0.1M sodium hydroxide</li> <li>Pre-Wash: 0.1 N sodium hydroxide</li> </ul>
Phenyl Sepharose 6 FF High Sub	<ul style="list-style-type: none"> <li>Wash: Water-for-Injection (WFI)</li> <li>Equilibration: 20mM sodium acetate, 2.0 M sodium chloride, pH 4.5</li> </ul>
(Phenyl, Phenyl High Sub)	<ul style="list-style-type: none"> <li>Load: Adjust sodium chloride content of Copper Eluate to 2 M, by adding 20mM sodium acetate, 5 M sodium chloride, pH 4.5</li> <li>Wash 1: 10 mM sodium phosphate, 2.0 M sodium chloride, pH 7.1</li> <li>Wash 2: 20mM sodium acetate, 2.0M sodium chloride, pH 4.5</li> <li>Elution: 20mM sodium acetate, 250mM sodium chloride, pH 4.5</li> <li>Regeneration: 20mM sodium acetate, pH 4.5</li> <li>Sanitization: 0.5 N sodium hydroxide</li> <li>Storage: 0.1 N sodium hydroxide</li> </ul>
UF/DF, DNA Filtration, Viral Filtration, Formulation	<ul style="list-style-type: none"> <li>Equilibration: 10mM sodium phosphate, 150mM sodium chloride, pH 5.8</li> <li>Concentration: Concentration to NMT 1.5 mg/mL</li> <li>Diafiltration: 10 mM sodium phosphate, 150 mM sodium chloride, pH 5.8</li> <li>DNA Filtration: Product filtered through a DNA filter</li> <li>Viral Filtration/Dilution: Product filtered through a 0.02 µm filter and diluted to 1.0 mg/ml</li> <li>Formulation: Polysorbate 80 at a concentration of 50 µg/mL is added</li> <li>Filtration: Filter the diluted product through a 0.2 µm filter into storage container</li> </ul>

All purification columns are regenerated prior to use, sanitized after use and stored in the appropriate buffers as indicated in Tables 15 and 16.

#### Purification Raw Materials

All materials are supplied by qualified vendors.

**Table 17: Raw Materials for Purification**

Ingredient	Grade
Glacial Acetic Acid	USP
Cupric Sulfate Pentahydrate	USP
Edetate Disodium	USP
Dehydrated Alcohol, USF (Ethanol, 200 Proof)	USP
Glycerine	USP
Sodium Acetate, Trihydrate	USP
Sodium Chloride	USP
Sodium Hydroxide 50% w/w Solution	Reagent Grade
Sodium Phosphate, Dibasic, Heptahydrate	USP/EP
Sodium Phosphate, Monobasic, Monohydrate	USP
Hydrochloric Acid, 6 N Volumetric Solution	Reagent Grade
Polysorbate 80, MF/EP (CRILLE 4 HP)	NF/EP
Water-for-Injection, Packaged in Bulk	USP

The glycerine to be utilized is to be derived from a synthetic process. All raw materials used are to be in compliance with the latest version of the CPMP/CVMP Note for Guidance entitled, "Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Human and Veterinary Medicinal Products," in which tallow derivatives such as glycerol and fatty acids manufactured by rigorous processes involving high temperatures and pressure conditions or chemical reactions known to be terminally hostile to the bovine spongiform encephalopathic (BSE) agent are thought unlikely to be infectious. Thus, the risk of BSE transmission from the glycerine is considered to be low.

The viral safety of rhASB is confirmed by a combination of selection and qualification of vendors, raw material testing, cell bank characterization studies, viral removal studies and inactivation capacity of the rhASB purification process, and routine lot release testing. Relevant US, EU, and ICH regulations and guidelines have been referenced to ensure the viral safety of rhASB.

#### Column Chromatography, DNA Removal and Viral Filtration

RhASB is now purified using a series of chromatography and filtration steps. The harvest fluid is concentrated to 20X by ultrafiltration, pH adjusted, filtered and loaded onto a Blue Sepharose Fast Flow chromatography column (45 cm x 16 cm). The Blue Sepharose Fast Flow eluate is filtered prior to loading on to a Copper Chelating Sepharose column. The Copper eluate is filtered prior to loading on to a Phenyl Sepharose Fast Flow High Sub column. All three column chromatography purification steps are run in a bind and elute mode. The Phenyl Sepharose column eluate is passed through an anion exchange filter and viral reduction filter prior to concentration and buffer exchange by ultrafiltration.

#### Viral Removal/Inactivation Studies

Two studies were conducted to assess the viral reduction capacity of the modified rhASB purification process. Studies were performed at BioReliance (Rockville, MD) using two model virus systems, Xenotropic murine leukemia virus (XMuLV) and Murine Minute Virus (MMV). XMuLV is an enveloped single-stranded RNA retrovirus with low resistance to physico-chemical inactivation. MMV is a small, non-enveloped single-stranded DNA virus with high resistance to physico-chemical agents.

These studies evaluated two chromatographic steps (Copper Chelating Sepharose FF and Phenyl Sepharose FF High Sub) and the viral filter (0.02 µm) used in the rhASB purification process. Spike and recovery studies were performed using scaled down versions of the process steps. The critical parameters maintained were retention times and matrix-solution interactions. This was achieved by replicating the buffers, linear flow rates and column heights but adjusting for column diameter. Materials used in the study (product and buffers) were collected from actual full scale production.

Chromatography columns (Copper Chelating Sepharose and Phenyl Sepharose) were packed and pre-run with either typical rhASB loads (blank) or loads spiked with viral buffer prior to shipping to BioReliance. Chromatograms and product yields in the presence of viral buffers were comparable to blank runs. Identical column loads were spiked with either XMuLV or MMV immediately prior to chromatography. The amount of viral reduction for each of the evaluated steps was determined by comparing the viral burden in the column loads and eluates. A summary of the results for this study are shown in Table 18.

**Table 18: Reduction Factors for XmuLV and MMV**

Process Step	XMuLV Log Reduction	MMV Log Reduction
Blue Sepharose	not tested	not tested
Copper Chelating (+ low pH hold)	$\geq 3.51 \pm 0.52$	$\geq 2.71 \pm 0.52$
Phenyl Sepharose	$\geq 3.54 \pm 0.36$	$\geq 1.72 \pm 0.64$
DNA Filtration	not tested	not tested

Process Step	XMuLV Log Reduction	MMV Log Reduction
Viral Filtration	$\geq 5.51 \pm 0.43$	$\geq 4.76 \pm 0.00$
Total log Reduction	$\geq 12.56$	$\geq 9.19$

#### In-Process Testing

In-process testing is performed throughout the process and is illustrated in Figure 3. In-process testing of the harvest cell culture fluid and the purification intermediates is described in Tables 19 and 20.

**Table 19: In-Process Testing of the Harvested Cell Culture Fluid**

Test	Action Levels
Bacterial Endotoxin by LAL (USP/EP)	$\geq 2$ EU/mL
Bioburden (USP/EP)	$\geq 1$ cfu/10 mL
Total Protein by Bradford and Activity	Results used for the calculation of the Blue Sepharose column load
Mycoplasma <sup>1</sup>	Negative (Release Specification)
<i>In vitro</i> Assay For The Presence of Viral Contaminants <sup>1</sup>	Negative (Release Specification)

<sup>1</sup> Sampling is performed at multiple time points during the cell culture harvest stage of manufacturing. The last sample removed prior to the termination of the cell culture process is tested and a negative result is required for lot release.

**Table 20: In-Process Testing of Purification Intermediates**

Test	Action Levels
Bacterial Endotoxin by LAL (USP/EP)	$\geq 3$ EU/mL in column eluates <sup>1</sup>
Bioburden (USP/EP)	$\geq 20$ cfu/mL pre-filtration <sup>1</sup> $\geq 1$ cfu/100 mL in Formulated Bulk Drug Substance (FBDS) <sup>2</sup>
Activity	Result used for the calculation of the Copper Chelating and Phenyl Sepharose column loads
Total Protein by UV-Vis Spectrophotometry	Result used for the calculation of the protein concentration for the UF/DF

<sup>1</sup>Results exceeding action levels are investigated per standard operating procedures.  
<sup>2</sup>If results exceed action level, the FBDS will be 0.2  $\mu$ m filtered into appropriate sterile storage containers and then quarantined pending release for shipment to filling sites.

#### Results of perfusion method of purifying precursor rhASB

Table 21 provides data on the degree of purity of rhASB obtained using the purification methods described in Tables 15 and 16. The "Purity by RP-HPLC" column indicates the degree of purity obtained for the combined amount of both the precursor and mature forms of rhASB.

**Table 21: rhASB Lot Release Results**

Lot	Purity by RP-HPLC	Presence of the processed forms*	Manufacturing process
AP60028	99.6	-	Batch process
AP60029	98.8	+	Batch process
AP60030	98.7	+	Batch process
AP60031	99.1	-	Batch process
AP60032	99.8	-	Batch process
AP60033	99.4	-	Batch process
AP60035	98.7	-	Batch process
AP60036	99.4	-	Batch process
AP60038	99.6	-	Batch process
AP60039	99.3	-	Batch process
AP60040	99.1	-	Batch process
AP60101	99.3	-	Batch process
AP60102	98.9	-	Batch process
AP60103	99.0	-	Batch process
AP60104	99.0	+	Batch process



Lot	Purity by RP-HPLC	Presence of the processed forms*	Manufacturing process
AP60105	99.0	-	Batch process
AP60106	99.2	-	Batch process
AP60107	99.4	-	Batch process
AP60108	99.7	-	Perfusion process
AP60109	99.8	-	Perfusion process
AP60201	99.0	N/A	Perfusion process
AP60202	100	-	Perfusion process
*"+" indicates the presence of the processed forms (estimated at 1-15%) in the formulated bulk drug substance; "-" indicates the absence of the processed forms.			

Figures 4A-4C provide the representative chromatograms of the three chromatographic steps of the perfusion method of purification (Table 16). Table 22 provides the average recoveries of precursor rhASB for each of these three chromatographic steps. Figure 5 provides the data for the purity analysis of in-process samples at each chromatographic step and of the purified final product (precursor rhASB).

**Table 22: Summary of Purification Recoveries.**

Step	Average Recovery (%)
pH Adjustment to 5.0	83
Blue Sepharose Column	84
Copper Chelating Sepharose Column	86
Copper to Phenyl Transition	86
Phenyl Sepharose Column	88
Overall	50

When purified products obtained using the batch process and the perfusion process are compared, the perfusion process clearly produces a purer precursor rhASB. Even attempts to further optimize the batch processes by selecting different wash fractions or varying the conditions did not result in the consistently pure product obtainable by the perfusion process. The perfusion process is able to produce consistent precursor rhASB purity of 99% or more, while the batch process does not (Table 21). Figure 6 provides a comparison between the products obtained using the old batch method wherein the cell culture is cultured using a medium that is supplemented with G418 and not supplemented with folic acid, serine and asparagine (lane 2), and the purified products purified using the perfusion process (lanes 3-9)). The samples of rhASB were denatured in SDS with a reducing agent and subjected to electrophoresis through 4-20% PAGE in SDS running buffer. The batch process purified products clearly contain far more impurities (especially near the 48 kDa size) than the perfusion process purified products. The batch process rhASB appear to detectable amounts of processed forms (estimated at 1-15%) (lane 2). The perfusion process purified lots are highly comparable to each other and are significantly less complex than the batch process purified rhASB standard, indicating a higher degree of purity for the perfusion process material.

Table 23 provides percent purity of precursor rhASB purified using the perfusion process purification method.

**Table 23: Purity of Final Product**

Lot	Purity by RP-HPLC (% main peak)
AC60109 UF #4	99.8
AC60109 UF #10	99.6
AC60109 UF #1	99.7
AC60109 UF #18	99.9
AC60109A UF #22	99.7
AC60109A UF #25	99.8
AC60109A UF #27	100
AC60109 BMK Manufacturing	99.8
AC60202 UF #4	ND
AC60202 UF #10	ND
AC60202 UF #18	ND
AC60202 BMK Manufacturing	100
Average	99.8
ND = not determined.	

Table 24 provides percent purity of lots of precursor rhASB purified using the perfusion process purification method, where purity has been evaluated by RP-HPLC and SEC-HPLC (which allows quantification of impurities of different molecular weights such as processed forms or multimers).

**Table 24: Purity by RP-HPLC and SEC-HPLC**

Lot	Purity by RP-HPLC (% Main Peak)	Purity by SEC-HPLC (% Main Peak)
AP60109	99.8	99.8
AP60202	100	>99
AP60204	99.7	99.8
AP60206	100	99.8
AP60301	100	99.6
AP60302	100	99.6
AP60303	100	99.5
AP60304	100	ND*
Average	99.9	99.6
* Not Determined		

#### Protease removal in the Perfusion Process

At the 180 L batch scale two relatively large 24 L DEAE Sepharose runs are required per lot. The perfusion process generates 10-fold larger harvest volumes that impose severe impractical DEAE Sepharose column sizes and buffer volumes for practical large scale purification. Therefore, elimination of the particularly untenable DEAE Sepharose step was evaluated in an effort to streamline the new perfusion purification train. As a consequence, the three column train (as described in Table 16) overcomes this large scale production obstacle.

The DEAE Sepharose chromatography step in the batch process (described in Table 15) was employed primarily to remove total protein (including proteases) and the pH indicator dye, Phenol Red. The latter issue is addressed in the new process by removal of Phenol Red from the 302 media formulation. The removal of total protein by DEAE sepharose step resulted in higher Blue sepharose capacity at pH 5.5. In the new process, Blue Sepharose conditions needed to be identified that balanced high rhASB capacity while limiting protease activity. Both capacity and proteolysis are enhanced at low pH. In addition, clearance of proteases should be demonstrable to improve the robustness of the subsequent Copper Chromatography step, where clearance of the protease, cathepsin, has been found problematic in the Batch process. Conditions were found which yielded the desired results, whereby, loading was performed at low pH, while wash and elution conditions were at higher pH, conditions that efficiently clear the protease, cathepsin, with an acceptable load of 0.8 mg rhASB/ml resin. Removal of the protease activity (e.g. cathepsin) is demonstrated in the chromatograms of Figure 8.

Comparison of rhASB purified from perfusion nm, 102PD0055, using either the new process (without DEAE FT chromatography) or old Batch purification process (PS Eluate (OP) indicate that the old process material purified (using the batch method) from the same reactor run, lane PS Eluate (OP), has significant more proteolysis, as demonstrated by the additional bands on silver-stained gel and detectable by anti-ASB antibodies (see Figure 9). The new process is much purer relative to the batch standard. The cathepsin protease is cleared by Blue chromatography. Anti-cathepsin cross-reactive material is seen in the Blue Strip fraction using the new process.

The pharmacokinetic results described below in Example 11 show that the purer rhASB preparation results in a longer half-life than the previous batch standard.

#### EXAMPLE 10

##### Surfactant Effects on Particle Formation

An experimental protocol was carried out to determine the effect of Polysorbate-80 on particle generation in vials of rhASB made with the new perfusion cell culture process. Visual and particle count assays were employed to determine the extent of particle generation. In addition, activity testing, polyacrylamide gel electrophoresis (PAGE), immunoblotting and size exclusion chromatography (SEC) were performed to confirm product quality in the presence of Polysorbate-80 and/or shaking relative to untreated controls. Vials and stoppers used for the rhASB batch process (Old, Wheaton 5ml, 2905-B24B/ West S-127 (4401/45)) were also compared to the proposed vials and stoppers to be used for the perfusion-derived material (New, Wheaton 5ml, 2905-B8BA/ West S-127 (4432/50)).

Vials were filled after rhASB had been passed through a 0.2µm filter using a 10 ml disposable pipette. Two vials were filled for each rhASB-containing test Both were used for the visual assay with one dedicated for particle counting and the other saved for the remainder of assays. Only one buffer control vial/test was filled. Few particles were introduced due to set-up, sample handling, filtration or filling. From visual and particle count analysis (results shown in Tables 25A-C and 26A-C below), on days 0, 1 and 14 days, no significant particulate formation was detected in unshaken vials of formulated drug relative to buffer-only controls.

After 18 hr of shaking at 4 degrees C; a large increase in particles was observed in both vial configurations with rhASB in formulation buffer alone (10 mM sodium phosphate, 150 mM sodium chloride, pH 5.8), without polysorbate. Fine flakes were clearly visible.

The particle counting was done using the HIAC/ROYCO Model 9703 Particle Counter. The liquid particle counter is capable of sizing and counting particles ranging from 2.0 µm to 150 µm. Three aliquots of not less than 5 mL each are put into the light obscuration sensor. The average particle counts from runs 2 and 3 are reported as particle counts per mL. Based on HIAC-Royko quantification of particles 10µm in size, 2069 and 1190 particles/mL were counted in old and new vials configurations, respectively. In the presence of 0.001% Polysorbate-80, a markedly lower count was found with 21 and 49 particles/mL. At 0.005% Polysorbate-80, essentially no difference between shaken and

unshaken vials could be detected.

In old vials/seals without Polysorbate-80, there was an unexpected drop in particle counts/ml from 2069 to 268 from days 1 to 14 days after shaking. The visual assay indicated larger flakes in the same 14 day vials in which lower counts were found; it is possible that the larger particles interfered with the HIAC-Royko assay, or that the old vial configuration contributes to increased particle formation with eventual coalescence and aggregation into larger, albeit fewer, particles.

Additional testing was done to confirm the integrity of the protein after shaking. Specific activity was unchanged. Size Exclusion Chromatography, a method that can detect changes in protein aggregation, which might be influenced by mechanical agitation, showed no increase in larger molecular weight species. In order to detect breakdown products, SDS-PAGE gels of rhASB were either silver stained or transferred onto nitrocellulose and blotted with Anti-rhASB antibody. No additional bands were detected in any of the samples.

**Table 25A. Visual Assay. Day 0**

		102PD0089-01		Buffer
Vial	[Polysorbate-80] %	No Shake		No Shake
Old	0	(-)	(-)	(-)
Old	0.005	(-)	(-)	(-)
Old	0.001	(-)	(-)	(-)
New	0	(-)	(-)	(-)
New	0.005	(-)	(-)	(-)
New	0.001	(-)	(-)	(-)

**Table 25B. Visual Assay. Day 1**

		102PD0089-08				Buffer	
Vial	[Polysorbate-80] %	Shake		No Shake		Shake	No Shake
Old	0	(++)	(++)	(-)	(-)	(-)	(-)
Old	0.005	(-)	(-)	(-)	(-)	(-)	(-)
Old	0.001	(+/-)	(-)	(-)	(-)	(-)	(-)
New	0	(++)	(++)	(+/-)	(-)	(-)	(-)
New	0.005	(-)	(-)	(-)	(-)	(-)	(-)
New	0.001	(+/-)	(-)	(+/-)	(-)	(-)	(-)

**Table 25C. Visual Assay. Day 14**

		102PD0089-01				Buffer	
Vial	[Polysorbate-80] %	Shake		No Shake		Shake	No Shake
Old	0	(+++)	(+++)	(-)	(-)	(-)	(-)
Old	0.005	(-)	(-)	(-)	(-)	(-)	(-)
Old	0.001	(+/-)	(+)	(-)	(-)	(-)	(-)
New	0	(++)	(++)	(+/-)	(-)	(-)	(-)
New	0.005	(-)	(-)	(-)	(-)	(-)	(-)
New	0.001	(+/-)	(-)	(+/-)	(-)	(-)	(-)

**Visual Assay:**

No visible precipitation	-
Borderline, uncertain about observation	-/+
Small amount of fine precipitate	+
Significant precipitation, cloudy	++
Very cloudy, large flakes of precipitate	+++

**Table 26A. Particulate Analysis, HIAC-Royko (Particles/mL). Day 0**

		102PD0089-01		Buffer	
Vial	[Polysorbate-80] %	No Shake		No Shake	
		10 µm	25 µm	10 µm	25 µm

		102PD0089-01		Buffer	
Vial	[Polysorbate-80] %	No Shake		No Shake	
Old	0	4.5	0.5	2.5	0.0
Old	0.005	2.0	0.5	1.5	0.0
Old	0.001	4.5	1.5	2.5	1.0
New	0	6.5	2.5	6.0	4.0
New	0.005	1.5	0.5	3.0	1.0
New	0.001	19.5	5.0	2.0	0.0

**Table 26B. Particulate Analysis, HIAC-Royko (Particles/mL). Day 1**

		102PD0089-01				Buffer			
Vial	[Polysorbate-80] %	Shake		No Shake		Shake		No Shake	
		10 µm	25 µm	10 µm	25 µm	10 µm	25 µm	10 µm	25 µm
Old	0	2069.0	74.5	3.0	0.5	0.5	0.5	2.5	2.0
Old	0.005	3.5	0.0	2.0	0.5	3.5	2.5	1.0	0.5
Old	0.001	21.5	2.5	2.0	1.0	2.5	1.0	1.5	1.5
New	0	1190.0	34.5	5.0	0.5	0.0	0.5	2.5	3.5
New	0.005	19.0	0.0	4.5	0.0	2.5	0.0	17.0	0.0
New	0.001	49.0	0.5	9.0	2.5	9.5	0.5	6.0	1.0

**Table 26C Particulate Analysis, HIAC-Royko (Particles/mL). Day 14**

		102PD0089-01				Buffer			
Vial	[Polysorbate-80] %	Shake		No Shake		Shake		No Shake	
		10 µm	25 µm	10 µm	25 µm	10 µm	25 µm	10 µm	25 µm
Old	0	268.0	18.0	0.5	0.0	4.5	2.0	0.0	0.5
Old	0.005	2.0	2.5	1.0	1.0	2.5	0.5	6.0	3.0
Old	0.001	72.0	6.5	7.0	3.0	7.0	1.0	1.0	2.0
New	0	1335.0	29.0	2.0	0.0	1.5	0.0	1.5	0.5
New	0.005	7.0	0.5	16.0	0.0	13.5	0.0	13.0	2.0
New	0.001	38.0	1.5	5.5	0.5	16.0	0.5	8.0	0.0

The results indicated that the manual fill procedure generates very few particles regardless of formulation. After constant and vigorous shaking at 180 rpm, particles were clearly visible in vials containing rhASB formulated without Polysorbate-80.

The presence of Polysorbate-80 was highly effective in inhibiting mechanically-induced particle formation. Protection was dramatic, with particle counts kept down to essentially background levels in shaken vials at 0.001% and 0.005% Polysorbate-80. The 0.005% Polysorbate-80 concentration shows slight improvement in reducing particle counts over the 0.001% Polysorbate-80 concentration.

Shaking or formulation was not observed to cause any product instability as evaluated by activity, protein concentration, purity or percent aggregation.

#### EXAMPLE 11

##### Clinical Study in Humans

A Phase 1/2, randomized, double-blinded clinical trial of recombinant human N-acetylgalactosamine-4-sulfatase (rhASB) in patients with mucopolysaccharidosis VI (MPS VI), Maroteaux-Lamy Syndrome, was conducted using drug product produced according to the batch process described herein. The rhASB was formulated at a concentration of 1 mg/mL, pH 5.8, 9mM monobasic sodium phosphate 1 H<sub>2</sub>O, 1 mM dibasic sodium phosphate 7 H<sub>2</sub>O, 150 mM sodium chloride. When administered to patients, the rhASB was prepared by diluting the appropriate amount of drug solution in an intravenous (IV) bag with normal saline at room temperature. All patients were premedicated with an antihistamine prior to infusion to reduce the potential for infusion-associated reactions.

The objectives of this study were to evaluate the safety, efficacy and pharmacokinetic profile of two doses of rhASB as ERT in patients with MPS VI. Patients were randomized in a double-blind fashion to one of two dose groups, 0.2 and 1.0 mg/kg. Drug was given once per week as a four-hour IV infusion. Measures of safety included complete chemistry panel, urinalysis, complete blood count (CBC) with differential, and tracking of AEs. Immune response and infusion-associated reactions, complement activation and antibody formation are assessed for all patients. Efficacy parameters included exercise tolerance/endurance (6-minute walk test), respiratory capacity (pulmonary function tests), joint range of motion (JROM), functional status (Childhood Health Assessment Questionnaire [CHAQ]), levels of urinary GAGs, and

changes in hepatomegaly, visual acuity, cardiac function, and sleep apnea.

Pharmacokinetic evaluations were conducted during infusions at Weeks 1, 2, 12, 24 and periodically thereafter to measure antigen (enzyme) levels during and after infusion. Pharmacokinetic evaluations were also conducted at Weeks 83, 84 and 96 of the open-label extension of the study, when patients were switched commencing at Week 84 to the new perfusion-process purified rhASB in a formulation with 0.005% polysorbate80.

A total of seven patients was enrolled, and included patients with a range of characteristics consistent with rapidly advancing or moderately advancing disease. One patient dropped out of the study at Week 3 for personal reasons and was replaced. A prespecified interim analysis was conducted following the completion of 24 weeks of treatment for the remaining six patients. Data from the Week 24 interim analysis showed that rhASB was well tolerated and the 1.0 mg/kg dose appeared to produce greater clinical benefit and to have a safety profile comparable to that of the 0.2 mg/kg dose.

Patients in the low-dose group were offered continued treatment at the 1.0 mg/kg dose level. Two patients initiated therapy at the higher dose level at intervals after Week 48 (Weeks 59 and 69). Patients treated through Week 96 have received the majority of planned rhASB infusions. No patient has missed more than two infusions total during this period.

Safety assessments performed during the initial 24, then 48 weeks of treatment showed that weekly treatment with rhASB at 0.2 or 1.0 mg/kg was well tolerated without significant differences between the two dose groups. Longer term treatment (through Week 96) at 1.0 mg/kg weekly also was well tolerated.

#### **Anti-rhASB Antibody Development and Complement Levels**

All six patients on study drug at Week 30 had developed antibody to rhASB. Levels for four of the six patients remained relatively low and declined from peak levels by Week 60. Of the two patients that developed higher levels of antibody than the other four patients, one patient's antibody levels declined from the peak level at Week 24, and the other patient's levels fell rapidly from the peak level at Week 60. No consistent changes in CH50, C3, or C4 complement levels have been observed during 96 weeks of treatment. Several patients have had modest intermittent declines in CH50 levels; however, no evidence of complement consumption or correlation between these low levels and infusion-associated symptoms were noted. Figure 10 shows antibody levels over 96 weeks of treatment.

#### **Urinary Glycosaminoglycans**

The level of urinary GAGs is a biochemical marker of the degree of lysosomal storage in MPS-affected individuals. By Week 24, urinary GAGs were reduced by an average of 70% in the high-dose group versus 55% in the low-dose group. The percentage of DS, the primary storage product of MPS VI, was similarly decreased by 44% and 18%, respectively. Examination of the time course of the change in urinary excretion of GAGs as a function of weeks on treatment showed a more pronounced drop in total urinary GAGs by Week 6 in the high-dose group. These data confirm that the higher dose produced a larger change in both urinary GAGs and DS. Urinary GAGs have continued to decline through Week 96 for the patients in both treatment groups who remained in the study. Figure 11 shows the reduction in total urinary GAG levels over the 96 weeks and Figure 12 shows a comparison of levels at week 96 to age-appropriate normal levels. Note that the two patients in the 0.2 mg/kg dose group who remained in the study, Patients 41 and 45, rolled over to the 1.0 mg/kg dose on

Weeks 69 and 59, respectively. At Week 96, these two patients had reductions in GAGs from screening of 85% and 63%. These were similar to reductions seen in the patients on 1.0 mg/kg through the entire 96 weeks. Patients 42, 43, and 44 had reductions of 64%, 77%, and 86% from screening, respectively. In both groups, the patients who had higher baseline GAG levels had higher percent reductions than patients with GAG levels that were closer to the normal range (<5 times upper limit of normal, i.e., mean plus 2 SD).

#### **Endurance**

The 6-minute walk test served as the primary clinical measure of endurance. The two patients unable to walk >100 m at baseline, who were randomized to receive 1.0 mg/kg of study drug (#43 and #44), had large improvements in the total distance walked by Week 24. All patients except #44, who developed C1-C2 cord compression, showed improvement in their distance walked by Weeks 48 and 96. Figure 13 shows the improvement in results of the 6-minute walk test over 96 weeks of treatment

#### **Additional Measures of Efficacy**

Modest improvements in a number of other efficacy parameters were seen during the 96 weeks of study drug treatment. Shoulder ROM, particularly for flexion, improved in five of the six study patients at the Week 24 assessment. Three of the four patients (#41, 43 and 45) with assessments at Week 96 continued to show increases in shoulder flexion. Slight improvements were also seen in the measures of respiratory function, forced vital capacity (FVC) and forced expiratory volume for one second (FEV1), in three of four patients, at Week 96. Visual acuity, measurable in four out of six patients enrolled, improved by one or more lines in three of these four patients, although one patient had changes in his prescription lenses during this period. Hepatomegaly, measured by CT scan, although not a prominent feature of this disease, also declined modestly, particularly in those patients with larger livers at baseline.

Little change was seen in the remaining efficacy parameters, including cardiac function by ECG, grip and pinch strength, CHAQ questionnaire, height and weight, bone mineral density, and sleep studies. No significant deterioration was observed in any of these parameters over the 96 weeks.

#### **Pharmacokinetics**

Pharmacokinetic evaluations were conducted during Weeks 1, 2, 12 and 24 of the double-blind study and during Weeks 83, 84 and 96 of the open-label extension. Beginning with Week 84, patients began to receive rhASB manufactured by the perfusion process described in Example 9.

Blood samples were collected during the infusion at 0, 15, 30, 60, 90 and 180 minutes, at the end of infusion at 240 minutes, and post-infusion at 5, 10, 15, 30, 45, 60, 90, 120 and 240 minutes. Pharmacokinetic parameters for rhASB were calculated using non-compartmental

methods. Areas under the plasma concentration-time curve to infinity and first moment were calculated using the linear trapezoidal method to the last time point with a concentration above the qualified limit of quantitation.

For patients administered 0.2 mg/kg/week, the mean values for  $AUC_{0-t}$  were relatively consistent from Week 1 through Week 24 ( $10,009 \pm 5,107$  at Week 1,  $11,232 \pm 3,914$  at Week 2,  $13,812 \pm 9,230$  at Week 12, and  $13,812 \pm 9,230$  at Week 24). For the 1.0 mg/kg/week cohort, the mean values for AUC were  $94,476 \pm 13,785$  at Week 1,  $180,909 \pm 46,377$  at Week 2,  $157,890 \pm 45,386$  at Week 12 and  $251,907 \pm 201,747$  at Week 24. For this 1.0 mg/kg/week group the increase in values for  $AUC_{0-t}$  and  $AUC_{\infty}$  and large standard deviation was due to a single patient whose AUCs were about 2-fold higher than the other two patients in the group.

Comparison of the mean values for  $AUC_{0-t}$  between the 0.2 and 1.0 mg/kg/week cohorts showed an increase far in excess of the 5-fold increase in dose, indicating that the pharmacokinetics of rhASB are not linear over this dose range.

Pharmacokinetic parameters at Weeks 83, 84 and 96 are shown in Table 27 below.

**Table 27**

Parameter	Week 83	Week 84	Week 96
C <sub>max</sub> (ng/mL)	1,143 $\pm$ 284	1,367 $\pm$ 262	1,341 $\pm$ 523
T <sub>max</sub> (min)	180	120	121
AUC <sub>0-t</sub> (min-ng/mL)	172,423 $\pm$ 49,495	213,713 $\pm$ 45,794	200,116 $\pm$ 76,506
AUC <sub><math>\infty</math></sub> (min-ng/mL)	173,570 $\pm$ 49,969	215,383 $\pm$ 47,018	201,157 $\pm$ 77,248
CL (mL/min/kg)	6.23 $\pm$ 2.10	4.81 $\pm$ 0.99	5.54 $\pm$ 2.09
V <sub>z</sub> (mL/kg)	67.6 $\pm$ 22.0	122 $\pm$ 60.2	123 $\pm$ 17.4
V <sub>ss</sub> (mL/kg)	266 $\pm$ 52.3	236 $\pm$ 21.1	233 $\pm$ 27.6
t <sub>1/2</sub> (min), half-life	8.49 $\pm$ 4.68	19.0 $\pm$ 13.2	17.3 $\pm$ 8.26
MRT (min)	44.4 $\pm$ 7.61	50.9 $\pm$ 12.9	46.1 $\pm$ 16.1
Arithmetic mean and standard deviation are reported except for T <sub>max</sub> which is the median. Individual patient values were reported if n<3.			

Parameters for Week 83 were comparable to those obtained from Weeks 2 through 24 (excluding one patient with high AUCs), indicating consistency in rhASB pharmacokinetics after weekly exposure for approximately 18 months. From Week 83 to Week 84, mean AUC values trended upward by approximately 25% while total body clearance trended downward by approximately 25%. From Week 83 to Week 84, the half-life increased from about 8.5 minutes to 17-19 minutes. Parameters from Week 96 were comparable to those from Week 84. Although this pharmacokinetic data was from a small number of patients and there was substantial overlap of individual values, these results indicated that the high purity rh ASB preparation obtained from the modified process described in Example 9 had a longer half-life than the original rhASB preparation.

## EXAMPLE 12

### Further Clinical Study in Humans

A Phase 2 open-label clinical trial of recombinant human *N*-acetylgalactosamine-4-sulfatase (rhASB) in patients with mucopolysaccharidosis VI (MPS VI), Maroteaux-Lamy Syndrome, was conducted using drug product produced according to the new perfusion process described in Example 9. The rhASB was formulated at a concentration of 1 mg/mL, pH 5.8, 9mM sodium phosphate, monobasic, -1 H<sub>2</sub>O, 1 mM sodium phosphate, dibasic, -7 H<sub>2</sub>O, 150 mM sodium chloride, 0.005% polysorbate 80.

The objectives of this study were to evaluate the safety, efficacy and pharmacokinetics of 1.0 mg/kg rhASB given as a weekly IV infusion. The inclusion criteria were modified for this study to include a relatively uniform set of patients with impaired endurance. Patients had to be able to walk at least 1 m but no more than 250 m in the first six minutes of a 12-minute walk test at baseline. The other inclusion and exclusion criteria were similar to those of the Phase 1/2 Study in that patients had to be at least five years old and have a documented biochemical or genetic diagnosis. In addition to the walk test, a number of endpoints not previously studied in MPS patients were included in this study.

Measures of safety included clinical laboratory evaluations, urinalysis, CBC with differential, ECG and tracking of AEs. Infusion-associated reactions as well as complement consumption and antibody formation are being assessed for all patients. Efficacy parameters included measures of endurance and mobility, including a 12-minute walk test, the Expanded Timed Get Up and Go test, the 3-minute stair climb test and physical activity. Subjective effects, such as joint pain and joint stiffness, were assessed with a questionnaire, and other measures in the Denver Developmental scale such as time to tie shoes, touch top of head, pull over sweater, and pick up coins and put them in a cup were also assessed. Additional Efficacy measures included measures of visual acuity, bone density studies, assessment of grip and pinch strength, shoulder ROM, functional status, pulmonary function, urinary GAG excretion, visual exams, cardiac function, and oxygen saturation during sleep. Pharmacokinetic evaluations were conducted during infusions at Weeks 1, 2, 12 and 24 to measure antigen (enzyme) levels during and after infusion. Ten patients were enrolled-five in the U.S. and five in Australia. All patients completed Week 24 of treatment. There were no deaths or discontinuations. All patients received all 24 study drug infusions; therefore, all patients were included in the safety and efficacy analyses.

As in the Phase 1/2 study, there was wide heterogeneity of disease severity among the MPS VI patients enrolled in the Phase 2 Study. Several patients also had neurological features of the disease, including communicating hydrocephalus, cervical spine instability, and spinal disc disease.

### Clinical Laboratory Assessments

No clinically significant laboratory abnormalities were observed in these patients over the 24-week period.

#### **Anti-rhASB Antibodies and Complement Studies**

Eight of the 10 patients developed antibody to rhASB by Week 24. Of the three patients that developed higher levels of antibody than the other patients, two patients' antibody levels continued to rise after Week 24 but appeared to have stabilized by Week 48. These two patients were either the "null" genotype or had a mutation in the presumed major antigenic site for ASB. The reduction in urinary GAGs was less for these two patients compared to the others. Figure 14 shows antibody levels over 48 weeks of treatment.

At Week 24, one patient had decreased C4 and CH50 values, both pre- and post-Week 12 infusion, which were considered to be clinically significant by the investigator. C4 and CH50 were slightly below the lower limit of normal prior to infusion and showed a greater decrease following the infusion. Although these results suggested the possibility of complement consumption, no clinical signs or symptoms consistent with such consumption were observed. Clinically significant abnormalities in complement levels were not observed in any of the other patients in the study.

#### **Urinary glycosaminoglycans**

The mean reduction in urinary GAG levels was rapid, reaching a nadir at Week 6, and remaining relatively constant from Week 6 through Week 24. By Week 24, urinary GAGs were reduced by an average of 71%, and by Week 48 urinary GAGs were reduced by an average of 76%. All ten patients showed a decrease in urinary GAG levels that approached the normal range after 24 weeks of treatment. These data are consistent with the results seen for the seven patients in the Phase 1/2 study. Figure 15 shows the reduction in total urinary GAG levels over the 48 weeks and Figure 16 shows a comparison of levels at week 48 to age-appropriate normal levels.

#### **Measures of Endurance**

Distance walked was assessed in a 12-minute walk test for all patients at baseline and every six weeks thereafter. Distance was recorded as the mean of two separate measures at each evaluation and was determined at both 6 minutes and 12 minutes.

At baseline, the mean distances walked at 6 and 12 minutes were 152.4 ( $\pm 75.0$ ) and 264.0 ( $\pm 161.7$ ) meters, respectively. At Week 24 the mean distance walked at 6 minutes had improved by 57.3 ( $\pm 59.0$ ) meters (62% per patient), while the mean distance walked at 12 minutes had improved by an average of 155 meters (98% per patient). All patients improved their distance walked at 12 minutes, and all but one patient improved in the first 6 minutes of the walk. At Week 48, the average improvement in the 12-minute walk test was 212 meters (138% per patient). Figure 17 shows the improvement in the 12-minute walk test results over the 48 weeks.

The 3-minute stair climb was the second measure of endurance tested. At baseline, patients could climb a mean of 50.3 ( $\pm 29.5$ ) stairs. By 24 weeks, the mean number of stairs climbed had increased to 98.1 ( $\pm 62.5$ ) stairs, an improvement of 48 stairs or 110% per patient ( $\pm 116\%$ ). By 48 weeks, the average number of stairs climbed had increased by 61 stairs to a total of 111 stairs, an improvement of 147% per patient. Figure 18 shows the improvement in the 3-minute stair climb results over the 48 weeks. Patients varied greatly in terms of the number of stairs climbed, and performance at baseline did not necessarily correlate with the degree of improvement at Week 24.

#### **Additional Efficacy Measures**

A number of additional efficacy parameters were evaluated during the first 24 weeks of this study. The Expanded Timed Get Up and Go Test was used as a measure of general functional ability. Overall, there was no significant reduction in total time needed for this test between baseline and Week 24, but there was a modest improvement by Week 48. Figure 19 shows results of the Expanded Timed Get Up and Go Test over 48 weeks of treatment.

Forced Expiratory Time (FET), FVC and FEV<sub>1</sub> were performed to assess pulmonary function. Table 28 below displays pulmonary function values at baseline, Week 24 and Week 48 and also shows improvement in height and organomegaly at Week 48. No significant changes in these parameters were seen over the 24-week period. At Week 48, 5/10 patients improved in FVC and FEV<sub>1</sub>, of whom 3 of the 5 showed improvement despite less than 2% change in growth. At Week 48 there was also an average 43% improvement in Forced Expiratory Time (>1 second).

**Table 28**

<b>Pulmonary Function versus Growth &amp; Organomegaly</b>										
Patient	Age	Baseline Height (cm)	Baseline FVC (liters)	Wk 24 FVC	Wk 48 FVC <sup>1</sup>	Change in Height [cm] (%)	%Δ Liver Wk 48 <sup>2</sup>	Relative %Δ Liver Wk 48	%Δ Spleen Wk 48 <sup>3</sup>	Relative %Δ Spleen Wk 48
200	18	96.2	0.47	0.44	0.48	-0.05(<1)	-6.3	-15.2*	-5.88	-13.90
201	9	93.2	0.52	0.54	0.60	6.65 (7.1)	-2.3	-14.4*	-8.82	-20.17
202	17	120	0.75	ND	0.92	0.55 (<1)	-6.7	-5.3	-27.81	-26.69
203	15	107	0.28	0.27	0.38	1.9 (1.8)	-8.6	-12.5	-16.45	-20.22
204	7	87	0.52	0.60	0.50	3.8 (4.4)	-1.3	-4.5	-6.74	-9.71
300	22	102.4	0.37	0.39	0.37	2.5 (2.4)	-15.5	-20*	-16.58	-21.09
301	9	121.1	1.40	1.46	1.55	4.9 (4.0)	+26.3	+3	19.67	-2.56
302	6	99.7	0.81	0.85	0.83	4.8 (4.8)	+5.7	-13	-1.92	-19.99
303	8	84.6	0.16	0.27	0.31	1.1 (1.3)	-5.8	-13+	-45.33	-49.38

Pulmonary Function versus Growth & Organomegaly										
Patient	Age	Baseline Height (cm)	Baseline FVC (liters)	Wk 24 FVC	Wk 48 FVC <sup>1</sup>	Change in Height [cm] (%)	%Δ Liver Wk 48 <sup>2</sup>	Relative %Δ Liver Wk 48	%Δ Spleen Wk 48 <sup>3</sup>	Relative %Δ Spleen Wk 48
304	16	124.7	0.83	0.74	0.83	1.3 (1.0)	-2.8	-15*	6.93	-6.03
<sup>1</sup> Bolded values indicate FVC values that represent clinically meaningful increases <sup>2</sup> mean liver size at baseline 681.6 ± 118 cc <sup>3</sup> mean spleen size at baseline 146.8 ± 40 cc *Liver volumes above 95% limit age-adjusted to body weight at baseline, and within normal limits at week 48 +Liver volumes above 95% limit age-adjusted to body weight at baseline and week 48										

Table 29

Shoulder Range of Motion			
Shoulder Motion	Baseline	Change @ Week 24 (degrees of function)	Change @ Week 48
All patients (n=10)			
Active Flexion	102	6	3
Passive Flexion	116	4	(1)
Active Extension	52	5	5
Passive Extension	63	9	(6)
Active Lateral Rotation	59	7	6
Passive Lateral Rotation	69	9	(6)
Patients with baseline flexion <90° (n=3)			
Active Flexion	85	9	5
Active Extension	50	6	4
Active Lateral Rotation	52	7	7

Shoulder ROM (flexion, extension, rotation) was assessed both actively and passively. Overall for the group, a modest percent increase was seen in both active and passive ROM; however, some patients showed a decrease in some measures at 24 weeks. At 48 weeks, there was great variability in results due to several outliers; however, there continued to be improvement seen in shoulder ROM particularly in patients that had less than 90 degrees of flexion at baseline. Table 29 above displays the degrees of shoulder range of motion at baseline, Week 24 and Week 48.

Pain and stiffness were assessed using a questionnaire. While two patients had no change in pain score, seven patients showed improvement in pain by Week 24 (one patient had no baseline evaluation). All of the nine patients evaluated showed improvement in stiffness at Week 24. Pain and stiffness evaluated using the questionnaire continued to improve by Week 48. Results of the joint pain questionnaire and joint stiffness questionnaire over 48 weeks of treatment are shown in Figures 20 and 21, respectively.

Significant improvements in grip strength were seen across the entire group of patients. Seven patients had improvements in grip strength for one or both hands. Little change was seen in the pinch test over the 24-week period. There was little significant change seen in the other quality of life assessments, except that at Week 48, there was a modest improvement in Dexterity and Sensation (as measured by the coin pick-up test).

Activity levels were measured using the belt-attached ActiTrac® device. No significant changes in activity level were observed.

No significant changes were observed in cardiac function assessments (ECGs) or in standing height and supine length. Sleep studies performed in a subset of patients showed no clinically significant changes.



## SEQUENCE LISTING

&lt;110&gt; BIOMARIN PHARMACEUTICAL, INC.

&lt;120&gt; PRECURSOR OF N-ACETYL GALACTOSAMINE-4-SULFATASE, METHODS OF TREATMENT USING SAID ENZYME AND METHODS FOR PRODUCING AND PURIFYING SAID ENZYME

&lt;130&gt; 30610/30011A

&lt;150&gt; US 10/290,908

&lt;151&gt; 2002-11-07

&lt;160&gt; 22

&lt;170&gt; PatentIn version 3.2

&lt;210&gt; 1

&lt;211&gt; 1656

&lt;212&gt; DNA

&lt;213&gt; Homo sapiens

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (45)..(1643)

&lt;400&gt; 1

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tagctacagt cggaaaccat cagcaagcag gtcattgttc caac atg ggt ccg cgc      56
                                     Met Gly Pro Arg
                                     1

ggc gcg gcg agc ttg ccc cga ggc ccc gga cct cgg cgg ctg ctc ctc      104
Gly Ala Ala Ser Leu Pro Arg Gly Pro Gly Pro Arg Arg Leu Leu Leu
5                               10                               15                               20

ccc gtc gtc ctc ccg ctg ctg ctg ctg ctg ttg ttg gcg ccg ccg ggc      152
Pro Val Val Leu Pro Leu Leu Leu Leu Leu Leu Leu Ala Pro Pro Gly
25                               30                               35

tcg ggc gcc ggg gcc agc cgg ccg ccc cac ctg gtc ttc ttg ctg gca      200
Ser Gly Ala Gly Ala Ser Arg Pro Pro His Leu Val Phe Leu Leu Ala
40                               45                               50

gac gac cta ggc tgg aac gac gtc ggc ttc cac ggc tcc cgc atc cgc      248
Asp Asp Leu Gly Trp Asn Asp Val Gly Phe His Gly Ser Arg Ile Arg
55                               60                               65

acg ccg cac ctg gac gcg ctg gcg gcc ggc ggg gtg ctc ctg gac aac      296
Thr Pro His Leu Asp Ala Leu Ala Ala Gly Gly Val Leu Leu Asp Asn
70                               75                               80

tac tac acg cag ccg ctg tgc acg ccg tcg cgg agc cag ctg ctc act      344
Tyr Tyr Thr Gln Pro Leu Cys Thr Pro Ser Arg Ser Gln Leu Leu Thr
85                               90                               95                               100

ggc cgc tac cag atc cgt aca ggt tta cag cac caa ata atc tgg ccc      392
Gly Arg Tyr Gln Ile Arg Thr Gly Leu Gln His Gln Ile Ile Trp Pro
105                               110                               115

tgt cag ccc agc tgt gtt cct ctg gat gaa aaa ctc ctg ccc cag ctc      440
Cys Gln Pro Ser Cys Val Pro Leu Asp Glu Lys Leu Leu Pro Gln Leu
120                               125                               130

cta aaa gaa gca ggt tat act acc cat atg gtc gga aaa tgg cac ctg      488

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Leu	Lys	Glu	Ala	Gly	Tyr	Thr	Thr	His	Met	Val	Gly	Lys	Trp	His	Leu	
		135					140					145				
gga	atg	tac	cgg	aaa	gaa	tgc	ctt	cca	acc	cgc	cga	gga	ttt	gat	acc	536
Gly	Met	Tyr	Arg	Lys	Glu	Cys	Leu	Pro	Thr	Arg	Arg	Gly	Phe	Asp	Thr	
	150					155					160					
tac	ttt	gga	tat	ctc	ctg	ggt	agt	gaa	gat	tat	tat	tcc	cat	gaa	cgc	584
Tyr	Phe	Gly	Tyr	Leu	Leu	Gly	Ser	Glu	Asp	Tyr	Tyr	Ser	His	Glu	Arg	
165					170					175					180	
tgt	aca	tta	att	gac	gct	ctg	aat	gtc	aca	cga	tgt	gct	ctt	gat	ttt	632
Cys	Thr	Leu	Ile	Asp	Ala	Leu	Asn	Val	Thr	Arg	Cys	Ala	Leu	Asp	Phe	
				185					190						195	
cga	gat	ggc	gaa	gaa	ggt	gca	aca	gga	tat	aaa	aat	atg	tat	tca	aca	680
Arg	Asp	Gly	Glu	Glu	Val	Ala	Thr	Gly	Tyr	Lys	Asn	Met	Tyr	Ser	Thr	
		200						205					210			
aac	ata	ttc	acc	aaa	agg	gct	ata	gcc	ctc	ata	act	aac	cat	cca	cca	728
Asn	Ile	Phe	Thr	Lys	Arg	Ala	Ile	Ala	Leu	Ile	Thr	Asn	His	Pro	Pro	
		215				220						225				
gag	aag	cct	ctg	ttt	ctc	tac	ctt	gct	ctc	cag	tct	gtg	cat	gag	ccc	776
Glu	Lys	Pro	Leu	Phe	Leu	Tyr	Leu	Ala	Leu	Gln	Ser	Val	His	Glu	Pro	
	230					235					240					
ctt	cag	gtc	cct	gag	gaa	tac	ttg	aag	cca	tat	gac	ttt	atc	caa	gac	824
Leu	Gln	Val	Pro	Glu	Glu	Tyr	Leu	Lys	Pro	Tyr	Asp	Phe	Ile	Gln	Asp	
245					250					255					260	
aag	aac	agg	cat	cac	tat	gca	gga	atg	gtg	tcc	ctt	atg	gat	gaa	gca	872
Lys	Asn	Arg	His	His	Tyr	Ala	Gly	Met	Val	Ser	Leu	Met	Asp	Glu	Ala	
			265						270					275		
gta	gga	aat	gtc	act	gca	gct	tta	aaa	agc	agt	ggg	ctc	tgg	aac	aac	920
Val	Gly	Asn	Val	Thr	Ala	Ala	Leu	Lys	Ser	Ser	Gly	Leu	Trp	Asn	Asn	
			280					285					290			
acg	gtg	ttc	atc	ttt	tct	aca	gat	aac	gga	ggg	cag	act	ttg	gca	ggg	968
Thr	Val	Phe	Ile	Phe	Ser	Thr	Asp	Asn	Gly	Gly	Gln	Thr	Leu	Ala	Gly	
		295					300					305				
ggt	aat	aac	tgg	ccc	ctt	cga	gga	aga	aaa	tgg	agc	ctg	tgg	gaa	gga	1016
Gly	Asn	Asn	Trp	Pro	Leu	Arg	Gly	Arg	Lys	Trp	Ser	Leu	Trp	Glu	Gly	
	310					315					320					
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Gly	Val	Arg	Gly	Val	Gly	Phe	Val	Ala	Ser	Pro	Leu	Leu	Lys	Gln	Lys	
325					330					335					340	
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Gly	Val	Lys	Asn	Arg	Glu	Leu	Ile	His	Ile	Ser	Asp	Trp	Leu	Pro	Thr	
				345					350					355		
ctc	gtg	aag	ctg	gcc	agg	gga	cac	acc	aat	ggc	aca	aag	cct	ctg	gat	1160
Leu	Val	Lys	Leu	Ala	Arg	Gly	His	Thr	Asn	Gly	Thr	Lys	Pro	Leu	Asp	
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Gly	Phe	Asp	Val	Trp	Lys	Thr	Ile	Ser	Glu	Gly	Ser	Pro	Ser	Pro	Arg	
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att Ile	gag Glu	ctg Leu	ctg Leu	cat His	aat Asn	att Ile	gac Asp	cca Pro	aac Asn	ttc Phe	gtg Val	gac Asp	tct Ser	tca Ser	ccg Pro	1256															
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tgt Cys	ccc Pro	agg Arg	aac Asn	agc Ser	atg Met	gct Ala	cca Pro	gca Ala	aag Lys	gat Asp	gac Asp	tct Ser	tct Ser	ctt Leu	cca Pro	1304															
405					410					415					420																
gaa Glu	tat Tyr	tca Ser	gcc Ala	ttt Phe	aac Asn	aca Thr	tct Ser	gtc Val	cat His	gct Ala	gca Ala	att Ile	aga Arg	cat His	gga Gly	1352															
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aat Asn	tgg Trp	aaa Lys	ctc Leu	ctc Leu	acg Thr	ggc Gly	tac Tyr	cca Pro	ggc Gly	tgt Cys	ggt Gly	tac Tyr	tgg Trp	ttc Phe	cct Pro	1400															
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cca Pro	ccg Pro	tct Ser	caa Gln	tac Tyr	aat Asn	gtt Val	tct Ser	gag Glu	ata Ile	ccc Pro	tca Ser	tca Ser	gac Asp	cca Pro	cca Pro	1448															
			455				460					465																			
acc Thr	aag Lys	acc Thr	ctc Leu	tgg Trp	ctc Leu	ttt Phe	gat Asp	att Ile	gat Asp	cgg Arg	gac Asp	cct Pro	gaa Glu	gaa Glu	aga Arg	1496															
	470					475					480																				
cat His	gac Asp	ctg Leu	tcc Ser	aga Arg	gaa Glu	tat Tyr	cct Pro	cac His	atc Ile	gtc Val	aca Thr	aag Lys	ctc Leu	ctg Leu	tcc Ser	1544															
485					490					495					500																
cgc Arg	cta Leu	cag Gln	ttc Phe	tac Tyr	cat His	aaa Lys	cac His	tca Ser	gtc Val	ccc Pro	gtg Val	tac Tyr	ttc Phe	cct Pro	gca Ala	1592															
				505					510					515																	
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atg Met	taggatttca ggg															1656															
<210> 2																															
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1				5					10					15																	
Arg	Leu	Leu	Leu	Pro	Val	Val	Leu	Pro	Leu	Leu	Leu	Leu	Leu	Leu	Leu																
			20					25					30																		
Ala	Pro	Pro	Gly	Ser	Gly	Ala	Gly	Ala	Ser	Arg	Pro	Pro	His	Leu	Val																
		35					40					45																			
Phe	Leu	Leu	Ala	Asp	Asp	Leu	Gly	Trp	Asn	Asp	Val	Gly	Phe	His	Gly																
50						55					60																				

Ser Arg Ile Arg Thr Pro His Leu Asp Ala Leu Ala Ala Gly Gly Val  
 65 70 75 80  
 Leu Leu Asp Asn Tyr Tyr Thr Gln Pro Leu Cys Thr Pro Ser Arg Ser  
 85 90 95  
 Gln Leu Leu Thr Gly Arg Tyr Gln Ile Arg Thr Gly Leu Gln His Gln  
 100 105 110  
 Ile Ile Trp Pro Cys Gln Pro Ser Cys Val Pro Leu Asp Glu Lys Leu  
 115 120 125  
 Leu Pro Gln Leu Leu Lys Glu Ala Gly Tyr Thr Thr His Met Val Gly  
 130 135 140  
 Lys Trp His Leu Gly Met Tyr Arg Lys Glu Cys Leu Pro Thr Arg Arg  
 145 150 155 160  
 Gly Phe Asp Thr Tyr Phe Gly Tyr Leu Leu Gly Ser Glu Asp Tyr Tyr  
 165 170 175  
 Ser His Glu Arg Cys Thr Leu Ile Asp Ala Leu Asn Val Thr Arg Cys  
 180 185 190  
 Ala Leu Asp Phe Arg Asp Gly Glu Glu Val Ala Thr Gly Tyr Lys Asn  
 195 200 205  
 Met Tyr Ser Thr Asn Ile Phe Thr Lys Arg Ala Ile Ala Leu Ile Thr  
 210 215 220  
 Asn His Pro Pro Glu Lys Pro Leu Phe Leu Tyr Leu Ala Leu Gln Ser  
 225 230 235 240  
 Val His Glu Pro Leu Gln Val Pro Glu Glu Tyr Leu Lys Pro Tyr Asp  
 245 250 255  
 Phe Ile Gln Asp Lys Asn Arg His His Tyr Ala Gly Met Val Ser Leu  
 260 265 270  
 Met Asp Glu Ala Val Gly Asn Val Thr Ala Ala Leu Lys Ser Ser Gly  
 275 280 285  
 Leu Trp Asn Asn Thr Val Phe Ile Phe Ser Thr Asp Asn Gly Gly Gln  
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 Thr Leu Ala Gly Gly Asn Asn Trp Pro Leu Arg Gly Arg Lys Trp Ser  
 305 310 315 320

Leu Trp Glu Gly Gly Val Arg Gly Val Gly Phe Val Ala Ser Pro Leu  
 325 330 335  
 Leu Lys Gln Lys Gly Val Lys Asn Arg Glu Leu Ile His Ile Ser Asp  
 340 345 350  
 Trp Leu Pro Thr Leu Val Lys Leu Ala Arg Gly His Thr Asn Gly Thr  
 355 360 365  
 Lys Pro Leu Asp Gly Phe Asp Val Trp Lys Thr Ile Ser Glu Gly Ser  
 370 375 380  
 Pro Ser Pro Arg Ile Glu Leu Leu His Asn Ile Asp Pro Asn Phe Val  
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 405 410 415  
 Ser Ser Leu Pro Glu Tyr Ser Ala Phe Asn Thr Ser Val His Ala Ala  
 420 425 430  
 Ile Arg His Gly Asn Trp Lys Leu Leu Thr Gly Tyr Pro Gly Cys Gly  
 435 440 445  
 Tyr Trp Phe Pro Pro Pro Ser Gln Tyr Asn Val Ser Glu Ile Pro Ser  
 450 455 460  
 Ser Asp Pro Pro Thr Lys Thr Leu Trp Leu Phe Asp Ile Asp Arg Asp  
 465 470 475 480  
 Pro Glu Glu Arg His Asp Leu Ser Arg Glu Tyr Pro His Ile Val Thr  
 485 490 495  
 Lys Leu Leu Ser Arg Leu Gln Phe Tyr His Lys His Ser Val Pro Val  
 500 505 510  
 Tyr Phe Pro Ala Gln Asp Pro Arg Cys Asp Pro Lys Ala Thr Gly Val  
 515 520 525  
 Trp Gly Pro Trp Met  
 530

## Patentkrav

1. Præparat, der omfatter precursor til human N-acetylgalactosamin-4-sulfatase, hvor precursoren til human N-acetylgalactosamin-4-sulfatase har en renhed på mindst lig med eller over 99 % baseret på totalt protein, hvor renheden er målt ved anvendelse af omvendt fase-HPLC (RP-HPLC)-metoden, og hvor præparatet er fri for påviselige bånd på ca. 47-48 kDa ved natriumdodecylsulfat-polyacrylamidgelelektroforese (SDS-PAGE) med Coomassie-farvestof.
2. Præparat ifølge krav 1, hvor precursoren til human N-acetylgalactosamin-4-sulfatase er en rekombinant precursor til human N-acetylgalactosamin-4-sulfatase.
3. Præparat ifølge krav 1 eller krav 2, hvor precursoren til human N-acetylgalactosamin-4-sulfatase har en renhed på mindst lig med eller over 99,2 % baseret på totalt protein, hvor renheden er målt ved anvendelse af RP-HPLC-metoden, eller hvor renheden er lig med eller over 99,5 %, eller hvor renheden er lig med eller over 99,9 %.
4. Præparat ifølge krav 1 eller krav 2, hvor precursoren til human N-acetylgalactosamin-4-sulfatase er glycosyleret.
5. Præparat ifølge et hvilket som helst af ovennævnte krav, hvor precursoren til human N-acetylgalactosamin-4-sulfatase har en renhed på mindst lig med eller over 99 % baseret på totalt protein, hvor renheden er målt ved anvendelse af størrelseskromatografi-HPLC (SEC-HPLC)-metoden, eller hvor renheden ved hjælp af SEC-HPLC er lig med eller over 99,5 %, eller hvor renheden ved hjælp af RP-HPLC er lig med eller over 99 %, og renheden ved hjælp af SEC-HPLC er lig med eller over 99,5 %.
6. Farmaceutisk præparat, der omfatter præparatet ifølge krav 1 eller krav 2 og et farmaceutisk acceptabelt bæremateriale.

7. Farmaceutisk præparat ifølge krav 6, der yderligere omfatter en natriumchloridopløsning og et ikke-ionisk detergent.

5

8. Farmaceutisk præparat ifølge krav 6, hvor precursoren til human N-acetylgalactosamin-4-sulfatase er til stede ved en koncentration på ca. 1-5 mg/ml eller ca. 50 til ca. 250 enheder/ml.

10

9. Farmaceutisk præparat ifølge krav 6, hvor bufferen er en natriumphosphatbuffer ved en koncentration på ca. 10-50 mM.

15

10. Farmaceutisk præparat ifølge krav 6, hvor pH i opløsningen opretholdes ved ca. 5,8.

11. Farmaceutisk præparat ifølge krav 6, der omfatter en polyoxyethylensorbitan.

20

12. Farmaceutisk præparat ifølge krav 11, hvor polyoxyethylensorbitanen er polyoxyethylensorbitan 20 eller 80, og koncentrationen af polyoxyethylensorbitan 20 eller 80 er ca. 0,005 % (vægt/volumen).

25

13. Præparat ifølge et hvilket som helst af kravene 1 til 12 til anvendelse til behandling af en sygdom, der er forårsaget helt eller delvist af en defekt i N-acetylgalactosamin-4-sulfatase, hvor sygdommen er mukopolysakkaridose, MPS VI eller Maroteaux-Lamys syndrom.

30

14. Præparat til anvendelse ifølge krav 13, hvor patienten har ca. 10 % eller mindre af en normal N-acetylgalactosamin-4-sulfataseaktivitet.

35

15. Anvendelse af præparatet ifølge et hvilket som helst af kravene 1 til 12 til fremstilling af et medikament til behandling af en sygdom, der er forårsaget helt eller delvist af en defekt i N-acetylgalactosamin-4-sulfatase, hvor

sygdommen er mukopolysakkaridose, MPS VI eller Maroteaux-Lamys syndrom.

16. Anvendelse ifølge krav 15 eller farmaceutisk præparat til  
5 anvendelse ifølge krav 13, hvor mindst ca. 50 enheder/kg eller  
mindst ca. 1 mg/kg af precursoren til human N-  
acetylgalactosamin-4-sulfatase skal administreres ugentligt  
til patienten, eller hvor mindst ca. 100 enheder eller 2,0  
10 mg/kg af precursoren til human N-acetylgalactosamin-4-  
sulfatase skal administreres ugentligt til patienten.

17. Anvendelse ifølge krav 15 eller farmaceutisk præparat til  
anvendelse ifølge krav 13, hvor medikamentet er til behandling  
af et menneske.

15

18. Anvendelse ifølge krav 15 eller farmaceutisk præparat til  
anvendelse ifølge krav 13, hvor mængden af precursoren til  
human N-acetylgalactosamin-4-sulfatase er effektiv til  
tilvejebringelse af en eller flere gunstige virkninger, der er  
20 udvalgt fra gruppen, der består af ledbevægelighed, ledsmerter,  
ledstivhed, arbejdstolerance, arbejdsudholdenhed,  
lungefunktion, synsstyrke og daglige gøremål.

19. Farmaceutisk præparat, der omfatter precursor til human  
25 N-acetylgalactosamin-4-sulfatase ifølge et hvilket som helst  
af kravene 1-5 og en polyoxyethylensorbitan ved en  
koncentration i intervallet fra ca. 0,002 % til ca. 0,008 %  
(vægt/volumen).

30 20. Farmaceutisk præparat ifølge krav 19, hvor  
polyoxyethylensorbitanen er polyoxyethylensorbitan 80 ved en  
koncentration på 0,005 % (vægt/volumen).

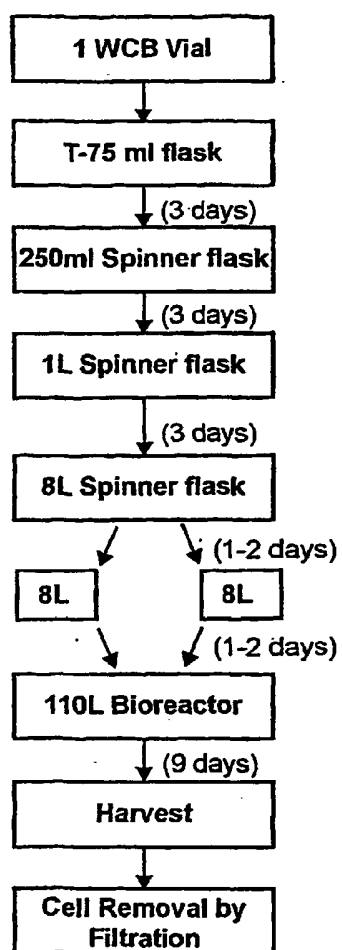
21. Anvendelse ifølge krav 15 eller farmaceutisk præparat til  
35 anvendelse ifølge krav 13, hvor medikamentet er til behandling  
af et menneske, og precursoren til human N-acetylgalactosamin-  
4-sulfatase skal administreres ved en dosis på mindst  
0,2 mg/kg pr. uge, eller hvor precursoren til human N-



acetylgalactosamin-4-sulfatase skal administreres ved en dosis på 1 mg/kg pr. uge.

22. Anvendelse ifølge krav 15 eller farmaceutisk præparat til  
5 anvendelse ifølge krav 13, hvor precursoren til human N-acetylgalactosamin-4-sulfatase skal administreres ved en infusion en gang ugentligt over et tidsrum på 2 til 4 timer, eller hvor precursoren til human N-acetylgalactosamin-4-sulfatase skal administreres ved en infusion en gang ugentligt  
10 over et tidsrum på 4 timer.

**FIG. 1**



**FIG. 2**

**Outline of the rhASB Drug Substance  
Purification Process**

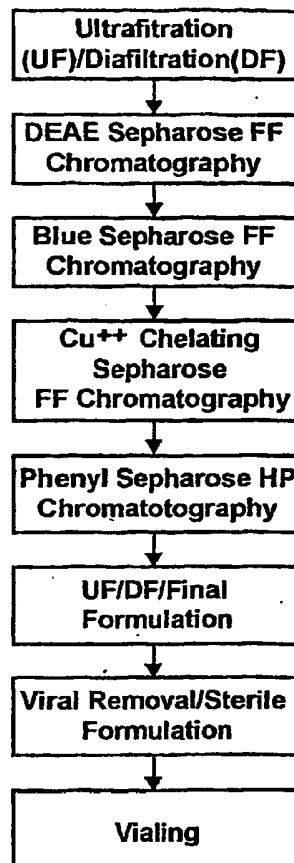


FIG. 3

## Process Flow Chart - Sampling Plan

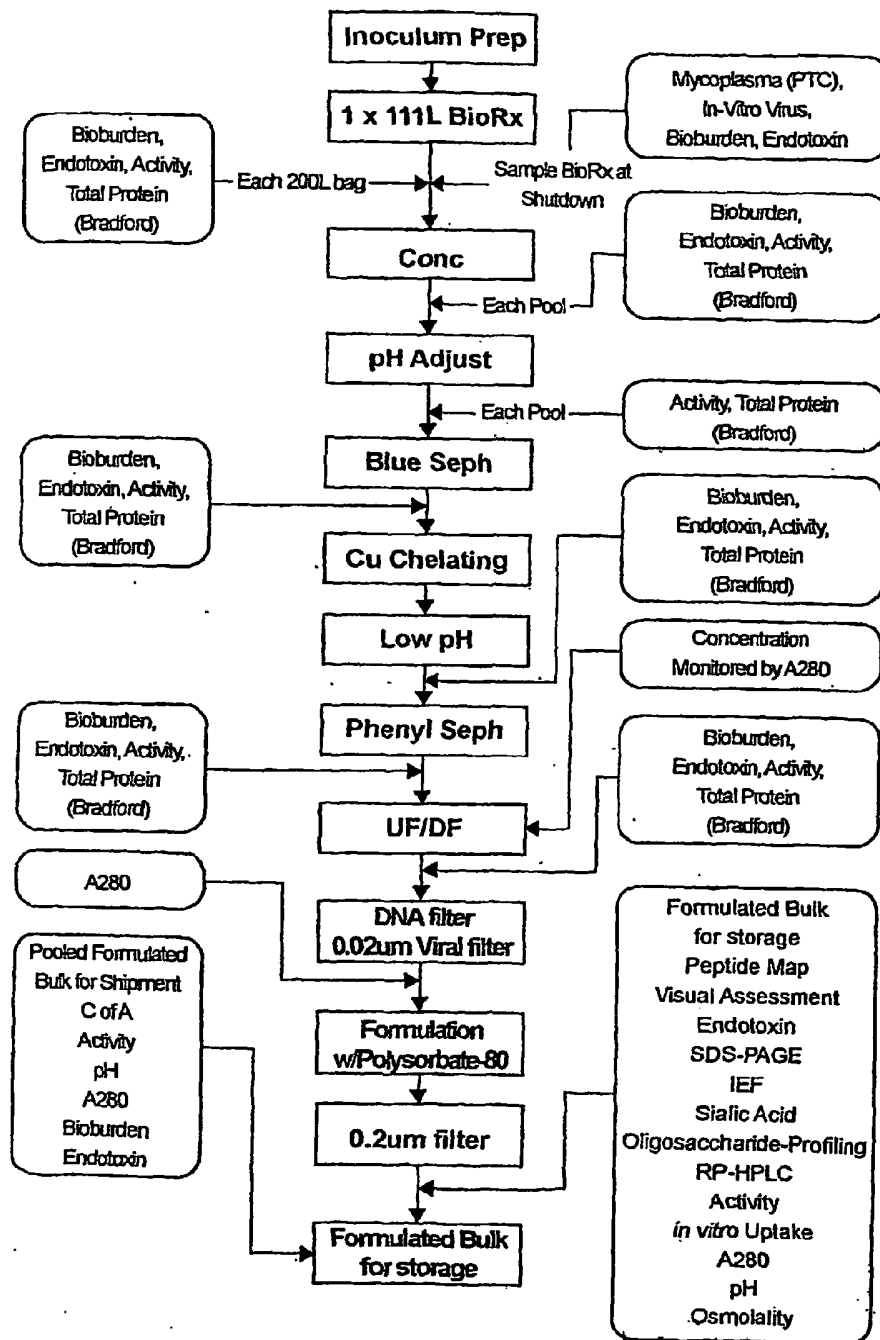
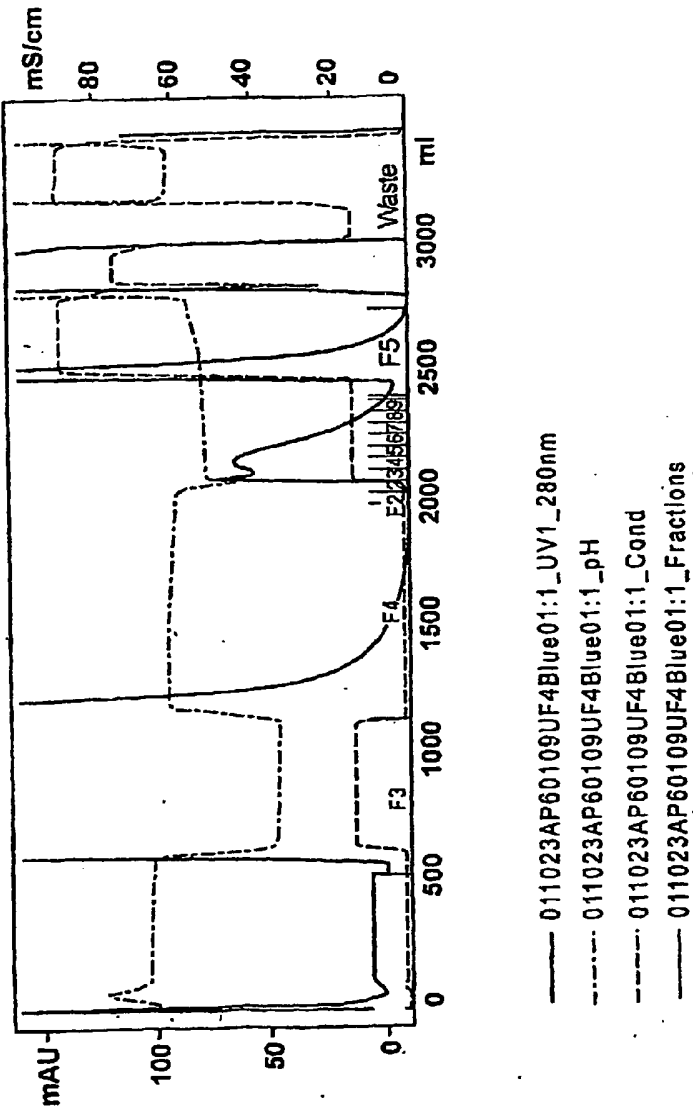


FIG. 4A



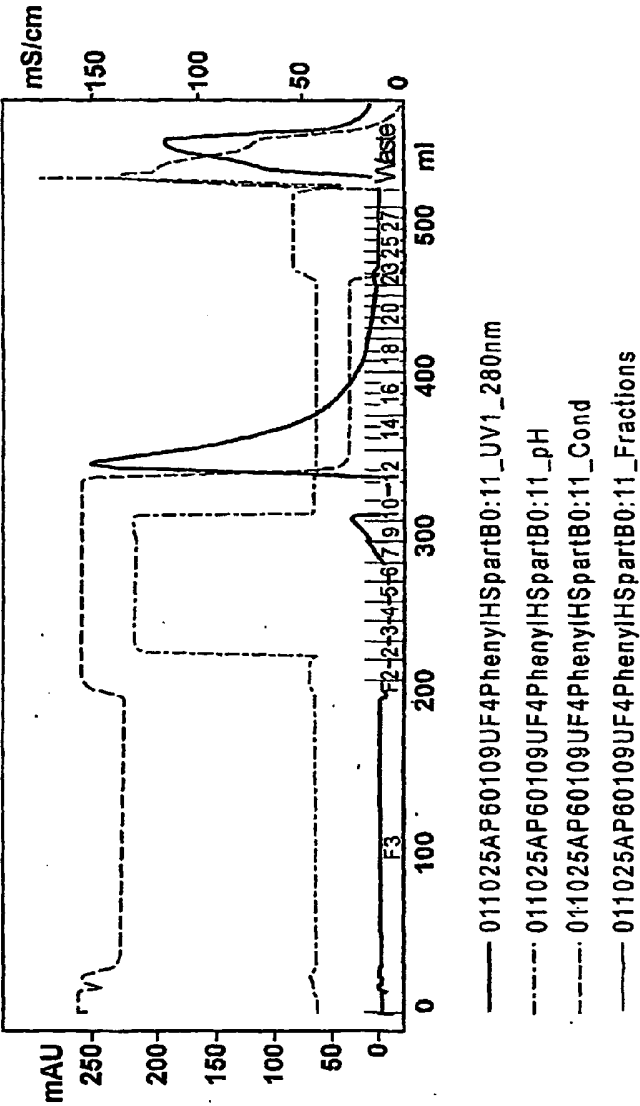
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----- 011024AP60109UF4CC01:1\_pH

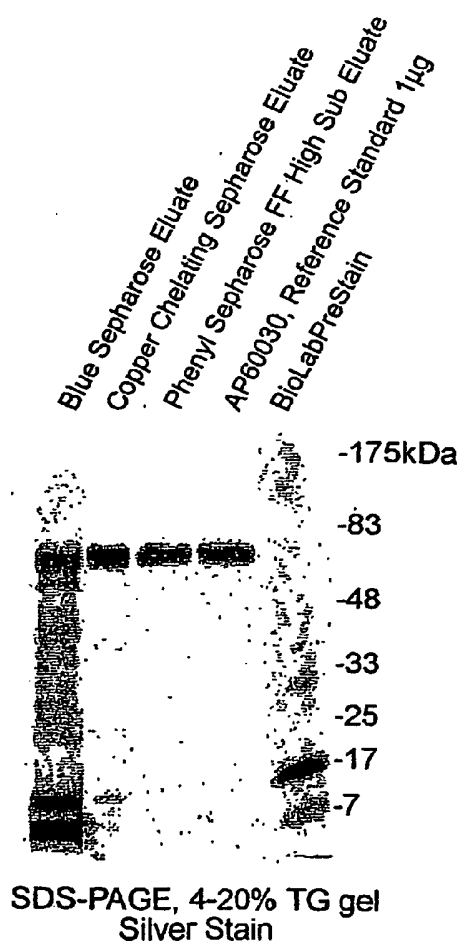
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011024AP60109UF4CC01:1\_Fractions

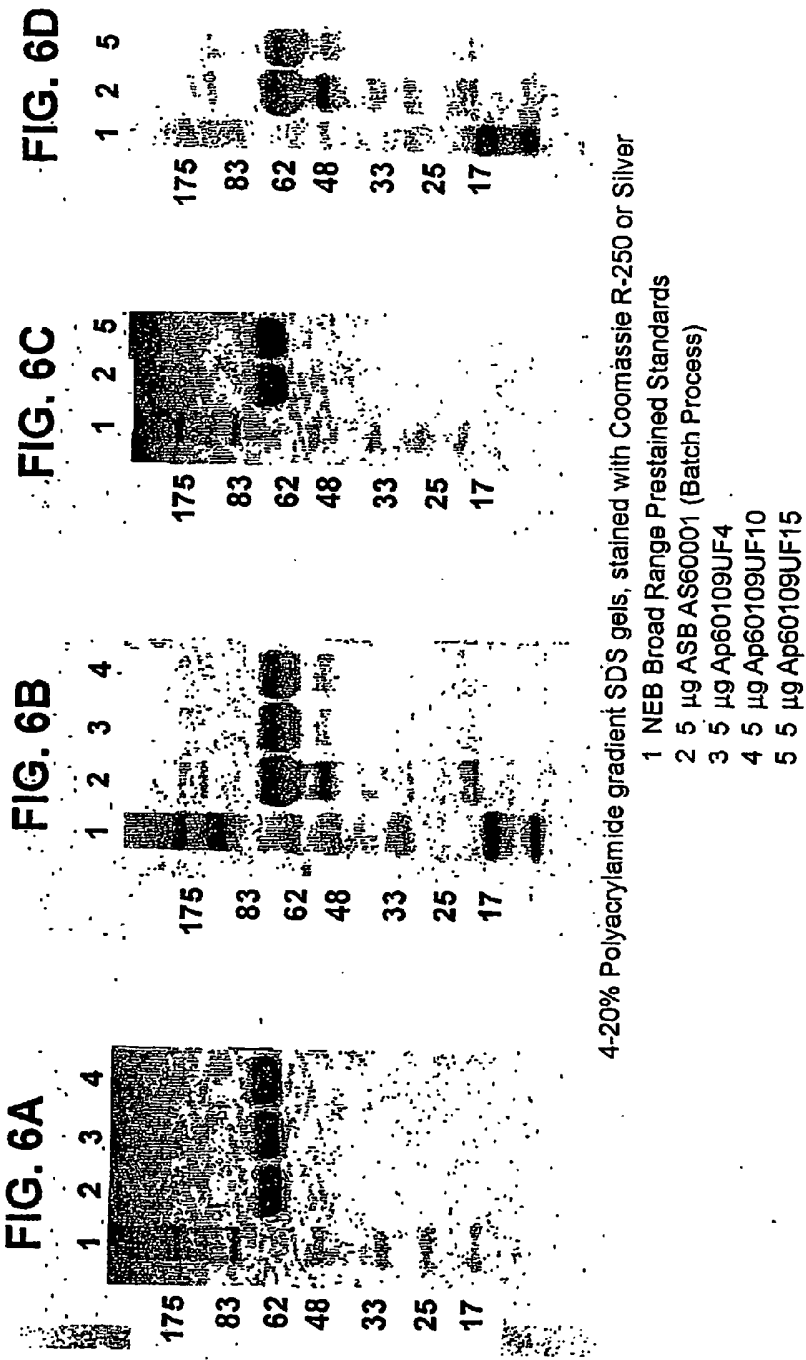
FIG. 4C

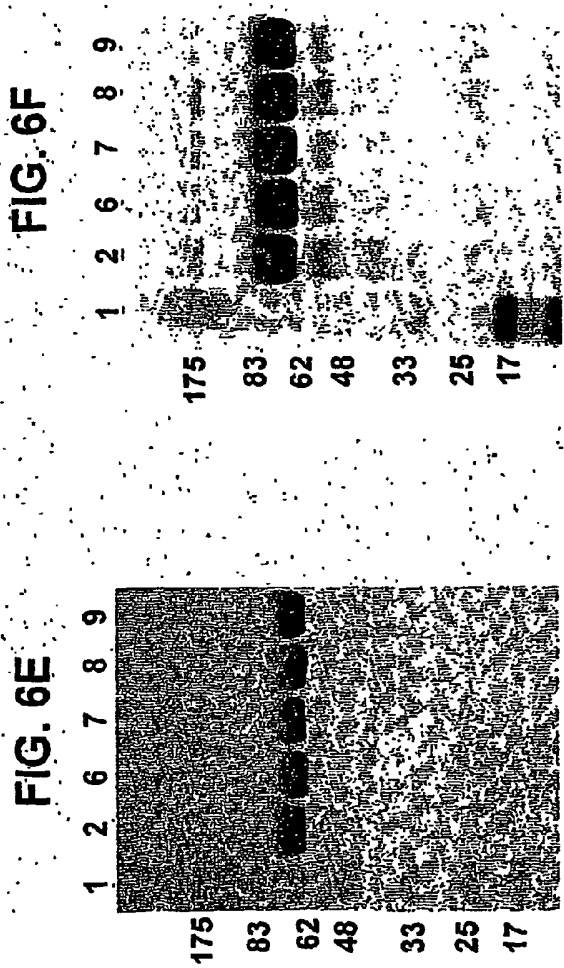


**FIG. 5**





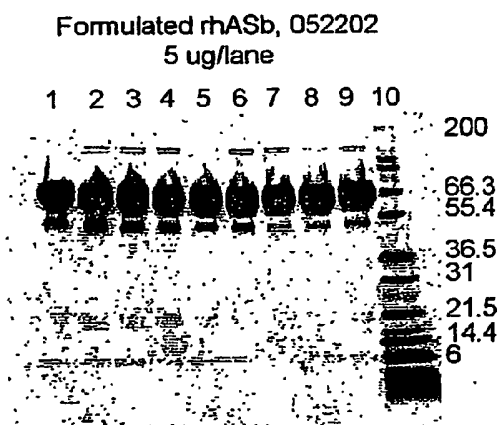




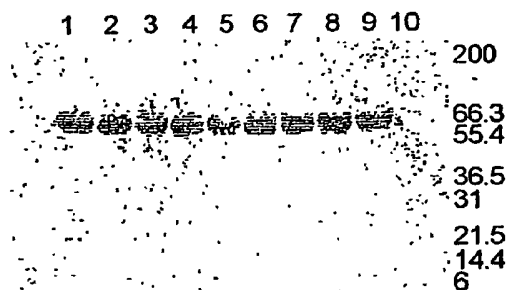
4-20% Polyacrylamide gradient SDS gels, stained with Coomassie R-250 or Silver

- 1 NEB Broad Range Prestained Standards
- 2 5  $\mu$ g ASB AS60001 (Batch Process)
- 3 5  $\mu$ g Ap60109UF4
- 4 5  $\mu$ g Ap60109UF10
- 5 5  $\mu$ g Ap60109UF15
- 6 5  $\mu$ g Ap60109UF18
- 7 5  $\mu$ g Ap60109UF22
- 8 5  $\mu$ g Ap60109UF25
- 9 5  $\mu$ g Ap60109UF27

**FIG. 7A**



**FIG. 7B**



- |                  |                      |
|------------------|----------------------|
| 1. AP60202 UF 4  | 6. 102PD0139xB5      |
| 2. AP60202 UF 10 | 7. rhASB-202-002     |
| 3. AP60202 UF 18 | 8. 102PD0136 P1      |
| 4. AP60202 (BMK) | 9. 102PD0136 P2      |
| 5. 102PD0139xB3  | 10. Mark 12 Standard |

FIG. 8A

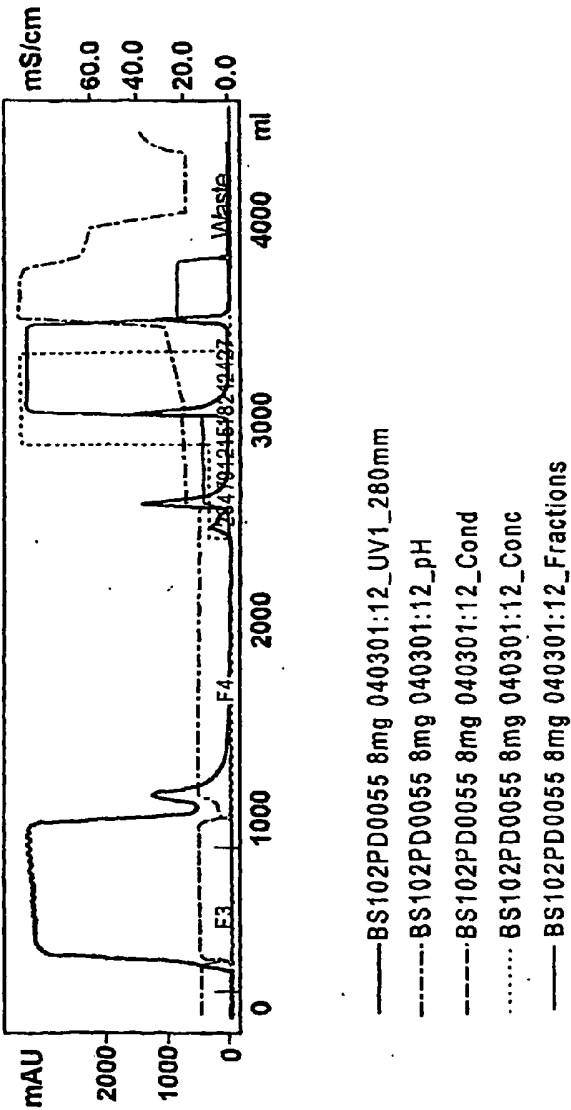


FIG. 8B

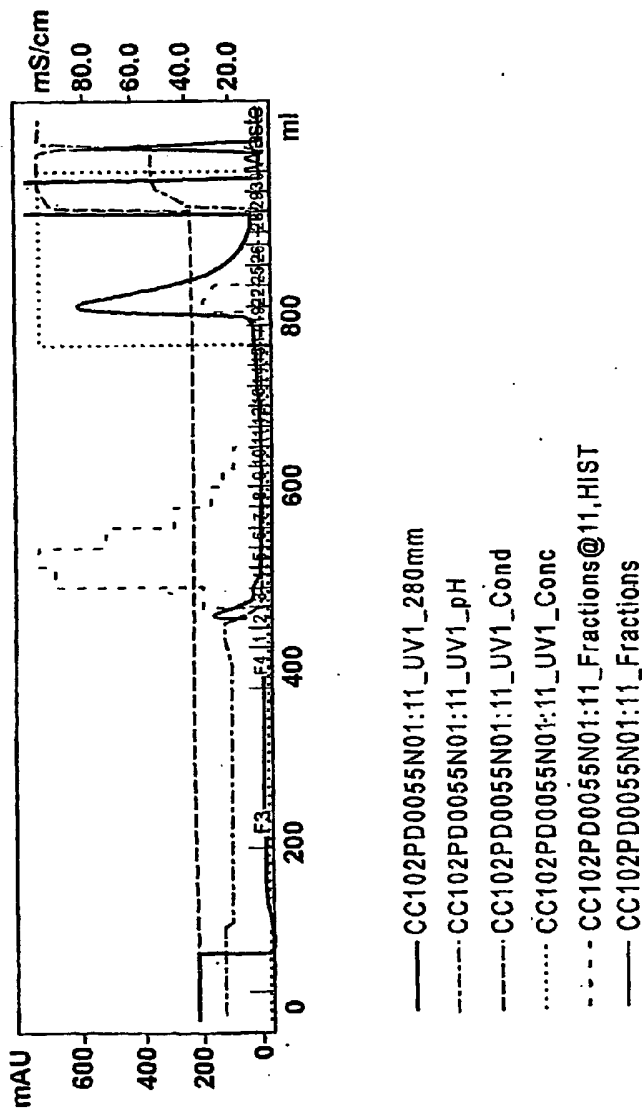


FIG. 8C

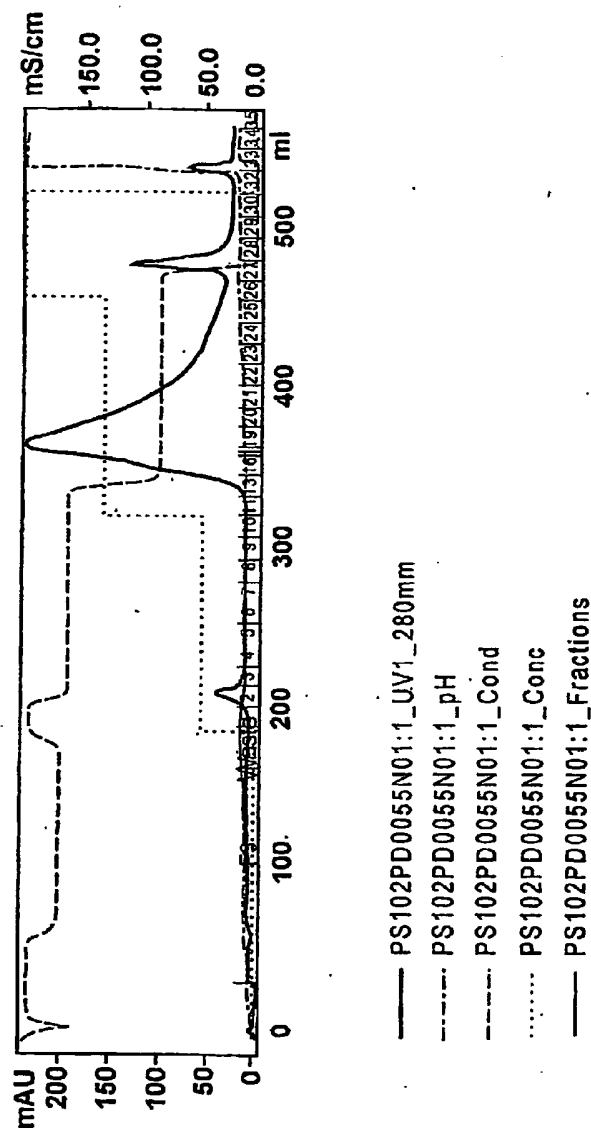


FIG. 9A

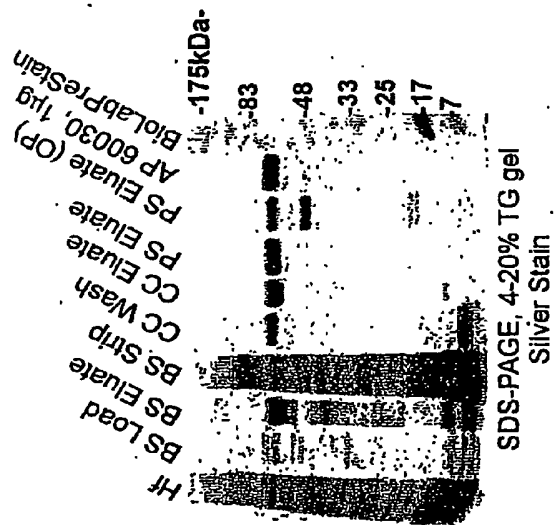
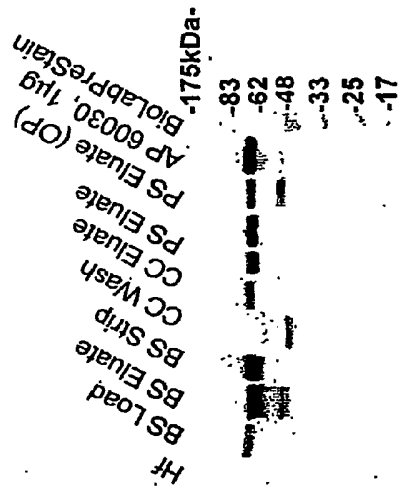


FIG. 9B



Western Blot, Anti-Asb, BP15 BL2, 1:5,000  
Anti-Rabbit IgG (H+L) AP Conjugate 1:3,000

FIG. 9C

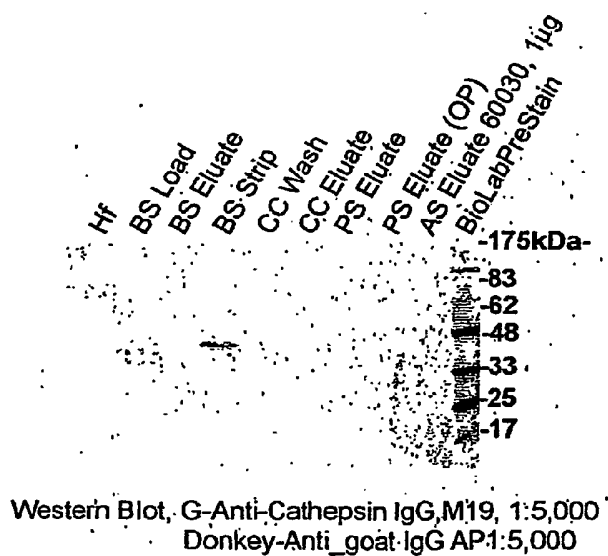




FIG. 10

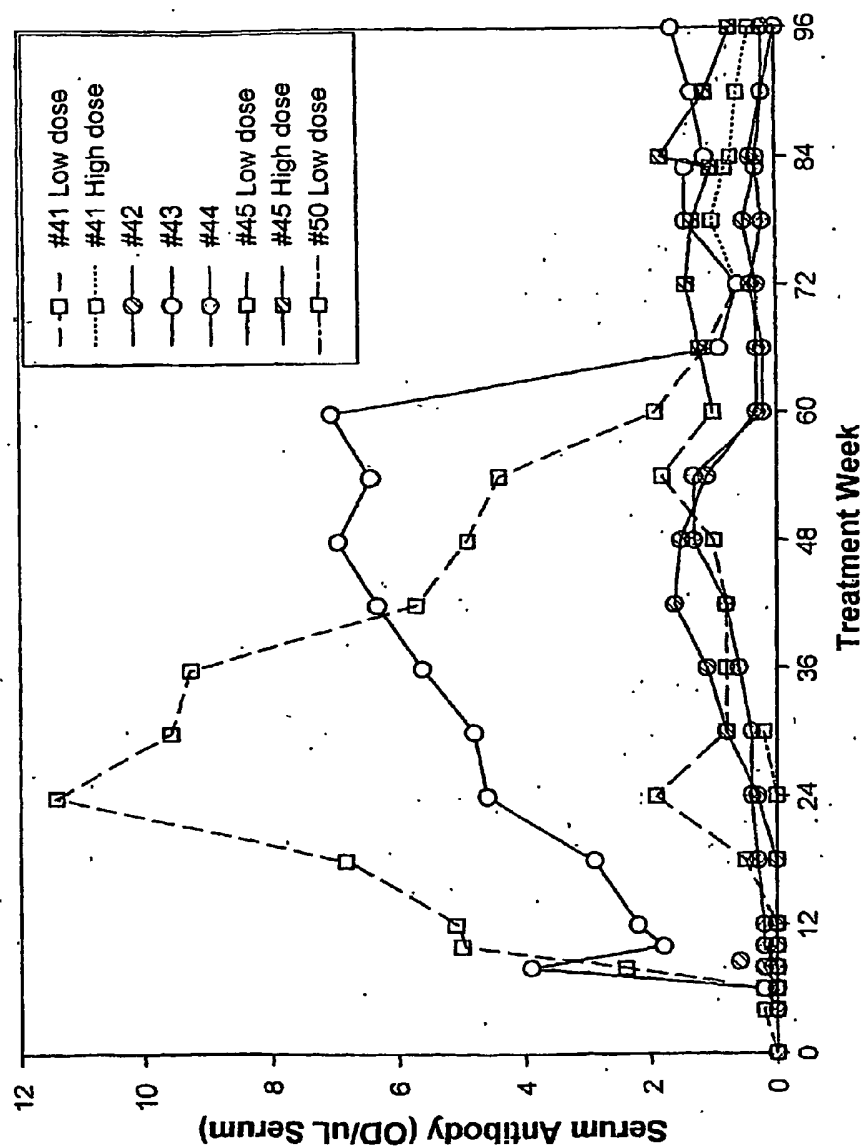


FIG. 11

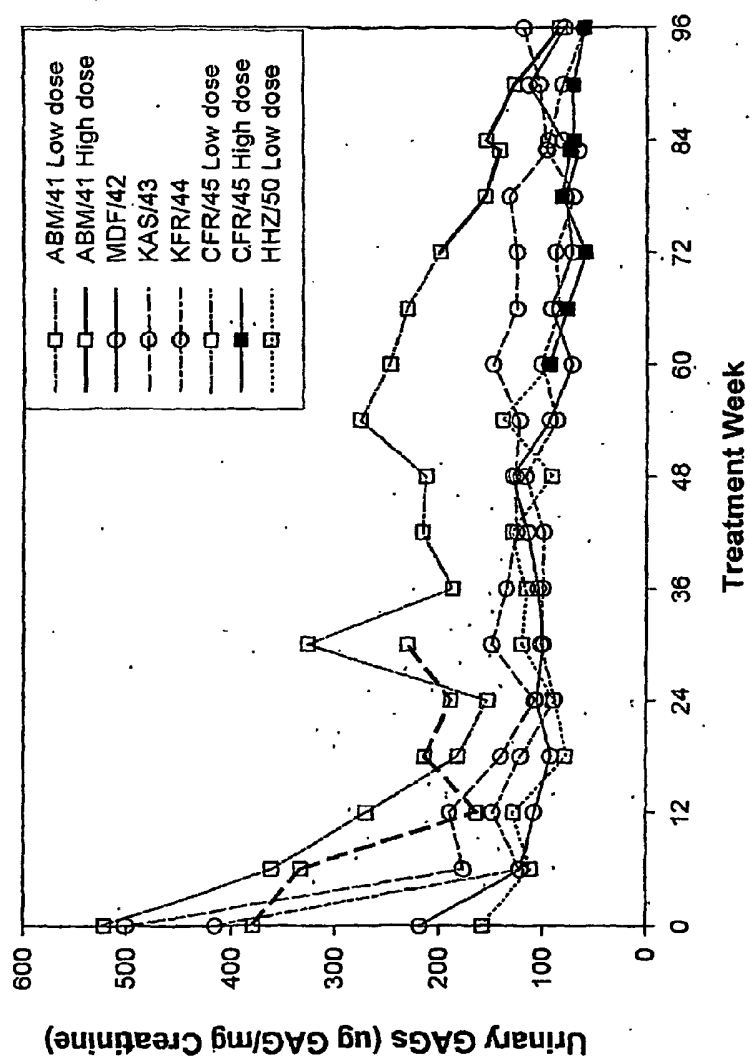
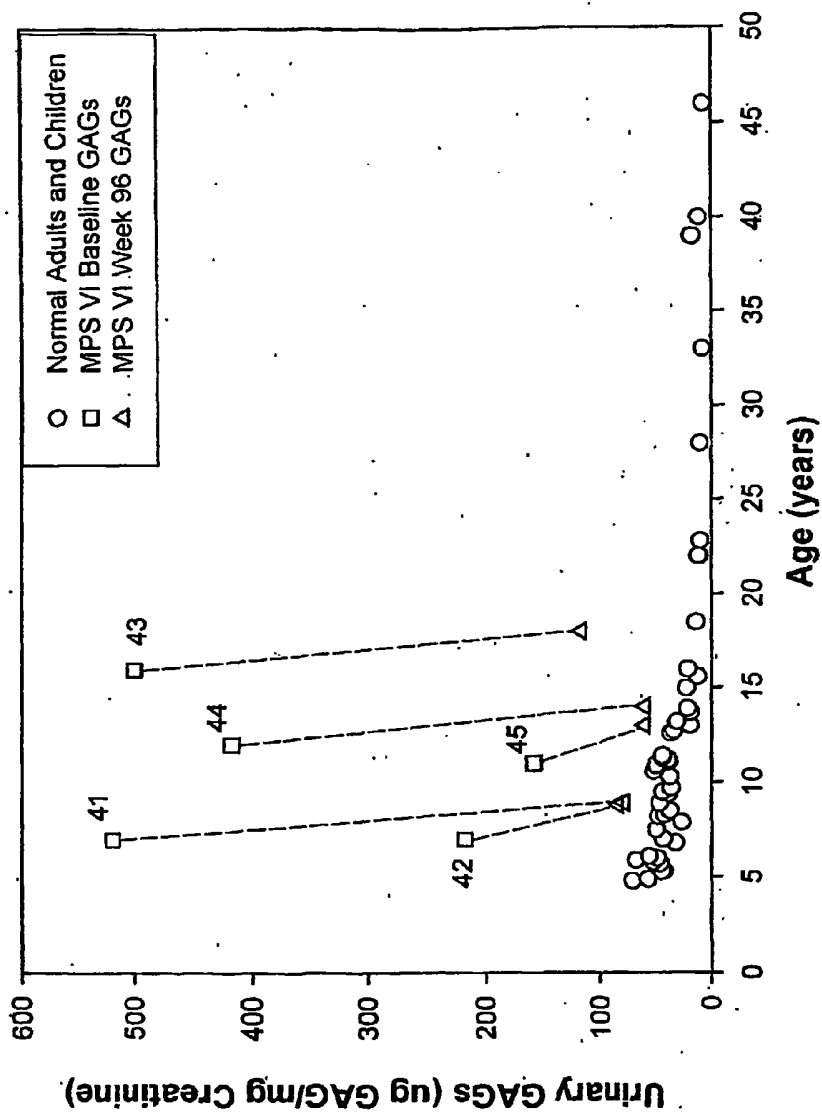
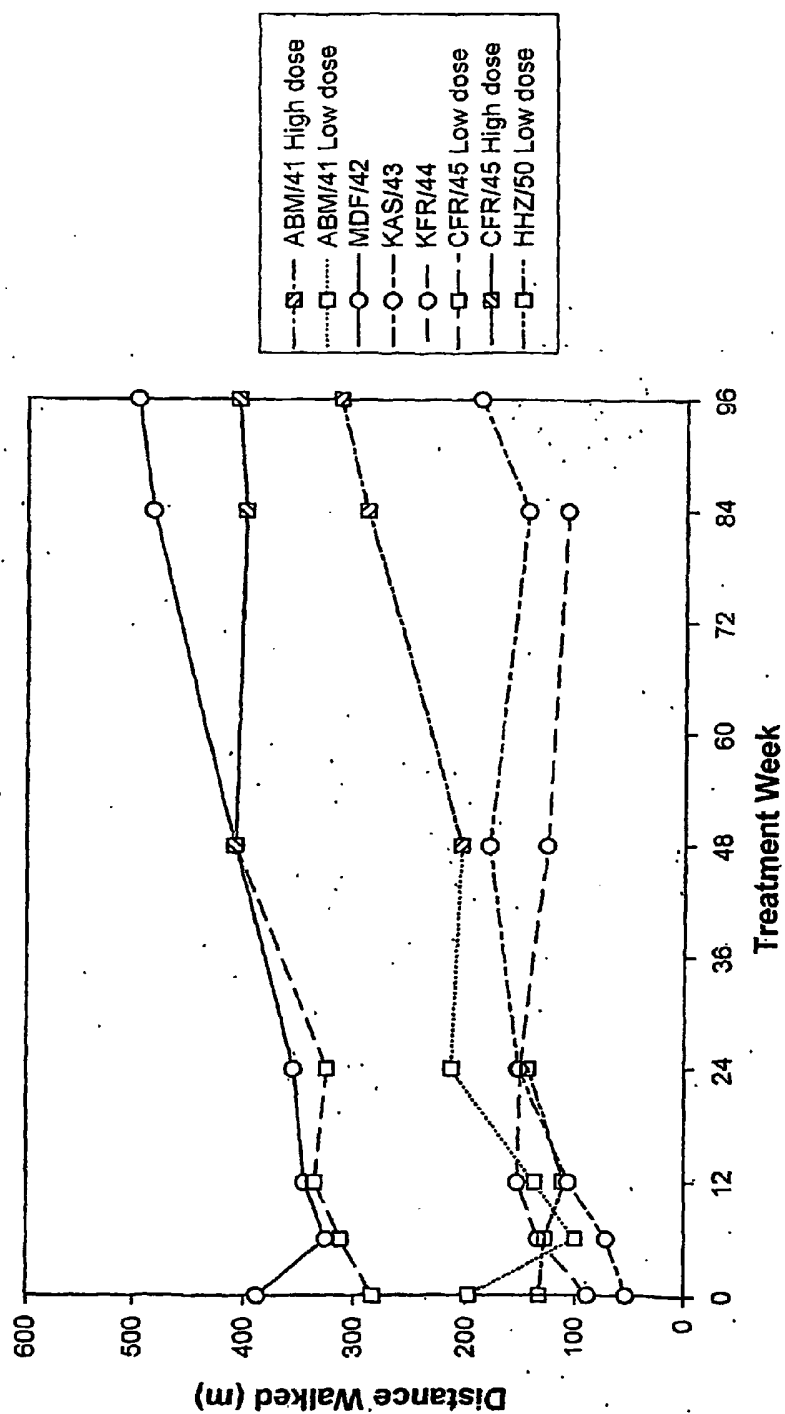


FIG. 12





**FIG. 14**

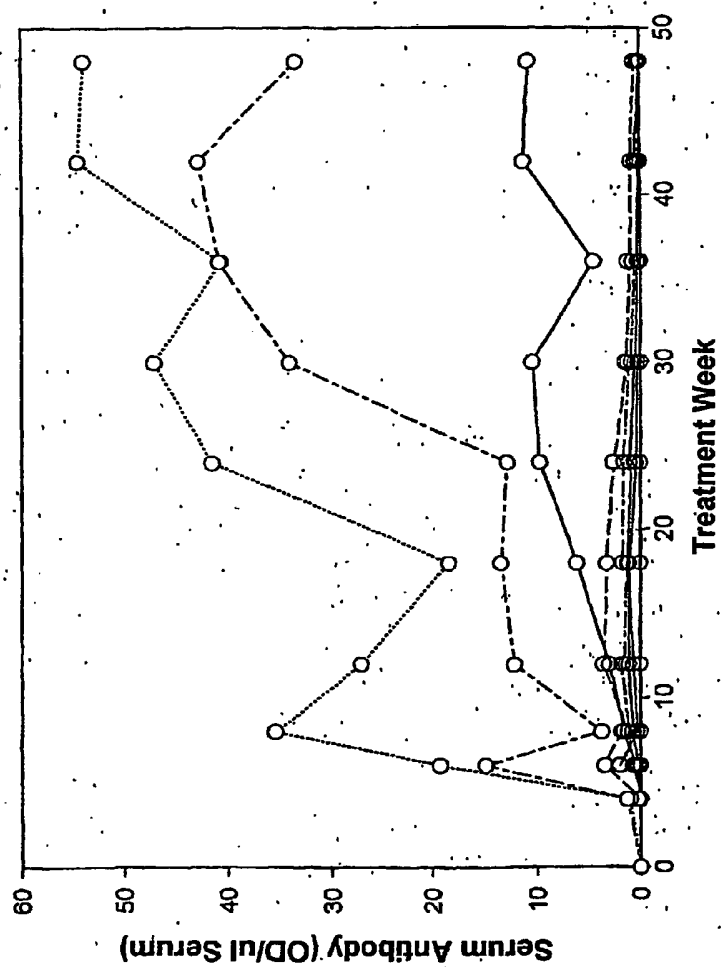


FIG. 15

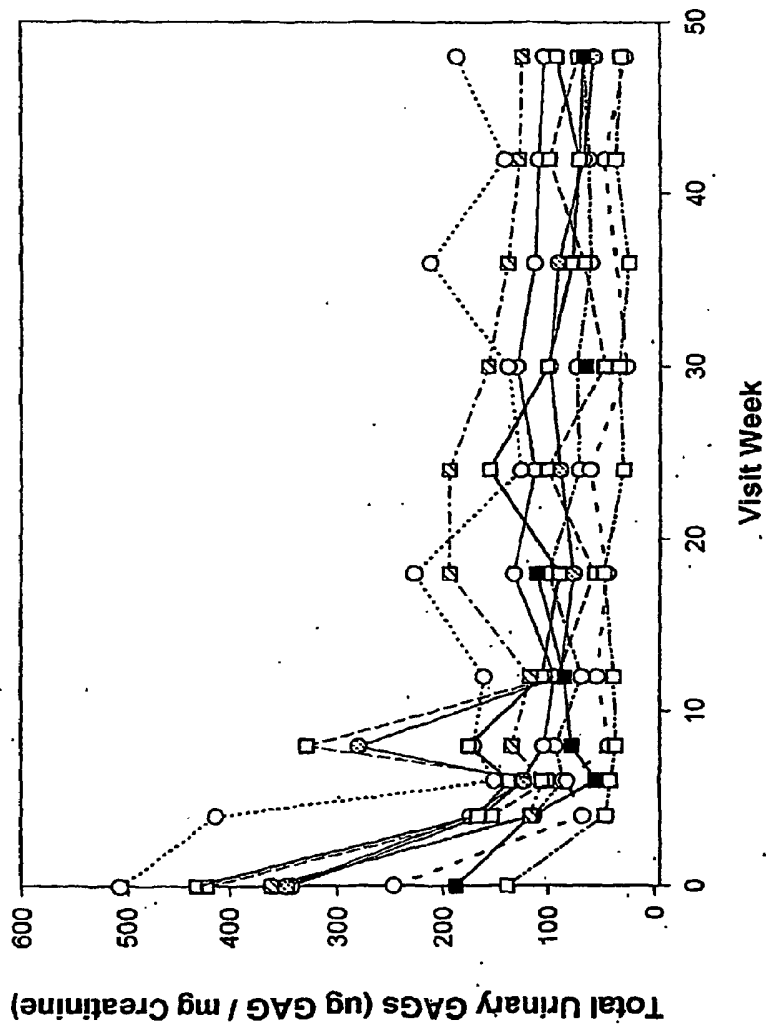


FIG. 16

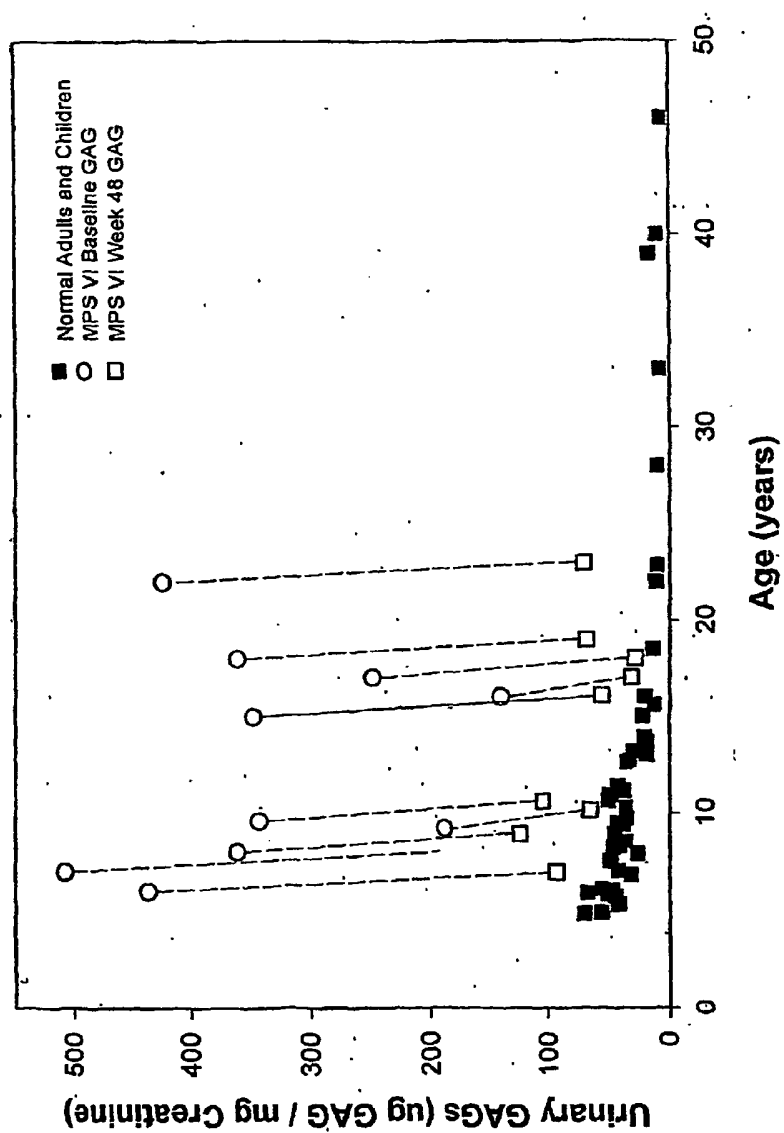


FIG. 17

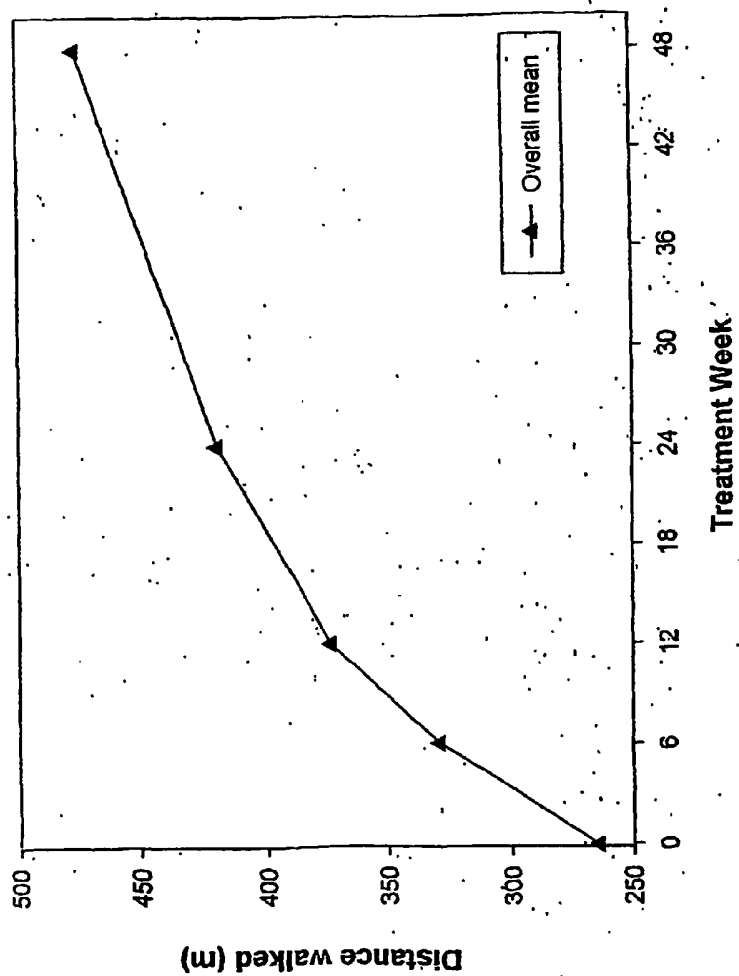




FIG. 18

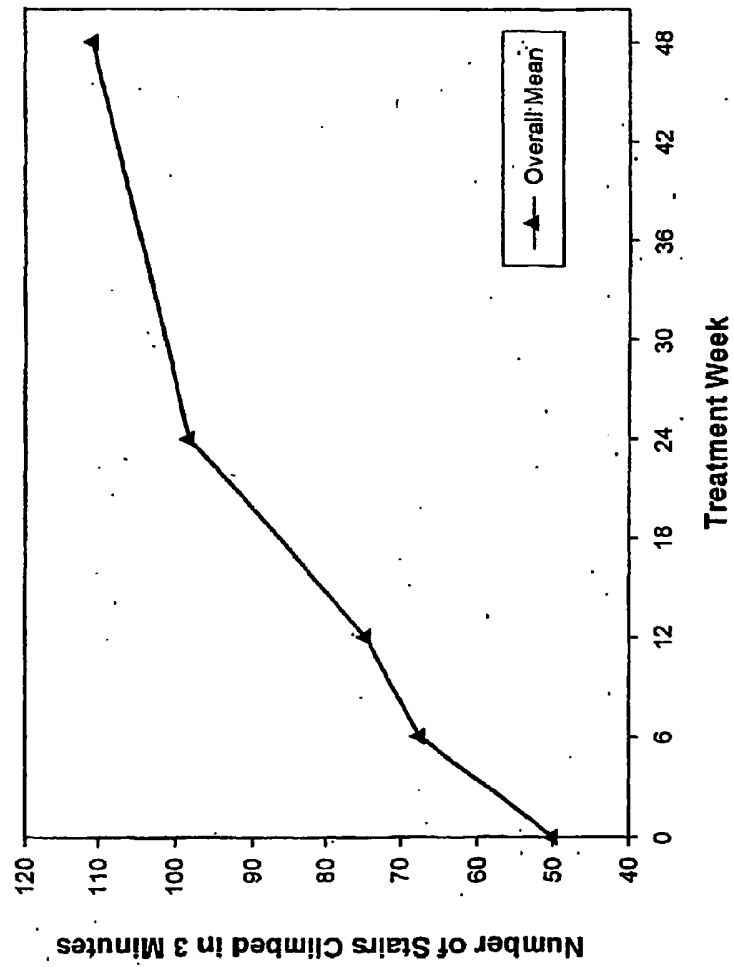


FIG. 19

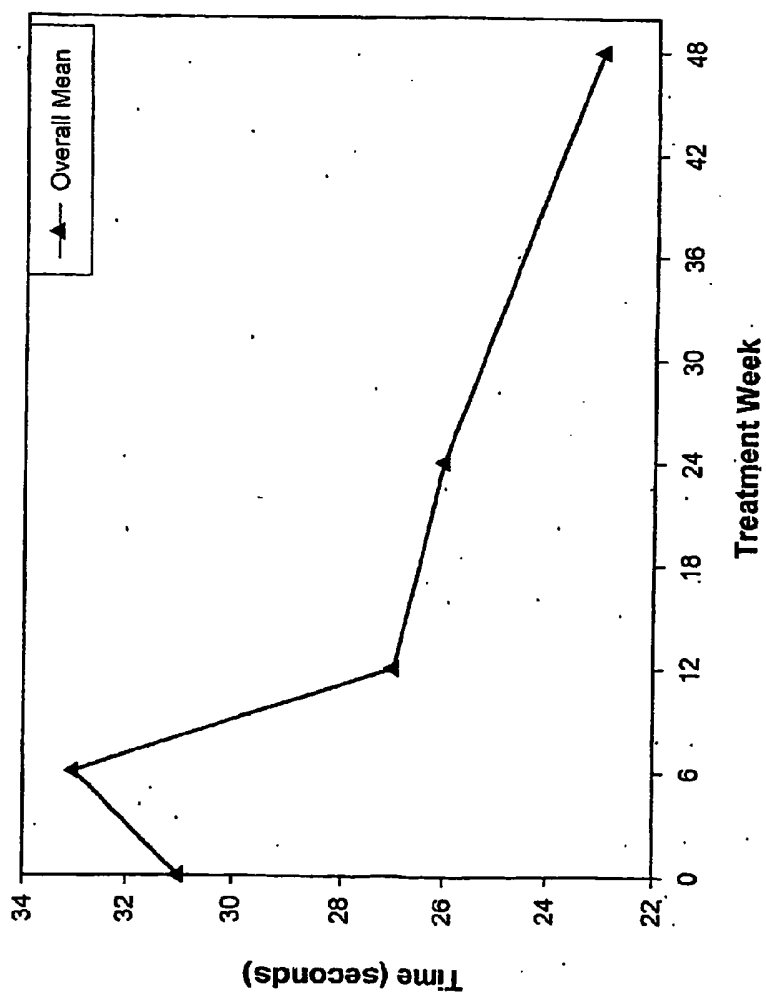


FIG. 20

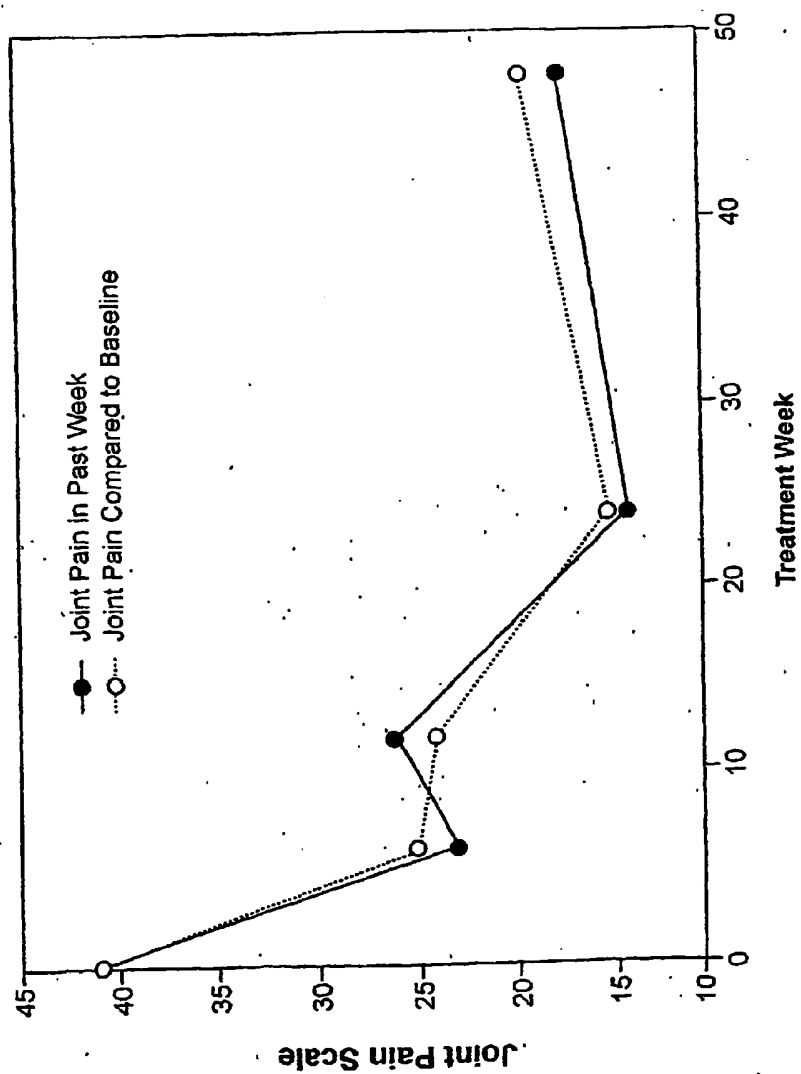


FIG. 21

